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Laboratory Evaluation of Seven Pakistani Strains of Entomopathogenic Nematodes Against a Stored Grain Insect Pest, Pulse beetle *Callosobruchus chinensis* (L.)

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Abstract: Seven Pakistani strains of entomopathogenic nematodes belonging to the genera *Steinernema* and *Heterorhabditis* were tested against last instar and adult stages of the pulse beetle, *Callosobruchus chinensis* (L.). These nematodes included *Steinernema pakistanense* Shahina, Anis, Reid and Maqbool (Ham 10 strain); *S. asiaticum* Anis, Shahina, Reid and Rowe (211 strain); *S. abbasi* Elawad, Ahmad and Reid (507 strain); *S. siamkayai* Stock, Somsook and Reid (157 strain); *S. feltiae* Filipjev (A05 strains); *Heterorhabditis bacteriophora* Poinar (1743 strain); and *H. indica* Poinar, Karunakar and David (HAM-64 strain). Activity of all strains was determined at four different nematode densities in Petri dishes and in concrete containers. A significant nematode density effect was detected for all nematode species tested. Overall, *Heterorhabditis bacteriophora, S. siamkayai*, and *S. pakistanense* were among those that showed the highest virulence to pulse beetle larvae and adults. For all nematode species, the last larval stage of the pulse beetle seems to be more susceptible than the adult. LC_{50} values in Petri dish and concrete containers were 14-340 IJs/larvae and 41-441 IJs/larvae, respectively, and 59-1376 IJs/adult and 170-684/adult, respectively.

Key words: entomopathogenic nematode, *Callosobruchus chinensis, Steinernema, Heterorhabditis,* concrete containers.

Insect pests can damage stored grains by reducing dry weight, nutritional value, and seed viability (Sample et al., 1992). Pulses (Fabaceae [Leguminosae]) are important sources of protein, fats, carbohydrates, sugars and vitamin. B. The pulse beetle,(*Callosobruchus chinensis* (L.)) is the most serious pest of pulses in tropical and subtropical countries. They infest pulses both in the field and in storage, causing considerable economic losses (Srivastava and Pant, 1989; Ramzan et al., 1990). Field infestation by *C. chinensis* has been observed in *Vigna angularis* (Wild.) Ohwi and Ohashi, *Vigna radiata* (L.) Wilckez, *Lens culinaris* Medikus, and *Vigna unguiculata* (L.) Walp (Yamamoto, 1990; Yoshida, 1990; Raina, 1972; Hariri, 1981; Olubayo, 1993). In the field, infestation of *C. chinensis* is characterized by its insidious nature (Taylor, 1981). Eggs are typically glued on the pod as it dries and matures, the young first instars bore into seeds and, at threshing, seeds either show slight or no apparent external damage (Booker, 1967; Caswell, 1968; Southgate, 1978). Although infestation and damage in the field is generally low, such infestation has serious implications because the insects multiply very rapidly and consequently cause a high level of damage once the infested seeds are stored (Taylor, 1981).

For sound management of stored agriculture products, there is an increasing interest in biological control, which reduces negative impacts on the environment (Arbogast, 1984; Guedes, 1990; Brower et al., 1996). Entomopathogenic nematodes (genera: *Steinernema* and *Heterorhabditis*) possess tremendous potential as biological control agents of economically important insect pests (Georgis, 1990). These nematodes kill insects through mutualistic symbiosis with bacteria (*Xenorhabdus* and *Photorhabdus* spp for steinernematids and heterorhabditids, respectively). Infective juveniles (IJs), the only free-living stage, enter hosts through natural openings, such as the mouth, anus and spiracles or in some cases through the cuticle, and release bacteria that kill the host within 48 h (Poinar, 1990). The nematodes molt and complete up to three generations within the host after which IJs exit the cadaver to find new hosts (Kaya and Gaugler, 1993).

Entomopathogenic nematodes have been most successful at suppressing populations of soil–dwelling pests or pests in other protected environments e.g., greenhouses (Shapiro-Ilan et al., 2002). The nematodes require moisture to move and prevent desiccation. Thus, entomopathogenic nematodes may appear to be a poor fit for the dry stored-product environment, and as a result, applications of nematodes in stored product commodities have received very limited attention. Indeed, the application of nematodes to bulk grain bins or storage areas could be highly problematic due to environmental limitations. However, entomopathogenic nematodes may offer considerable potential for hidden refuge and outside spillage areas or product accumulations. Chemical insecticides are typically applied in an aqueous solution; if nematodes can be applied in a similar amount of liquid to provide sufficient moisture for the

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nematodes to find and infect the target insects, then they could be used as biological insecticides in these stored product situations (Ramos-Rodriquez et al., 2006, 2007).

There is no information available about efficacy of entomopathogenic nematodes against *C. chinensis*, but a few studies provided data on nematode efficacy against other stored-product insects such as the red flour beetle *Tribolium castaneum* (Herbst), Indian meal moth *Plodia interpunctella* (Hübner), Mediterranean flour moth *Ephestia kuehniella* (Zeller), sawthoothed grain beetle *Oryzaephilus surinamensis* (L.), yellow mealworm *Tenebrio molitor* (L.), and the warehouse beetle *Trogoderma variable* (Ballion) (Athanassiou et al., 2008; Morris, 1985; Ramos-Rodriguez et al., 2006). Mbata and Shapiro-Ilan (2005) also evaluated efficacy of various heterorhabditids against larvae and adult stage *P. interpunctella*, under laboratory conditions. Trdan et al. (2006) reported susceptibility of the young adult of granary weevil, *Sitophilus granaries* (L.), and the saw-toothed grain beetle, *O. surinamensis* (L.), to four entomopathogenic nematode species.

The present study was conducted to evaluate the virulence of seven Pakistani strains of entomopathogenic nematodes to adult and last instar *C. chinensis* in Petri dishes and in concrete containers. The concrete containers were used to mimic situations in field applications; such containers contain cracks or crevices that may be used as refugia by the target pest.

Materials and Methods

Insects: Callosobruchus chinensis were obtained from heavily infested black gram, *Vigna mungo* (L.). Insects were maintained in a 1000 ml glass jar containing 500 gm fresh black gram and incubated in a rearing cage (200 x 120 x 350) at 35 ± 2°C with 12:12 h day: night cycle.

Nematode culture: Entomopathogenic nematodes used in the experiments were *Steinernema pakistanense* Shahina, Anis, Reid and Maqbool (Ham 10 strain); *S. asiaticum* Anis, Shahina, Reid and Rowe (211 strain); *S. abbasi* Elawad, Ahmad and Reid (507 strain); *S. siamkayai* Stock, Somsook and Reid (157 strain); *S. feltiae* Filipjev (A05 strain); *Heterorhabditis indica* Poinar, Karunakar and David (HAM-64 strain) and *H. bacteriophora* Poinar (1743 strain) obtained from the storage unit of the National Nematological Research Center (NNRC) at the University of Karachi, Karachi, Pakistan. All nematodes were propagated in last instar *Galleria mellonella* (L.) using the method outlined by Dutky et al., (1974). Infective juveniles were collected in White traps (White, 1927), harvested, and stored in sterilized distilled water at 10-15°C for no more than two weeks before they were used.

Virulence in Petri dishes: Last instar (10-12 days from egg-hatch) and freshly emerged adult pulse beetles (emerged after 28 d from pupal case) were used at the time of inoculation. The experiment was conducted in Petri dishes (90 mm diameter) lined with Whatman No. 1 filter paper in the bottom. Each dish was inoculated with one of the seven nematode species using 50, 150, 250 and 350 IJs per insect in a 1 ml water suspension. Ten last instars and adults were collected from the colony and released separately in a Petri dish having 20 pulse grains as a food material. Control treatments received 1 ml water without nematodes. Applications were carried out using a micropipette. Pipette tips were changed after every treatment. Petri dishes were wrapped with Parafilm and kept in a chamber maintained at 30 ± 5°C. There were four replicates for each treatment combination (dose, insect, and nematode) and the experiment was carried out twice. After 48 hours, dead larvae and adults were individually transferred to White traps to evaluate nematode infectivity and emergence of nematode progeny.

Virulence in concrete containers: To mimic storage structure applications that could be made into refugia (cracks and crevices), concrete containers were made by mixing the sterilized crushed stone, sand, gravel, and cement in the ratio of 1:2:4:1. Material was mixed by hand on wood sheets with enough water to create consistency among the experimental units. A one inch deep base and wall layer was prepared by pouring the mixture in plastic containers (28 x 16 x 8 cm). The surface of the concrete was made smooth with a piece of glass rod. Containers were allowed to dry at 40-45°C. After 24 h, twenty individuals of each of last instars and adults were placed separately in concrete containers with 20 pulse grains as a food material. Four different densities of nematodes were applied: 2000 IJs (100 IJs/insect), 4000 IJs (200 IJs/insect), 6000 IJs (300 IJs/insect), and 8000 IJs (400 IJs/insect). Nematodes were applied in a 20 ml water suspension that was pipetted onto the concrete arena, while changing pipette tips after every treatment. Control treatments received 20 ml distilled water. There were four replicates for each treatment combination (dose, insect, and nematode) and the experiment was carried out twice. Concrete containers were covered with lids and incubated at 30 ± 5°C. Observations were taken after 72 h and each insect was transferred individually in White traps to evaluate nematode infectivity and emergence of nematode progeny.

Statistical analysis: The statistical design of experiments was completely randomized. Treatment effects were analyzed by multifactor analysis of variance (ANOVA); if the ANOVA was significant, differences in treatments were elucidated through Duncan's multiple range test ($P < 0.05$) using SPSS statistical software. Percentage data were arcsine of square root transformed. LC_{50} values were analyzed with probit analysis by using the PROC PROBIT routine of SAS 2000.

Results

Virulence in Petri dishes: Mortality in larval stages was significantly influenced by nematode density (ANOVA:

F = 95.77; df = 3, 96; $P < 0.001$), entomopathogenic nematode species (ANOVA: F = 168.53; df = 7.96; $P < 0.001$), and the interaction between these factors (ANOVA: F = 3.07; df = 21, 96; $P < 0.001$). In all nematode treatments the total mortality was significantly higher than the mortality in control treatment (Fig. 1A). At densities of 250 and 350 IJs/larva, *H. bacteriophora* (1743 strain) and *S. siamkayai* (157 strain) caused 100% mortality while *H. indica* (HAM-64 strain) and *S. pakistanense* (Ham 10 strain) exhibited 100% mortality only at the highest nematode density. At 150 IJs per insect, the virulence of *H. bacteriophora* was higher than all other nematode species except *S. siamkayai* and *S. pakistanense* (Fig. 1A). The least virulent nematode was *S. feltiae* (A05 strain). Generally, mortality of *C. chinensis* larvae was higher than that of adults. LC_{50} values calculated from probit analysis of all tested nematode strains are given in Table 1. The highest LC_{50} value is associated with *S. feltiae* (340 IJs /larva), and lowest with *H. bacteriophora* (14 IJs/larva).

The mortality in adult stages was also significantly influenced by nematode density (ANOVA: F = 63.19; df = 3, 96; $P < 0.001$), entomopathogenic nematode species (ANOVA: F = 79.72; df = 7, 96; $P < 0.001$), and the interaction between nematode density and nematode species (ANOVA: F = 1.82 df = 21, 96; $P < 0.001$). Among all nematode strains *H. bacteriophora*, *S. siamkayai*, *S. pakistanense*, and *H. indica* caused the highest mortality rate where as *S. feltiae* was the least virulent at all four densities (Fig. 2A). LC_{50} values calculated from probit analysis of

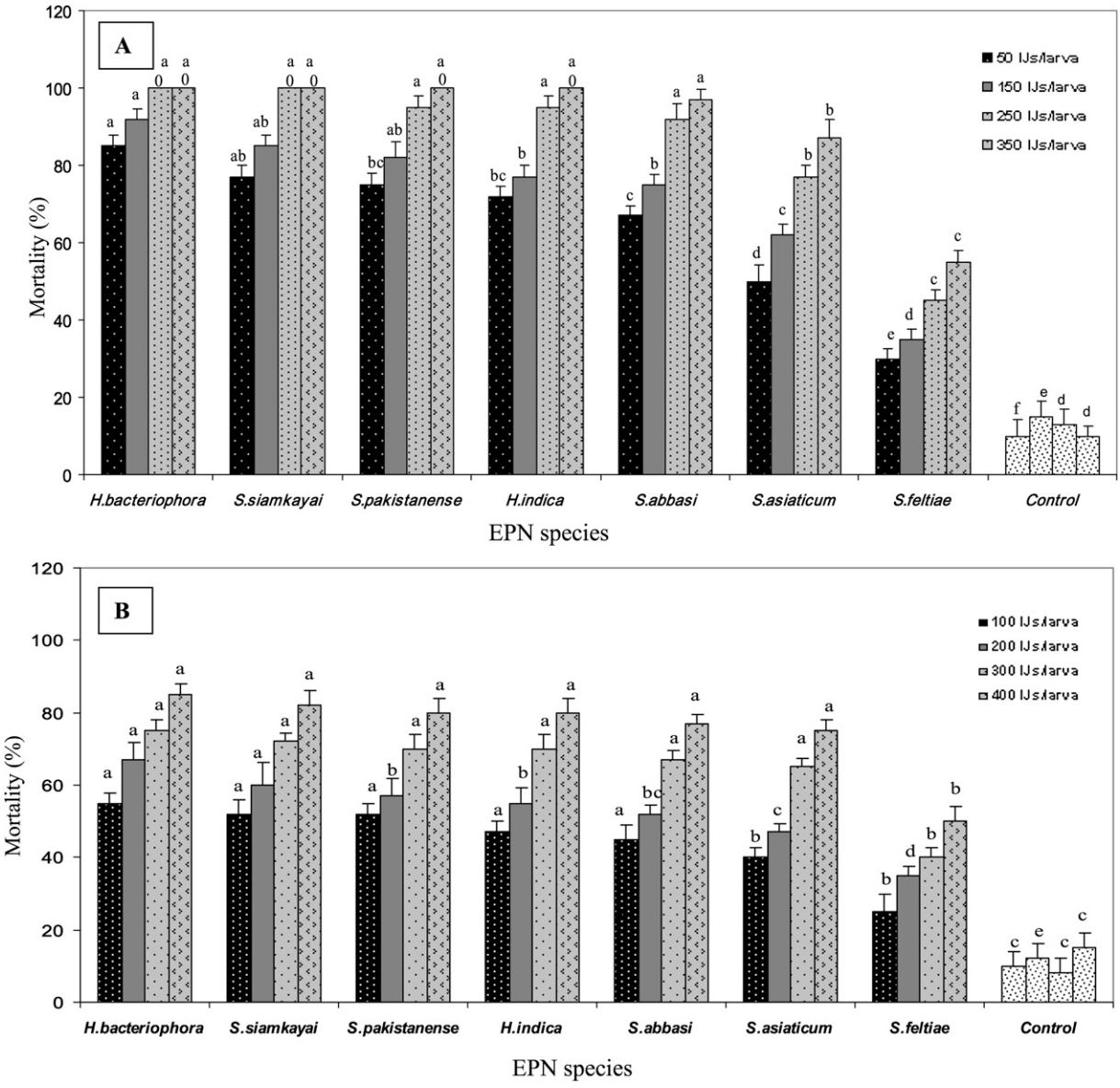

FIG. 1. **(A-B)-**: Larval mean mortality of *C.chinensis* treated with seven different species of entomopathogenic nematodes (EPN) in **A**. Petri dish and **B.** concrete container depending on dose concentration. Data were analyzed by multifactor ANOVA followed by Duncan's multiple range test ($P<0.05$) for separation of means. The same letters above are not significantly different and vertical lines represent standard errors of the mean.

TABLE 1. Effect of seven Pakistani strains of entomopathogenic nematodes (*Heterorhabditis* spp. and *Steinernema* spp.) on last instar and adult stage of *Callosobruchus chinensis* at different concentrations.

	Petri dish		Concrete container	
	LC_{50} (95% CL)		LC_{50} (95% CL)	
Nematode Species	Last instar larvae	Adult stage	Last instar larvae	Adult stage
H. bacteriophora	14.20 (0.49-31.80)	59.30 (16.59-95.58)	41.32 (2.87-79.12)	170.20 (56.52-512.55)
S. siamkayai	22.10 (2.38-39.15)	66.63 (16.82-108.1)	58.90 (10.41-100.19)	186.36 (65.4 – 529.57)
S. pakistanense	23.12 (4.19-42.75)	77.73 (16.27-128.5)	60.76 (6.53-107.17)	219.65 (75.60-637.77)
H. indica	25.36 (4.76-45.85)	81.10 (18.35-133.2)	70.90 (15.32-116.92)	230.30 (68.50-773.78)
S. abbasi	29.29 (5.92-52.72)	123.8 (56.9-197.74)	82.42 (21.01-133.63)	294.61 (216.53-511.3)
S. asiaticum	58.26 (18.51-92.20)	154.8 (82.6-271.90)	107.63 (45.26-167.01)	313.68 (234.37-530.9)
S. feltiae	340.84 (5.12-167.47)	1376.0(51.38-3686.0)	441.32 (226.36-23663.1)	684.99 (406.99-4029.13)

LC_{50} expressed as No. of IJs per insect.
Confidence limit, CL, are given in parenthesis.

all tested nematode strains are given in Table 1. The highest LC_{50} value is associated with *S. feltiae* (1376 IJs/adult) and the lowest with *H. bacteriophora* (59 IJs/adult). In White traps, all species of *Heterorhabditis* and *Steinernema* showed emergence of new generations from last instars and adult stage insects, which confirmed the suitability of nematode treatments in the target pest.

Virulence in concrete container: Statistical analysis of the larval stage in concrete containers showed that percentage mortality was significantly influenced by nematode density (ANOVA: F = 58.35; df = 3, 96; $P < 0.001$), nematode species (ANOVA: F = 108.13; df = 7.96; $P < 0.001$), and the interaction between nematode density and nematode species (ANOVA: F = 3.42; df = 21, 96; $P < 0.001$). Generally, mortality of the nematode treatment was higher than that of control treatment (Fig.1B). The most virulent strains were *H. bacteriophora* and *S. siamkayai*, followed by *H. indica* and *S. pakistanense* (Fig. 1B). The least virulent nematode was *S. feltiae* (Fig.1B). LC_{50} values calculated from probit analysis of all tested nematode strains are given in Table 1. The highest LC_{50} value is associated with *S. feltiae* (441 IJs/larva) and lowest with *H. bacteriophora* (41 IJs/larva).

For adult stages, significant effects were detected in dose concentrations (ANOVA: F = 175.13; df = 3, 96; $P < 0.001$), nematode species (ANOVA: F = 34.27; df = 7, 96; $P < 0.001$), and interaction between dose concentrations and nematode species (ANOVA: F = 4.08; df = 21, 96; $P < 0.001$). The effect of the nematode strain on adult mortality is shown in Fig. 2B. Mortality of adults was higher at 300 and 400 IJs/adult than at 100 and 200 IJs/adult. Among the most virulent nematode species were *H. bacteriophora*, *S. siamkayai*, *S. pakistanense*, and *H. indica* at highest nematode density, and at 300 IJs per insect, *H. bacteriophora and S. siamkayai* caused greater mortality than all other nematodes except *S. pakistanense*. *S. feltiae* caused the lowest mortality. LC_{50} values calculated from probit analysis of all tested nematode strains are given in Table 1. The highest LC_{50} value is associated with *S. feltiae* (684 IJs/adult) and lowest with *H. bacteriophora* (170 IJs/adult).

DISCUSSION

Both last larval instars and adult stage beetles of *Callosobruchus chinensis* are excellent targets for all tested nematode species since all nematodes caused significantly higher mortality than control treatments. *C. chinensis* is one of the most destructive insect pests of pulses in storage. According to Qayyum and Zafar, (1978) pulse beetles cause a maximum loss of 90% in gram. Attempts have been made by different scientists to biologically control *C. chinensis* with parasitoids (Aslam et al., 2006). Entomopathogenic nematodes have other attributes that may contribute to their successful use against stored product insects: (1) They can tolerate warm and cold conditions (Cabanillas et al., 1994), (2) they possess varying foraging strategies, i.e., they can seek out or ambush their hosts, and (3) they are currently produced commercially (Campbell and Gaugler, 1997).

Both stages of the insect were universally susceptible to six of the nematode species/strains: *S. pakistanense* (Ham 10 strain), *S. asiaticum* (211 strain), *S. abbasi* (507 strain), *S. siamkayai* (157 strain), *H. bacteriophora* (1743 strain) and *H. indica* (HAM-64 strain, while *S. feltiae* (A05 strain) was notably less virulent. Application of these biological control agents were performed at 30 ± 5 °C. The poor virulence of *S. feltiae* may have been due to the fact that this nematode is a naturally cold tolerant species with optimum infectivity closer to 20 °C (Hominick and Briscoe, 1990; Wright, 1992). This implies that management of stored product species with nematodes may be possible year round in certain storage facilities but with different strains of nematodes (Mbata and Shapiro-Ilan, 2005).

In the Petri dish bioassay, *H. bacteriophora*, *S. pakistanense* and *S. siamkayai* resulted in 100% mortality in last larval and adult stages at 350 IJs/larva. Similarly, high levels of entomopathogenic nematode virulence were observed in the red flour beetle *T. castaneum*, *T. molitor, Ephestia kuehniella*, and *P. interpunctella* (Ramos–Rodriquez et al., 2006). Athanassiou et al. (2008) found that *S. feltiae* (Hawaii strain) caused 100% larval mortality of the confused flour beetle *Tribolium confusum* du

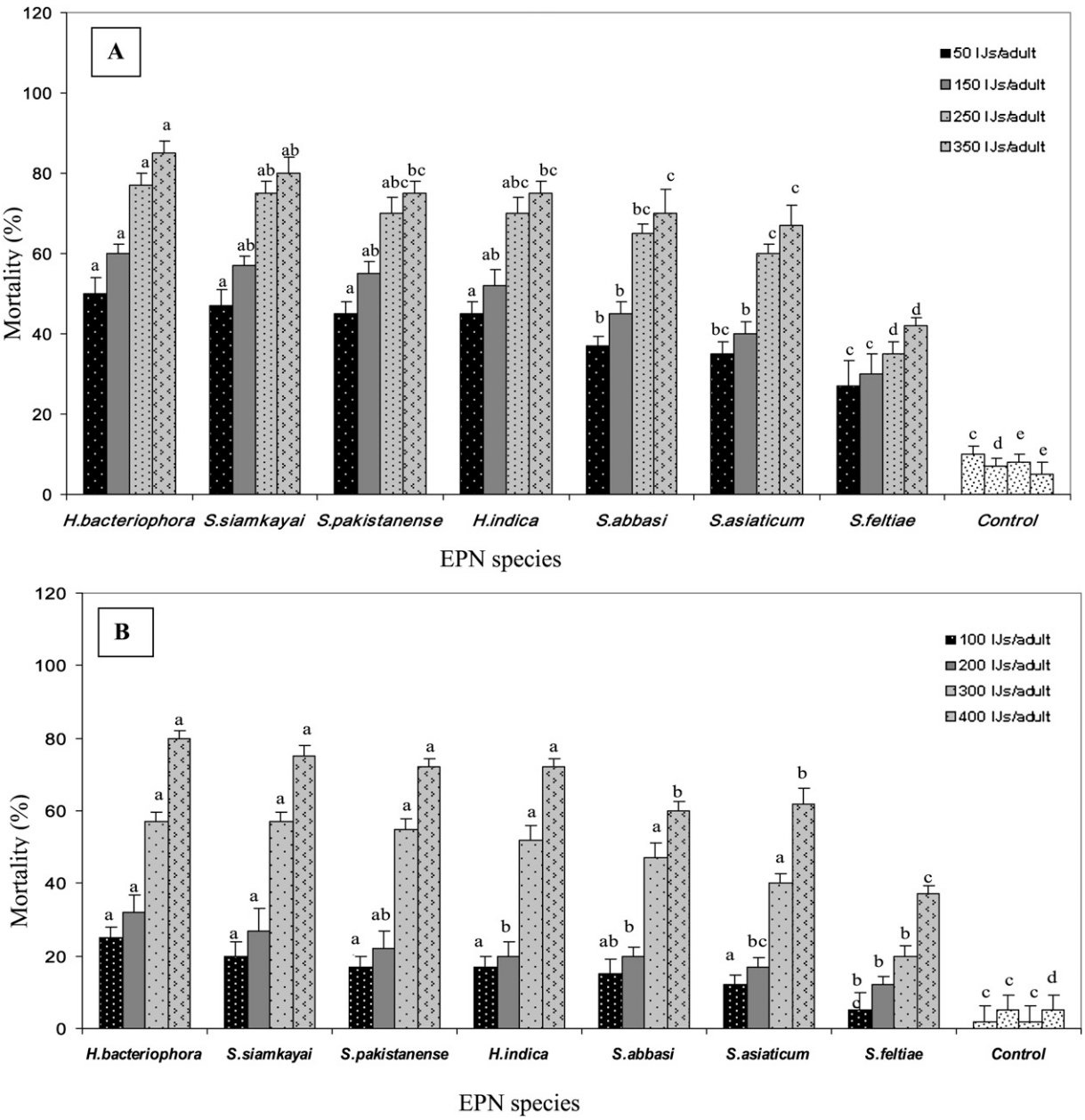

FIG. 2. **(A-B)-**: Adult mean mortality of *C. chinensis* treated with seven different species of entomopathogenic nematodes (EPN) in **A**. Petri dish and **B.** concrete container depending on dose concentration. Data were analyzed by multifactor ANOVA followed by Duncan's multiple range test ($P<0.05$) for separation of means. The same letters above bars are not significantly different and vertical lines represent standard errors of the mean.

Val after 14 d of exposure to 900 IJs/larva. Pupal stage and fourth instar larvae of *Lasioderma serricorne* (F.) also showed almost 100% mortality at the rate of 250 and 350 IJs/insect with *H. bacteriophora* (1743 strain) and *S. siamkayai* (157 strain) (unpublished data).

In concrete containers, sufficient moisture was applied in hidden refugia such as cracks and cervices, which increased the chance of nematode survival, infectivity, and maximum mortality response ranging between 50-85 and 37-80 percent in last instar and adult stage respectively at 8000 IJs/insect. This result demonstrates that if sufficient moisture is applied in hidden refugia such as cracks and crevices, it increases the chance of nematode survival and infectivity. It is well known that entomopathogenic nematodes act efficiently in humid environments (Ebssa et al., 2004; Kaya and Gaugler, 1993).

Our results indicate that the larval stage is more susceptible than the adult stage. Similar to our results, larval stages have been found more susceptible than adults for quite a number of insect species (Fuxa et al., 1988; Mannion and Jansson, 1992; Shapiro-Ilan et al., 2002; Ramos–Rodriquez et al., 2006; Trdan et al., 2009). Further investigations are required for the management of stored grain pests in stored product arenas. In conclusion, our study indicates that entomopathogenic nematodes have the potential to be applied successfully

against *C. chinensis* but still require further investigation. Additional research is needed to conduct trials in warehouses against relevant target pests so that realistic nematode applications in stored-product pest management programs can be initiated.

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