ANA MARIA GUIMARÃES BERNARDO

SEED YIELD AND QUALITY OF SOYBEAN FIELDS INFESTED BY *Euschistus heros* SUBLETHALLY EXPOSED TO IMIDACLOPRID

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitotecnia, para obtenção do título de *Doctor Scientiae*.

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Fabrício Rainha Ribeiro

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Eugênio Eduardo de Oliveira
(Coorientador)

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(Orientadora)

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Dedico

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BIOGRAFIA

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RESUMO

BERNARDO, Ana Maria Guimarães, D.Sc., Universidade Federal de Viçosa, julho, 2019. **Produção e qualidade de sementes de soja provenientes de campos infestados por *Euschsius heros* subletalmente exposto a imidaclopride.** Orientadora: Denise Cunha Fernandes dos Santos Dias. Coorientador: Eugênio Eduardo de Oliveira.

O percevejo marrom da soja, *Euschistus heros*, é a praga mais presente nos campos brasileiros de soja e o controle desta praga tem sido feito com pesticidas químicos, como os neonicotinóides. No entanto, alguns estudos reportaram que em condições controladas, esses percevejos têm alta reprodução quando expostos a doses subletais de imidaclopride (neonicotinóide). Esse incremento populacional pode causar aumento na ocorrência de danos às sementes e reduzir a sua qualidade fisiológica. Portanto, objetivou-se avaliar se a população de *E. heros* em campo pode aumentar quando exposta a doses subletais de imidaclopride e quais as possíveis consequências para a produção, qualidade fisiológica e atividade de ezimas antioxidantes das sementes. Adicionalmente, investigou-se se *E. heros* exposto a doses subletais interfere no sistema de defesa da planta, avaliando-se a atividade de inibidor de proetase. Inicialmente, adultos recém-emergidos (≤ 72 h) foram expostos por 48 horas a resíduos secos de imidacloprid ou água destilada (controle). Foram conduzidos experimentos em campo e em casa de vegetação em delineamento inteiramente casualizado com 4 repetições. Em campo, um casal/planta foi liberado em gaiolas com 14 plantas durante os estádios R_5-R_6. A população de *E. heros* foi quantificada com 15, 22, 30 e 45 dias após a infestação. Plantas não infestadas foram utilizadas como controle. A colheita das vagens foi realizada no estádio R_8. A produção, porcentagem de vagens cheias e vazias, a qualidade das sementes e a atividade de enzimas antioxidantes foram determinadas. No experimento em casa de vegetação, plantas de soja no estádio R_5-R_6 foram infestadas com um casal de

percevejo por planta ou por vagem, em gaiolas, onde permaneceram por sete dias. Plantas não infestadas foram utilizadas como controle. Em seguida, foi realizada a colheita e determinou-se a atividade de inibidor de protease nas sementes. A população da praga foi significativamente maior quando esses foram previamente expostos a imidacloprid comparado ao controle. Plantas de soja infestadas por *E. heros* exposto a imidacloprid apresentaram redução significativa no rendimento e na qualidade das sementes. Além disso, descobriu-se que o percevejo marrom pode modificar o sistema de defesa da semente de soja. Conclui-se que a população de *E. heros* aumenta em condições de campo em resposta a doses subletais de imidaclopride e, consequentemente, há redução na produção e na qualidade da semente. Além disso, essa praga possivelmente pode alterar o sistema de defesa das sementes de soja.

ABSTRACT

BERNARDO, Ana Maria Guimarães, D.Sc., Universidade Federal de Viçosa, July, 2019. **Seed yield and quality of soybean fields infested by *Euschistus heros* sublethally exposed to imidacloprid.** Adviser: Denise Cunha Fernandes dos Santos Dias. Co-adviser: Eugênio Eduardo de Oliveira.

Neotropical brown stink bugs, *Euschistus heros,* is the most present pest in Brazilian soybean crop fields. Chemical pesticides are used to reduce this pest on soybean crops such as neonicotinoids. However, some studies reported that, under controlled conditions, these stink bugs demonstrated higher reproduction when exposed to sublethal doses of imidacloprid (neonicotinoid). Thus, the soybean seeds physiological may decrease with these higher *E. heros* population. Though, plant defence sytem can be induced by herbivores to defend themselves against this pest. Thus, we aimed to known the effects of exposure to imidacloprid sublethal dose on *E. heros* population under field conditions and possible consequences of such exposure on yield, physiological quality and antioxidant enzymes activity of soybean seeds. Additionally, we investigated whether *E. heros* exposed to imidacloprid can interfere on the soybean plant defence system by proteinase inhibitor activity. Newly emerged *E. heros* adults (≤ 72 h) were exposed to distilled water (control) or imidacloprid dried residuals for 48h. After that, one couple per plant was released in field cages with fourteen plants at pod filling (R_5-R_6). *E. heros* population was quantified at 15, 22, 30 and 45 days after infestation. Yield and seed quality were assessed at harvest (R_8). In order to study plant defence system a couple of stink bugs per plant or per pods was released in small cages, where they remained for seven days in a greenhouse. *E. heros* population was higher when stink bugs were previously exposed to imidacloprid than the control (distilled water). Infested soybean plants by *E. heros* exposed to imidacloprid had significant reduction in yield and lower quality seeds. Moreover, we found out *E. heros* can interfere soybean

plant defence system. Therefore, our results showed that imidacloprid sublethal exposure may increase *E. heros* population and consequently reduce yield and seeds quality. In addition, this pest can likely change soybean seeds defence system.

GENERAL INTRODUCTION

Soybean *Glycine max* (L.) Merril, is one of the most important crops in the world (Oerke and Dehne, 2004; Oliveira and Schneider, 2016). Brazil is the largest exporter of soybeans grains of the world and the second largest producer, with around 35,149.3 thousands of hectares in planted area in 2017/2018 season, being only behind of the United States (CONAB, 2018; USDA, 2018). The productivity in Brazil is around 3,394 $Kg.ha^{-1}$ with an estimated production of 119,427.5 thousands of tons for the next season (CONAB, 2018). However, this production could be higher if the damage caused by pest was reduced (Oerke, 2006).

During growth this crop can be attacked by different pest such as caterpillars, aphids, thrips and stink bugs. However, currently stink bugs species from Pentatomidae family are the focus of growers in Brazil (Panizzi, 2013), since they feed on pods and consequently damage seeds and reduce their quality (Corrêa Ferreira and Azevedo, 2002; Panizzi and Slansky Jr, 1985; Turnipseed and Kogan, 1976). Stink bugs feed by inserting their stylets into pods to suck up nutrients resulting in many injuries to plant tissues and might also cause abortion of pods and seeds (Panizzi et al., 2000). There are twenty five species of stink bugs on soybean crops in Brazil, but only *Piezodorus guildinni* (Westwood), *Nezara virudula* (Linnaeus) and *Euschiustus heros* (Fabricius) are considered economically important (Panizzi and Slansky Jr, 1985).

A lot of studies have shown that when plants are attacked by stink bugs the quality of seeds are gradually reduced. Hence, seeds have low germination rate, vigor and oil content are reduced (Corrêa-Ferreira and Azevedo, 2002; Daugherty et al., 1964; McPherson et al., 1979; Panizzi et al., 1978; Silva et al., 2012). In addition,

stink bugs can also transmit fungi and diseases (Daugherty, 1967; Hoffmann-Campo et al., 2000; Panizzi et al., 1978; Villas Bôas et al., 1990). Corrêa Ferreira and Azevedo (2002) compared damage caused by *P. guildinni*, *N. virudula* and *E. heros*, with four stink bugs/m plant rows and during 15 days in pod filling. Results from tetrazolium test indicated that infested plants with *P. guildinii* had a higher percentage of non-viable seeds than infested plants with *N. virudula* or *E. heros*. Thus, they concluded that infested plants with *P. guildinii* had the greatest number of damaged seeds when compared to damaged plants by *N. viridula* or *E. heros*. Therefore, it is known that seeds attacked by *E. heros* were less damaged compared to *P. guildinii*; however, it is important to highlight that, *E. heros* is the most abundant and prevalent stink bug on soybean crops (Corrêa Ferreira and Azevedo, 2002; Panizzi et al., 2014; Silva et al., 2011).

Stink bugs are mostly controlled by the use of chemical insecticides, which are often excessively and erroneously applied (Song and Swinton, 2009; Panizzi, 2013). Neonicotinoid insecticides are generally used to control stink bugs *E. heros* on soybean fields in Brazil (Sosa-Gómez et al., 2009; Sosa-Gómez et al., 2010; Panizzi et al., 2014; Silva et al., 2011; Tuelher et al. 2016). However, excessive use of chemical control might bring negative consequences such as pest resurgence, secondary pest outbreaks, selection resistant pest, natural enemy populations reduction or induce hormesis (Hardin et al., 1995; Ripper, 1956; Desneux et al., 2007; Guedes and Cutler, 2014; Castellanos et al., 2018).

Hormesis is described as biphasic dose-response phenomenon, characterized by a low dose of stimulation and a high dose of inhibition (Calabrese and Baldwin 2003; Calabrase et al. 2008; Guedes and Cutler, 2014). Therefore, when pests are exposed to sublethal doses of insecticidal compounds they have a stimulant response

(e.g. high oviposition rate) (Calabrese et al., 2008; Cutler, 2013; Guedes and Cutler, 2014; Santos et al., 2016). Many studies have identified hormesis in several arthropods like green peach aphid (*Myzus persicae*), maize weevil (*Sitophilus zeamais*), red spider mite (*Oligonychus ilicis*) and Mexican bean weevil (*Zabrotes subfasciatus*) (Christopher Cutler et al., 2009; Guedes et al. 2010; Cordeiro et al., 2013; Mallqui et al., 2014). Santos et al., (2016) has recently showed that *E. heros* females exposed to sublethal doses of imidacloprid had higher fecundity and fertility rate. Haddi et al., (2016) reported that *E. heros* males, when exposed to sublethal doses of imidacloprid increased their sexual fitness. Moreover, it is well known that the presence of low insecticide doses almost always occurs in fields due to insecticide degradation (Guedes and Cutler, 2014). Thus, in the field conditions recommended doses of pesticides are used to control pest and when a chemical compound is applied many insects can be controlled. However, at the time of application some insects can move to another area or farm. Subsequently, these insects can return to the area they were before and keep contact with sublethal doses due to the insecticide degradation. So, the occurrence of hormesis may help elucidating recent outbreaks of *E. heros* on Brazilian soybean fields where the use of insecticides is frequent, and pests can be exposed to lower doses of insecticides.

Plants have constitutive and induced defence mechanisms to offer protection against herbivores and pathogens (Walling, 2008; Dangl and Jones, 2001). Thus, when plants are attacked by arthropods successive cascades of biochemical compounds are triggered (Maffei et al., 2007). Reactive oxygen species are one example of that which are known as an adaptation under biotic and abiotic stress conditions and responsible for the signaling activation of plant defence (Miller et al., 2008; Shetty et al., 2008). Reactive oxygen species and oxidative enzymes are

accumulated after herbivory (Bi and Felton, 1995). Moreover, induced plant defence such as proteinase inhibitors occur on higher concentration in seeds which has antinutritional effects and consequently offer protection against arthropods (Koiwa et al., 1997; Ryan, 1990; War et al., 2012). Nevertheless, there are few studies demonstrating that arthropods are able to manipulate plant defences (Zarate et al., 2007; Sarmento et al., 2011; Musser et al., 2002; Lawrence et al., 2008).

Thus, we aimed to know the effects of exposure to sublethal dose of imidacloprid on *E. heros* population and possible consequences of such exposure on yield and quality of soybean seeds under field conditions. Furthermore, we investigated whether *E. heros* in different proportions might interfere on soybean plant defence sytem response and whether this response may vary when *E. heros* is exposed to sublethal doses of imidacloprid.

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CHAPTER 1

Sublethal exposure to imidacloprid can increase *Euschistus heros* population on soybean field: Impact on yield and seed quality

Abstract

Many insecticides are used to avoid decreasing the soybean crops productivity. The neonicotinoids insecticides are generally used to control different species of stink bug in soybean fields. It is known that Neotropical brown stink bug *Euschistus heros* (Hemipetera: Pentatomidae) is the most present in Brazilian soybean crops. It has been demonstrated under controlled conditions that this pest exhibit higher reproductive abilities after sublethal neonicotinoid insecticide exposure, imidacloprid. Therefore, we aimed to know whether the population of *E. heros* in the field can be increased when exposed to sublethal doses of imidacloprid and what are the possible consequences for yield and quality of soybean seeds. Newly emerged adults (≤ 72 h) were exposed for 48 h to dry imidacloprid residuals or distilled water (control). After that, one couple/plant was released in field cages for 45 days, during the pod filling (R_5-R_6). Fourteen plants were allowed in each cage. Yield, empty and fullpods, and seed quality by tetrazolium test were assessed at harvest (R_8). *E. heros* population was significantly higher when stink bugs were previously exposed to imidacloprid than the control (distilled water). Soybean plants infested by *E. heros* exposed to imidacloprid had significant reduction in yield and lower seed quality. This shows that populations of *E. heros* in the field can increase in response to imidacloprid sublethal exposure and it may also reduce yield and quality of the seeds. This finding could explain the recent out-breaks of *E. heros* on soybean fields in Brazil.

Keywords: Neonicotinoids, Stink bugs, Sublethal doses, Hormese, Productivity

Introduction

In agricultural ecosystems around the world, populations of insect pests are mostly controlled by using chemical insecticides (Song and Swinton, 2009; Panizzi, 2013). However, excessive use of chemical control can have negative consequences, such as selection of resistant pest, pest resurgence, secondary pest outbreaks and reduction of natural enemy populations (Hardin et al., 1995; Ripper, 1956; Desneux et al., 2007; Guedes and Cutler, 2014; Castellanos et al., 2018). Furthermore, the presence of low insecticide doses always occurs in fields due insecticide degradation (Guedes and Cutler, 2014) and exposure to such low doses can also affect physiological and life-history traits of targeted and non-targeted insect populations (Guedes et al., 2016; Desneux et al., 2007). Of particular interest here are stresses incurred from the exposure to sublethal dosages of insecticides that can lead to hormetic responses (Calabrese et al., 2011). Such responses are well-known bi-phasic phenomena from toxicological studies characterized by stimulatory effects of biological functions at small dosages whereas large dosages are detrimental or lethal (Calabrese et al.,2011; Calabrese and Baldwin 2003; Calabrase et al. 2008; Guedes and Cutler, 2014). Many studies have identified hormetic response in several species of arthropods after exposure to chemical (Cutler and Guedes, 2017) and also botanical insecticides (Haddi et al., 2015; Papanastasiou et al., 2017).

Among the chemical classes of insecticides registered for agricultural applications, neonicotinoids are increasingly used due to their low levels of cross resistance compared to other classes of insecticides (carbamates, pyrethroids, organophosphates, organochlorides) (Jeschke et al., 2013). Molecules of this class of insecticides act selectively as agonists of nicotinic acetylcholine (nAChRs) receptorsin the central nervous system (Jeschke et al., 2013). In Brazilian soybean

fields neonicotinoids are widely used, alone or in combination with pyrethroids, for controlling infestation of soybean Pentatomids (stink bugs) and especially the most abundant specie the Neotropical brown stink bug *Euschiustus heros* (Fabricius) (Sosa-Gómez et al., 2009; Sosa-Gómez et al., 2010; Panizzi et al., 2014; Silva et al., 2011; Tuelher et al. 2016).

However, in recent study, sublethal exposure to imidacloprid stress in adulthood has been shown to hormetically increase the sexual fitness of males of the *E. heros* (Haddi et al., 2016) as their contact with dry residues of imidacloprid (at 1% of field label rate) did not affect insect survival, but led to higher mating frequencies when male or female of stink bugs was exposed. In addition, Santos et al., (2016) also reported that females of *E. heros* exposed to sublethal doses of imidacloprid had lower survival, but showed higher fecundity and fertility rate, compared to females not exposed. The occurrence of such hormetic responses could help elucidating the recent outbreaks of *E. heros* on Brazilian soybean fields where the use of insecticides is frequent. Since both above cited studies have been carried out under laboratory conditions, fields studies are needed to confirm such hypothesis.

Therefore, in the present investigation, we assessed the effects of exposure to sublethal dose of imidacloprid on population of *E. heros* and the possible consequences of such exposure on yield and quality of soybean seeds under field conditions.

Material and Methods

Insect rearing and exposure to insecticide

The colony of *Euschistus heros* was initially started from eggs provided by Embrapa Genetic Resources and Biotechnology (Brasília, DF, Brazil). Rearing was performed as described elsewhere (Borges et al., 2008; Silva et al., 2008) under controlled conditions at 27 ± 2°C; 75 ± 5 % R.H. and 14: 10h (L: D). Artificial lighting was maintained between 08:00 and 22:00 h to avoid diapause. In order to increase genetic variability, new individuals from soybean fields at Federal University of Viçosa (UFV; Viçosa, Minas Gerais, Brazil) and from farms in Tangará da Serra Region (Mato Grosso, Brazil) were routinely introduced into rearing.

The neonicotinoid insecticide imidacloprid (water-dispersible granules at 700 g active ingredient (a.i.)/L; Bayer Crop Science, São Paulo, SP, Brazil) was used at a concentration of 1% of the field label rate (equivalent to 0.042 a.i.µg/cm²). Exposure to insecticide was done as previously described by Santos et al., (2016). Briefly, the imidacloprid solution was applied as 2 mL aliquots using a micropipette into the inner wall of transparent glass-vials (250 ml) and left to dry. Control treatment consisted of application of distilled water only. Three hundred of newly emerged male and female adult (≤ 72 h) were transferred to the treated glasses that were kept closed with a piece of organza veil and a rubber band to prevent the insects from escaping. After 48 hours exposure period, surviving stink bugs were transferred to a clean plastic cup (250 ml) and provided *ad libitum* with fresh green beans pods (*Phaseolus vulgaris* L.) until used for the following experiments.

Soybean culture installation

Soybean plantation was done in November 2017 at the experimental farm of the Univértix Campus, Matipó, Minas Gerais, Brazil (20º 18' 40.6"S, 42º 19' 16.4"W). Soybean seeds (TEC 7849 IPRO, COOPADAP, São Gotardo, MG, Brazil) were sowed at 0.5 m spacing between rows and at 16 seeds/m within the row. Mineral fertilization was performed according to local agricultural practices and following the recommendations to soybean culture and soil fertility (Alvarez et al., 1999). Thirty days after emergence, soybean plants received a 2kg/ha of foliar fertilization (5-10-5 of NPK and micronutrients) (Heringer/FH SOJA foliar).

Effect of sublethal exposure to imidacloprid on infestation capacity of stink bugs under field conditions

At stage R_5-R_6 (pod filling stage), fourteen plants were isolated with a cage made of plastic pipes (2.5 cm in diameter) covered with organza (1.20 x 1.20 x 1.0 m). Four replicates (cages) were used respecting a minimum distance of 2.0 m between cages. Three treatments were set up by releasing one couple stink bug adults/plant as follows: *i)* plants without insect (control); *ii)* plants infested with unexposed stink bugs to imidacloprid; *iii)* plants infested with stink bugs exposed to 1% of the field label rate of imidacloprid. After fifteen, twenty-two, thirty and forty-five days of release, total number of different life stages of stink bug individuals (eggs, nymphs at third, fourth and fifth instar and adults) was recorded. In the last evaluation, all stink bugs were removed from the cages and soybean plants were kept in the field without bugs until harvest (at R8 stage). Yields, full and empty pods per plant with 13% of humidity were assessed after harvesting.

Effect of infestation by stink bugs unexposed and sublethaly exposed to imidacloprid on seed viability

To estimate the viability of soyben seeds, the tetrazolium test was used. This test is based on the activity of dehydrogenase enzymes that reduce the 2,3,5 triphenyltetrazolium chloride in the living tissues of the seeds resulting in the formation of a red non-diffusible compound, referred to as triphenylformazan indicating respiratory activity. Four replicates of 50 seeds each were sampled from different treatments after harvesting and were conditioned on paper towels moistened with distilled water and left in a germination chamber at 25°C for 16 hours. Thereafter, seeds were transferred to 100 mL Becker and immersed in Tetrazolium (2,3,5- triphenyl tetrazolium chloride) at concentration of 0.075%. The seeds were kept in a germination chamber at 40°C for 2h and 30 min in the dark. After this time, seeds were washed with distilled water and kept in distilled water until evaluation. The seeds were individually evaluated according as described by França Neto et al., (1998) and the percentage of sucked (1-8 categories) and non-viable seeds (6-8) were determined.

Statistical analysis

The total number of individuals (eggs, nymphs and adults) was subject to repeated-measures (multivariate) analyses of variance, since the individuals numbers per cage was evaluated at the same plots each time (Green, 1993; Paine, 1996). The analyses were performed using the PROC MANOVA procedure with the PROFILE statement (von Ende, 1993). The means from soybean plants yields were analyses by the PROC ANOVA procedure and *post hoc* Tukey's HSD tests (α = 0.05) were realized to

compare means. All analyses were performed with statistical software SAS/STAT software for Windows (SAS Institute, Cary, NC).

The means from soybean seeds non-viable and sucked seeds were analysed by the PROC ANOVA procedure followed bypost hoc Tukey's HSD tests (α = 0.05) to compare means. All analyses were performed with statistical software SAS/STAT software for Windows (SAS Institute, Cary, NC).

Results

Effect of sublethal exposure to imidacloprid on infestation capacity of stink bugs under field conditions

The sublethal exposure to imidacloprid affected the cumulated number of eggs laid by females of *E. heros* (Fig 1A, Table 1) and the total of nymphs plus adults over time (Fig 1B, Table 2). No statistical differences were found from the number of nymphs plus adults among treatments during 15, 22 and 30 days, but differed significantly at 45 days (Fig 1B, P <0,001). The total number of eggs (Fig. 1C, F= 9.21, *df*=1, P=0.02) and nymphs plus adults was significantly higher due the sublethal exposure to imidacloprid (Fig. 1D, F= 8.11, *df*=1, P=0.02). These individuals differed significantly between two treatments with time and interactions among treatments and time were significant (Table 2).

Table 1. Repeated measures ANOVA for the average cumulated number of eggs laid by females of stink bugs *Euschistus heros* unexposed and exposed to 1% of the field label rate of imidacloprid under field conditions.

Variation source	***df***	**F**	**P**
Treatment (T)	1	9.61	0.021*
Evaluation Time (ET)	3	6.08	0.005*
T vs ET	3	0.282	0.83
Error	18	-	-

Table 2. Repeated measures ANOVA for infested soybean plants by stink bugs *Euschistus heros* unexposed and exposed to 1% of the field label rate of imidacloprid under field conditions.

Variation source	***df***	**F**	**P**
Treatment (T)	1	12.25	0.013*
Evaluation Time (ET)	3	45.33	<0.001*
T vs ET	3	4.84	0.012*
Error	18	-	-

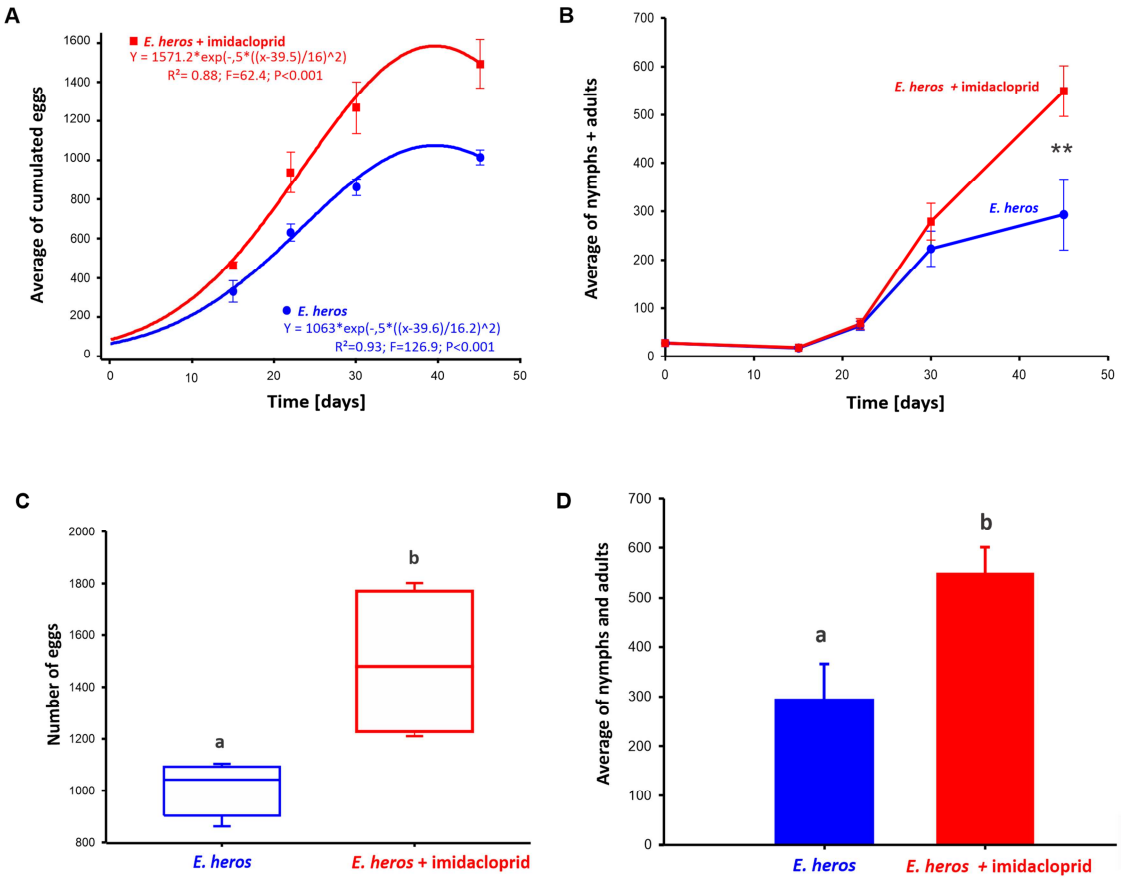

Fig. 1 Average (mean ± SE) of *E. heros* exposed (red square) and unexposed (blue cycles) to imidacloprid obtained at 15, 22, 30 and 45 days after infestation under field condition. Average of cumulated eggs (**A**); average of nymphs at third, fourth, and fifth instars and adults (**B**); total number of eggs (**C**); average of total nymphs and adults (**D**). The newly emerged adults (≤ 72 h) were exposed for 48 h to imidacloprid and the imidacloprid concentration was 0.042 a.i.µg/cm². Different letters above the bars indicate significant among treatments ($P < 0.05$).

The yield of soybean plants infested with *E. heros* previously exposed and non-exposed to imidacloprid was similar, but significantly lower when compared with no infested plants (control) (Fig. 2A, $F = 19.25$; *df* =2; $P< 0.001$). Soybeans mean yield varied from 22.72 g plant^{-1} in control plants to 10.45 g plant^{-1} in plants infested with stink bugs (Fig. 2A). The average of full pods was similar between plants infested with *E. heros* independently of imidacloprid exposure, and

significantly lower compared to control plants (Fig. 2B, F = 7.70; *df* =2; P= 0.01). Mean of empty pods was lower in control plants, intermediate in plants infested with non-exposed and higher in plants with *E. heros* previously exposed to imidacloprid (Fig. 2C, F = 8.06; *df* =2; P= 0.009).

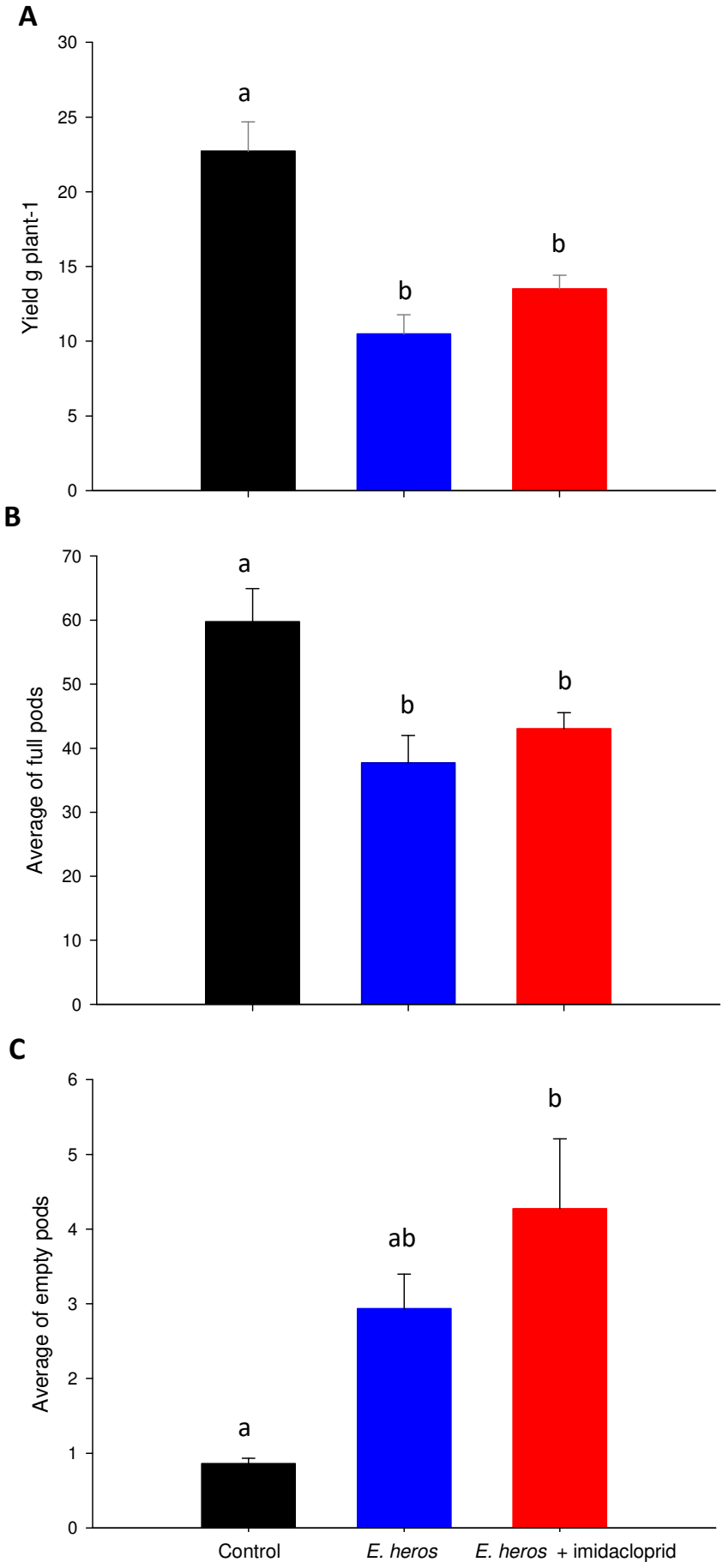
A
Yield g plant-1
30
25
20
15
10
5
0
a
b
b
B
Average of full pods
70
60
50
40
30
20
10
0
a
b
b
C
Average of empty pods
6
5
4
3
2
1
0
a
ab
b
Control
E. heros
E. heros + imidacloprid

Fig. 2 Average yield (**A**), full pods (**B**) and empty pods (**C**) from plants in the absence (black bars) and presence of non-exposed (blue bars) and sublethaly exposed to imidacloprid (red bars), during pod filling and maturation stage in fields. The bars represent the mean of four replicates (mean ± SE). The newly emerged adults (≤ 72 h) were exposed for 48 to imidacloprid and the imidacloprid concentration was 0.042 a.i.µg/cm². Different letters above the bars indicate significant among treatments ($P < 0.05$).

Effect of infestation by stink bugs unexposed and sublethaly exposed to imidacloprid on seed viability

The non-viable seeds percentage was higher in seeds damaged by *E. heros* previously exposed to imidacloprid compared with non-exposed (Fig. 3A, $F = 104.46$; *df* =2; $P<0.001$). However, the percentage of sucked seeds were higher between treatments with *E. heros* independently of imidacloprid exposure and lower in control (Fig. 3B, $F = 2009.3$; *df* =2; $P<0.001$).

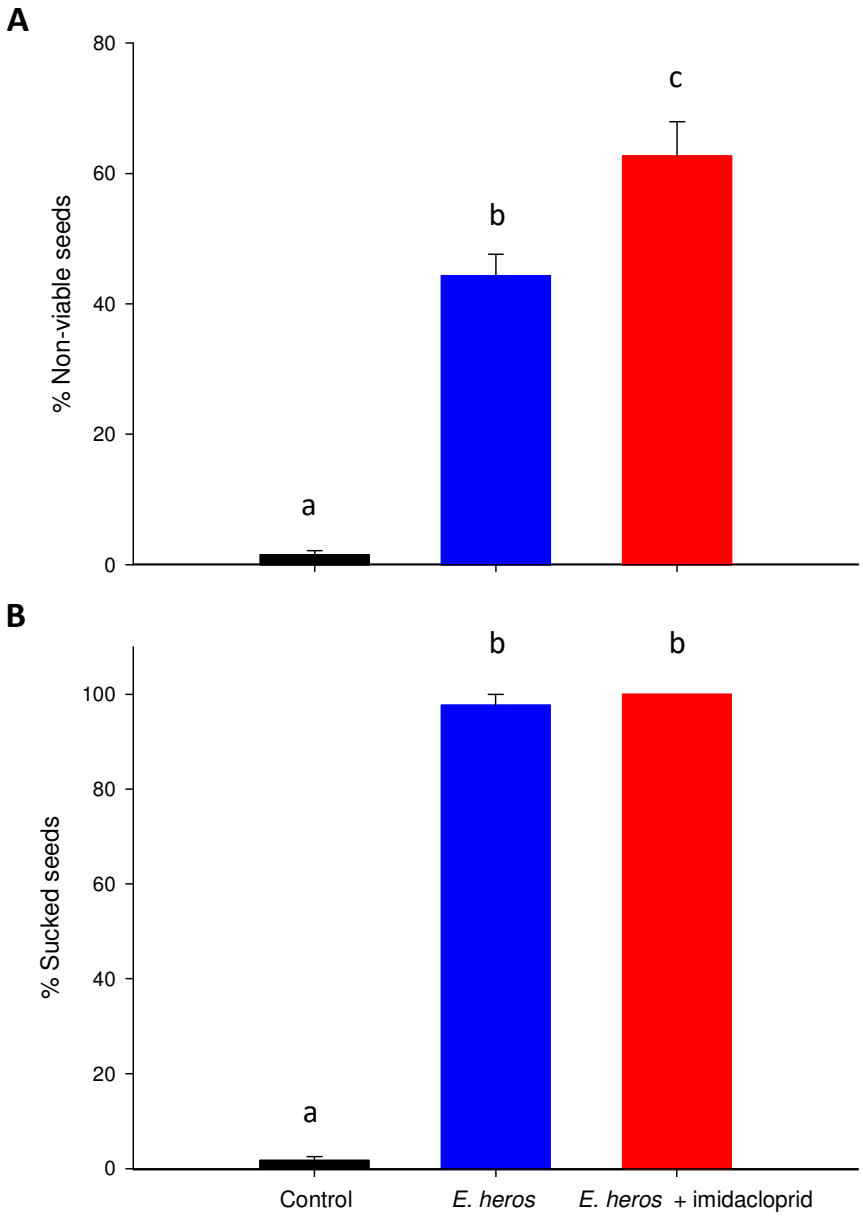

Fig. 3 Percentage non-viable (**A**) and sucked seeds (**B**) (mean ± SE) from soybean plants in the absence of *E. heros* (black bars), infested by *E. heros* non-exposed (blue bars) and previously exposed to imidacloprid (red bars), during pod filling and maturation stage in fields cages. The newly emerged adults (≤ 72 h) were exposed for 48 to imidacloprid and the imidacloprid concentration was 0.042 a.i.µg/cm². Different letters above the bars indicate significant among treatments ($P < 0.05$).

Discussion

According to the results, sublethal exposure to imidacloprid can increase *E. heros* population in the field (Fig. 1). It is known that sublethal exposure to imidacloprid can increase reproductive responses in some arthropods (Christopher Cutler et al., 2009; Haddi et al., 2016; James and Price, 2002; Santos et al., 2016; Szczepaniec and Raupp, 2013; Yu et al., 2010). To the best of our knowledge, this is a potential evidence to explain the massive outbreaks of *E. heros* frequently seen on soybean crops in Brazil, it may be a consequence of neonicotinoid exposure combined with the suppression of natural enemies (James and Coyle, 2001). The exposure to sublethal doses of insecticides in fields is a common situation, as a consequence of environmental degradation or via heterogeneous spatial coverage on plants, e.g. poor spray distribution on leaves and others parts of the plants (Christopher Cutler et al., 2009). Thus, an hormetic response may be triggered as a response to insecticide sublethal doses (Calabrese et al., 2008; Cutler, 2013; Guedes and Cutler, 2014; Santos et al., 2016).

Here, it was demonstrated that soybean plants infested with one couple per plant had lower yield per plant (Fig. 2A) and full pods (Fig. 2B) compared to control plants. There were more deformed and damaged seeds in attacked plants by *E. heros* what contributed with the lower production parameters compared to the control. The largest population was found on soybean plants infested by exposed *E. heros* (Fig. 1B); however, the yield at the end of the study was similar to plants infested by unexposed insects. It is known that the reduction of soybean yield caused by stink bugs depends on infestation times, population densities or soybean developmental stage (Corrêa-Ferreira and Azevedo, 2002; Galileo and Heinrichs, 1978; McPherson et al., 1979; Panizzi et al., 1978; Panizzi and Slansky Jr, 1985; Tood and Turnipseed,

1974). The first two weeks the stink bugs population had predominantly eggs and nymphs in first, second and third instar, which cause less damaged to plants when compared to fourth, fifth instar and adult (Bowling, 1980; Corrêa-Ferreira and Panizzi, 1999). Differences between population of exposed and unexposed insects, considering the most harmful stages were recorded from 30 days after infestation (Fig. 1B). In addition, seeds are more susceptible to deformation during pod-fill, which occurred in the first two weeks of the experiment.

Our results showed that plants infested by *E. heros* exposed to sublethal doses and unexposed had approximately the same number of empty pods (Fig. 2C). Corrêa-Ferreira and Azevedo (2002) also have not found significant differences among empty pods from plants infested by *E. heros* and control plants. Nevertheless, in our experiment plants infested by stink bugs exposed to imidacloprid had more empty pods than control plants. Thus, the exposure to imidacloprid can also negatively affect the soybean productivity. In short, the most susceptible stage of the plants were exposed to the less harmful stages of the pest, this might be reflected in the lack of difference between both treatments on yield at the end of experiment. Despite the similar soybean yield, the bigger population of exposed *E. heros* reduced the seed quality (Fig. 3).

Non-viable seeds percentage was significantly higher in plants damaged by *E. heros* exposed to imidacloprid (Fig. 3A), as this treatment had a major number of insects at 3º, 4º e 5º instar compared with stink bugs not exposed (Fig. 1B). However, the percentage of sucked seeds was similar between plants infested by *E. heros* non-exposed and exposed to imidacloprid, but different from control (Fig. 3B). Some researchers also confirmed that soybean plants infested by *E. heros* with two stink bugs per plant had more sucked seeds and non-viable seeds when compared to

control plant (Corrêa-Ferreira and Azevedo, 2002; Nunes and Corrêa-Ferreira, 2002). Here, it has been demonstrated that the exposure of *E. heros* to imidacloprid may increase its population and reduce seed quality as a consequence of the higher number of individuals that may damage soybean seeds.

It is known that imidacloprid insecticide is used largely in Brazil for controlling a large range of pests on crops. Our study suggested that applications of imidacloprid on soybean crops in Brazil may significantly increase *E. heros* population. Hence, it may contribute to additional insecticide treatments and consequently has a higher cost to famers. In addition, the greater number of spraying may intensify environmental contamination (Christopher Cutler et al., 2009). It could also collaborate with pest resurgence, selection of resistant pest population and harm beneficial organisms (e.g. natural enemies, pollinators) (Christopher Cutler et al., 2009; Cutler et al., 2005; Cutler and Rix, 2015; Guedes et al., 2009; Guedes and Cutler, 2014; Castellanos et al., 2018).

Our research suggested that imidacloprid may enhance *E. heros* population and as a result reduce yield and seeds quality. It might also help explaining recent outbreaks of *E. heros* on soybean crops in Brazil. However, only a concentration of 1% of the field rate was tested. Further investigations are necessary to study in which range of imidacloprid concentration it might occur and investigate whether other classes of insecticides can present the same results. Moreover, the indiscriminate use of chemical insecticides with preventive spraying, just may contribute to increase *E. heros* population, because imidacloprid residue can be degraded and it can have stimulatory effect in pest.

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CHAPTER 2

Sublethal exposure to imidacloprid can increase damage by *Euschistus heros* in soybean seeds

Abstract

Neotropical brown, *E. heros*, is currently the most abundant and prevalent pest on soybean crops in Brazil and their reproduction can increase after sublethal neonicotinoid pesticide exposure. It is known that physiological quality of soybean seeds such as germination and vigor can decrease with attack of stink bugs. Moreover, mechanisms of protection (e.g. antioxidant enzymes) against damage can occur which is an adaptation on condition of biotic stress. Likewise, plants present some strategies that offer protection against herbivory such as proteinase inhibitors which interfere with digestion of proteins that are present in the insect gut. Thus, we aimed to know whether: i) *E. heros* exposed to sublethal doses of imidacloprid in the field condition can decrease physiological quality of soybean seeds and change antioxidant enzymes activity; ii) *E. heros* can interfere on defence system of soybean seeds and whether this response can variable when this stink bug is exposed to sublethal doses of imidacloprid. Newly emerged adults (≤ 72 h) were exposed to dry imidacloprid residuals or distilled water for 48h. Afterwards, two stink bugs per plant were released in the field cages for 45 days, during the pod filling (R_5-R_6). Plants without stink bugs were considered as control. Physiological quality of the seeds was assessed after harvest by germination, accelerated aging, first count, speed of seedling emergence and electrical conductivity tests. Antioxidant enzymes activities were also determined: superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX). To study proteinase inhibitor activity, stink bugs were realed in cages in

proportion of, two stink bugs per plant or two stink bugs per pods, at R5-R6 stage, where they remained for seven days. After, seeds were harvested and proteasis inhibitor activity was assayed. Physiological quality was higher in control plants, intermediate in infested plants with non-exposed and lower in infested plants with *E. heros* previously exposed to imidacloprid. SOD activity decreased in seeds from *E. heros* infested plants exposed to insecticide. Nevertheless, CAT and POX activity from soybean seeds damaged by stink bug exposed to insecticide enhanced compared infested plant with this pest without exposition to imidacloprid and control plant. It was found out that damaged plants by *E. heros* in different proportion independently of imidacloprid exposure, presented less than half proteinase inhibitor activity compared to non-damaged seeds. Thus, *E. heros* exposed to imidacloprid can decrease the physiological quality of soybean seeds and change antioxidant enzymes activity. *E. heros* independently of imidacloprid exposure can interfere soybean seeds defence. These findings suggest that sublethal exposure to imidacloprid can increase *E. heros* population and consequently reduce soybean seeds quality and this stink bug can also interfere soybean seeds defence.

Keywords: Vigor, Antioxidant, Neonicotinoids, Stink bugs, Sublethal doses

Introduction

Stink bugs is the most important pest on soybean crops in different countries of the world (Panizzi and Slansky Jr, 1985; Turnipseed and Kogan, 1976; Panizzi et al., 2012). There are three species from Pentatomidae family that are more harmful to soybean crop and economically important, *Piezodorus guildinii* (Westwood), *Nezara virudula* (Linnaeus) and *Euschistus heros* (Fabricius) (Panizzi & Slansky Jr, 1985). It is known that *P. guildinii* cause more damage in soybean seeds compared to others species (Corrêa-Ferreira and Azevedo, 2002). However, Neotropical brown, *E. heros*, is currently the most abundant and prevalent pest on soybean crops (Panizzi et al., 2012; Saluso et al., 2011). This pest can have multiple generations on the same season (Sosa-Gómez and Omoto, 2013), can also survive on native plants or/and other crops present in the field during soybean intercrop period (Panizzi et al., 2012). This pest are dominant competitors among others species (Tuelher et al., 2016) and can induce severe damage on soybean seed (Silva et al., 2012). In addition, in a recent study was presented that *E. heros* sublethally exposed to insecticide can enhance their reproduction and possibly explain recent outbreaks of this pest in the field (Santos et al., 2016).

The occurrence of these species on soybean crop can cause seed abortion, decrease yield, oil content and seed quality (e.g. germination and vigor) (Corrêa-Ferreira and De Azevedo, 2002; Daugherty et al., 1964; McPherson et al., 1979; Musser et al., 2011; Silva et al., 2012; Tood and Turnipseed, 1974). These pest can puncture different plant parts, but it prefers to feed on young developing seeds (McPherson et al., 1994). The feeding creates opportunity for pathogens cololonization in the plant, which can also contribute with reduction of yield and seed quality (McPherson et al., 1994). Severe damage caused by stink bugs is

attributed to their morphology of mouth parts, salivary enzymes and feeding behavior (Depieri and Panizzi, 2010). The damage intensity is variable according to stink bugs population level and soybean development stage (McPherson et al., 1993; McPherson, 1996; Panizzi et al., 1979). It is known that from pod development to pod filling is the most critical stage for stink bugs attack (Corrêa-Ferreira and Panizzi, 1999).

Soybean seeds with high physiological quality have an important role on crops establishment and also for storage period of seeds. It is well known that seed vigor defines its ability to germinate and establish seedlings rapidly and uniformly across diverse environmental conditions (Finch-Savage and Bassel, 2016). Deterioration process in seeds involves a series of physiological, biochemical and physical changes that cause progressive and irreversible decline in the seed quality, ending in its death (Delouche and Baskin, 1973). Attack of stink bug can contribute to deterioration process and reduce physiological quality of soybean seeds. Moreover, it is known that damage in soybean seeds is variable for each stink bugs, for example, infested plants by *P. guildinii* have more non-viable seeds compared to infested plants with *N. virudula* or *E. heros* (Corrêa-Ferreira and Azevedo, 2002). However, *E. heros* is the most abundant and prevalent pest on soybean crops (Panizzi et al., 2012; Saluso et al., 2011) which can harm seeds quality.

Euschistus heros is mostly controlled by chemical insecticides such as neonicotinoids and they have been widely used in Brazil and in another countries in order to control arthropod pests (Jeschke et al., 2013; Sosa-Gómez et al., 2009; Sosa-Gómez and Silva, 2010). The use of neonicotinoid insecticides has increased in Brazil, as it has been used to replace other compounds such as carbamates, organophosphates and endosulfan (Jeschke et al., 2013). Furthermore, there is few

registered number of insecticides and sometimes with a frequent overuse, which can contribute to stink bugs control failure (Tuelher et al., 2016).

Plants can also induce a defense mechanism when injured by herbivores (Walling, 2008), which is variable according to the type of stressor and genetic background (Courtois et al., 2009). It is known that reactive oxygen species (ROS) is an adaptation on biotic and abiotic stress conditions, which can be responsible for signaling activation of plant defense or programmed cell death (Miller et al., 2008; Shetty et al., 2008). Oxidative enzymes and reactive oxygen species are accumulate following herbivory attack (Bi and Felton, 1995). Reactive oxygen species were noted in response to insect injury (De Ilarduya et al., 2003; Maffei et al., 2006). In addition, several studies suggest that degradation and inactivation of antioxidative enzymes activity as superoxide dismutase, catalase and peroxidase ocurrs in deteriorated seeds (Bailly, 2004; Goel et al. 2003; Lehner et al., 2008). However, there is no study of soybean seeds redox response induced by Neotropical brown stink bug.

In addition, plants have been developing many strategies that offer protection against pathogens and arthropods during evolution (Dangl and Jones, 2001; Walling, 2008). The defence mechanisms might vary with type of stressor and plant species (Courtois et al., 2009). It is well known that plants have defence systems to counter herbivore attack such as direct defences which involve structural components (e.g. thorns and trichomes) or secondary metabolites (e.g. flavonoids, tannins and proteinase inhibitors) that affect herbivore performance (Arimura et al., 2009; Dudareva et al., 2006; Howe and Jander, 2008; War et al., 2012). Besides that, indirect defence that might attract natural enemies by the production of volatile compounds (Dicke et al., 1990; Price et al., 1980; War et al., 2012). When plants are

attacked by an herbivore successive cascades of biochemical are triggered with genes activation in a few minutes (Maffei et al., 2007). Proteinase inhibitors (PI) are induced by herbivore injury which have antinutritional effects and consequently interfere on the digestion of proteins that are present in the insect gut and subsequently offer protection against herbivory. PI occur on higher concentration in seeds and they are induced by herbivore attacked (Koiwa et al., 1997; Ryan, 1990; War et al., 2012). The accumulation of inhibitors is important to maintain seeds integrity, which is crucial for the survival of species (Koiwa et al., 1997). Additionally, studies have been done with soybean plants aiming to find herbivore resistant varieties (Moraes et al., 2009; Vieira et al., 2013; Timbó et al., 2014).

In addition, Santos et al., (2016) observed that female *E. heros* exposed to sublethal doses of imidacloprid, classified as neonicotinoid, can enhance their reproduction. Several studies also demonstrated that sublethal neonicotinoid exposure can enhance reproduction in other arthropods (Christopher Cutler et al., 2009; James and Price, 2002; Szczepaniec and Raupp, 2013; Yu et al., 2010). This phenomenon is called hormesis, biphasic dose-response: when a low dose leads stimulation and high dose to inhibition (Calabrese et al., 2008; Calabrese and Baldwin, 2003; Guedes and Cutler, 2014). In addition, it is known that occurrence of low pesticide doses always happens with crops due to compounds degradation on the environment (Guedes and Cutler, 2014).

Many studies investigate whether sublethal exposure to pesticides affects reproduction and survival of arthropods species (Cutler, 2013; Desneux et al., 2007; Guedes et al., 2016; Haddi et al., 2016; Santos et al., 2016), but there is not any research that presents its consequences for seeds quality. Thus, we aimed to know whether *E. heros* exposed to sublethal doses of imidacloprid in the field condition

can affect soybeans seeds physiological quality, antioxidant enzymes activity and proteinase inhibitors.

Material and Methods

Rearing methods

Euschistus heros was provided by Embrapa Genetic Resources and Biotechnology (Brasília, DF, Brazil). Rearing was performed as described by Borges et al., (2008) and Silva et al., (2008). Rearing of *E. heros* was kept under controlled conditions at 27 ± 2°C; 75 ± 5 % R.H. and 14: 10h (L: D). Artificial lighting was maintained between 08:00 and 22:00 h to avoid diapause. New individuals from soybean fields at Federal University of Viçosa (UFV; Viçosa, Minas Gerais State, Brazil) and from farms of Tangará da Serra Region (Mato Grosso, Brazil) were introduced in rearing to increase genetic variability.

Exposure to insecticide

Exposure to imidacloprid was done as described by Santos et al., (2016). Imidacloprid (water-dispersible granules at 700 g active ingredient (a.i.)/L; Bayer Crop Science, São Paulo, SP, Brazil) was used at a concentration of 1% of the field rate (at 0.042 a.i.µg/cm²). Stink bugs that were exposed to distilled water were considered as control. After this period of exposure to imidacloprid stink bugs were introduced in a clean cup (250 ml) covered with fine mesh in order avoid escaping of insects. Green bean fresh pods (*Phaseolus vulgaris* L.) were supplied as food until installing experiments in the field.

Soybean culture installation

Soybean seeds ('TEC 7849 IPRO' - late cycle, indeterminate growth and maturity group 7.8) were sown in November 2017, within 0.5 m row spacing and sowed 16 seeds/m at Univértix Campus, Matipó, Minas Gerais, Brazil (20º 18' 40.6"S, 42º 19' 16.4"W). Mineral fertilization was performed according to the recommendation for the crop and to soil fertility (Alvarez V. et al., 1999). Thirty days after emergence, soybean plants received a 2kg/ha of foliar fertilization (5-10-5 of NPK and micronutrients) (Heringer/FH SOJA foliar).When plants were at R_5-R_6 stage (pod filling) just fourteen plants were allowed in a cage (1.20 x 1.20 x 1.0 m, covered with fine gauze). Cages were randomly distributed with four replicates and a minimum of 2.0 m of distance between cages. Plants were infested with a rate of two newly emerged adults per plant. Treatments were set up as it follows: *i)* plants without insect (control); *ii)* bugs without previous exposure to imidacloprid; *iii)* bugs exposed to imidacloprid. After forty-five days all stink bugs were removed and soybean plants were kept in the cages until harvest (R8 stage). Posteriorly, the seeds from different treatments were harvested and submitted to tests below.

Seeds experiment

Seed moisture content: was determined by oven method at 105 ± 3ºC for 24 hours as described in the Rules for Seed Testing (Brasil, 2009).

Germination: was done with four replicates of 50 seeds, which were sown on paper towels moistened with distilled water volume equivalent to 2.5 times the dry paper weight. Rolls were kept in a germination chamber at 25°C. Percentage of normal seedlings was determined on the 5^{th} and 8^{th} day after sowing (Brasil, 2009).

First count: was carried out together with germination test, counting the number of normal seedlings present 5th day (Brasil, 2009). Results were expressed in percentage.

Accelerated aging: Two hundred seeds were uniformly distributed on wire mesh in plastic boxes (10 x 10 x 10 cm), where 40 ml of distilled water was added and kept at 41°C for 48 hours. Afterwards, four replicates of 50 seeds for each treatment were sown on towel paper and moistened with distilled water. The rolls were kept as described above for germination test and normal seedlings were determined on the eighth day.

Speed of seedling emergence: Fifty seeds were sown in Styrofoam trays (30 x 23 x 4.5 cm) containing soil and sand on 1:1 (w:w) ratio and moistened to 70% retention capacity. Trays were kept under 25 ± 5°C in a greenhouse. Seedling emergence was daily quantified for 16 days. Speed of seedling emergence was calculated according to Maguire (1962).

Electrical conductivity: was done with four replicates of 50 seeds, which was weighed and immersed in 75 mL distilled water. It was kept in a chamber at 25°C for 24 hours. Posteriorly, the conductivity meter was used to determine the solutions electrical conductivity. Result was expressed in $\mu S.cm^{-1}.g^{-1}$ of seeds.

Enzymatic activit experiment

Antioxidant enzymes activity was determined in order to give information about soybean seed quality, such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (POX). Seeds were imbibed for 24 hours at similar germination test condition. Afterward, the embryos (cotyledons and embryonic axes) were extracted and it was frozen in liquid nitrogen and stored at −20°C until the enzyme assays.

After this time, 0.2 g of cotyledons and embryonic axes were extracted and grounded in a mortar containing liquid nitrogen. Posteriorly, 2 ml of extraction medium was added, 0.1 M of potassium phosphate buffer (pH 6.8), containing 0.1 mM of ethylenediaminetetraacetic acid (EDTA), 1.0 mM of phenylmethanelsulfonyl fluoride (PMSF) and 1 % of polyvinylpolypyrrolidone (PVPP) (w/v) (Peixoto et al., 1999). Thereafter, homogenized product was centrifuged at 19000 xg during 15 min at 4 ºC. Soluble protein content of the enzymatic extracts was quantified Bradford Method (1976) using BSA (Bovine Serum Albumin) as standard. Bradford reagent (1mL) was added to enzyme extract (10 µL) followed by shaking. After 20 minutes the sample was read with a spectrophotometer at 595 nm.

SOD activity was assessed by adding crude enzyme extract (50 µL) to reaction medium (2.95 mL), which had sodium phosphate (50 mM) (pH 7.8), methionine (13 mM), p-nitro blue tetrazolium (NBT) (75 µM), riboflavina (2 µM) and EDTA (0.1 mM) (Del Longo et al. 1993). Analysis was realized at 25 ºC in a chamber under 15-W fluorescente lighting during five minutes. After that, light was off and blue formazan was produced by the photoreduction of NBT, which was assessed by absorbance at 560 nm. Absorbance value of a reaction medium was equal to the previous medium, however, it was kept in the dark at the same time that it was considered as control and it was subtracted from the absorbance reading sample that was exposed to light (Giannopolitis and Ries, 1977). It is well known that one SOD unit is the quantity of enzyme required to inhibit NBT photoreduction by 50% (Beauchamp and Fridovich, 1971). The result was expressed in $U^{-1}min^{-1}$ mg^{-1} protein.

CAT activity was assessed by addition the of crude enzyme extract (50 µL) to the reaction medium (2. 95 mL), which had potassium phosphatase buffer (50 mM) (pH 7.0) and hydrogen peroxide (12.5 mM) (adapted from Havir and McHale, 1987). Reduction in absorbance at 240 nm at 25°C was measured during the first minute of reaction and activity was quantified by molar extinction coefficient of 36 M^{-1} cm^{-1} (Anderson et al. 1995) and result was expressed in µmol min^{-1} mg^{-1} protein.

POX activity was assessed by the addition of crude enzyme extract (100 µL) to reaction medium (2.90 mL), which had potassium phosphate buffer (25 mM) (pH 6.8), pyrogaloll (20 mM) and hydrogen peroxide (20 mM) (adapted from Kar and Mishra, 1976). Purpurogallin production was calculated by the increment in absorbance at 420 nm at 25 ºC and its activity by 2.47 M^{-1} cm^{-1} was quantified by molar extinction coefficient (Chance and Maehly, 1955) and result was expressed in µmol min^{-1} mg^{-1} protein.

Greenhouse experiments

Soybean seeds (TEC 7849 IPRO - late cycle, indeterminate growth and maturity group 7.8) were sown in January 2018 in pots (3 liters). The pots were kept in a greenhouse (mean temperature: 25 ± 5°C, 70% ± 10% R.H., 11: 13 h L: D). Plants were fertilized according to the recommendation to soybean crops and soil fertility (Alvarez V. et al., 1999) and thirty days after emergence, the soybean plants received a 2kg/ha of foliar fertilization (5-10-5 of NPK and micronutrients) (Heringer/FH SOJA foliar). Plants at R_5 (pod filling) reproductive stage were used for experiments below.

Two stink bugs per plant: In this experiment just one plant was allowed in a cage (0.45 x 0.35 x 0.80 m, covered with fine gauze) with a couple of newly emerged

adults. These cages were randomly distributed with four replicates. Treatments were: *i)* plants without insect (control); *ii)* damaged plants by *E. heros*; *iii)* damaged plants by *E. heros* previously exposed to imidacloprid. After seven days, two pods from fourth or fifth leaf with damage were collected. Posteriorly, seeds from different treatments was frozen in liquid nitrogen and stored at -80°C until proteinase inhibitor assays.

Two stink bugs per pods: In this experiment just the two pods from fourth or fifth leaf per plant were damaged by a couple of *E. heros*. Plastic cup was cut in the bottom (200 ml). After that, top and bottom of the cup were covered with fine gauze to allow ventilation and avoid stink bugs scape. Plants were randomly distributed with four replicates. Treatments were the same as described above. All seeds from these cages were collected and stocked in the same condition as describe above until proteinase inhibitor assays.

Proteinase inhibitor (PI) activity assays

Proteinase inhibitor activity was quantified in non-damaged seeds (control), damaged by *E. heros* and damaged by *E. heros* previously exposed to imidacloprid. An amount of 0.2 g cotyledons and embryonic axes were extracted and ground in a mortar containing liquid nitrogen. Posteriorly, 2 ml of extraction medium was added, 0.1 M of potassium phosphate buffer (pH 6.8), containing 0.1 mM of ethylenediaminetetraacetic acid (EDTA), 1.0 mM of phenylmethanelsulfonyl fluoride (PMSF) and 1% of polyvinylpolypyrrolidone (PVPP) (w/v) (Peixoto et al., 1999). It was centrifuged at 12000 xg during 30 min at 4 ºC and supernatant was diluted 10 times in distilled water to obtain the sample.

After that, 40 µL trypsin was mixed with 5 µL of sample and 655 µL incubation buffer (0.1 m Tris-HCl buffer, pH 8.2 and 20 mm $CaCl_2$), which was incubated at room temperature during 5 min. Controls consisted of 660 µL incubation buffer and 40 µL of trypsin. A 700 µL aliquot of this mixture was added to 300 µL Na-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride (1.2 mm). The trypsin activity was monitored with a spectrophotometer (410 nm). Difference between measured absorbance at 150 and 30 s was used to determine trypsin activity.

Soluble protein content of enzymatic extracts was quantified by Bradford Method (1976) using BSA (Bovine Serum Albumin) as standard. Sample was obtained by centrifugation at 12000 xg during 30 min at 4 ºC and the supernatant was diluted 4 times in distilled water. Then, 1 µL of sample was added to distilled water (799µL) and Bradford reagent (200 µL) followed by shaking. It was monitored with spectrophotometer (595 nm). Measurements were performed in triplicate per sample. Results were expressed as Intensive Trypsin Units (TIU) per gram of protein (e.g., 1 TIU correspond to the inhibition of 1 arbitrary unit of trypsin).

Statistical Procedure:

Experiment was conducted in a completely randomized design with four replications. Data was submitted to analysis of variance (ANOVA) and means were compared by Tukey test ($P<0.05$). All analysis was performed with R statistical software (R Development Core Team 2017).

Results

Seeds from infested plants with *E. heros* previously exposed to imidacloprid presented the lowest germination percentage (25.0 %) (Fig. 1; F = 180.61; d.f.=2;

P<0.001). Seeds from infested plants by *E. heros* not exposed to insecticide also presented lower germination rate (41.2 %). However, when plants were infested by *E. heros* exposed to imidacloprid they presented 16.16 % less normal seedlings when compared to infested plants by *E. heros* not to exposed to insecticide. These results were also observed by the tetrazolium test (Fig. 3; chapter 1). Infested plants with *E. heros* exposed to imidacloprid showed lowest vigor by accelerated aging test (Fig. 2 A; F = 323.5; d.f.=2; P<0.001). Germination speed index was lower in the seeds from infested plants with *E. heros* exposed to imidacloprid than in control plants (Fig. 2B; F = 11.51; d.f.=2; P=0.006). Additionally, the percentage of germination at first count (Fig. 2C; F = 337.63; d.f.=2; P<0.001) was higher in control plants, but was similar among *E. heros* treatments regardless imidacloprid exposure. Electrical conductivity were higher in seeds from infested plants with stink bugs (Fig. 2D; F = 23.52; d.f.=2; P<0.001).

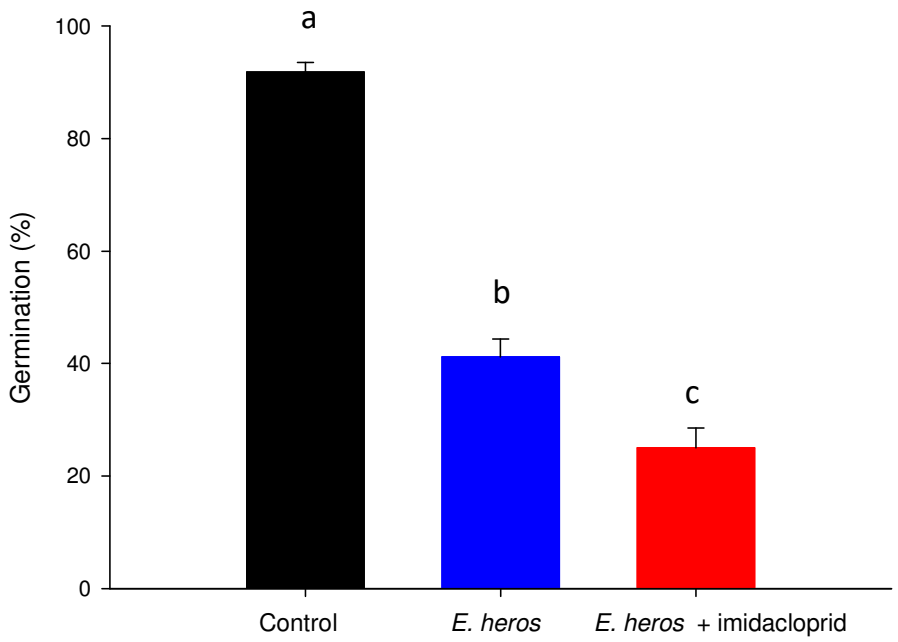

Fig. 1 Mean values of germination percentage of soybean seeds from clean plants (black bar), infested plants by non-exposed *E. heros* (blue bar) and previously

exposed to imidacloprid (red bar), during pod filling and maturation stage in field cages. Individuals were exposed to imidacloprid at concentration of 0.042 µg a.i./cm ² during 48 hours. The bars represent the average of four replicates ± standard error. Bars followed by the same letter did not differ by Tukey test ($P < 0.05$).

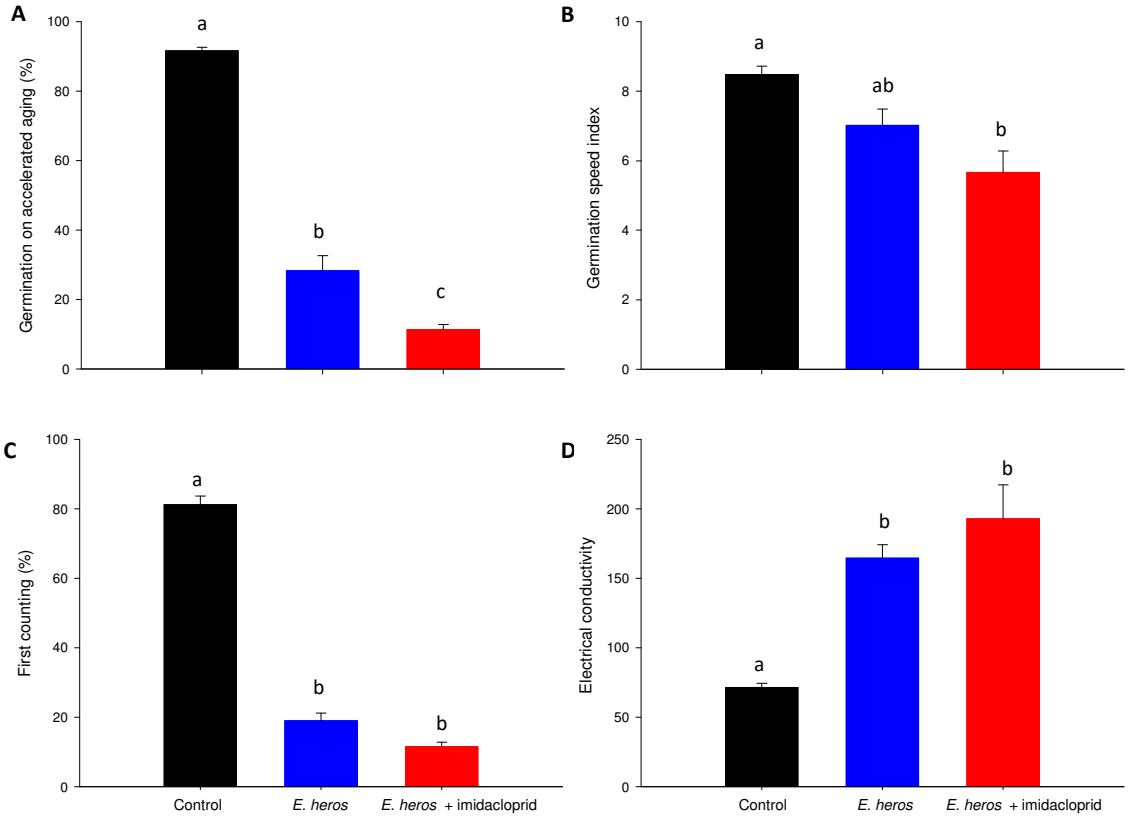

Fig. 2 Accelerated aging (**A**), germination speed index (**B**), first germination count (**C**) and electrical conductivity (**D**) of soybean seeds from clean plants (black bar), infested plants by non-exposed *E. heros* (blue bar) and previously exposed to imidacloprid (red bar), during pod filling and maturation stage in field cages. Individuals were exposed to imidacloprid at concentration of 0.042 µg a.i./cm ² during 48 hours. Bars represent the average of four replicates ± standard error. Bars followed by the same letter did not differ by Tukey test ($P < 0.05$).

It was observed a reduction in SOD activity in seeds from infested plants with *E. heros* exposed to imidacloprid unlike other treatments (Fig. 3A, $F = 12.08$; d.f.=2; $P<0.01$). In contrast, CAT activity was higher in seeds from infested plants with *E. heros* exposed to imidacloprid when compared to seeds from infested plants with non-exposed *E. heros* and control plants (Fig. 3B, $F = 9.77$; d.f.=2; $P<0.01$). POX activity was also higher in damaged seeds by *E. heros* previously exposed to imidacloprid compared to control plants (Fig. 3C, $F = 6.8$; d.f.=2; $P=0.02$).

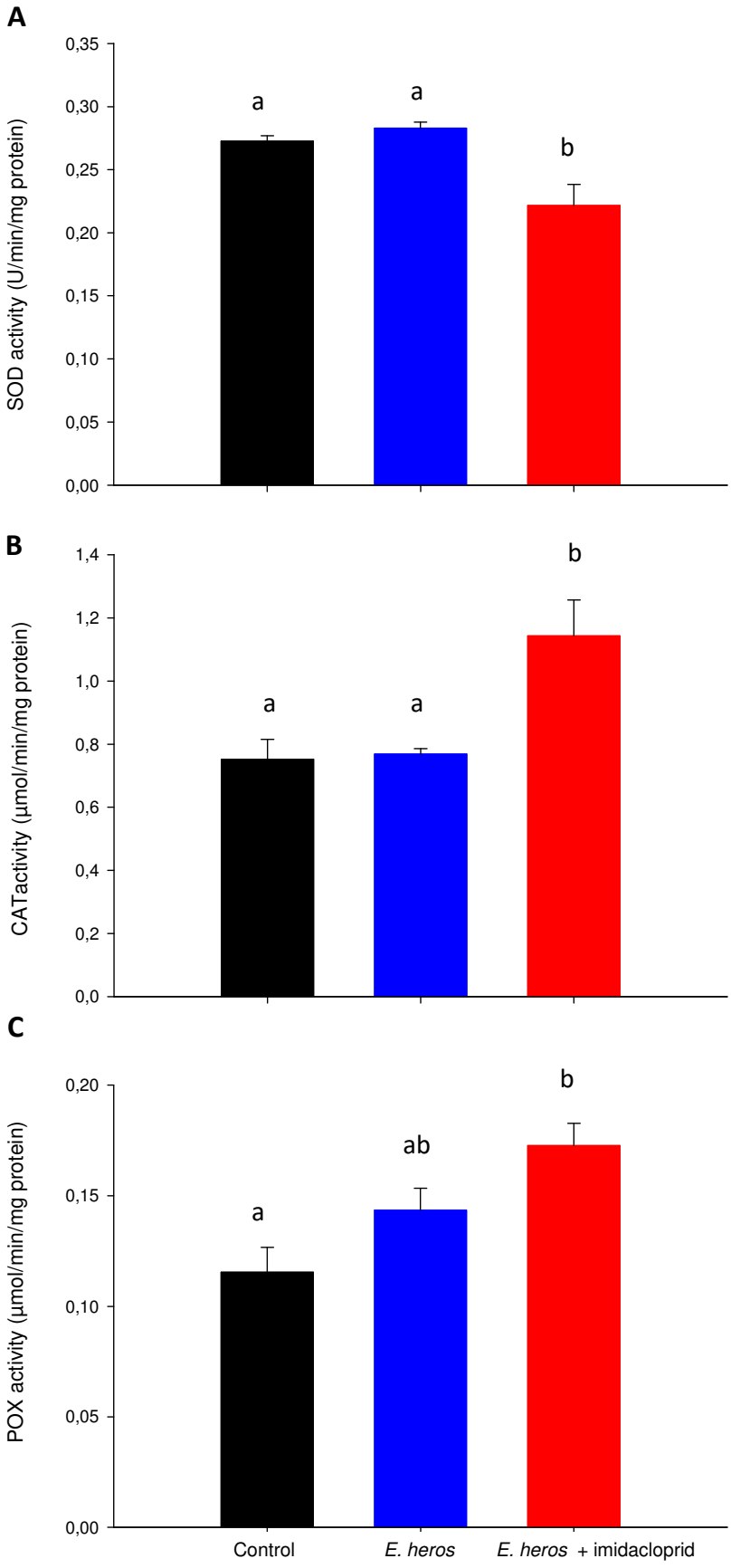
A
SOD activity (U/min/mg protein)
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
a
a
b
B
CATactivity (µmol/min/mg protein)
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
a
a
b
C
POX activity (µmol/min/mg protein)
0,20
0,15
0,10
0,05
0,00
a
ab
b
Control
E. heros
E. heros + imidacloprid

Fig. 3 Activity of the enzymes superoxide dismutase (SOD)(**A**), catalase (CAT)(**B**) and peroxidase (POX)(**C**) of soybean embryos seeds from clean plants (green bar), infested plants by non-exposed *E. heros* (blue bar) and previously exposed to imidacloprid (red bar), during pod filling and maturation stage in field cages. Individuals were exposed to imidacloprid at concentration of 0.042 µg a.i./cm ^{2} during 48 hours. Bars represent the average of four replicates ± standard error. Bars followed by the same letter did not differ by Tukey test ($P < 0.05$).

Proteinase inhibitor (PI) activity in soybean damaged seeds by a couple of *E. heros* per plant independently of imidacloprid exposure presented less than half the value when compared to non-damaged seeds (Fig. 1A, $F=25.30$; d.f.=2; $P<0.001$). PI activity in soybean damaged seeds by couple of *E. heros* especifically in pods from fourth or fifth leaf also demonstrated less than half the value when compared to the one found in non-damaged plant seeds (Fig. 1B, $F=21.54$; d.f.=2; $P<0.0001$).

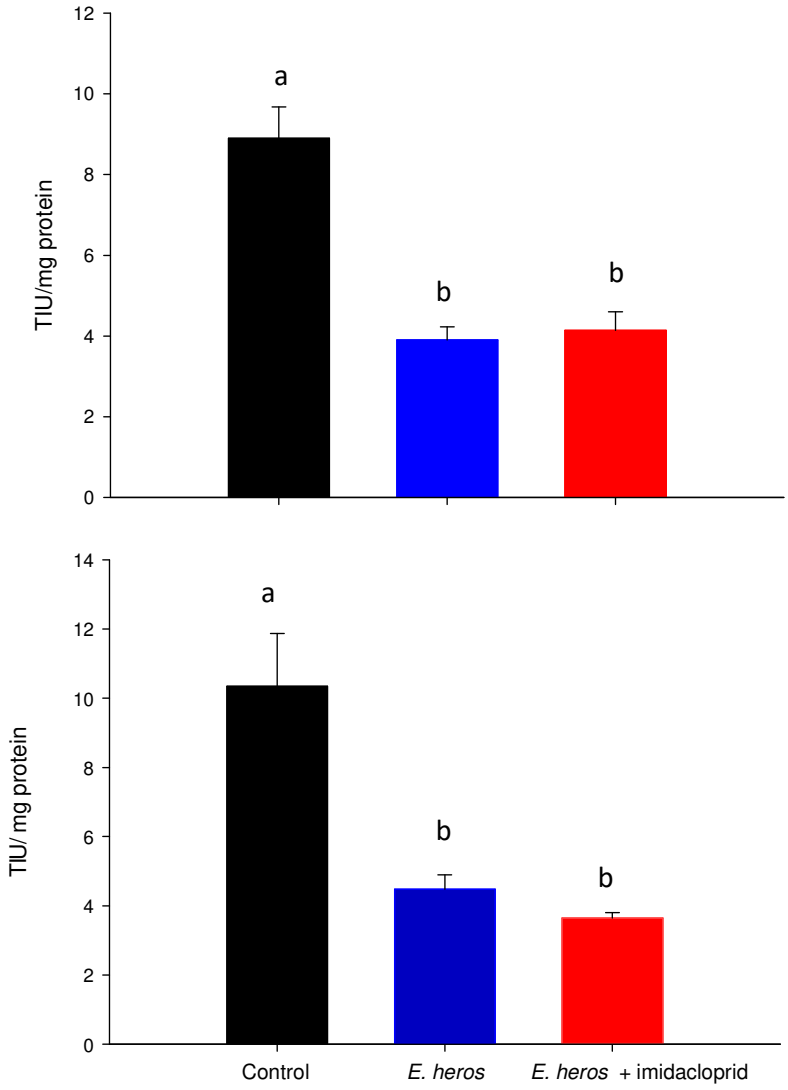

Fig. 4 Damaged plants by a couple of *E. heros* per plant (**A**) and damaged pods by a couple of *E. heros* in pods from fourth or fifth leaf (**B**). Mean of Trypsin Inhibitory Units (TIU) per gram of protein of soybean seeds from non-damaged plants (control - black bar), damaged by non-exposed *E. heros* (blue bar) and damaged by previously exposed to imidacloprid (red bar), during pod filling. Individuals were exposed to imidacloprid at concentration of 0.042 µg a.i./cm ² for 48 hours. Bars represent the average of four replicates ± standard error. Bars followed by the same letter did not differ by Tukey test ($P < 0.05$).

Discussion

Soybean seeds quality was higher in control plants, intermediate ininfested plants with non-exposed and lower in infested plants with *E. heros* exposed to imidacloprid (Fig.1; Fig.2). It is known that germination and vigor can be reduced in infested plants with two *E. heros* per plant (Nunes and Corrêa-Ferreira, 2002). This research have found out that soybean infested plants with two stink bugs per plant in a greenhouse during fifteen days at the pod filling stage had seed quality negatively affect. Russin et al., (1987) also showed that germination percentage and seed vigor evaluated by accelerated aging decrease with different levels of stink bugs infestation. It is known that *E. heros* induce severe damage in cotyledons soybean seeds (Silva et al., 2012). Furthermore, the location of damage is more important than the amount of damage (e.g. a damage in the radicle-hypocotyl axis can inviability germination, but several damages in the cotyledons cannot affect germination, just vigor) (Jensen and Newsom, 1972).

Germination speed index and first germination count were lower in soybean infested plants by *E. heros* exposed to imidacloprid when compared to control plants. In addition, seeds from control plants showed high vigor because of their electrical conductivity values under 70-80 µS cm-1 g-1 (Fig. 2D) (Vieira and Krzyzanowski, 1999). The same cannot be stated in damage seeds by stink bugs, where the electrical conductivity values were greater with values around 100 µS cm-1 g-1 (Vieira et al., 2004). These seeds are most likely deteriorating as a consequence they present poor reorganization membrane system (Hampton and Tekrony, 1995). It is possibly observed that seeds damage caused by stink bugs can reduce seed germination and vigor. Furthermore, it was reported that exposure of *E. heros* to imidacloprid can

negatively affect soybean seeds quality due to their higher population (Fig. 1; chapter 1).

Seeds from soybean damaged plants by *E. heros* previously exposed to imidacloprid showed the lowest germination rate (Fig. 1) due to the major damage in seeds, which had also the lowest SOD activity (Fig. 3A). The SOD activity was reduced when regarding seeds from soybean infested plants with stink bugs exposed to insecticide due the lower vigor of these seeds. SOD enzyme catalyzes O_2^- to formation of hydrogen peroxide (H_2O_2). Reactive oxygen species (ROS) such as O_2^-, H_2O_2 and OH^- accumulate following herbivore attack and enzymes from salivary glands can also contributed to higher ROS at the site of damage (Karuppanapandian et al., 2011). Moreover, H_2O_2 is responsible for the expression of defense genes to prevent subsequent attack by herbivores and their levels remain during herbivore (Torres, 2010).

It is known that CAT activity can increase in order to control hydrogen peroxide levels formed from attack of pest (Karuppanapandian et al., 2011). In addition, this enzyme is important to remove the ROS excess during stress, which decomposing hydrogen peroxide to water and oxygen (Mittler, 2002). CAT activity was higher in seeds damage by *E. heros* exposed to insecticide compared to stink bug without exposed (Fig. 2B), probably due the major damage in seeds. CAT activity from herbivore in different plant species can also persist unchanged or decreased (Bi and Felton, 1995; Chen et al., 2009; Gomez et al., 2004; Timbó et al., 2014). POX activity was higher in the seeds from infested plants with *E. heros* exposed to imidacloprid compared to control, but seeds from infested plants by stink bug without exposure to insecticide showed the same activity (Fig. 2C). This enzyme under stress condition is responsible to keep hydrogen peroxidase homeostasis by its

elimination and also organic hydroperoxides (Singh et al., 2017). It is possible to observe that soybean infested plants by stink bugs exposed to imidacloprid may present an increased activity of CAT and POX. However, these enzymes activity were not associated with lower vigor presented by these seeds (Fig. 2A).

Proteinase inhibitor activity in soybean damaged seeds by couple of *E. heros* per plant, independently of imidacloprid exposure was significantly lower than non-damaged seeds (control) (Fig. 4A). The same result was observed when pods from fourth or fifth leaf were damaged by a couple of *E. heros* (Fig. 4B). Thus, soybean seeds defence system response was not variable with damage intensity (2 stink bugs/plant or 2 stink bugs/pods) which confirm that infested plants with different damage intensity may present similar defence system response (De Oliveira et al., 2016). These evidences demonstrated that unlike other herbivores (Bede et al., 2006; Kant et al., 2007; Turlings and Erb, 2018; Walling, 2008) *E. heros* might interfere plant defence sytem. This is can be another evidence that arthropods may supress plants defence (Lawrence et al., 2008; Musser et al., 2002; Sarmento et al., 2011; Zarate et al., 2007). However, more researches are necessary to understand whether this arthropod can suppress soybean plant defence and what strategy *E. heros* uses to interfere in seeds defence system. Dillon and Dillon (2004) discussed that microbial communities present in the insect gut might develop symbiotic relationships. This microbiota could be linked to the defence system suppression. In addition, it is necessary more studies in order to know whether this response may occur in other plant varieties. Timbó et al., (2014) reported that two soybean varieties responded differently to the *E. heros* attack.

Therefore, *E. heros* exposed to sublethal doses of imidacloprid in the field condition can reduce soybean seeds physiological quality and change antioxidant

enzymes activity due to the major population that contributed to a higher damage in seeds. Moreover, *E. heros* might interfere with seeds defence system. Growers control the pest for reducing losses (Zalucki et al., 2009) and chemical insecticides is the predominant method to control pest in soybean crops, which are often used excessively and erroneously (Panizzi, 2013; Song and Swinton, 2009). Hence, the increase in *E. heros* population and reduction in the seed quality may contribute with major insecticide applications. Currently, the economic threshold used to control stink bugs in soybean seed crops in Brazil is one insect per meter (Bueno et al., 2013), which is safe (de Freitas Bueno et al., 2015). It is known that *E. heros* is predominant on soybean crops, but is important to follow economic threshold used to control stink bugs in order to reduce the use of chemical insecticide and consequently reduce environment impact (de Freitas Bueno et al., 2015). Thus, it is important to find other efficient alternatives to control this pest such as resistant plant and biological control. Besides being able to interfere plant defence, *E. heros* is a dominant competitor (Tuelher et al., 2016) which also increase its reproduction when sublethaly exposed to neonicotinoid (Santos et al., 2016). Therefore, we suggest that all these evidence may explain out-breaks of this pest on soybean crops in Brazil.

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GENERAL CONCLUSIONS

- Sublethal exposure to imidacloprid can increase *Euschistus heros* population on soybean field.
- Physiological quality of soybean seeds decreases due to these greater number of individuals.
- Superoxide dismutase activity from antioxidant system decreases in seeds from infested plants with *E. heros* exposed to imidacloprid.
- *E. heros* can interfere in seed defence system due to low proteinase inhibitor activity in different proportions and independently of imidacloprid exposure.