

Resistance of important bean genotypes to the Mexican bean beetle [*Zabrotes subfasciatus* (Bohemann)] during storage and its control with chemical synthetic and botanical insecticides**Carlos E.A. Luz¹, Tamiris A. Araujo¹, Arthur V. Ribeiro¹, Cristina S. Bastos^{2*}, Jorge B. Torres³, Yann S.T. Krieger³**¹Departamento de Entomologia (DDE), Universidade Federal de Viçosa (UFV), Campus Universitário s/no., 36570-000, Viçosa, MG, Brazil²Universidade de Brasília (UnB), Faculdade de Agronomia e Medicina Veterinária (FAV), Instituto Central de Ciências Ala Sul (ICC-SUL), Campus Darcy Ribeiro, 70910-900, Asa Norte, Brasília, DF, Brazil³Universidade Federal Rural do Pernambuco (UFRPE), Departamento de Agronomia-Entomologia (DEPA), 52171-900, Dois Irmãos, Recife, PE, Brazil***Corresponding author: cshetino@unb.br; cshetino@gmail.com****Abstract**

During storage, beans can be infested with many insect-pests including *Zabrotes subfasciatus*, a key pest of these crops. This study aimed to identify bean genotypes demonstrating antixenosis and/or antibiosis to *Z. subfasciatus* and to test their integration with chemical control. The ultimate goal of assessment was to distinguish whether genotypic and insecticidal factors can provide effective beetle control. The tested genotypes were (a) *Phaseolus vulgaris* group: CCB, BSCB, BCB and PCB; (b) *Vicia faba* group: YBB, WBB and SBB; and (c) *Vigna unguiculata* group: C and GC. Initial assays were run to select genotypes (without insecticide treatment) that would be further tested with insecticides. Final assays included genotypes with varying degree of antibiosis and antixenosis treated with a neem formulation (Natuneem®) and distilled water (control) plus deltamethrin (Decis®) which latter was used only in the final antibiosis assay. The insecticides were used at the rates of 3 and 0.1 mL of Natuneem® and Decis®, respectively, per 30 mL of distilled water. There were no differences in preference of *Z. subfasciatus* adults among non-treated genotypes (initial assays), although neem-treated genotypes altered the preference and reduced infestation from 40.54-100% (final assays). In antibiosis tests, oviposition and density of emerged adults were reduced among C and SBB, and SBB also reduced the weight of emerged adults. Insecticides reduced oviposition in 53-100% and yielded half to five-fold fewer emerging insects weighting 35%-40% less in antibiotic genotypes. SBB was the most antibiotic genotype and this and other genotypes possessing antibiosis had a synergistic effect with neem or deltamethrin.

Keywords: *Zabrotes subfasciatus*, antibiosis, antixenosis, neem, deltamethrin.**Abbreviations:** BCB_Black Common Bean; BSCB_Brown Spotted Common Bean; CCB_Carnival Common Bean; C_Cowpea; GC_Greenly Cowpea; PCB_Purple Common Bean; SBB_Spotted Broad Bean; WBB_White Broad Bean; YBB_Yellow Broad Bean.**Introduction**

The common bean (*Phaseolus vulgaris* L. Fabaceae) possesses high protein content (Hosfield, 1991) and is a dietary staple in the Americas and Africa countries. The broad bean (*Vicia faba* L. Fabaceae) and cowpea (*Vigna unguiculata* L., Walp. Fabaceae) are also commonly consumed in northeastern Brazil. They serve as an alternative protein source to the Brazilian population, reducing the nation's dependence on common beans (Frota et al., 2008). Together, these beans have a special importance in the diet of Brazilians and throughout the tropics due to their low cost of production compared with animal protein. The protein content in these Fabaceae species varies from 16% to 33% (Frota et al., 2008). Appropriate storage helps to reduce the effects of seasonality and variation in prices over the growing season and to maintain the quality of the seeds over time (Brackmann et al., 2002). However, it can also increase the chances of pest infestation and dissemination. The Mexican bean beetle (MBB), *Zabrotes subfasciatus* (Bohemann) (Coleoptera: Chrysomelidae: Bruchinae), is a key pest of stored beans, attacking beans belong to the genera *Phaseolus*,

Vicia and *Vigna* (Hill, 2002). In Brazil, MBB also infests other types of beans such as *Vicia* and *Vigna* and other legumes such as peas (*Pisum sativum* L. Fabaceae) probably because of the presence of a great number of digestive proteinases that exist in the insect's midgut (Magalhães et al., 2007). Infested seeds lose mass, quality and nutritional value, the latter two due to the presence of frass, eggs and dead insects in the product. In Brazil, losses attributed to this pest can vary from 7% to 15% of the seeds, reaching up to 40% in the northeast of the country (Mazzoneto and Vendramim, 2002). The control of this beetle is mainly insecticide-based, involving the use of fumigant (aluminum and magnesium phosphide) and contact (deltamethrin) products, which together, comprise all of the molecules registered for this purpose (Brasil, 2017). Although the control level achieved by these chemicals is satisfactory, the limited amount of registered molecules increases the risk of resistance of insects to these products. Also, toxicological issues limit their widespread use (Gutierrez et al., 1981). An alternative approach to reduce this array of problems is the adoption of

resistant cultivars. Such an approach may have several advantages, including seed cost that partially encompasses the cost of arthropod control, reduction in insecticide costs and residues, higher net return per dollar invested and synergistic effects with other traditional pest management tactics, to mention only a few (Smith, 2005). Some previous studies have considered the exploitation of genotypes possessing variable degrees of arcelin (Acosta-Gallegos et al., 1998; Barbosa et al., 2000; Moraes et al., 2011). However, none of them have considered the resistance of different groups of beans belonging to different plant genera to this pest. Another possibility that should be considered in the pest control is the use of plant extracts or formulations possessing insecticidal activity, such as neem-based (*Azadirachta indica* A. Juss. Meliaceae) extracts or formulations. Commercial formulations are already available in Brazil and are currently in use for the control of field insect-pests (Brasil, 2017). One of the components of neem-based formulations, azadirachtin-A, which has known insecticidal properties, degrades in light after 2.47 days, on average, on foliar surfaces (Johnson et al., 2003). Such formulations have not yet been tested against pests of stored products and their effectiveness under the prevailing conditions of stored environments (i.e., darkness) is of particular interest. However, the relative effectiveness of implementing control measures individually or in combination should be taken into consideration. The synergistic effects of combining pest control measures, such as using both Mexican bean beetle-resistant varieties and azadirachtin formulations, may increase pest mortality rates and improve the efficacy of pest control. The goal of this study was to select varieties of common beans, broad beans and cowpeas that have antixenosis or antibiosis resistance to the Mexican bean beetle and examine whether using bean genotypes with some levels of resistance in combination with neem and deltamethrin-based chemical formulations can provide more effective control of this key pest.

Results

Non-preference

Selection of genotypes for further tests (initial assay)

Bioassays run to select genotypes that would be further tested with neem-based formulation showed that the interaction of genotypes and time of evaluation was not significant according to repeated measures analysis of variance ($F_{40, 192} = 1.20$, $P = 0.25$). Additionally, there was no significant effect of the genotypes ($F_{5, 24} = 0.92$, $P = 0.49$). However, although cowpea (0.85 ± 0.04 adults) and greenly cowpea (0.94 ± 0.05 adults) had lower densities of *Z. subfasciatus* per 20 g of seeds, black common bean (1.12 ± 0.18 adults), purple common bean (1.07 ± 0.07 adults) and white broad bean (1.06 ± 0.07 adults) had higher densities. Because of the absence of statistical significance and need to select the genotypes that should be included in subsequent assays, we chose the most and least preferred genotypes, based on differences on the numerical density of adult insects.

Neem-treated seeds tests (final assays)

Based on the results of the assays to select genotypes that should be further tested with neem formulation, three additional antixenosis bioassays were performed, in which we tested the following genotypes: black common bean (BCB), cowpea (C) and white broad bean (WBB) in the first bioassay; black common bean (BCB), cowpea (C) and purple

common bean (PCB) in the second; and black common bean (BCB), greenly cowpea (GC) and cowpea (C) in the third.

In the first bioassay (Assay 1), we detected significant differences in the density of adults for the last two evaluation times (36 h: $\chi^2 = 7.49$, $p < 0.0236$; 48 h: $\chi^2 = 7.62$, $p < 0.0222$). At 36 and 48 h from the beginning of the assay, white broad bean (WBB) and black common bean (BCB) had the greatest densities of *Z. subfasciatus*, while cowpea (C) had the lowest density (Fig 1A). However, the mean density of *Z. subfasciatus* across all evaluation times showed that while white broad bean (WBB) was most preferred (149 adults), black common bean (BCB) (72 adults) and cowpea (C) (49 adults) were the least preferred ($\chi^2 = 28.65$; $p < 0.0001$). In the second antixenosis bioassay (Assay 2), the only significant effect detected was that associated with the treatment of seeds with neem. The neem-treated seeds were significantly less preferred by *Z. subfasciatus* (i.e., had lower densities of adult insects) than seeds that were treated with distilled water (control) at 0.5 ($\chi^2 = 6.00$, $p = 0.0143$), 2 ($\chi^2 = 6.93$, $p = 0.0084$), 12 ($\chi^2 = 4.06$, $p = 0.0438$), 24 ($\chi^2 = 4.97$, $p = 0.0297$) and 48 ($\chi^2 = 4.59$, $p = 0.0321$) hours after the beginning of the assay (Fig 1B). The same happened to the mean density of *Z. subfasciatus* across all evaluation times ($\chi^2 = 34.09$, $p < 0.0001$). In the third bioassay (Assay 3), there was a significant effect only for the treatment of seeds with neem. The neem-treated seeds were preferred less than those treated with water in most of the evaluation times, with the exception of the first three (0.5, 1 and 2h after the beginning of the assay) that did not promote any significant differences (Fig 1C).

Antibiosis bioassay (initial assay)

Selection of genotypes for further tests

The tested genotypes contained different numbers of *Z. subfasciatus* eggs ($F_{7, 32} = 4.89$, $P = 0.0008$) and yielded different numbers ($F_{7, 32} = 4.73$, $P = 0.0010$) and masses of emerging adults ($F_{7, 32} = 5.48$, $P = 0.0003$). The number of eggs of *Z. subfasciatus* was highest in brown spotted common bean (BSCB); intermediate in white broad bean (WBB), yellow broad bean (YBB), carnival common bean (CCB), black common bean (BCB) and purple common bean (PCB) and least in cowpea (C) and spotted broad bean (SBB) (Fig 2A). The same pattern held for emerged *Z. subfasciatus* adults, excepting for black common bean (BCB) that grouped with cowpea (C) and spotted broad bean (SBB) (Fig 2A). However, the mean weight of emerged adults was highest at carnival common bean (CCB), brown spotted common bean (BSCB), white broad bean (WBB) and yellow broad bean (YBB), intermediate in black common bean (BCB), purple common bean (PCB) and cowpea (C) and lowest in spotted broad bean (SBB) (Fig 2B). Hence, based on these results, we selected spotted broad bean (SBB) and cowpea (C) as the two most resistant genotypes and yellow broad bean (YBB), brown spotted common bean (BSCB) and white broad bean (WBB) as the most susceptible genotypes.

Insecticides-treated seeds tests (final assay)

In analyzing density of *Z. subfasciatus* eggs, a significant interaction was found between genotypes and treated seeds ($F_{8, 60} = 4.61$, $P = 0.0002$). Treating the seeds with both neem and deltamethrin significantly reduced the number of eggs laid by *Z. subfasciatus* in the majority of the genotypes tested. Exceptions to this pattern were found for brown spotted common bean, which had equal numbers of eggs in all treatments, and the spotted broad bean, which had reduced

Table 1. Total number (\pm standard error of the mean) of *Zabrotes subfasciatus* eggs obtained in an antibiosis bioassay according to the bean genotype treated with different solutions.

Genotypes	Seed Treatments ^a		
	Control	Deltamethrin	Neem
Yellow Broad Bean	67.0 \pm 17.56 aA	25.4 \pm 7.02 bA	25.2 \pm 7.92 bA
Cowpea	39.2 \pm 15.82 aB	12.2 \pm 3.35 bB	13.6 \pm 5.55 bABC
White Broad Bean	17.4 \pm 10.09 aBC	0.6 \pm 1.34 bC	0.6 \pm 0.89 bC
Brown Spotted Common Bean	22.4 \pm 10.06 aBC	12.4 \pm 4.50 aB	18.2 \pm 12.70 aAB
Spotted Broad Bean	13.6 \pm 6.80 aC	5.4 \pm 3.13 bBC	6.4 \pm 2.07 abBC

^aMeans followed by at least one letter in common do not differ by Tukey's test ($\alpha = 0.05$). Capital letters stand for different genotypes in the column within each seed treatment, while the lowercase letters provide comparisons among treatments for a given genotype within a row.

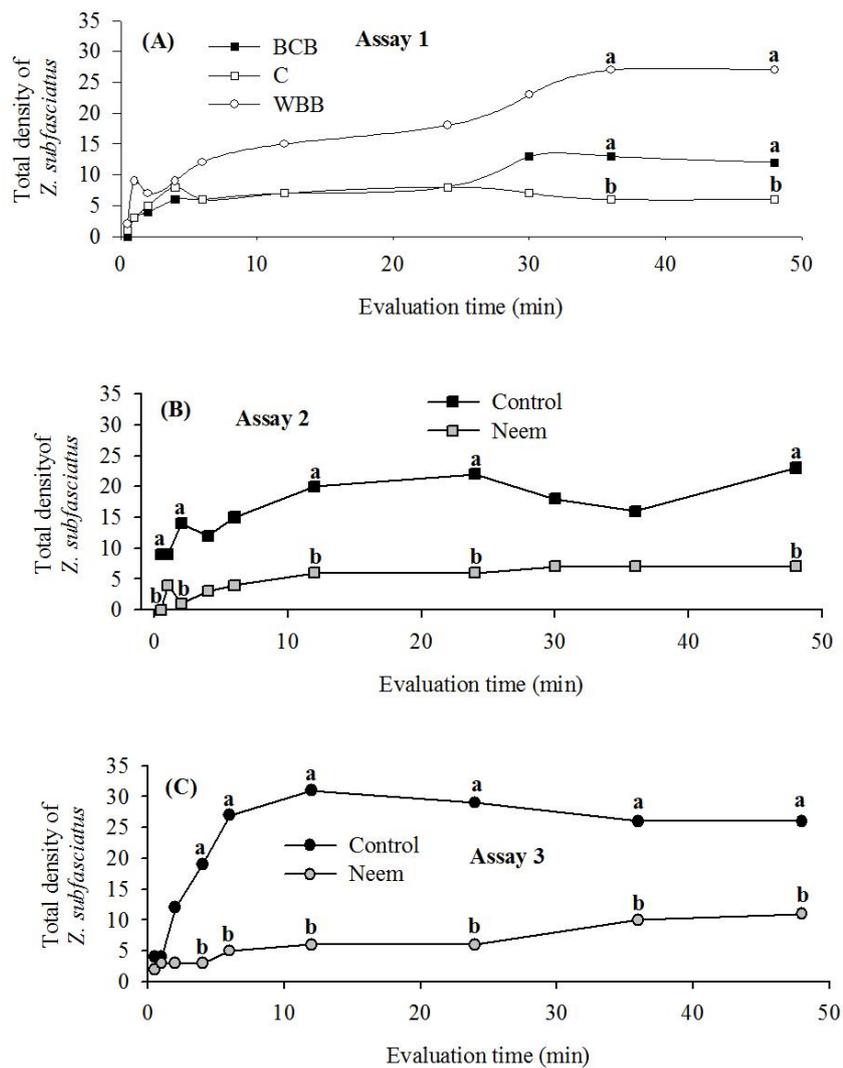


Fig 1. Total density of *Zabrotes subfasciatus* adults in preference (antixenosis) tests: (A) choosing a given bean genotype (20 grams samples) at different evaluation times; (B) as a function of the treatments used in the seeds; (C) as a function of the treatments used in the seeds at different evaluation times. Means followed by the same letter within a given evaluation time do not differ according to Chi-square test at $\alpha = 0.05$. (Black common bean [BCB], cowpea [C] and White broad bean [WBB]).

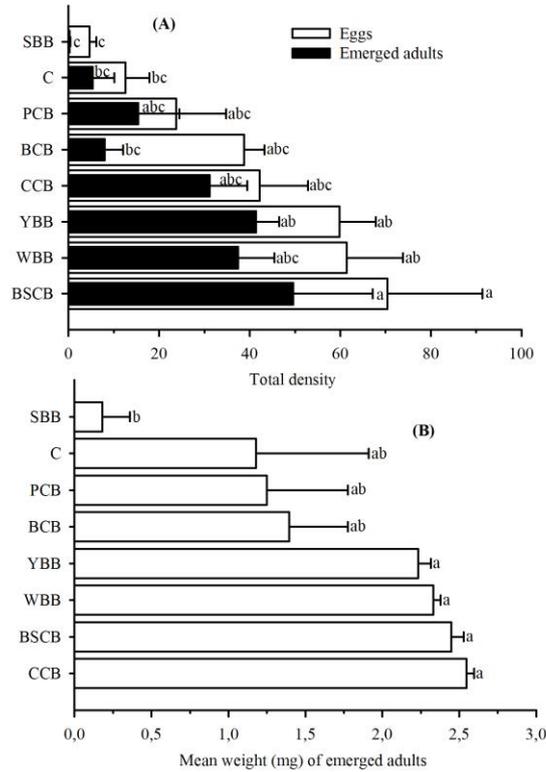


Fig 2. Total density (\pm standard error of the mean) of eggs (white horizontal bars) and of emerged adults (black horizontal bars) (A) of *Zabrotes subfasciatus* in an antibiosis initial bioassay and mean weight of the emerged adults (B) according to the bean genotype tested (Black common bean [BCB]; Brown spotted common bean [BSCB]; Carnival common bean [CCB]; Cowpea [C]; Purple common bean [PCB]; Spotted broad bean [SBB]; White broad bean [WBB]; Yellow broad bean [YBB]). Means in bars of the same color followed by at least one letter in common do not significantly differ according to Tukey's test at $\alpha = 0.05$.

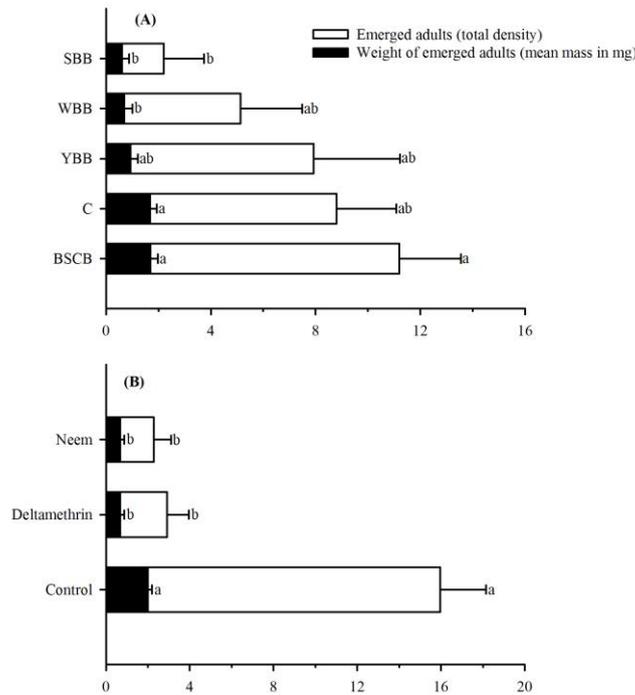


Fig 3. Total density and mean weight (\pm standard error of the mean) of the emerged adults of *Zabrotes subfasciatus* obtained from an antibiosis assay as a function of: (A) the genotypes tested; (B) the treatments of the seeds. Means in bars of the same color followed by at least one letter in common do not differ by Tukey's test ($\alpha = 0.05$). *Brown spotted common bean (BSCB); Cowpea (C); Spotted broad bean (SBB); White broad bean (WBB); Yellow broad bean (YBB).

numbers of eggs compared with control when the seeds were treated with deltamethrin (Table 1). The yellow broad bean genotype had the highest number of eggs regardless of treatment. However, when treated with neem, cowpea and brown spotted common bean had similar numbers of eggs to those observed in yellow broad bean. White broad bean had the lowest number of eggs when the seeds were insecticide-treated; spotted broad bean was the least oviposited when the seeds were water-treated (Table 1).

No significant interactions were found between insecticide treatment and genotypes for adult *Z. subfasciatus* emerged ($F_{8,60} = 1.3$, $P = 0.2601$) or the mean weight of emerged adults ($F_{8,60} = 1.8$, $P = 0.0946$). However, there were significant main effects of both beans genotypes and insecticide treatment on the densities (genotype: $F_{4,60} = 3.95$, $P = 0.0065$; treatment: $F_{2,60} = 32.52$, $P = 0.0001$) and mean mass (weight) (genotype: $F_{4,60} = 6.08$, $P = 0.0004$; treatment: $F_{2,60} = 20.49$, $P = 0.0001$) of emerged adults.

The density of the emerged adults was maximum in brown spotted common bean (BSCB) and minimum in spotted broad bean (SBB) genotypes (Fig 3A). However, concerning mean weight, it was maximum in both brown spotted common bean (BSCB) and cowpea (C) and minimum in both spotted broad bean (SBB) and white broad bean (WBB) (Fig 3A). Deltamethrin and neem significantly reduced the density and weight of emerged adults from treated seeds compared with the control (Fig 3B).

Seed density

Seed density varied from 1.19 to 1.50 g/dm³. No significant correlations were detected between this characteristic and the number of eggs ($r = -0.62$, $P = 0.1032$), density ($r = -0.66$, $P = 0.0730$) or mass ($r = -0.68$, $P = 0.0658$) of emerged adults of *Z. subfasciatus*.

Seed color intensity

The values detected for the hue angle of the different genotypes varied from 16.04 to 74.81 and the values of chroma (C) varied from 0.59 to 41.28. There was no significant correlation between the number of adults of *Z. subfasciatus* choosing a given genotype and its hue angle ($r = -0.19$, $P = 0.36$) or chroma ($r = -0.24$, $P = 0.25$).

Discussion

The interaction between Bruchids and legume seeds is highly specific, so that only seeds of a very few species can be attacked by any of insect species (Sales et al., 2005). Despite this fact, *Z. subfasciatus* is recognized as being one of the few Bruchids able to feed on a variety of beans, including those belonging to the genera *Phaseolus* (common beans), *Vigna* (cowpea) (Sales et al., 2005; Bifano et al., 2010) and *Vicia* (broad beans) (Pacheco and Paula, 1995; Toledo et al., 2013). Differences in resistance of bean species to *Z. subfasciatus* have been well documented and are attributed to many causes, including the presence of trypsin and proteinase inhibitors, lecithins and tannins, all of which are deleterious to the development of *Z. subfasciatus* (Osborn et al., 1986; Posso et al., 1992; Pereira et al., 1995; Guzmán-Maldonado et al., 1996; Acosta-Gallegos et al., 1998; Barbosa et al., 2000; Aguiar et al., 2006; Moraes et al., 2011). However, many of these defenses do not seem to affect *Z. subfasciatus*, as its larvae are able to secrete α -amylases that are insensitive to the α -amylase inhibitor found in seeds of *P. vulgaris* (Bifano et al., 2010). This strategy has not been reported for other bruchid species feeding on *P. vulgaris*, which may

explain why this host is unsuitable for them (Ishimoto and Kitamura, 1989; Hivrale et al., 2011).

Despite the relative plasticity shown by *Z. subfasciatus* during host selection, slight differences may exist in *Z. subfasciatus* preference for or performance on different beans because it is well accepted that inhibitors can be very efficient in blocking enzymes from organisms that are not specialized on that plant (Aguiar et al., 2006).

In the present study, we aimed to measure the preference of Mexican bean beetle for genotypes of different colors and found, mostly that preference did not differ significantly among the tested genotypes. Only in one assay we observed alteration in preference of two out of ten measures taken (Fig 1A), with the white broad bean and the brown common bean being the most and cowpea the least preferred genotypes. However, cowpea and spotted broad bean altered some biological characteristics of *Z. subfasciatus*, greatly reducing the oviposition and density of emerged adults (Fig 2A). Additionally, spotted broad bean greatly reduced the weight of emerged adults of *Z. subfasciatus* (Fig 2B), making it the most antibiotic genotype tested. Previous work has also demonstrated that different cultivars of cowpea can alter the damage caused by another bruchidae (*Callosobruchus maculatus* [Fabricius]), reducing insect emergence and population growth, and increasing mortality (Torres et al., 2016). Hence, the cowpea genotype used in this work must have been unsuitable to *Z. subfasciatus* development the same way that the cultivars BRS Tapaihum and BRS Acauã were to *C. maculatus*.

We observed no relationship between the density of seeds and biological characteristics of the beetle. Hence, some other causes, not measured here, might be involved including the chemical composition of seeds (Barbosa et al., 2000; Minney et al., 1990). This and other possibilities should be investigated in future work.

Despite this finding, some of the patterns that we detected suggest potential synergism of control tactics included in an IPM approach. The use of multiple strategies and tactics is the basic principle of IPM (Gray et al., 2009). This integration, if synergistic, may result in efficacy of combined tactics that would fail in isolation. Thus, one can search for moderate resistance levels and pesticides causing lower rates of mortality without discarding either tactic for further use in pest control. Such an approach is especially useful for cosmopolitan pests and systems with few management options, such as the case for *Z. subfasciatus* infesting bean seeds.

When we treated genotypes possessing variable degrees of antibiosis resistance with one botanical and one synthetic insecticide, we noticed a large reduction in pest density – including a 20–50% reduction in oviposition, even among highly susceptible genotypes, such as the brown spotted common bean (Table 1). For moderately and highly resistant genotypes, such as white and spotted broad bean, the reduction in oviposition varied from 53% to nearly 100% (Table 1) and was reflected in the number of adults emerging from these genotypes, which were half and five times less numerous than those emerging from the susceptible genotype (brown spotted common bean) (Fig 3A). Additionally, the adults emerging from those genotypes had only approximately 35% to 40% of the mass of those emerging from the susceptible genotype (Fig 3A).

Our results, therefore, suggest a synergistic effect of using genotypes possessing moderate and high level of resistance and chemical control with alternative pesticides (to the fumigants that are currently used). The synergistic effect observed here is similar to that described by Mazzoneto and Vendramin (2002). According to these results, the adoption

of alternative measures of control in isolation should be avoided because they are not expected to yield effective control of the pest.

The comparison between a neem formulation and control in preference tests showed that it may greatly reduce the infestation from 53.33% to 100% (Fig 1B) and from 40.54% to 72.72% (Fig 1C) depending on the evaluation time. Also, in the antibiosis assay the neem-based formulation was as efficient as deltamethrin in reducing infestation (Table 1).

The results showing comparable efficiency of neem's formulation to a synthetic insecticide (deltamethrin) used to control *Z. subfasciatus* agree with results from Barbosa et al. (2002), who compared a neem oil formulation with malathion. Hence, neem-based formulations should be preferred over synthetic pesticides because neem-based formulations degrade rapidly (Johnson et al., 2003) and thus may reduce the amount of residues on the final product, which will be consumed by humans.

Materials and Methods

Plant materials

The tested genotypes comprised the following groups: a) *Phaseolus vulgaris* group: carnival common bean (CCB), brown spotted common bean (BSCB), black common bean (BCB) and purple common bean (PCB); b) *Vicia faba* group: yellow broad bean (YBB), white broad bean (WBB) and spotted broad bean (SBB); and c) the *Vigna unguiculata* group: cowpea (C) and greenly cowpea (GC). These samples were obtained from open markets, cataloged and morphologically described for the following characteristics: 1) husk and hilo colors; 2) width x length ratio; 3) diameter; 4) 100-seed mass; and 5) shape of the seed [data not shown in this manuscript], according to the methodology described in Silva (2005). Seeds were stored in a freezer to prevent infestation.

Insects

Insects used in the assays were manually transferred from mass rearing colonies maintained on *P. vulgaris* seeds (group *Carioquinha*), a variety not included among the tested genotypes. The mass rearing procedures followed those described by Baldin and Pereira (2010) which used transparent glass vial of 1 L volume covered with a lid containing a screen (35 mesh) and that was filled with 300 g of grains and received around 300 insects. Colonies started with insects obtained from infested beans sold at open markets which were totally infested and damaged.

Experimental design and conditions

The treatments of the antibiosis and antixenosis bioassays were arranged in a randomized design with five replications. Antibiosis bioassays were run under controlled environment within a B.O.D. set to $25 \pm 2^\circ\text{C}$ and a 12:12 h L:D photophase. Further, non-preference bioassays were run under normal lab conditions. We kept record of that data using an Instrutemp ITLog 80 data logger, with measurements taken every 30 minutes.

Non-preference (antixenosis) bioassays

In the initial assay (without insecticide treatment), six genotypes [white broad bean (WBB), spotted broad bean (SBB), cowpea (C), greenly cowpea (GC), black common bean (BCB) and purple common bean (PCB)] were tested. A

foam arena containing 30 holes, designed to precisely fit plastic container (50 mL volume), was built. The edges of the cups were leveled with the surface of the foam platform.

To each plastic container, we added 20 g of seeds of one of the bean species (i.e., five replicate cups per each of the six genotypes). Then, 60 *Z. subfasciatus* adults were released in the center of the arena. The number of insects on each genotype was evaluated after 0.5, 1.0, 2.0, 4.0, 6.0, 12.0, 22.0, 30.0 and 48 h from the beginning of the assay. These data were used to select the most and least preferred genotypes to be further tested in combination with a neem formulation in three other bioassays. Then, in the final assays (with insecticide treatment) for each tested genotype, 20 g of seed samples were treated with an oil-based formulation of neem (Natuneem[®], Manufacturer: Natural Rural Ind. e Com. de Produtos Biológicos Ltda., Araraquara, SP, Brazil) at the rate of 3 mL of commercial product (formulation) per 30 mL of distilled water or distilled water (control) using manual polyethylene sprayers with a capacity of 250 mL. The seeds were maintained at room temperature and humidity ($25 \pm 3^\circ\text{C}$ and $50 \pm 20\%$ r.h.) until they were dry and were then added into the arena. The experimental conditions, the number of insects released and variables measured followed the same procedure as that described for the initial assay. The evaluations were performed at 0.5, 1.0, 2.0, 4.0, 6.0, 12.0, 22.0, 30.0 and 48 h after the beginning of the third bioassay run with neem-treated seeds. The other two bioassays included an additional evaluation time, at 36 h after the beginning of the assay.

Antibiosis bioassays

In the initial assay (without insecticide treatment), eight genotypes [black common bean (BCB), brown spotted common bean (BSCB), carnival common bean (CCB), cowpea (C), purple common bean (PCB), white broad bean (WBB), spotted broad bean (SBB) and yellow broad bean (YBB)] were tested. A single glass vial (20 mL volume), containing 20 g of seeds, was infested with five female adults of *Z. subfasciatus*. We chose only females to be used in experimental units since they are easily recognized for their body size and color pattern (Kaur et al., 1999). The vials were lidded and kept within a B.O.D set up to the aforementioned conditions for seven days. After this time, the adults were removed and the eggs counted. Then, the vials were returned to the B.O.D and the emergence of adults was recorded daily. After the emergence of the first adult, evaluations were maintained for the following 20 days. Emerged adults were recorded daily and weighed in an analytical electronic balance (model FA-2104N). The total mass was divided by the total number of adults obtained and used to estimate the mean mass, which was used in the statistical analysis.

The results obtained in the initial bioassay were used to select five genotypes having variable degrees of resistance or susceptibility, which were tested in a subsequent assay (final assay – with insecticide treatment). Each of the selected genotypes were exposed to one of three treatments: distilled water (as a control), deltamethrin (Decis 25 CE, Manufacturer: Bayer S/A, São Paulo, SP, Brazil) at the rate of 0.1 mL of commercial product (formulation) per 30 mL of distilled water and an oil based neem formulation (Natuneem[®]) at the rate of 3 mL of commercial product (formulation) per 30 mL of distilled water. The condition and number of insects confined and seeds used, as well as the variables measured, followed the same procedure described for the initial assay.

Seed density

Samples of 50 g of each of the genotypes included in the initial antibiosis assay were added to dry graduated cylinders, which then received 150 mL of distilled water. The volume of samples + water were recorded and these measures were used to calculate real density (RD) according to the following formula: $RD = (\text{mass of seeds}) / [(\text{volume of distilled water} + \text{seeds}) - (\text{volume of distilled water})]$. All determinations were carried out with five replicates for each genotype.

Seed color intensity

Color intensity of the seeds included in the initial antixenosis assay was determined using a Minolta Colorimeter CR-200 (Minolta Camera Co., Osaka, Japan). Data were presented as *L*-, *a*- and *b*-values of the Hunter color system, where *L* represents lightness (values range from 0 to 100, dark to light) and the *a*- and *b*-values are chromatic components of (+) redness to (-) greenness and (+) yellowness to (-) blueness, respectively. All determinations were carried out with five replicates for each genotype. The *a*- and *b*-values were used to calculate Hue angle (*h*) and chroma (*C*) according to the following equations (Maskan, 2001):

$$h = \tan^{-1}(b/a)$$
$$C = (a^2 + b^2)^{1/2}$$

Data analysis

Because data from the three different initial antixenosis assays showed no significant differences among the same treatments of different assays [by PROC GLM of the SAS System (SAS, 2002)], they were reduced to overall means. Initial antixenosis assay was transformed to square root ($x+0.5$) to fit the analysis of variance assumptions and submitted to repeated measures ANOVA [by PROC GLM of the SAS System (SAS, 2002)]. This was followed by comparisons of means by Tukey's test at $\alpha = 0.05$. The data coming from the subsequent antixenosis assays (final assays with neem-formulation treatment) were submitted to Chi-square test to evaluate differences between the numbers of insects present in neem treated seeds in different evaluation times. Also, it was used to detect differences among the genotypes in different evaluation times. In this analysis, the expected values of Chi-square were taken assuming an equal distribution of *Z. subfasciatus* among treatments and the observed values were derived from the number of insects found on each treatment within a given evaluation time.

The remaining data were subjected to one-way (antibiosis initial assay) or two-way (insecticide-treated antibiosis assay) ANOVA followed by comparison of means by Tukey's test at $\alpha = 0.05$. The data were also tested for a relationship of mean density and color intensity of seeds using Pearson correlation analysis at $\alpha = 0.05$. Color intensity was correlated with overall mean density of insects throughout the evaluations' time in the initial preference (antixenosis) assay, since in this test the insect had the chance to choose among materials possessing different colors while the same did not happen in no-chance tests. Density of the seeds was correlated with total number of eggs and emerging adults and with the mean weight of the emerged adults in the initial antibiosis assays, in a way to try to account for the possible antibiosis effects observed.

Conclusion

Z. subfasciatus adults exhibited similar preference among non-treated bean genotypes, although neem-treated genotypes altered the preference and reduced infestation from 40.54–100%. The less preferred genotype was cowpea (C). The most antibiotic genotype was spotted broad bean (SBB) once oviposition, density and the weight of emerged adults of *Z. subfasciatus* was reduced while in this genotype. Insecticides (both deltamethrin and neem) reduced oviposition about 53–100% and yielded half to five-fold fewer emerging insects weighting 35%–40% less in antibiotic genotypes. Both insecticides synergized with the genotypes possessing high degrees of antibiosis, reducing oviposition in 53–96%.

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