Pathogenicity of Native Entomopathogenic Fungus *Nomuraea rileyi* Against *Spodoptera litura*

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Abstract

The entomopathogenic fungus *Nomuraea rileyi* was isolated from *Spodoptera litura* and *Helicoverpa armigera* insect cadavers collected from different sampling sites. Pathogenicity of ten *N. rileyi* isolates against *Spodoptera litura* was studied by exposing third instars to topical application of a spore concentration of 10^8 conidia/ml. All ten isolates of *N. rileyi* were active against third instars of *S. litura*, resulting in 85 to 97% mortality. However, there were statistically no significant differences among the isolates with respect to the pathogenicity levels. Median lethal time (LT_{50}) values of *N. rileyi* isolates against third instars of *S. litura* ranged from 5.5 to 6.6 days, which were not statistically different. This strongly suggests that the isolates from different geographical locations are equally pathogenic against *S. litura*.

Introduction

Spodoptera litura Fabricius is a major pest of subtropical and tropical agricultural crops. Control relies mainly on the use of chemical insecticides including carbamates, pyrethroids, and organophosphates (18). Frequent use of these insecticides poses increasing problems for human health and the environment. Moreover, certain insects have developed resistance to some of these insecticides (8). The reduced effectiveness of insecticides has resulted in an increase in the number of applications and the doses for desired level of control (22). Consequently, there is renewed interest in utilizing biological agents such as bacteria, viruses, and fungi for control of insect pests. These biological agents including entomopathogenic fungi can provide an alternative and more environmentally friendly approach for the control of pests. Fungal diseases are known to cause insect mortality naturally (10,25,33). Entomopathogenic fungi are potential agents for pest control due to their specificity, mode of action and ease of application. This has promoted the evaluation of the entomopathogenic fungi as biocontrol agents in many countries.

Unlike bacteria or viruses, fungi infect insects by breaching the host cuticle. The insecticidal activity *Nomuraea rileyi* (Farlow) Samson to lepidopteran pests was reviewed by Ignoffo (11), and he concluded that the fungus was a candidate for further development as a microbial insecticide. A recent study on the pathogenesis of *N. rileyi* against *S. litura* showed that the infection process starts with adhesion of conidia on the insect cuticle (27). The germ tube penetrates through the cuticle, causes lysis of endocuticle, followed by development of hyphal bodies in the hemocoel which convert to invasive mycelia and causes death of the host. At the end of the infection cycle, mycelia

emerge from the cuticle and produces conidiophores (27). Also, *N. rileyi* secretes a proteinaceous substance inhibiting larval molt and metamorphosis (16). Entomopathogenic fungi also possess added advantage over other microbial control agents as they are capable of attacking all developmental stages of insects including pupal stages (1,6). Most importantly, these entomopathogenic fungi are the principal pathogens on sucking pests since these hosts cannot ingest other pathogens like bacteria or viruses that infect through gut wall. Apart from this, virtually all insect orders are susceptible to fungal diseases. However, of the 700 species of entomopathogenic fungi currently known, only ten species have been are presently being developed for biocontrol (24). The full potential of entomopathogenic fungi has not been approached. However, previous studies indicate that the insect mycopathogen *N. rileyi* a dimorphic hypomycete causes fungal epizootics in population of several noctuid pests (14,28,31). Moreover, previous bioassays have shown that *N. rileyi* isolates from different geographical locations and different hosts vary in their virulence and specificity (3,12,30,34). However, the virulence of entomopathogenic fungi is affected by environmental factors such as temperature, humidity, light, and solar radiation. The optimum temperature for conidia to germinate and penetrate into noctuid insect body for *N. rileyi* is 20 to 30°C (7,29). Also, the optimum humidity for conidial germination, infection and sporulation is 95 to 100% (29). Therefore, it is important to identify potential entomopathogenic fungal isolates which further can be exploited as myco-insecticide for specific geographic locations. In this paper, our main objective was to study the insecticidal activity of *N. rileyi* isolates obtained from different geographic locations against *S. litura*. Thus, the efficient isolates can be exploited as myco-insecticide for the control of *S. litura* which is a major pest against many vegetable and commercial crops like cabbage, okra, cotton, tobacco, soybean, and tomato in these specific geographic locations.

Isolation and Identification of *N. rileyi* Isolates

N. rileyi was isolated from *S. litura* and *Helicoverpa armigera* (Hübner) cadavers collected from different geographic locations based on the symptoms of infection in the cadavers at the Institute of Agri Biotechnology, University of Agricultural Sciences, Dharwad, which is situated in the northern transitional zone of Karnataka State, India. Additionally, two isolates were available from other geographic locations for this study. The host insects, collection locations, isolate names, and geographical coordinates are listed in Table 1. The dead larvae collected from the fields were surface sterilized by immersing in 0.1% HgCl₂ for 1 min followed by rinsing in three changes of sterile water. The surface sterilized, diseased specimens were cut in a sterile watch glass and a small portion of the infected tissue was transferred to a sterile culture plate containing Sabourauds Maltose Agar Media fortified with 1% yeast extract (SMAY) (2). The plates were incubated at 28°C and the colonies that came up were further purified by repeated subculture on SMAY media. The isolates that came up on the SMAY medium were identified as *N. rileyi* by microscopic examination according to the out-lines given by Samson et al. (1988) (26).

Table 1. Geographical Isolates of *N. rileyi* used in this study.

Sl. no.	Isolate name	Location of collection	Host insect	Geographical coordinates
1	NR-A	Arabhavi	<i>S. litura</i>	16°12'0"N, 74°54'0"E
2	NR-BL	Bailhongal	<i>S. litura</i>	15°49'1.2"N, 74°52'1.2"E
3	NR-DH	Devihosur	<i>S. litura</i>	14°47'8.7"N, 75°20'0.74"E
4	NR-D	Dharwad	<i>S. litura</i>	15°28'0"N, 75°1'0"E
5	NR-S	Siruguppa	<i>S. litura</i>	15°37'48"N, 76°54'0"E
6	NR-B ^x	Bangalore	<i>H. armigera</i>	12°58'0"N, 77°34'0"E
7	NR-HB	Hirebagewadi	<i>H. armigera</i>	16°17'24"N, 74°36'0"E
8	NR-H ^x	Hyderabad	<i>H. armigera</i>	17°22'31"N, 78°28'28"E
9	NR-N	Nalavadi	<i>H. armigera</i>	15°8'0"N, 75°3'0"E
10	NR-R	Ron	<i>H. armigera</i>	15°40'12"N, 75°43'48"E

^x Isolates available for study from other geographic locations.

Isolation of the fungus on the SMAY medium yielded ten *N. rileyi* isolates. The microscopic examination revealed that the vegetative hyphae of *N. rileyi* appeared smooth septate, hyaline and conidiophores were erect and septate. Branches formed near a septum were developed in whorls each giving rise to 2 to 4 phialides. Conidia were single smooth ellipsoidal as shown in Figure 1. All the *N. rileyi* isolates sporulated on SMAY media after 7 days of incubation as shown in Figure 2.

Fig. 1. Microscopic examination of *N. rileyi* isolates: (A) vegetative hyphae and conidiophores of *N. rileyi* isolate (400× magnification); and (B) conidia of *N. rileyi* isolate (400× magnification).

Fig. 2. In vitro growth of *N. rileyi* on SMAY medium: (A) growth of fungal hyphae (white in color); and (B) spore production (green in color). Note the fungal vegetative growth in panel A (white) and sporulation in panel B (green).

Preparation of Fungal Culture of *N. rileyi* Isolates

SMAY media was used for the multiplication of the fungus as well as spores. The cultured plates were incubated at 28°C for 7 to 10 days. Spores obtained on the SMAY media for the 10 isolates were screened for their insecticidal activity against third instars of *S. litura*. The fungal spores from SMAY plates were mixed with distilled water and 0.02% Tween-80 to produce the spore suspension. The number of conidia was determined with a Neubauer haemocytometer and a compound microscope. Finally, through serial dilution method spore suspensions containing 1×10^8 conidia/ml were obtained for the 10 individual isolates. The *S. litura* culture that was used for the bioassays was raised from field collected larvae and maintained under laboratory conditions in the Department of Entomology at University of Agricultural Sciences, Dharwad.

Bioassays of *N. rileyi* Isolates

Insecticidal activity of *N. rileyi* isolates to *S. litura* was studied through larval bioassays. 500 µl of spore suspension of 1×10^8 conidia/ml of all the 10 isolates was topically applied on dorsal side of third instars with a small fine painting brush. They were fed with fresh castor leaves. Three replicates of 20 larvae were tested. Controls consisted of larvae applied with 500 µl of distilled water and 0.02% Tween-80 solution. The number of dead larvae was recorded on the fifth day of spore inoculation and finally the percent mortality for each of the isolates was computed. The data were subjected to statistical analysis of variance using SAS OnlineDoc 9.1.3. (SAS Institute Inc., Cary, NC) and mean separation was carried out using DMRT (Duncan's Multiple Range Tests) to find significant difference among the isolates. Median lethal time (LT₅₀) was estimated using ProcLifeReg procedure and data were fitted to a Weibull distribution (SAS OnlineDoc 9.1.3). The differences in pathogenicity and LT₅₀ among *N. rileyi* isolates against *S. litura* were compared using the Fisher's least significant difference test ($\alpha \leq 0.05$).

Pathogenicity Levels of *N. rileyi* Isolates Against *S. litura*

All ten isolates of *N. rileyi* were pathogenic against *S. litura*. The mortality of *S. litura*, topically applied with the spore suspension is shown in Table 2, Table 3, and Figure 3. Median lethal time (LT₅₀ in days) values of *N. rileyi* isolates against third instars of *S. litura* are shown in Table 4 and Table 5.

Table 2. Effect of different *N. rileyi* isolates of *S. litura* origin on the mortality of third instars of *S. litura*.^x

Sl. no.	Isolates	Percent mortality at indicated days after spore inoculation ^y					
		5	6	7	8	9	10
1	NR-A	45.0 ab	56.7 ab	73.3 bc	83.3	91.7	91.7
2	NR-BL	51.7 a	61.7 ab	70.0 bc	83.3	88.3	95.0
3	NR-DH	46.7 a	60.0 ab	78.3 ab	86.7	90.0	93.3
4	NR-D	53.3 a	75.0 a	86.7 a	91.7	96.7	96.7
5	NR-S	33.3 b	48.3 b	65.0 c	78.3	85.0	88.3

^x Conidial suspension: 1×10^8 conidia/ml.

^y Means followed by the same alphabet within the same column are not significantly different ($P \leq 0.05$) by Duncan's multiple range test.

Table 3. Effect of different *N. rileyi* isolates of *H. armigera* origin on the mortality of third instars of *S. litura*.^x

Sl. no.	Isolates	Percent mortality at indicated days after spore inoculation					
		5	6	7	8	9	10
1	NR-B	33.3	53.3	66.7	78.3	81.7	85.0
2	NR-HB	38.3	48.3	61.7	73.3	86.7	91.7
3	NR-H	38.3	43.3	58.3	75.0	90.0	90.0
4	NR-N	31.7	50.0	71.7	78.3	85.0	86.7
5	NR-R	45.0	56.7	66.7	86.7	93.3	93.3

^x Conidial suspension: 1×10^8 conidia/ml.

Table 4. LT_{50} values of *N. rileyi* isolates of *S. litura* origin on mortality of third instars of *S. litura*.^x

Sl. no.	Isolates	LT_{50} (days) $\pm$ SD	95% confidence limits	
			Lower limit	Upper limit
1	NR-A	6.1 ± 0.48	5.21	7.15
2	NR-BL	6.0 ± 0.47	5.15	7.02
3	NR-DH	5.9 ± 0.48	5.06	6.94
4	NR-D	5.5 ± 0.38	4.80	6.29
5	NR-S	6.5 ± 0.54	5.56	7.71

^x Conidial suspension: 1×10^8 conidia/ml.

Table 5. LT_{50} values of *N. rileyi* isolates of *H. armigera* origin on mortality of third instars of *S. litura*.^x

Sl. no.	Isolates	LT_{50} (days) $\pm$ SD	95% confidence limits	
			Lower limit	Upper limit
1	NR-B	6.6 ± 0.6	5.52	7.89
2	NR-HB	6.5 ± 0.52	5.59	7.63
3	NR-H	6.6 ± 0.48	5.13	7.02
4	NR-N	6.5 ± 0.56	5.51	7.69
5	NR-R	6.1 ± 0.46	5.24	7.08

^x Conidial suspension: 1×10^8 conidia/ml.

Fig. 3. The mortality of *S. litura* topically applied with *N. rileyi* spores. Note the larvae at different stages of infection and mortality.

Bioassays of *N. rileyi* isolates of *S. litura* origin against *S. litura* showed that the Dharwad and Devihosur were the best in terms of optimum larval mortality of 78.3 and 86.7%, respectively, within 7 days after spore inoculation (Table 2). This is similar to the observation made by other researchers that isolates from different geographic locations vary in their virulence and optimum larval mortality (34). However, there were no significant differences among the isolates in terms of efficacy from 8 to 10 days after spore inoculation. A similar level of mortality with different geographical isolates of *N. rileyi* has been reported (34). Initial mortality was observed at 5 days after treatment (Table 2). The median lethal time (LT_{50}) values ranged from 5.5 to 6.5 days among *N. rileyi* isolates of *S. litura* origin (Table 4), which were not statistically different. However, geographical isolate from Dharwad location showed high pathogenicity against *S. litura* with a LT_{50} (confidence interval) value of 5.5 days (4.8 to 6.29). This is consistent with observations made by other authors regarding LT_{50} values of *N. rileyi* isolates against *S. litura* and other lepidopteran pests (4,19,28,29).

In case of larval bioassays with *N. rileyi* isolates of *H. armigera* origin, all isolates showed similar optimum larval mortality (73.3 to 86.7%) within 8 days after spore inoculation (Table 3). However, there were no statistical significant differences among the isolates in terms of efficacy against *S. litura* from 5 days itself after spore inoculation (Table 3). The LT_{50} values ranged from 6.1 to 6.6 days among *N. rileyi* isolates of *H. armigera* origin (Table 5), which were not statistically different. However, the geographical isolate from the Ron location showed a high pathogenicity against *S. litura* with a LT_{50} (confidence interval) value of 6.1 days (5.24 to 7.08). This is consistent with the observations made by other authors (4,19,28,29). Time taken for causing larval death was more with isolates of *H. armigera* origin as compared to isolates of *S. litura* origin. A similar variation in terms of susceptibility of *S. litura* and *H. armigera* origin to different geographical isolates of *N. rileyi* has been reported (34).

Among all ten *N. rileyi* isolates of *S. litura* and *H. armigera* origin, Dharwad and Bailhongal isolates of *S. litura* origin showed the highest percent mortality of 96.7 and 95%, respectively, after ten days of post inoculation (Table 2). The lowest mortality (85%) was recorded in the Bangalore isolate of *H. armigera* origin (Table 3). Isolates of entomopathogenic fungi from different host insects have varying degrees of virulence as measured by percent mortality in bioassays (13,15). The mortality of the third instars treated with *N. rileyi* ranged from 85 to 96.7% at spore concentration of 1×10^8 spores/ml, 10 days after inoculation (Table 2 and Table 3). This is similar to the observation to others that *N. rileyi* was pathogenic to *S. litura* and other lepidopteran pests (9,23,32,33,34). They observed the pathogenicity levels ranging from 70 to 100% in *S. litura*. However, in our research after ten days of spore inoculation, there was no significant difference among the isolates with respect to their insecticidal activity. This strongly suggests that the isolates from different geographical

locations are equally pathogenic. This is in line with the observations of other researchers (5,15,17,20,21) who observed similar insecticidal activity among the isolates obtained from different locations. The sequential stages of the larval death showing different symptoms are shown in Figure 4.

Fig. 4. Progress of infection of *N. rileyi* on *S. litura*: (A) healthy larvae (8 days old; (B) dead larvae (after 5 days of spore application); (C) dead larvae with thick white fungal hyphae (after 7 days of spore application); and (D) dead larvae covered with green colored conidia (after 10 days of spore application).

Conclusion

The most important criteria for commercial exploitation of any entomopathogenic fungus is its virulence against the target insect pest. High percent of mortality observed in this study, indicates the potential of *N. rileyi* isolates of *S. litura* and *H. armigera* origin from different geographic locations for controlling *S. litura*. However, isolates of *S. litura* origin cause higher percent mortality and also take less time to cause larval death compared to isolates of *H. armigera* origin. *N. rileyi* isolates of Dharwad location followed by Devihosur are the most virulent isolates from our collection within 7 days after spore inoculation. However due to the observed high susceptibility of *S. litura* to *N. rileyi*, all isolates of *N. rileyi* from our collection can be used against *S. litura*. Detailed studies about differences in conidial germination time, spore yields, ease and cost effective method of mass production, chitinolytic enzyme profiles, and optimum environmental conditions for infectivity have to be done to identify isolates with best traits for further exploitation as myco-insecticides. There is also a need to screen the pathogen against other insect pests to exploit its full potential. In addition, there is a need to assess their toxicity on non-target insects.

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