HIGHLIGHTS

- Control alternatives are needed for S. zeamais given its high resistance to pesticides
- *Anisopteromalus calandrae* can effectively limit the growth of the weevil population
- *A. calandrae* can locate weevil larvae even down in the bottom of 500kg bags of paddy rice

Graphical Abstract

A. calandrae can parasitize larvae of weevils located in the bottom of a 500kg bags of paddy rice

Releases of the parasitoid *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae) can control *Sitophilus zeamais* (Coleoptera: Curculionidae) in big bags of paddy rice

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Abstract

Sitophilus zeamais is a key pest in stored rice in Spain and other Mediterranean countries. Today, it is mostly controlled with a few pesticides, such as pyrethroids or phosphine. Apart from the problem of pesticide resistance, this can lead to the accumulation of toxic residues in the stored rice. Therefore, alternative control methods are needed. Biological control with the parasitoid *Anisopteromalus calandrae* is a feasible and sustainable alternative since it is very effective at limiting the growth of the weevil population. In this study, we evaluated the dispersal capacity and effectiveness of this parasitoid in controlling *S. zeamais* larvae located deep in 500 kg bags of paddy rice during two seasons, summer and autumn. The parasitoid was easily able to reach the bottom of the bags (1 m) in both seasons. At the released parasitoid-to-host ratio, the parasitoid was also able to limit weevil population growth by around 60% compared to the control treatment, based on the released parasitoid-to-host ratio, indicating that it offers an effective alternative control method.

Keywords: Biological control, grain weevil, larval parasitoid, parasitoid dispersal, stored products.

1. Introduction

More than 778,000 metric tons of rice (*Oryza sativa* L, Poaceae) were produced in Spain in 2019, and it is among Spain's main grain crops (FAOSTAT, 2019). When stored, it is frequently attacked by a range of insect pests; of these, *Sitophilus* spp. (Coleoptera: Curculionidae) are the most concerning species, causing significant quantitative and qualitative losses (Carvalho et al., 2013; Pascual-Villalobos et al., 2006; Riudavets et al., 2002, Trematerra et al., 2004). In warmer zones, such as the Mediterranean region, the maize weevil, *S. zeamais* (Motschulsky), is generally more abundant in rice than the other two *Sitophilus* species, *S. oryzae* (L.) and *S. granarius* (L.) (Carvalho et al., 2012). Conventional insecticides remain the dominant pest-management approach for stored-product insects, particularly in warmer climates. However, it is important to develop alternatives to chemical control methods due to the problem of pesticide resistance, such as to the pyrethroids commonly used to control the grain weevils *S. granarius* and *S. zeamais* (Correa et al., 2011; Kavallieratos et al., 2015).

Biological control could offer an effective alternative for preventing insect populations from reaching pest status. Natural enemies offer several benefits: They leave no toxic residues on stored commodities, pests 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 (Riudavets et al., 2018, 2020; van Lenteren et al., 2020). In addition, the environmental conditions in storage facilities are more stable than those in open fields or greenhouses, where biological control has already been widely adopted.

Anisopteromalus calandrae (Howard) (Hymenoptera: Pteromalidae) is one of the most promising natural enemies for regulating weevils. This species is a generalist, solitary ectoparasitoid that attacks late-instar coleopterans, such as *Sitophilus* spp., that develop concealed inside a grain kernel (Smith, 1992, 1993). Several studies have reported the significant potential of this parasitoid to suppress weevil populations in stored wheat (Mahal et al., 2005,), maize (Wen and Brower, 1994), and rice (Belda and Riudavets, 2012; Chaisaeng et al., 2010; Nam et al., 2011).

A previous study using small containers (2 kg) of brown rice showed that *A. calandrae* significantly limited *S. zeamais* population growth (up to 99% compared to the control treatment) and associated damage to rice grains (insect-damaged kernels, frass production, and mold presence), at 23ºC and 28ºC (Solá et al., 2020). However, no data are available on the effectiveness of this parasitoid on a larger scale. One important factor to consider when increasing the experimental scale is the dispersion capacity of the females. *Lariophagus distinguendus*, another pteromalid parasitoid of stored weevil pests, can locate larvae of *S. granarius* in a sitting open pile of stored wheat grain of 4m wide and 4m tall (Steidle and Schöller, 2002). *Anisopteromalus calandrae* females can disperse in a 2.2 m wheat column to locate *S. oryzae* larvae (Press, 1988); they can also disperse within chickpeas to find their host, *Callosobruchus chinensis* (Iturralde-García et al., 2020). Pulses are bigger than rice kernels, so the spaces between them are also bigger, allowing the female parasitoid to easily move through them. In paddy rice, stored rice prior to being milled, the spaces between kernels are smaller than those between individual large beans, so the movement of female parasitoids could be more difficult. We hypothesized that the parasitoid would be able to disperse and parasitize hosts up to a depth of 1.5 m in paddy rice. The aim of the present study was to evaluate the capacity of *A. calandrae* to find and parasitize *S. zeamais* larvae in paddy rice stored in large bags, a common method of rice storage. An initial study was first conducted to test the ability of the parasitoid to locate its host at different depths inside polyvinyl chloride tubes (PVC), and then a subsequent experiment was done with parasitoids released in large commercial bags, both filled with paddy rice.

2. Methods and materials

Sitophilus zeamais and *A. calandrae* populations were obtained from warehouses in Tarragona, Spain and maintained in a climatic chamber under controlled environmental conditions of temperature (28 ± 2ºC), relative humidity (75 ± 5%) and photoperiod (16h:8h light-to-dark). *S. zeamais* was reared on rice (japonica cv. Antara) and *A. calandrae* on rice infested with *S. zeamais* larvae. To obtain rice samples infested with second- and third-instar *S. zeamais* larvae, we released 225 *S. zeamais* adults in sets of 550 g of rice for one week and incubated them in the climatic chamber for an additional week.

2.1. PVC pipes. This experiment was conducted in PVC pipes with an internal diameter of 20 cm and lengths of 40 cm, 100 cm, and 150 cm. The pipes were placed vertically and filled to the top with paddy rice, containing either 6.8 kg, or 17.0 kg, or 25.5 kg, respectively. A stainless-steel screened cylindrical cage (7 cm high, 5 cm internal diameter and 1x2.5 mm screen) containing 25 g of rice infested with second- and third-instar *S. zeamais* larvae was placed at the bottom of each PVC pipe. A fourth treatment was tested with the tallest pipe (150 cm): Three cylindric cages filled with 8.3 g of rice infested with second- and third-instar *S. zeamais* larvae were simultaneously placed at depths of 40 cm, 100 cm, and 150 cm in the same PVC pipe.

Next, three male-female pairs of *A. calandrae* adults (0–7 days old) were released on the surface of the grain in all treatments, together with a small tube containing sugary water, and the pipes were sealed with fabric mesh. After a week in a climatic chamber maintained at 28ºC, the PVC pipes were poured off, the parasitoids were removed, and the screened cylindric cages containing the infested rice were isolated in plastic containers. The emergence of adult *S. zeamais* and/or parasitoids was then recorded twice, within a three-week lapse. Six replicates were conducted for each pipe height. For the control treatment, eight 710 mL plastic containers with 25 g of rice infested with second- and third-instar *S. zeamais* larvae were placed outside the PVC pipes and maintained in the same climatic conditions. In addition, 50 *S. zeamais* and 50 *A. calandrae* adults (N = 3 replicates) were weighed to estimate the individual adult weight of each species; this estimate was used to determine the biomass of each species in each replicate.

2.2. Big commercial bags. This experiment was conducted in nine large, woven polypropylene bags that were maintained under ambient conditions in a warehouse during summer and autumn. The bags measured 90 cm x 90 cm x 110 cm (height) and were filled with 500 kg of paddy rice. The same type of stainless-steel cylindric cages were used as in the first experiment. The cages contained either 25 g of rice infested with second- and third-instar *S. zeamais* larvae and 35 g of non-infested rice (high pest density treatment [HD]) or 5 g of infested rice and 55 g of non-infested rice (low pest density treatment [LD]). In both cases, the cages were placed at the bottom of the bag. Three pairs of *A. calandrae* adults (0–7 days old) were released on the surface of the grain, together with a tube with sugary water to provide food for the females. Next, all the bags were closed with a rope and covered with a polyester mesh. After one week, the screened cages were removed from the bags, and the rice inside the cages was incubated at 25ºC. Two rounds of experiments were carried out, one in mid-July to early August and another in late September. Two control treatments were placed outside the bags. The first, weevils only, with 25 g or 5 g of rice infested with second- and third-instar *S. zeamais* larvae and complemented with 35 g or 55 g of non-infested rice, respectively; all rice was placed inside 710 mL plastic containers. The second control treatment, parasitoids + weevils, was conducted in the same type of containers and used the same pest density, but three pairs of *A. calandrae* (0–7 days old) were added to each container. In the first round, nine replicates were conducted for each pest density and for the two control treatments. In the second round, five and four replicates of the low and high host density were conducted, respectively, as well as the same number of replicates for both controls (Table 2).

2.3. Data analysis. The number of *S. zeamais* and parasitoid adults, the percentage of reduction in host emergence, and the total biomass of hosts and parasitoids when *A. calandrae* was released in the PVC pipes with host larvae at different depths were analyzed using a one-way analysis of variance (ANOVA). Post-hoc comparisons were conducted using the Tukey correction for multiple comparisons. The proportion of *A. calandrae* females emerging from *S. zeamais* at different depths were determined using Student's *t*-test. In the big bags experiment, only the data from the control treatments and from the bags from which pests or parasitoids emerged were included in the analysis. The percentage of reduction in pest emergence in the summer and autumn and the two host densities in the parasitoid control treatment and in the big bags were analyzed using a two-way ANOVA. All statistical analyses were conducted using JMP 14.2.0 (SAS Institute, 2018).

3. Results

3.1. PVC pipes. *A. calandrae* was able to parasitize *S. zeamais* larvae at all depths tested in the PVC pipes (Table 1). Parasitism was similar at all depths; there were no significant differences in the emergence of pest or parasitoid individuals at any depth, even when the host larvae were offered simultaneously at three different depths (F = 3.62; df = 4, 31; P = 0.01). The parasitoid sex ratio was significantly male biased at 40 cm and in the mixed heights treatment (Table 1). Pest reduction compared to the pest control treatment was high and similar among all depths, ranging from 90% to 93% (F = 1.25; df = 1, 25; P = 0.319) (Figure 1A). Based on pest and parasitoid biomass, a similar drastic reduction compared to the control was observed at all depths tested: While the total biomass in the control treatment was 56.3 ± 3.30 mg, in the parasitoid treatments the mean biomass ranged from 8.0 mg to 9.6 mg (F = 88.67; df = 4,31; P < 0.0001). The mean pest biomass ranged from 3.9 mg to 5.8 mg, and that of the parasitoid ranged from 3.8 mg to 4.7 mg of fresh weight (Figure 1B).

3.2. Big commercial bags. Temperatures in the warehouse during the summer test ranged from 23.9 ºC to 28.6ºC, with a mean of 26.2 ± 0.08ºC. During the autumn test, the temperature ranged from 18.1ºC to 22.3ºC, with a mean of 20.2 ± 0.08ºC. In this experiment, female parasitoids were able to move freely through the rice in the big bags. They were also able to find and parasitize the host larvae at a depth of 100 cm (Figure 2). The mean infestation of the rice offered to the parasitoid in the summer test was 11.4 ± 1.49 larvae in the LD treatment and 63.7 ± 3.08 larvae in the HD treatment (Figure 2). In the autumn test, a higher mean infestation of rice was offered to the parasitoid: 23.4 ± 1.86 larvae in the LD treatment and 79.5 ± 3.57 larvae in the HD treatment (Figure 2).

In the summer test, pest adults were recovered from all replicates of the parasitoids + weevils' treatment, and parasitoids were recovered from 89% (eight of nine replicates) of the LD treatment. Pest adults were recovered from 89% (eight of nine replicates) of the large LD bags and from all HD bags, while parasitized samples were found in 78% (seven of nine replicates) of the LD bags and in 44% (four of nine replicates) of the HD bags (Table 2). The effective parasitoid-to-host ratio was 1:4 to 1:21 for the LD and HD treatments.

In the autumn test, pest and parasitoid adults were recovered from all replicates of the parasitoids + weevils' treatment; pest adults were recovered from all the bags, while parasitoids were recovered from 80% (four of five replicates) of the LD bags and from all HD bags. The effective parasitoid-to-host ratio was 1:8 and 1:30 for the LD and HD treatments.

In the parasitoids + weevils' treatment, *A. calandrae* was able to significantly reduce the growth of the weevil population (F = 43.35; df =3, 23; P < 0.0001). This reduction was affected by the season and the host density (season F = 120.0; P < 0.0001; density F = 15.76; df = 3, 23; P = 0.0006); there was no interaction between these two factors (F = 3.15; df = 1; P = 0.089) (Figure 3). Pest reduction reached 97% in summer but was lower in autumn; reduction was higher (87%) in the LD treatment than in the HD treatment. A slightly lower reduction (up to 62%) of pest emergence was observed in the samples in the big bags; here, reduction was similar in summer and autumn and at the two tested pest densities (F = 0.35; df = 3, 15; P = 0.790) (Figure 3).

4. Discussion

Female parasitoids were able to move freely among the rice kernels up to a depth of 1.5 m from the release point. They were also able to locate the host larvae in the PVC tubes, since no differences in parasitism were observed among all the depths tested. Even when hosts were offered simultaneously at three depths, parasitism was similar at the top and at the bottom of the tube. Comparable results were obtained when females of *A. calandrae* were released in a similar setting (PVC tubes) with *Callosobruchus chinensis* larvae as the host and chickpeas (*Cicer arietinum* L, Fabaceae) as the commodity (Iturralde-García et al., 2020). In that study, *A. calandrae* females were able to locate the host and efficiently parasitize it, also limiting the growth of the pest population by 90% or more compared to the control treatment without parasitoids. This high efficacy could be partly explained by the high temperature (28ºC) used in that experiment, an optimal condition for the development of *A. calandrae* (Chun et al., 1992; Menon et al., 2002; Smith, 1992, 1993, 1994). These findings reveal the high potential of this parasitoid species for controlling rice weevils.

Similar efficacy has been described for *S. zeamais* and other *Sitophilus* species (Chaiseng et al., 2010), as well as for other weevils, such as *C. chinensis* (Iturralde-García et al., 2020).

One drawback commonly discussed in the use of biocontrol with macrobials (arthropods) in stored commodities is that grain owners are reluctant to introduce beneficial arthropods to their warehouses since any type of arthropod is considered grain contamination. The reality, however, is that even supposedly clean commodities contain undetected arthropods (Trematerra et al., 2011). Therefore, it is unrealistic to expect that there will be no arthropods present in stored foods. To limit pest development, parasitoids must use hosts to reproduce, and, at the same time, they will produce some parasitoid biomass. However, this biomass is much less than that produced by weevils, as demonstrated in the present experiment with PVC tubes (up to 5 mg of fresh parasitoid weight compared to 53 mg of pest weight in the control treatment). Furthermore, these insects are tiny and dry out quickly, and after a few days, they are very difficult to distinguish from dust particles with the naked eye. This is not the case for the pest.

The male-biased sex ratio observed in two treatments (40 cm and mixed depths) may be related to the size of the offered larvae. Since immature *S. zeamais* develop within the rice grain, the proportion of offered hosts and their developmental stages cannot be precisely evaluated and must be estimated. *A. calandrae* females choose large host larvae to produce females and smaller larvae to produce males (Choi et al., 2001; Ji et al., 2004).

In the big bags, female parasitoids were able to move successfully between the paddy rice kernels and parasitize *S. zeamais* larvae located at the bottom of the bags at a depth of 1 m from the surface. A similar reduction in pest emergence was observed in all bags (53% to 63%) compared to the weevils' treatment, despite the different parasitoid-to-host ratios, which were 1:4 and 1:8 in the LD treatment and 1:21 and 1:27 in the HD treatment. However, a higher reduction in pest emergence was observed in the PVC tubes, where the parasitoid-to-host ratio (1:12) was between those of the LD and HD treatments.

There were two main differences between our two experiments: the mean temperatures and the amount of rice that females had to explore to find the larval host. The PVC tubes were placed in a climatic chamber with a mean temperature of 28ºC; the mean temperature in the warehouse where the bags were placed was 26.7ºC in the summer and 20.2ºC in the autumn.

However, the PVC tubes contained a maximum of 25 kg of rice, while the bags contained 500 kg, 20 times more rice. *A. calandrae* adults find hosts by following olfactory and other stimuli produced by the host larvae (Belda and Riudavets, 2010; Ghimire and Phillips, 2008). Therefore, the odor of the host was more diluted in the large bags than in the PVC tubes, giving female parasitoids a weaker signal for locating host larvae. The fact that some parasitoids failed to reproduce in some bags in the summer may be because they escaped from the bags when they were released or because they died before they were able to parasitize.

The temperatures in the warehouse were more appropriate for the parasitoid's development in summer than in autumn. This was reflected in the reduction of pest development in the parasitoid control treatment, which was significantly lower in autumn than in summer. However, pest reduction in the bags was similar in summer and autumn. This is likely due to the more stable temperatures inside the rice: Measurements at the beginning of December showed that as the temperature dropped in the warehouse (13ºC to 14ºC), it remained warm at a depth of 1 m inside the bags (16ºC to 17ºC).

In conclusion, *A. calandrae* is a promising biological agent for controlling *S. zeamais* in paddy rice stored in big bags. Parasitoid females can move through the rice kernels and locate the host larvae even at the bottom of the bags to parasitize them. Even better weevil control may be achieved by increasing the parasitoid release rate and/or by releasing the parasitoid several times to span the parasitoid time lapse of action. Further studies should be conducted to explore these possibilities.

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Authors declare that they have No competing interests.

Table captions

Table 1. Number (mean ± SEM) of *S. zeamais* and *A. calandrae* adults and percentage of *A. calandrae* females that emerged (mean ± SEM) from samples with the host larvae located at three depths in PVC tubes (20 cm diameter) filled with paddy rice. N = 6 for treatments with parasitoids; n = 8 for the control with no parasitoids.

Table 2. Number of replicates in which the pest (*S. zeamais*) and the parasitoid (*A. calandrae*) were recovered from the parasitoids + weevils treatment and from the big bags. The table also shows the parasitoid-to-host ratios for the summer and autumn tests.

Figure captions

Figure 1. A) Mean (± SEM) percentage of reduction in *S. zeamais* emergence at different depths in the PVC pipes vs. the control treatment (without parasitoids). **B)** Mean (± SEM) insect biomass (mg) of emerged *S. zeamais* (blue bars) or *A. calandrae* (dotted orange bars) in treatments where host larvae were provided at three different depths (n = 6) or in the control treatment (no parasitoids, n = 8). Treatments with the same lowercase letter are not significantly different (Tukey's test, $P < 0.05$).

Figure 2. The number of adults (*S. zeamais*, blue bars, and *A. calandrae*, orange bars) (mean ± SEM) that emerged in the weevils, in the parasitoids + weevils (samples outside the large bags) and in the big bags (samples at the bottom of the bag). The samples contained 25 g (HD) or 5 g (LD) of infested rice, and the parasitoid was released in summer and in autumn (N = 4 to 9).

Figure 3. Mean (± SEM) percentage of reduction in the emergence of *S. zeamais* in the large bags and in the parasitoids + weevils treatment *vs*. the weevils treatment.
*Denotes significant differences between factors ($P < 0.05$).

Figure 1.

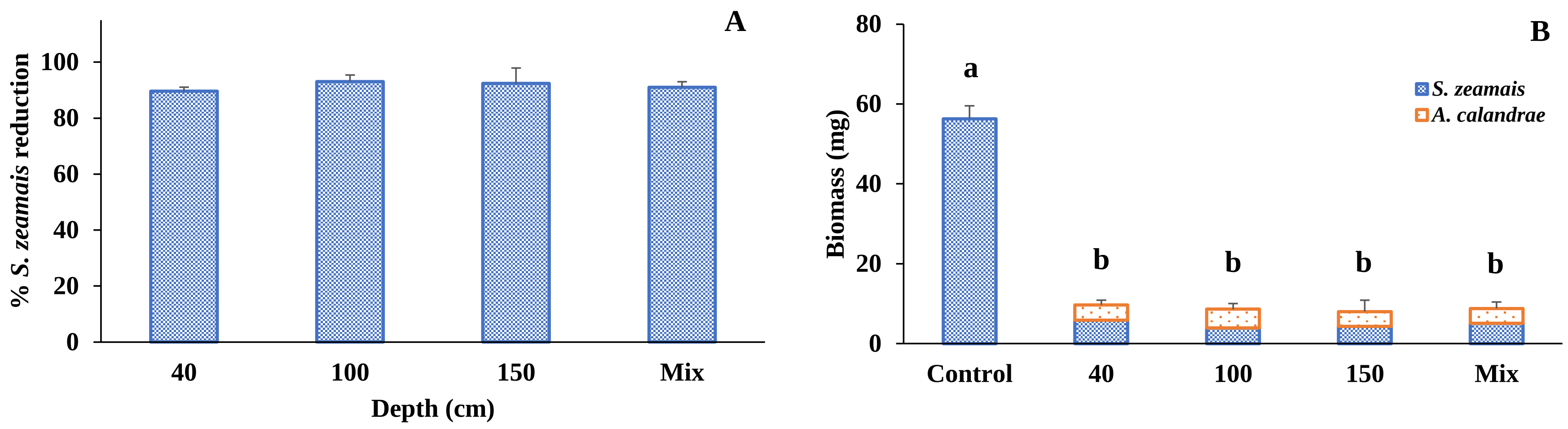
A
% S. zeamais reduction
0
20
40
60
80
100
40
100
150
Mix
Depth (cm)
B
Biomass (mg)
0
20
40
60
80
a
b
b
b
b
S. zeamais
A. calandrae
Control
40
100
150
Mix

Figure 2

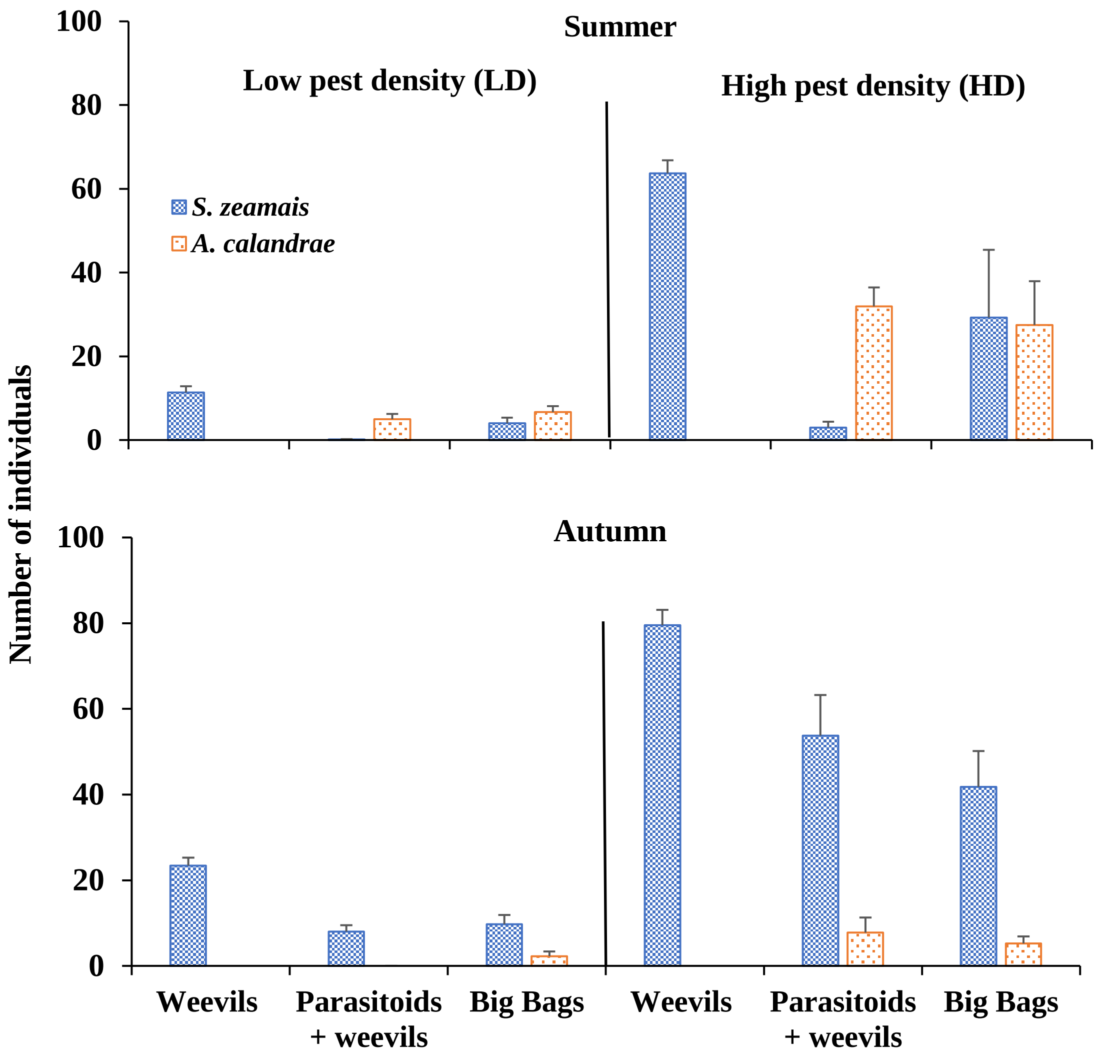
Summer
Low pest density (LD)
High pest density (HD)
S. zeamais
A. calandrae
Autumn
Number of individuals
0
20
40
60
80
100
Weevils
Parasitoids + weevils
Big Bags
Weevils
Parasitoids + weevils
Big Bags

Figure 3

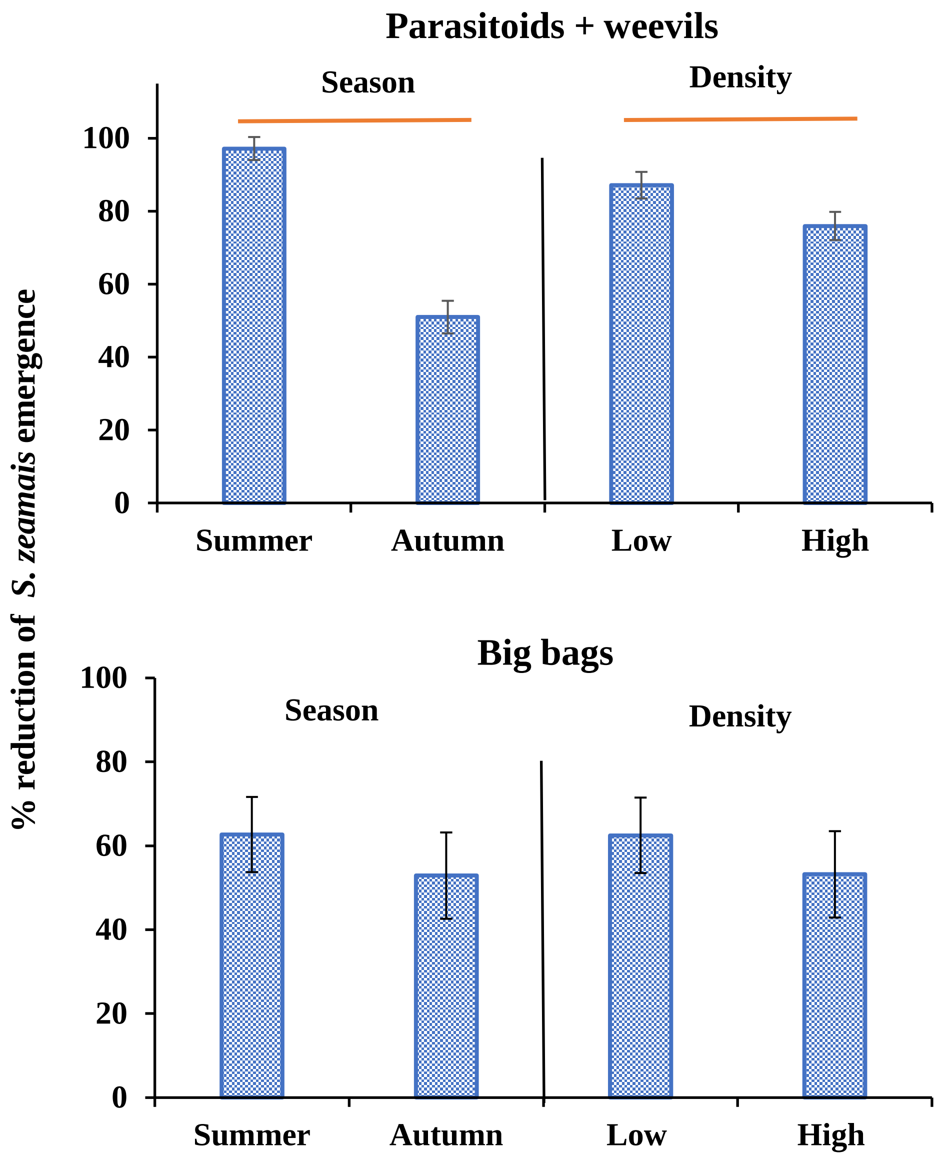
Parasitoids + weevils
Season
Density
100
80
60
40
20
0
Summer
Autumn
Low
High
% reduction of S. zeamais emergence
Big bags
Season
Density
100
80
60
40
20
0
Summer
Autumn
Low
High

Table 1.

Treatment	Nº emerged adults		% *A. calandrae* females	Student's *t*-test	
	S. zeamais	*A. calandrae*		*t*	*P*
Control	24.0 ± 1.4a				
40 cm	2.5 ± 0.3b	12.0 ± 3.3a	23.9 ± 5.7*	3.90	< 0.05
100 cm	1.7 ± 0.6b	14.8 ± 1.8a	34.8 ± 9.4	1.55	0.18
150 cm	1.8 ± 1.3b	11.7 ± 3.3a	37.1 ± 9.3	1.53	0.19
Mixed depths	2.2 ± 0.5b	11.7 ± 2.7a	31.3 ± 4.7*	4.11	< 0.05
	F = 106.80	F = 0.289			
	df = 4, 27	df = 3, 20			
	P < 0.0001	P = 0.833			

Values in the same column followed by the same letter are not significantly different (Tukey test, P > 0.05).
* Denotes a significant deviation from 1:1 female-to-male (Student's *t*-test).

Table 2.

Season	Pest density	Nº of parasitoids + weevils replicates with		Nº of big bag replicates with		Parasitoid-to-host ratio
		S. zeamais	*A. calandrae*	*S. zeamais*	*A. calandrae*	
Summer	Low	9	8	8	7	1:4
	High	9	9	9	4	1:21
Autumn	Low	5	5	5	4	1:8
	High	4	4	4	4	1:30

N = 9 for LD and HD in summer, N = 5 for LD in autumn, and N = 4 for HD in autumn for both controls (pest and parasitoid) in the large bags.

Authors declare that they have No competing interests.

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