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Esterase and catalase isozymes of temperature tolerant *Steinernema* isolates recovered from Meerut and Ghaziabad region of western Uttar Pradesh, India

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Abstract
In the present study, we undertook laboratory investigations on the effect of temperature exposure (ranging from 5-45 ^{0}C for 72 h treatment) on survival and infectivity of isolates of *Steinernema* species, which were recovered from Meerut and Ghaziabad regions of Western Uttar Pradesh, India. The study was conducted to find out the temperature tolerant isolate, which can withstand the semi-arid and sub-tropical climatic conditions of western Uttar Pradesh. Biochemical characterization of 4 temperature tolerant isolates of *Steinernema* was undertaken, on the basis of isozymic profiles of esterase and catalase. The isozymic patterns of esterase of 4 isolates of *Steinernema* species by mini slab gel polyacrylamide electrophoresis revealed species specific enzyme phenotypes for CCS-EPN-14S, CCS-EPN-30S and CCS-EPN-53S characterized by having two, one and three species-specific bands respectively. Isozyme profiles of catalase also revealed species-specific phenotypes with one band at Rf 0.24 for the strain CCS-EPN-*13S,* at Rf 0.31 for the strain CCS-EPN-14S, at Rf 0.29 for strain CCS-EPN-30S and at Rf 0.34 for the strain CCS-EPN-53S.

Keywords: *Steinernema*, temperature tolerant, species specific, isozymic profiles

1. Introduction

Entomopathogenic nematodes (EPNs) are soft bodied, non-segmented roundworms that are obligate or sometimes facultative parasites of insects. They occur naturally in soil environments and locate their host in response to carbon dioxide and other chemical cues [12]. They form a complex nematode-bacterium mutualistic symbiosis. The bacteria are carried in the body of entomopathogenic nematodes and vectored onto hosts [18]. The proliferation of the bacteria causes septicemia in the insect. Entomopathogenic nematodes are used to control several agriculturally important insect pests of different orders. According to the Global Nematicide Market Report (2014-2019), nematicide market is expected to grow 1329.5 million dollar by 2019 by virtue of a healthy compound annual growth rate (CAGR) of 3.2% between 2014 and 2019. Considerable progress has been made during the last 20 years on the subject dealing with taxonomy, biology, genetics, ecology, host range, production, application technologies, laboratory and field trials and commercialisation of EPNs and their symbiotic bacteria [4]. The genus *Steinernema* Travassos, 1927 is the most intensively studied group of entomopathogenic nematodes. Temperature affects the dispersal, infectivity, reproduction and development of entomopathogenic nematodes [17, 13, 8, 7, 16, 20]. Semi-arid and sub-tropical climatic conditions persist in western Uttar Pradesh, where the temperature varies between 28-40 ^{0}C in summer and 5-25 ^{0}C in winter. The present study was conducted to find out the temperature tolerant isolate, which can withstand these temperature extremes and work efficiently.

The taxonomic study of these nematodes involves morphological, biological, biochemical and molecular parameters [10]. Although DNA based technologies such as RFLPs and base sequencing of the entire ITS region of rDNA, have been successfully applied for distinguishing species of *Steinernema*, they remain of limited use for large surveys because they are costly and time consuming procedures [15]. Instead, isozyme patterns may prove to be an excellent tool for the identification of newly isolated strains of *Steinernema* species during routine surveys. The present investigation was undertaken to know the isozymic profiles of esterase (EST, E. C. 3.1.1.1) and catalase (CAT, E. C. 1.11.1.6) of four temperature tolerant isolates of *Steinernema* to differentiate the species.

2. Materials and Methods
The experiments were carried out at Nematology Laboratory, Department of Zoology, Chaudhary Charan Singh University, Meerut, U P from October 2011 to November 2014.

2.1 Nematode culture
Isolates of third stage juveniles (Only free living stage and Infective in nature) of entomopathogenic nematodes were obtained by baiting of soil samples taken from different agricultural sites of Meerut and Ghaziabad region of Western Uttar Pradesh. All these local isolates were cultured on fully grown *Galleria mellonella* larvae as per the procedure of [23]. Freshly emerged infective juveniles were surface sterilized in 0.1% hyamine solution and stored in distilled water in tissue culture flasks. Up to one week old culture was used in the experiments.

2.2 Bioassay to test the temperature tolerance
To test the temperature tolerance, the final instar larvae of *G. mellonella* were exposed to 100 third stage juveniles (Infective stage) of each isolate in a 6 well plate, having one larva in each well. Each isolate was replicated 10 times with 5 replications of control in each treatment. The plates were incubated in biological oxygen demand incubator at 5 different temperature regimes viz. 5, 15, 25, 35 and 45 ^{0}C for 72 h. These temperatures were chosen because in western Uttar Pradesh, the temperature varies between 28-40 ^{0}C in summer and 5-25 ^{0}C in winter. The standard test duration for the nematode bioassay is a period of 24 h. The testing period can be extended to 72 h for more authentic results. Observations on their mortality were recorded at 6 hrs interval for 72 h. Dead larvae of test insects were removed from each well and kept on a White trap [22] at 5, 15, 25, 35 and 45 ^{0}C in the BOD incubator to observe the emergence of entomopathogenic nematodes from the body of dead insect. Statistical analysis of temperature tolerance of entomopathogenic nematodes was done by ANOVA. The two way repeated measure ANOVA analysis for all these nematode isolates at five exposed temperatures were analyzed through SPSS.

2.3 Sample preparation, electrophoresis and isozyme visualization of temperature tolerant isolates
About 5,000 infective juveniles in 1 ml of suspension were put in 1.5 ml eppendorf tubes and centrifuged at 2,147 g for 5 min. The supernatant was discarded and the pellets were homogenized with 40 ul of extraction buffer containing 20% glycerol and 2% triton-X-100 [5]. Additional extraction medium was added to increase the total volume of crude samples to about 100 ul and centrifuged at 3,354 *g* for 10 min at 4 ^{0}C. The clear supernatant was introduced immediately into the electrophoretic cell or stored at -80 ^{0}C until use [15]. The isozymes of infective juveniles were separated by native polyacrylamide electrophoresis in a mini slab gel apparatus. 5% acrylamide stacking gel and 7% acrylamide separation gel were used. About 25 ul of the samples were loaded into the wells of the gel. The electric potential was set at 35 volts for the first 40-45 min to allow stratification of proteins in the sample according to their relative charge. Afterwards, voltage was increased to 150 volts until the marker dye moved to the lower end of the gel. Esterase activity was determined by incubating the gel at 37 ^{0}C for 1 h in a freshly prepared solution of fast blue RR salt (15 mg), EDTA (7.5 g) dissolved in 25 ml of 0.1 M sodium phosphate buffer pH 7.1 and naphthyl acetate. Stain solution was poured off when the enzyme bands were sufficiently visible. Distilled water was added to wash the gel from any remaining stain solution [9]. Catalase activity was determined by chilling the gel in a dish resting on a bed of ice for 10 min followed by treatment with 0.01% solution of hydrogen peroxide. The gel was rinsed with two changes of distilled water and covered with stain solution (0.5% potassium ferricyanide + 0.5% ferric chloride) for 1-2 min. Gel was again rinsed with distilled water and stored in 7% acetic acid. Regions of catalase activity appeared as clear areas on a blue- green background [9]. The stained gels were dried, scanned by means of a scan jet (Hewlett Packard), analyzed as digital images and then printed on photo quality paper. The species specific enzyme phenotypes were named with capital letter of the respective species followed by the number of isozymes, while non-specific enzyme phenotypes were labeled as 'N' followed by the number of isozymes. For each phenotype, the presence of a band (1) or its absence (0) was scored. Cluster analysis was conducted using NTSYS software and UPGMA method based on Jaccard's similarity matrix.

3. Results and Discussion
The infective juveniles of indigenous populations of *Steinernema* sp. were found efficacious at all the studied temperature regimes except 5 ^{0}C. Temperature range 25-35 ^{0}C was found best for the nematode pathogenicity against *G. mellonella* as infective juveniles killed maximum number of *G. mellonella* larva within this range. The overwhelming results with respect to high mortality were obtained with four nematode isolates 13S, 14S, 30S from Meerut region and 53S from Ghaziabad region (Table 1, Figure 1).

Table 1: Susceptibility of test insect by different nematode isolates at exposed temperatures.

Sr. No.	Isolate	Percent mortality of insect (mean±SE) at different temp (^{0}C) exposure				
		5 ^{0}C	15 ^{0}C	25 ^{0}C	35 ^{0}C	45 ^{0}C
1	5S	-----------	-----------	27.5±8.0	56.7±10.4	-----------
2	7S	-----------	-----------	-----------	44.2±8.7	16.7±2.24
3	12S	---------	5.83±1.5	-----------	28.3±5.5	------------
4	13S	---------	16.7±5.9	66.7±8.99	15.0±2.61	13.3±5.7
5	14S	---------	20.8±7.4	83.3±11.2	43.3±8.5	16.7±5.9
6	21S	---------	----------	2.50±1.31	44.4±7.8	----------
7	28S	---------	-----------	45.0±7.0	39.2±8.4	----------
8	30S	---------	9.2±4.2	57.5±12.9	65.0±13.1	-----------
9	35S	---------	-----------	----------	4.42±0.86	8.2±0.74
10	37S	---------	13.3±5.7	32.5±9.9	47.5±8.2	-----------
11	46S	---------	2.5±1.3	13.3±5.7	26.7±4.8	-----------
12	51S	-----------	-----------	1.25±0.3	3.67±0.8	-----------
13	52S	-----------	-----------	1.67±0.48	2.50±0.78	-----------
14	53S	-----------	0.92±0.26	3.50±0.76	3.08±0.87	-----------

*Mean value of 10 insects treated with 100 IJs irrespective of exposure time

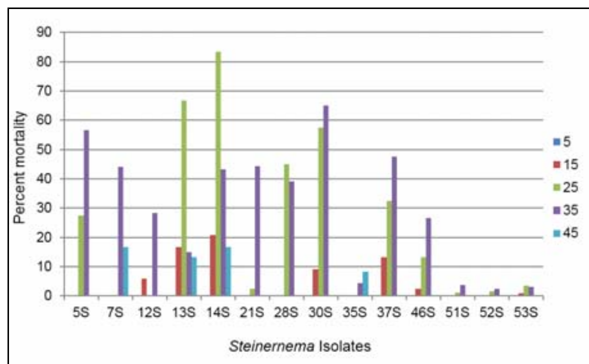

Fig 1: Susceptibility of test insect by *Steinernema* isolates at five different temperatures**.**

Isozymic patterns of esterase (EST) and catalase (CAT) showed polymorphism, which could differentiate all the three CCS-EPN-*13S,* CCS-EPN-14S, CCS-EPN-30S and species/ strains of *Steinernema* of Meerut and one CCS-EPN-53S from Ghaziabad region. EST patterns (Figure 2) showed good polymorphism among *Steinernema* species. Strain CCS-EPN-*13S* exhibited four distinct bands at Rf 0.41, 0.47, 0.76 and 0.83. Strain CCS-EPN-14S possessed two species specific bands at Rf 0.29, and 0.44. Strain CCS-EPN-30S showed one species specific bands of Rf 0.59 and rest two bands at Rf 0.29 and 0.85. Strains CCS-EPN-53S exhibited three bands of Rf values 0.35, 0.58, 0.85 but had one unique band at Rf 0.28. Isozyme profiles of all the four strains of CAT (Figure 3) revealed species-specific phenotypes with one band at Rf 0.24 for the strains CCS-EPN-*13S,* at Rf 0.31 for the strainCCS-EPN-14S, at Rf 0.29 for strain CCS-EPN-30S and at Rf 0.34 for the strain CCS-EPN-53S.

The dendrogram obtained from hierarchical cluster analysis based on esterase and catalase isozymes (Figure 4) placed strains CCS-EPN-14S,CCS-EPN-30S and CCS-EPN-53S in one cluster (Cluster-I) showing 71% similarity between CCS-EPN-14S and CCS-EPN-30S and both of these showed 50 % similarity with CCS- EPN-53S and 36 % similarity between fourth strain CCS- EPN-*13S* which form Cluster-II. The present study has demonstrated the utility of esterase and catalase isozymes as diagnostic markers for differentiating 4 strains/species of *Steinernema*, as is evident from the species-specific enzyme phenotypes obtained for almost all the strains studied using both the enzymes. This is in conformity with the earlier findings of Sha and Artyukhovsky [19, 3], who differentiated the species based upon enzyme phenotypes. These findings are also in accordance with the work done by Ganguly *et al.* [6] who also reported the utility of catalase and esterase isozymic profiles for differentiating 6 strains/species of *Steinernema*.

The results of the temperature bio-assay in the present study widens our knowledge of the impact of the temperature and show how it might be possible to use these findings and implement them into the practical use of EPNs. Enzyme electrophoresis has proved to be a useful taxonomic tool for nematode species and even strains and also for the bacterial associates of entomopathogenic nematodes. Starch gel [1], polyacrylamide gel [19, 14] and cellulose acetate gel [11] electrophoresis were applied in nematode taxonomy. Phenotypes of isoenzymes such as esterase (EST), malate dehydrogenase (MDH), glucose oxidase (GOD), superoxide dismutase (SOD) and isocitrate dehydrogenase (IDH) have been used to identify and differentiate various nematodes until now [2, 24].

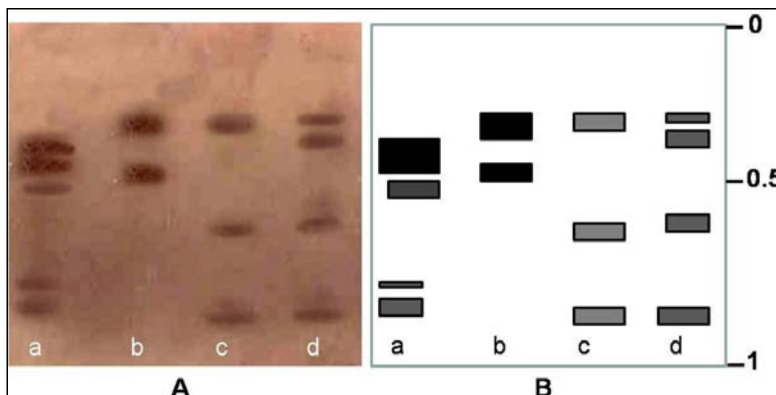

Fig 2: Esterase isozyme profiles (A) and their enzyme phenotypes (B), of infective juveniles of four Indian species of *Steinernema*. a: CCS-EPN-*13S*; b: CCS-EPN-*14S*; c: CCS-EPN-*30S*; d: CCS-EPN-*53S.*

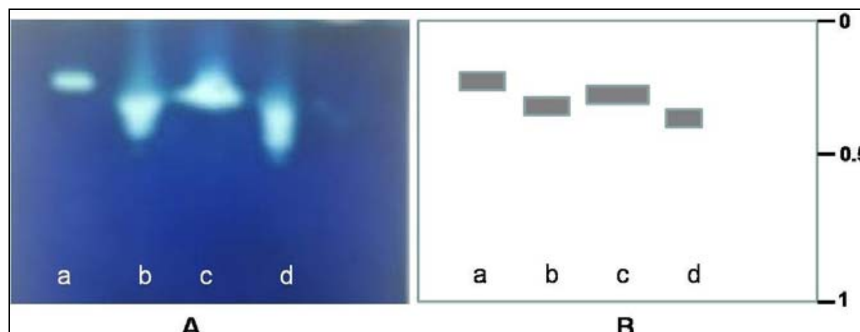

Fig 3: Catalase isozyme profiles (A) and their enzyme phenotypes (B), of infective juveniles of four Indian species of *Steinernema*. a: CCS-EPN-*13S*; b: CCS-EPN-*14S*; c: CCS-EPN-*30S*; d: CCS-EPN-*53S.*

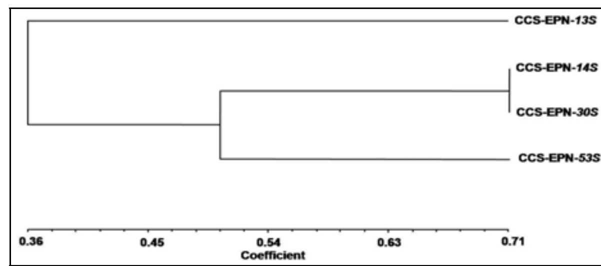

Fig 4: Dendrogram showing the inter-relationships among the *Steinernema* species based upon esterase and catalase isozymic profiles.

The present study has demonstrated the utility of esterase and catalase isozymes as diagnostic markers for differentiating four strains/species of *Steinernema*, as is evident from the species-specific enzyme phenotypes obtained for almost all the strains studied using both the enzymes. This is in conformity with the earlier findings of Sha and Artyukhovsky *et al.* [19, 3] who differentiated the species based upon enzyme phenotypes. These findings are also in accordance with the work done by Ganguly and Pandey [6]. who also reported the utility of catalase and esterase isozymic profiles for differentiating six strains/species of *Steinernema*. Further studies on isozymic profiles of several populations of *Steinernema* comprising different species and strains, will yield useful information for the revalidation of these diagnostic markers.

Keeping in view the high biocontrol potential of *Steinernema* species, their strains are continuously being isolated from different parts of the world and several strains are already in queue waiting for identification. Combination of esterase and catalse isozymic profiles from infective juveniles, supplemented with morphological details, can be useful for preliminary screening and differentiation of *Steinernema* species.

Conclusively, further studies on host range of these strains will be of immense value for exploiting their biocontrol potential against specific insect pests prevailing in their respective niche agro-climatic zones.

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