

Beauveria bassiana (Ascomycota: Hypocreales) Introduced as an Endophyte in Corn Plants and Its Effects on Consumption, Reproductive Capacity, and Food Preference of *Dichroplus maculipennis* (Orthoptera: Acrididae: Melanoplinae)

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Subject Editor: Fangneng Huang

Received 13 January 2017; Editorial decision 8 March 2017

Abstract

In this study, the effects of strain *Beauveria bassiana* (LPSC 1067) as an endophyte in corn plants on consumption, fecundity, and food preference of *Dichroplus maculipennis* were examined. We observed that the daily consumption by grasshoppers fed with control plants was almost twice that of those that were fed treated plants. Significant differences in fecundity of grasshoppers that were fed with treated plants compared with those that only fed on control plants were also observed. The number of eggs laid per female fed with control plants was 27.2, while the number of eggs laid per female that were fed during 15 d with treated plants was 17.7. Similar results were observed when the number of embryonated eggs was evaluated. The highest number of embryonated eggs were recorded in those females that only fed on control plants (96%) while fewer embryonated eggs were recorded in grasshoppers fed for 15 d with treated plants only (25%). In relation to food preference the average consumption rate for *D. maculipennis* females on control corn plants was 303.8 ± 24.5 mg while it was only 25 ± 2.1 mg on plants treated with *B. bassiana* as an endophyte. In summary, we observed that *B. bassiana* as a corn plant endophyte negatively affected the daily consumption rate, fecundity and food preference of *D. maculipennis*.

Key words: *Beauveria bassiana*, grasshoppers, endophytic, corn plants, *Dichroplus maculipennis*

Melanoplinae grasshoppers are of central relevance among the Acrididae of Argentina as they constitute the subfamily with the largest number of known species. Many of them are often numerically dominant in grasshopper communities of different regions of the country and several species are considered agricultural pests (Lange et al. 2005, Cigliano et al. 2014). One of these species is *Dichroplus maculipennis* (Blanchard) which causes damage in grasslands and in economically important crops such as maize, soybean, and wheat, among others (Carbonell et al. 2006, Mariottini et al. 2013). These grasshoppers mainly consume leaves but they often also eat the stems (Mariottini et al. 2011a). *Dichroplus maculipennis* occurs in much of Argentina and Chile, Uruguay, and

southernmost Brazil (Carbonell et al. 2006, Mariottini et al. 2011b). In recent years, an outbreak (up to 75 individuals per square meter) occurred in southern Buenos Aires province, causing important economic losses to farmers and ranchers (Mariottini et al. 2012). Chemical insecticides are still the only available option for *D. maculipennis* control in Argentina, but their use is of serious environmental concern (Gonzalez et al. 2010). The fungal species *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin s.l. is commonly used for the biological control of insect pests of agricultural crops (Jaronski 2010, Vega et al. 2012). In addition to colonizing insects, some entomopathogenic fungi may endophytically colonize plants (Vega et al. 2008). Fungal endophytes can live within plants and, in

general, do not cause obvious harm to the host (Van Bael et al. 2005, Gurulingappa et al. 2010). *Beauveria bassiana* has been isolated from many naturally colonized plants (Vega et al. 2008), as well as following inoculation in corn (Lewis et al. 2001, Russo et al. 2015). The presence of endophytic entomopathogens in plants may influence the population dynamics of herbivores. Strains of endophytic *B. bassiana* reduced the population of *Iraella luteipes* (Thompson) (Hymenoptera: Cynipidae) feeding on *Papaver somniferum* L. (Quesada-Moraga et al. 2009). Increased adult mortality and reduced larval damage by *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) were attributed to the endophytic establishment of *B. bassiana* in banana (Akello et al. 2008). Similarly, reduction of tunnelling in maize by *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) (Bing and Lewis 1991) and *Sesamia calamistis* (Hampson) (Lepidoptera: Pyralidae) (Cherry et al. 2004) were attributed to endophytic *B. bassiana*. Furthermore, in laboratory bioassays several authors have observed the ability of *B. bassiana* as endophyte to control *Helicoverpa armigera* in *Vicia faba* (Jaber and Vidal 2010); Diptera in *V. faba* (Acutse et al. 2013), *Sesamia nonagrioides* in sorghum plants (Mantzoukas et al. 2015); Coleoptera in *Ophiomyia phaseoli* (Diptera: Agromyzidae) (Mutune et al. 2016); *H. armigera* on tomato plants (Qayyum et al. 2015) and *H. zea* in cotton plants (Lopez and Sword 2015). In a previous study, Pelizza et al. (2013) evaluated the survival and fecundity of *D. maculipennis* when sublethal doses of *B. bassiana* were applied. However, the effects produced by *B. bassiana* on consumption, fecundity and food preference of *D. maculipennis* have never been studied when used as an endophyte microorganism in corn. Therefore the objective of this study was to evaluate the effects of strain *Beauveria bassiana* (LPSC 1067) as an endophyte in corn plants on consumption, fecundity, and food preference of *Dichroplus maculipennis* were examined.

Materials and Methods

Insect

Individuals of *D. maculipennis* used in this study belonged to the first laboratory generation [F1] of specimens originally collected in the southern Pampas region (Laprida county, Buenos Aires province, Argentina, 37° 32'60" S, 60° 49'00" W) and maintained in a rearing room under controlled conditions (30°C, photoperiod 14:10 (L:D) h, 40% RH) as described in previous studies (De Wysiecki et al. 1997, Mariottini et al. 2011c).

Fungal Isolate

The fungal strain used was *B. bassiana* LPSC 1067 (accession number KF500409) from the culture collection of the Spegazzini Institute (LPSC), La Plata, Argentina. The choice of this fungal strain was based on its laboratory efficacy against other pest grasshopper and locust species (Pelizza et al. 2012a,b) and its ability to act as an endophyte in corn plants (Russo et al. 2015). Conidia were obtained from cultures maintained on potato dextrose agar (PDA; Britania S.A., Buenos Aires) for 10 d at 25°C in darkness. Conidia were harvested and placed in test tubes containing 0.01% (v/v) polyoxyethylene sorbitan monolaurate (Tween 80, Merck). Suspensions were adjusted to 1×10^8 conidia per milliliter according to Gurulingappa et al. (2010) and Russo et al. (2015) using a Neubauer hemocytometer. The viability of conidia used in the tests was determined after 24 h as described by Goettel and Inglis (1997). This germination test was repeated for each stock suspension to maintain the constancy of the viability assessments. In all cases, the average viability of the conidia was over 95%.

Inoculation of Corn Plants With *B. bassiana* conidia

About 300 corn plants were inoculated for the consumption test, 350 for the endophyte effects on the fecundity test, and 50 for the food preference test. The same number of control plants were utilized in every test, respectively.

The technique used to inoculate *B. bassiana* conidia in corn plants was leaf spray according to Russo et al. (2015) because it proved most successful for introducing this entomopathogenic fungus as an endophyte. Seeds of corn were planted in 330 cm³ plastic pots at a depth of 4 cm, filled with a sterile mixture of perlite, vermiculite, soil, equally ground (1:1:1) and maintained in a greenhouse at 25°C and a photoperiod of 12:12 (L:D) h. Corn plants were inoculated at 3 wk of age, when they presented two true leaves. The floor of each of the pots was covered with foil to prevent runoff of conidia (Tefera and Vidal 2009). A glass hand sprayer (30 cc capacity) was used to spray each seedling with 3 ml conidial suspension, spraying the adaxial surface of all leaves only (Posada et al. 2007). Control plants were sprayed with a conidia-free solution of 0.01% (v/v) Tween 80. Treated and control seedlings were watered as needed. Colonization of plants by *B. bassiana* (LPSC 1067) was evaluated at 7-d postinoculation according to the technique described by Russo et al. (2015). In order to assess endophytic colonization of *B. bassiana* in corn plants six pieces of leaves for each treated and control plants were used. Leaves were surface-sterilized by immersing in 0.5% sodium hypochlorite for 2 min, followed by 2 min in 70% ethanol and rinsing in sterile distilled water (Arnold et al. 2001, Vega et al. 2008, Muvea et al. 2014, Parsa et al. 2016). To ensure total disinfection of the leaves, 100 µl of the last rinse water of each sample was cultured on plates containing PDA. The leaves were dried on sterile paper towels in a laminar flow cabinet and the edges were cut to remove dead tissue ensuing from the sterilization process. The leaf pieces of ~1 cm² were placed on Petri dishes containing PDA, added with antibiotics according to Vega et al. (2008). The presence or absence of *B. bassiana* growth on the pieces was recorded after 7 d at 25°C. The data are expressed as colonization frequencies, where colonization frequency = (number of plant pieces colonized/total number of plant pieces) × 100 (Petrini and Fisher 1987). Plants utilized in the tests were those colonized by *B. bassiana*, and were offered to the insects 14 d after inoculation.

Consumption Test

Three treatments of 30 *D. maculipennis* nymphs each were fed for a period of 2, 7, and 15 d, respectively, with a fresh ration of corn daily (one leave) that was inoculated with *B. bassiana* as an endophyte, while another treatment (control) of 30 *D. maculipennis* was fed with a fresh ration of corn daily (one leave) that was not inoculated with *B. bassiana*. These grasshoppers were kept individually in cages (20 cm long × 10 cm diameter) under controlled conditions (25°C, photoperiod 14:10 (L:D) h, 40% RH). After 24 h, the remains of corn that were not consumed were oven dried at 60°C. Thirty control rations were prepared and oven-dried. The average dry weight of these control rations was used as a correction factor which was obtained by dividing the product of the dry weight of control rations/fresh weight of control rations and applied to the initial fresh weight of each of the offered rations to calculate the dry weight of offered food (Pereyra 1995). Difference in weight between the offered rations and the remaining material after a trial represented the consumption during the test (Sánchez and De Wysiecki 1990, Mariottini et al. 2011b).

Table 1. Results of analysis of variance for consumption, number of egg-pods, number of eggs, and number of embryonated eggs laid per female of *D. maculipennis*, fed for 2, 7, and 15 d with control plants and plants treated with *B. bassiana* as an endophyte

Variables	Consumption			Number of egg-pods			Number of eggs			Number of embryonated eggs		
	df	F value	P	df	F value	P	df	F value	P	df	F value	P
Treatment	1,1439	523.34	<0.0001	1,239	73.84	<0.0001	1,437	271.77	<0.0001	1,437	544.23	<0.0001
Days	2,1439	17.95	<0.0001	2,239	1.09	0.3387	1,437	1.53	0.2178	1,437	37.90	<0.0001
Days×treatment	2,1439	1.38	0.2527	2,239	1.09	0.3387	1,437	1.53	0.2178	1,437	37.90	<0.0001

Endophyte Effects of *B. bassiana* on Fecundity of *D. maculipennis*

Three treatments of 40 *D. maculipennis* nymphs each were fed for a period of 2, 7, and 15 d with a fresh ration of corn daily that presented *B. bassiana* as an endophyte. After completion of each respective treatment period grasshoppers were fed with control plants and kept individually in cages (20 cm long×10 cm diameter) under controlled conditions (25°C, photoperiod 14:10 (L:D) h, 40% RH) until the end of trial. Another treatment (control) of 40 *D. maculipennis* was fed with a fresh ration of corn plants daily that did not have *B. bassiana* as an endophyte. Immediately after moulting to adults, females and males of both treatments were separated into couples (1♂, 1♀). Each couple was placed in a wire-screened, aluminium cage (12 cm×12 cm×16 cm). Number of egg-pods, number of eggs per pod, and number of embryonated eggs were recorded throughout their lives (Uvarov 1966). Dead grasshoppers were removed and immediately deposited in high-humidity chambers (sterile Petri dishes with filter paper dampened with sterile distilled water). Mycosis was confirmed by microscopical examination of the dead grasshoppers.

Food Preferences

Laboratory tests to determine whether the grasshoppers prefer to consume corn plants endophyte-infected or control plants were carried out as follows. Thirty adult females of *D. maculipennis* were individually placed in 30 wire-screened, aluminium cages (20 cm×20 cm×30 cm) along with two corn plants each, one with *B. bassiana* as an endophyte and another plant without the fungus as control. After 24 h, consumption was assessed for each adult female as mentioned previously.

Data Analysis

All data obtained for consumption and fecundity tests were subjected to analysis of variance two-way (ANOVA). Data obtained for food preference test, were subjected to analysis of variance one-way (ANOVA). Prior to the analysis, the data were tested for normality and homogeneity of variance. In both bioassays, for later comparisons to Tukey test ($P=0.05$) was used. Analyzes of variance were performed with the InfoStat 2007 software (InfoStat 2001).

Results

Colonization percentages for the plants utilized in the tests were 86.6% for the one on consumption, 88.3% for the one on fecundity, and 83.3% for the one on food preference. *Beauveria bassiana* was not detected in any of the control plants. Significant differences were observed at all time intervals tested with respect to the rate of daily consumption of *D. maculipennis* when insects were offered a control fresh corn ration and a fresh corn ration treated with *B. bassiana* as an endophyte (Table 1). The rate of daily consumption carried out by the grasshoppers fed with control plants at each of the time intervals studied (2, 7, and 15 d) was higher than the rate of daily consumption by those grasshoppers that were fed only with

corn rations treated with *B. bassiana* (Fig. 1). The average daily consumption rate of *D. maculipennis* was 55.5 ± 4.2 mg, 60.42 ± 4.72 , and 64.46 ± 4.47 mg at 2, 7, and 15 d, respectively, for those grasshoppers fed with control plants, and 23.5 ± 2.4 mg, 33.71 ± 4.31 mg, and 33.66 ± 4.58 mg, at 2, 7, and 15 d, respectively, for those *D. maculipennis* fed with a fresh corn ration treated with *B. bassiana* as an endophyte. We also observed significant differences in fecundity on those grasshoppers that were fed for 2, 7, and 15 d with control plants with respect to those that were only fed with treated plants (Table 1). The highest number of egg-pods laid per female was in those fed only with control plants (2.55 ± 0.32) while the lowest number of egg-pods per female was observed in individuals that were fed for 15 d with corn plants treated with *B. bassiana* (1.25 ± 0.36) (Table 2). Regarding the number of eggs per pod again females fed with control plants only laid more (27.25 ± 5.85) while the lowest number of eggs per pod was observed in grasshoppers which were fed for 15 d with treated plants (17.69 ± 2.03) (Table 2). Similar results were observed when the number of embryonated eggs laid by *D. maculipennis* fed with treated and control plants were measured. The highest number of embryonated eggs was in those females that only fed on control plants (26.82 ± 6.01), while fewer embryonated eggs were recorded in grasshoppers fed for 15 d with treated plants only (4.56 ± 1.42) (Table 2).

In relation to food preference significant differences were observed ($F=38.03$; $df=1,59$; $P<0.0001$). The average consumption rate for *D. maculipennis* females on control corn plants was 303.8 ± 24.5 mg while it was only 25 ± 2.1 mg on plants treated with *B. bassiana* as an endophyte (Fig. 2). *Beauveria bassiana* mycelial development was observed in 70% of the grasshoppers fed for 15 d with plants treated with *B. bassiana* but was not recorded in grasshoppers fed for 2 and 7 d with *B. bassiana*-treated plants.

Discussion

Several genera of entomopathogenic fungi have been isolated as endophytes in different plant species. Some of them have been reported as natural endophytes while others have been artificially introduced into the plant using different techniques (Vega et al. 2008, Greenfield et al. 2015). This is the first report of the effects on consumption, fecundity and food preference produced by *B. bassiana* as an endophyte in corn plants on the grasshopper pest *D. maculipennis*. In this study, we observed a significant reduction in the daily consumption rate in nymphs of *D. maculipennis* when they were offered a fresh ration of corn plants inoculated with the entomopathogenic fungi *B. bassiana*. At all time intervals studied (2, 7 or 15 d) consumption by grasshoppers that were fed with control plants was almost twice that of those fed only with treated plants. Similar results were observed by Lopez and Sword (2015) in *H. zea* fed with cotton plants inoculated with *B. bassiana*. Mutune et al. (2016) found a reduction in the oviposition rate of *Ophiomyia phaseoli* fed with *Phaseolus vulgaris* plants also inoculated with

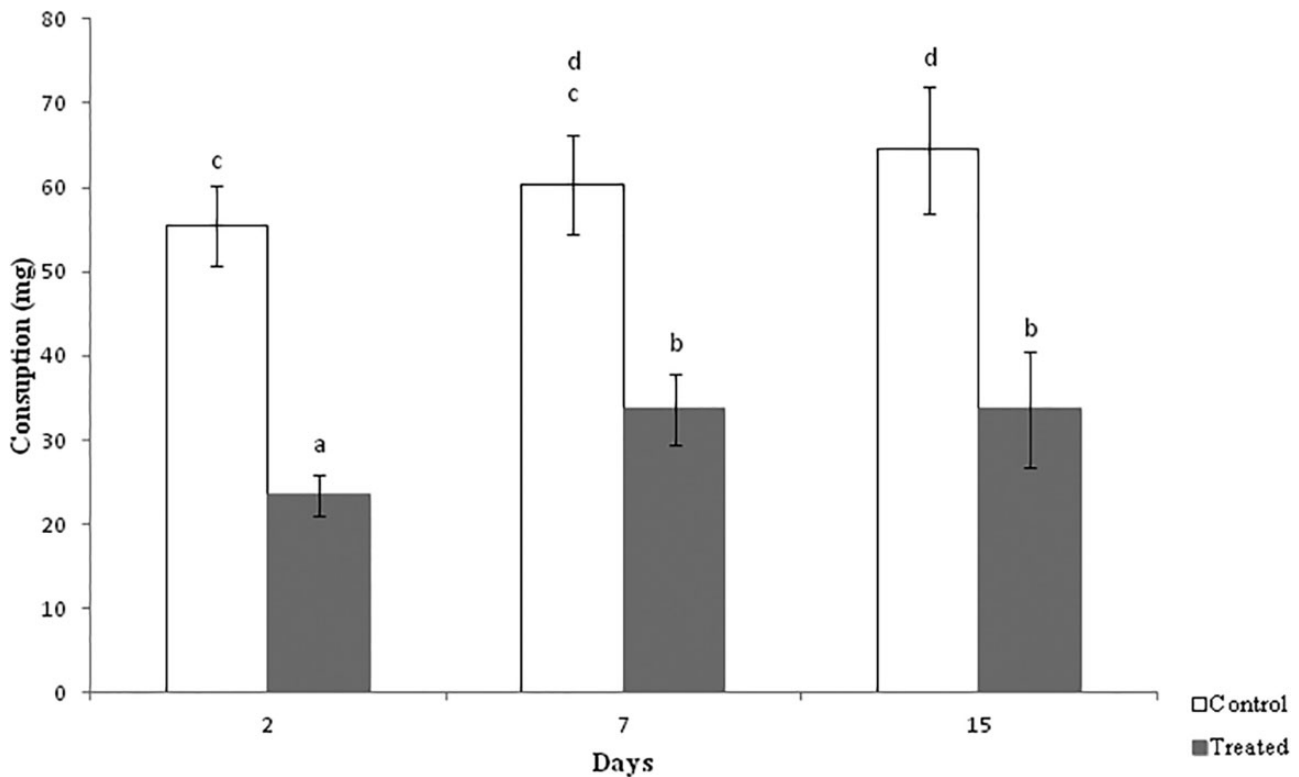


Fig. 1. Rate of daily consumption (means \pm SD) of *D. maculipennis* on control corn plants and corn plants inoculated with *B. bassiana* as an endophyte (treated). For 2, 7 and 15 d. Different letters indicate significant differences according to the Tukey test ($P=0.05$).

Table 2. Number of egg-pods, number of eggs per egg-pod and number of embryonated eggs per egg-pod in *D. maculipennis* fed with control plants and with treated plants with *B. bassiana* as endophyte for 2, 7, and 15 d

Number of days fed treated plants	Number of egg-pods	Number of eggs per egg-pod	Number of embryonated eggs per egg-pod
0 (Control)	2.55 \pm 0.32 a	27.25 \pm 5.85 a	26.82 \pm 6.01 a
2	1.45 \pm 0.41 b	19.85 \pm 4.39 b	15.71 \pm 6.79 b
7	1.27 \pm 0.45 b	19.33 \pm 3.05 b	10.19 \pm 4.51 b
15	1.25 \pm 0.36 b	17.69 \pm 2.03 b	4.56 \pm 1.42 b

Different letters denote significant differences between treatments in the same column according to the Tukey test ($P < 0.05$).

B. bassiana. Furthermore, Akutse et al. (2013) observed a reduction of fertility in Diptera fed with *V. faba* and *P. vulgaris* presenting *B. bassiana* as an endophyte. Although there are a number of studies that claim that secondary metabolites produced by different species of entomopathogenic fungi could discourage consumption by herbivorous insects, another possible cause could be an induced systemic response of plant defense that confers resistance to herbivorous insects (Quesada-Moraga et al. 2009, Gurulingappa et al. 2010). The mechanism by which entomopathogenic fungi as endophytes discourage plant consumption is not clear. Production of toxic metabolites by endophytic *B. bassiana* is attributed to damage reduction by *I. luteipes* larvae on plants of *Opium poppy* L. (Quesada-Moraga et al. 2009), by *C. sordidus* in banana (Akello et al. 2008), and by both *O. nubilalis* (Bing and Lewis 1991) and *S. calamistis* (Cherry et al. 2004) in corn. Gurulingappa et al. (2010) observed that the presence of *Aspergillus parasiticus* (Speare) and *B. bassiana* as endophytes in wheat plants did not cause direct mortality on Australian plague locust nymphs *Chortoicetes terminifera* (Walker) (Orthoptera: Acrididae). However, they mentioned a significant reduction in the rate of development and a large decrease in the rate of

consumption of those locusts that were fed wheat plants with fungi as endophytic. Another example of some endophytes fungi that may discourage plant consumption by herbivorous insects is the case of *Muscodor vitigenus* (Daisy, Strobel, Ezra and Hess) endophyte fungi of *Paullinia paullinioides* Radlk, producing naphthalene which repels the action of the adult stage of *Cephus cinctus* (Norton) (Hymenoptera: Cephidae) (Daisy et al. 2002).

A big difference in the number of pods and eggs per egg-pod, especially between females that were fed with control plants and those that were fed for 15 d with *B. bassiana*-treated plants, was observed. But even a higher difference was observed in egg embryonation: a higher number of eggs were embryonated in *D. maculipennis* fed only with control plants than those that were fed for 15 d with treated plants. In other pests such as aphids, survival and reproduction was adversely affected when the insects were brought into contact with conidia or secondary metabolites produced by *B. bassiana* or *M. anisopliae* present as natural endophytes from different crops (Gurulingappa et al. 2011, Akello and Sikora 2012). Similar results in terms of reduced consumption, fecundity, and fertility in the *Ophiomyia phaseoli* (Tyron) (Diptera: Agromyzidae) were observed

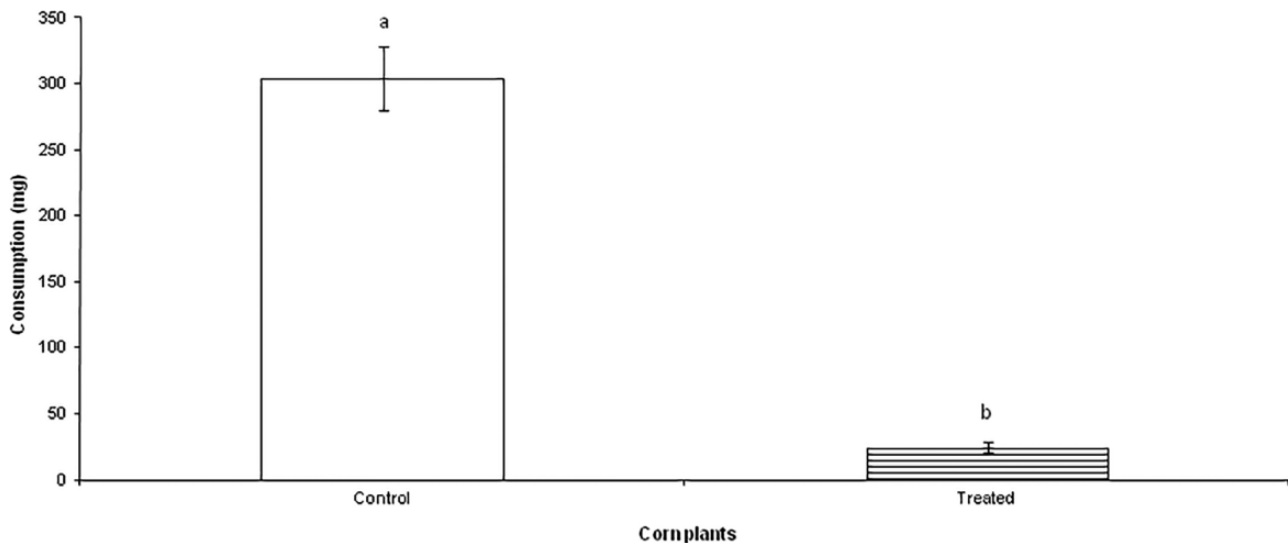


Fig. 2. Food preferences (expressed in mg of consumption) of adult females of *D. maculipennis* when they had the choice to consume both control corn plants and corn plants treated with *B. bassiana* as an endophyte. Different letters indicate significant differences according to the Tukey test ($P = 0.05$).

with *Phaseolus vulgaris* L. plants inoculated with *B. bassiana* and *M. anisopliae* as endophytes (Mutune et al. 2016). We have observed in previous studies (Pelizza et al. 2013) that when sublethal doses of *B. bassiana* are applied on *D. maculipennis* nymphs and another melanopline *Ronderosia bergi* (Stal), the resulting adults were unable to copulate or lay eggs. Similar results were observed by Hornbostel et al. (2004) after applying a sublethal dose of *M. anisopliae* on engorged larvae of *Ixodes scapularis* (Say) (Acari: Ixodidae). When assessing the food preferences of *D. maculipennis* we observed that when offered the two options (control and treated plants) the grasshoppers choose to consume control plants. To our knowledge, similar tests on other species of grasshoppers or locusts have not been conducted. However, food preference tests were performed on other insect pests. Lopez and Sword (2015) observed that the presence of *B. bassiana* and *Purpureocillium lilacinum* (Thom) Luangsa-Ard, Houbraken, Hywel-Jones and Samson as endophytes of cotton affected negatively the consumption, survival and development of *H. zea* (Boddie) (Lepidoptera: Noctuidae) when fed on cotton plants that were colonized by these. A study carried out by Kerri et al. (2010) showed that the grasshopper *Schistocerca americana* (Drury) (Orthoptera: Acrididae) did not consume grasses that had *Epichloë* or *Neotyphodium* as natural endophytes but did so on the same grasses that were free of the endophytes.

Another important point of this study is that we observed development of *B. bassiana* on 70% of the grasshoppers after death when fed for 15 d with treated plants. It is conceivable that *B. bassiana* was present in the body of these insects (latently) and this could be one reason whereby females of *D. maculipennis* were affected in their reproductive capacity. Although other studies are necessary to assess whether *B. bassiana* as an endophyte in corn plants produce toxic substances that may affect humans, this appears an interesting tool that could be considered to control this species of grasshopper pest.

Acknowledgments

This study was partially supported by Consejo Nacional de Investigaciones Científicas y Tecnológicas (PIP 0018), Comisión de Investigaciones Científicas de la provincia de Buenos Aires (CICPBA), Universidad Nacional de La Plata

(UNLP, 11/N 651) and Fondo para la Investigación Científica y Tecnológica (FONCYT; PICT 2015-1146).

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