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Molecular characterization and toxic evaluation of *Lysinibacillus sphaericus* crude extract against *Tetranychus urticae* and its predator *Neoseiulus zaheri*

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Abstract

The present study was targeted to; i) assess the production of *Lysinibacillus sphaericus* crude extract (*Ls* CE) on different solid-state fermentation, ii) assess the acaricidal activity of *Ls* CE against *Tetranychus urticae*, iii) evaluate the prey mite mediated effects of *Ls* CE on the predatory mite, *Neoseiulus zaheri*, and iv) study the genetic diversity using ISSR marker. The bio-production of *Ls* CE was carried out on five medium substrates; and the most promising substrates for alkaline protease production were wheat bran and fodder yeast. The efficacy of *Ls* CE produced on wheat bran was investigated against eggs and females of both the prey and predatory mite species at different concentrations of 100%, 50%, 25% and 12.5%, and exposure times of 24h, 48h and 144h under laboratory conditions. Females were significantly influenced and mortality percentages increased as the *Ls* CE concentrations and exposure time increased. Mortality of *T. urticae* was 87.50 ± 2.06% and *N. zaheri* was 6.00 ± 1.07% at 100% concentration after 144h. Additionally, the population densities and reduction percentages of *T. urticae* stages were evaluated after 1, 3, 7, 10, and 16 days post- LC_{50} values reported to *T. urticae* females by *Ls* CE treatment under semi-field- trials. The highest decrease was observed after 3-days of indirect exposure and 7-days post direct exposure. Also, the consumption rate and fecundity of *N. zaheri* females were conducted through prey mite mediated effects of the same LC_{50} value. The consumption rate and fecundity of *N. zaheri* females were slightly influenced in indirect treatments. Finally, the genetic diversity of treated prey and predatory mites were resulted in 51% polymorphism in *T. urticae*, and 16% polymorphism in *N. zaheri* treatments. In conclusion, current findings recommend using *Ls* CE from wheat bran in biological control applications.

Keywords: Biological control, predator-herbivore interaction, biopesticides, alkaline protease, ISSR, SDS-PAGE

Introduction

Agrochemicals, such as chemical pesticides contribute significantly to the management of food production by increasing agricultural yield through eliminating pests. Nonetheless, pesticides have the capacity to seriously harm both human health and the environment (Jepson *et al*., 2020) [24]. The two-spotted spider mite (TSSM), *Tetranychus urticae* Koch (Acari: Tetranychidae) is considered the most economically important pest in the world (Migeon and Dorkeld, 2024) [28]. It develops resistance mechanisms toward various chemical groups of pesticides (Han *et al*., 2024), as it can be increasing the activity of metabolic detoxification (van Leeuwen and Tirry, 2007) [7], or boosting of metabolic proteins (Xu *et al*., 2021) [49].

Predatory phytoseiid mites were successfully used in the biological control applications against wide range of pest mite and insect species globally (Barbar *et al*., 2024) [7], however, in heavy pest infestations, predatory mites are unable to keep the population of pests below the injury level (Miresmailli and Isman, 2006) [29]. Moreover, their susceptibility to most chemical pesticides also poses an issue (Duso *et al*., 2020) [13]. *Neoseiulus zaheri* (El-Borolossy) (Acari: Phytoseiidae) is an indigenous predator which has been originally recorded from Upper Egypt (El-Borolossy, 1979) [13]. Its biological properties and life table parameters have been tested with eriophyid and tetranychid mite species, as well as herbivore insect species (i.e., the tomato whitefly and scale insect) and date palm pollens (Rasmy *et al*., 2003) [33]. Besides, it has been studied as a potential bio-agent to control *Thrips tabaci* (Lind.) (Insecta: Thysanoptera: Thripidae) (Abou-Elella, 2003) [2].

With the expansion growth of the green products (bio-products) industry and the global strategies aimed at bringing about an ecological transition and green revolution, the demand for creative solutions is always growing (Feijao *et al*., 2022) [18]. Microbial-based products are one type of sustainable agriculture method, gradually becoming more viable substitutes for agrochemicals (Sabbahi *et al*., 2022) [38].

Lysinibacillus sphaericus strain (*Ls*) is a microbial control agent of the *Bacillus* group (Chen *et al*., 2024) [11]. It has been used as an insecticidal bio-agent to combat mosquitoes since the mid of the 20th century (Berry, 2012) [8]. It has been reported to have a lethal potentiality against the root-knot nematode (RKN), *Meloidogyne incognita* (Kofoid & White) (Nematoda: Heteroderidae) (Soliman *et al*., 2019) [41], the German cockroach, *Blattella germanica* L. (Insecta: Blattodea: Ectobiidae), the common cutworm, *Spodoptera litura* (Fabricius) (Insecta: Lepidoptera: Noctuidae) (Nishiwaki *et al*., 2007) [31], the itch mite, *Sarcoptes scabiei*

(L.) (Acari: Sarcoptidae) (Pérez *et al*., 2024) [32] and the two-spotted spider mite, *T. urticae* (Emam, 2019).

Recently, solid-state fermentation (SSF) has been thought of for an innovative method for microbial growth instead of synthetic growth environments (Fayad *et al*., 2022) [17]. Its economic value is concluded in in turning waste and byproducts into useful material (Mattedi *et al*., 2023) [27]. One of the SSF microbial growth environments is the wheat bran which considered a rich source of carbon, nitrogen and other nutrients (Fayad *et al*., 2022) [17].

Moreover, *L. sphaericus* gained an economic benefit, due to its ability to produce a variety of metabolites that are effective as insecticides (Ruiu, 2015[37]; Williamson *et al*., 2023) [48]. The microbial metabolites composition may consist of live bacteria, their spores, toxins, or enzymes, and perhaps their engineered protein (Ayilara *et al*., 2023) [6]. It is necessary to understand the mechanism of interaction at the molecular and biochemical level (Chattopadhyay *et al*., 2017) [10].

DNA technology enhances the efficiency of understanding the genes of living organisms to select biotoxins, to reach the industrial level in biopesticide production (and Gopalakrishna, 2004) [42]. The Inter-Simple Sequence Repeat markers (ISSR) were introduced by Zietkiewicz *et al*., (1994) [51], these markers were employed to conduct genetic diversity studies (Hashem, 2016; Reddy *et al*., 2002) [22, 34], this technique is simple and efficient (Goulão and Oliveira, 2002) [20]. No previous knowledge of the genome is required to use this technique. Despite being dominant markers, they can be used to assess multiple loci in a single reaction (Souza *et al*., 2017; Tsumura *et al*., 1996) [43, 46]. Therefore, the present study aimed to i) assess the production of *L. sphaericus* crude extract (*Ls* CE) on different solid-state fermentation medium substrate, ii) assess the acaricidal activity of *Ls* CE produced from the most promised medium substrate against *T. urticae*, iii) evaluate the prey mite mediated effects of *Ls* CE on the predatory phytosiied mite, *N. zaheri*, and iv) study the genetic diversity using ISSR marker.

Material and Methods

1. Bacterial treatment assay

1.1. Bacterial strain and Inoculum preparation

Lysinibacillus sphaericus Amira strain, Microbial Chemistry Dept., NRC (GenBank accession number KT361851.1). According to Roshdy *et al*., (2022) [35], *L. sphaericus* inoculum were prepared by inoculating in nutrient broth yeast extract medium at 30 °C for 24 h. The colony forming units achieved 22×10^7 CFU/mL, by plating 10-fold serial dilutions in nutrient broth agar plates (Teng *et al*., 2017) [45].

1.2. Production of bacterial extract on solid state fermentation

Five grams of less than 1 mm particle size of the solid growth media substrates named fodder yeast, rice husk, wheat bran, rice straw, and treated rice straw (0.5% casein) substrates. Each substrate mixed with 15 mL of tap water, and autoclaved at 121°C and 15 lbs pressure for 15 min, cooled to room temperature, inoculated with 5 mL of prepared inoculum under sterile static conditions, and incubated at 30°C temperature for 3 days. Fermented substrates were mixed with 125 mL tap water, by stirring on a magnetic stirrer for 30 min at room temperature. The slurry was squeezed through cheesecloth followed by centrifugation of the whole content at 4000 rpm for 10 min at 4°C to remove the insoluble matters.

The clear supernatant of the most promising substrate was used as a crude extract for 1) alkaline protease assay, in glycine NaOH buffer at pH 10 following Foda *et al*., (2013) [19]0, and 2) the acaricidal bioassay. The *Ls* CE was prepared at 100%, 50%, 25%, and 12.5% (w/v)).

2. Acaricidal bioassay

2.1. Mite rearing

The mite cultures of *T. urticae* and *N. zaheri* were maintained at the Acarology laboratory of the NRC. Both mites were collected from unsprayed eggplant leaves, *Solanum melongena* L. (Solanaceae) in the NRC experimental station at Wadi El-Natrun, Beheira Governorate, Egypt (30°29'50.2"N 30°19'14.2"E). Cultures were reared under controlled condition (27 ± 2 °C, 65 ± 5 % RH, and 16:8 h L:D photoperiod) for three generations prior experiments. *Tetranychus urticae* was transferred onto up-side-down cooper acalypha leaves, *Acalypha wilkesiana* Müll. Arg. (Euphorbiaceae), in water saturated cotton in 10 cm-in-diameter (cm Ø) Petri dishes. *Neoseiulus zaheri* were placed on detached cooper acalypha leaves infested with *T. urticae* placed up-side-down on moist cotton wool in Petri-dishes. Fresh leaves that were infested were introduced to give *T. urticae* a constant feeding source.

2.2. Experimental procedures

2.2.1. The acaricidal impact of *L. sphaericus* crude extract against eggs and females of both *T. urticae* and *N. zaheri* under laboratory conditions

For *T. urticae* eggs, ten mated females were placed on wet cotton pads in Petri plates as discs made of acalypha leaves (3 cm Ø). The females were then removed, and the eggs were sprayed directly with glass atomizers that contained aqueous quantities of the tested extract. A total of 50 eggs were replicated five times for each concentration. Six days after spraying, the treated eggs' hatchability was recorded. The same procedure was used to estimate the toxicity effect of the extract on females, but here there were 25 females of the same age were replicated eight times for each concentration. Lethal and sub-lethal concentrations were computed, and the death % was noted after 24, 48 and 144h. Distilled water was used as a control. Every bioassay was done twice on different time. For *N. zaheri*, the procedure and recording data was the same as it was described for *T. urticae*; but here (25 eggs and 15 females each / replicate) were used. Egg's hatchability was calculated after 72 h from treatment.

To assess the *T. urticae* mediated effects of *Ls* CE on *N. zaheri*, newly emerged 5-days females were collected after mating in two separated sets of experiments as follow.

- ***The first set* (*Ls* CE treat 1):** *T. urticae* protonymphal stages (n=20) transferred into the experimental arenas (3 cm Ø) of *A. wilkesiana* discs and were treated with *Ls* CE of LC_{50} value reported to *T. urticae* female. The untreated *N. zaheri* females were moved individually to the treated substrates (n= 15). *Neoseiulus zaheri* females were supplied with treated *T. urticae* protonymphal stages daily until the end of the experiment.

- ***The second set* (*Ls* CE treat 2):** *N. zaheri* females were sprayed directly with *Ls* CE of LC_{50} value recorded to *T. urticae* female. Treated females were transferred individually into untreated arenas of *A. wilkesiana* discs (n= 15), and were supplied with 20 untreated *T. urticae* protonymphs daily.

Each experimental set was kept at laboratory conditions and checked every 24h. Consumption and fecundity rates of each replicate have been recorded for 10 days.

2.2.2. Semi-field trials- impact of *L. sphaericus* crude extract against *T. urticae*

The trial was conducted in open air at five leaf stage seedlings of Lettuce, *Lactuca sativa* var. *romana* L. (Asteraceae). Plants were cultivated in a mixture of soil and vermiculite in plastic pots. Ten plant seedlings were considered for each treatment and the control. About 50 females of *T. urticae* were transferred to each plant seedling. Two modalities used; 1) *treat 1* (*T. urticae* + *Ls* CE): a day after infestation, bacterial extract was applied (with about 20 ml of LC_{50} concentration reported to *T. urticae*) and 2) *treat 2* (*Ls* CE + *T. urticae*): bacterial extract (the same concentration) was sprayed then infested *T. urticae* was transferred after 1-day post- treatment). Five leaves were randomly selected from each plant after 1-, 3-, 7-, 10-, and 16-days post –treatments. The average number of mites detected on each of the five leaves was calculated. Eggs as well as all motile stages of *T. urticae* were counted and the mortality of the last was recorded.

3. Molecular characterization

3.1. DNA extraction

Genomic DNA was extracted and purified from treated and untreated *T. urticae* and *N. zaheri* by LC_{50} value recorded to *T. urticae* female using Wizard®Genomic DNA Purification Kit (Promega). The DNA samples were stored at -20ºC.

3.2. Molecular markers ISSR for *T. urticae* and *N. zaheri* control and treated samples

PCR amplification: was performed by using Inter simple sequence repeats (ISSR) analysis was applied following Zietkiewicz *et al*., (1994) [51], and procured from UBC (University of British Columbia, biotechnology laboratory, Vancouver, Canada) based on core repeats anchored at the 5' or 3' end as follows,

Table 1

No.	Primer	Sequence	annealing
1	HB12	5′-GTGGTGGTGGC-3′	40°C
2	HB09	5′-(GT)6GG-3′	40°C
3	17899B	5′-(CA)6 GG-3′	40°C
4	17899A	5′-(CA)6 AG-3′	38°C
5	17898B	5′-(CA)6 GT-3′	38 °C

Tetranychus urticae and *N. zaheri* DNA were amplified using Taq DNA polymerase chain reaction (PCR) as in the manufacturer's instructions Taq (Go Taq Promega kit) for ISSR primers, the PCR reaction consisted of a 5 min incubation period at 94°C followed by 40 cycles of 94°C/30s, (38-40) °C/45sec and 72°C/1.30 min, with a final extension step of 72°C/7 min. The PCR product was separated by 1.5% agarose gel electrophoresis using a TAE buffer and 0.004% red safe dye. The gel was photographed by gel documentation (Bio-Rad) and analysed by Total Lab program version 1.10 software based on DNA ladder to find out the molecular weight of each band and that to compare the presence and absence of the band among samples.

4. Statistical analysis

The corrected mortality of females was not computed as the control mortality was zero. Statistical differences in mortality means between the treatments were assessed using SPSS version 26.0 (SPSS, 2019) [44], followed by Duncan Multiple Range test. The following equation, established by Henderson and Tilton, (1955) [23] was used to compute the reduction percentages of *T. urticae* population density

$$= \left(1 - \frac{n \text{ in } C \text{ before treatment } x \text{ n in } T \text{ after treatment}}{n \text{ in } C \text{ after treatment } x \text{ n in } T \text{ before treatment}}\right) x\ 100)$$

Where: *n* = pest mite population, *T*= treated, *C* = control.

Results

1. Alkaline protease assay

Figure (1) shows that the wheat bran and the fodder yeast were the best growth media for alkaline protease production with values 20683.60 ± 2.88 and 20683.60 ± 2.88, (Unit: µgram Tyrosine / mL / min), respectively, while the rice husk ranked the last (8208.60 ± 11.73) (U / mL / min).

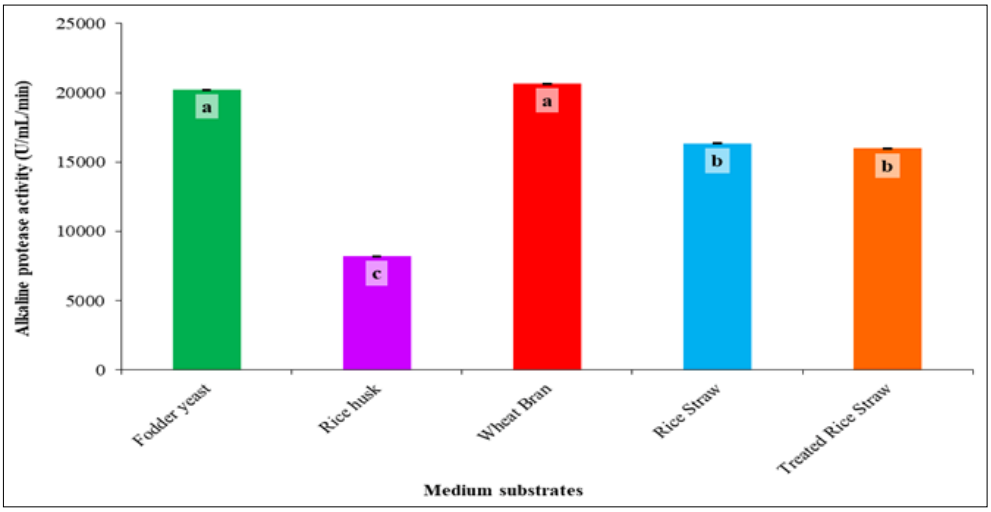

Fig 1: Effect of solid-state fermentation media substrates on *L. sphaericus* alkaline protease production.

2. Acaricidal bioassay

2.1. The impact of *L. sphaericus* crude extract against eggs and females of *T. urticae* and *N. zaheri*

The acaricidal impact of *Ls* CE against eggs and females of both *T. urticae* and *N. zaheri* was concluded in Table 1. Resulted data showed that *T. urticae* females were significantly influenced (*P*=0.000). The mortality percentages increased as *Ls* CE concentrations and exposure time increased. The highest mortality of the pest mite was 87.50 ± 2.06 % at 100% concentration after 144h post – treatment. In contrary, the mortality of *N. zaheri* was 6.00 ± 1.07% at the same concentration and time.

A higher hatchability rate for both the pest and predator eggs at all tested concentrations has been resulted in Table (1), although the emergence rate of larvae was observed after 6- and 3-days post- treatments, respectively.

Calculating the relationship between the females of *T. urticae* mortality and *Ls* CE concentrations, the toxicity regression equation was Y=22.48+0.68x, and the concentration-response correlation was highly positive (R= 0.946) *F*= 96.07, *df*= (1, 30), *P* = 0.000). The resulted LC_{50} was 38.79 (at confidence limits of 95% = 31.48-45.74) and LC_{90} was 102.11 (at confidence limits of 95% = 89.22-121.72) when the calculated $\chi 2$ was 243.65, *P*= 0.000 at $P \leq 0.05$.

Table 2: The acaricidal impact of *L. sphaericus* crude extract against females (mortality) and eggs (hatchability percentages) of both *T. urticae* and *N. zaheri*

Mite species	Female mortality% (Means ± SE)			
	Conc.%	24h	48h	144h
T. urticae	12.5	13.50 ± 1.68^{d}	15.00 ± 1.65^{d}	24.00 ± 2.00^{d}
	25	45.50 ± 2.26^{c}	54.00 ± 2.00^{c}	63.00 ± 1.81^{c}
	50	53.00 ± 2.36^{b}	63.00 ± 1.81^{b}	63.50 ± 1.92^{b}
	100	74.00 ± 2.27^{a}	86.00 ± 2.83^{a}	87.50 ± 2.06^{a}
	$F_{df\ (3,28)}$	135.07	194.44	181.63
	P	0.00	0.00	0.00
N. zaheri	12.5	0.00 ± 0.00	0.00 ± 0.00^{a}	0.50 ± 0.50^{c}
	25	0.00 ± 0.00	0.00 ± 0.00^{a}	1.00 ± 0.65^{bc}
	50	0.00 ± 0.00	0.50 ± 0.50^{a}	3.5 ± 1.18^{ab}
	100	0.00 ± 0.00	1.00 ± 0.64^{a}	6.00 ± 1.07^{a}
	$F_{df\ (3,28)}$	-	1.35	7.99
	P	-	0.28	0.001

Egg hatchability %			
Conc.%	*T. urticae* (Means ± SE)		*N. zaheri* (Means ±SE)
	6th day	10th day	3rd day
12.5	94.00 ± 1.41^{b}	98.00 ± 0.63^{ab}	100.00 ± 0.00
25	83.60 ± 1.72^{c}	97.20 ± 1.02^{b}	100.00 ± 0.00
50	74.00 ± 1.41^{d}	94.80 ± 0.80^{c}	100.00 ± 0.00
100	70.00 ± 1.41^{e}	90.40 ± 0.75^{d}	100.00 ± 0.00
Control	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00
$F_{df\ (4,20)}$	90.91	25.67	
P	0.000	0.000	

Means (±SE) values with different letters within the same hour are significantly different at $P \leq 0.05$.

2.2. Population density of *T. urticae* (active stages and eggs) on *L. sativa* leaves post- *L. sphaericus* crude extract treatments

Data in Tables (2) showed a significant decrease in the mean number of *T. urticae* movable stages by treat 1 and treat 2 on leaves compared to that of control at all indicated times ($P \leq 0.05$). The highest decrease of *T. urticae* movable stages was observed at 3rd (2.20 ± 0.66) and 7th days (3.40 ± 0.60) by treat 2 and treat 1, respectively. Similarly, in decreasing the mean number of *T. urticae* eggs as of what observed in movable stages in comparable with control.

Treat 2 showed a highest decrease in eggs number (3.40 ± 0.93) after 1st day compared to that of another treatment (8.60 ± 1.03) and control (16.60 ± 1.21).

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Table 3: Mean number of *T. urticae* eggs and movable stages post- *L. sphaericus* crude extract treatments at indicated days on Lettuce (*Lactuca sativa* var. *romana* L.)

Treats	Indicated time	Mean number ± SE/ leaf				
		1st day	3rd day	7th day	10th day	16th day
T. urticae eggs						
Treat 1 (*T. urticae* + *Ls* CE)		8.60 ± 1.03^{b}	14.60 ± 1.03^{b}	7.00 ± 0.71^{b}	21.00 ± 3.74^{b}	35.20 ± 2.87^{b}
Treat 2 (*Ls* CE + *T. urticae*)		3.40 ± 0.93^{c}	16.20 ± 1.66^{b}	17.60 ± 2.71^{b}	23.40 ± 2.98^{b}	40.40 ± 3.68^{b}
Control		16.60 ± 1.21^{a}	55.00 ± 4.28^{a}	84.80 ± 7.42^{a}	88.00 ± 11.24^{a}	72.40 ± 8.03^{a}
$F_{df=\ (2,12)}$		39.24	71.04	84.90	29.06	14.14

P	0.000	0.000	0.000	0.000	0.001
T. urticae movable stages					
Treat 1 (*T. urticae* + *Ls* CE)	8.80 ± 0.66[a]	6.80 ± 0.86[b]	3.40 ± 0.60[b]	6.00 ± 0.71[b]	13.40 ± 0.93[b]
Treat 2 (*Ls* CE + *T. urticae*)	5.40 ± 1.03[b]	2.20 ± 0.66[c]	5.20 ± 0.86[b]	15.00 ± 1.61[a]	25.60 ± 2.99[a]
Control	10.60 ± 0.51[a]	11.80 ± 1.07[a]	16.20 ± 1.98[a]	19.20 ± 2.06[a]	27.40 ± 3.61[a]
F df= (2,12)	11.89	29.81	28.58	18.59	7.61
P	0.001	0.000	0.000	0.000	0.007
Different letters within each day denote to a significant difference among treatments.					

2.3. Mortality and reduction percentages of *T. urticae* stages on Lettuce post- *L. sphaericus* crude extract treatments

Table (3) clarified that the treat 2 showed a significant reduction reached 81.74 %, 77.24% for movable stages and eggs at 3rd and 1st days, respectively. Treat 1 showed a considerable reduction of movable stages (79.44%) and eggs (90.83%) after 7 days post-treatment. Regarding to the mortality of movable stages, treat 1 showed the highest effect and the highly percentage was 41.18 after 7th day.

Table 4: Mortality and reduction percentages of *T. urticae* stages post- *L. sphaericus* crude extract treatments at indicated days on *L. sativa*

Indicated times	Reduction % at checked days /leaf				Mortality %	
	Movable stages		Eggs		Movable stages	
	***T. urticae* + *Ls* CE**	***Ls* CE + *T. urticae***	***T. urticae* + *Ls* CE**	***Ls* CE + *T. urticae***	***T. urticae* + *Ls* CE**	***Ls* CE + *T. urticae***
1st day	18.68	50.10	42.44	77.24	13.64	7.41
3rd day	43.55	81.74	70.51	67.27	29.41	18.18
7th day	79.44	68.65	90.83	76.94	41.18	4.00
10th day	69.39	23.47	73.48	70.45	16.67	0.78
16th day	45.66	8.48	45.98	38.00	0.00	0.00

2.4. The impact of the prey mite mediated *L. sphaericus* crude extract effects on consumption and fecundity of *N. zaheri* females

Consumption and fecundity rates of *N. zaheri* predatory females have been estimated at probability level of $P \leq 0.05$. There were no significant differences in the consumption rate between *Ls* CE treat 1 = 13.50 ± 0.52 protonymphs/female/10 days (F = 5.30, df (14, 102), P = 0.000) and *Ls* CE treat 2 = 13.40 ± 0.42 protonymphs/female/10 days (F = 4.60, df (14, 121), P = 0.000). While, differences were detected in the comparison with control (mean = 16.65 ± 0.27 protonymphs/female/10 days, F = 2.50, df (14, 109), P = 0.004) (Figure 2 a.i).

Similarly, fecundity rates were almost the same in both experimental sets, where *Ls* CE treat 1 = 1.39 ± 0.08 eggs/female/10 days (F = 8.33, df (14, 102), P = 0.000) and *Ls* CE treat 2 = 1.41 ± 0.09 eggs/female/10 days (F = 5.81, df (14, 121), P = 0.000). Differences were recorded in comparison with control which was 1.79 ± 0.07 eggs/female/10 days (F = 6.98, df (14, 110), P = 0.000) (Figure 2 a.ii). Figure 2b shows the mortality to survival percentages of *N. zaheri* females after the indirect (*Ls* CE set 1) and direct (*Ls* CE Set 2) exposure within and after the experimental duration. Besides, the bacterial effects on set 1 included female escaping from the arenas and no egg deposited (uncalculated data).

3. Molecular characterization

3.1. Molecular depiction of *T. urticae* and *N. zaheri*

Molecular description of *T. urticae* has been conducted using ISSR primers ISSR HB12, ISSR HB09, ISSR-17899B ISSR-17899A and ISSR-17898B. ISSR analysis profile has resulted in five ISSR band patterns (Fig. 3a) which produced a total of 63 markers, 34 bands of them were polymorphic with unique bands or unique bands displayed 51% polymorphism (Table 4). While, the molecular description of *N. zaheri* has been carried out using ISSR primers ISSR HB12, ISSR-17899A ISSR-17899B and ISSR-17898A. The ISSR analysis profile resulted in four band patterns (Fig. 3b). The ISSR primers produced a total of 46 markers, 8 bands of them were polymorphic with unique bands or unique bands displayed 16% polymorphism (Table 4).

The highest number of total bands were displayed in *T. urticae* control and *T. urticae* + *Ls* CE (T+B) treatments, with 50 while 42 were displayed control. The number of polymorphic with unique bands or unique were 13 and 21 bands were appeared in control and treatment respectively (Table 5). While, the highest number of total and polymorphic with unique bands were displayed in control *N. zaheri* with 43 and 5 bands compared with *N. zaheri* treated with *L. sphaericus* (N+ B) were 41 and 3 respectively (Table 5).

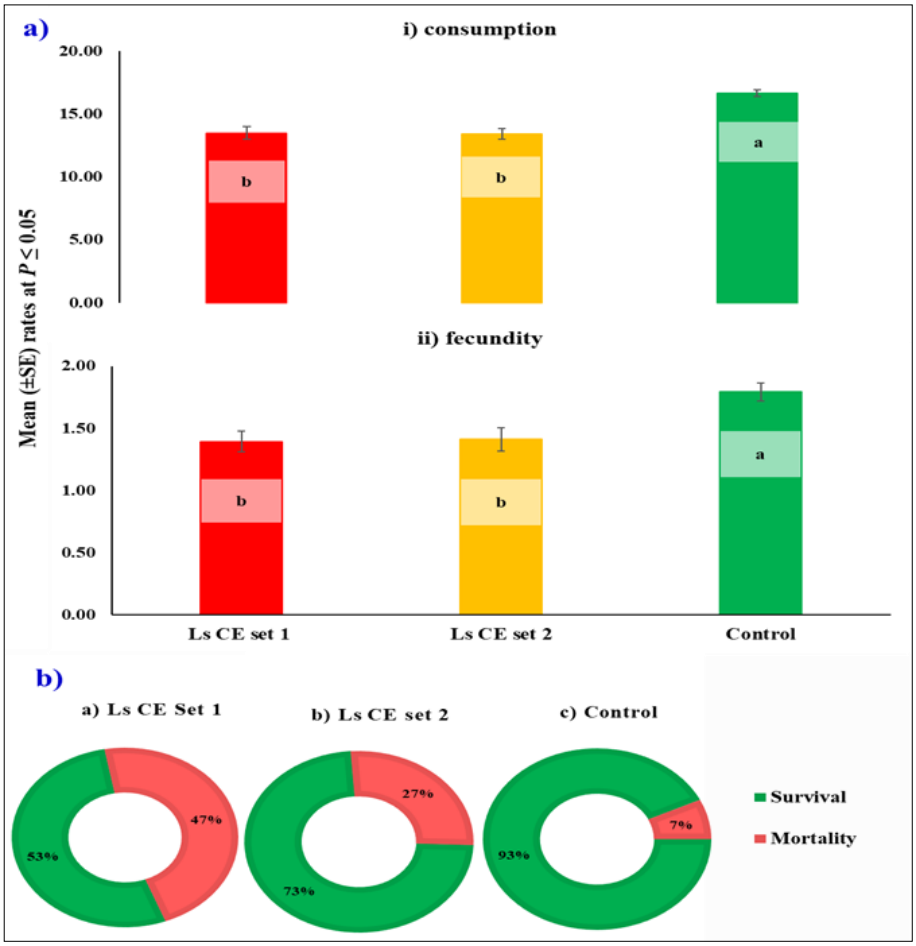

Fig 2: Neoseiulus zaheri females mean (± SE) a.i) consumption and a.ii) fecundity rates after Ls CE treatments during the experimental period of 10 days at P ≤ 0.05, and b) female survival and mortality percentages after Ls CE treatments during the experimental period of 10 days at P ≤ 0.05.

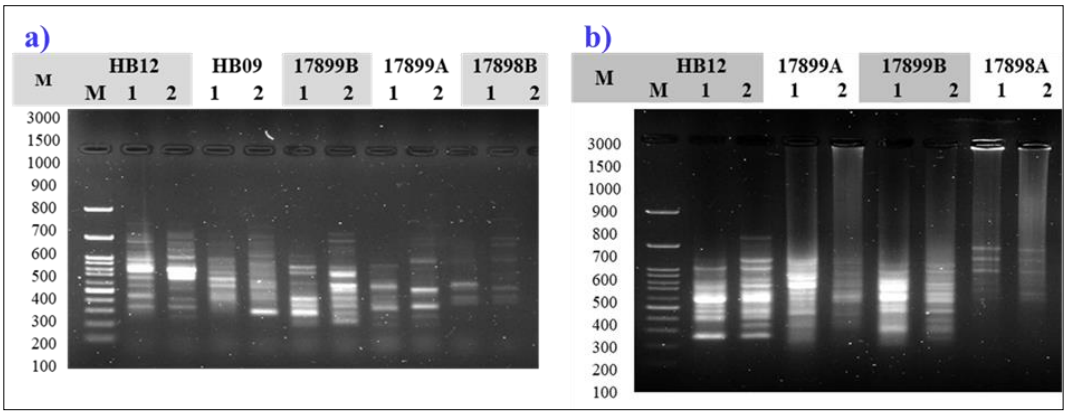

Fig 3: Inter-Simple Sequence Repeats (ISSR) amplification pattern obtained of the a) five ISSR patterns of *T. urticae* control and *T. urticae* + *Ls* CE (T+B) samples; primers were HB09, HB12, ISSR-17899B ISSR-17899A and ISSR-17898B, and b) four ISSR patterns of *N. zaheri* control and *N. zaheri* and *Ls* CE (N+B) samples; primers were HB12, ISSR-17899A ISSR-17899B and ISSR-17898A.

Table 5: Band variation and polymorphism percentage in *T. urticae* and *N. zaheri* control and treated with *L. sphaericus* crude extract using ISSR primers.

ISSR Primers	Total Bands	Molecular Size (bp)	Number of Monomorphic	Polymorphic bands or unique bands	Polymorphism %
T. urticae					
HB09	11	1509-504 bp	7	4	36%
HB12	18	1986-292 bp	6	12	66%
17899B	12	1652-286 bp	7	5	41%
17899A	13	1467-252bp	5	8	61%
17898B	9	2205-395bp	4	5	55%

Total	63		29	34	51%
N. zaheri					
HB12	12	177-287	9	3	25%
17899A	13	122-273	11	2	15%
17899B	12	1089-274	10	2	16%
17898A	9	1512- 594	8	1	11%
Total	46		38	8	16%

Table 6: The total and polymorphic and unique bands of ISSR primers produced from each primer for all amplified fragments from *T. urticae* and *N. zaheri* control and treatments of *T. urticae* + *L. sphaericus* CE and *N. zaheri* + *L. sphaericus* CE

T. urticae						
Total Bands						
Genotypes	HB12	HB09	17899B	17899A	17898B	Total
Control	13	8	9	7	5	42
T. urticae + Ls CE	11	10	10	11	8	50
Number of Polymorphic with Unique Bands or Unique Bands						
Genotypes	HB12	HB09	17899B	17899A	17898B	Total
Control	7	1	2	2	1	13
T. urticae + Ls CE	5	3	3	6	4	21
N. zaheri						
Total Bands						
Genotypes	HB12	17899A	17899B	17898A	Total	
Control	10	13	11	9	43	
N. zaheri + Ls CE	11	11	11	8	41	
Number of Polymorphic with Unique Bands or Unique Bands						
Genotypes	HB12	17899A	17899B	17898A	Total	
Control	1	2	1	1	5	
N. zaheri + Ls CE	2	0	1	0	3	

Discussion

The present study concluded that the solid growth media wheat bran and fodder yeast were the best two for the production of alkaline protease from *L. sphaericus* crude extract. It was found that the production of alkaline protease tripled compared to using fodder yeast in the submerged fermentation, from 718 units (Afify *et al*., 2009) [3] 0to more than 20,000 units (current study). Similarly, Zhang *et al*., (2023) [50] indicated that the enzymatic activity of alkaline protease for the BL10-aprE strain increased from 8491.23 units /ml to 15435.1 units /ml, when using the optimal growth environment. Also, Fashola *et al.*, (2021) [16] found that enzyme activity was 6848.171 units/ml, which confirming that *Bacillus subtilis* C3a-FIIRO can produce sustainable alkaline protease at industrial scale. Moreover, Roshdy *et al.*, (2023) [36] reported that *Bacillus thuringiensis dendrolimus* IP 4A/4B had the best alkaline protease production of 269 U/mL/min when fermented wheat bran under solid state fermentation conditions.

The increase in the productivity of metabolites in the wheat bran growth medium may be due to its rich components of various nutrients, vitamins and minerals (Teng *et al.*, 2017) [45]. Adopt using waste of agricultural industries, like wheat bran for microbial growth is considered economic more than synthetic one (Fayad *et al*., 2022) [17]. The present study revealed that, *Ls* CE showed toxic effect against *T. urticae* with highly mortality percentages and little toxic effect on its predatory mite, *N. zaheri*, based on concentrations and exposure times. *Bacillus* group has been reported to have highly acaricidal activity against *T. urticae* (Emam, 2021; Al-Azzazy *et al*., 2020) [4, 15].

Also in the current study, *L. sphaericus* crude extract resulted in reduction percentages of *T. urticae* active stage and eggs (79.44% and 90.83%) in direct trial and (68.65% and 76.94%) in the indirect trials, respectively after 7 days. Similarly, *Bacillus subtilis* and *B. qassimus* resulted in *T. urticae* reduction (72.22% and 70.74 %), respectively, after 7 days of direct exposure (Al-Azzazy *et al*., 2020) [4]. Also, the foliar application using *B. amyloliquefaciens* and *L. sphaericus* against *T. urticae* infestations resulted in a reduction percentage of about 37% (Abou Zaid *et al*., 2018) [1]. Again, in the present study, direct spray led to lower mortality rates of the *N. zaheri* females (27%) comparing to high mortality rates at indirect exposure (47%), which reflects the *L. sphaericus* effects on the predatory digestion system (Berry, 2012) [8]. Similarly, low side effects on the predatory mite, *Phytoseius plumifer* (Canestrini & Fanzago) have been recorded by *B. subtilis*, where the mortality after 7 days of exposure was 13.56% and *B. qassimus* was 12.87% (Al-Azzazy *et al*., 2020) [4]. Furthermore, indirect spray of *B. thuringiensis* led to lower toxicity than direct spray on the adult females of *Euseius scutalis* Athias-Henriot (Awad *et al*., 2020) [5].

Different metabolism and mode of entry of the crude extract by *T. urticae* and its predatory phytoseiid mite could account for variations in the toxicity levels between the two species (Dogan *et al*., 2017) [12]. Moreover, the lethal activity of *L. sphaericus* may depend on hetero-dimer powerful binary toxic crystals that produced during its sporulation phase and other toxins (Mtx) during vegetative growth. These toxins are existed in the midgut of the host and together are responsible for pesticidal activity, killing cells, retarding growth, and decreasing fecundity (Charles *et al*., 1996; Berry, 2012) [8, 9]. Correspondingly, β-exotoxin dimers of *B. thuringiensis* have been reported to cause high mortality in the adult stages of the citrus red mite, *Panonychus citri* (McGregor) (Al-Azzazy *et al*., 2020) [4].

Furthermore, Abou Zaid *et al*., (2018) [1] found that gas chromatography mass spectrometry (GC/MS) analysis contained the most dominant organic compound of *L.*

sphaericus and *B. amyloliquefaciens* crude extracts was Bis(2-propylpentyl) phthalate ($C_{24}H_{38}O_4$) which known as di(2-ethylhexyl) phthalate or DEHP. Consequently, miticidal effects of *L. sphaericus* and *B. amyloliquefaciens* crude extracts against *T. urtica* have been resulted due to DEHP antilarval activities (Abou Zaid *et al*., 2018) [1].

A genetic diversity was conducted between *T. urticae* control and (*T. urticae* + *Ls* CE) treatments, as well as between *N. zaheri* control and (*N. zaheri* + *Ls* CE) using the ISSR markers. ISSR analysis displayed 51% polymorphism in *T. urticae* treatments, and 16% polymorphism in *N. zaheri* treatments. Similarly, ISSR has been used in the genetic diversity of white-backed planthopper, *Sogatella furcifera* (Hemiptera: Delphacidae) (Liu *et al*., 2010) [26], *Heterorhabditis* sp. isolates (Khashaba and Abd El Azim, 2018), *Oryzaephilus surinamensis* L. (Coleoptera: Silvanidae) (Nilly *et al*., 2021) [30], *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) (Sabit *et al*., 2021) [39], and different *Aspergillus* strains (Salim *et al*., 2019) [40].

Conclusions

ISSR genetic markers confirmed the highly potentiality of *L. sphaericus* against *T. urticae* and lower side effects to the predatory females, *N. zaheri.* The current findings support using wheat bran medium as a growth medium for alkaline protease production. *Lysinibacillus sphaericus* crude extract is a promising method that would be recommended in the future biological control applications.

Conflict of interest

Authors declare there is no conflict of interest**.**

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