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Evaluation of *in vitro* and *in vivo* nematicidal potential of a multifunctional streptomycete, *Streptomyces hydrogenans* strain DH16 against *Meloidogyne incognita*

Talwinder Kaur[a], Shivam Jasrotia[b], Puja Ohri[b], Rajesh Kumari Manhas (Dr. Associate Professor)[a,*]

[a] Department of Microbiology, Guru Nanak Dev University, Amritsar, 143005 Punjab, India
[b] Department of Zoology, Guru Nanak Dev University, Amritsar, 143005 Punjab, India

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ABSTRACT

The present work demonstrated the nematicidal potential of *Streptomyces hydrogenans* strain DH16 (a strain with strong antagonism against fungal phytopathogens and insect pest) against *Meloidogyne incognita*. The culture supernatant and solvent extract significantly inhibited egg hatching (almost 100%) along with J2 mortality of more than 95% after 96 h. The nematicidal activity of 10-(2,2-dimethyl-cyclohexyl)-6,9-dihydroxy-4,9-dimethyl-dec-2-enoic acid methyl ester (SH2; a new antifungal compound) purified from this streptomycete was also evaluated using different concentrations. The juvenile mortality of the nematode increased with increasing concentration and exposure time and reached the maximum (95%) after 96 h at concentration of 100 μg/ml. After 160 h of incubation, egg hatch of 16% was observed at concentration of 100 μg/ml as compared to control where 100% egg hatching was achieved. However, at the highest concentration of the compound (200 μg/ml), 100% J2 mortality and 0% egg hatching were observed after 72 and 160 h of incubation, respectively. *In vivo* pot experiments further revealed the nematicidal potential of *S. hydrogenans* where soil drenching with its culture supernatant and cells effectively controlled root galls, egg masses in nematode infested tomato plants and at the same time promoted the growth of tomato plants. Additionally, in the absence of nematodes, soil drenching with culture supernatant and cells significantly enhanced the various agronomic traits of plants as compared to control plants. Thus, the outcomes of the current study endorse the potential of *S. hydrogenans* strain DH16 and its metabolites to be developed as safe nematicidal and plant growth promoting agents.

1. Introduction

Among plant parasitic nematodes, root knot nematodes belonging to genus *Meloidogyne* are one of the important microscopic obligate biotrophic plant parasites with worldwide distribution (Trudgill and Blok, 2001). They cause severe damage to a wide variety of economically important agricultural crops by attacking their roots and thus result in distinctive swellings on the roots (galls), root dysfunction, and reduced efficiency of water and nutrients utilization. Therefore, species of *Meloidogyne* account for more than 10% loss in worlds total crop production where *Meloidogyne incognita* alone accounts for global loss of 78 billion dollars (Chen et al., 2004). Chemical nematicides are used to protect the crops from these harmful root knot nematodes but these chemicals have severe negative impact on environment and human health along with their limited availability and high costs (Thomason, 1987). In addition, these nematicides become unreliable at high densities of nematode populations. So, there is a great need to find better solution to cut the use of these chemical nematicides for sustainable agriculture.

Now a days, there is an increasing trend towards the use of microorganisms as biocontrol agents in agriculture as main tools for controlling various crop pests, and enhancing crop yields. Thus, the search for new potential microbial antagonists and their metabolites has gained momentum as safe and ecofriendly alternative to the agrochemicals. Of these microbial antagonists, actinobacteria (Gram positive bacteria) have been reported as potential biocontrol agents against various plant pathogens (fungi and bacteria) and pests (insects, mites). In addition, there are reports which demonstrated that actinobacteria, especially *Streptomyces* spp. can also be

* Corresponding author.
E-mail address: rkmanhas@rediffmail.com (R.K. Manhas).

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used to control plant parasitic nematodes because of their ability to produce nematicidal compounds (Mishra et al., 1987; Sun et al., 2006; Ruanpanun et al., 2011; Yang et al., 2013; Zeng et al., 2013; Rashad et al., 2015). Avermectins obtained from fermentation broth of *Streptomyces avermitilis* is commercially used as seed treatment in corn, cotton, beans, *etc.* to control a wide variety of plant parasitic nematodes under the trademark name of AVICTA® (Cabrera et al., 2013; Khalil, 2013).

Because of the immense environmental problems resulted from the use of chemicals in agriculture, the quest for microbial strains and their metabolites with multiple properties to improve agricultural productivity, has gained attention of the research workers. *Streptomyces* spp. exhibit several different biological activities. But these properties have been mostly described as scattered in various *Streptomyces* spp. rather than in a single species. Therefore, to meet the increasing demand for sustainable and eco friendly agriculture, preference would be given to those *Streptomyces* spp. which encounter more than one activities.

In response, the present work was planned to assess the nematicidal activity of a soil streptomycete, *Streptomyces hydrogenans* strain DH16 which also exhibits activity against fungal phytopathogens and insect pest (Kaur and Manhas, 2014; Kaur et al., 2014). *In vivo* pot experiments were also conducted to evaluate the nematicidal potential of this strain under green house conditions. The nematicidal activity of the compound **SH2,** purified from this strain and characterized as 10-(2,2-dimethyl-cyclohexyl)-6,9-dihydroxy-4,9-dimethyl-dec-2-enoic acid methyl ester, was also determined. This new compound with significant antagonistic potential against fungal phytopathogens was reported for the first time from this streptomycete (Kaur et al., 2016).

2. Materials and methods

2.1. Streptomyces hydrogenans strain *DH16*

S. hydrogenans strain DH16 (GenBank accession no. JX123130) was recovered from soil and maintained on starch casein nitrate agar slopes at temperature of 4 °C (Kaur and Manhas, 2014).

2.2. Nematode culture

Meloidogyne incognita was obtained from pure cultures maintained in roots of tomato plants (*Lycopersicon esculentum*). *M. incognita* egg masses were extracted from severely infected tomato roots using a 1.5% NaClO solution (Hussey and Barker, 1973), and subsequently washed with distilled water. The surface-sterilized egg masses were either used for *in vitro* assay of egg hatch or incubated in sterile water for 3–5 days at 25 °C using Baermann funnel method (Siddiqui and Alam, 1990) to obtain second-stage juveniles (J2s). The freshly hatched J2s were used for nematicidal assay.

2.3. Production of bioactive metabolites

The production of bioactive metabolites by *S. hydrogenans* was carried out as described by Kaur and Manhas (2014). The fermentation was carried out at temperature of 28 °C at 180 rpm. After incubation of 3 days, the culture broth was centrifuged for 20 min at 4 °C at 10,000 rpm. The filter sterile cell free supernatant was then used to determine nematicidal activity.

Active metabolites in the cell free supernatant were extracted in ethyl acetate and, concentrated under vacuum. The resulted brown colored residue was re-dissolved in 0.5% of dimethyl sulfoxide (DMSO) and stored at 4 °C for further use. The new non-mutagenic, non-cytotoxic and non-phytotoxic nematicidal compound SH2 was purified from culture supernatant of strain DH16 by silica gel chromatography and semi-preparative HPLC, and characterized as 10-(2,2-dimethyl-cyclohexyl)-6,9-dihydroxy-4,9-dimethyl-dec-2-enoic acid methyl ester on the basis of various spectroscopic techniques (IR, NMR, LC–MS) (Kaur et al., 2016).

2.4. In vitro *assays*

To evaluate the nematicidal potential of *S. hydrogenans* strain DH16, culture supernatant (CS), solvent extract (SE) and purified antifungal compound (SH2) were tested on egg masses and J2s of *M. incognita*. Culture supernatant was used directly. Different concentrations of solvent extract (50, 100, 150, 200 and 250 μg/ml) and purified compound (12.5, 25, 50, 100 and 200 μg/ml) were used for determining egg hatching and juvenile mortality.

2.4.1. Nematicidal activity

Nematicidal activity of all the samples was determined using second stage juveniles. Nematode juvenile suspension (200 J2s/50 μl) was added to 1 ml of sample solution in 35 mm Petri plates. 0.5% DMSO in water was used as negative control. The plates were incubated at room temperature (25 ± 2 °C) for 96 h and were daily examined for dead J2s. Three replicates were used for each sample. Living and dead juveniles were counted under the light microscope and mortality was calculated according to the formula:

$$\text{juvenile mortality} = 100 \times \text{dead juveniles/totaljuveniles (Sunet al., 2006).}$$

The malformed, immobile or motionless juveniles when probed with a fine needle were considered to be dead.

2.4.2. Hatching inhibition analysis

Egg masses from root-knot nematode cultured on tomato plants were separated and surface-disinfected with 1% sodium hypochlorite (NaClO). Three consecutive washings were given to these egg masses with sterile distilled water to remove residual NaClO. Single egg mass (approximately 200 eggs/50 μl) was treated with each sample (1 ml). As control, 1 ml of 0.5% DMSO was used. The plates were then incubated at room temperature (25 ± 3 °C) for 7 days and daily examined for egg hatch rate. Three replicates were done for each sample. Percentage of egg hatch was determined by counting all eggs and juveniles under the microscope and calculated according to the formula:

$$\text{percentage of egg hatch} = 100 \times \text{juveniles/(eggs + juveniles) (Sunet al., 2006).}$$

2.5. . In vivo *effect of the* S. hydrogenans *strain DH16 on the control of* M. incognita *in pot experiments*

In vivo pot experiments were conducted to investigate the potentiality of cells as well as culture supernatant of *S. hydrogenans* strain DH16 for their antagonistic activity towards *M. incognita* under greenhouse conditions. Seeds of tomato (*Lycopersicon esculentum* Mill., variety Pusa Ruby, susceptible to *M. incognita*) were sown in sterilized soil at 28 ± 2 °C. Tomato seedlings with true stage leaves were then transplanted singly into pots of 8 cm diameter containing 100 g of sterilized soil. After 2 days of transplantation, plants were divided into 6 groups and each group was given different treatments. Group1: each plant was inoculated with 10 ml of *M. incognita* suspension containing 100 vigorous J2s/ml, Group 2: each plant was inoculated with 10 ml of *M. incognita* suspension and 10 ml of culture supernatant obtained from 3 day old fermentation broth of *S. hydrogenans* strain DH16, Group 3: each plant was inoculated with 10 ml of *M. incognita* suspension and 10 ml of cell suspension (1×10^6 cells/ml) of *S. hydrogenans* strain DH16 grown on SCNA medium at 28 °C for 7 days, Group 4: each plant was treated with 10 ml of water only, Group 5: each plant was treated with 10 ml of culture supernatant Group 6: each plant was inoculated with 10 ml of cell suspension (1×10^6 cells/ml) of