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Evidence of Multiple Pesticides Resistance in the Tomato Leaf Miner *Tuta Absoluta* Meyrick (1917) from Savannah Region of northern Nigeria

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Abstract

In 2016 northern Nigeria experienced a devastating infestation by the tomato leaf miner, leading to soaring in prices of tomato across the country. Unfortunately, information on the bionomics and resistance status of this pest is lacking in northern Nigeria, hampering appropriate control measures. Here, we identified to species level, and using conventional and synergist bioassays characterised pesticides resistance profile of a field population of a tomato leaf miner from northern Nigeria. Highest resistance was obtained with λ-cyhalothrin (Type II pyrethroid) with a low mortality (18.52% at 56hr) and LD_{50} of 7461.474ppm. Resistance was also established toward propoxur and chlorpyrifos-methyl with average mortalities each of 56% and LD_{50s} of 1023.51ppm and 106.351ppm, respectively. Highest susceptibility was seen with abamectin with mortality of 86% and LD_{50} of 0.034ppm. Pre-exposure to piperonyl butoxide significantly recovered λ–cyhalothrin susceptibility (mortality = 90% and LD_{50} = 0.92ppm) implicating the P450 monoxygenases in the resistance. No significant changes in mortalities were obtained on pre-exposure to diethyl maleate and triphenyl-phosphate- inhibitors of glutathione S-transferases and carboxylesterases, respectively. The finding of resistance to these agricultural pesticides will sensitize stakeholders across Nigeria to take action to manage the resistance at an early stage before it gets out of hand.

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Introduction

The leaf miner, *Tuta absoluta* (Meyrick 1917) (Lepidoptera: Gelechiidae), native to South/Central America [1], is one of the most devastating pests of tomato (*Solanum lycopersicum L.*), potato (*Solanum tuberosum L.*) and other solanaceous plants [2]. Since its first appearance in Spain in 2006 [3] *T. absoluta* has expanded its geographic range outside Americas (https://www.cabi.org/isc/datasheet/49260), and is now found in far flung places in Europe [4, 5], middle East/Asia [6, 7] and Africa [8, 9]. Following its first appearance in Africa at Senegal [10], this pest has expanded southward and in the recent years and have been reported in East [11] and southern African countries [12]. Africa is a continent of which agricultural sector accounts for more than 60% of total labour force [13] and Nigeria is the largest producer of tomato in the continent (http://www.fao.org/faostat/en/#data/QC). However, following the 2016 *T. absoluta* invasion (nicknamed '*tomato ebola*') more than 80% of the tomato produce was lost in northern Nigeria [14], leading to purported 125-400% increase in tomato price as reported in the local news. In Kano State alone farmers lost more than 2 billion Nigerian Naira in the 2016 season alone (https://punchng.com/tomato-farmers-in-kano-lose-n2bn-to-tuta-absoluta/).

As usual farmers responded by increasing the quantities of pesticides they apply (personal communication), often in mixtures of classes having similar mode of actions. At Kadawa farms in Kano farmers were found to be using the following pesticides for control of the leaf miner: (i) Expert 50 WDG, Emacot 050 WG and Caterpillar Force, all three made of Emamectin benzoate; and (ii) TEMA, made of emamectin benzoate (60g/kg) + Teflubenzurone (75/kg). They claimed that TEMA is the most effective of all four formulations. This heavy and unscientific reliance on pesticides could have placed intense selective pressure on this pest. Coupled with the short generation time of *T. absoluta* [2] may have led to increased resistance to the pesticides commonly used by the farmers making it impossible to control the pest even as of present.

Unfortunately, prior to this study little is known of the actual species identity of the leaf miner ravaging northern Nigeria. In addition, information on the nature and mechanism of the pesticide resistance was non-existent. Elsewhere, significant progress has been reported on the bionomics of this pest, its pesticides resistance status and the underlying molecular mechanisms driving the resistance in the field. Studies in different countries

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have shown that *T. absoluta* has developed resistance to insecticide classes in use for its control [15, 16]. Cases of insecticide resistance in *T. absoluta* and its underlying molecular mechanisms have been reported in Southern America and Europe, e.g. as in Chile and Brazil [17, 18], and as reported in Greece, Italy, Spain and Portugal [19, 20]. In Africa reliable studies which describe pesticide resistance in this pest are from Ethiopia, e.g. the recent work by Ayalew and Shiberu with their colleagues [21, 22].

Since insecticide resistance could be heterogenous even over short distances as observed in other species like mosquitoes [23] it is not wise to extrapolate findings from other countries to the local populations in Nigeria and/or Africa. To fill these gaps in knowledge and provide information to the relevant agricultural authorities we characterised a field population of *T. absoluta* from sudan Savanna of northern Nigeria. Following field collection and morphological identification, the *T. absoluta* were identified to species level using molecular approach. Insecticides resistance profile was then established and the possible enzymes systems responsible for metabolic resistance identified using synergist bioassays. The *T. absoluta* populations were resistant to pyrethroid, carbamate and organophosphate insecticides, with also resistance suspected to abamectin. Synergist bioassays significantly recovered susceptibility with mortalities increasing fourfold on average; revealing that the P450 monooxygenases are possibly involved in the pyrethroid resistance.

Materials and Methods

Materials

Chemicals and Reagents

The four different insecticides classes used for the bioassays: λ-cyhalothrin (a type II pyrethroid), propoxur (a carbamate), chlorpyrifos-methyl (an organophosphate) and abamectin (an ivermectin), were purchased from SIGMA ALDRICH, UK (Dorset, United Kingdom). The triton X-100 and the synergists piperonyl butoxide (PBO) and diethyl maleate (DEM) and triphenyl phosphate (TPP) were all purchased from the SIGMA, UK. For molecular analyses KAPA*Taq* polymerase kit was used (https://www.kapabiosystems.com/). Other chemicals used were to make a LIVAK DNA extraction buffer and are given in the methods section.

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Methods

Field collection and Rearing of Insects

Tomato leaves infested with *T. absoluta* larvae and eggs at different stages of development were collected from tomato farms at Kadawa [(11.6457ºN, 8.4479ºE), Figure 1] in GarunMallam Local Government Area, Kano, Nigeria. Collection was done for two days in three farms in April 2017.

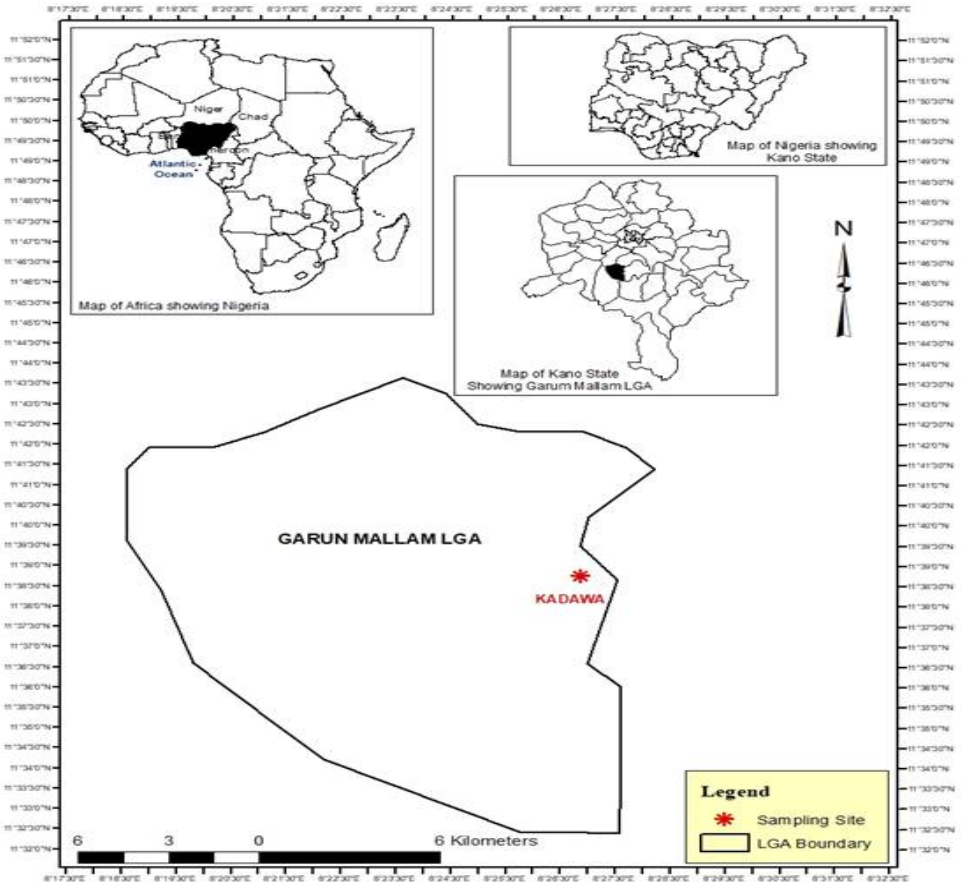

Figure 1: Sampling site, showing the location of Kadawa, Kano State.

Eggs and larvae were placed into large and wet jute bags. Approximately 300–350 larvae were collected. The samples were transferred to net cages measuring 70cmx50cmx50cm (height x width x depth, respectively), locally constructed following established procedure [24]. This was done within an hour of collection to avoid stressing the larave. Cages were maintained at 22-25°C and a photoperiod of 12:12h (light:day cycle) at Wellcome-Bayero Insectary at Bayero University Kano, Nigeria. Relative humidity was

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maintained as 70-75% using a humidifier. Adults that emerged were provided daily with fresh tomato leaves and allowed to mate. After laying eggs the F_0 parents were transferred into other cages and killed for morphological identification and molecular identification. Fresh foliage was provided daily for the newly emerged F_1 larvae and allowed to grow for 3-5 more days until the 2nd instar stage (after second moulting). The tomato leaves were continually sprayed with water to keep them from wilting.

Morphological Identification of T. absoluta life stages

Identification of eggs, larvae, pupae and the field collected adults was carried out morphologically using a stereomicroscope and following the protocol of EPPO [2].

Molecular Identification of T. absoluta to species level

Following morphological identification 16 F_0 parents were used for DNA extraction using the protocol described by LIVAK [25]**.** Buffer was made by dissolving 5.48g sucrose, 1.57g Tris, 1.6ml of 5M sodium chloride in 10.16ml of 0.5M EDTA. This was then followed by a 2.5ml of 20% SDS, and the volume was finally made up to 100ml in a volumetric flask. The buffer solution was then filtered and sterilised. 5ml aliquots were stored at -20ºC which was heated in a water bath and whirled to re-dissolve precipitate before use. Larvae were homogenized individually using a battery-operated mortar and pestle (SIGMA) in 50µl preheated grind buffer in 1.5ml Eppendorf. The pestle was rinsed with a further 50µl of the buffer to a total of 100µl. Homogenate was incubated at 65ºC for 30minutes. Condensation was collected by microfuging and 14µl of 8M of potassium acetate added to a final concentration of 1M. Samples were vortexed and incubated for 30min on ice. Tubes were centrifuged for 20min at 4ºC after which the supernatant was transferred carefully to a 1.5ml Eppendorf. At this point, 200µl of 100% ethanol was added and mixture spun for 15 mins at 4ºC. Pellets was rinsed in approximately 100µl ice cold 70% ethanol, air-dried for two hours and then re-suspended in 100µl of distilled water. Tubes were finally incubated at 65ºC for 10min.

Identification to species level was carried out by amplifying Cytochrome Oxidase subunit I (COI) gene using polymerase chain reaction [26, 27]. The universal forward and reverse primers: LCO1490 (5´-GGTCAACAAATCATAAAGATATTGG-3´ and HCO2198 (5´-

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TAAACTTCAGGGTGACCAAAAAATCA-3´) were used for PCR using 1µl each of the genomic DNA in a total reaction volume of 15µl. Reaction mix comprise 1.5µl of 10x TaqA Buffer, ~0.4µM (0.5µl) of each of forward and reverse primers, 1.25mM (0.75µl) of $MgCl_2$, 0.25mM (0.15µl) of dNTP mixes and 0.12µl of *Taq* DNA polymerase, in ddH_20. Amplification was carried out using the following conditions: initial denaturation of 5min at 95ºC, followed by 35 cycles each of 30s at 94ºC (denaturation), 30s at 57ºC (primer annealing) and 1min at 72ºC (extension). This was followed with 10min final extension at 72ºC. PCR products were separated in a 1.5% agarose gel stained with ethidium bromide.

Pesticides (Leaf-Dip) Bioassay

For conventional bioassays 810 L2 larvae were utilised, 702 for tests with pesticides and 108 for control. Larvae were sorted using a fine brush according to sizes to select those at the 2nd instar stage. Pesticides were tested using the leaf-dip bioassay protocol of the Insecticide Resistance Action Committee (IRAC test method # 022) (http://www.irac-online.org/methods/tuta-absoluta-larvae/) with minor modifications. Initially, stock solutions at different concentrations were prepared for the various insecticides: 0.1% abamectin, and 0.5% each of propoxur, chlorpyrifos-methyl and λ-cyhalothrin. These were next serially diluted into decreasing concentrations (1:6) in water containing 0.01% Triton X-100. Fresh tomato leaves were cut to equal sizes of 2.5cm diameter using hand-held punch. For each insecticide, for the six different concentrations 27 tomato leaves were dipped individually, for 3s with gentle agitation. After treatment the leaves were placed individually on wire net for 60s to dry. Individual leaves were transferred into bioassay trays (ref: RT32W) containing slightly moistened filter paper. A fine soft brush was used to transfer the 2nd instar larvae into a cell in the bioassay tray individually and the trays covered with a lid (ref: RTCV4). Trays were closed carefully, sealing the cells with their lids and then stored at about 25± 2°C, 60-70% relative humidity, and 12:12h light: dark photoperiod. Leaf damage as well as larvae mortality were then evaluated after 56 hours. Larval mortality was recorded with regards to those which were unable to make coordinated movement from gentle stimulus with fine pointed forceps to the posterior body segment (considered dead or seriously affected by the insecticide) (IRAC

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method # 022). Leaf damage was evaluated by physical examination to determine extent of damage as percentage of total leaf area mined. For control, the same procedure as above was followed with 27 larvae set for each experiment with different pesticides, except that the leaves were dipped into water containing 0.01% Triton X-100.

Synergists Bioassay

To determine the possible contribution of metabolic resistance in the *T. absoluta* populations, synergist assay was conducted with piperonyl butoxide (PBO: an inhibitor of P450 monooxygenases), DEM and TPP against λ-cyhalothrin (the insecticide to which the larvae exhibited the highest resistance). 324 L2 larvae were used for the test and 81 larvae as control. 27 larvae per replicate of insecticide concentration were placed in cell units containing a leaf dipped into either 4% PBO, 8% DEM or 10% TPP for 1hr, as done in some other insects [28, 29]. The larvae were then immediately transferred into bioassay cells containing leaves individually dipped into the various concentrations (0.643ppm, 3.853ppm, 23.15ppm and 138.89ppm) of λ-cyhalothrin- concentrations at which lowest mortalities were previously observed in the conventional bioassay described above.

For control, 27 larvae were first treated each with either PBO, DEM or DEF and then placed in cells containing untreated leaves. Mortality was assessed 56hr after exposure.

Data Analysis

The intensity of resistance was estimated by calculating the LD_{50} for the various insecticides using probit analysis as implemented in MASS package of R version 3.5.0 (https://cran.r-project.org/bin/windows/base/). All figures were prepared with and the results of synergised and un-synergised tests with λ-cyhalothrin compared using a two-tailed Chi-Square test of independence as implemented in GraphPad Prism 7.02 (GraphPad Inc., La Jolla, CA, USA).

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Results

Morphological and Molecular Identification of T. absoluta to species level

The field collected F_0 larvae and adults and the F_1 larvae were morphologically identified as *T. absoluta* (Meyrick 1917) (Lepidoptera: Gelechiidae), based on the following characteristics as explained in previous publications [2, 30]: early instars were white/creamy with black heads which changed into greenish from second instar with heads turning to brown/dark brown. The 1st instar larvae were <1mm and there is gradual increases in length until the 4th instar which was in average 7-8mm long. 2nd instar larvae were 4-5.5mm. Pupae were brown in colour and were folded into leaves singly. Adults were about 9-10mm long, with filiform antennae, silver-grey scales and evidence of black spots on anterior wings.

All the 16 F_0 parents were identified as *T. absoluta* Meyrick from the PCR amplification of Cytochrome Oxidase I fragment with a characteristic band of ~658bp [31] (Figure 2).

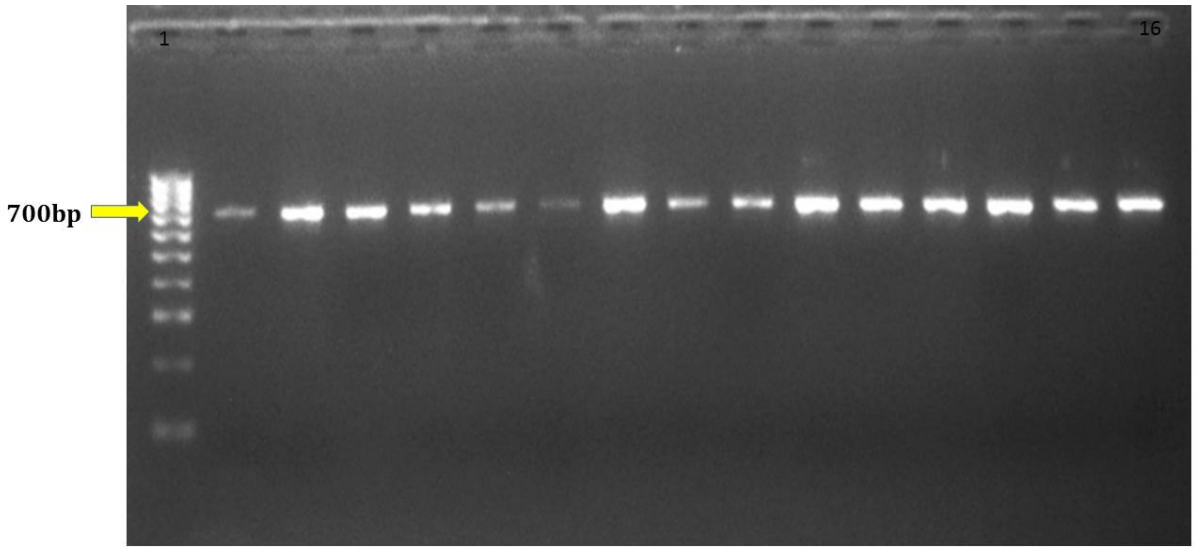

Figure 2: PCR species identification of *T. absoluta* species. A characteristic band of 658bp typical to metazoans is evident in lanes 2-16. Lane 1 represents HypperLadder IV from Bioline (~1013bp).

Insecticides Resistance Profile of T. absoluta Populations

The leaf-dip bioassay revealed highest resistance towards the type II pyrethroid λ-cyhalothrin with average mortality of only 18.52% ± 2.0 after 56hr (Figure 3A, Table S1) and an LD_{50} of 7461.474ppm ± 1213.793 (Table S1), greater than top concentration used

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for this insecticide. Highest leaf damage (~15% of the leaves destroyed) was also observed with this insecticide (Figure 3B, and Table S1), though not significantly different from the other pesticides tested.

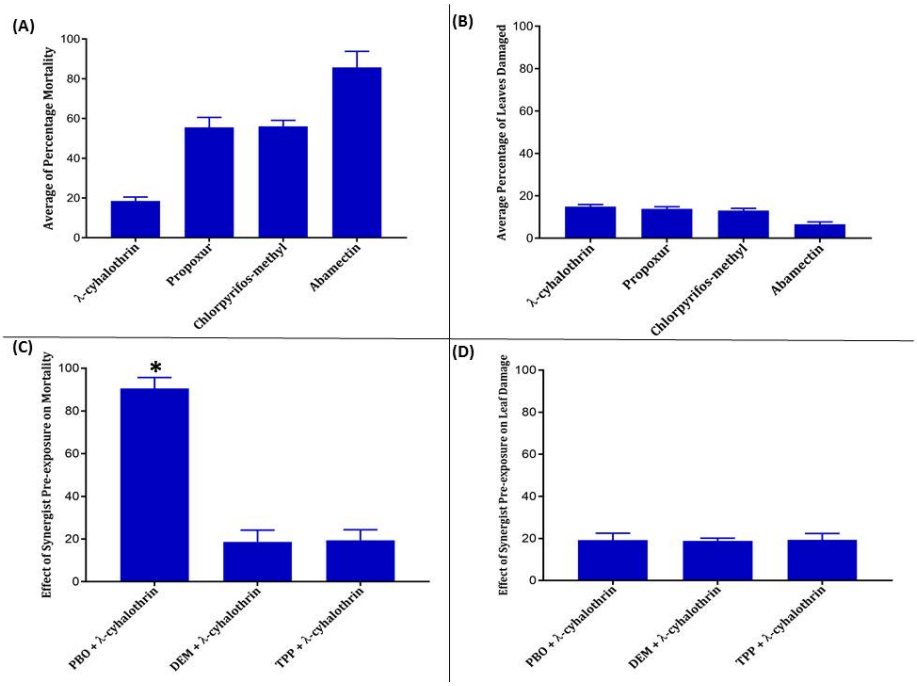

Figure 3: Results of conventional and synergist bioassays with various concentration of insecticides. Average of percentage for six different concentration ranges for each insecticides ± standard error of mean, for (A) mortalities, (B) leaves damaged, (C) mortalities with pre-exposure to synergists PBO, DEF and DEM, and (D) leaves damaged from synergist bioassay with PBO. *Significantly different from conventional test with λ-cyhalothrin only, $\chi^2 = 124.9$, $p<0.0001$.

In contrast with observation with λ-cyhalothrin, approximately 56% mortalities were recorded respectively, for propoxur and chlorpyrifos-respectively (Figure 3A, Table S1). However, the LD_{50} of propoxur (1023.35ppm ± 218.69) was on average ten times higher than obtained with chlorpyrifos-methyl (106.30ppm ± 13.09) due to the higher mortalities obtained at lower concentrations with the latter.

Lowest resistance was obtained with abametin, with average mortality of 85.71% ± 8.1 and a very low LD_{50} of only 0.034ppm ± 0.0036 (Figure 3A, Table S1). Highest foraging inhibition was also observed with this pesticide with only ~7% of the leaves damaged at the end of the experiment (Figure 3B).

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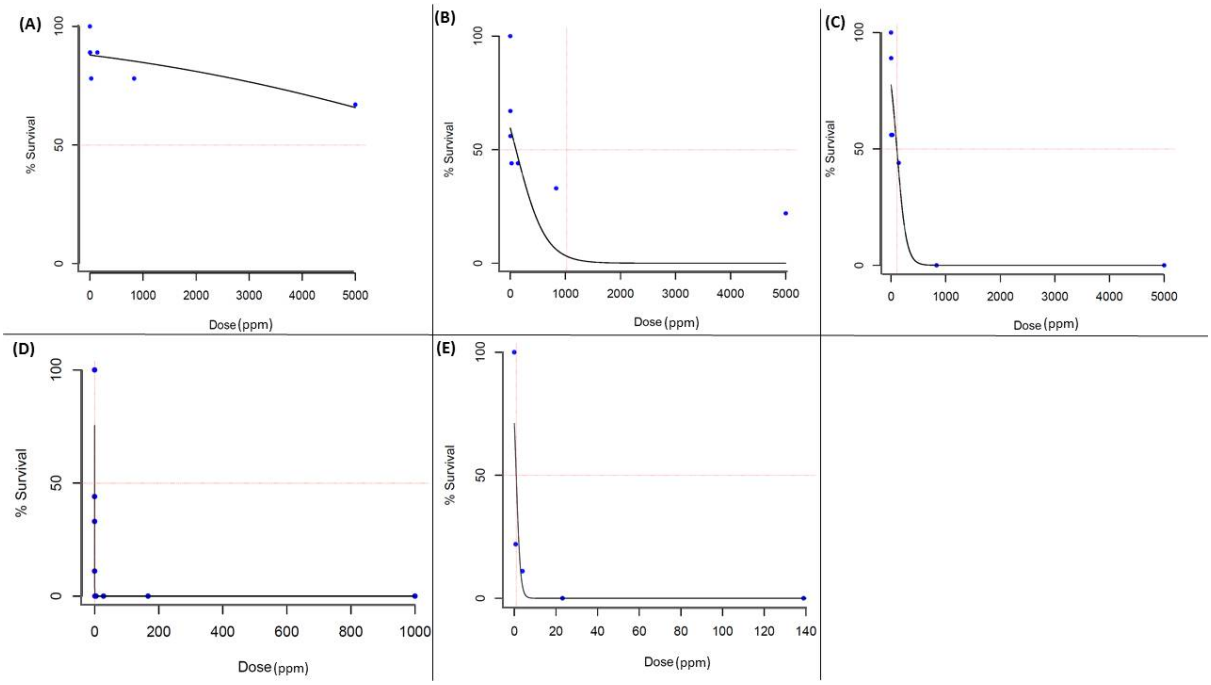

Figure 4: Results of dose-response bioassay to establish the LD_{50} for *T. absoluta* L2 larvae with (A) λ-cyhalothrin, (B) propoxur, (C) chlorpyrifos-methyl, (D) abamectin, and (E) PBO pre-exposure with λ-cyhalothrin.

Identification of Possible Mechanism of Resistance Using Synergist

To establish the possible major enzyme systems responsible for the pyrethroid resistance synergist assays were conducted for λ–cyhalothrin with PBO, DEM and TPP. In contrast with the observation from pre-exposure to DEM ($\chi^2 = 0.222$, $p = 0.64$) and TPP ($\chi^2 = 0.712$, $p = 0.399$), pre-treatment with PBO significantly recovered susceptibility ($\chi^2 = 124.9$, $p<0.0001$) with on average a five-fold increase in mortalities from 18.5% in the conventional bioassays to ~90% in synergised assay (Figure 3C). The LD_{50} plummeted down to only 0.92ppm ± 0.15, more than 8000 times lower than obtained in the conventional bioassay (Table S2). Thus a synergist ratio was calculated for the PBO- λ–cyhalothrin as 8154.35.

Surprisingly, for all the three synergists tested no major difference in foraging capability was observed between the synergised tests and conventional treatment with λ–cyhalothrin (Figure 3D, Table S2).

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Discussions

Tomato is the second most important vegetable crop in the world, next to potato, and Nigeria is its largest producer in Africa (and ranked 14th largest produder in the world). However, *T. absoluta* invasion is threatening the sustainability of tomato farming in Nigeria with millions of Nigerian Naira lost in recent years. The abrupt expansion and destruction of tomatoes by the leaf miner caught Nigerian farmers and other stakeholders unprepared. Continous invasion is reported here and there in northern Nigeria, e.g. (https://www.premiumtimesng.com/regional/nnorth-east/230931-disastrous-tomato-pest-tuta-absoluta-returns-destroys-tomatoes-three-local-govts.html) with no clear plans on ground on how to control this pest. In contrast to studies carried out elsewhere in Europe and Americas, except for few reports from Ethiopia most of the studies done on *T. absoluta* from sub-Saharan Africa did not describe the insecticides resistance profile of this pest. For, example, published data from Senegal [32], Burkina Faso [33], Niger [9], Tanzania [11], Angola [34] and Botswana [12] described only ecology and/or bionomics of this pest. To facilitate plans for control of this pest and manage its resistance in Nigeria and neigboring regions we investigated the pesticides resistance profile of one field population of the leaf miner from northern Nigeria and interrogated the possible enzymes system driving the resistance.

Pyrethroids being cheap and safest insecticides for mammals are the frontline pesticides farmers prefer to apply. However, the high pyrethroid resistance in agricultural pests, as observed in this northern Nigerian populations resulted in a shift to other more effective but expensive pesticides. High resistance to type I pyrethroid permethrin and type II pyrethroids, λ-cyhalothrin, deltamethrin and α-cypermethrin have been previously described for the Brazilian and Iranian populations of *T. absoluta* [18, 20, 35]. In this study, comparable resistance was observed between the organophosphate chlorpyrifos-methyl and the carbamate propoxur, though at average mortalities of ~56% lower than obtained with λ-cyhalothrin. Organophosphate and carbamate resistance had been described for *T. absoluta* populations previously, e.g. for chlorpyrifos and methamidorphos in Iranian and Brazilian populations [35, 36], and towards methamidorphos in the Brazilian populations [36]. Just as established in our study the above studies described multiple resistance to pyrethroids, carbamates and

organophosphates in the *T. absoluta* populations from different parts of the world [35, 36].

The lowest resistance we observed towards abamectin was in keeping with a report from Argentine populations with abamectin exhibiting lowest LD50 in three different populations compared to methamidorphos and deltamethrin [37]. In a recent study [22] resistance to emamectin benzoate (a derivative of abamectin) has also been shown to exist in populations from Ethiopia.

Synergists PBO, DEM and TPP have been used by Sequeira and colleagues [38] to synergize abamectin in bioassays with results implicating P450s the most, in comparison to the other two synergists with much lower synergism. This is in agreement with our finding which suggests that P450s are the major drivers of metablic resistance to λ-cyhalothrin. However, using biochemical assays of enzyme activities another study conducted with Brazilian populations of *T. absoluta* established greater correlation between pyrethroids resistance and increased levels of both monooxygenases and GSTs [39].

Conclusion

In northern Nigeria, farmers desperate to control *T. absoluta* often mix and increase the amount and frequency of pesticides they apply. Unfortunately, these possibly is placing selective pressure in this pest populations exacerbating the already high pesticide resistance. Resistance towards four classes of pesticides in use for agricultural control of pests is present in the field in *T. absoluta* from northern Nigeria. However, the least resistance observed with abamectin suggests its possible potency in the field. But this insecticide as well as diamide insecticides like chloranthraniliprole are very expensive and possibly unaffordable by subsistence farmers from Nigeria. The claim by farmers of better kill with a formulation containing a mixture of emamectin benzoate and teflubenzurone could possibly be due to lower or absence of resistance to the teflubenzurone, which is an an insect growth regulator benzoylurea.

There is urgent need for the Nigerian Ministry of Agriculture and other stakeholders to educate farmers on best practices such as resistance management strategies using pesticides rotation to slow down its progress in the field. In addition there is an urgent

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need to investigate the molecular mechanism of the resistance by genotyping for the voltage-gated sodium channels knockdown resistance mutations which have been described in *T. absoluta* populatios elsewhere, as well as transcriptional analyses to establish the major metabolic resistance genes responsible for the resistance in the field.

Supplementary Materials

Table S1: Results of insecticides bioassay and leaf damage with various concentration of insecticides.

Table S2: Results of synergist bioassay and leaf damage with λ-cyhalothrin.

Acknowledgments

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Authors contribution

Conceived and designed by SSI. IB and MMM collected samples from field and carried out pesticides bioassays. SSI analysed the data with the help of IB and SKH. SSI did the molecular analyses and wrote the manuscript. All authors read and approved the manuscript.

Conflicts of interest

The authors declare no competing interests.

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Figure Legends

Figure 1: Sampling site, showing the location of Kadawa, Kano State.

Figure 2: PCR species identification of *T. absoluta* species. A characteristic band of 658bp typical to metazoans is evident in lanes 2-16. Lane 1 represents HypperLadder IV from Bioline (~1013bp).

Figure 3: Results of conventional and synergist bioassays with various concentration of insecticides. Average of percentage for six different concentration ranges for each insecticides ± standard error of mean, for (A) mortalities, (B) leaves damaged, (C) mortalities with pre-exposure to synergists PBO, DEF and DEM, and (D) leaves damaged from synergist bioassay with PBO. * Significantly different from conventional test with λ-cyhalothrin only, $\chi^2 = 124.9$, $p<0.0001$.

Figure 4: Results of dose-response bioassay to establish the LD_{50} for *T. absoluta* L2 larvae with (A) λ-cyhalothrin, (B) propoxur, (C) chlorpyrifos-methyl, (D) abamectin, and (E) PBO pre-exposure with λ-cyhalothrin.

Table Legends

Table S1: Results of insecticides bioassay and leaf damage with various concentration of insecticides.

Table S2: Results of synergist bioassay and leaf damage with λ-cyhalothrin