RNAi in *Tuta absoluta* management: effects of injection and root delivery of dsRNAs

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Abstract

The South American tomato pinworm, *Tuta absoluta*, is considered worldwide as one of the most important tomato pests. Insecticide resistance reported on the moth and the awareness about the negative impact of agrochemicals on the environment and human health, has raised the demand for new control tools. Among these, RNA interference (RNAi) can represent a valid tool to be included into new control strategies against this pest. Here we report the results of trials aimed at evaluating the effects of dsRNAs targeting Acetylcholinesterase (AChE), Nicotinic Acetylcholine alpha 6 (nAChRs), Ryanodine (RyR) and receptors by injection and root delivery. In the injection procedure 2 and 5 µg of dsRNA were able to reduce the gene expression in a range of 62.7-75.4%, inducing a maximum mortality rate of 92.59%. The dsRNAs administered at 5 µg by root adsorption revealed the potential of this delivery system to affect the gene expression (47-69% reduction of) and the mortality (ranging from 67.1 to 80.5%) of treated specimens. The delivered dsRNAs (both injections and root administered) affected the weight of developed *T. absoluta* both prepupae and pupae. The selected AChE, nAChRs and RyRs genes can be suitable targets for *T. absoluta* control by means of *in planta* delivery dsRNAs.

Keywords: RNA interference, Hydroponic, Ryanodine Receptor, Acetylcholinesterase, Nicotinic Acetylcholine Receptor, Tomato

Key message

- RNAi is a promising tool for the control of insect pest.
- We tested three dsRNAs against T. absoluta preimaginal stages.

- The dsRNAs had effect on moth mortality both by injection and root delivery.
- The dsRNA root adsorption represent a potential route to develop control strategy to be applied in real field conditions.

Introduction

Agricultural pests cause annual crop losses worth billions of US dollars (Bingsohn et al. 2017). In many cases, insecticide applications are one of the major pest control approaches. Despite its negative effects, chemical control is normally considered quicker and more effective compared to other pest control methods. (Geiger et al. 2010; Pimentel et al. 2005). The wide spread of insecticides has led to the rapid development of insecticide resistance and also to a negative effects on non-target organisms, persistence and biomagnifications in the environment, which cause environmental as well as health related problems (Desneux et al. 2007; Weston, et al. 2015; Cimino et al. 2017)

The South American Tomato Pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is a small oligophagous Lepidoptera that feeds of many solanaceous species and has a particular preference for tomato (*Solanum lycopersicum* L.) (Desneux et al. 2010; Campos et al. 2017; Biondi et al. 2018), causing up to 100 % damage if not controlled (Desneux et al. 2011). It can develop on other cultivated Solanaceae such as potato, eggplant, sweet pepino (*Solanum muricatum* L.) and tobacco (*Nicotiana tabacum* L.), as well as on wild plant species [Jimson weed (*Datura stramonium* L.), black nightshade (*Solanum nigrum* L.) and deadly nightshade (*Atropa belladonna* L.)] (Tropea Garzia et al. 2012; Cherif et al. 2018).

This pest was first reported in Perù at the beginning of the 20th century and it is currently recorded in more than 80 countries around the world and affecting nearly 60% of all cultivated tomatoes (Biondi et al. 2018). After its first report in Spain in 2006, it spread quickly in Europe

87 and North Africa and has subsequently invaded the Middle East, Central and South Asia
88 (Desneux et al. 2011; Sankarganesh et al. 2017; Sylla et al. 2017; Mansour et al. 2018)
89 threatening China, i.e., the major tomato producer country (Xian et al. 2017; Han et al. 2018).

90 In many tomato cropping systems, chemical applications are necessary and often resulted in
91 overuse of these chemicals. This caused the disruption of integrated pest control strategies
92 where natural enemies are largely employed (Biondi et al. 2012; 2013; Zappalà et al. 2013).
93 Moreover, because of the high reproductive potential (up to 300 eggs per female) and short
94 generation cycle (10 to 12 generations per year) (Tropea Garzia et al. 2012; Biondi et al.
95 2018), this moth can rapidly develop resistant populations to insecticides belonging to
96 different chemical classes (Roditakis et al. 2017). The insecticide resistant populations of the
97 pest were detected in the early 2000s in Chile, Brazil and Argentina and include resistance to
98 organophosphates (OPs), pyrethroids, abamectin, cartap, spinosad and diamides (Siqueira et al.
99 2000; Lietti et al. 2005; Haddi et al. 2012; Roditakis et al. 2015, 2017; Zibaee et al. 2018).
100 Therefore, the development of novel and effective alternative control methods are urgently
101 needed (Cocco et al. 2013; Sohrabi et al. 2016; Campolo et al. 2017).

102 Recently, biotechnology has provided additional techniques to decrease the damage caused by
103 insect pests while at the same time it has given solutions for the limitations of traditional and
104 hazardous chemical methods (Christou et al. 2006). Therefore, new strategies for pest control
105 are required imperatively and one promising approach is the use of RNA interference
106 (Bingsohn et al. 2017).

107 RNA interference (RNAi) is a biological mechanism which leads to post transcriptional gene
108 silencing directed by the presence of double-stranded RNA (dsRNA) molecules to prevent the

expression of specific genes occurs widely among plants, animals and microorganisms resulting in the degradation of the targeted mRNA (Kim et al. 2015). Since the discovery that providing double-stranded RNA (dsRNA) to a wide variety of organisms, including insects, can cause RNAi, this technique has become a common tool for functional genomic studies, especially in non-model systems. It has been exploited successfully as a powerful reverse genetic tool to study the function of genes and for biological control of various agricultural insect pests and pathogens (Price and Gatehouse 2008; Zotti and Smagghe 2015; Mamta and Rajam 2017). Because biological compartments are regulated by numerous proteins and enzymes, the identification of the most promising gene targets is one of the major challenges to exploit RNAi in pest control strategies (Laudani et al. 2017).

In this context, the aim of this research was to evaluate the effects on *T. absoluta* of three dsRNA delivered by injection and root adsorption in tomato plants. The target genes for this study were the Ryanodine Receptors (>KX519762.1)(RyRs), Acetylcholinesterase subunit 1 (>KU985167.1)(AChE) and Nicotinic Acetylcholine Receptor alpha 6 (>KP771859.1)(nAChRs). Owing to the involvement of these genes in nerve transmission and other metabolic processes, they have been effective targets for controlling insect pests of crops by applying chemical insecticides.

Materials and Methods

Insects

129 The population of *T. absoluta* used in the study originated from field collected specimens from
130 tomato fields of Hashtgerd Region, Karaj, Albarz Province, Iran. The moth was reared on tomato
131 plants or leaves at the Department of Agriculture of Mediterranean University of Reggio
132 Calabria, Italy, under laboratory conditions (25 ± 2 ºC; 60 ± 10% relative humidity; 14 h
133 photoperiod) for several generations according to Zappalà et al. (2013). Cherry-type pesticide-
134 free tomato plants (cv Shiren) were used for rearing and experiments.

135

136 **RNA extraction and cDNA synthesis**

137 Total RNA was extracted and purified from 100 mg of the third instar larvae of *T. absoluta* using
138 TRIzol®(Invitrogen; Carlsbad, CA, USA) and Pure Link® RNA Mini Kit (Cat. No. 12183025, Life
139 Technology). The quality and quantity of the extracted RNA was determined by using a
140 Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA).cDNA synthesis are
141 performed in 20µl reactions comprised of components of the ImProm-II™Reverse Transcription
142 Kit (#A3800, Promega) by using about 1µg experimental RNA, according to the manufacturer's
143 instruction. The synthesized cDNA was used as a template to amplify a portion of the coding
144 sequence of AChE, nAChR and RYAR genes selected from the NCBI database, using the primers
145 reported in Table 1. The synthesized cDNA was then stored at-20 °c.

146

147 **dsRNA synthesis**

148 Target genes, selected from the NCBI database and primer pairs were designed using Primer-
149 BLAST software® (Table S1). For dsRNA Synthesis, a T7 promoter sequence (5´-
150 TAATACGACTCACTATAGGG- 3´) was ligated to the amplicons, including this sequence at the 5´

151 ends of each primer. Amplifications were performed using a Mastercycler Ep Gradient S
152 thermocycler (Eppendorf, Germany) with the following conditions: 5 cycles at 94°C for 2 min,
153 then 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, followed by 35 cycles at 94°C for 30 s, 60°C
154 for 30 s and 72°C for 45 s, with a final extension step of 10 min at 72°C. A non-template control
155 in which target cDNA was replaced by nuclease-free water was also included in all PCR
156 reactions. PCR products were analyzed by Nanodrop and gel electrophoresis in TBE buffer (1X)
157 using 1% agarose gel. The dsRNA fragments were generated using MEGAscript®RNAi Kit
158 (Ambion) according to manufacturer's instructions. The final concentration and quality of
159 dsRNA products were measured by Nanodrop. The quality and integrity of dsRNA was also
160 checked by electrophoresis on 1% agarose gel.
161 The negative control groups were injected with the same concentration of a 495 bp-long dsRNA
162 molecule targeting the green fluorescent protein being absent in *T. absoluta* to assess the
163 effect of injecting and feeding the target-less dsRNA. Molecular biology grade water was
164 administered as further negative control. As internal controls, ribosomal protein 18S (Rp18)
165 were used.
166

167 **Administration of dsRNA by injection and through root delivery**

168 Newly molted third instar larvae were used for assays because they are the easier instar to
169 manipulate in this kind experiment. In the injection delivery procedure, the dsRNA of each gene
170 was injected with 2 different concentrations (2 and 5 µg per each larva) on the dorsal side of
171 the first or second abdominal segment of each larva (Tomoyasu and Denell 2004) using
172 Eppendorf Microinjector®InjectMan NII. Injected larvae were transferred on tomato untreated

173 leaflets and kept in a climatic chamber (Cavallo 1400 CFU, Milano, Italy) under the same
174 climatic conditions adopted for the rearing procedures.
175 Control larvae were injected with dsGFP (Green Fluorescent Protein) and nuclease-free water
176 as negative controls and kept in the same condition as the other treatments. Dead larvae within
177 the first 48 hours after the injection were removed and excluded from the experiments. The
178 injection was carried out for all three genes separately, employing, for each treatment, groups
179 of 5 individuals. The experiment was replicated three times.
180 In the administration of dsRNAs through roots, the petiole of detached two weeks old fully
181 expanded tomato leaves (cherry-type, cv Shiren) was immersed for 6 days in oxygenated sterile
182 water. This ensured a complete rooting of the leaves. The larvae used in the experiments were
183 left starving for two hours prior starting the tests. Then the roots were immersed in 200 µL of
184 water containing 5 µg of dsRNA (the most effective dose in the injection procedure, see results
185 below) from each target gene. The rooted leaves were left until the dsRNA solution was
186 completely absorbed, and this took around 3hours.Control treatments were treated with
187 nuclease-free water and dsGFP solution adopting the same procedure above described.
188 Immediately after the uptake, five third instar larvae were gently placed onto the leaves for
189 feeding. The trial was replicated three times.
190 For both dsRNAs delivery routes, the effects of RNAi on the larvae were evaluated, after 72h, by
191 quantitative amplification of reversed transcripts (RT-qPCR) of each target gene compared to
192 the controls.
193

Effects of dsRNA administration on mortality, biometric and morphometric traits of T. absoluta.

To evaluate the effects on mortality, biometric and morphometric traits due to the dsRNA on T. absoluta two separate experiments were carried out.

In the first experiment, 2 or 5 µg dsRNAs of the three targeted genes, were injected in each of fifteen newly molted 3rd instar larvae, after the injection of dsRNAs each larvae was individually placed in a small petri dish and fed with untreated leaflets.

In the second experiment, the delivery of dsRNAs was carried out by root adsorption. The roots were immersed in 200 µl of water containing either 5 µg of dsRNA of each target gene and left to absorb all the dsRNA solution for 3 hours. Then a newly molted third instar larva was gently placed onto each rooted leaf for feeding.

The rate of mortality and any changes in larval stages, weight and phenotypic changes were recorded every 24 hours, and observations were carried out until all the adults emerged. Controls with dsRNA from the GFP gene sequence and nuclease-free water were run in parallel as well. Each trial was replicated three times.

Cloning

The template cDNA was denatured at 95ºCfor 5 min followed by 25 cycles at 95 ºC for 30 seconds and annealing temperature at 55 ºC for another 30 seconds followed by 30 seconds extension time at 72 ºC, the PCR reaction was ended at 72 ºC for 7min. The PCR products were subsequently purified using MinElute Extraction Kit (QIAGEN® Venlo, Netherlands) and cloned using Strata cloning® kit (Agilent Technologies® Santa Clara, USA). Three insertion-positive *E.*

215 *coli* colonies for each amplified gene were selected, the fragment was cut from the vector using
216 EcoRI enzyme (Thermo Fisher Scientific, USA), and it was sequenced by MacroGen Laboratories
217 in order to check for the amplification of the correct target. dsRNAs for each target was
218 synthesized starting from 1000 ng/µl cDNA, and through specific primers using the
219 MEGAscript® RNAi Kit (Thermo Fisher Scientific®, USA) according to the manufacturer's
220 instructions. The quality and integrity of dsRNA was also checked by electrophoresis on 1%
221 agarose gel. Products were also quantified using a Nanodrop 2000 (Thermo Fisher Scientific®,
222 USA), obtaining an average concentration ranging between 700-1000 ng/µl.

223

224 **Gene expression analysis with quantitative reverse transcription PCR (RT-qPCR)**

225 The total RNA was extracted and processed into cDNA as described above. RT-qPCR was carried
226 out using primers listed in Table 1, designed to detect a segment of each gene selected to be
227 external to the segment targeted by the dsRNA. The ribosomal protein Rp18 was used as an
228 internal reference gene. The levels of mRNA were measured by StepOnePlus™ Real-Time PCR
229 System (Life Technologies®) using the GoTaq- qPCR MasterMix (Promega, USA, #A6001)
230 according to the manufacturer's instructions. Reaction were performed using the following
231 conditions: 95°C for 3 min, 50 cycles of 95 °C for 3 seconds and 60 °C for 30 seconds, and a final
232 dissociation stage. The relative expression of the target gene was normalized using the ΔΔCq
233 method (Livak and Schmittgen 2001) towards both the reference gene (Rp18) and negative
234 control insects treated with nuclease-free water. A further negative control was performed
235 with GFP.

236

237 **Data analysis**

238 Insect mortality data was corrected and normalized against the untreated control treatments
239 using Abbott's formula (Abbott 1925). Dependent variables were subjected to Levene and
240 Shapiro-Wilk tests in order to assess the homogeneity and normality of variance across the
241 groups, respectively, and transformed whenever needed. Data were subjected to univariate
242 analysis of variance following the GLM procedure with the relative expression level and
243 percentage mortality as dependent variables and the target genes and the application rates as
244 fixed factors. Multiple comparisons among treatments were carried out by using the Tukey–
245 Kramer (HSD) post hoc test. The cumulative mortality response from the treated larvae and the
246 respective controls were further analyzed using Kaplan–Meier survival analysis. The differences
247 among the factors (i.e target genes or application rate) were tested for significance using the
248 Mantel-Cox log rank test.
249 Differences in gene knockdown and mortality among the targeted genes and different doses
250 were assessed using Tukey's test for multiple comparisons. All statistical analyses were
251 performed using Microsoft Excel® 2016 and SPSS® 22.

252

253 **Results**

254 **RNAi effects by dsRNAs injection**

255 As confirmed by qRT-PCR, a significant dose dependent reduction in the expression of the three
256 target genes was observed in both concentrations (2 and 5 µg) after 72 h of injection in larvae
257 (Fig. 1A-C). At the lowest concentration (2 µg), the transcript levels were reduced of 27.60%,

34.50%, and 48.80% whereas at 5 µg the gene expressions were reduced of 62.73%, 65.36% and 75.42 % for AChE, nACHRs and RyRs respectively, compared with the negative control (dsGFP).

The administration of dsRNAs caused a significant decrease in the survivorship on specimens treated with the dsRNAs of selected genes, whereas both in the negative controls (GFP and water) only few larvae (n = 1 specimen in both controls) did not reach the adult stage. The survival analysis highlighted statistical differences (Log-Rank Mantel-Cox χ^2= 20.593; df=7; p=0.004) among the two controls (GFP and water) and the mortality observed in the larvae injected with the dsRNA target genes.

In detail, the most effective treatment was the one where larvae were treated with 5µg of dsRyRs; indeed in this treatment, 92.59±5.73% specimens did not reach to the adult stage (Figure 2). Conversely, the lowest mortality was achieved in larvae treated with 2 µg of dsAChE (7.40±2.86% mortality). Overall, the mortality caused by the injection of each dsRNA was always significantly dose dependent (AChE: F=16.200; df=1; p=0.016 – nACHRs: F=18.000; df=1; p=0.013 – RyRs: F=9.800; df=1; p=0.035).

The dsRNAs injection had negative effects both in pre-pupal as well as in pupal stages weight increase (Table 2). Both pre-pupae and pupae injected with 5µg of AChE dsRNA were lighter than those treated with the lowest dsRNA application rate and the GFP control (pre-pupae: F=17.028; df=2; p<0.001 – pupae: F=13.231; df=2; p<0.001). In contrast, larvae treated with nACHRs dsRNA at both application rates (2 and 5µg), when reached the pre-pupal stage, were statistically lighter than the control (F=31.051; df=2; p<0.001); instead, pupal weight decreased proportionally to the dsRNA amount injected (F=20.579; df=2; p<0.001). The effects on weight

of RyRs dsRNA injection were dose dependent both in pre-pupal ($F=107.240$; $df=2$; $p<0.001$) as well as in pupal stages ($F=67.242$; $df=2$; $p<0.001$).

RNAi effects by dsRNAs delivered by roots

Larvae that fed on leaves that had been administered dsRNAs radically, highlighted a reduction of the expression of the three target genes (Figure 3). In all cases, the relative gene expression of the target genes were significantly lower ($p>0.05$) than the controls (GFP and water). The reduction of the transcript level ranged from 47% (RyRs) to 69% detected in the larvae that fed on plants treated with the dsRNA of nACHRs.

Also in these cases the dsRNAs had a significant effect on *T. absoluta* survival. The survival analysis showed no significant difference in the mortality between the two controls (GFP and water mortality = 1 specimen), whereas significant differences (Log-Rank Mantel-Cox $\chi^2=$ 17.862; $df=4$; $p=0.001$) were highlighted among the control and the mortality observed in larvae fed on plants in which the dsRNAs were delivered by root adsorption.

In detail, the mortality induced by the dsRNA delivered by plants, were significantly higher ($F=17.806$ $df=3$; $p=0.001$) in the silenced genes than in the GFP negative control (Figure 4). Multiple comparisons highlighted no significant differences among the three target genes ($p>0.05$). Overall the mortality registered in the dsRNAs treated plants ranged from 67.15% (RyRs) to 80.47% (nAChRs).

The weight of the pre-pupae fed on plants in which the dsRNAs of the target genes were administered (Table 3) was significant affected by the treatments ($F=10.032$ $df=3$; $p<0.001$). The specimens fed on plants treated with AChE dsRNA, had a pupal weight similar to the control

(GFP) ($p>0.05$) whereas, prepupae fed on plants administered both with nAChRs and RyRs dsRNAs were lighter than the control.

Pupae (Table 3) developed in treated plants in all case weighted less than in the GFP negative control ($F=72.579$ $df=3$; $p<0.001$) with the lowest mean weight (1.71±0.01 mg) recorded in pupae developed in plants treated with RyRs dsRNA.

Discussion

The invasive pest *T. absoluta* is considered in many parts of the world as one of the key pests of tomato. Insecticide resistance reported for several native and invaded areas (Biondi et al. 2018), has raised the demand to new control tools. In this scenario, RNAi can be the perfect candidate. However, serious efforts in outreach and education are needed to better inform the different stakeholders including the public and agricultural industry, leaders as well as decision makers in the regulatory and political communities to help expedite the release and adoption of RNAi products and technology (Andrade and Hunter 2016).

Our study investigated the RNAi efficacy in silencing selected target genes in *T. absoluta* dsRNAs by injection and root delivery. All the target AChE, nAChRs and RyRs genes were successfully silenced by dsRNAs we synthetized and provided to the3rd instar larvae. In the injection procedure, the administered dsRNAs was able to reduce the gene expression, inducing high mortality rate in the treated larvae. Both application rates have been shown to significantly reduce the gene expression while lower doses (i.e. 1 µg) were ineffective for all the tested genes (data not shown). Also the weight of preimaginal stages was affected by the dsRNAs provided to

larvae; probably this aspect could have negative consequences on some biological parameters (fecundity, flight capacity, longevity, etc.) of adults.

Our target genes (AChE, nAChRs and RyRs) were selected for their importance and the involvement in nervous system and nerve transmission and for their effectiveness in RNAi experiments against other insect pests. Acetylcholinesterase (AChE) is a key enzyme in the insect central nervous system by hydrolysing the neurotransmitter acetylcholine into acetate and choline (Ye et al. 2017). Most insects have two different acetylcholinesterases (AChEs): AChE1 and AChE2 (encoded by ace1 and ace2 genes, respectively). Between these two AChEs, AChE1 has been suggested as the main catalytic enzyme based on its higher expression level (approximately 2- to 250-fold higher) and frequently observed point mutations correlated with insecticide resistance (Kim and Lee 2013). AChE gene silencing has been successfully tested on a number of insects such as *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), *Blattella germanica* L. (Blattodea: Blattellidae), *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), affecting the survival, the female reproduction, the embryo development and growth of offspring (Lu et al. 2012; Kumar et al. 2009; Revuelta et al. 2009; Saini et al. 2018). Our results, apart from reducing gene expression, prove the effectiveness of AChE gene silencing in *T. absoluta* by increasing mortality, and reducing the weight of both the prepupae and the pupae either by ingestion or radical absorption. In *H. armigera* the down-regulation AChE induced by dsRNA ingestion had negative effects, apart on the survival, also on the reproductive capacity of surviving adults whereas no effects were highlighted in pupal weight (Kumar et al. 2009).

nAChRs are ligand-gated ion channels that mediate fast synaptic transmission in the insect nervous system owing to the involvement of AChE in nerve transmission and other metabolic

346 processes (Jones et al. 2007). The α6 Nicotinic Acetyl-choline receptor subunit is the target of
347 two groups of insecticides (spinosins and neonicotinoids) but the role of individual nAChRs in
348 modulating responses to insecticides are not well known. (Rinkevich and Scott 2013) studying
349 the effects of the silencing of α6 nAChR in *Drosophila melanogaster* (Diptera: Drosophilidae)
350 and *T. confusum* concluded that this gene is not useful to understand the resistance mechanism to
351 spinosad. The same Authors attributed these limits, among the others, to intrinsic properties of
352 the target species. Our results, suggested that α6 nAChR is an effective target for RNA
353 silencing aimed at evaluating the effectiveness against *T. absoluta*. The corrected mortality
354 registered in the specimens injected with nAChRs dsRNA was higher than that recorded in the
355 specimens in which the target gene was AChE whereas, when the specific dsRNA was delivered
356 by root adsorption, the moth mortality reached about 80%.
357 Ryanodine receptors (calcium-induced calcium release channels) (RyRs) play an essential role in
358 most cell types. These receptors are located in the sarcoplasmic/endoplasmic reticulum
359 membrane mediating the release of intracellular Ca^{2+} during the excitation and contraction in
360 both cardiac and skeletal muscles (Hong et al. 2002; Lanner et al. 2010). Since calcium
361 signalling has a key role in most vital processes of insects, RYRs are molecular target for novel
362 diamide insecticides that have notably activity against Lepidopteran species (Sun et al. 2016).
363 Wan et al (2014) reported that oral supply of 0.5 µg of RyR dsRNA significantly reduced the
364 mRNA levels in *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) larvae and adults
365 without affecting the mortality and the larval and adult movement. Yang et al. (2014) reported
366 similar results in *Sogatella furcifera* (Horváth) (Hemiptera: Delphacidae) in which the oral
367 administration of dsRNA of Ryanodine receptors significantly reduced the mRNA level of *SfRyR*
368 without affecting the mortality. In our study we used a higher amount of dsRNA; this could

369 explain the effect on mortality of insects treated at both 2 and 5 µg. We suppose that the dsRNA
370 application rate play an important role since in our preliminary trials 1 µg of dsRNA did not
371 affected the mortality pf treated specimens.
372 The RNA interference approach in Lepidoptera was first reported in 2002 when the effects of
373 dsRNA on *Bombix mory* L (Lepidoptera: Bombycidae), H*yalophora cecropia* L (Lepidoptera:
374 Saturnidae) and *Spodoptera litura* Fabr. (Lepidoptera: Noctuidae) were reported (Bettencourt et
375 al. 2002; Quan et al. 2002; Rajagopal et al. 2002). However, most studies about RNAi targeted to
376 Lepidoptera were aimed to understand a number of systems, particularly developmental
377 processes and immunity (Terenius et al. 2011). In this scenario, the studies on the application of
378 RNAi method for the control of Lepidoptera and specifically on *T. absoluta* are limited. The first
379 experiments on this pest were conducted by Camargo et al. (2016) that reported the V-ATPase
380 and Arginine kinase gene silencing by dsRNAs. Camargo et al. (2016) investigated a delivery
381 approach in which *T. absoluta* larvae fed in leaflets (not rooted) left to uptake the dsRNA from
382 an aqueous solution.
383 The main objective of our experimentation was the potential use of effective dsRNAs delivered
384 by root adsorption. To date the main methods of dsRNAs administration were injection into the
385 haemolymph and oral delivery. Although these methods are valid for basic research, they are far
386 from being applied under field conditions. To date the dsRNA root adsorption route has not yet
387 been verified against *T. absoluta*. Both the delivery methods we used (injection and root
388 adsorption) were successful in larvae gene silencing, increased mortality and reduced both the
389 pre-pupal and pupal weight. The root adsorption delivery may have advantages over traditional
390 methods (injection and oral delivery via artificial diet) as this method takes into account the
391 entire plant system (root adsorption, dsRNA translocation in the plant and other variables,

392 difficult to study singularly, which may affect the degradation or bioavailability of dsRNA). Our
393 results highlighted the ability of dsRNAs to migrate from water solution to internal parts of roots
394 and then translocate in the plant. Despite we didn't measure the dsRNAs in plant, in larvae fed
395 on treated plants a significant reduction were detected in the target gene expressions. The
396 movement of dsRNAs in plant was studied by Camargo et al. (2016) by using labelled dsRNAs
397 which were strongly detected both in leaflet petioles and blades 6 h after the treatment and
398 throughout the leaf blade after 24h. Similarly to Camargo et al. (2016), our results, highlighted,
399 72 h after the treatments, a significant reduction in the transcript levels for all the target genes in
400 larvae suggesting that the ability of plant to translocate dsRNAs is similar both in detached
401 leaves as well as in rooted shoots.
402 Because the dsRNA root adsorption we adopted could well simulate what happened in the
403 hydroponic crops, this delivery method could be considered as an insecticide treatment methods
404 to be applied in the field. The potential of RNAi as a tool for insect pest control is widely
405 recognized, since this approach has specificity and flexibility that cannot be matched in
406 traditional control methods (chemicals, Biological control, GMO plants) (Scott et al. 2013). One
407 of the major issues that limited the use of RNAi as a control tool is the cost related to the
408 production and delivery of the effective dose of dsRNAs in field conditions. The ability of the
409 dsRNA to be delivered through roots and the recent advances in dsRNA production technology
410 allowing for the production of high doses at a reasonable cost, gives good reason to hope for its
411 use on a large scale (Hunter et al. 2010, 2012; Scott et al. 2013).
412
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420

421 **Author contribution statement**

422 SM, VP, FL and OC conceived and designed the research. SM carried out the experiments. OC,
423 LZ analyzed the data. SM, OC, LZ and FL wrote the MS. VP supervised the project. R.F.P.A
424 helped supervise the project. S.R. and A.M. contributed to the final version of the manuscript.
425 All authors read and approved the manuscript.

426

427 **Ethical approval**: This article does not contain any studies with human participants
428 or animals performed by any of the authors.

429

430 **Conflict of Interest**: Authors declare no conflict of interests.

431

432

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599

600

601 Table 1: Primers used in PCR, synthesizing dsRNA, and performing qRT-PCR.

Gene	experiment		Sequence (5'-3')	Product Size
RyRs	qRT-PCR	F	CGAAGAAGGCAAGATGGACG	198
		R	TCCTCCAAGCACATGACCAT	198
	PCR	F	CGAGATGGTCATGTGCTTGG	450
		R	TTGATGTGCTCGTCCCTCAT	450
	dsRNA	F	TAATACGACTCACTATAGGGAAAACAAGTTCCGTGCCCTC	256
		R	TAATACGACTCACTATAGGGAGCCAGTTGAGACGATTGGA	256
AchE	qRT-PCR	F	GACCCTTTAGTCGTTCGCAC	154
		R	ATCCTTCAGTGGGTCTTGGG	154
	PCR	F	ATGTCCCTGGAAATGCTGGA	276
		R	CGGACAATGTACAGCTTCGG	276
	dsRNA	F	TAATACGACTCACTATAGGGAATGTTATGGGTGTTCGGCG	430
		R	TAATACGACTCACTATAGGGCGGACAATGTACAGCTTCGG	430
nACHRs	qRT-PCR	F	GAGGTGTAAAGGACGTTCGC	163

		R	GTGCTCTTGAATATGCCCGG	163
	PCR	F	CTCCGATGCTATCCCCTTGT	439
		R	GTCCTCCATAGTAGACGGCC	439
	dsRNA	F	TAATACGACTCACTATAGGGGCCATGGATCTTGAGGATGT	236
		R	TAATACGACTCACTATAGGGTGCGAAATATTGAGCACCTG	236
18S ribosomal RNA gene	qRT-PCR	F	TATGTTGTGAGGCGACGATG	155
		R	GATCCACCGTCCAGGGTAAT	155
GFP	qRT-PCR	F	TAATACGACTCACTATAGGGAGATACGGCGTGCAGTGCT	495
		R	TAATACGACTCACTATAGGGAGATGATCGCGCTTCTCG	495

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Instar	Dose/treatment	AChE	nACHRs	RyRs
pre-pupae	GFP	1.85±0.05a	1.85±0.06a	1.85±0.07a
	2 µg	1.90±0.01a	1.56±0.01b	1.45±0.01b
	5 µg	1.62±0.05b	1.46±0.03b	1.24±0.04c
	sign.	P<0.01	P<0.01	P<0.01
pupae	GFP	2.68±0.05a	2.68±0.05a	2.68±0.05a
	2 µg	2.60±0.05a	2.43±0.07b	1.55±0.05b
	5 µg	2.31±0.02b	1.94±0.06c	0.66±0.03c
	sign.	P<0.01	P<0.01	P<0.01

605

606 Table 2 – Effects of RNAi on *T. absoluta* pre-pupae and pupae weight (mean±SE), after 2 and 5
607 µg of dsRNA injection. Different letters indicate statistical differences (p<0.05) among the
608 treatments (Tukey HSD post-hoc test)

609

610

Treatment	pre-pupae	Pupae
GFP	1.82±0.03a	2.12±0.03a
AChE	1.77±0.02ab	1.98±0.02b
nACHRs	1.71±0.01bc	1.83±0.02c
RyRs	1.67±0.02c	1.71±0.01d
sign.	P<0.01	P<0.01

611

612 Table 3 – Effects (mean±SE) of RNAi on *T. absoluta* pre-pupae and pupae weight, fed on on
613 plants administered with 5 µg of dsRNA. Different letters indicate significant differences
614 ($p<0.05$) among the treatments (Tukey HSD post-hoc test) ($p<0.05$).

615

Figure captions

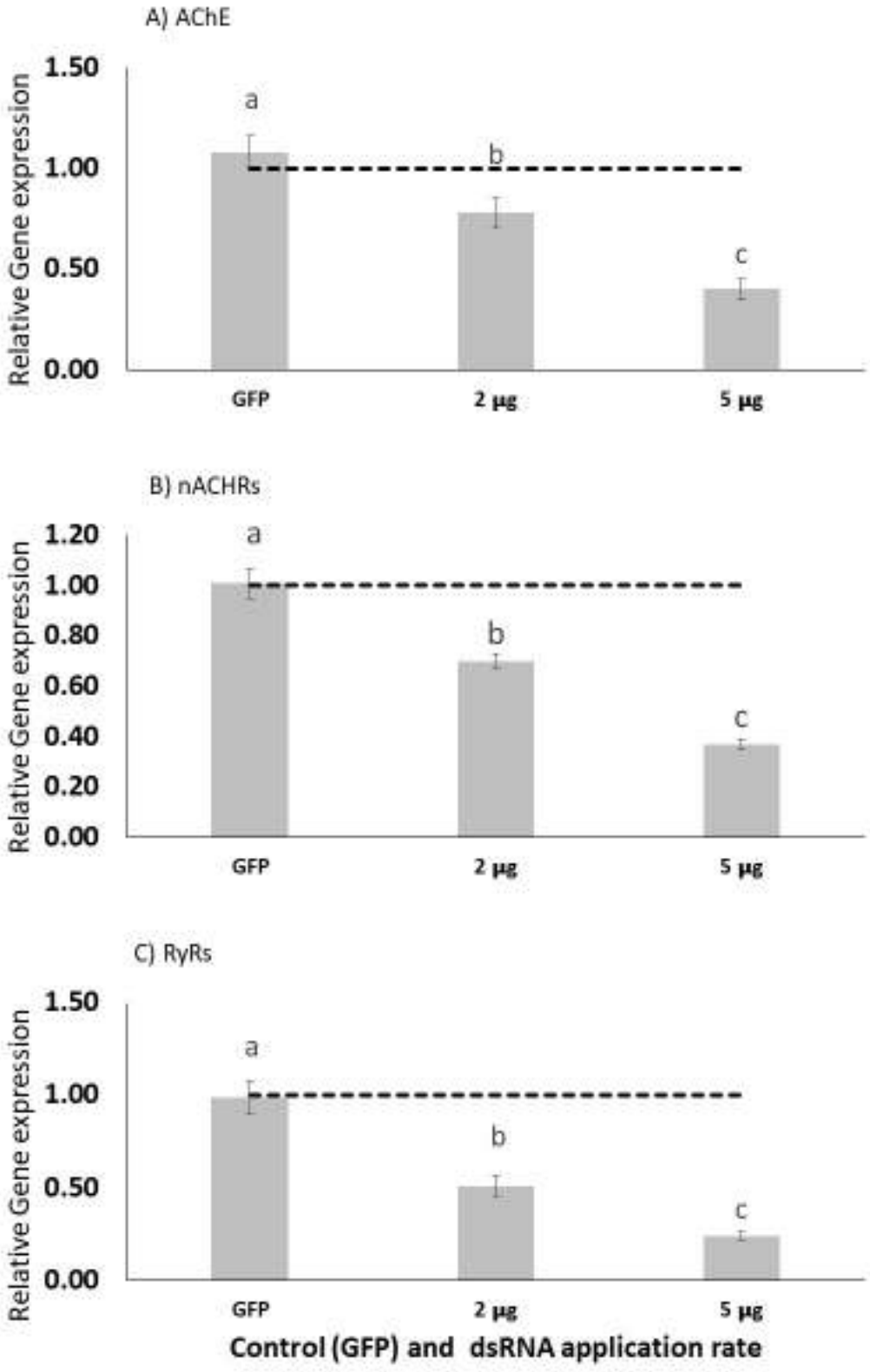
Figure 1 - Inhibition of the expression of three target genes after 72h of injection of 2 and 5 **µ**g of dsRNA in *T. absoluta* third instar larvae. Values represent the mean±SE of three replicates. Gene expressions were relative to the control GFP normalised to the internal control (18S rRNA). Dotted line represent the value of nuclease-free water. Different letters indicate significant differences among treatments (Tukey HSD post-hoc test; $p<0.05$).

Figure 2 – Effects of RNAi on *T. absoluta* mortality (mean±SE) from third instar larvae to adults injected with2 and 5 **µ**g of dsRNA. Values represent corrected mortality (Abbott's formula); Different letters indicate significant differences between the two doses within the same treatment group (GLM UNIANOVA; $p<0.05$).

Figure 3 - Inhibition of the expression (mean±SE) of three target genes in *T.absoluta* third instar larvae after 72h of feeding in rooted leaflets administered with 5 µg dsRNAs. Gene expressions were relative to the control GFP normalised to the internal control (18S rRNA). Dotted line represent the value of nuclease-free water. Asterisks indicate significant differences between each gene and the control (GLM UNIANOVA $p<0.05$).

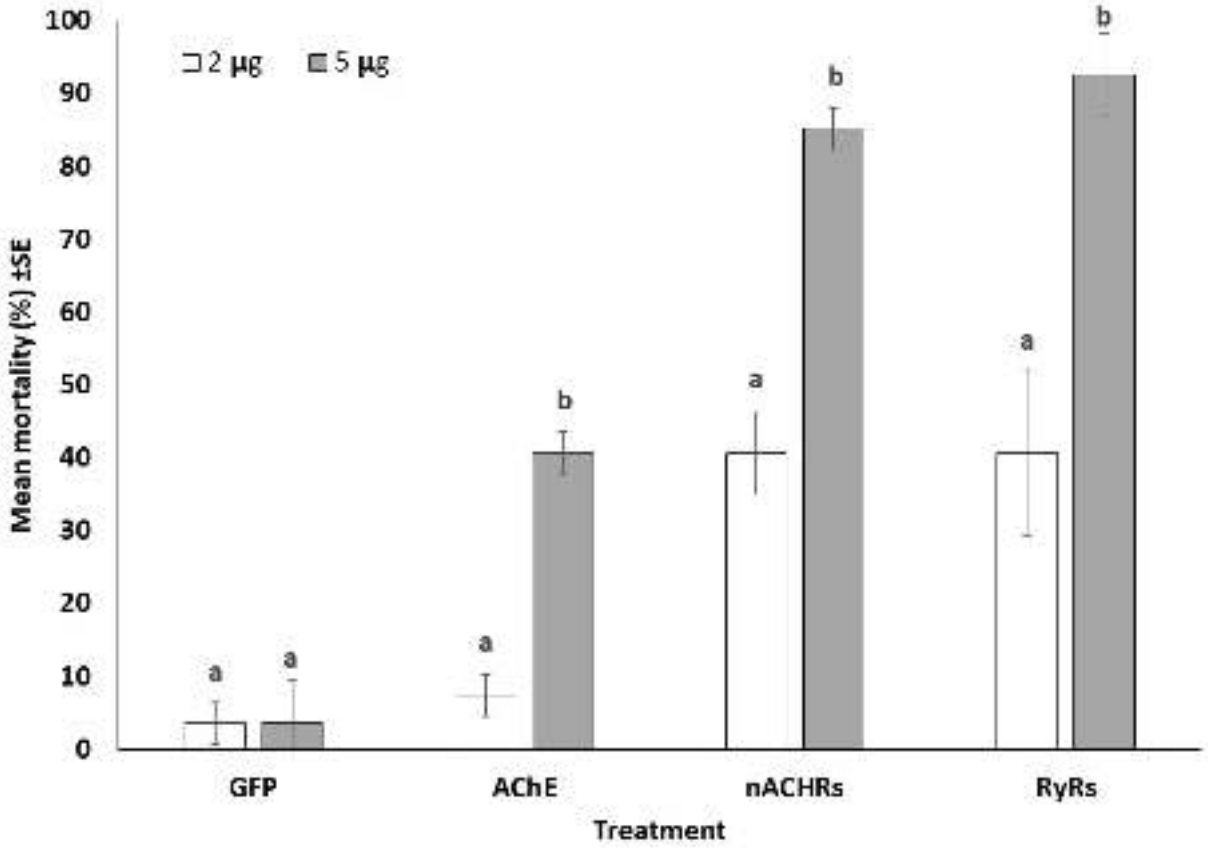
Figure 4 – Effects of RNAi on *T. absoluta* mortality (mean±SE) fed on plants administered with5 **µ**g of dsRNA. Values represent corrected mortality (Abbott's formula); Different letters indicate significant differences among the treatments (Tukey HSD post-hoc test; $p<0.05$)

638 Figure 1



639

640 Figure 2



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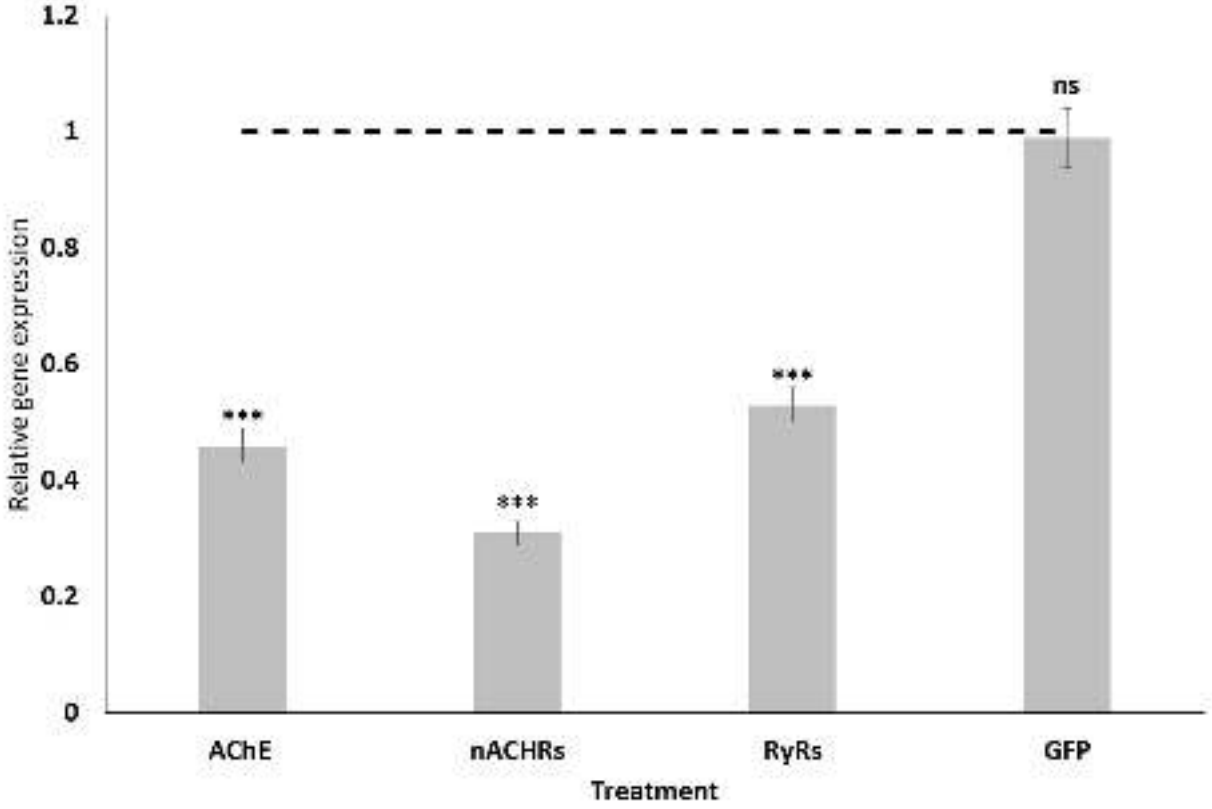
643 Figure 3



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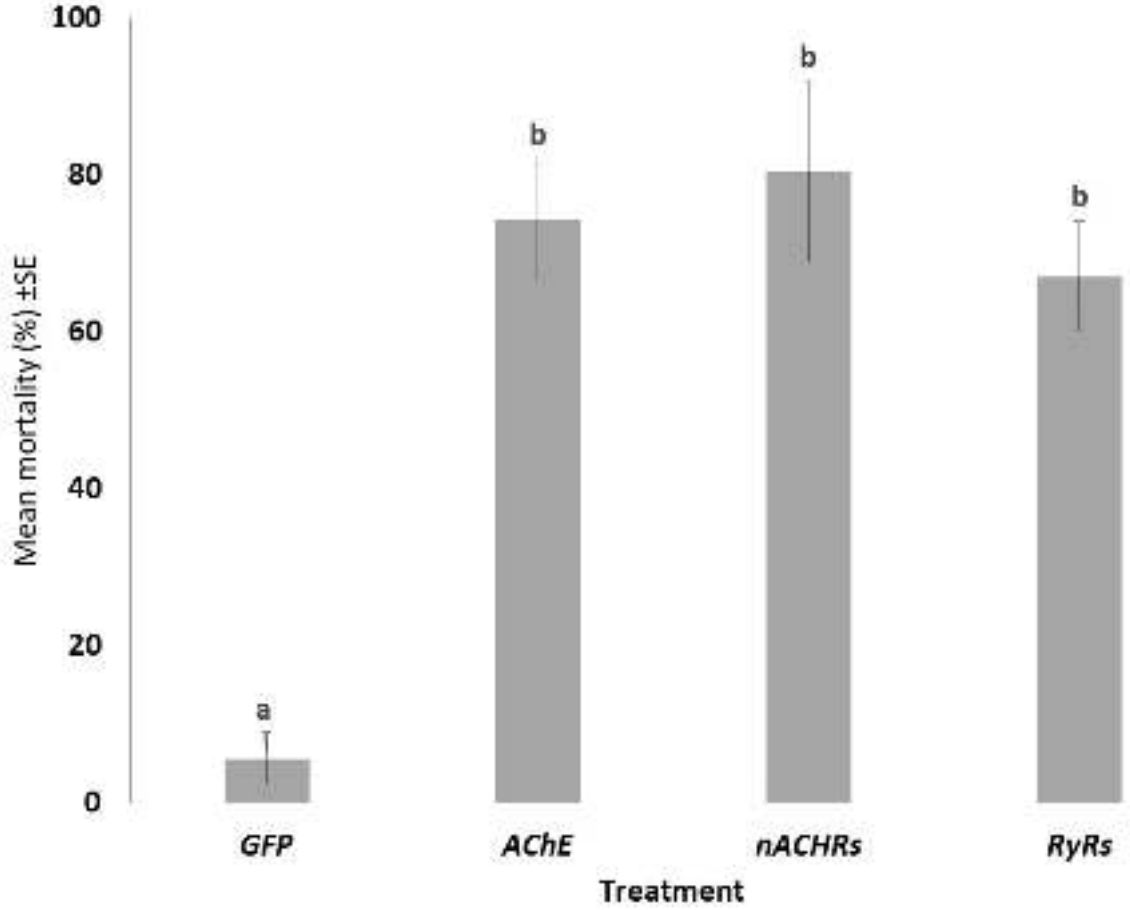
646 Figure 4



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