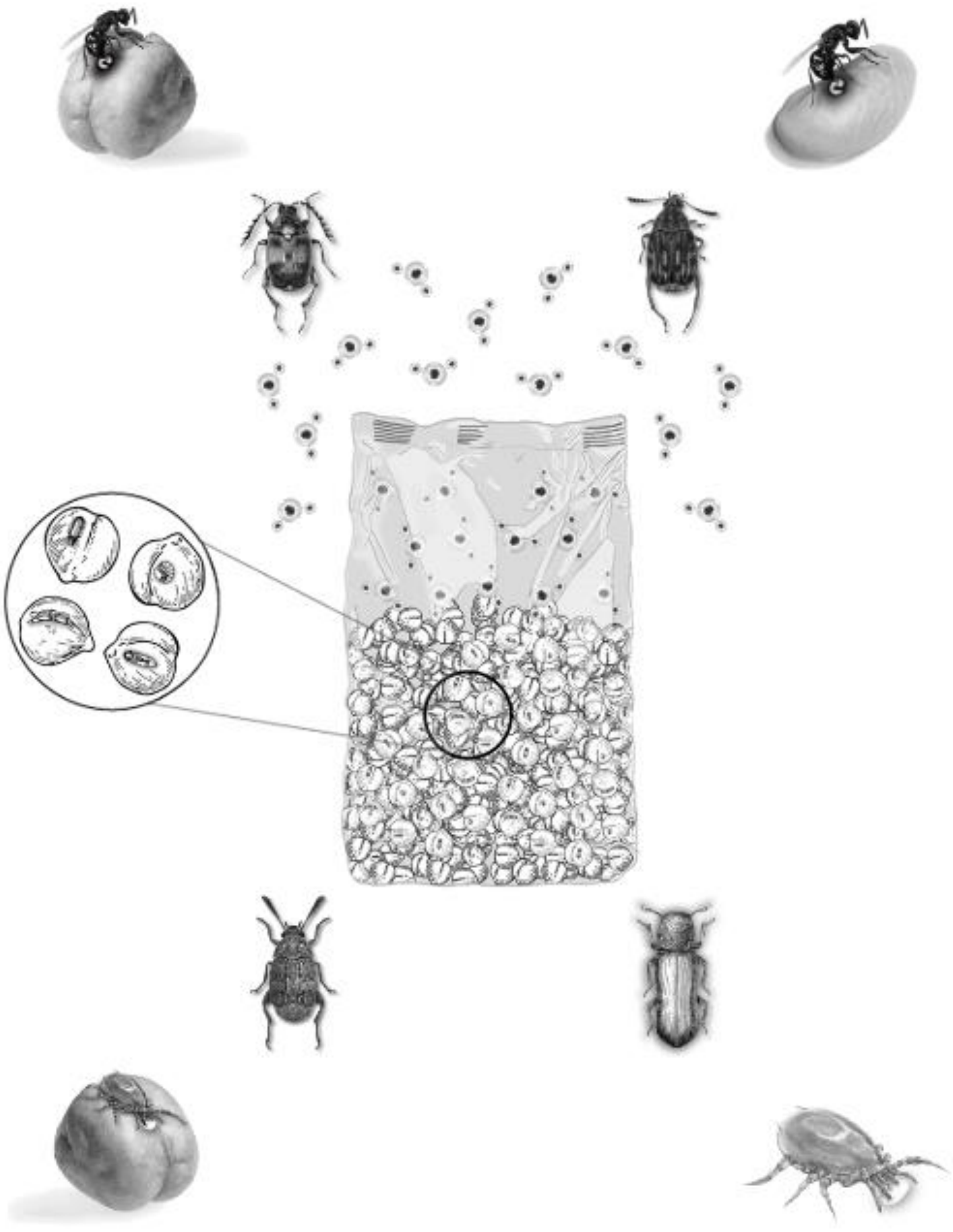
Manejo Integrado de Plagas en Legumbres Almacenadas Mediante el Uso de Atmósferas Modificadas y de Control Biológico



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Desarrollo de nuevas alternativas para el manejo integrado de plagas en judía y garbanzo almacenado

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En cada uno de nosotros hay caos y orden, bien y mal. Pero esto se puede y se debe controlar. Hay que aprenderlo. Y tú estás aprendiéndolo.

Geralt de Rivia.

Aquellas personas que ya no están.

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Resumen

España es uno de los principales países productores de garbanzos y de judías en Europa, y también es el principal importador. El control de las plagas de las legumbres almacenadas se hace principalmente fumigando con fosfina, pero se están buscando alternativas debido a los riesgos que tiene sobre la salud y el medio ambiente. En la presente tesis se ha profundizado en la implementación de las atmósferas modificadas (AM) y del control biológico (CB) como alternativas para el control de *Callosobruchus chinensis*, *Rhyzopertha dominica*, *Acanthoscelides obtectus* y *Zabrotes subfasciatus,* plagas de garbanzos y judías.

Las AM se aplican en envases impermeables a los gases. La sorción del gas por el producto afecta a la cantidad de gas dentro de los envases, produciendo el vacío. Los dos primeros capítulos, están dedicados a evaluar la sorción de CO_2 por los garbanzos envasados con AM, y a evaluar su efecto sobre la mortalidad de *R. dominica*. Los ensayos se realizaron en envases de plástico rígido a tres proporciones de llenado con garbanzo, con tres AM y durante diferentes tiempos de exposición. Para evaluar el efecto de la sorción sobre la mortalidad de *R. dominica* se compararon dos proporciones de llenado extremas. La máxima sorción se obtuvo con la proporción de llenado baja del envase y esta se redujo al disminuir la concentración de CO_2. El tiempo de equilibrio de la sorción disminuyó al aumentar la concentración de CO_2 y la proporción de llenado del envase. Además, produjo una presión negativa (vacío) que aumentó con el incremento del llenado y de la concentración de CO_2. El tiempo de exposición a las AM para alcanzar el 50% de mortalidad (TL_{50}) de *R. dominica* fue estimado para la proporción baja de llenado. Al aumentar el llenado del envase y aplicando el periodo exposición estimado de TL_{50}, la mortalidad de los huevos y los adultos de *R. dominica* disminuyó, mientras la de larvas y pupas no cambió.

Para la implementación del CB en legumbres, en el tercer y cuarto capítulo se evaluaron diversas especies de ácaros depredadores y de avispas parasitoides para el control de *C. chinensis* en garbanzo y de *A. obtectus* y *Z. subfasciatus* en judías. En garbanzo, *Amblyseius swirskii* consumió huevos de *C. chinensis,* pero *Blattisocius tarsalis* no fue capaz de depredarlos. Sin embargo, *Anisopteromalus calandrae* y *Lariophagus distinguendus* fueron muy efectivos sobre las larvas de *C. chinensis*, parasitando al huésped a profundidades de hasta 150 cm, a distintas proporciones parasitoide-huésped (*A. calandrae*) así como cuando se liberaron (*A. calandrae*) dentro de bolsas de 25 kg de garbanzos en condiciones simuladas de almacén. En judías, *B. tarsalis* y *A. swirskii* fueron capaces de depredar huevos de *A. obtectus* en las condiciones ensayadas. Aunque *A. calandrae* y *L. distinguendus* fueron efectivos en limitar el crecimiento de las poblaciones del brúquido, cuando *A. calandrae* se combinó con *B. tarsalis*, se mejoró de forma sustancial el control.

Los resultados de esta tesis indican que al envasar garbanzos con AM a altas proporciones de llenado se esperaría una disminución en la mortalidad de los huevos y adultos de *R. dominica* con respecto a la LT_{50} calculada en envases con bajas proporciones de llenado, debido a la sorción de CO_2 por la leguminosa. Por otro lado, *A. calandrae* y *L. distinguendus* son agentes de CB efectivos para el manejo integrado de *C. chinensis* en garbanzos almacenados. Además, la combinación de *A. calandrae* con *B. tarsalis* promete ser una estrategia para controlar poblaciones de *A. obtectus*. Sin embargo, se necesitan más pruebas para identificar a otros enemigos naturales que puedan reducir poblaciones de *Z. subfasciatus.*

Palabras claves: Envasado, Legumbres, Sorción de CO_2, Vacío, Concentración de CO_2, Proporción de llenado, Plagas, Ácaros depredadores, Parasitoides larvarios, Almacenamiento.

Abstract

Spain is one of the main producing countries of chickpeas and beans in Europe and is also the main importer. Nowadays, pest control is mainly based on the application of phosphine. However, the risks on human health and on the environment of phosphine, demands to implement alternatives for pest control. The present thesis was carried out to improve the application of modified atmospheres (MA) and biological control (BC) as alternatives for the control of *Callosobruchus chinensis*, *Rhyzopertha dominica*, *Acanthoscelides obtectus* and *Zabrotes subfasciatus* in stored chickpeas and beans.

MA are used in gas-tight packages, and sorption by the commodity produces a vacuum effect in the package. The first two chapters of this thesis are devoted to evaluate CO_2 sorption by chickpeas packed with MA, and the effect of CO_2 on the mortality of *R. dominica* in packaged infested chickpeas with three MA at different exposure times. Trials to determine the CO_2 sorption by chickpeas were conducted in rigid plastic containers with three filling ratios. While, two extreme cases of filling ratios were compared to evaluate sorption effect on the mortality of *R. dominica*. The maximum sorption was obtained with the lower filling ratio and sorption decreased with the decline in the CO_2 concentration. The time to reach the equilibrium sorption depended on the CO_2 concentration and the filling ratio. The vacuum effect produced by sorption resulted in a negative pressure that increased with the increase in the filling ratio and the CO_2 concentration. The exposure time to reach 50% mortality of *R. dominica* (LT_{50}) was estimated at a low filling ratio. The mortality of eggs and adult of *R. dominica* decrease at high filling ratio, while for the larvae and pupae the susceptibility remained at the exposure time estimated of LT_{50}.

To implement BC in legumes, the third and fourth chapter of this thesis was focused on the evaluation of various species of predatory mites and parasitoid wasps on the control of *C. chinensis* in chickpeas and of *A. obtectus* and *Z. subfasciatus* in beans. In chickpeas the results obtained indicate that while *Amblyseius swirskii* preyed on the weevil eggs *Blattisocius tarsalis* did not. However, *Anisopteromalus calandrae* and *Lariophagus distinguendus* were very effective against *C. chinensis* larval population, and were able to parasitize the host at depths until 150 cm. *A. calandrae* was effective at reducing the bruchid population at different parasitoid-to-host ratios tested. Moreover, *A. calandrae* efficiently reduced *C. chinensis* populations when released in 25-kg commercial bags of chickpeas in simulated warehouse conditions. In beans, *B. tarsalis* and *A. swirskii* were able to prey on *A. obtectus* eggs, reducing the bruchid population. *Anisopteromalus calandrae* and *L. distinguendus* were effective at suppressing bruchid populations, but when *A. calandrae* was combined with *B. tarsalis* the control efficacy was substantially improved.

The results of this thesis indicate that a decrease in the mortality of eggs and adults of *R. dominica* could be expected when chickpeas are packaged with MA at high filling ratios, due to CO_2 sorption by the legume. On the other hand, *A. calandrae* and *L. distinguendus* are effective BC agents for the management of *C. chinensis* in stored chickpeas. Furthermore, the combination of *A. calandrae* with *B. tarsalis* seems to be a promising strategy to control populations of *A. obtectus*. However, more tests are needed to identify other natural enemies that may reduce *Z. subfasciatus* populations.

Key words: Packaging, Legumes, CO_2 sorption, Vacuum, CO_2 concentration, Filling ratio, Pest, Predatory mites, Larval parasitoids, Storage.

Tabla de contendidos

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Listado de abreviaturas y símbolos

Abreviaturas	**Descripción**
AC / CA	Atmósferas controladas / Controlled atmospheres
ADN	Ácido desoxirribonucleico
AM / MA	Atmósferas modificadas / Modified atmospheres
CB / BC	Control biológico / Biological control
cm	Centímetros
cm^3	Centímetros cúbicos / Cubic centimeters
CO_2	Dióxido de carbono / Carbon dioxide
g	Gramos / grams
h	Horas / Hours
HR (h.r.) / RH (r.h.)	Humedad relativa / Relative humidity
IDK	Insect damage kernel
kg	Kilogramos / kilograms
kPa	Kilo pascales / Kilo pascals
LT	Lethal time
MIP / IMP	Manejo integrado de plagas / Integrated pest management
mL	Miliitros / Milliliters
Mt	Millones de toneladas
N_2	Nitrogeno / Nitrogen
nm	Nanómetros
O_2	Oxígeno / Oxigen

PET	Polyethylene terephthalate
PH_3	Fosfuro de hidrógeno / Hydrogen phosphide
PVC	Polyvinyl chloride
UE	Unión Europea
ºC	Grados Celsius / Celsius degree
µm	Micrometros / Micrometers

INTRODUCCIÓN GENERAL

La civilización humana siempre ha necesitado producir y almacenar fuentes de alimentos, y en la medida que aumenta la población mundial se incrementa su demanda. Se ha valorado que la producción de los alimentos tendría que crecer un 60% para poder alimentar una población estimada de10.5 billones en el 2050 (Alexandratos y Bruinsma, 2012). Entre las principales fuentes de alimentos se encuentran las legumbres, con una producción que se ha incrementado de 77.6 a 92.4 millones de toneladas (Mt) entre el 2014 hasta el 2018, siendo las legumbres más producidas la judía, con alrededor de 30.4 Mt, y el garbanzo, con 17.2 Mt. (Food and Agriculture Organization of the United Nations. Statistical Division [FAOSTAT], 2020). Estás leguminosas son almacenadas para garantizar su disponibilidad cuando son requeridas, ya que debido a la alta producción estacional no pueden ser consumidas en su totalidad de forma inmediata, y además cuentan con una larga vida de anaquel.

Problemática de plagas en productos almacenados

El almacenamiento consiste en depositar la producción del grano en las estructuras de almacenamiento que son ecosistemas donde se acumulan grandes cantidades de alimentos en un espacio reducido bajo condiciones de temperatura y humedad adecuadas. Estas condiciones son también adecuadas para el desarrollo de ciertas especies de artrópodos que viven en condiciones similares en sus hábitats naturales (Nayak y Daglish, 2018). La mayoría de estas especies son insectos adaptados al ecosistema de almacenaje, tienen distribución cosmopolita y pueden dañar todo tipo de productos como granos, legumbres, especias, frutas desecadas, semillas y material seco de origen animal (Hill, 2002). Causan una proporción significativa de las pérdidas que sufren estos productos durante la pos-cosecha y pertenecen principalmente al orden Coleoptera (escarabajos), Lepidoptera (polillas) y Psocoptera (psocidos). Se categorizan como plagas primarias y secundarias de acuerdo al nivel de daño que producen (Stejskal et al., 2014). Las plagas primarias se encuentran representadas por insectos altamente especializados con la capacidad de perforar la testa de los granos enteros alimentándose del embrión, endospermo o cotiledones de las leguminosas o cereales. Las plagas secundarias son insectos poco especializados que poseen un espectro de alimentación más amplio, alimentándose del grano dañado por las plagas primarias o por los daños mecánicos ocasionados durante su cosecha, manejo y transporte; otras especies tienen preferencias por productos molidos como la harina, o por los alimentos procesados y manufacturados como los cereales de desayuno, chocolates y los alimentos de mascota (Stejskal et al., 2015).

Los escarabajos de la familia Chrysolemidae (sub familia Bruchinae) han sido reconocidos durante siglos por la capacidad de ciertas especies en destruir leguminosas: alrededor de 30 especies en el mundo son plagas serias y al menos 9 de ellas se han convertido en cosmopolitas debido a las actividades comerciales. Entre estas especies de brúquidos sobresalen las del género *Acanthoscelides*, *Bruchus*, *Callosobruchus*, *Caryedon* y *Zabrotes* que son plagas primarias de diferentes legumbres almacenadas, y que frecuentemente son asistidos por los humanos en su dispersión al facilitarles su movilidad y proporcionándoles una gran fuente de alimento en los métodos de almacenamiento (Kingsolver, 2004). Las especies plagas más importantes en garbanzos y judías son *Callosobruchus chinensis* L., *Acanthoscelides obtectus* Say y *Zabrotes subfasciatus* Boheman (USDA, 2004). Otra especie que se ha encontrado infestando garbanzo almacenado es *Rhyzopertha dominica* Fabricius (Coleoptera: Bostrichidae), que es una plaga primaria en cereales y causa daños de manera discreta en leguminosas (Wong 1987). Estas cuatro especies primarias son de desarrollo interno, es decir los adultos ponen los huevos en el exterior de las legumbres y las larvas de primer estadio penetran y se desarrollan en el interior del grano hasta alcanzar el estado adulto que emerge del grano y se dispersa.

El término "perdida de pos-cosecha" en los productos almacenados se ha implementado hace dos décadas y se refiere a las perdidas cuantitativas (de peso por grano dañado, reducción en la capacidad germinativa y valor nutritivo de los alimentos) y cualitativas (presencia de restos y residuos de insectos, fomento de la presencia de microorganismos y toxinas) (Ofuya y Reichmuth, 1992; de Lucia y Assennato, 1994; Hodges et al., 2011). Las perdidas cuantitativas pueden deberse a los daños físicos causados por el consumo directo por las plagas primarias seguido por una infestación de las plagas secundarias, reduciendo la capacidad germinativa de los granos (daños en el embrión o germen) y de su valor nutritivo (daños en el endospermo). Las pérdidas cualitativas son más complejas de medir y pueden ocasionar que el producto almacenado quede inservible tanto para el consumo humano como animal. Se deben a la presencia de cuerpos muertos, productos de desperdicio y polvo producidas por la actividad del insecto (Fleurat-Lessard, 2002). Las actividades alimenticias del insecto pueden añadir altas cantidades de ácido úrico produciendo la rancidez del grano (Mason y McDonald, 2012) así como producir focos de calor incrementando la temperatura hasta 57 ºC (Mills, 1989). Estos focos de calor en combinación con la humedad dentro del producto favorecen la actividad fúngica, produciendo cambios de sabor, olor, color, reducción del valor nutricional y reducción de la capacidad germinativa del grano (Sauer et al., 1992). Además, la actividad fúngica de algunas especies de los géneros *Aspergillus* y *Fusarium* pueden producir metabolitos secundarios (micotoxinas) que son muy tóxicos para la salud humana y animal (Stejskal et al., 2018).

Aunque la legislación de la Unión Europea (UE) tiene tolerancia cero respecto a la presencia de artrópodos o restos de los mismos en los alimentos procesados, no existen programas de vigilancia ni de evaluación sistemática del riesgo que representan las plagas de almacén para la seguridad alimentaria (Stejskal et al., 2015). Este nivel de tolerancia cero comporta que no existan niveles de acción o umbrales respecto a la presencia de artrópodos en las materias primas que se utilizan para la elaboración de alimentos (Stejskal et al., 2018). Otros países industrializados como Canadá y Australia, también tienen tolerancia cero respecto a la presencia de insectos vivos en granos almacenados (Rajendran, 2008). En contraste, en los Estados Unidos de América la Administración de Alimentos y Medicamentos (FDA por sus siglas en inglés) ha desarrollado leyes estrictas que no prohíben sino que limitan el número permisible de insectos vivos en los productos almacenados o la presencia de granos dañados por insectos en el grano que representan un riesgo para la salud de las personas. Por ejemplo, en una partida de garbanzos o judías destinadas al consumo se permite un 5% de granos infestados y/o dañadas por insectos en una muestra de 100 g de un mínimo de 12 muestras (United States Food and Drug Administration [FDA], 2020)

Métodos de control de plagas mayoritarios aplicados en la actualidad

El manejo de plagas tiene como objetivo proteger los granos (legumbres y cereales) durante su almacenamiento para reducir los daños económicos causados por las plagas, proteger el medio ambiente y la salud de los aplicadores, trabadores y consumidores. Actualmente el método mayoritario de control de plagas en productos almacenados es la utilización de tratamientos insecticidas fumigantes y residuales, en ocasiones aplicados de forma excesiva y poca efectiva (Riudavets, 2017). Suele ser complicado combinar las demandas de los consumidores por alimentos libres de artrópodos y de residuos químicos, ya que los insecticidas utilizados por la industria para obtener una protección a largo plazo de un amplio espectro de insectos suelen dejar residuos.

Entre los fumigantes la fosfina es el más utilizado en la mayoría de países, incluidos España y México, para desinfestar legumbres almacenadas, aunque en algunos países también hay registrados otros fumigantes como el ozono, el fluoruro de sulfurilo, el óxido de propileno o el formato de etilo (Rajendran, 2019). Estos gases no tienen un efecto residual y después del tratamiento el grano se encuentra expuesto para ser colonizado de nuevo por los artrópodos. Pero el empleo excesivo de la fosfina ha incrementado la preocupación sobre su utilización por el desarrollo de resistencias en diversas especies plagas de almacén, como por ejemplo *R. dominica* (Lorini et al., 2007; Nayak et al., 2013; Holloway et al., 2016; Gautam et al., 2016). Otro problema sobre el uso de fosfina es que es un fumigante muy tóxico; aunque existe la creencia de que los riesgos asociados con la fosfina son bajos por su lenta liberación en las tabletas o perdigones, el envenenamiento por fosfina a escala global sigue siendo muy elevado por las malas prácticas o por accidentes (Stejskal et al, 2018). Hasta hace poco años otro fumigante que se utilizaba de forma habitual sobre productos almacenados era el bromuro de metilo, pero actualmente se encuentra prohibido a nivel mundial debido al Protocolo de Montreal, y solo está autorizada su utilización para el control de plagas cuarentenarias en fronteras. El Protocolo de Montreal acordó en retirarlo por su efecto negativo sobre la capa de ozono (World Meteorological Organization [WMO], 2018). El fluoruro de sulfurilo es un fumigante muy utilizado en el control de polillas, pero para el control de escarabajos la información se encuentra muy limitada (Jagadeesan et al., 2014). En España este producto solo está autorizado para su uso en estructuras de almacenaje y procesado que estén vacías pero no se puede aplicar directamente sobre productos alimentarios.

Los insecticidas residuales tienen un rol importante para la protección a largo plazo de las legumbres durante su almacenamiento, ya sea sobre legumbres infestadas como recién cosechados para su protección de los ataques de los insectos. Entre los más utilizados se encuentran el metil pirimifos y piretroides como la deltametrina. En España solo está permitido el uso de deltametrina para el tratamiento directamente sobre leguminosas (Registro de Productos Fitosanitarios). Debido a su amplio uso, diversas especies plaga como *R. dominica* han desarrollado resistencia a estas sustancias activas (Carvalho-Guedes et al., 1996; Lorini y Galley, 1999).

Métodos de control de plagas alternativos al uso de plaguicidas

El futuro del control de las plagas de almacenes pasa por el desarrollo de programas de Manejo Integrado de Plagas (MIP) en los que se combinen medidas de prevención y de control, minimizando o evitando totalmente el uso de plaguicidas convencionales. Entre los métodos destacados de MIP se encuentran los tratamientos con calor y frío, la aplicación de atmósferas modificadas (AM), la utilización de extractos de aceites esenciales, de tierras diatomeas y de nanopartículas, las estrategias de interrupción del apareamiento (confusión sexual) y el control biológico (CB). Los tratamientos con calor consisten en incrementar la temperatura ambiente en las instalaciones de almacenaje hasta alcanzar el nivel letal para los artrópodos, que es de unos 50 ºC durante un tiempo de exposición determinado (1-3 horas) (Mahroof et al., 2003). Por otro lado, tenemos los tratamientos con frío que son aplicados sobre productos infestados para matar a los insectos con la reducción de la temperatura, pero primeramente es necesario calcular la temperatura y el intervalo de exposición requerida para obtener un control completo y sin afectar al producto, debido a que los insectos generalmente son tolerantes a las bajas temperaturas (Fields, 1992; Flinn et al., 2015). Los extractos de aceites esenciales proceden en general de una amplia variedad de especies de plantas y son utilizados para el control de artrópodos por su toxicidad o también como repelentes. Las tierras diatomeas están formadas por fósiles de organismos unicelulares (fitoplancton) compactados en una roca calcárea amorfa. Actúan adhiriéndose a la cutícula del artrópodo y produciéndole una pérdida de agua hasta causarle la muerte por desecación (Subramanyam y Roesli, 2000). Las nanopartículas (óxido de aluminio, óxido de zinc, óxido de titanio, y de plata) se encuentran dentro de un rango de 1 y 100 nm y afectan a los artrópodos de una manera similar a las tierras diatomeas (Stadler et al., 2012; Buteler et al, 2015). La estrategia de la confusión sexual se basa en la saturación del ambiente del almacén con la feromona sexual de la especie plaga a controlar, lo que impide que se encuentren los machos y las hembras, y que estas no se reproduzcan (Trematerra et al., 2011, 2013). Las AM se basan en reducir la concentración de oxígeno (O_2) mediante el uso de gases como el dióxido de carbono (CO_2) y/o nitrógeno (N_2) (Navarro, 2012), provocando la anoxia en los artrópodos. Pueden aplicarse sobre el producto a presión atmosférica o bien a alta presión. Actualmente el empleo de las AM en combinación con las altas temperaturas, forman parte de las nuevas técnicas de aplicación que han permitido reemplazar con gran éxito a los fumigantes convencionales (Bell y Conyers, 2002; Navarro, 2006; Athanassiou et al., 2016). El CB implica la utilización de enemigos naturales de las plagas para reducir sus poblaciones (Schöller et al., 2018). El CB es una excelente alternativa del MIP ya que consigue mantener las poblaciones de los artrópodos por debajo de los niveles de daño económico dentro de un marco de protección de los trabajadores y del medio ambiente (Dent, 2000). En esta tesis nos hemos centrado en el estudio de algunos aspectos de las AM a presión atmosférica y del CB con artrópodos aplicados al control de las plagas de las leguminosas almacenadas.

Atmósferas modificadas

Es una de las alternativas seguras y amigables con el medio ambiente que se ha desarrollado para el control de diversas plagas. La técnica consiste reemplazar el aire dentro del envase o recinto de tratamiento con una mezcla alterada de los gases atmosféricos principales (N_2, O_2 y CO_2) y saturar los productos alimenticios, por el tiempo de exposición necesario para controlar las plagas. De forma habitual se diferencian dos tipos de atmósferas modificadas según si se mantiene la concentración de gases constante (atmósferas controladas, AC) o cambia durante el tratamiento (Atmósferas modificadas, AM). En caso de las AC la composición inicial de gases se mantiene contantes conectando el envase o recinto de tratamiento a un regulador de gases (atmósfera controlada). En el caso de las AM, una vez llenado el envase o el recinto de tratamiento con la atmósfera deseada se deja interactuar durante el periodo de exposición sin modificar las concentraciones de gases. En este caso las concentraciones de gases variarán con el tiempo de exposición debido a la difusión de los gases en el producto, la permeabilidad del envase a los gases y los efectos de la respiración del alimento y/o del metabolismo microbiano (Church, 1994; Philips, 1996). El uso de las AM pueden representar un alto costo por los materiales utilizados y su sellado, ya que deben asegurar la estanqueidad de los gases en su interior. Aunque la aplicación a limitado su uso para el manejo de recintos de granos, actualmente esta técnica se ha desarrollado para aplicar en bolsas grandes (250-1000 kg) o “cocoons” utilizadas para el almacenamiento hermético de granos que se mantienen en el suelo (De Groote et al., 2013; Moussa et al., 2014). Otra aplicación de las AM es para envases pequeños (de 500 g a 2 kg) que van destinados directamente al consumidor. Entre las distintas AM utilizadas, destacan las ricas en CO_2 y deficientes en O_2. Las altas concentraciones de CO_2 producen hipercarbia y las bajas concentraciones de O_2 tienen un efecto de hipoxia sobre el artrópodo (Navarro, 2012). La hipercarbia por tiempos prolongados tiene efectos tóxicos sobre el sistema nervioso y suele acidificar la hemolinfa produciendo fallas en las membranas de algunos tejidos (Nicloas y Sillans, 1989; White et al., 1995). Los niveles de CO_2 por encima del 10% provocan la apertura permanente de los espiráculos que conduce la muerte de los insectos por pérdida de agua (Navarro, 2012). La hipoxia generalmente tiene un mayor efecto sobre los adultos que sobre los otros estados del desarrollo (Navarro, 1978). Pero por debajo de una concentración del 1% de O_2 es necesario incrementar el periodo de exposición para tener un efecto letal, porque los artrópodos suelen cerrar sus espiráculos (Navarro, 2012; Levy-de la Torre, et al., 2018).

Se conoce bien la efectividad del CO_2 para controlar las diferentes etapas del desarrollo de diversas especies de plagas de cereales y de leguminosas en condiciones experimentales de laboratorio, esto es con una cantidad reducida de grano y un gran volumen de gas en los recipientes (Riudavets et al., 2009; Wong-Corral et al., 2013). Pero poco se sabe de cuál es la efectividad cuando se aplica el CO_2 en condiciones prácticas, esto es en envases llenos de grano con muy poco volumen de espacio disponible para el gas. En condiciones prácticas, sabemos que gran parte del gas que se introduce en los contenedores es absorbido por el garbanzo, y que esta absorción disminuye la concentración de gas libre en el espacio disponible del envase (Iturralde et al., 2016). Sin embargo, se desconoce la cantidad de CO_2 que puede sorber el garbanzo, cuestión importante a la hora de calcular la cantidad de gas necesaria para que los tratamientos sean efectivos; además, al absorber el gas de los intersticios de los granos la sorción tiende a colapsar los envases con producto por el vacío que se produce dificultando su estibado y transporte. También, se desconoce el que efecto puede tener la disminución del gas libre en el envase debido a la sorción del CO_2 sobre el control de las plagas, dado que queda poco gas libre en el contenedor.

Control Biológico

Es otra herramienta importante en las estrategias MIP por su efectividad, inocuidad para los usuarios y consumidores, y por ser respetuosa para el medio ambiente. El CB es comúnmente utilizado contra plagas en invernaderos o en campos agrícolas (Bigler, 1994), pero para plagas de almacén su uso es todavía muy restringido. Sin embargo, en instalaciones de almacenamiento las condiciones ambientales son generalmente estables y al ser recintos confinados hay buenos elementos para que sea una técnica efectiva. La técnica de CB consiste en liberar a los enemigos naturales sobre los insectos plagas de almacén de manera sincronizada con el crecimiento de la población de la plaga. Para lo cual es necesario monitorear con trampas la población plaga dentro de las instalaciones y determinar el mejor momento de realizar las liberaciones (Flinn y Schöller, 2012). Los enemigos naturales estudiados en el control de plagas de productos almacenados incluyen a los Himenópteros parasitoides, Hemípteros y ácaros depredadores, y en menor medida a los microorganismos como los hongos entomopatógenos, las bacterias, los baculovirus, los protozoos y los nematodos entomopatógenos (Collatz et al., 2007; Meierhofer et al., 2007; Coelho et al., 2008, Wakefield, 2018).

Entre los microorganismos uno de los más estudiados es la bacteria *Bacillus thurigensis,* produce durante su esporulación una proteína que, una vez digerida por el insecto, forman endotoxinas que causan la parálisis y la muerte del huésped. La bacteria ha sido estudiada extensivamente en diversas plagas de insectos de almacén, principalmente en especies de Coleópteros y Lepidópteros (Wakefield, 2018). Los Baculovirus son un grupo de virus con una doble cadena de ADN, que son patógenos de especies de Lepidópteros. Una vez ingeridos por el insecto infectan el sistema digestivo y detienen su alimentación conduciéndolo hasta la muerte (Wakefield, 2018). Estos Baculovirus tienden a tener un huésped especifico y los más utilizado son los granulovirus de *Plodia interpunctella* Hübner y *Ephestia kuehniella* Zeller (Mcvean et al., 2002; Vail et al., 2003). Los Protozoos son organismos celulares que entran en el huésped por ingestión o por una transmisión vertical de la hembra a su progenie. Entre las especies con mayor potencial para el control de insectos plaga de almacenes se encuentran las del género *Mattesia* y *Nosema* sobre algunos Coleópteros como *Oryzaephilus surinamensis* L. y *Cryptolestes ferrugineus* Stephens (Lord 2003; Lord et al., 2010). Los hongos entomopatógenos producen enfermedades en escarabajos y polillas al adherirse a su cutícula para poder proliferar dentro del huésped, y la muerte por inanición. Actualmente existen alrededor de 1000 especies de hongos que son patógenos para los insectos, siendo los más estudiados *Metarhizium anisopliae* (Metchnikoff) Sorkin y *Bauveria bassiana* (Balsamo) Vuillemin (Wakefield, 2018). Estás especies se han utilizado para controlar *Callosobruchus maculatus* Fabricius, *E. kuehniella*, *P. interpunctella*, *Prostephanus truncatus* Horn, *R. dominica*, *Sitophilus oryzae* L., *Sitophilus zeamais* Motchuslky y *Tribolium castaneum* Herbst (Padin et al., 1996; Bischoff y Reichmuth, 1997; Kassa et al., 2002; Mahdneshin et al., 2009; Mahdneshin et al., 2011; Sabbour et al., 2012; Zidan, 2013). Los nematodos entomopatógenos llevan bacterias simbióticas que son patogénas al ser liberados dentro del insecto causándole la muerte por septicemia o toxemia (Wakefield, 2018). Los miembros de la familia Steinernemetidae y Heterorhabditae son los más estudiados para el control biológico. Las especies del género *Steinernema* y *Heterorhabdits* se encuentran asociados con las bacterias del género *Xenorhabdus* y *Photorhabdus*, respectivamente (Boemare, 2002). La eficiencia de los miembros de ambas familias de nematodos se han evaluado contra algunas especies plaga como *E. kuehniella*, *O. surinamensis*, *P. interpunctella*, *R. dominica* y *S. oryzae* (Mbata y Shapiro-Ilian, 2005; Ramos-Rodriguez et al., 2006; Trdan et al., 2006; Ramos-Rodriguez et al., 2007; Athanassiou et al., 2010). Ninguno de estos microorganismos están autorizados en Europa para su uso sobre productos almacenados

En el caso del uso de macrorganismos, es decir de artrópodos, es esencial que tengan una capacidad suficiente de búsqueda del huésped o presa para ser considerados como buenos agentes de CB de plagas (Noldus, 1989). Los enemigos naturales liberados en un lugar, se dispersan para localizar y atacar activamente a las plagas ubicadas en el interior de las grietas o dentro de una masa de granos. Además, las estructuras de almacenamiento limitan su salida de las instalaciones. Es necesario conocer el momento correcto de su liberación, ya que se recomienda realizarlo de manera temprana en el ciclo de crecimiento de las plagas para que el agente biológico pueda limitar el crecimiento de la población de las plagas (Flinn y Schöller, 2012). Una de las ventajas del uso enemigos naturales es que pueden continuar reproduciéndose desde el momento de ser liberados hasta que los huéspedes dejen de encontrarse disponibles, prolongando su acción en el tiempo. Algunas de las limitaciones de la técnica es que muchos de los enemigos naturales son específicos del huésped, por lo que es necesario determinar al enemigo natural apropiado para controlar a la especie plaga presente, y coexistiendo en un mismo almacén suele haber varias posibles especies plaga. Una posible desventaja que se ha mencionado en el uso de enemigos naturales para el control de plagas en productos almacenados es la contaminación del producto con los cuerpos de los mismos enemigos naturales aplicados. Sin embargo, en realidad esta contaminación es prácticamente despreciable ya que, si el control es efectivo, el tamaño de las poblaciones de estos artrópodos es muy pequeño. Además, por lo general, sus cuerpos son mucho más pequeños que los de sus huéspedes o presas y, una vez secos, no se detectan.

Entre los macroorganismos que se han ensayado en el control de plagas de almacén destacan los Hemípteros, los ácaros depredadores y los parasitoides. Entre los Hemipteros depredadores utilizados en almacenes destaca *Xylocoris flavipes* Reuter (Hemiptera: Lyctocoridae). Esta especie es un depredador polífago muy eficiente que consume los huevos y los estados del desarrollo temprano de escarabajos y polillas tales como *Tribolium confusum* Jaqueline du Val, *T. castaneum*, *S. zeamais*, *Sitophilus granarius* L., *Lasioderma serricorne* F., *P. interpunctella*, *Sitrotoga cerealella* Oliver y *Corcyra cephalonica* Stainton (Press et al., 1975; Reichmuth, 2000; Reichmuth et al., 2007; Rabinder y Singh, 2011; Schöller y Prozzel, 2011; Suma et al., 2013). Los ácaros depredadores suelen alimentarse de los huevos y larvas de sus presas y los más utilizados son de la familia Phytoseiidae, Ascidae, Bdellidae, Tydeidae, Cheyletidae y Stigmaeidae (Mirabal, 2003). En el ámbito de los almacenes de grano se encuentra de forma habitual *Blattisocius tarsalis* Berlese (Acari: Ascidae) sobre cereales y leguminosas infestadas con *C. ferrugineus*, *P. intercpunctella*, *L. serricorne*, *T. confusum* y *Blatella germanica* L. En condiciones experimentales *B. tarsalis* ejerce un control natural efectivo sobre huevos de diversas especies plagas de almacén como *E. kuehniella*, *Ephestia cautella* Walker, *P. interpunctella* y *L. serricorne*, también puede consumir huevos de *T. castaneum*, *C. ferrugineus* y *A. obtectus* (Haines, 1981; Nielsen, 2003; Riudavets et al., 2002; Stejskal et al., 2006). Otra especie estudiada para el control de plagas de almacén es *Cheylitus eruditus* Schrank (Trombidiformes: Cheyletidae), una especie cosmopolita que se ha utilizado para reducir poblaciones de otros ácaros y de pequeños artrópodos en productos almacenados (Mullen et al., 2019). Su utilización como agente de CB no es recomendable porque especies de este género pueden producir dermatitis que pueden ser graves para el personal que está en contacto con el grano en los almacenes (Stejskal y Huber, 2008). Las especies de parasitoides más frecuentemente utilizadas pertenecen a las familias Braconidae, Ichneumonidae, Trichogrammatidae, Pteromalidae y Bethylidae (Grieshop et al. 2004). Los parasitoides suelen clasificarse de acuerdo al tipo de desarrollo que tienen sobre el huésped: endoparasitoides para aquellos que se desarrollan en el interior del huésped, ectoparasitoides para aquellos que se desarrollan en el exterior del huésped y mesoparasitoides para los que tienen un desarrollo mixto interno y externo. Uno de los parasitoides de plagas de almacén más estudiados es *Habrobracon hebetor* Say (Hymenoptera: Braconidae), un ectoparasitoide cosmopolita que se desarrolla sobre las larvas de varios Lepidópteros, principalmente de la familia Pyralidae (Schöller, 1998). Otro parasitoide bastante estudiado *Theocolax elegans* Westwood (Hymenoptera: Pteromalidae), ectoparasitoide de larvas de *R. dominica,* que permite reducir los fragmentos de insectos presentes en la harina (Flinn y Hagstrum, 2001). *Anisopteromalus calandrae* Howard (Hymenoptera:

Pteromalidae) es uno de los parasitoides más ampliamente distribuido y que se encuentra más frecuentemente en granos almacenados (Schöller et al., 2018). Este es un ectoparasitoide que ataca los últimos estadios larvarios y las pupas tempranas de muchas especies de escarabajos de desarrollo interno (Shin et al., 1994). Para ello, las hembras primero paralizan la larva huésped y luego depositan el huevo sobre su superficie; la larva del parasitoide se alimenta de la hemolinfa del huésped hasta consumirlo (Ahmed, 1996; Arbogast and Mullen, 1990). Se ha reportado como enemigo natural de diversas plagas de almacén como *S. granarius*, *S. zeamais*, *R. dominica*, *Stegobium paniceum* L., *L. serricorne, A. obtectus* y *C. maculatus* (Williams y Floyd 1971; Arbogast and Mullen 1990; Reichmuthm 2000; Reichmuth et al., 2007; Ngamo et al., 2007). *Lariophagus distinguendus* Förster (Hymenoptera: Pteromalidae) es también un ectoparasitoide de larvas y prepupas que se ha utilizado contra una amplia variedad de escarabajos que infestan granos almacenados como *S. oryzae*, *S. granarius*, *R. dominica*, *L. serricorne*, *S. paniceum*, *A. obtectus* y *Ptinus clavipes* Panzer (Reichmuth, 2000; Lucas y Riudavets, 2002; Steidle y Schöller, 2002; Menon et al., 2002; Steidle et al., 2006).

En el caso del control de los brúquidos con enemigos naturales, aunque hay algunos estudios sobre la biología de los parasitoides *Anisopteromalus calandrae* y *Lariophagus distinguendus* con diferentes especies, poco se sabe de su capacidad de control sobre las mismas (Schmale et. al., 2001; Ngamo et. al., 2007; Cox et al., 2007). El número de especies de ácaros depredadores que se conoce que colonizan los almacenes son limitadas, pero existen otras especies utilizadas comercialmente para el control de plagas de invernaderos que pueden ser una opción para el ámbito de los almacenes. Entre estas especies más comunes se pueden citar *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae), *Neoseiulus cucumeris* Oudemans (Acari: Phytoseiidae) y *Stratiolaelaps scimitus* Berlese (Acari: Laelapidae) (Jess and Schweizer, 2009; Rahman et al., 2011; Delisle et al., 2015; Kakkar et al., 2016). Estas son especies que no se han evaluado como enemigos naturales de las especies plaga en leguminosas almacenadas. Parasitoides y ácaros depredadores podrían combinarse ya que atacan estadios diferentes y su acción podría ser complementaria.

Objetivos

El objetivo general de la presente tesis doctoral se centra en mejorar las técnicas de las atmósferas modificadas y de control biológico para el manejo integrado de algunas de las plagas de garbanzos y judías almacenadas.

Para llevar a cabo dicho objetivo general, se establecieron cuatro objetivos específicos plasmados en los capítulos presentados:

Objetivo del Capítulo 1. Cuantificar la sorción y la presión negativa producida por esta sorción cuando se envasan garbanzos con diferentes concentraciones de CO_2 y con diferentes proporciones de llenado del envase.

Objetivo del Capítulo 2. Evaluar el efecto de las distintas proporciones de llenado del envase y de las diferentes concentraciones de CO_2 aplicadas a los garbanzos sobre la mortalidad de los estados del desarrollo de *Rhyzopertha dominica*.

Objetivo del Capítulo 3. Evaluar la efectividad de los ácaros depredadores *Blattisocius tarsalis* y *Amblyseius swirskii*, y las avispas parasitoides *Anisopteromalus calandrae* y *Lariophagus distinguendus* como agentes de control biológico para mantener reducida una población de *Callosobruchus chinensis* en garbanzo almacenado.

Objetivo del Capítulo 4. Evaluar la efectividad de los ácaros depredadores *Blattisocius tarsalis*, *Amblyseius swirskii*, *Neoseiulus cucumeris* y *Stratiolaelaps scimitus,* y las avispas parasitoides *Anisopteromalus calandrae* y *Lariophagus distinguendus*, solos o combinados, para mantener reducidas las poblaciones de *Zabrotes subfasciatus* y *Acanthoscelides obtectus* en judías almacenadas.

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CAPÍTULO 1

Sorption of carbon dioxide by chickpeas packaged in modified atmospheres

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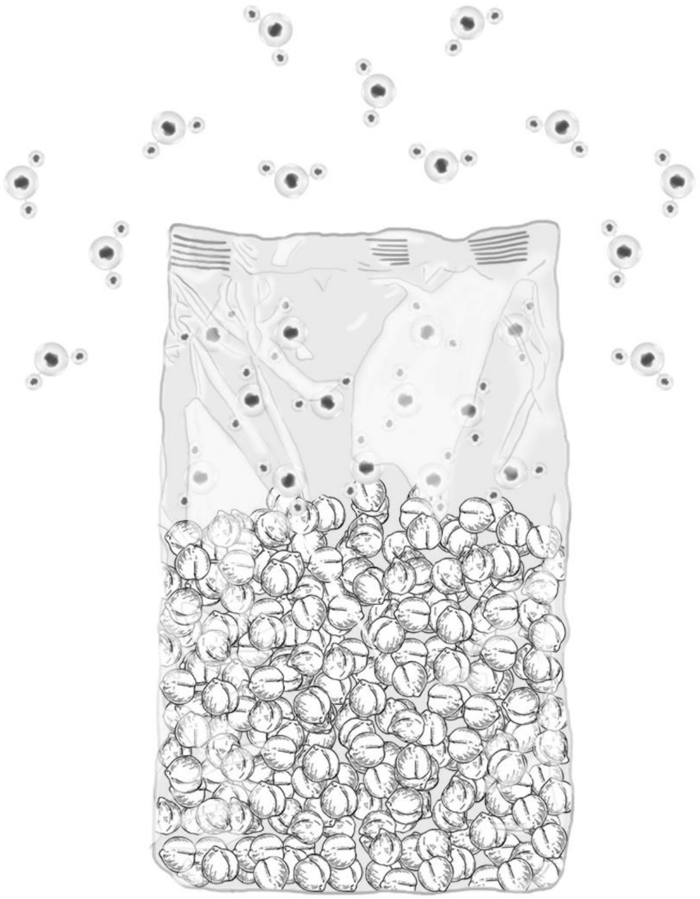

Introduction

Chickpea (*Cicer arietinum* L.) is the third most important grown grain legume around the world, after beans and peas. In 2016, there was a worldwide production of 12.1 million tons of chickpea from 12.7 million hectares of harvested areas (FAOSTAT, 2018). Chickpeas are grown in several countries, with the leading exporters being India, Australia and Mexico. After harvesting, chickpea storage can be extended for more than a year due to the seasonal variability in market prices. During this storage period, chickpeas are susceptible to insect attack, particularly by *Callosobruchus maculatus* (Fab.) (Col. Bruchidae). The larvae of *C. maculatus* are internal feeders; the neonate larvae bore holes on the kernels where they develop until the emergence of the adults (CABI, 2018), and they produce qualitative and quantitative losses (weight loss, a decrease in the grain's nutritional value and the failure of seed germination (Ofuya and Reichmuth, 1992). The control of this species is currently based on fumigation with synthetic insecticides, hydrogen phosphide (PH_3) being the most effective and widely used. However, the long storage period of chickpeas results in a high number of PH_3 fumigations, which promotes the development of resistant populations of the weevil. It also poses a threat to the health of operators handling the fumigant, represents a risk to consumers from the accumulation of chemical residues in the grain legume and pollutes the environment (Garry et al., 1989; Chaudry, 1997; Sousa et al., 2009). Therefore, it is important to develop alternative control methods that are effective and environmentally safe.

Modified atmospheres (MAs) with low oxygen (O_2) and high carbon dioxide (CO_2) are one of the alternatives to synthetic chemicals for the control of legume pests, such as *C. maculatus*, *Acanthoscelides obtectus* (Say), *Zabrotes subfasciatus* (Boheman) and *Rhyzopertha dominica* (Fab.) (Donahaye et al., 1996; Navarro, 2006a, b; Riudavets et al., 2009). MAs with 50%–90% CO_2 are effective for the control of *C. maculatus*, *A. obtectus* and *Z. subfasciatus* at exposure times of 3 d, 5 d, 9 d and 2 d for eggs, larvae, pupae and adults, respectively (Wong-Corral et al., 2013; Iturralde-García et al., 2016). Also, the quality of the grain (water absorption, cooking time, texture, colour and flavour) and the vigour of germination are preserved after exposure to CO_2 (Navarro, 2006b; Carvalho et al., 2012; Iturralde-García et al., 2016).

The application of MAs requires the use of gas-tight structures to maintain gas concentrations during the exposure time necessary to achieve the effective control of pests. One problem with the application of this technique when using high-CO_2 MAs with durable food commodities (legumes, cereals, dried fruits, etc.) in flexible packages is the negative pressure caused by CO_2 sorption in the commodity, which gradually decreases the volume of the package. This causes a vacuum effect in the package, which adopts a random shape, and makes it impossible to reshape it without opening the package. Also, the increase of the negative pressure inside the package by sorption eliminates the gas available in the free space of the package, produces a progressive decrease in the gas concentration and, possibly, affects pest control. CO_2 sorption depends on different factors, such as temperature, atmospheric pressure, moisture content, CO_2 initial concentration and the type of commodity (Brunaeur, 1943; Mitsuda et al., 1973; Cofie-Agblor et al., 1995; 1998; Navarro, 1997; Jian et al., 2014). The pattern of CO_2 absorption with different contents of a product within the package (filling ratios) was previously investigated for meat products (Zhao et al., 1995; Jakobsen and Bertelsen, 2004; Rotabakk et al., 2007), but few studies have been conducted on grains (Banks and Annis, 1990; Navarro, 1997).

The present study aimed to measure the sorption of CO_2 by chickpeas and the negative pressure caused by this sorption when they are packaged with different CO_2 MAs and at different filling ratios. Our hypothesis was that sorption by chickpeas and negative pressure inside the package will increase with increasing filling ratios and CO_2 concentrations.

Materials and Methods

Chickpeas (cv. Blanco Lechoso) were purchased from Burcol (Guadalajara, Spain) and were all from the same batch. The water activity of the chickpea was 0.600 (Aqualab pre, Labferrer, Cervera, Spain), and the physical properties provided by the supplier were 6.8 % of fat, 57 % of carbohydrates and 23 % of protein.

To assess the sorption of CO_2 by chickpeas, three MAs with different initial CO_2 concentrations were tested: MA1: 90% CO_2, 3% O_2 and 7% N_2; MA2: 70% CO_2, 6% O_2 and 24% N_2; MA3: 50% CO_2, 10% O_2 and 40% N_2. They were previously prepared before starting the experiments using a gas mixer (Witt Km 100-3M/MEM, Witt Gasetechnick, Witten, Germany). The experiments were conducted at room temperature (20 ± 3°C). They consisted of filling a semi-rigid plastic container (710 mL capacity, 500 µm thickness, polyethylene terephthalate [PET]) with 125 g, 250 g or 500 g of chickpeas (bulk density of 0.74 g/cm^3), which occupied filling ratios of 24%, 48% and 96%, respectively. Afterwards, the lid was sealed with hot glue, the desired MA was introduced with a needle in the top of the container and then the gas inlet and outlet holes were sealed with hot glue. The gas concentrations inside the containers were measured with a gas analyser (OXYBABY®, Witt Gasetechnick, Witten, Germany) to verify the CO_2 and O_2 content inside the plastic containers. It was measured at the beginning, without any delay after sealing the container, and at the end of the different periods of exposure tested: 24 h, 48 h, 240 h or 384 h. An aliquot of 6 ml of the headspace gas was collected with a gas analyser using a foam rubber seal (Witt Gasetechnick, Witten, Germany) to avoid the introduction of the exterior atmosphere. A control treatment without chickpeas was also included for each MA concentration, and the exposure time was tested. Ten replicates were done for each combination of initial gas concentration, filling ratio and exposure time.

The gas volume available after introducing the chickpeas was determined by the volume of water displaced when dropping 125 g, 250 g or 500 g of chickpeas into 710 mL water (1.04 g/cm^3 of density) and was calculated as follows:

$$V_{gas} = V_{total} - V_{chickpeas} , \quad (1)$$

where

V_{gas} = gas volume available in the container (mL);

V_{total} = total volume of the container (mL);

$V_{chickpeas}$ = volume of water displaced after chickpeas were dropped into the water.

Data Analysis

Assuming the amount of oxygen, nitrogen and water vapour in each container remained constant in each replicate, the volume of CO_2 sorbed at different times was calculated as follows:

$$V_s = (L_{CO2} V_{gas}) / 100, \quad (2)$$

where

V_s = volume of CO_2 sorbed by the chickpeas (mL);

L_{CO2} = loss of CO_2 concentration (%) (Initial concentration – final concentration).

Mass (g) of CO_2 sorbed by the chickpeas at different gas volumes, exposure times and initial CO_2 concentrations were calculated using the equation from Jian et al. (2014):

$$S = (\rho_{CO2} V_s) / M_{chickpeas} , \quad (3)$$

where

S = sorption of CO_2 (g) per tested mass of chickpea (kg);

ρ_{CO2} = CO_2 density of 0.00182952176 g/mL, according to the equation of the density of gases (Chang & College, 2002);

$M_{chickpeas}$ = tested chickpeas mass (kg).

Accumulative CO_2 sorption was fitted to the sorption duration, as proposed by Brunauer (1943):

$$S_C = S_E (1- exp (-B\Theta^C)), \quad (4)$$

where

S_C = accumulative sorption of CO_2 (g) per mass chickpea (kg);

S_E = equilibrium sorption of CO_2 (g) per mass chickpea (kg);

Θ = sorption duration (h);

B and C = constant.

Equilibrium sorption time (Θ_E), which is the time needed to reach 97% of equilibrium sorption (S_E), was calculated following Jian et al.'s (2014) equation:

$$\Theta_E = - \ln 0.03/B. \tag{5}$$

To evaluate the negative pressure, we first obtained the values of the total mass of CO_2 sorbed in the experiment, calculated as follows:

$$m = S\, M_{chickpeas}, \tag{6}$$

where

m = total mass of CO_2 sorbed in the container (g).

Then, Eq. (5), proposed by Cofie-Agblor et al. (1995), was used to calculate the negative pressure created by the CO_2 sorption:

$$P_f = ((m\, R\, T) / (V_{gas}\, M_{CO2})) – Pi, \tag{7}$$

where

P_f = final pressure (kPa);

R = universal gas constant (8.314472 L kPa/ K mol);

T = temperature (°K);

V_{gas} = gas volume available in the container (L);

M_{CO2} = molar mass of the CO_2 (g/mol);

P_i = initial pressure (Kpa).

Paired t-tests were conducted to compare the percentage of CO_2 sorbed by chickpeas (all pair comparison combinations) for each filling ratio (24, 48 and 96%) at 384 h of exposure. Two-way analyses of variance (ANOVA) followed by a Tukey's multiple range test were used to compare the CO_2 sorption and negative pressure among the different initial CO_2 concentrations and filling ratios for each exposure time. Statistical analyses were done with JMP® 13.1.0 (SAS Institute Inc. 2016). Accumulative sorption was fitted with SigmaPlot curve fitting (SigmaPlot Scientific Graph System, Janel Scientific, 2010).

Results

Analysis of gases

The percentage of CO_2 available in the control treatment showed a maximum reduction of 2% at the end of the test, indicating that the containers were highly gas-tight. The percentages of CO_2 in the containers substantially decreased for all the initial concentrations of CO_2 with all filling ratios, with a greater decrease in the 96% filling ratio (Fig. 1 A-C). As a general pattern, the levels of CO_2 in the sealed plastic containers declined sharply during the first 24 h and continued declining more smoothly until 240 h of exposure, at which time the CO_2 content stopped decreasing and remained at the same level until the last tested exposure period (384 h). The CO_2 treatments were significantly different at the end of the exposure time when comparing the percentage of CO_2 loss in the headspace of containers filled with the same chickpea filling ratio (Table 1), except for the comparison between 70% and 90% with a filling ratio of 24% and the treatments between 50% and 70% with filling ratios of 24% and 48%.

CO_2 sorption by chickpeas

Volume of CO_2 sorbed (V_s)

The initial gas volume (V_{gas}) available in the 710 mL container with 125 g, 250 g and 500 g of chickpeas (filling ratios of 24%, 48% and 96%, respectively) was 608 ± 0.70 mL, 505 ± 0.98 mL and 300 ± 0.75 mL, respectively. Therefore, true density of chickpeas used was 1.22 g/mL. The volume of CO_2 sorbed after the different exposure times varied with the CO_2 available in the headspace of the containers with the different filling ratios and initial CO_2 concentrations tested (Table 2).

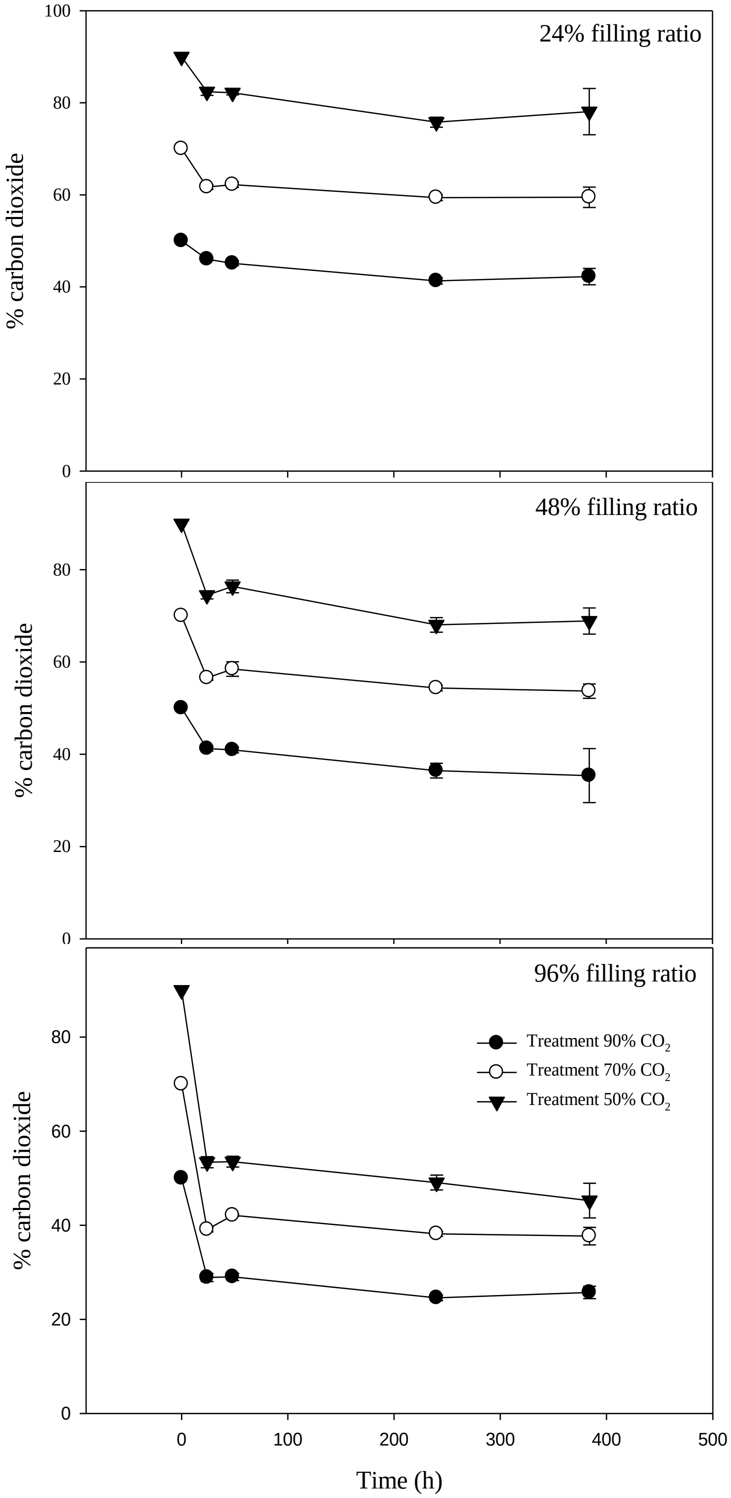

Fig. 1. Changes in carbon dioxide content (means ± standard error) within the sealed plastic containers during exposure to three different modified atmospheres (MA1: 90% CO_2; MA2: 70% CO_2; MA3: 50% CO_2) with different filling ratios of chickpeas at 24 h, 48 h, 240 h and 384 h of exposure time.

Table 1. Paired t-test comparing percentage of CO_2 loss in the headspace of containers filled with different initial CO_2 concentrations after 384 h exposure and for each chickpea filling ratio tested

Filling ratio (%)	Between 50% and 70% initial CO_2		Between 50% and 90% initial CO_2		Between 70% and 90% initial CO_2	
	t	*P*	t	*P*	t	*P*
24	17.2	0.051	2.5	< 0.05	1.4	0.195
48	1.3	0.213	4.3	< 0.001	6.1	< 0.001
96	11.3	< 0.001	17.2	< 0.001	10.1	< 0.001

Degrees of freedom (d.f.) = 1.19

Table 2. Mean volume (± standard deviation) of CO_2 available for 125 g, 250 g and 500 g of chickpeas (filling ratios of 24%, 48% and 96%, respectively) when at a 50, 70 and 90% initial CO_2 concentrations and at different exposure times (h)

Initial CO_2 concentration (%)	Filling ratio (%)	V_s (mL)				
		0 h	24 h	48 h	240 h	384 h
50	24	608	581(9.1)	576 (11.7)	555 (6.3)	558 (10.7)
	48	505	455 (5.6)	465 (9.1)	432 (16.9)	435 (31.1)
	96	300	233 (8.9)	242 (7.3)	225 (4.4)	227 (4.2)
70	24	608	563 (10.7)	569 (10.8)	538 (15.5)	546 (13.7)
	48	505	442 (7.6)	450 (25.5)	417 (11.4)	422 (8.1)
	96	300	210 (5.0)	218 (2.0)	198 (10.0)	203 (5.5)
90	24	608	501 (17.8)	552 (12.9)	524 (16.9)	532 (27.7)
	48	505	421 (10.8)	433 (21.3)	395 (24.8)	391 (14.4)
	96	300	193 (8.3)	187 (11.2)	176 (15.4)	164 (10.6)

Mass of CO_2 sorbed (S)

Chickpeas sorbed a large quantity of CO_2 that varied according to the initial CO_2 concentration, filling ratio and exposure time. The highest CO_2 sorption (above 50% of the total CO_2 sorption) occurred in the first 24 h of exposure for all the initial CO_2 concentrations and for the different filling ratios tested (Table 3, Fig. 2). The predicted curves of accumulative CO_2 sorption (S_C) from Eq. (4) continued to smoothly increase over time (Fig. 2).

Table 3. Factorial tests among CO_2 sorption (*S*) of chickpeas at different chickpea filling ratios and initial CO_2 concentrations

	Statistical parameter	Time of exposure to CO_2 (h)			
		24	48	240	384
Initial CO_2 concentration (%)	*F*	40.6	33.3	38.9	30.7
	P	< 0.001	< 0.001	< 0.001	< 0.001
	df	2	2	2	2
Filling ratios (%)	*F*	47.7	42.9	137.6	76.0
	P	< 0.001	< 0.001	< 0.001	< 0.001
	df	2	2	2	2
Initial CO_2 concentration × filling ratios	*F*	3.8	1.4	3.4	1.2
	P	< 0.01	0.25	< 0.05	0.33
	df	4	4	4	4

The degrees of freedom (d.f.) of the replicates for the factors and their interaction were 89.

When comparing CO_2 sorption (*S*) by chickpeas in containers with 24% and 48% filling ratios, significant differences were observed between the initial CO_2 concentrations of 90% and 50% for the all exposure times analysed, with intermediate values for 70% (Fig 2). For the 96% filling ratio, CO_2 sorption was higher in treatments with initial concentrations of 90% and 70% CO_2 compared with 50% during the first 48 h of exposure. Afterwards, no significant differences among all the initial CO_2 concentrations (Fig. 2) were noted.

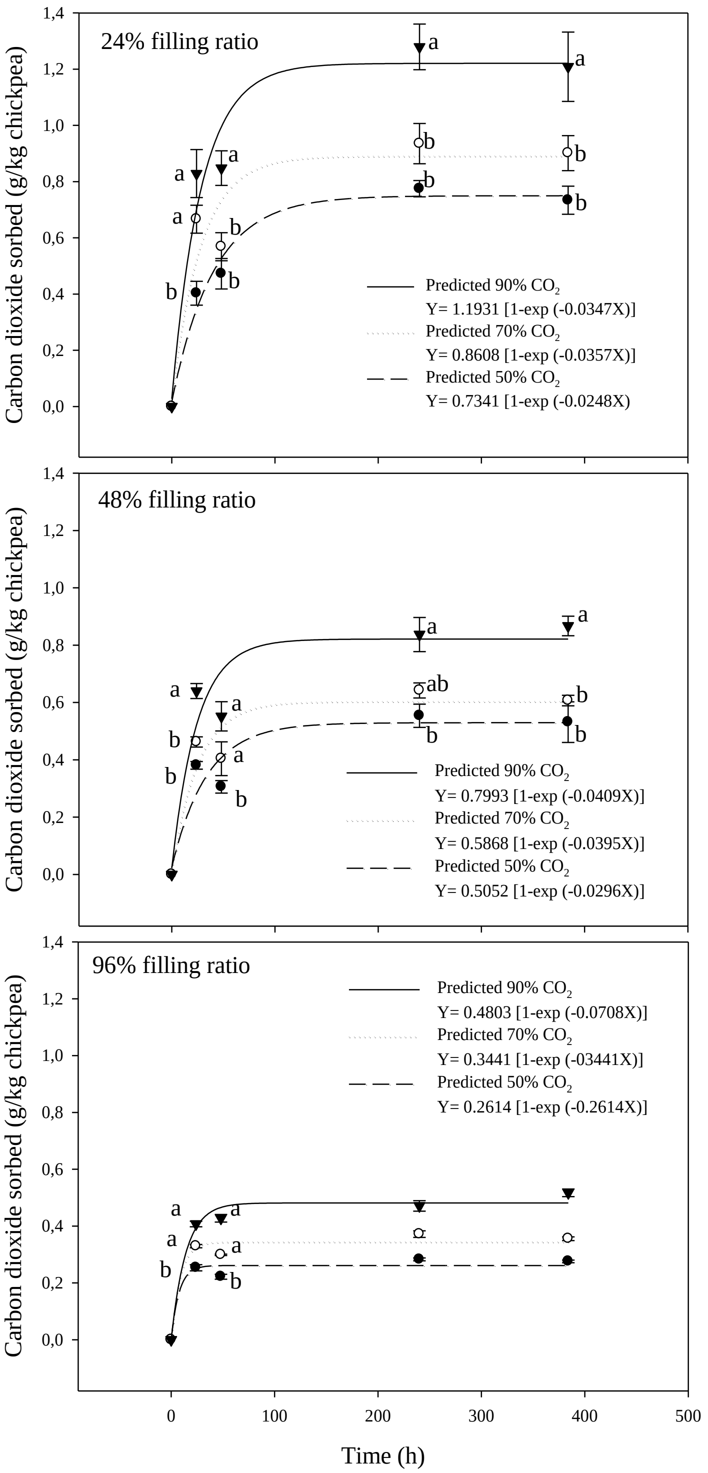

Fig. 2. Carbon dioxide sorption (S) (means ± standard error) at different chickpea filling ratios in 90%, 70% and 50% initial CO_2 concentrations with predicted curves of the amount sorbed (Sc) from Eq. (2). When significant differences were found, means followed by different letters between the initial CO_2 concentrations for each exposure time are shown ($P > 0.05$, Tukey range test).

Equilibrium sorption of CO_2 (S_E)

Sorption gradually stabilised until it reached the equilibrium. For the filling ratios of 24% and 48%, the exposure time to reach equilibrium (Θ_E) tended to decrease as the initial CO_2 concentration increased. However, for the 96% filling ratio, increasing the initial CO_2 concentration increased the exposure time needed to reach the sorption equilibrium (Table 4).

Table 4. Equilibrium sorption (S_E) (± standard deviation) and time (Θ_E) under different chickpea filling ratios and initial CO_2 concentrations

Filling ratio (%)	Nominal initial CO_2 (%)	R^2	S_E (g/kg)	Θ_E (h)
24	50	0.98	0.71 (0.061)	141.4
	70	0.91	0.83 (0.159)	98.2
	90	0.96	1.16 (0.145)	101.1
48	50	0.89	0.49 (0.104)	118.5
	70	0.92	0.57 (0.104)	88.8
	90	0.92	0.78 (0.144)	85.7
96	50	0.96	0.25 (0.033)	26.9
	70	0.97	0.33 (0.038)	29.6
	90	0.98	0.47 (0.039)	49.5

The R^2 value for the regression Eq. (4).

Negative pressure due to CO_2 sorption (P_f)

Negative pressure depended on the filling ratio and on the initial CO_2 concentration; however, a significant interaction was apparent between both factors at all the exposure times tested (Table 5). Negative pressure due to CO_2 sorption (*S*) was greater in treatments with a 96% chickpea filling ratio than with 24% at all the initial CO_2 concentrations (50%, 70% and 90%). Similarly, negative pressure was greater in the treatments with 90% than with the 50% CO_2 concentrations at the same filling ratio (Fig. 3).

Table 5. Factorial test among negative pressure of chickpeas at different chickpea filling ratios and initial CO_2 concentrations

	Statistical parameter	Time of exposure to CO_2 (h)			
		24	48	240	384
Initial CO_2 concentration (%)	*F*	110.7	82.3	70.0	106.1
	P	< 0.001	< 0.001	< 0.001	< 0.001
	df	2	2	2	2
Filling ratios (%)	*F*	861.5	436.8	370.6	415.7
	P	< 0.001	< 0.001	< 0.001	< 0.001
	df	2	2	2	2
initial CO_2 concentration × filling ratios	*F*	13.5	17.1	8.4	16.6
	P	< 0.001	< 0.001	< 0.001	< 0.001
	df	4	4	4	4

The degrees freedom (d.f.) of the replicates for the factors and their interaction were 89.

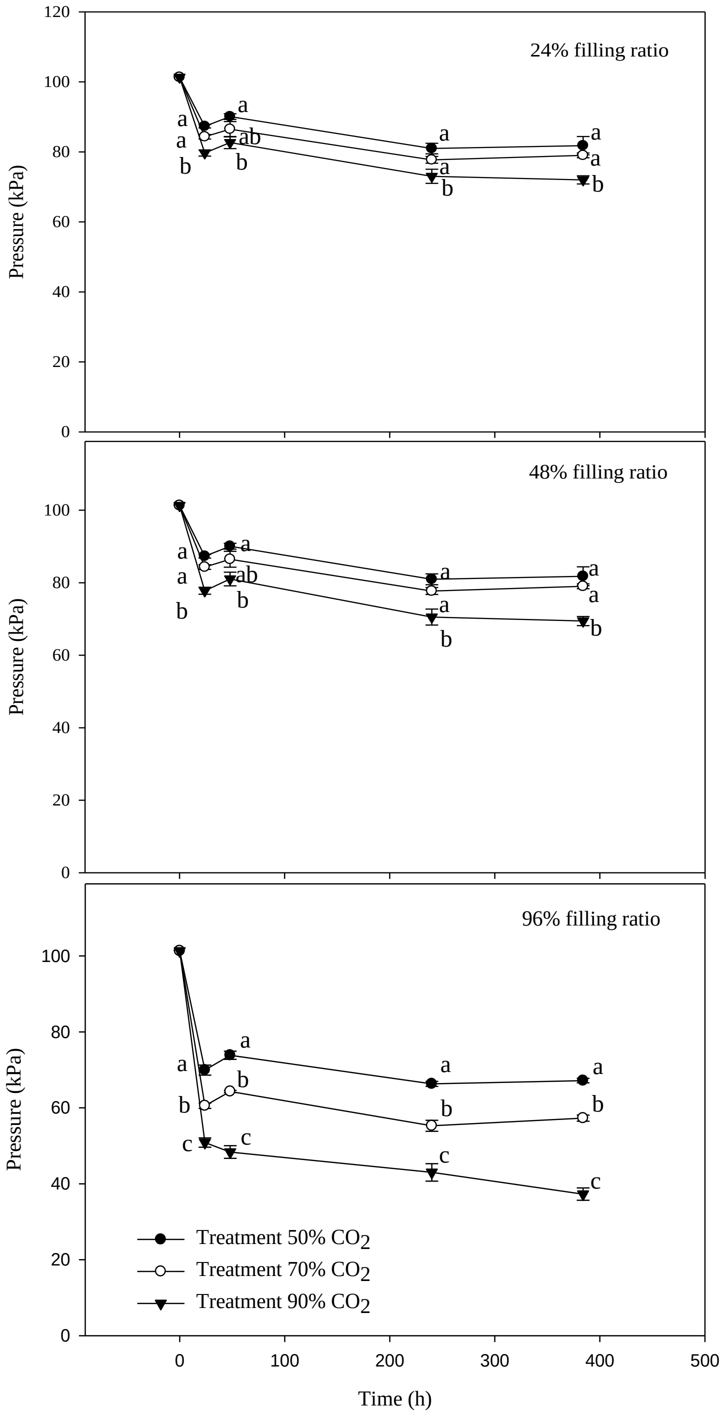

Fig. 3. Negative pressure (means ± standard error) caused by CO_2 sorption at different chickpea filling ratios and different initial CO_2 concentrations. When significant differences were found, means followed by different letters between the initial CO_2 concentrations for each exposure time are shown ($P > 0.05$, Tukey range test).

Discussion

Sorption is assumed to be caused by the diffusion of CO_2 into the kernel pores and by the formation of carbamate when reacting with the functional groups of proteins in the kernel. A phenomenon occurs called van der Waals adsorption, in which the CO_2 is quickly absorbed by the carbon atoms on the surface of the grain, forming a layer of CO_2 molecules. This layer attracts more CO_2 molecules, resulting in the accumulation of several layers together at the surface of the grain. Carbamate formation is a weak and reversible interaction (Brunauer, 1943; Yamamoto and Mitsuda, 1980). This interaction is a chemisorption process similar to a reaction between a free radical and a gas molecule, which does not always require activation energies (Hartel and Polanyi, 1930; Eyring, 1931). CO_2 also binds with other molecules of the grain, such as carbohydrates, fatty acids and amino acids, and has a uniform distribution in the kernel (Mitsuda et al., 1973).

The most important decrease in the CO_2 content in the packed chickpeas occurred during the first 24 hours of exposure for all the filling ratios and the initial CO_2 concentrations tested. Afterwards, sorption slowly increased until it reached stability (Fig. 1). Using data from other studies with different commodities and gases (hydrogen, chloropicrin and nitrogen), Brunauer (1943) concluded that gas molecules are sorbed as rapidly as they can reach the surface by van der Waals adsorption followed by a chemisorption reaction.

In this study, CO_2 sorption and negative pressure were influenced by the filling ratios and the initial CO_2 concentrations. In general, the highest CO_2 sorption and negative pressure were obtained at the 90% initial CO_2 concentration. However, the filling ratios differentially affected CO_2 sorption more than negative pressure: the highest CO_2 sorption was obtained at a 24% filling ratio (Fig. 2), and the highest negative pressure occurred at a 96% filling ratio (Fig. 3).

The highest filling ratio was expected to have the highest sorption of CO_2 due to the highest mass of chickpeas. However, our results showed that CO_2 sorption by chickpeas was lower at the 96% filling ratio than at 48%, and both were lower than that at the 24% filling ratio (Fig. 2). This lower sorption observed with the 96% filling ratio was due to the lower amount of gas volume available in the container. At a filling ratio of 48%, the CO_2 volume available was greater, but not enough for the chickpeas to sorb all they could. At a filling ratio of 24%, more gas was available, and the mass of chickpeas was able to sorb more CO_2 than at the other two filling ratios. The sorption of CO_2 was not only affected by the filling ratio of the container but also by the initial CO_2 concentration; a higher sorption of gas with 90% of CO_2 was observed than with the 50% and 70% levels after 384 h of exposure (Fig. 2). This was true for the 24% and 48% filling ratios, while for the 96% filling ratio, no differences could be observed among the initial CO_2 concentrations since the quantity of CO_2 remaining was low. A slightly lower sorption (around 0.33 g of CO_2 at 20°C) was obtained when using wheat at an initial CO_2 concentration of 99.8% in the containers filled up to 93% of their capacity (Navarro, 1997).

CO_2 sorption also depends on temperature, moisture content and type of grain packed. In one study, the CO_2 sorption in canola varied according to temperature from 3 g of CO_2/kg at 10°C to 1.2 g at 30°C and according to moisture content from 2 g of CO_2/kg at 8% to 1.6 g at 14%, with an initial CO_2 concentration of 100% (Jian et al., 2014). Various commodities also absorb CO_2 differently, and oiled seeds absorb more CO_2 than cereals and legumes (Mitsuda et al., 1973). A higher CO_2 sorption was found in 250 g of canola (0.63 g of CO_2/kg of canola) than in cereals (0.38 g of CO_2/kg of wheat and 0.45 g of CO_2/kg of hull-less oats) at a 69% CO_2 initial concentration, 14% moisture content and 20°C (Cofie-Agblor et al., 1998). These different CO_2 sorption amounts, compared with the 0.61 g of CO_2/kg of chickpeas that we obtained at an initial concentration of 70% CO_2 and a filling ratio of 48% (250 g of chickpeas), indicated that chickpeas have a similar CO_2 sorption to canola, and both have a greater CO_2 sorption than cereals.

Equilibrium sorption occurs when the amounts of CO_2 sorbed remain constant over time at a given temperature and pressure. This is specific for the interaction of chickpeas with CO_2 due to the physical structure of the chickpea (the extent of the surface, size, shape and the distribution of pores), its chemical constitution and the physical and chemical properties of the CO_2 (Brunauer, 1943). In our study, the equilibrium sorption was reached at 101 h to 49.5 h at the different filling ratios for a 90% initial CO_2 concentration, which is in agreement with results for wheat, with the equilibrium sorption occurring at 95 h for a filling ratio of 93% (Navarro, 1997). In contrast, the equilibrium time of the sorption occurs quickly at 2 h in oilseeds, such as canola, with 100% CO_2 initial concentration due to the influence of the oil content in the rate and the amount of CO_2 diffusion into the seed (Jian et al., 2014).

Sorption equilibrium time tends to increase with the increasing initial CO_2 concentration at the filling ratio of 96% (Table 4). This is due to the high negative pressures generated by the small amount of CO_2 available in the headspace (Fig. 3). This negative pressure in the package generated a decrease in the multimolecular sorption of CO_2 in the surface of the chickpeas (Mitsuda et al., 1973). Mitsuda et al. (1973) found a negative correlation between the negative pressure and the volume of CO_2 sorbed in brown rice. The same pattern was found in our study; the highest negative pressure (37.3 kPa) was obtained with a filling ratio of 96% of chickpeas at a 90% initial CO_2 concentration. Lower negative pressures were obtained when using wheat: 73 kPa at a filling ratio of 93% at a 100% initial CO_2 concentration (Navarro, 1997). The filling ratio had more influence on the negative pressure than the initial CO_2 concentration (Fig. 3).

In conclusion, and in agreement with our hypothesis, the negative pressure increased with the increasing chickpeas filling ratios and the initial concentrations of CO_2, and the CO_2 sorption increased with the increasing initial concentrations of CO_2. However, the CO_2 sorption by chickpeas did not increase with the increasing filling ratio, which is in disagreement with our hypothesis. This is caused by the lower amount of gas available in the headspace as the filling ratio increases.

This is the first study to evaluate CO_2 sorption by chickpeas when packaged at different filling ratios and to identify the negative pressure produced. Further studies are necessary to test whether the amount of CO_2 that is available in packages after gas sorption occurs at the different filling ratios is required to produce the desired insect mortality. Also, future researchers could determine which conditions can be improved to prevent or reduce the vacuum inside the packages due to CO_2 sorption by the different commodities.

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CAPÍTULO 2

Susceptibility of *Rhyzopertha dominica* to High CO_2 Modified Atmospheres in Packaged Chickpeas

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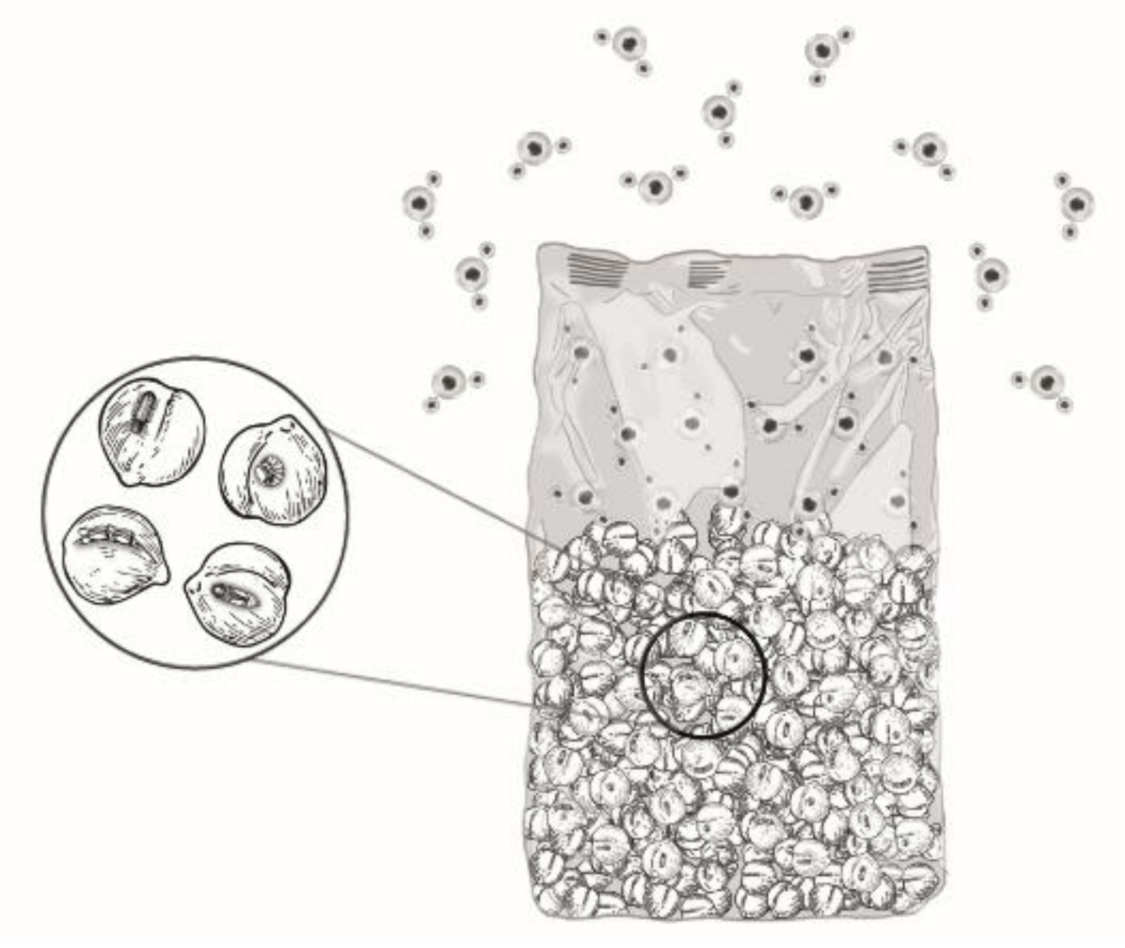

Introduction

Rhyzopertha dominica (F.) (Col. Bostrichidae) is a primary pest of stored grain that is widely distributed globally, especially in warmer regions between latitude 40º N and latitude 40º S of the equator. Females deposit eggs on grain kernels, and the newly hatched larvae bore into the kernels and develop within until they reach the adult stage. The mature adult bores out of the kernel by a large exit hole; the kernel is then referred to as an "insect-damaged kernel" (IDK) (Potter, 1935; Arbogast, 1991; Rees, 1995). *Rhyzopertha dominica* attacks a wide range of grains, such as rice, wheat, corn, and others containing starch (Chujo,1958; Rees,1995), as well as to chickpeas (Wong-Corral et al., 1987; Batta, 2005). The most common method of controlling *R. dominica* is through the use of organosphosphate, pyrethroid and juvenile hormone analogue pesticides, and fumigants such as phosphine. However, *R. dominica* has been shown to have developed resistance to both methodologies (Bengston et al., 1975; Zettler and Cuperus, 1990; Collins, 1998; Opit et al., 2012; Jagadeesan and Nayak, 2016; Daglish and Nayak, 2018). The problem of resistance to insecticides and the consumer concern over the use of pesticides in food has resulted in the search for alternative control methods.

An alternative control measure is the treatment of grains with modified atmospheres (MAs), which has the advantage of avoiding pesticide residues in food commodities (Navarro, 2012). MAs with high carbon dioxide (CO_2) and low oxygen (O_2) contents can be applied to stored raw materials, to semi-processed commodities, and/or to final food products (Fleurat-Lessard, 1990; Adler et al., 2000; Navarro, 2006; Riudavets et al., 2007). Previous studies with high CO_2 MAs showed that larvae and adults of weevil pests are susceptible stages, while eggs and pupae are more tolerant (Riudavets et al., 2009; Wong-Corral et al., 2013). These studies were done in flexible packages with a low proportion of the commodity in relation to the total volume of the package (low filling ratio), and the loss of CO_2 content in the package was negligible. However, in practical situations (e.g., packages of grains and pulses), the proportion of the total volume of the package occupied by the commodity is much higher (high filling ratio), causing gas sorption that decreases the concentration of CO_2 in the headspace. This sorption produces a vacuum effect inside the package, and most of the sorption occurs during the first hours of exposure to CO_2 (Cofie-Agblor et al., 1995, 1998; Navarro, 1997). The higher the volume occupied by the commodity (filling ratio), the greater the vacuum and the loss of CO_2 in the headspace of the package (Iturralde-García et al., 2019). This loss of CO_2 in packages headspace after gas sorption by the commodity might affect pest control efficacy, and this effect remains to be tested.

The hypothesis of the present study was that the control efficacy of the CO_2 MAs on the developmental stages of *R. dominica* will decrease as the volume of the commodity increases inside the package due to sorption and loss of CO_2 in the headspace. To test this hypothesis, we evaluated the mortality of *R. dominica* eggs, larvae, pupae, and adults in chickpeas packaged with different CO_2 concentrations and with different filling ratios.

Materials and methods

Chickpeas (cv. Blanco Lechoso) were purchased free of pesticides and insect infestation. The pulse had a water activity of 0.650 measured with Aqualab pre (Labferrer, Cervera, Spain), and consisted of 57% carbohydrates, 6.8% fat, and 23% protein (properties provided by the supplier).

A stock colony of *R. dominica* was reared on chickpeas and maintained at 28 ± 2º C, 70 ± 10% r.h., and a photoperiod of 16:8 h of Light: Dark. Experiments were performed in the same environmental conditions. To daily collect fresh eggs, fifty 3-day-old adults were offered 200 g of chickpeas in ventilated cages during 24 h. Some of these newly deposited eggs were confined with 50 g of chickpeas and kept for 9-15 days to obtain mature larvae or kept 34-39 days to obtain pupae. The presence of penetration holes on the surface of the pulse was an indication that they held an immature stage of *R. dominica*.

Mortality of *R. dominica* when chickpeas were packaged at a 4% filling ratio

Mortality of *R. dominica* was assessed by exposing each developmental stage inside a cage (7 cm diameter x 4 cm high) with 4% filling ratio to 3 MA at different exposure intervals. For treating eggs, 1-3-day-old eggs of *R. dominica* were placed inside a gelatin capsule (three loose eggs per capsule), and 5 of these capsules were deposited in a small ventilated cage. For treating larvae or pupae, 15 chickpeas infested with a minimum of one individual of *R. dominica* were deposited in each cage. For treating adults, 15 three-day-old individuals were placed in each cage. Each cage was placed inside a 300 x 210mm (59 µm-thick) plastic bag (Cryovac BB4L, Sealed Air, Elmwood Park, NJ, USA). The plastic bags had permeabilities to O_2 and CO_2 of 30 and 150 cm^3 m^{-2} day^{-1} bar^{-1} respectively, measured under conditions standardized at 23º C and 0% r.h. Bags were filled with three CO_2 concentrations prepared using a gas mixer (Witt KM 100-3M/MEM, Witt Gasetechnick, Witten, Germany), and filled with a vacuum packaging machine (EVT-7-TD, Tecnotrip, Terrassa, Spain). The MAs tested were: i) 50% CO_2, with a residual of 10% O_2 and a 40% balance of N_2; ii) 70% CO_2, with a residual of 6% O_2 and a 24% balance of N_2; and iii) 90% CO_2, with a residual of 3% O_2 and a 7% balance of N_2. An aliquot of 6 mL of the headspace gas was collected with a gas analyzer (OXYBABY®, Witt Gasetechnick, Witten, Germany) to verify the CO_2 and O_2 contents inside the plastic bags at the beginning and at the end of the different periods of exposure tested. A foam rubber seal (Witt Gasetechnick, Witten, Germany) was used when punching the package to avoid introducing any outside atmosphere. Five different exposure times were tested for eggs (6, 24, 48, 72 and 96 h), larvae (6, 10, 18, 24 and 48 h), pupae (24, 72, 120, 168, 240 h) and adults (24, 30, 48, 72 and 96 h). Three replicates were done for each CO_2 concentration and exposure time. Sets of cages with the same number of individuals as in the tests were maintained without MAs for the same exposure intervals in order to determine control mortality. Adult mortality was assessed 24 h after opening the bags.

Cages with eggs were kept up to 10 days to allow their hatching, while cages with larvae and pupae were kept until the emergence of adults.

Mortality of *R. dominica* when chickpeas were packaged at a 96% filling ratio

Mortality of developmental stages was assessed at time intervals of the LT_{50} that was previously calculated with the three modified atmospheres for a filling ratio of 4%. Each set of individuals was deposited in a semi-rigid plastic container (710 mL capacity, 500 µm thickness, polyethylene terephthalate [PET]) filled with 500 g of chickpeas (bulk density of 0.74 g/cm^3), which occupied a filling ratio of 96%. The lid was sealed with hot glue and the MAs tested were flushed through an inlet hole at the top of the container removing the air from inside package. Gas concentrations were measured at the start and at the end of the exposure time. Test performed with the same CO_2 concentrations and developmental stages using the same intervals for mortality measurement as previously describe at 4% filling ratio. The whole procedures were repeated ten times (10 replications).

Data analysis

Percentage of mortality was calculated using the Abbott's formula (1925). Data were analysed with Probit analysis (Poloplus, LeOra Software) and estimated exposure times to achieve 50% and 90% mortality were compared using the fiducial limits as the criterion to determine significant differences: when fiducial limits did not overlap between modified atmospheres for each developmental stage, the comparison was significant (Robertson et al., 2017). A chi-square (X^2) test was used to compare mortality of each developmental stage obtained when in chickpeas packed at high filling ratio with that of chickpeas packed at a low filling ratio at time intervals of the estimated LT_{50}. The available gas volume in the semi-rigid package, the volume of CO_2 sorbed by chickpeas, and the negative pressure (vacuum) produced at the filling ratio of 96% were calculated according to the methodology described in Iturralde-García et al. (2019):

-CO_2 sorption using the equation from Jian et al. (2014):

$$S = (\rho_{CO2}\ V_S) / M_{chickpea} \qquad (1)$$

where.

S = sorption of CO_2 (g) per mass of chickpea (kg);

ρ_{CO2} = CO_2 density of 0.00182952176 g/mL, according to the equation of the density of gases (Chang and College, 2002);

V_S = volume of CO_2 sorbed by the chickpeas (mL);

$M_{chickpeas}$ = chickpeas mass (kg).

$$P_f = ((m\ R\ T) / (V_{gas}\ M_{CO2})) - P_i \qquad (2)$$

where.

Pf = final pressure (kPa);

R = universal gas constant (8.314472 L kPa/K mol);

T = temperature (K);

V_{gas} = gas volume available in the container (L);

M_{CO2} = molar mass of the CO_2 (g/mol);

P_i = initial pressure (Kpa).

m = total mass of CO_2 sorbed in the container (g).

-Total mass of CO_2 sorberd (m) was previously calculated as follows:

$$m = S\ M_{chickpeas} \qquad (3)$$

Results

Mortality of *R. dominica* when chickpeas were packaged at a 4% filling ratio

Percentage of CO_2 content within the sealed plastic bags during the exposure to three MAs are shown in Fig. 1. Carbon dioxide declined slowly during exposure time: after 240 h of dosing, the CO_2 content in the bags filled with 50%, 70%, and 90% CO_2 were 46.6 ± 0.2%, 62.4 ± 1.1%, and 84.5 ± 0.9%, respectively.

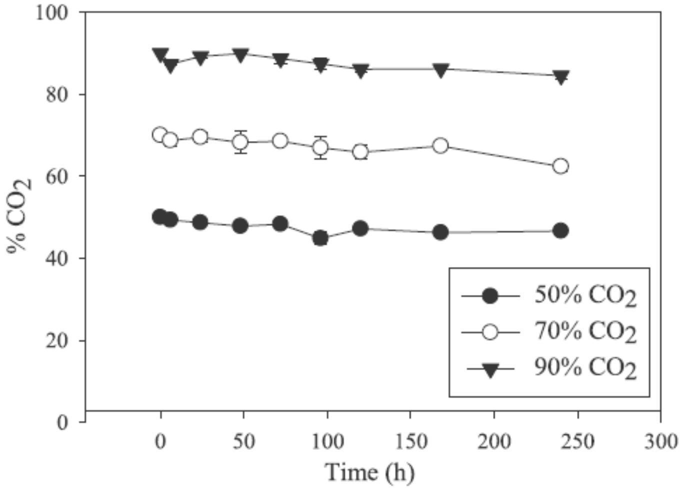

Fig. 1. Percentage of CO_2 in the plastic bags with a 4% filling ratio of chickpeas for the three CO_2 concentrations and at the different exposure times tested.

Mortality of *R. dominica* developmental stages produced by the three CO_2 MAs are shown in Fig. 2. Larvae was the most sensitive developmental stage, reaching total mortality after 48 h of exposure to all CO_2 concentrations tested. In contrast, pupae was the most tolerant, needing 240 h of exposure to 70 and 90% CO_2 concentrations for reaching a 100% mortality; a 96.4% mortality was obtained at 50% CO_2 with the same exposure time. Eggs and adults had an intermediate sensitivity needing 96 h of exposure to all CO_2 concentrations tested to obtain complete mortality.

Parameters of the Probit analysis for eggs, larvae, pupae, and adults with three CO_2 concentrations tested are shown in Table 1. Mortality in the control treatments were 0% (adults), 11.1 ± 3.8% (eggs), 16.4 ± 6.6% (larvae) and 16.0 ± 3.7% (pupae). Mortality of larvae and adults had a positive dose-response and fiducial limits of the two extreme CO_2 concentration tested (50% and 90%) did not overlap, while intermediate values were obtained with 70% CO_2, both at LT_{50} and LT_{90}. However, mortality of eggs and pupae was not significantly different among the three CO_2 concentrations tested, both at LT_{50} and LT_{90}.

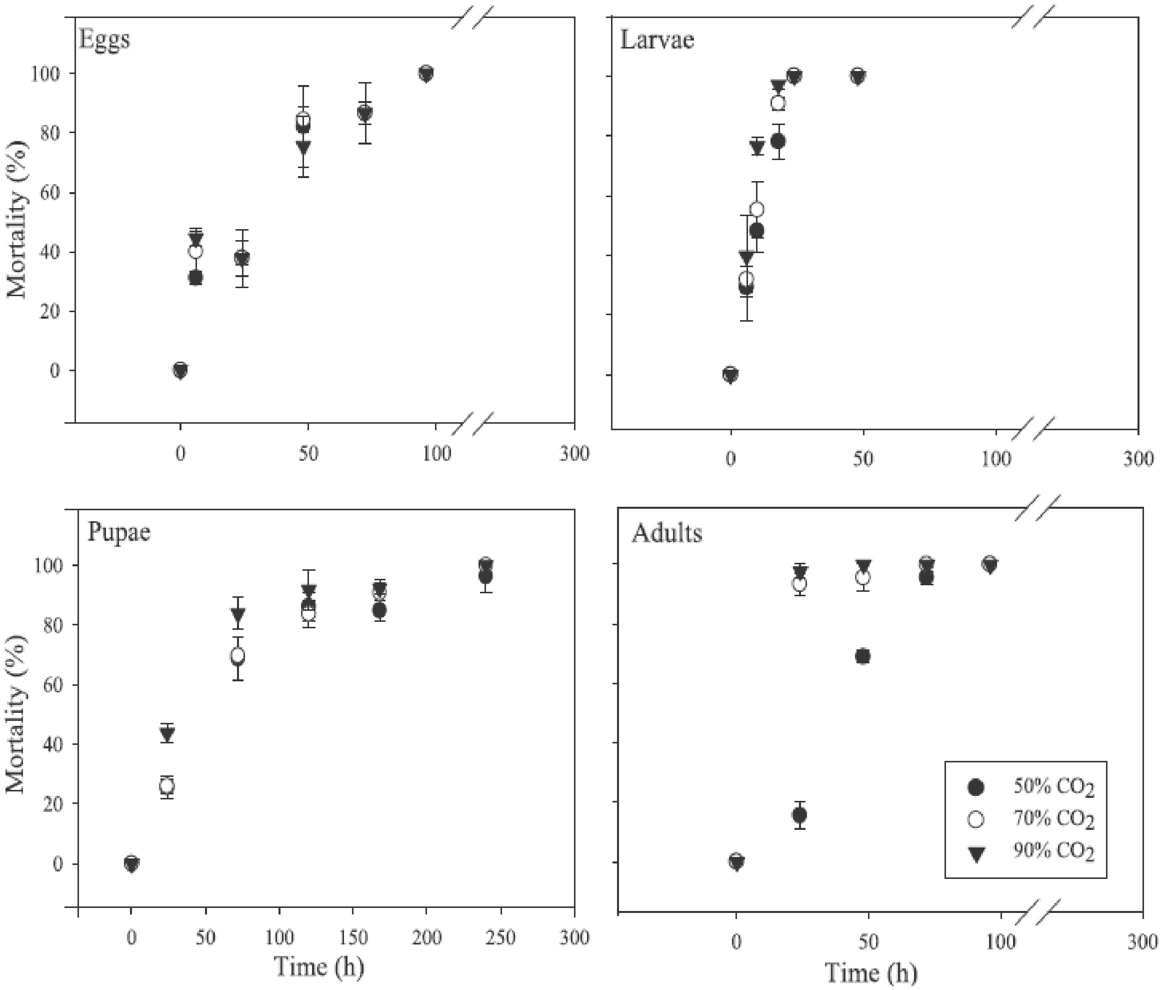

Fig. 2. Percentage mortality of the developmental stages of *R. dominica* exposed to 50%, 70%, and 90% CO_2 concentrations during different exposure intervals

Table 1. Parameters of the Probit analysis with modified atmospheres of 50%, 70%, and 90% CO_2 for the eggs, larvae, pupae, and adults of *R. dominica*.

Stage	CO_2 (%)	Slope (SE)	LT_{50} (h)[a]	95% fiducial limits	LT_{90} (h)[a]	95% fiducial limits	X^{2b}
Eggs	50	1.96 (0.22)	16.8 a	9.0-25.2	76.0 a	49.2-162.7	43.9
	70	1.72 (0.21)	14.6 a	7.8-21.6	81.1 a	53.1-166.5	31.76
	90	1.55 (0.20)	14.3 a	5.8-23.3	96.4 a	56.6-289.7	42.72
Larvae	50	3.78 (0.42)	9.4 a	7.6-11.1	20.4 a	16.6-28.7	22.94
	70	4.27 (0.48)	7.8 ab	6.8-8.6	15.5 ab	13.6-18.5	5.46
	90	4.58 (0.61)	6.7 b	5.8-7.5	12.8 b	11.3-15.5	12.37
Pupae	50	2.27 (0.21)	45.6 a	35.0-55.4	166.9 a	134.8-225.8	18.04
	70	2.61 (0.24)	44.3 a	35.1-53.0	137.2 a	115.1-172.1	14.73
	90	2.04 (0.23)	29.7 a	17.6-40.9	125.9 a	97.3-179.4	20.13
Adults	50	4.28 (0.55)	32.6 a	28.1-36.7	64.9 a	55.4-82.9	13.10
	70	5.25 (0.73)	16.2 ab	11.1-50.5	28.4 b	22.1-52.4	43.54
	90	4.83 (0.51)	10.5 b	8.2-13.4	19.4 b	15.0-30.4	36.38

[a] Values with different letters in a column for each developmental stage are significantly different ($P < 0.05$, confident interval for the ratio of LTs).

[b] Eggs, df = 4; Larvae, df = 4; Pupae, df = 4; Adults, df = 4. Chi-square testing the linearity of dose-dependent mortality indicated that normal distribution provided an adequate fit for the model in all cases.

Mortality of *R. dominica* when chickpeas were packaged at a 96% filling ratio

When the rigid plastic containers were filled at the 96% of their capacity with chickpeas and exposed to three CO_2 concentrations for the LT_{50} calculated for each developmental stage, sorption of CO_2 took place together with a negative pressure (Table 2). Mortality in the control treatments were 0% (adults), 11.7 ± 3.0% (eggs), 18.0 ± 2.1% (larvae) and 0% (pupae).

Table 2. Gas changes that occurred inside the rigid plastic containers with a filling ratio of 96% by the application of different CO_2 modified atmospheres at the exposure time at which the stages of *R. dominica* reached the LT_{50}.

Initial CO_2 (%)	Exposure time of LT_{50} (h)	Loss of CO_2 (%)	CO_2 sorption (g/Kg chickpea)	Negative pressure (kPa)
50	9.4 (larvae)	17.19	0.1955	77.12
	16.8 (eggs)	17.58	0.1965	76.99
	32.6 (adults)	17.68	0.2010	76.43
	45.6 (pupae)	18.08	0.2057	75.86
70	7.8 (larvae)	23.44	0.2665	68.32
	14.6 (eggs)	24.62	0.2800	66.66
	16.2 (adults)	25.50	0.2900	65.42
	44.3 (pupae)	25.80	0.2934	64.99
90	6.7 (larvae)	28.29	0.3217	61.49
	10.5 (adults)	27.25	0.3099	62.96
	14.3 (eggs)	28.88	0.3285	60.65
	29.7 (pupae)	27.31	0.3106	62.87

For the pupae, the most tolerant stage, initial concentrations of 50%, 70%, and 90% lost a total of 18.08%, 25.80%, and 27.31% of CO_2, respectively. The rate of CO_2 sorption follow a logarithmic curve: at 50%, CO_2 sorption was of 0.1955 g/Kg chickpea at 9.4 h and of 0.2057 g/Kg chickpea at 45.6 h; at 70%, CO_2 sorption was of 0.2665 g/Kg chickpea at 7.8 h and of 0.2934 g/Kg chickpea at 44.3 h; and at 90%, CO_2 sorption was of 0.3217 g/Kg chickpea at 6.7 h and of 0.3285 g/Kg chickpea at 14.3 h. The negative pressure produced inside the containers was proportional to the CO_2 sorption, increasing as the CO_2 concentration increases. Vacuum ranged between 77.12-75.86, 68.32-64.99, and 62.96-60.65 kPa for 50%, 70%, and 90% CO_2 concentrations, respectively. Mortality data of *R. dominica* developmental stages in the containers with a filling ratio of 96% of chickpeas are shown in Fig. 3 and Table 3. Maximum egg and adult mortality observed in this assay reach up to 19.8% and 12.7%, respectively, and were significantly lower than the 50% mortality estimated on the flexible package trials at the same exposure time (LT_{50}). However, mortality of larvae with 50% and 70% CO_2 concentrations and of pupae with all CO_2 concentrations was not significantly different from the 50% calculated on the flexible packages (LT_{50}) with the Probit model. Surprisingly, mortality of larvae with a 90% CO_2 concentration was significantly greater (64.1% of mortality) than that estimated on the flexible package trials at the LT_{50} (Fig. 3, Table 3).

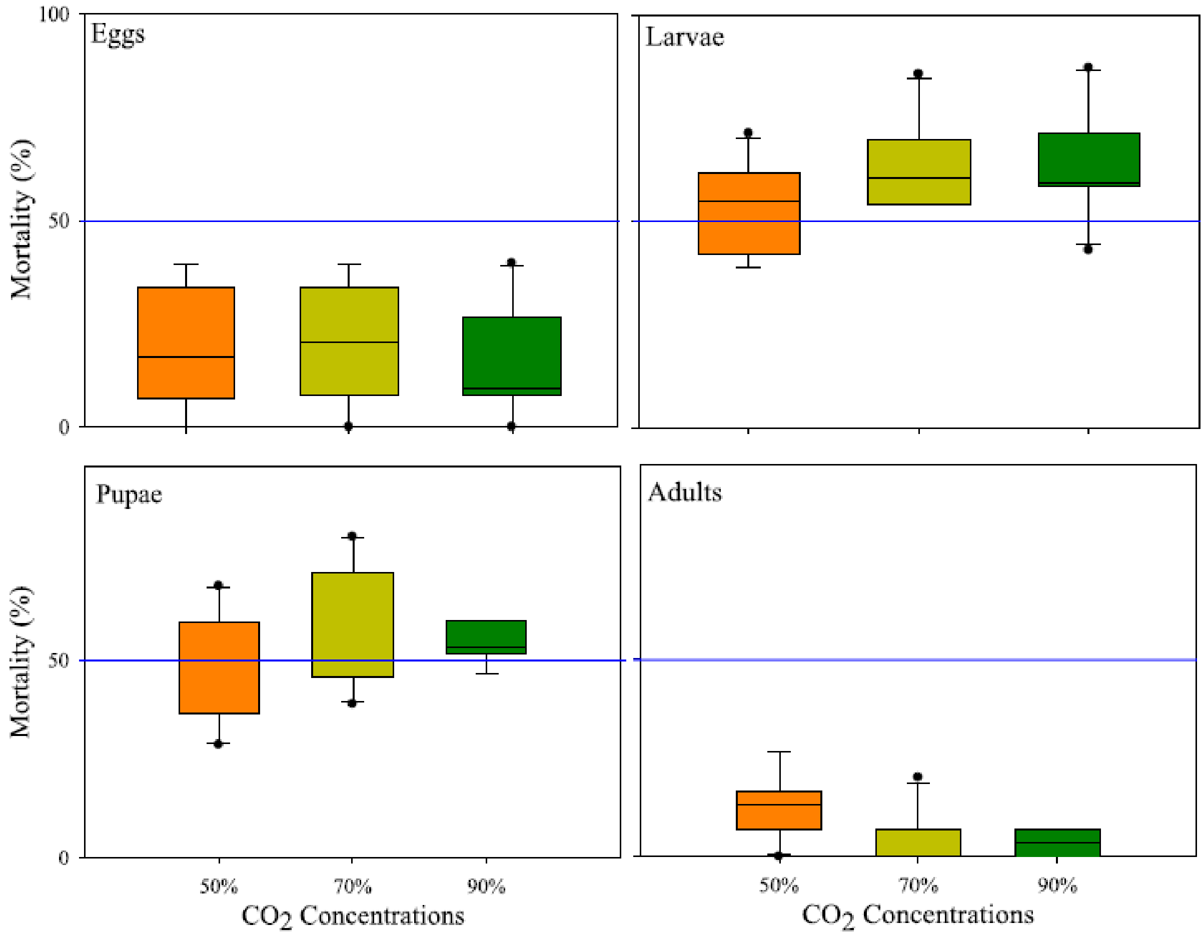

Fig. 3. Mortality (%) of the four developmental stages of *R. dominica* when treated in semi-rigid packages (96% filling ratio) with different modified atmospheres at the LT_{50} calculated with the flexible package (4% filling ratio). Boxplot with median (solid line), the inter-quartile range (box length), and the minimum and maximum values (whiskers).

Table 3. Mortality (%, mean ± ET) of the developmental stages of *R. dominica* when treated in semi-rigid packages with a filling ratio of 96% of chickpeas and different modified atmospheres at exposure times equivalent to reach 50% of mortality (LT_{50}) in flexible packages with a filling ratio of 4%.

Developmental stage	Initial CO_2 concentration (%)	Exposure time (h)	Mortality ± ET (%)	X^2 (df)	*P**
Eggs	50	16.9	19.6 ± 4.8	24.33 (9)	<0.01
	70	14.6	19.8 ± 4.6	20.60 (9)	<0.05
	90	14.3	16.0 ± 4.2	22.47 (9)	<0.01
Larvae	50	8.3	53.5 ± 3.4	10.74 (9)	0.294
	70	7.7	63.3 ± 3.4	15.05 (9)	0.090
	90	6.7	64.1 ± 4.1	19.39 (9)	<0.05
Pupae	50	45.6	49.2 ± 4.2	4.72 (9)	0.858
	70	44.3	56.9 ± 4.8	8.56 (9)	0.479
	90	29.5	54.7 ± 1.7	1.40 (9)	0.998
Adults	50	33.1	12.7 ± 2.7	43.80 (9)	<0.001
	70	15.9	4.0 ± 2.0	64.60 (9)	<0.001
	90	10.6	3.3 ± 1.1	65.67 (9)	<0.001

* The *P* value > 0.05 indicates that the observed assay data are not significantly different from the expected data for the Probit model.

Discussion

While the decrease of CO_2 in flexible packages (4% filling ratio) was negligible (Fig.1), it was significant in the semi-rigid containers with a 96% filling ratio (Table 2). Similar losses of CO_2 have been reported for semi-rigid packages with a filling ratio of 96% with chickpeas, in which the CO_2 content decreased around 45%, 40%, and 25% from the initial concentrations of 90%, 70%, and 50%, respectively, after 10 d of exposure (Iturralde-García et al., 2019). These differences in CO_2 losses between low and high filling ratio were due to the sorption of CO_2 caused mainly by the diffusion of the gas into the kernel pores by the van der Waals adsorption and by the formation of carbamate when reacting with the functional groups of proteins in the kernel (Brunauer, 1943; Mitsuda et al., 1973; Yamamoto and Mitsuda, 1980).

The sorption of CO_2 observed in this study were in the range of 0.19-0.32 g/Kg of chickpea at the three concentrations of CO_2 tested (Table 2). Similar sorptions were obtained in corn and coffee beans (0.306 g/kg and 0.203 g/kg); other grains such as rice, wheat, and red beans had lower sorption values (0.126 g/kg, 0.135 g/kg, and 0.115 g/kg), while peanuts, soybeans, and sesame seeds had higher sorptions (1.008 g/kg, 0.792 g/kg, and 0.414 g/kg) (Mitsuda et al., 1973). Iturralde-García et al. (2019) reported the equilibrium sorption of CO_2 to be of 0.47 g/Kg of chickpea at 49.5 h in semirigid packages with a filling ratio of 96% and 90% of CO_2. In comparison, the CO_2 sorption recorded in the present study represented 68% of the total capacity to sorb CO_2 of the chickpeas.

The negative pressure was mainly influenced by the filling ratio and the initial concentration of CO_2. The main increase in vacuum occurs during the 14.3 h of interaction at 90% CO_2 (60.65 kPa) (Table 2), which corresponded to the 70% of vacuum reported by Iturralde-García et al. (2019) at the equilibrium (49.5 h of exposure) in a similar study with chickpeas at 96% filling ratio and same initial concentration of CO_2.

The susceptibility to CO_2 of *R. dominica* developmental stages in flexible packages with a low filling ratio showed variations in sensitivity among them for the MAs tested (Fig. 2). Larvae were the most sensitive to hypercarbia and hypoxia, needing 1 day of exposure to obtain complete mortality, while the pupae presented the greatest tolerance, since they only reached complete mortality after 10 days of exposure to concentrations of 70% and 90% of CO_2. Other studies have reported the differences susceptibility of the developmental stages of *R. dominica* in wheat and rice. For wheat, with 50% CO_2 and a low filling ratio in the package, a total of 5 days are needed to obtain complete mortality of 1st instar larvae and 15 days to control pupae (Gonçalves et al., 2000). For rice, with 50% and 90% CO_2 and a low filling ratio, the oldest larvae and pupae were killed after 12 days and 8 days, respectively (Riudavets et al., 2009). Our results show similar mortalities of *R. dominica* developmental stages with other type of grains. For example the control with MAs of the common pest of the chickpea *Callosobruchus maculatus* (F.) (Col. Bruchidae) and *R. dominica* can be achieved together, since a total of 10 days were needed for reaching total mortality of *R. dominica* with 70% and 90% CO_2 (present study), and a total of 9 days are needed for killing all developmental stages of *C. maculatus* with 50%, 70%, or 90% CO_2 (Wong-Corral et al., 2013; Iturralde-García et al., 2016).

The fact that the susceptibility of larvae and adults of *R. dominica* were affected by the different concentrations of CO_2 while eggs and pupae were not can be related to the metabolic activity of each developmental stage. Active stages are more sensitive to hypoxia and hypercarbia than inactive stages, such as eggs and pupae (Hoback and Stanley, 2001; Navarro, 2006). Active stages, such as the young larvae and adults, present a high respiratory activity and have the ability to change their metabolism from aerobic to anaerobic in response to hypoxia and hypercarbia (Emekci et al., 2004). During the first 24 h, they try to adapt to the low oxygen concentrations by decreasing their metabolic activity and, therefore, the oxidation of the substrates decreased. But after 24 h, the process stops impeding the utilization of the O_2 and they became susceptible to MAs (Blomberg and Siegbahn, 2014; Levy-de la Torre et al., 2018).

Eggs have a different sensitivity to CO_2 during their developmental period, with the young and old eggs being more susceptible than intermediate developed eggs. Recently laid eggs lose more water or oxygen through the soft chorion than unexposed eggs, while in older eggs, the susceptibility to MAs is due to the high respiratory activity of the pre-emergent larvae (Mbata et al., 2004). Finally, the pupae are less disturbed by the reduction of the O_2 concentrations than the other developmental stages (Emekci et al., 2004).

The mortality of the developmental stages located outside the pulse in the treatments with a filling ratio of 96% were significantly lower than those of the filling ratio of 4%, reaching up to 20% (eggs) and 13% (adults) of the expected 50% mortality in the different concentrations of CO_2. This reduction was caused by the decline in the concentration of CO_2 in the headspace of the package caused by the sorption of the gas by the chickpeas. Mortality of the internal development stages (larvae and pupae) in the semi-rigid container was not significantly different from the expected 50% mortality on the flexible package, with exception of the larvae with a mortality of 64% at 90% of CO_2 that was significantly greater. This similitude between the mortality obtained and expected was due to the fact that the amount of CO_2 sorbed by each chickpea was similar in the flexible and in the semi-rigid packages. In the case of the larvae at 90% of CO_2, the greater mortality was probably due to the increase of the negative pressure in the package combined with the higher CO_2 concentration. Mbata et al. (2005) also has shown that the active larval stage of *C. maculatus* is sensitive to negative pressures.

In conclusion, and in agreement with our hypothesis, mortality of eggs and adults was lower in packages with a high filling ratio than in those with a low filling ratio. This reduction in mortality of the external developmental stages was due to the decline of CO_2 in the headspace of the package caused by its sorption on the chickpeas. However, and in disagreement with our hypothesis, mortality of larvae at concentration of 50% and 70% CO_2 and of pupae at all CO_2 concentrations tested were similar in packages with low and high filling ratios. This was due to the fact that all internal developmental stages were similarly exposed to CO_2 since the amount sorbed by each chickpea was similar at any filling ratio. The increment of the larval mortality at 90% of CO_2 in packages with high filling ratios could be due to the combined effect of the modified atmospheres and the negative pressure produced in the packages.

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3

CAPÍTULO 3

Biological Control of *Callosobruchus chinensis* (Coleoptera: Chrysomelidae) in Stored Chickpeas through the Release of Natural Enemies

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Introduction

The adzuki bean weevil, *Callosobruchus chinensis* (L.) (Coleoptera: Chrysomelidae), is a primary pest of stored chickpeas. Adults attach eggs to the surface of the pulse, and larvae develop concealed inside the pulse. *C. chinensis* can build significant populations within a short period of time (Islam and Kabir, 1995) and can inflict serious damage on chickpeas, including loss of mass, product contamination, and decreased seed germination. Currently, a limited number of highly toxic pesticides are used to control this pest during storage (Iturralde-García et al., 2016). However, interest in sustainable alternative control methods is increasing, and biological control could offer an effective alternative for preventing insect populations from reaching pest status. Natural enemies such as parasitoids and predators have many advantages over chemical control. They leave no toxic residues on the stored commodities, populations cannot develop resistance to them, they are safe for workers and the environment, and their use has been proven to be economically feasible for controlling several pest species. In addition, storage facilities are suited to the use of biological control since they are closed environments from which natural enemies cannot escape (Schöller and Flinn, 2000; Riudavets, 2018). The environmental conditions in storage facilities are also milder than those in open fields or greenhouses, where biological control has been widely adopted. The present study evaluated the effectiveness of two types of natural enemies for controlling *C. chinensis*. Two species of predatory mites that prey on eggs, *Blattisocius tarsalis* (Berlese) (Acari: Ascidae) and *Amblyseius swirskii* (Athias- Henriot) (Acari: Phytoseiidae), were evaluated, as were two larval parasitoids, *Anisopteromalus calandrae* Howard and *Lariophagus distinguendus* Förster (Hymenoptera: Pteromalidae).

Many predatory mites are easy to raise, cheap to purchase, and highly voracious; they are widely used in augmentative biological control strategies. *Blattisocius tarsalis* is a cosmopolitan species that is commonly found in food-storage habitats; it can feed on several Acari, Psocoptera, Lepidoptera, and Coleoptera species (Haines, 1974, 1981a, 1981b; Nielsen, 1999; Riudavets et al., 2002). *Amblyseius swirskii* is very effective for the control of different horticultural pests, including whiteflies, thrips, eriophyid mites, spider mites, and broad mites (Gerson and Weintraub, 2007; Chow et al., 2010; Park et al., 2011; Onzo et al., 2012; Buitenhuis et al., 2014; Soleymani et al., 2016). In addition, *A. swirskii* preys on the eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) (Riahi et al., 2017). *Anisopteromalus calandrae* and *L. distinguendus* are ectoparasitoids that attack the larvae and pupae of stored-product pests that develop concealed within the host substrate. These parasitoids are cosmopolitan; they commonly attack several coleopteran species such as *Sitophilus oryzae* (L.), *S. granarius* (L.), *S. zeamais* (Motschulsky) and *Rhyzopertha dominica* (F.) (van den Assen et al., 1984; Wen and Brower, 1994; Steidle and Scöller, 2002; Adarkwah et al., 2012; Belda and Riudavets, 2013; Castañé and Riudavets, 2015). Both parasitoids have been reported to attack the larvae and pupae of *Callosobruchus* species (Onodera et al., 2002; Ghimire and Philips, 2007).

The general objective of this study was to test the effectiveness of biological control for maintaining a low *C. chinensis* population. We hypothesized that the release of natural enemies would maintain the bruchid population below 10% that of the control. We therefore evaluated the efficacy of four natural enemies in reducing the pest's population growth and the ability of the two parasitoids to penetrate a pile of chickpeas to different depths. We also tested different parasitoid-to-host ratios with *A. calandrae,* and we evaluated the efficacy of *A. calandrae* in controlling pest populations in commercial polypropylene bags of chickpeas.

Materials and Methods

Insect and mite colonies

Colonies were maintained and experiments performed at a constant temperature of 28 ± 2ºC, 75 ± 5% RH and a photoperiod of 16 hours of light to 8 hours of darkness. Stock colonies of all insects were initiated with samples collected in warehouses located in Spain. To raise Callosobruchus chinensis, unsexed adults were placed in plastic containers with chickpeas every week (cv. Blanco Lechoso) to obtain weevils of known ages. Anisopteromalus calandrae and L. distinguendus were reared by offering C. chinensis larvae (aged 8 to 14 days) to newly emerged adults. Two plastic tubes, each containing sugary water and a cotton plug, were supplied as additional food. After three weeks, a new generation of adults was available for experiments. Predatory mites, B. tarsalis and A. swirskii, were supplied by Agrobio SL (Almería, Spain).

Effectiveness of predatory mites and larval parasitoids in suppressing *C. chinensis*

To test the predatory capabilities of *B. tarsalis* and *A. swirskii,* a total of 45 *C. chinensis* eggs were offered to 15 females over three weeks as follows: chickpeas with 15 eggs per week were offered to three (in the first week), six (in the second week) and six (in the third week) predatory females of each species. The final proportion was one female predatory mite for every three *C. chinensis* eggs. To test *A. calandrae* and *L. distinguendus*, chickpeas containing 15 *C. chinensis* eggs were introduced each week for three weeks; these eggs were offered to three pairs of adults that were released on the third week. A total of 45 eggs was introduced with a proportion of one female parasitoid for 15 hosts. A plastic tube containing sugary water and a cotton plug was also used to provide additional food. After three additional weeks, the number of weevils and/or parasitoids was evaluated weekly. Plastic 710-mL containers containing 100 g of chickpeas were used for all experiments. Ten replicates were conducted for each predatory mite and parasitoid species and for the untreated control.

Searching ability of the parasitoids at different depths

To assess the ability of *A. calandrae* and *L. distinguendus* to locate its host in a vertical arena, *C. chinensis* larvae were offered at the bottom of polyvinyl chloride (PVC) pipes measuring 40, 100, and 150 cm high (20 cm internal diameter). These pipes were filled to the top with chickpeas (containing a total of 9.3, 23.2, and 34.9 kg of chickpeas, respectively). A stainless steel screened cage (7 cm high, 5 cm internal diameter) containing 60 g of chickpeas infested with 15 two-day-old eggs, 15 first-instar larvae, and 15 second-instar larvae of *C. chinensis* (a total 45 individuals) was located at the bottom of each PVC pipe. An additional treatment was tested with the tallest pipe (150 cm); three infested cages were placed in that pipe at depths of 40, 100, and 150 cm. Each cage contained 60 g of chickpeas infested with 5 two-day-old eggs, 5 first-instar larvae, and 5 second-instar larvae for a total of 15 individual *C. chinensis* instars per cage and 45 individual *C. chinensis* instars per pipe. Next, three pairs of *A. calandrae* or *L distinguendus* adults (0 to 7 days old) were released on the surface of the grain. A tube containing sugary water was also placed on the surface, and the pipes were sealed with fabric mesh. After a week, the PVC pipes were poured off; the parasitoids were removed, and the screened cages containing infested chickpeas were isolated in plastic containers. The emergence of adult *C. chinensis* and/or parasitoids was then recorded. Six replicates were conducted for each parasitoid species and pipe height. For the control group, plastic 710-mL containers containing 60 g of chickpeas infested with 15 two-day-old eggs, 15 first-instar larvae, and 15 second-instar larvae of *C. chinensis* (a total 45 individuals) were located outside the PVC pipes. Six replicates were conducted.

Effectiveness of *A. calandrae* at different host ratios

To assess the efficacy of *A. calandrae* at different host ratios, an increasing number of hosts were offered to a fixed number of parasitoids. Forty-centimeter PVC pipes were used for this experiment, which followed a similar methodology as that described in the previous section: Infested screened cages were deposited at the bottom of the pipe. The parasitoid-to-host ratios were tested. Hosts were offered at ratios of 1:7 (7 eggs, 7 first-instar larvae, and 7 second-instar larvae), 1:30 (30 eggs, 30 first-instar larvae, and 30 second-instar larvae) and 1:60 (60 eggs, 60 first-instar larvae, and 60 second-instar larvae), for a total 21, 90, and 180 *C. chinensis* individuals offered at each ratio. Three pairs of *A. calandrae* were released at the surface of the grain. Six replicates were conducted with each ratio; for the control group, plastic 710- mL containers containing 60 g of chickpeas infested with a total 21, 90, and 180 *C. chinensis* individuals (eggs, first-instar larvae, and second-instar larvae) were located outside the PVC pipes. Six replicates were also conducted. The results of the previous experiment with 40-cm PVC pipes (single depth) and a 1:15 parasitoid-to-host ratio were also compared to those of this experiment. Fifty *C. chinensis* individuals and 50 *A. calandrae* individuals (with three replicates) were weighed to estimate the individual mass for each species; this number was multiplied by the number of adults that emerged to determinate the biomass of both species in each replicate.

Effectiveness of *A. calandrae* in commercial chickpeas bags

To assess the ability of *A. calandrae* to locate its host in commercial woven polypropylene bags (42 x 66 cm, 25 kg of chickpeas) a test was done in an experimental storage facility at ambient temperature and humidity (27 ± 2ºC and 65 ± 4% RH). One infested screened cage, similar to those previously described, was placed at one end of a bag. The cage contained 30 two-day-old eggs, 30 first-instar larvae, and 30 second-instar larvae of *C. chinensis* in 60 g of chickpeas. At the opposite end of the bag, three pairs of *A. calandrae* (1:30 parasitoid: host ratio) were released; one tube of sugary water was also placed there. One polypropylene bag was placed on the floor of each empty room (3 x 2 m). After one week, the bags were opened, and screened cages containing the infested chickpeas were placed in a climatic chamber (28 ± 2ºC; 75 ± 5% RH; 16 h: 8 h light: dark) to develop. Over the following weeks, the number of *C. chinensis* and *A. calandrae* that emerged were counted. Six replicates were conducted, each in a different room. A control treatment with plastic 710-mL containers containing 60 g of chickpeas infested with a total 90 individuals of *C. chinensis* but without parasitoids was also conducted. Six replicates were done.

Data analysis

Data normality was analyzed using the Shapiro–Wilk test. The following data sets were analyzed using a one-way analysis of variance (ANOVA): a) the number of *C. chinensis* and parasitoid progeny obtained when predatory mites or parasitoids were released; b) the percentage by which the number of *C. chinensis* and *A. calandrae* were reduced and the biomass of these species at different parasitoid-to-host ratios; and c) the number of *C. chinensis* that emerged when *A. calandrae* were released in commercial bags. Two-way ANOVAs were conducted to analyze the number of *C. chinensis* and parasitoids that emerged in the experiments testing the parasitoids' ability to find the host at different depths. Two-way ANOVAs were also conducted to analyze the number and biomass of *C. chinensis* and the parasitoids at different parasitoid-to-host ratios. Post-hoc comparisons were carried out with Tukey correction for multiple comparisons. The proportions of *A. calandrae* females emerging from *C. chinensis* at different depths, at different parasitoid-to-host ratios, and from commercial bags were determined by a Student's t-test. All statistical analyses were conducted with JMP (JMP 8.0.1, 2009, SAS Institute, Inc).

Results

Effectiveness of predatory mites and larval parasitoids in suppressing *C. chinensis*

The predatory mite *A. swirskii* and the parasitoids *A. calandrae* and *L. distinguendus* significantly reduced the progeny of *C. chinensis* compared to the control treatment. Only the treatment with the predatory mite *B. tarsalis* did not show any significant differences with the control treatment (Table 1). Both parasitoid species were much more effective than the predatory mites; a mean number of zero or nearly zero *C. chinensis* emerged in the treatments with parasitoids; with the mite treatments, many more emerged. Of the 30 parasitoid pairs introduced in the ten replicates, most were recovered alive after one week of exposure (around 92% for both parasitoid species). *A. calandrae* and *L. distinguendus* were similarly effective; the mean number of emerged *C. chinensis* did not significantly differ between them (Table 1). There were no significant differences in the proportion of *A. calandrae* females and males that emerged ($t = 0.36$, $P = 0.727$); however, for *L. distinguendus*, more females emerged ($t = 12.54$, $P < 0.001$) (Table 1).

Table 1. Mean (± SEM) number of *C. chinensis*, *A. calandrae*, or *L. distinguendus* that emerged in the predatory mite, larval parasitoid, and control groups (no predators or parasitoids were released). The sex ratio of the emerged parasitoids is also shown (n = 10).

Treatments	No. of emerged adults		
	C. chinensis	Parasitoids	Female parasitoids (%)
Control	36.3 ± 0.70 a	-	-
B. tarsalis	33.2 ± 1.15 ab	-	-
A. swirskii	30.1 ± 1.07 b	-	-
A. calandrae	0 c	12.2 ± 1.09 a	52.4 ± 6.71
L. distinguendus	0.3 ± 0.21 c	14.6 ± 0.75 a	76.9 ± 2.15 *
	$F_{4, 49} = 552.75$; $P < 0.001$	$F_{1, 19} = 3.28$; $P = 0.087$	

Values in the same column followed by a different lowercase letter are significantly different (Tukey test, $P < 0.05$). * Denotes significant deviation from 50% (Student's t-test)

Searching ability of the parasitoids at different depths

A mean of 41.3 ± 0.8 *C. chinensis* emerged in the control treatment, indicating natural mortality of 8.2%. Both parasitoids were able to reduce the emergence of *C. chinensis* at all depths tested, leading to global mortality over 90%. *A. calandrae* and *L. distinguendus* were similarly effective at locating hosts at the three depths tested;

there were no significant differences among the numbers of *C. chinensis* that emerged from the samples at different depths (Tables 2 and 3). Furthermore, both parasitoids were similarly effective at suppressing *C. chinensis* whether the same number of hosts was offered at a specific depth or spread across three different depths, indicating that the adult parasitoids had no difficulty finding the hosts under 1.5 m of chickpeas.

Table 2. Statistical parameters of the two-way analysis of variance (ANOVA) for the number of hosts and parasitoids that emerged when *C. chinensis* larvae were offered to adult *A. calandrae* or *L. distinguendus* at three depths (40, 100, and 150 cm) in a pile of chickpeas.

Factors	No. of emerged *C .chinensis*		No. of emerged parasitoids	
	F	*P*	*F*	*P*
Parasitoid species	3.79	0.059	18.91	< 0.001
Depth	2.44	0.079	1.38	0.261
Parasitoid species x Depth	0.43	0.732	2.43	0.080

Degrees of freedom: Parasitoid species: 1; Depth: 3; Parasitoid species x Depth: 3

Table 3. Mean (± SEM) number of adult *C. chinensis*, *A. calandrae*, and *L. distinguendus* that emerged, with parasitoid sex ratios, when host larvae were offered at three depth levels (40, 100, and 150 cm) in a pile of chickpeas (n = 6).

Treatments	No. of emerged adults		Female parasitoids (%)	Student’s t-test	
	C. chinensis	Parasitoids		*t*	*P*
A. calandrae					
40 cm	1.7 ± 1.75	13.0 ± 1.75	62.8 ± 5.39	2.38	0.063
100 cm	3.0 ± 2.68	16.2 ± 2.52	72.4 ± 4.27*	5.26	< 0.050
150 cm	2.5 ± 2.97	16.2 ± 1.01	60.7 ± 5.0	1.25	0.268
Mixed depths	4.3 ± 1.45	12.2 ± 2.13	66.4 ± 5.35*	3.06	< 0.050
L. distinguendus					
40 cm	1.3 ± 1.62	5.7 ± 1.54	47.5 ± 12.21	-0.21	0.844
100 cm	0.5 ± 0.22	7.0 ± 1.32	74.2 ± 6.26*	3.87	< 0.050
150 cm	0.8 ± 1.65	6.8 ± 1.76	60.4 ± 13.42	0.78	0.472
Mixed depths	3.3 ± 1.63	12.3 ± 0.55	60.7 ± 5.55	1.93	0.112

* Denotes significant deviation from 50% (Student’s t-test)

Significantly more *A. calandrae* adults than *L. distinguendus* adults emerged at all depths, alone or combined. In addition, more female *A. calandrae* progeny emerged at one depth and in the mixed combination of depths; more female *L. distinguendus* progeny emerged only at one depth (Tables 2 and 3).

Successful parasitism, the emergence of adult parasitoids, accounted only for 12.6% to 36.0% host mortality with both parasitoid species. Other causes, such as natural host mortality, host feeding by adult parasitoids, immature mortality of parasitoid progeny, or a combination of these, also contributed to the total reduction in host emergence.

Effectiveness of *A. calandrae* at different host ratios

A. calandrae was able to reduce the emergence of *C. chinensis* adults and to reproduce in all tested parasitoid-to-host ratios (Table 4). As expected, the mean number of *C. chinensis* that emerged in the control groups significantly increased as the initial number of hosts released increased, ranging from 17.0 to 148.7 adults for the 1:7 to 1:60 ratio treatments. When *A. calandrae* was released, the total number of both *C. chinensis* and *A. calandrae* that emerged increased significantly as the parasitoid-to-host ratios were reduced (Figure 1A). In the parasitoid treatment, *C. chinensis* emergence ranged from 0.7 to 17.8 adults, and *A. calandrae* emergence ranged from 1.8 to 47.8 at the 1:7 and 1:60 ratios, respectively. Furthermore, as the parasitoid-to-host ratio decreased, the bias toward female progeny increased (Table 5).

Table 4. Statistical parameters of the two-way analysis of variances (ANOVA) for the number of emerging adults (host and parasitoids) and their biomass; comparison of control to treatments in which *A. calandrae* was released at four parasitoid-to-host ratios in a 40-cm pile of chickpeas.

Factors	No. of emerged adults		Biomass	
	F	*P*	*F*	*P*
Treatment	793.51	< 0.001	1245.33	< 0.001
Parasitoid: host ratio	820.20	< 0.001	402.43	< 0.001
Treatment x Parasitoid: host ratio	101.21	< 0.001	182.11	< 0.001

Degrees of freedom: Treatment: 1; Parasitoid-to-host ratio: 3; Treatment x Parasitoid-to-host ratio: 3

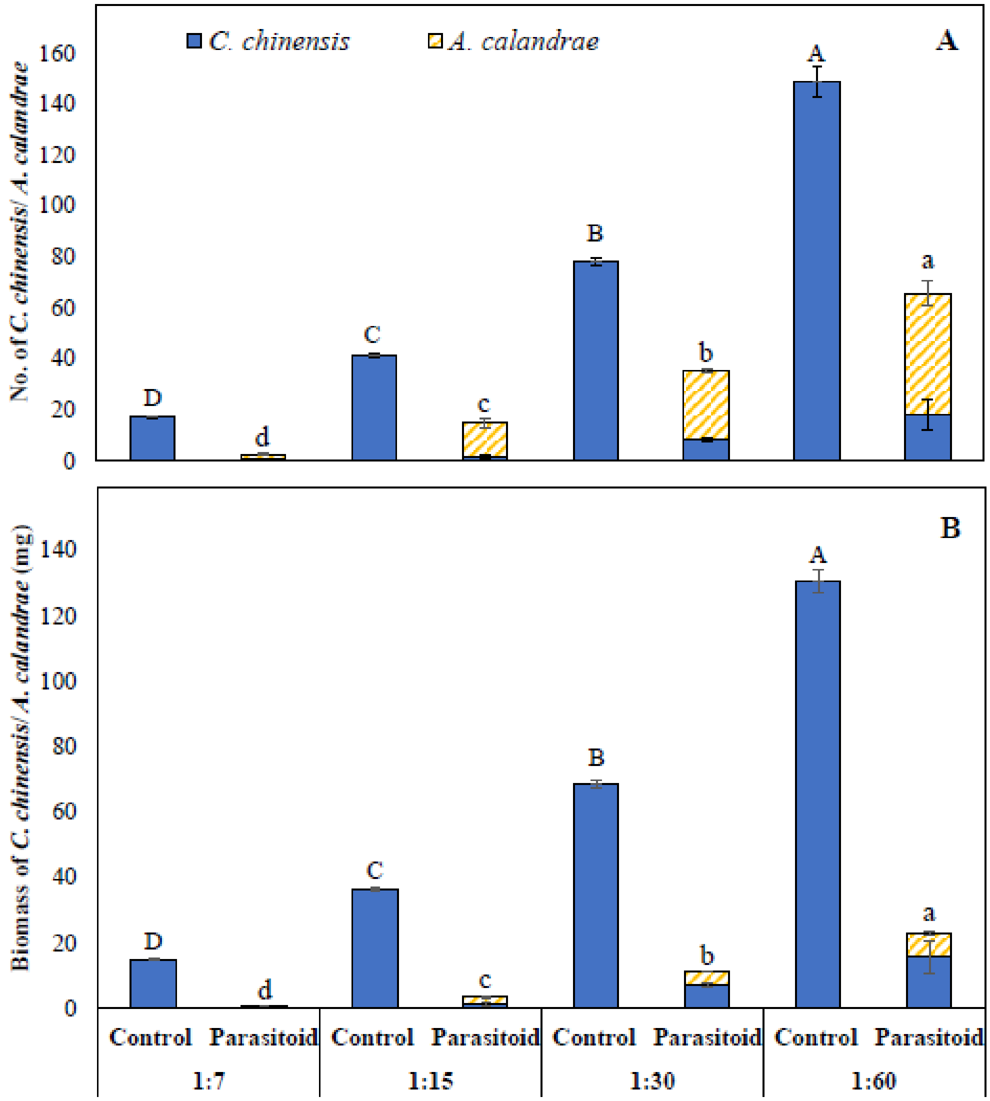

Figure 1. Mean (± SEM) number (A) and biomass (B) of *C. chinensis* in the control and of *C. chinensis* and *A. calandrae* in the treatment in which the parasitoid was released at different parasitoid-to-host ratios in 40-cm PVC pipes filled with chickpeas (n = 6). The number and biomass of emerged *C. chinensis* at different parasitoid-to-host ratios in the control followed by the same uppercase letter do not differ significantly. The number and biomass of emerged *C. chinensis* and *A. calandrae* at different parasitoid-to-host ratios followed by the same lowercase letter do not differ significantly (Tuckey test, $P < 0.05$).

Table 5. Mean percentage (± SEM) of *A. calandrae* female progeny at different host ratios in 40-cm PVC pipes filled with chickpeas.

Host ratio	Females (%)	Student's t-test	
		t	*P*
1:7	36.1 ± 11.72	-1.18	0.289
1:15	62.8 ± 5.39	2.38	0.063
1:30	64.3 ± 3.35*	4.28	< 0.010
1:60	73.2 ± 1.93*	12.02	< 0.001

* Denotes significant deviation from 50% (Student's t-test)

The emergence of adult parasitoids from a commodity can be perceived as a contaminant similar to the pest, even though the parasitoid control strategy resulted in a significant reduction in the total number of insects (pests plus parasitoids) contaminating the commodity compared to the untreated control. However, parasitoids are usually smaller than their hosts, resulting in a smaller total insect biomass. As expected, there were significant differences in the biomass of emerged adults in the control and in groups in which the parasitoid was released at different parasitoid-to-host ratios (Table 4, Figure 1B). The total biomass reductions in the treated groups were significantly greater than the reductions in the number of emerging adults achieved by decreasing the parasitoid-to-host ratios (Table 6).

Table 6. Mean (± SEM) percentage of reductions in the total number of emerged adults (*C. chinensis* + *A. calandrae*) and in biomass compared to the control in four parasitoid-to-host ratios in 40-cm PVC pipes filled with chickpeas (n = 6).

Parasitoid: host ratio	Percentage of reduction		Statistical parameters	
	No. of emerged adults	Biomass	*F*	*P*
1:7	85.3 ± 3.64	94.2 ± 2.16	4.45	0.061
1:15	64.5 ± 4.13	90.6 ± 1.69	34.11	< 0.001
1:30	65.5 ± 2.14	83.6 ± 3.19	134.26	< 0.001
1:60	55.83 ± 2.26	82.5 ± 3.47	41.36	< 0.001

Degrees of freedom: 1

Effectiveness of *A. calandrae* in commercial chickpeas bags

Significantly fewer *C. chinensis* and *A. calandrae* emerged in total when *A. calandrae* was released than in the control (76.2 ± 1.97 versus 29.5 ± 1.34) (F = 383.56; df = 1, 11; $P < 0.001$). When contamination is expressed in terms of insect biomass rather than the number of insects, contamination was significantly lower with the parasitoid treatment than in the control (67.0 ± 1.73 mg versus 9.4 ± 1.09 mg) (F = 789.80; df = 1, 11; $P <$ 0.001) (Figure 2). The parasitoid progeny were female-biased (75.4 ± 3.46%; t = 7.36, $P < 0.001$), as already observed in the parasitoid-to-host ratios experiment.

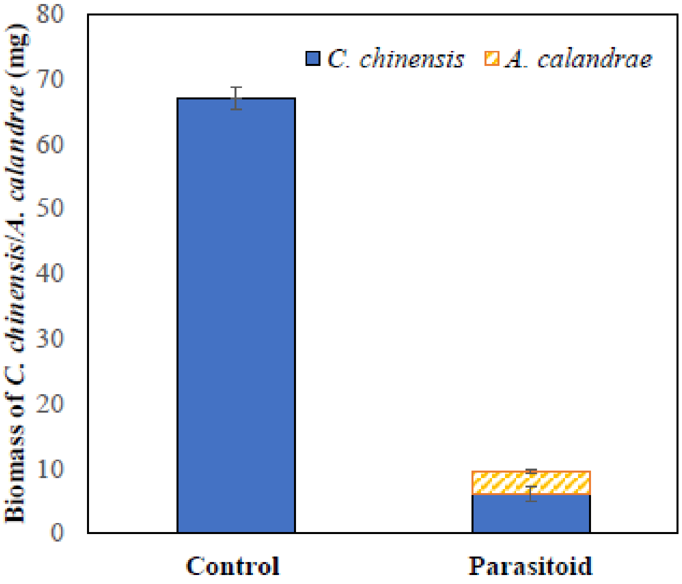

Figure 2. Mean (± SEM) biomass (mg) of *C. chinensis* in the control and of *C. chinensis* and A. calandrae when the parasitoid was released into commercial bags of chickpeas (n = 6). There were significant differences between the parasitoid treatment and the control (Student's t-test).

Discussion

Neither *B. tarsalis* nor *A. swirskii*, the two predatory mites tested here, have been previously reported to attack *C. chinensis* eggs. Although *B. tarsalis* did not effectively reduce *C. chinensis* in our experiment, it has been reported to effectively control other storage pests such as *Ephestia cautella* (Walker), *E. kuehniella* (Zeller), *Tribolium castaneum* (Herbst), *Plodia interpunctella* (Hüber), *Lasioderma serricorne* (Fabricius), *Cryptolestes ferrugineus* (Stephens), and *Acanthoscelides obtectus* (Say) (Haines, 1981b; Nielsen, 2001; Riudavets et al., 2002). While *A. swirskii* was able to prey on *C. chinensis* to some extent, its control efficacy was low, as can be seen in Table 1. The low or null effectiveness of predatory mites may be because *C. chinensis* attach their eggs to the surface of the chickpeas instead of leaving them loose to mix with the debris at the bottom of the container like other storage pests. However, further studies may determine whether these predatory mites can still positively contribute to the biological control of this pest species.

The two parasitoids tested were very effective at controlling *C. chinensis* populations: *A. calandrae* eliminated the weevil population, and *L. distinguendus* eliminated a mean of 44.7 individuals from an initial population of 45 individuals (from second-instar larvae to pupae) in our first experiment. A single *A. calandrae* female can kill about 20 *C. maculatus* (Fabricius) final-instar larvae during a 24-hour period (Ghimire and Phillips, 2007); a pair of *L. distinguendus* can kill 12 to 20 *C. chinensis* individuals or 10 to 15 *C. maculatus*, depending on the developmental stage, over 24 hours (Bellows, 1985). We released three pairs of *A. calandrae* or *L. distinguendus* for a longer period (at least one week), which explains the effectiveness we observed.

The sex ratios of *A. calandrae* progeny were balanced, but those of *L. distinguendus* were female-biased. This could be because the former exhausted the available hosts while the latter did not. Females of both species use large larvae to oviposit female eggs and smaller larvae to oviposit male eggs and for feeding (Choi et al., 2001; Lebreton et al., 2009). The higher activity of *A. calandrae* females may have superparasitized large larvae from which only one adult would emerge; with the *L. distinguendus*, large larvae may have been less superparasitized (Wen and Brower 1995).

Both parasitoids were similarly effective (96-99% host mortality) at penetrating a chickpea column 40, 100, or 150 cm deep to find host larvae, even when larvae were offered at mixed depths (Table 2). Both parasitoids seemed to be able to move freely within the chickpea pile, probably because the interstitial spaces between pulses are larger than those in smaller grains such as wheat and maize.

A. calandrae can locate and parasitize *S. oryzae* in a wheat column 220 cm deep, but they have low downward mobility, and, in a previous study, it was necessary to increase the number of parasitoids to adequately suppress *S. oryzae* (Press, 1988). *L. distinguendus* can penetrate 100 cm of maize or 4 m of wheat to locate *S. zeamais* and *S. granarius*, respectively. However, suppression is reduced from 74% to 34% at depths deeper than 90 cm (Steidle and Schöller, 2002; Adarkwah et al., 2012). These authors concluded that the density of parasitoids per unit volume of grain might significantly affect how deeply the parasitoids penetrate the grain.

In the present study, *A. calandrae* effectively controlled *C. chinensis* very well at all parasitoid-to-host ratios tested, achieving more than 90% reduction of host emergence at a ratio of one parasitoid to 60 hosts, the lowest ratio tested. These results are in line with other studies. *A. calandrae* effected 90% suppression of *S. oryzae* in wheat at parasitoid-to-host ratios of 1:15 and 1:20 (Press et al., 1984), and the parasitoid *Dinarmus basalis* (Rond.) achieved 78% suppression of *C. chinensis* in red lentils at a parasitoid-to-host ratio of 1:60 (Islam and Kabir, 1995). While F1 progeny of *A. calandrae* were low at the highest ratio tested (1:7) and had a malebiased sex ratio, progeny increased at the lowest ratio (1:60) and were female-biased. A similar trend has been reported for *D. basalis* with *C. chinensis* (Islam and Kabir, 1995). These differences in reproduction could be attributed to superparasitism that is documented occurs at high ratios (Wen and Brower, 1995).

Although *A. calandrae* females recognize hosts that they have parasitized, they cannot identify hosts parasitized by other females (Benkhellat et al., 2015), and the probability that a female will lay eggs on a host parasitized by another female increases with elevated parasitoid-to-host ratios. When several eggs are deposited on a single host larvae, no more than one parasitoid reaches maturity, resulting in a lower total number of offspring. Therefore, when the parasitoid-to-host ratio is high, either because of low host availability or a high number of parasitoids, competition among female parasitoids leads to high host mortality and decreased parasitoid offspring. When the females also feed on hosts, a behavior common to many parasitoids, this further reduces the number of hosts available for reproduction.

While the number of *A. calandrae* progeny increased when the parasitoid-to-host ratios were reduced, the emergence of *C. chinensis* decreased, both in terms of number of individuals and of biomass. However, this reduction in host emergence was significantly higher when expressed as biomass than when expressed as the number of individuals, particularly at low parasitoid-to-host ratios (Table 6, Figure 1A and B). This indicates that merely counting the number of individuals in the grain would lead to an overestimation of the insect contamination at the end of this process. Although adult parasitoids can also be a source of grain contamination, the total insect biomass is significantly reduced by parasitoid treatment.

This parasitoid demonstrated good potential for controlling *C. chinensis* in warehouse conditions since we observed similarly low final contamination (in terms of insect biomass) under warehouse conditions as in PVC pipes with the same parasitoid-to-host ratios but under controlled conditions. *A. calandrae* suppression of *C. chinensis* was high (93%) in woven propylene bags, even though the parasitoid's movement is more restricted in packaged commodities than in bulk grain (Press and Mullen, 1992).

In summary, under the conditions tested, parasitoid wasps alone show great potential for controlling *C. chinensis*; our hypothesis was confirmed as the bruchid population in the experimental groups was maintained at less than 10% of that in the control groups. These results indicate that *A. calandrae* and *L. distinguendus* are effective biological control agents for the integrated management of *C. chinensis* in stored chickpeas and can represent an alternative to pesticides for maintaining low bruchid population levels. Our study also suggests that the predatory mite *A. swirskii* could play a role in the control of *C. chinensis*, alone or in combination with other natural enemies, and deserves further investigation.

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4

CAPÍTULO 4

Biological Control of *Acanthoscelides obtectus* and *Zabrotes subfasciatus* in Stored Dried Beans

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Introduction

Acanthoscelides obtectus Say and *Zabrotes subfasciatus* Boheman (Coleoptera: Chrysomelidae) are major pests of stored dried beans *Phaseolus vulgaris* L. (Fabales: Fabaceae). These pests cause significant damage to the legume during long periods of storage (Hill 1990). *Acanthoscelides obtectus* is neotropical in origin but currently has a cosmopolitan distribution. *Zabrotes subfasciatus* is widespread in several countries in Africa, Asia and the Americas. *Acanthoscelides obtectus* attack dry bean seeds in the field and subsequently infest storage units; *Z. subfasciatus* attack only stored seeds (Cardona 1989; Oliveira et al. 2013; CABI 2019). The larvae of both bruchids are internal feeders; they can bore into seeds. Larvae develop inside the seeds until they reach adulthood, when they emerge from the legume and actively disperse (Subramanyam and Hagstrum 1996; Soares et al. 2015).

Pest management in stored dried beans commonly involves treatments with pesticides (fumigants and/or residual insecticides) (Paul et al. 2009). However, the use of pesticides poses a threat to the health of operators handling the fumigant. In addition, there are significant environmental concerns about the use of pesticides, and consumer demand for residue-free products is increasing. In addition, the number of active substances available is shrinking due to changes in legal approval processes and to the emergence of resistant pest populations (Benhalima et al. 2002; Opit et al. 2012; Pimentel et al. 2008; Clarke et al. 2011). Therefore, there is a demand for alternative, environmentally safe methods of pest control. Among these, the use of natural enemies such as predatory mites and parasitoid wasps stands out. Biological control using beneficial insects and mites is an effective pest management tool that does not induce resistance and involves no risks for operators (Niedermayer and Steidle 2013).

The present study evaluates the effectiveness of two types of natural enemies in the control of these two weevils: four polyphagous predatory mite species that prey on eggs and two polyphagous larval parasitoid species. The predatory mite *Blattisocius tarsalis* Berlese (Acari: Ascidae) is frequently found in storage areas of different commodities (Haines 1981; Riudavets et al. 2002a, b). However, the capacity of the other mite species to control storage pests is unknown. *Amblyseius swirskii* Athias-Henriot, *Neoseiulus cucumeris* Oudemans (Acari, Phytoseiidae) and *Stratiolaelaps scimitus* Berlese (Acari, Laelapidae) are commercially available biological control agents for different pests in many vegetable crops grown in open fields and greenhouses (Jess and Kilpatrick 2000; Messelink et al. 2006; Guerson and Weintraub 2007). *Amblyseius swirskii* is able to prey on the eggs of stored pests such as *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) and *Callosobruchus chinensis* L. (Coleoptera: Chrysomelidae) in the laboratory, and the predator *N. cucumeris* is able to consume *E. kuehniella* eggs (Deslise et al. 2015; Riahi et al. 2017; Iturralde et al. submitted). The larval parasitoids *Anisopteromalus calandrae* Howard and *Lariophagus distinguendus* Förster (Hymenoptera: Pteromalidae) often attack coleopteran larvae that develop inside the seeds of different stored commodities (Belda and Riudavets 2013; Castañé and Riudavets 2015; Berger et al. 2017).

The main objective of this study was to evaluate the effectiveness of these biological control agents on these two bruchid populations under controlled and warehouse conditions. We hypothesized that combining parasitoids with predatory mites would control both bruchid populations better than the use of either biological agents alone. Therefore, we first selected predatory mites based on their consumption of bruchid eggs and their ability to survive at different relative humidity levels. Predatory mites and parasitoid wasps were then tested, alone and in combination, on infested dried beans in small cages. Finally, the dispersion capacity of predators and parasitoids was evaluated on a larger scale: in 12-kg containers of beans under controlled conditions and in commercial 25-kg bags of beans under warehouse conditions.

Materials and methods

Insect and mite colonies

Insect colonies were maintained at a constant temperature of 28 ± 2ºC, 75 ± 5% relative humidity (RH) and a photoperiod of 16 hours of light to 8 hours of darkness. Colonies of *A. obtectus*, *A. calandrae* and *L. distinguendus* were initiated with samples collected from warehouses in Spain; a colony of *Z. subfasciatus* was started with samples from the University of Sonora (México). Every week, unsexed adults of *A. obtectus* or *Z. subfasciatus* were placed in plastic containers with dry bean seeds (*P. vulgaris*; cv. Riñón) to obtain weevils of known ages. *A. calandrae* was reared by offering *A. obtectus* or *Z. subfasciatus* larvae (aged 15 to 21 days) to newly emerged adults; *L. distinguendus* was reared similarly but was only offered *A. obtectus* larvae. Sugar water in a cotton plug was supplied as additional food. After three weeks, a new generation of adult parasitoids was available for experiments. The predatory mites *B. tarsalis*, *A. swirskii*, *N. cucumeris* and *S. scimitus* were supplied by Agrobio SL (Almería, Spain).

Survival and consumption of bruchid eggs by predatory mites

To assess the ability of the four species of predatory mites to prey on bruchid eggs, 15 two-day-old eggs of *A. obtectus* or *Z. subfasciatus* were offered to one female in a small cage (2.5 cm diameter). After 24 h, the female mite's survival was assessed and the number of damaged eggs recorded. A control treatment using 15 two-day-old eggs of *A. obtectus* or *Z. subfasciatus* but no predatory mites was also done. Ten replicate experiments of each combination of predator and bruchid species and of the control treatment were conducted. Experiments with *A. obtectus* were carried out at 28 ± 2ºC and at two levels of RH, 80% and 75%; *Z. subfasciatus* was kept at 80% RH.

Effectiveness of predatory mites and larval parasitoids in reducing bruchid populations

To further test the predatory capabilities of *B. tarsalis* and *A. swirskii*, 45 two-day-old eggs of *A. obtectus* or *Z. subfasciatus* were offered to 15 mite females over three weeks as follows: 15 two-day-old eggs per week were offered to three (in the first week), six (in the second week) and six (in the third week) predatory females of each species. The final proportion was one female predatory mite to three bruchid eggs. After four additional weeks, the number of weevils was evaluated. To test *A. calandrae* and *L. distinguendus*, beans containing 15 two-day-old eggs of *A. obtectus* or *Z. subfasciatus* were introduced weekly for three weeks, and, on the fourth week, three pairs of adults were released, resulting in a total proportion of one female parasitoid to 15 hosts. A plastic tube containing sugar water and a cotton plug was also included as additional food. After three additional weeks, the number of weevils and/or parasitoids was evaluated until no more emergence was recorded. To test the combined effect of the predatory mites *B. tarsalis* and *A. swirskii* with the parasitoid *A. calandrae* on the populations of *A. obtectus* or *Z. subfasciatus,* a similar test was performed in which female predatory mites were introduced during the first three weeks and parasitoid pairs in the fourth week, resulting in the same proportions of prey to hosts as previously described. A control treatment with 45 two-day-old eggs of *A. obtectus* or *Z. subfasciatus* but without predatory mites or parasitoids was also done. For all experiments, 710-mL plastic containers containing 100 g of beans, including the seeds with bruchid eggs, were used. Ten replicate experiments were conducted for each predatory mite, parasitoid species or combination and for the control treatment. Experiments were carried out at a constant temperature of 28 ± 2ºC, 75 ± 5% RH and a photoperiod of 16 h of light to 8 h of darkness.

Searching ability of predatory mites and *A. calandrae*

To assess the ability of *B. tarsalis* and *A. swirskii* females to locate their prey in a more realistic arena, bruchid eggs were offered in 12-kg containers of beans. Two predator-to-prey ratios were tested, 1:3 and 2:1. A fixed number of prey (45 two-day-old eggs of *A. obtectus*) were offered to 15 or 90 female predatory mites. PVC pipes (40 cm tall, 20 cm diameter) filled with beans were used in this experiment. A stainless-steel screened cage (7 cm high, 5 cm internal diameter) containing 60 g of beans and infested with 45 two-day-old eggs of *A. obtectus* was located at the bottom of the PVC pipe. The appropriate number of predatory mites was released at the top. After four additional weeks, the number of weevils that had emerged was counted.

The combination of the predatory mite *B. tarsalis* with the parasitoid *A. calandrae* was assessed in a similar arena. A fixed number of prey/hosts (45 two-day-old eggs of *A. obtectus*) was offered to 90 female predatory mites (2:1 predator-to-prey ratio) and to three pairs of *A. calandrae* (1:15 parasitoid-to-host ratio). Predatory mites were released the same week as the bruchid eggs, while the parasitoids were released four weeks later. After three additional weeks, the number of emerging weevils and parasitoids was evaluated. For the control treatment, 710-mL plastic containers containing 60 g of beans infested with 45 two-day-old eggs of *A. obtectus* were used. Six replicates were conducted of each treatment, including the control. The experiments were carried out at a constant temperature of 28 ± 2ºC, 75 ± 5% RH and a photoperiod of 16 h of light to 8 h of darkness.

To assess the ability of *B. tarsalis* to locate its prey in larger arenas, a similar test was done with commercial woven polypropylene bags (42 x 66 cm containing 25 kg of beans) in experimental rooms (23.5 ± 1.5ºC, 68 ± 10% RH). One infested screened cage containing 45 two-day-old eggs of *A. obtectus* in 60 g of beans was placed at one end of the bag. At the opposite end, 9 ml of commercial diet substrate containing *B. tarsalis* (90-180 individuals) was released. One polypropylene bag was placed on the floor of each empty room (3 x 2 m). After one week, the bags were opened, and the infested chickpeas were allowed to develop in screened cages under controlled conditions (28 ± 2ºC; 75 ± 5% RH; 16 h: 8 h light: dark). Over the following weeks, the emerging *A. obtectus* were counted. The control treatment without predators used 710-mL plastic containers containing 60 g of beans infested with 45 two-day-old eggs of *A. obtectus*. Six replicates were conducted, each in a different room.

Data analysis

An arcsine square root transformation was performed on the percentage of mortality rates so that these data complied with the normality and homoscedasticity requirements of parametrical tests. The following data sets were analysed using a one-way analysis of variance (ANOVA): a) percentage of mortality of *Z. subfasciatus* and *A. obtectus* eggs after one female predatory mite was released; b) percentage of mortality of *Z. subfasciatus* and *A. obtectus* when predatory mites, parasitoids or a combination of both were released; c) percentage of mortality of *A. obtectus* when two predatory-to-prey ratios of *A. swirskii* and *B. tarsalis* were tested alone, or when *B. tarsalis* was combined with *A. calandrae* in PVC pipes or when 9 ml of commercial *B. tarsalis* were released in a propylene bag. Post-hoc comparisons were conducted using Tukey corrections for multiple comparisons. All statistical analyses were conducted using JMP (JMP 8.0.1, 2009, SAS Institute, Inc).

Results

Survival and consumption of bruchid eggs by predatory mites

All females of *B. tarsalis, A. swirskii* and *N. cucumeris* survived at 80% RH; no *S. scimitus* female survived when offered eggs of *Z. subfasciatus*, and only 10% of females survived when offered *A. obtectus* eggs. At 75% RH, most females of *B. tarsalis* and *A. swirskii* survived, but no *N. cucumeris* survived. Only *N. cucumeris* consumed *Z. subfasciatus* eggs, resulting in a significant difference in egg mortality compared to the control (Table 1). However, when *A. obtectus* eggs were offered, all predatory mites tested produced significantly higher mortality than that observed in the control, at both 75% and 80% RH.

Table 1. Mean (± SEM) percentage of mite survival and egg mortality when 15 two-day-old *A. obtectus* or *Z. subfasciatus* eggs were offered to one female mite in a small cage (2.5 cm diameter) for 24 h at 28ºC and 80% or 75% RH.

Predatory mites	*Z. subfasciatus*		*A. obtectus*			
	80% RH		80% RH		75% RH	
	Mite survival	Egg mortality	Mite survival	Egg mortality	Mite survival	Egg mortality
Control	-	25.2 ± 2.42b	-	20.5 ± 4.40b	-	19.6 ± 3.46b
B. tarsalis	100	31.7 ± 1.91ab	100	51.8 ± 2.39a	83.3 ± 16.67	45.7 ± 4.24a
A. swirskii	100	34.4 ± 2.84ab	100	40.5 ± 4.06a	90.0 ± 10.00	42.1 ± 1.85a
N. cucumeris	100	40.3 ± 3.43a	88.9 ± 11.11	40.8 ± 4.18a	0	33.5 ± 3.97a
S. scimitus	0	30.5 ± 3.88ab	10 ± 10.00	47.6 ± 1.92a	-	-
		$F_{4, 49} = 3.29$; $P < 0.050$		$F_{4, 49}$ = 11:31; $P < 0.001$		$F_{3, 39} = 11.40$; $P < 0.001$

Values in the same column followed by a different lowercase letter are significantly different (Tukey test, $P < 0.05$).

Effectiveness of predatory mites and larval parasitoids in reducing bruchid populations

Neither predatory mites nor the parasitoid *L. distinguendus* were able to reduce the emergence of *Z. subfasciatus* adults compared to the control treatment. Only the parasitoid *A. calandrae* significantly reduced the *Z. subfasciatus* population when interacting for at least one week ($F = 12.17$; df = 6, 79; $P < 0.001$) (Fig. 1A). As expected, when predatory mites were combined with the parasitoid *A. calandrae*, the mortality of *Z. subfasciatus* was not significantly different than that observed with *A. calandrae* alone. However, immature stages of *A. obtectus* were more susceptible to attack by these natural enemies (F = 64.61; df = 6, 79; $P <$ 0.001). Both parasitoids, *A. calandrae* and *L. distinguendus*, and both predatory mites, *B. tarsalis* and *A. swirskii*, significantly impacted the mortality of *A. obtectus* (Fig. 1B). The combination of predatory mites with the parasitoid *A. calandrae* had a synergistic effect, producing an *A. obtectus* mortality between 52% and 65%. The combination of the parasitoid with *B. tarsalis* was significantly more effective than its combination with *A. swirskii* (Fig. 1B). Parasitoid reproduction was low, with less than 0.7 individuals produced per host.

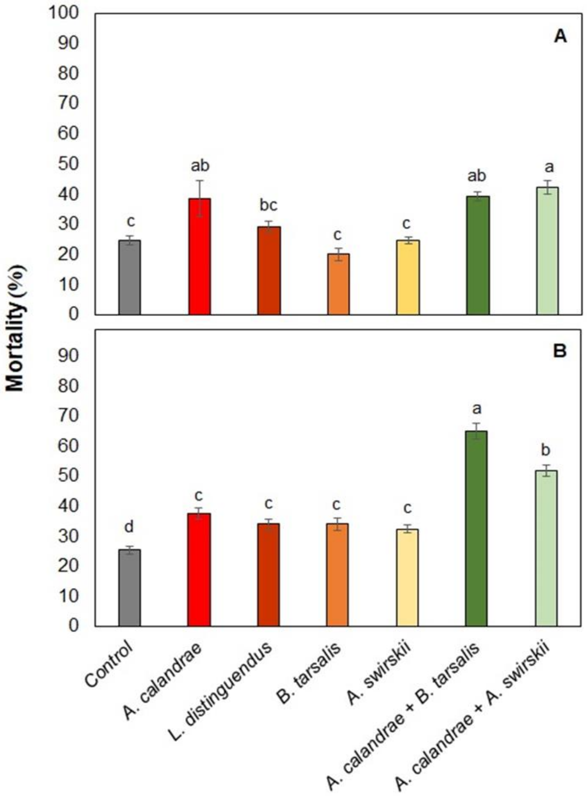

Fig. 1. Percentage (mean ± SEM) of mortality of bruchids when predatory mites and parasitoids were released, alone or in combination, in 100-g containers of beans. The predator-to-prey and parasitoid-to-host ratio was 1:15. A) *Z. subfasciatus*; B) *A. obtectus*. Values followed by a different lowercase letter are significantly different (Tukey test, P < 0.05).

Searching ability of predatory mites and *A. calandrae*

When *A. obtectus* eggs were offered 40 cm deep in a 12-kg pile of beans, both *B. tarsalis* and *A. swirskii* were able to locate and prey upon them. *A. obtectus* mortality was significantly higher than in the control treatment when either predatory mites or a combination with *A. calandrae* were released at the two predator-to-prey ratios tested (*F* = 55.58; df = 8, 53; *P* < 0.001) (Table 2). No significant differences were observed among the predator-to-prey ratios tested; *B. tarsalis* caused *A. obtectus* mortality ranging from 60% to 67% in PVC pipes and polypropylene bags. *Amblyseius swirskii* caused similar bruchid mortality with the high ratio tested in the PVC pipes, but bruchid mortality was significantly lower with a lower ratio. The best results were obtained with the combination of *B. tarsalis* with the parasitoid *A. calandrae*, which resulted in 81% mortality of *A. obtectus* (Table 2).

Table 2. Percentage of mortality (mean ± SEM) of *A. obtectus* when: a) *B. tarsalis* or *A. swirskii* were released at two predator-to-prey ratios or when *B. tarsalis* (2:1 predator-to-prey ratio) was combined with *A. calandrae* (1:15 parasitoid-to-host ratio) in 40-cm PVC pipes filled with 12 kg of dried beans (28 ± 2ºC, 75 ± 5% RH); b) when 9 ml of *B. tarsalis* enriched diet were released (90-180 individuals) in commercial bags containing 25 kg of dried beans (23.5 ± 1.5ºC, 68 ± 10% RH).

Treatments	PVC pipes (12 kg beans)		Commercial bags (25 kg beans)
	15:45 pred: prey	90:45 pred: prey	90-180:45 pred: prey
Control	26.1 ± 1.65d	25.3 ± 2.52d	26.3 ± 0.49d
A. swirskii	41.5 ± 1.94c	60.8 ± 2.67b	-
B. tarsalis	67.2 ± 3.51b	65.8 ± 3.59b	65.7 ± 4.15b
B. tarsalis + *A. calandrae* (1:15 par: host ratio)	-	81.1 ± 3.43a	-

Values followed by a different lowercase letter are significantly different (Tukey test, *P* < 0.05)

Discussion

Two of the predatory mites tested, *S. scimitus* and *N. cucumeris*, had difficulty surviving at high temperatures and low humidity, typical conditions in storehouses. *Stratiolaelaps scimitus* is used as a biocontrol agent of a number of pest species, including *Frankliniella occidentalis* Pergande and *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) under greenhouse conditions (16-25ºC and 24-32ºC with 64-98% and 35-78% RH, respectively) (Wu et al. 2014). *Stratiolaelaps scimitus* survive high temperatures for short periods, but when the temperature is constantly high, as in the present experiment, this predator is unable to survive. *Neoseiulus cucumeris* is often used to control thrips and spider mites on horticultural plants, and it can prey on storage pests such as *E. kuehniella* in a laboratory under controlled conditions (25ºC and 60-70% RH) (Sarwar et al. 2009; Deslise et al. 2015). Although *N. cucumeris* can survive high temperatures (25ºC), our study used an even higher temperature (28ºC), making it difficult for this predator to survive. However, *A. swirskii* was able to survive the high temperatures and low humidity common to orchards in the Middle East. It coexists with whiteflies and tolerates low humidity and high temperatures better than nearly all other species of predatory mite (Nomikou et al. 2001). This species is commonly used for the biological control of thrips and whiteflies in greenhouse crops and feeds on *E. kuehniella* eggs under controlled conditions (25ºC and 65% RH) (Fathipour and Maleknia 2016; Riahi et al. 2017). *Blattisocius tarsalis* was also able to survive the tested conditions; this was expected since this predator is spontaneously present in warehouses and food processing facilities (Dizlek et al. 2019).

The predatory mites *A. swirskii* and *B. tarsalis* were unable to prey on *Z. subfasciatus* eggs. This is probably because the eggs are firmly attached to the bean and have a protective coating once they harden (Southgate 1979); this may impede the predator's perforation of the eggshell. However, both predatory mites were able to prey on *A. obtectus* eggs. Unlike *Z. subfasciatus*, this bruchid species lay eggs in the intergranular space of pulses, so predatory mites do not have to deal directly with the bean. According to Jimenez et al. (2017), the bean testa contain toxic compounds that cause paralysis or antixenosis in bruchid adults and antibiosis and antixenosis in larvae. These toxic compounds, which are associated with bean resistance to pests, can also have adverse effects on biological control agents (Velten et al. 2008). This might explain the differences observed in the predatory capacity of the two mite species on *A. obtectus* and *Z. subfasciatus*. Both predatory mites were able to locate and prey on *A. obtectus* eggs in PVC pipes, indicating that they can locate their prey at a distance of 40 cm in a total volume of 12,566 cm^3 of beans. *B. tarsalis* also demonstrated good potential for reducing *A. obtectus* density in infested commercial bags under warehouse conditions, where it effected a similar suppression rate (65% mortality). Similarly, good dispersion capacity was observed in commercial bags of beans as well, indicating that the mites were able to locate their prey at a distance of 60 cm in a total volume of 46,653 cm^3 of beans. Thus, the release of either of the predatory mites alone seems to hold promise for the biological control of *A. obtectus* eggs.

The two parasitoids tested were able to locate and, to some extent, to reduce *A. obtectus* populations: *A. calandrae* effected a 38% reduction and *L. distinguendus* a 34% reduction from an initial population of 45 eggs. *A. calandrae* was also able to reduce *Z. subfasciatus* populations by up to 39%. However, these reductions in weevil populations were low compared to the reductions these species have been reported to effect with the same weevils in different pulses and/or with other weevils. *Anisopteromalus calandrae* produced up to 95% *A. obtectus* mortality in the cowpea *Vigna unguiculata* L (Fabales: Fabaceae) (Berger et al. 2017). This legume has a rougher surface than the smooth beans used in the present study. *Phaseoulus vulgaris* seeds are a difficult substrate for the movement of parasitoid wasps; their slippery surface does not allow parasitoid females to get a firm grip during drilling and parasitization (Mitsunaga and Fujii 1999; Niedermayer and Steidle 2013). *Anisopteromalus calandrae* was able to reduce *Sitophilus zeamais* (Motschulsky) and *Rhyzopertha dominica* Fabricius populations by more than 95% when released in rice at 28ºC under similar conditions to those used in the present study (Solà et al. 2020). Another factor that may have contributed to the parasitoid's poor performance is the release ratio used in this study. Some authors have argued that weevil control is effective only when parasitoids are introduced in quantities of one parasitoid per ten hosts or higher (Arbogast and Mullen 1990, in corn; Sanon et al. 1998, in cowpeas). However, *A. calandrae* has been used at the same release ratio and under the same environmental conditions as the present study to control *Callosobruchus chinensis* L. in chickpeas, resulting in more than 90% bruchid control (Iturralde-García et al. submitted).

The combination of *A. calandrae* with predatory mites did not improve the parasitoid's efficacy in controlling *Z. subfasciatus*. This is not surprising since the mites were unable to prey on the eggs. However, the combination of *A. calandrae* with *A. swirskii* or *B. tarsalis* improved control of *A. obtectus* (52-65% suppression) when the predators were released in small cages. Control improved even more (81% suppression) when two natural enemies were released in PVC pipes containing 12 kg of beans (Figure 1A, B; Table 2). Improved control has previously been observed with the combined release of multiple natural enemies that attack the host at different developmental stages. The larval parasitoid *A. calandrae* and the predator *Xylocoris flavipes* Reuter (Hemiptera: Lyctocoridae) together effected 95% suppression of *A. obtectus* progeny (Berger et al. 2017); the egg parasitoid *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) and the larval parasitoid *Habrobracon hebetor* Say (Hymenoptera: Braconidae) effected 84% reduction of *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) populations (Brower and Press 1990). In our study, it is important to mention that there was no direct competition between the two natural enemies since one (parasitoids) attacked the beetles at a developmental stage located inside the beans and the other (predatory mites) attacked the prey outside the beans. The combination of *B. tarsalis* with *A. calandrae* can be recommended for the biological control of *A. obtectus* populations. This method should not be a problem in bulk beans, since the dead bodies of parasitoids and predators are almost imperceptible due to the small size of both natural enemies. Furthermore, they can be separated from the beans using standard cleaning procedures.

In conclusion, the predatory mites *A. swirskii* and *B. tarsalis* were able to prey on *A. obtectus* eggs, reducing the bruchid population by more than 60% under both controlled and warehouse conditions. Therefore, they demonstrate good potential as biological agents for controlling this pest. Both parasitoids were moderately effective (34-39% reduction) in suppressing both bruchid populations, but significant improvement (81% reduction) was observed when *A. calandrae* was combined with *B. tarsalis*. The combined use of both natural enemies to control *A. obtectus* seems promising. However, further research is needed on the use of other natural enemies in combination with *A. calandrae* to reduce *Z. subfasciatus* populations.

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CONCLUSIONES

Las conclusiones generales que se extraen de esta tesis doctoral, de acuerdo a los objetivos planteados y resultados obtenidos, son las que se presentan a continuación:

Capítulo 1

1. La sorción de CO_2 producida por el garbanzo al ser envasado en paquetes de plástico de 500 g con atmósferas modificadas con un contenido de CO_2 superior al 50% tiende a incrementar al aumentar la concentración inicial de CO_2 aplicada.

2. El tiempo para alcanzar el equilibrio de sorción del CO_2 se reduce al incrementar las proporciones de llenado del envase con garbanzos.

3. La presión negativa (vacío) producida por la sorción del CO_2 por el garbanzo tiende a incrementar al aumentar la proporción de llenado del envase por la leguminosa.

Capítulo 2

4. Al aplicar las atmósferas modificadas con alto porcentaje de CO_2 (>50%) en paquetes de 500 g con una proporción de llenado del 4% con garbanzo infestado, la mortalidad de los diferentes estados de desarrollo de *R. dominica* tienen diferentes niveles de susceptibilidad a la exposición con el $CO_{2.}$ Las pupas son el estado de desarrollo más tolerante, seguido de los huevos y adultos con una tolerancia intermedia, y las larvas son el estado más susceptible.

5. El aumento de la sorción del CO_2 por el garbanzo al aumentar la proporción de llenado del envase reduce el contenido de CO_2 que se encuentra libre en el envase y en los intersticios entre granos, y esta reducción en el CO_2 libre sólo altera la mortalidad de los estados del desarrollo de *R. dominica* que se localizan en el exterior del grano. Así:

   a. La mortalidad de los huevos y los adultos de *R. dominica* (localizados en el exterior del grano) por la exposición al CO_2 disminuye al incrementar las proporciones de llenado (del 4% al 96%) del envase con garbanzos.

   b. La mortalidad de las larvas y pupas (localizados en el interior del grano) por la exposición al CO_2 no varía al incrementar las proporciones de llenado (del 4% al 96%) del envase con garbanzos.

Capítulo 3

6. Los ácaros depredadores *B. tarsalis* y *A. swirskii* consumen muy pocos huevos de *C. chinensis* en condiciones controladas (28 ± 2º C, 75 ± 5% HR), no resultando muy prometedores como agentes de control biológico para esta especie.

7. Los parasitoides de larvas *A. calandrae* y *L. distinguendus* son muy efectivos reduciendo las poblaciones de *C. chinensis* por encima del 80 % con respecto a la población control sin parasitoide.

8. Ambos parasitoides se dispersan bien sobre el grano y son capaces de localizar y parasitar las larvas del huésped a una profundidad de hasta 150 cm en una columna de 25 kg garbanzos.

9. Las relaciones *A. calandrae: C. chinensis* de 1:30 tiene una alta eficiencia de control tanto en condiciones controladas como en condiciones de almacén en sacos.

Capítulo 4

10. Los ácaros depredadores *B. tarsalis*, *A. swirskii*, *N. cucumeris* y *S. scimitus* son capaces de consumir huevos de *A. obtectus*, pero solamente *B. tarsalis* y *A. swirskii* pueden sobrevivir y ser activos a humedades del 70%.

11. *B. tarsalis* y *A. swirskii* tienen un buen potencial como agentes de control biológico sobre *A. obtectus* tanto bajo condiciones controladas como de almacenamiento.

12. Los parasitoides *A. calandrae* y *L. distinguendus* reducen moderadamente (34-39%) las poblaciones de *A. obtectus* con respecto a la población control sin parasitoide.

13. La combinación del parasitoide *A. calandrae* con los ácaros depredadores *B. tarsalis* o *A. swirskii* tienen un efecto sinérgico aumentando hasta un 80% la reducción las poblaciones de *A. obtectus* con respecto a la población control sin parasitoide.

14. *B. tarsalis* y *A. swirskii* no consumen cantidades apreciables de huevos de *Z subfasciatus* y *A. calandrae* es el único parasitoide que produce una cierta mortalidad sobre la población de *Z. subfasciatus.*

PUBLICACIONES

Publicaciones en revistas revisadas por pares

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