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Identification of Heliothinae species (Lepidoptera: Noctuidae) in chickpeas in central Brazil

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ABSTRACT

Chickpea is a commercially exploited crop on a relatively new scale in Brazil, that is why, little information on the main pests is available. Mostly caterpillars showing Heliothinae morphological characteristics have emerged in production areas. In order to point out the species of these insects in chickpea fields produced in the Federal District and the surrounding areas, this study aimed to determine the morphological and molecular identification of these invertebrates in order to assist producers in pest management in a more sustainable way. Caterpillars were hand-collected directly from the plants in six sites and the moth collection was collected using light traps in four sites, monthly, in 2017 and 2018. The morphological identification was made through the adult insect genitalia and the subsequent molecular identification was performed using the cytochrome oxidase I enzyme with the quantitative PCR technique, which resulted in the development of an efficient and low-cost DNA extraction protocol to be used in samples under unfavorable storage conditions. Morphological and molecular studies showed the presence of *Helicoverpa armigera*, *Helicoverpa zea*, *Chloridea virescens* and *Chloridea subflexa*, with a predominance of *Chloridea virescens* in chickpea plants. *Chloridea subflexa* was verified for the first time feeding on chickpeas in Brazil.

Keywords: *Cicer arietinum*, light trap, quantitative PCR; cytochrome oxidase I.

RESUMO

Identificação de espécies de Heliothinae (Lepidoptera: Noctuidae) no grão-de-bico na região central do Brasil

O grão-de-bico é uma cultura com exploração comercial em escala relativamente nova no país e, por isso, há poucas informações disponíveis sobre as principais pragas, majoritariamente lagartas, com características morfológicas de Heliothinae, que têm surgido nas áreas de produção. Com o intuito de apontar as espécies desses insetos no grão-de-bico produzido no Distrito Federal e região produtora em seu entorno, este trabalho foi realizado para a identificação morfológica e molecular desses invertebrados para auxiliar os produtores no manejo de pragas de forma mais sustentável. Foram realizadas coletas manuais de lagartas diretamente nas plantas em seis locais e a coleta de mariposas com o uso de armadilhas luminosas em quatro locais, mensalmente, durante os anos de 2017 e 2018. A identificação morfológica foi feita através da genitália dos insetos adultos e a posterior identificação molecular foi realizada através da enzima citocromo oxidase I com a técnica de PCR quantitativo, o que resultou no desenvolvimento de um protocolo de extração de DNA eficiente e de baixo custo para o uso em amostras sob condições desfavoráveis de armazenamento. Os estudos morfológico e molecular resultaram na constatação da presença de *Helicoverpa armigera*, *Helicoverpa zea*, *Chloridea virescens e Chloridea subflexa*, com predominância de *Chloridea virescens* nas plantas de grão-de-bico. Foi registrada pela primeira vez *Chloridea subflexa* se alimentando em grão-de-bico no Brasil.

Palavras-chave: *Cicer arietinum*, armadilha luminosa, PCR quantitativo, citocromo oxidase I.

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Producing worldwide 10.1 million tons per year, chickpea (*Cicer arietinum*) is the third most produced legume worldwide, after peas (10.4 million t/year) and beans (21.5 million t/year) (Muehlbauer & Sarker, 2017). India is responsible for 70% of production, with 500 to 900 kg/ha productivity (Muehlbauer & Sarker, 2017; Nascimento *et al*., 2016). In Brazil, the production area was expanded about 1000% in 2018 and the productivity reached 3,000 kg/ha (MAPA, 2018).

The low productivity in the East is due to inappropriate management, low technology and infestations of the main pest, *Helicoverpa armigera* (Lepidoptera: Noctuidae: Heliothinae), during flowering and maturation of pods (Ahmed & Khalique, 2012). This species, detected in several Brazilian regions in 2013 (Czepack *et al*., 2013; Specht *et al*., 2013), belongs to *Helicoverpa* complex, which encompasses different species whose identification is based on external morphology (Queiroz-Santos *et al*., 2018), a technique difficult to implement and that can lead to unreliable results in

the field.

DNA-based molecular markers, using mitochondrial genes such as cytochrome oxidase I (COI), is used as a complement to identify the species belonging to this complex (Arnemann *et al*., 2016). The identification consists of the amplification of a gene fragment by PCR followed by sequencing (Arnemann *et al*., 2016) or a restriction enzyme digestion.

Due to the increasing growing expansion of chickpea cultivation in Brazil and consequent increase in caterpillar attacks on crops, this study aimed to identify the main species of Heliothinae, complex *Helicoverpa*, in chickpeas in the Federal District and surrounding areas (Goiás State), using the quantitative PCR to confirm the species identification.

MATERIAL AND METHODS

Manual collection of the caterpillars from the crops and capture in light traps

The caterpillars were zigzaged-hand-collected in chickpea crops, every single month, in the morning, in harvests 2017, 2018, in six sites of the Federal District and surrounding production areas. In all these sites, Aleppo cultivar was grown and the crops were conducted under conventional management, using chemical insecticides. The six sites, identified as LC collection sites, were the following: LC1 = Embrapa Hortaliças, experimental field pivot (15°56'11"S, 48°8'9"W), DF; LC2 = Fazenda Garbanzo, under pivot, (16°10'3"S, 47°26'43"W), surrounding; LC3 = Fazenda Alvorada, pivot 1 (16°14'17"S, 47°25'44"W), surrounding; LC4 = Fazenda Alvorada, pivot 2 (16°14'39"S, 47°23'19"W), surrounding; LC5 = Feira Agrobrasília, Vitrine Tecnológica da Embrapa Hortaliças (16°0'47"S, 47°33'37"W) DF; LC6 = Fazenda Sucupira (seed production) (15°54'53"S, 48°2'21"W), DF. The caterpillars were individualized in 30 mL pots with an artificial diet (Montezano *et al*., 2014) kept at 25±1°C temperature, 80±2% relative humidity and 12-h photophase. After metamorphosis, the pupae were kept in moistened vermiculite until the adults emerged, being killed by freezing and kept in 90ºGL alcohol.

In four sites (LC1, LC2, LC3 and LC4), a light trapping, "Luiz de Queiroz" model (Biocontrole Ltda., SP, Brazil), was installed. The trapping was positioned about three meters of the border of each pivot, 1.50 m high. The black light of the traps was turned on in the evening and off at dawn, for three days in a row, in the new moon phase of each month, between May 2017 and October 2018.

Morphological identification of adults

The hand-collected insects and the ones captured in the traps were separated and identified by gender, considering the external morphological characteristics, based on Coleção de Referência da Embrapa Cerrados (Reference Collection of Embrapa Cerrados), Planaltina-DF. Afterwards, the species were identified by morphological comparison of the genitalia, according to Specht *et al*. (2013) and Bentivenha *et al*. (2016). Data were analyzed using Bioestat 5.3 software (Ayres *et al*., 2007).

Molecular identification and obtainment of total nucleic acids

In order to confirm the insect species identified as belonging to Helicoverpa genus, a random sample was taken. We evaluated 105 insects, considering the ones captured in the light trappings, using the molecular method. The molecular analyses were carried out at Laboratório de Bactérias Entomopatogênicas e Controle Biológico, of Embrapa Recursos Genéticos e Biotecnologia (Laboratory of Entomopathogenic Bacteria and Biological Control, of Embrapa Genetic Resources and Biotechnology), Brasíli-DF.

Each insect was processed individually for obtaining the total nucleic acids. The insect was taken out from alcohol and dried for 5 minutes. Each insect was put in a 1.5 mL plastic tube and macerated (head, thorax and abdomen) with a plastic stick. We added 500 μL lysis buffer solution (Tris-HCl 100 mM pH 8.0, EDTA 10 mM pH 8.0, NaCl 1.4 M and Triton X-100 0.3%). Each tube was submitted to vortexing for 5 seconds adding proteinase K at a final concentration of 120 μg/mL. Then, the solution was homogenized by inverting the tubes 5 times and incubating at 37°C for 40 min. Finally, the authors added, slowly, 1 mL icy ethyl alcohol. The homogenate was kept at 4°C for 15 days. The precipitated DNA was collected with a disposable Pasteur pipette, centrifuged at 12000 rpm for 5 minutes, discarding the supernatant. Two washes were performed with 500 μL of 70% ethanol at 12000 rpm for 5 minutes. After centrifugation, the alcohol was discarded, the pellet with the DNA dried at room temperature and was resuspended in 20 μL of 0.1X TE buffer. The samples were quantified by fluorimetry using QUANTUS™ fluorometer (Promega) adjusted to 1 ng/μL. The purity of the DNA samples was checked using the Picodrop Microliter UV/Vis Spectrophotometer.

Species were identified using the mitochondrial cytochrome oxidase I gene (COI) by real-time PCR oligonucleotides HaF#4 (5'CCC TAT TAA ATT TAA ACT GGG A3') and HaR#4 (5'TAG ACG AAG TTT ATA CAT TAA TAA G3') for *Helicoverpa armigera* and *H. zea*. Each reaction was prepared adding 10 μL of the mix GoTaq qPCR Master Mix (Promega), 0.4 μL of each primer at 10 μM concentration, 0.2 μL of CXR Reference Dye (Promega), 2 μL of DNA (10 ng/μL) and enough nuclease-free water to complete 20 μL of reaction.

Real-time PCR amplification during thermocycling followed the protocol of denaturation at 95°C for 2 min, 44 cycles of denaturation at 95°C for 30 seconds and annealing at 50°C for 3 seconds in AB Applied Biosystems Step One Plus equipment Real Time PCR System®.

Standardization of qPCR curve

Using the DNA quantification, the authors prepared a 10 ng/μL stock solution both for *H. armigera* and *H. zea*. A serial dilution (1:2) was performed to obtain final values and concentrations of 5 ng/μL, 2.5 ng/μL, 1.25 ng/μL and 0.625 ng/μL. Each

point was performed in triplicate and the C_T, R^2 parameters, efficiency (eff%), Y-intercept and Slope were determined.

RESULTS AND DISCUSSION

Manual collection of the caterpillars and capture in light traps

We hand-collected 774 caterpillars which were feeding on chickpeas. We verified that 581 developed into the adult form and were first identified by genus, as *Helicoverpa* and *Chloridea*. The other caterpillars (193) died due to Diptera (Tachinidae) and Hymenoptera parasitism or entomopathogenic fungi.

Chloridea caterpillars totalized 404 collections (69.54%), considering that 40, after turned into adults, were dissected and morphologically identified, resulting 36 individuals of *C. virescens* and four *C. subflexa*.

We collected 177 (30.46%) of *Helicoverpa* genus, 51 of them were dissected, and 33 were identified as *H. armigera* and 18 as *H. zea.*

Using comparison chi-square test, we observed that the number of *Chloridea* caterpillars collected directly from the chickpea plants was greater than the *Helicoverpa* ones ($\chi^2 = 87.91$; gl = 1, $\rho<0.0001$).

The distribution by collection date is represented in Figure 1. The ratio between the caterpillars collected in chickpeas was 7 *Chloridea* for each 3 *Helicoverpa* (7:3).

In light traps, 7,068 moths were captured,1,411 belonged to genus *Helicoverpa* (19.96%), a significantly larger amount ($\chi^2 = 1067$; gl = 1; $\rho<0.0001$) when comparing to *Chloridea* (129 insects captured, 1.82%). The other 5,528 moths captured in the experiment were native species which were not characterized as agricultural pests. The specimens were stored at Embrapa Recursos Genéticos e Biotecnologia (Embrapa Genetic Resources and Biotechnology).

In 2018, Reyes *et al*. (2018) reported *H. armigera* feeding on chickpea plants in the Federal District for the first time. Lourenção *et al*. (1989) had reported *C. virescens* in chickpeas in Brazil, as well as Castelo Branco *et al*. (1989). In this study, for the first time, *C. subflexa* was verified in chickpeas in Brazil.

Chloridea virescens was the most abundant species found in chickpea plants in hand-collected caterpillars, with low capture of adults in light traps, though. This may show that this is not the best way to monitor adults of this species in this crop. For Cheng *et al*. (2016), females of some nocturnal species are less captured than males in these traps, the reason why is still unknown, though. Other traps shall be tested for monitoring moths of this species in chickpea harvests. Another hypothesis is that their nocturnal movement is low, conserving energy for copulation and daytime search for oviposition sites, as occurs with females of Geometridae, which are more captured in light traps if copulated, showing that the flight under these conditions may be related to the selection of the oviposition site (Deslisle *et al*., 1998).

For *Spodoptera exigua* (Lepidoptera: Noctuidae), the males are more active than the females during scotophase, because their flight behavior is about searching for and calling the female and these insects may have a different perception to the light of the trap. For *S. exigua*, female activity occurs shortly after sunrise, probably for oviposition. The highest number of immature *Helicoverpa* was found in full flowering and maturation of the pods, as they offer a foraging site, corroborating the results found by Ahmed & Khalique (2012) and Damte & Ojiewo (2017).

For Blum *et al*. (2018), migrations of *H. armigera* occur between maturing crops and laying developing ones. For Damte & Ojiewo (2017), mechanical barriers may restrict dispersal, increasing search time to finding a host for oviposition, which may have favored the presence of *H. armigera* in chickpea, as observed in this study. For these authors, growing host plants such as sorghum and soybeans before planting chickpeas in the same site and in adjacent areas gives the opportunity for the insect population to grow and appear earlier. This can explain the population peaks of *H. armigera,* in this study, outside the chickpea growing season (showing that the population is established in this area and migrates to chickpeas when opportune).

For Jallow *et al*. (1999), the presence of *H. armigera* in plants during reproductive phase is due to the fact that females prefer flower exudates as it presents higher concentration of oviposition-stimulating chemicals. For them, the presence of nectar influences olfactory responses and oviposition by the action of chemical compounds, a plausible behavior in a polyphagous insect. This hypothesis could also be true for *Chloridea.*

In this study, even among insect populations which were affected by chemical insecticide applications, plants continued to be infested by caterpillars. For Damte & Ojiewo (2017), the explanation can rely on the fact that the insects are resistant to

Table 1. Identity sequence analysis of real-time PCR primers used for the identification of *H. armigera*. Brasília, Embrapa, 2022.

Primer	Identity	Description	Access
HaF#4	100%	*Helicoverpa armigera armigera* isolate Ug32L mitochondrion, complete genome	MG437196.1
HaR#4	100%	*Helicoverpa armigera armigera* isolate M0001 mitochondrion, complete genome	MG437193.1

Table 2. Real-time PCR primer (HaF#4/HaR#4) amplification parameters for the identification *of H. armigera* and *H. zea.* Brasília, Embrapa, 2022.

Species	Slope	Y-inter	R2	Eff%	CT ± DP
H. armigera	-3.322	25.453	0.951	99.993	22.998 ± 0.065
H. zea	-3.292	31.652	0.958	101.264	29.105 ± 0.170

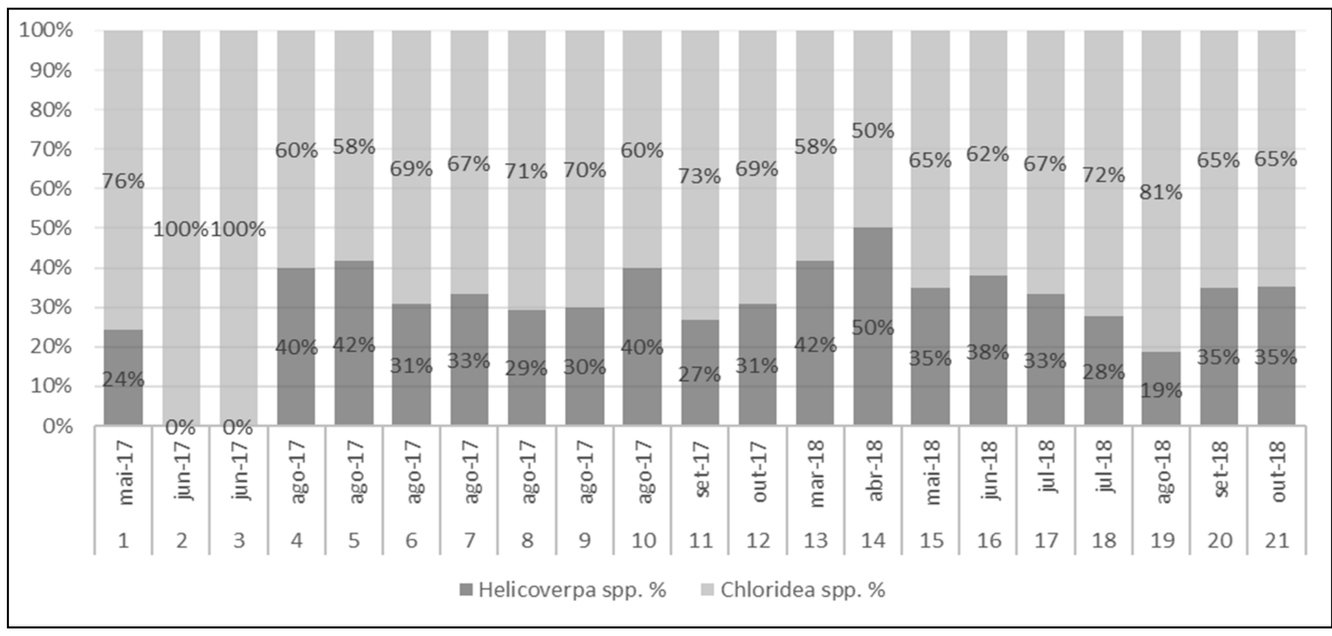

Figure 1. Proportion for genus *Helicoverpa* and *Chloridea* caterpillars in different collection dates of caterpillars on chickpea plants, in the Federal District and surroundings, Brazil. Brasília, Embrapa, 2022.

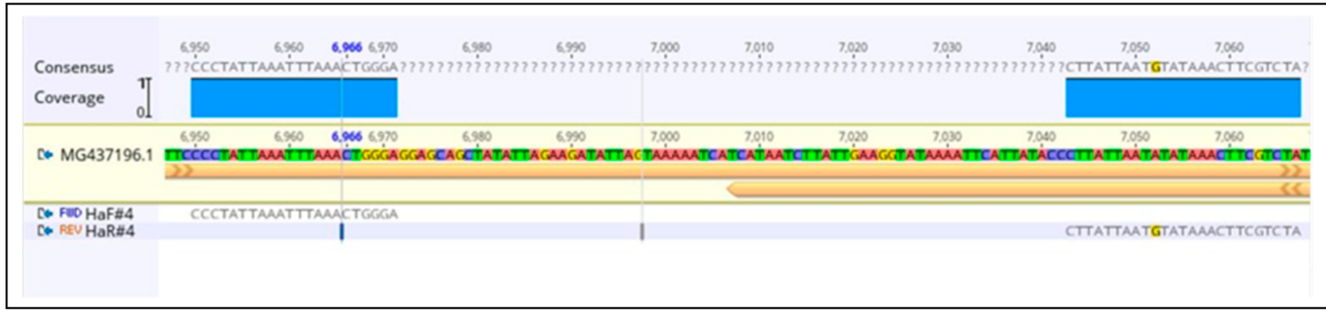

Figure 2. Real-time PCR primer (HaF#4/HaR#4) annealing sites in the internal regions corresponding to the cytochrome oxidase I gene (COI) of *H. armigera.* Brasília, Embrapa, 2022.

the active ingredient, short application time, low coverage, spray droplet size, poor equipment calibration and different caterpillar sizes. In this study, most caterpillars fed inside the pods, protected from surface applications, corroborating the data found by these authors.

Because *H. armigera* is exotic, the adaptive advantages represent an accelerated population growth, showing a life cycle 28% shorter than *H. zea.* This results in several generations per year, insects of different species and different development stages at the same time in the field (Barbosa *et al*., 2016).

Molecular identification of the insects

Real-time PCR primers were designed using the region corresponding to the cytochrome oxidase I gene (COI) of the isolate *H. armigera* Ug32L (GenBank: MG437196.1). The primers were designed with the PrimerQuest Tool program, which is available on the IDT Integrated DNA Technologies website (https://www.idtdna.com/pages). Then, we analyzed the operating conditions of the primers using the PCR Primer Stats program available on the Sequence Manipulation Suite website (https://www.bioinformatics.org/sms2/). After evaluating the functionality of real-time PCR primers, we analyzed the annealing capability with the target sequence using the Geneious program (Kearse *et al*., 2012) and analyzing the sequence identity using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Geneious program confirmed *in silico* the annealing of the primers with the internal region corresponding to COI gene (Figure 2). Sequence identity analysis of the PCR primers showed high identity for *H. armigera* (Table 1).

Identification curves were identified using DNA dilutions ranging from 10 ng/µL to 0.625 ng/µL obtaining the parameters established by Bustin *et al.* (2009). Surprisingly, the HaF#4/HaR#4 primer set produced amplification profiles able to discriminate *H. armigera* and *H. zea* (Table 2). The primer amplification standard curve was obtained for *H. armigera* and *H. zea* (Figure 3).

The primers designed to identify *H. armigera* also discriminated *H. zea*, with different amplification cycles (C_T), with C_T value = 22.998 ± 0.065 for *H.*

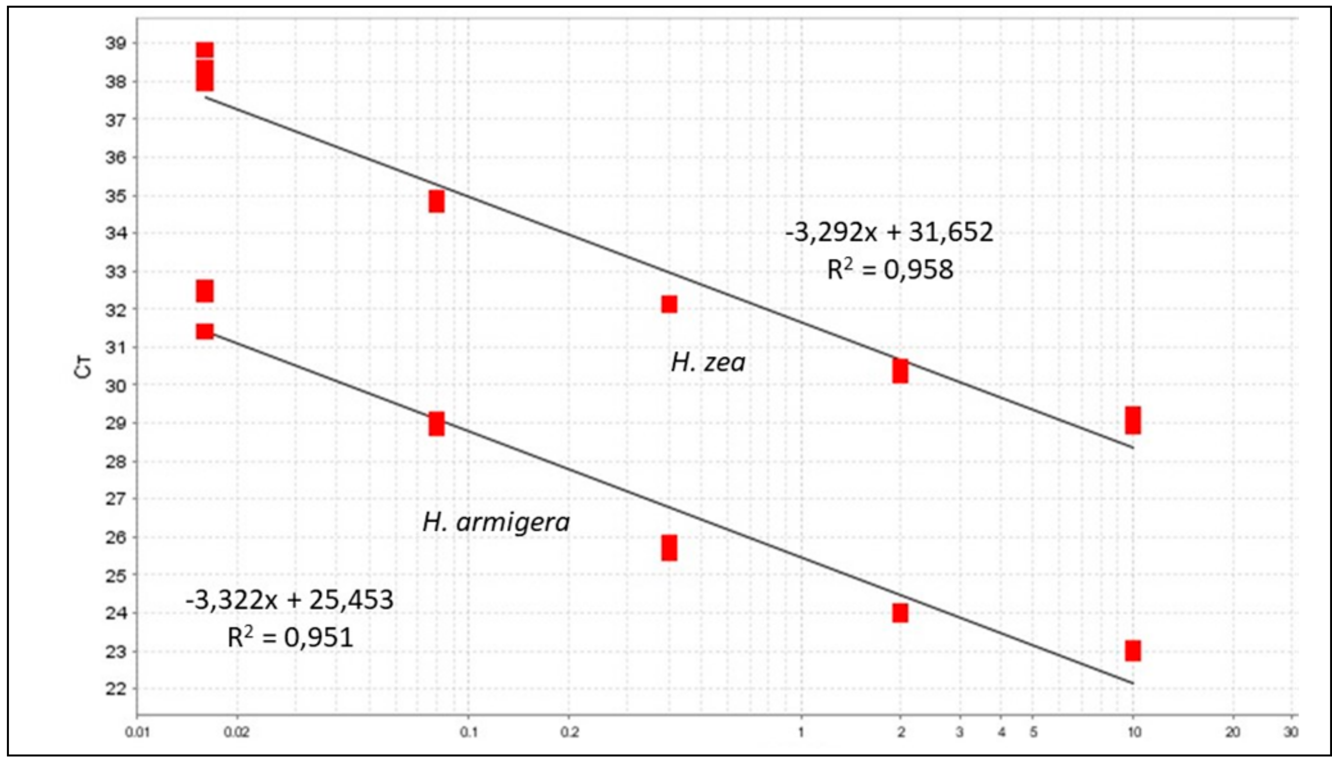

Figure 3. Standard curve established for molecular identification by real-time PCR for *H. armigera* and *H. zea* species. Brasília, Embrapa, 2022.

armigera and C_T = 29.105 ± 0.170 for *H. zea*.

With the amplification conditions established and standard curves determined, molecular identification of 105 samples from the light traps was performed. An amplification profile with C_T = 23 was obtained, corresponding to that established for *H. armigera* for 100% of the tested samples.

A region of the cytochrome oxidase I gene (COI) sequence can be a molecular marker that shows diversity, since their proteins contain conserved functional domains and variable regions (Miranda, 2019). According to this mentioned author, COI gene is widely used as DNA barcoding and it has potential for revealing cryptic species. This is an effective tool for identifying species and confirmed the presence of *H. armigera* or *H. zea* in 100% of the analyzed samples.

H. armigera and *H. zea* caterpillars are morphologically and molecularly similar. Precision molecular methods such as quantitative PCR facilitate the difference between *H. armigera* and other species occurring in Brazil (Specht *et al*., 2013).

The quantitative PCR system (qPCR) was used to identify the *Helicoverpa* species. Even being a more expensive method, qPCR is compensated by the speed in obtaining results and the high sensitivity for detecting genetic markers from samples with little DNA or in a state of degradation. To perform the analyses, amounts around 10 ng/µL were used for establishing the sample identification profile.

A surprising result verified in this study was that the *H. zea* samples were also amplified. Under conditions of establishing PCR reactions, co-amplification is a difficult problem to solve. But, in this case, *H. zea* samples used in this study as control showed a CT with difference of 7 cycles, ensuring an independent and different amplification of *H. armigera* samples. This allows to use only one set of PCR primers in different species, saving up time and reagents.

The qPCR primers developed in this study were based on highly conserved mitochondrial COI gene for amplifying short fragments (150 pb).

In conclusion, the Heliothinae species complex, *H. armigera*, *H. zea*, *C. virescens*, and the record for the first time of *C. subflexa*, at different development stages, pose challenges to manage these pests in the field. The molecular identification method by qPCR for the simultaneous identification of *H. armigera* and *H. zea*, presented in this study, proved to be an efficient tool to help manage these chickpea pests.

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