MANAGEMENT OF RENIFORM NEMATODE, *Rotylenchulus reniformis* LINFORD AND OLIVEIRA IN VEGETABLE COWPEA USING BACTERIAL ANTAGONISTS

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(2018-11-163)

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by

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(2018-11-163)

THESIS

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requirements for the degree of

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2020

DECLARATION

I hereby declare that this thesis entitled "**MANAGEMENT OF RENIFORM NEMATODE,** *Rotylenchulus reniformis* **LINFORD AND OLIVEIRA IN VEGETABLE COWPEA USING BACTERIAL ANTAGONISTS**" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.
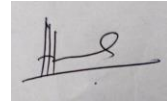
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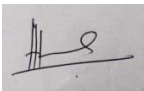
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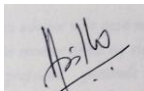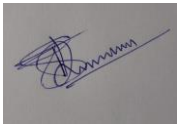
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LIST OF ABBREVIATIONS AND SYMBOLS USED

et al.,	And co-workers
@	At the rate
°C	Degree Celsius
cc	Cubic centimeter
CD	Critical difference
cm	Centimeter
CRD	Completely randomized design
cv	Cultivar
f. sp.	Forma specialis
Fig.	Figure
g	Gram
h	Hours
ha	Hectare
kg	Kilogram
L	Litter
m	Meter
mg	Milligram
min	Minutes
mL	Millilitter
mm	Millimeter
mmol	Millimole
rpm	Revolutions per minute
sec	Seconds
Sl	Serial
sp. or spp.	Species (singular and plural)
subsp	subspecies
T	Treatment
$v\ v^{-1}$	Volume to volume
viz	Namely
$w\ v^{-1}$	Weight by volume
$w\ w^{-1}$	Weight by weight
μg	Microgram
μL	Microlitter
μm	Micrometer
μmol	Micromole

Introduction

1. INTRODUCTION

The most abundant multicellular animals on planet are nematodes. Several species of nematodes are infecting crop plants and cause severe loss to agricultural system worldwide. These plant parasitic nematodes are known as "hidden enemy of farmers". Their microscopic size and sub terrain habitat leave them undetected. A yield loss of 12.30 per cent estimated annually on world's major crops due to plant parasitic nematodes (Abad *et al*., 2008). In India the economic loss due to plant parasitic nematodes are about 14.6 per cent (Khan *et al.,* 2010).

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important vegetable crop in Kerala. Cowpea is grown throughout the year under Kerala condition. Cowpea grain is a rich source of proteins, calories, minerals and vitamins.The major nematode pests of cowpea are root knot nematode (*Meloidogyne incognita* (Kofoid and White) Chitwood) and reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) (Sikora *et al*., 2005). They together cause a yield loss of 27.30 per cent on cowpea (Jain *et al*., 2007). Reniform nematode is gaining importance in India after root knot nematode. Reniform nematode is having wide host range of over 300 plant species in 77 families (Robinson, 2007). They are cosmopolitan in distribution and successfully infect many cultivable crops like cotton, castor, cowpea, papaya, okra, brinjal, tomato, pineapple, pigeon pea etc (Khan, 2005).

Efforts are being undertaken to develop good management strategy against phytonematodes. Any method that will bring down the nematode population below economic threshold level without harming nature and biodiversity are appreciated. Use of chemical nematicides increased in the past as the production method intensified to increase the output. Although chemical nematicides are effective, public health and environmental safety concerns compel for an alternative method. Moreover chemical nematicides *viz* carbosulfan and phorate are currently not available in Kerala. Here comes the importance of effective and eco-friendly method like biological control of nematodes.

Microorganisms like bacteria and fungi are mainly used as biocontrol agents. Bacteria being numerically abundant microorganism can be exploited considerably

against nematodes. Nematophagous bacteria present in soil *viz Pasteuria, Bacillus, Pseudomonas, Stenotrophomonas* and *Paenibacillus* posses diverse mode of action, like antagonism, competition, interfering host nematode recognition, altering composition of root exudates, inducing plant defence mechanism etc (Siddiqui and Mahmood, 1999).

In this context biocontrol using bacteria are gaining more consideration as novel, safe and potential tool which provide sustainable effect on management of phytonematodes. Nematode coexists with many bacterial antagonists in the rhizospheric soil of plants. Amplifying artificially the population of these bacterial antagonists will bring down the nematode population below economic injury level without harming environment. Considering all these facts, the present study entitled "Management of reform nematode, *Rotylenchulus reniformis* Linford and Oliveira in vegetable cowpea using bacterial antagonists" was undertaken with the following objectives

> To isolate indigenous bacterial antagonists from cowpea growing fields of Thiruvananthapuram district

> To evaluate the effect of cell free extracts of isolated bacterial antagonists on egg hatch inhibition and juvenile mortality of *R. reniformis*

> To evaluate biocontrol potential of promising bacterial isolates against *R. reniformis* in vegetable cowpea

> To identify the most effective bacterial isolates by molecular characterization

Review of Literature

2. REVIEW OF LITERATURE

A brief review on the related literatures on the study "Management of reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira in vegetable cowpea using bacterial antagonists" is done here.

2.1. PLANT PARASITIC NEMATODES AND CROP LOSSES

Nematodes are microscopic animals that inhabit the soil, fresh water, and marine environments. According to Ettema (2017), nematodes are numerous among animals and it represents about 80.00 per cent of all multicellular animals. They occur either as free living forms or parasites of animals and plants.

Nematodes that are parasitic to plants exist all over the world. They continue to threaten the efficiency of crop production and international trade. There are many species of plant parasitic nematodes; over 4100 species have been identified (Decraemer and Hunt, 2006). Most damage causing plant parasitic nematodes are root knot (*Meloidogyne* spp.), cyst (*Heterodera* and *Globodera* spp.), reniform (*Rotylenchulus reniformis*), root lesion (*Pratylenchus* spp.), bulb and stem (*Ditylenchus dipsaci* Kuhn), burrowing (*Radopholus similis* (Cobb) Thorne), bud and leaf (*Aphelenchoides* spp.), seed gall (*Anguina tritici* (Steinbuch) Chitwood) and citrus nematode (Tylenchulus semipenetrans Cobb) (Jonathan, 2009).

The economic losses due to plant parasitic nematode in various countries are enormous. According to IASRI, 2012 (Indian Agricultural Statistics Research Institute) annual yield loss of l0.70 per cent was estimated for the 20 major life sustaining crops that serve as man's primary food source. Estimated loss due to nematode in developing country (14.60 per cent) is more than that of developed country (8.80 per cent). Annual economic yield loss in major crops due to plant parasitic nematode has been estimated to be US dollar 173 billion based on a comprehensive survey on global scale by Elling (2013). A yield loss of 12.30 per cent was recorded worldwide due to plant parasitic nematodes and a loss of $ 40.30 is reported from India (Singh *et al.*, 2015). The per cent yield loss due to plant parasitic nematodes in horticultural crops (23.03 per cent) was estimated to be more than field

crops (18.23 per cent). It was estimated that 25.50, 19.60, 29.50, 18.80, 23.00, 11.80 and 19.75 per cent yield loss is recorded in fruits, vegetables, spices, cereals, pulses, oilseeds and fiber crops respectively (Kumar *et al.,* 2020).

Mostly encountered plant parasitic nematodes on cowpea are *Meloidogyne incognita* (Kofoid and White) Chitwood and *R. reniformis*. They together cause a yield loss of 27.30 per cent on cowpea, which is Rs.32 million as monetary loss (Jain *et al.*, 2007). In papaya *M. incognita* and *R. reniformis* together cause 30.00 per cent yield loss while in castor reniform nematode alone cause 15.00 per cent yield loss (Kumar *et al.,* 2020).

2.2. RENIFORM NEMATODE

2.2.1. Distribution and Host Range of Reniform Nematode

R. reniformis are obligate semi-endoparasitic nematodes which are polyphagous in nature. Reniform nematode was first observed in 1940 attacking cowpea, pineapple and several weeds in Hawaii (Linford and Oliveria, 1940). Reniform nematode is having wide host range with over 300 plant species in 77 families (Robinson, 2007). They infest many annual and perennial plants and are widespread in tropical and subtropical regions (Jones *et al.,* 2013).

R. reniformis prefers the hot semi arid and sub humid ecoregions. Even though *R. reniformis* is found distributed to almost all sates of India, where the optimum temperature (33°C) for reniform nematode is obtained at least a few months in a year (Kamra and Sharma, 2000).

Reniform nematodes are reported to infest many crops in India in the past years. From Andhra Pradesh reniform nematodes are identified as one among the prominent plant parasitic nematodes infecting groundnut (Mani and Kumar, 1990). Amaranatha and Krishnappa in 1990 reported that *R. reniformis* is the most predominant nematode associated with sunflower cultivation in Karnataka.

R. reniformis was reported as one of major nematode infesting tomato and is wide spread in tomato growing regions of Tamil Nadu. It is estimated that in tomato, a

yield loss of 42.25 to 49.02 per cent was caused by reniform nematode in Tamil Nadu (Subramaniyan *et al.,* 1990). Sundaram and Vadivelu (1995) identified six genera of plant parasitic nematodes infesting mandarin in Nilgiri Hill region and *R. reniformis* was one among the identified genera of nematodes in mandarin orange (*Citrus reticulate* Blanco). Mature females of *R. reniformis* were identified from feeder roots of small cardamom (*Elettaria cardamomum* (L.) Maton) grown in Karnataka. The number of mature females varied from 7-16 g root^{-1} (Eapen, 1995).

Khan and Dar (2002) found that reniform nematodes infects broccoli and two immature females of *R. reniformis* g soil^{-1} was found to be minimum damaging threshold level for broccoli. Reniform nematode was found as the most prominent nematode among 17 species of plant parasitic nematodes infecting cowpea and pea in a survey conducted in West Bengal (Roy *et al.,* 2007). Lone *et al.* (2008) found that reniform nematode is associated with almost all crops in Udaipur (Rajasthan) and castor, brinjal, tomato, chilli and okra were found more susceptible to *R. reniformis*. Reniform nematodes are found wide spread in the cotton growing areas of Punjab, Haryana and Uttar Pradesh. Absolute frequency of occurrence of *R. reniformis* was 56.50 per cent in Punjab, 30.00 per cent in Haryana and 42.30 per cent in Uttar Pradesh (Das and Gaur, 2009).

Even under high day time temperature and relative humidity condition in poly house, reniform nematodes are found to infect vegetable and ornamental crops (Sharma and Singh, 2009). Growth and development of bidi tobacco seedlings was delayed by infection of reniform nematode which caused 22.10 per cent avoidable loss at first pulling (Patel and Patel, 2009). Reniform nematodes alone causes 14.90, 8.10, 6.00, 13.20 and 8.70 per cent yield loss in cotton, maize, finger millet, cowpea and black gram respectively (Jonathan, 2009).

Cowpea was found to be a good host for reniform nematode in several parts of the world. In a pot experiment conducted by Crozzoli *et al.,* (2004) 20 per cent yield loss was recorded in cowpea with a population of two reniform nematodes cc soil^{-1}.

2.1.2. Symptoms of Infection of Reniform Nematode

Reniform nematode symptoms are often confused with symptoms of fertilizer deficiency or physiological disorders even some time as root disease caused by pathogens (Heald and Heilman, 1971). This is because the above ground symptoms of *R. reniformis* infection resemble moisture and nutrient deficiencies.

Reniform nematode infection also mediates changes in internal content of some biochemicals. In pigeon pea, reduced polyphenol oxidase (81.36 per cent) and β-glucosidase (59.36 per cent) activities as well as total phenol contents (47.07 per cent) were observed (Patel and Patel, 1990). On cowpea the common symptoms of reniform nematode infection are yellowing, wilting, root rotting etc. Some difference in nutritional status and biochemical changes also observed in cowpea infected with reniform nematode. Increased level of various amino acids, amides, total sugar, crude protein, nitrogen, potassium, calcium and magnesium content was observed in infected plants than uninfected plants while content of chlorophyll-a, chlorophyll-b, total chlorophyll, phosphorus, sulphur and micro nutrients *viz* Cu, Zn, Mn, Fe, B and Mo were found lesser in infected plants than healthy plants (Mohanty *et al.,* 1999).

Rajendran and Cannayane (2000) observed symptoms such as general stunting and brown to black discoloration on roots of finger millet due to reniform nematode infection. Numerous adult females also found attached to the roots with their head region embedded in the roots. In broccoli symptoms of reniform nematode infection includes drying of leaf tip and margin, entire leaf lamina become dry and brittle and the leaf sheds. Heavily infected plants become yellowish green and stunted at the time of harvest. Sprouting is delayed in infected plants. Roots of infected broccoli plants were brownish colour with several necrotic spots (Khan and Dar, 2002).

Considerable yield suppression was observed in reniform nematode infected cotton field. Cotton plants expressed nematode-induced nutritional deficiencies, fruit abortion, and abnormal maturation of the crop due to *R. reniformis* infection (Koenning *et al.,* 2004). According to Nicol *et al.* (2011) the nematode infection may not directly affect cell death or diseased tissue, it rather interfere with root system on uptake of nutrients and water and reduce their efficiency which is often misdiagnosed.

Reniform nematode infection in banana plants results in atrophic, black, rotten roots and stunted plants (Zhou *et al.,* 2012). According to Jones *et al.,* (2013) infection with reniform nematodes shows few symptoms on roots. It causes reduction in root growth with fewer secondary roots and root necrosis in some plants like pineapple and banana. Severely affected plants may be stunted and chlorotic. Losses of 40.00 to 60.00 per cent have been reported due to reniform nematode in different crops.

2.1.3. Biology of Reniform Nematode

Life stages of *R. reniformis* include eggs, juveniles (J_1 to J_4), adult male and female. Only the immature female stage is parasitic to plants. Generally reniform nematode takes around 25 days to complete the life cycle. This varies according to the host plant and the climatic conditions. *R. reniformis* required 23 days to complete the life cycle on susceptible cowpea plants (Haque and Padmavathy, 1985), while it takes only 15 days on soybean (Shekhar *et al.,* 1996) and 30 days on broccoli (Khan and Dar, 2002). The eggs are laid in gelatinous matrix. The first molting occurs in the egg itself.

The second stage juvenile emerges out from the eggs and second molting was observed within days. The second moult was observed on seventh day in soybean (Shekhar *et al.,* 1996), while it was observed on sixth day in bottle gourd (Khan and Ashraf, 2005). Third and fourth moulting occurs within three to four days after the second moult. The immature females after fourth moulting penetrate to the host plant roots and it occurs within one or two days after inoculation. The immature female then establish feeding site and mature in to adult females. They protrude out to the root with head region embedded in roots (Khan and Ashraf, 2005).

2.3. MANAGEMENT

Management of plant parasitic nematodes is a challenge to agricultural production. Their microscopic size and soil inhabitant nature make them difficult to control (Stirling, 1991). Studies have been conducted for several years for developing a strong strategy for managing plant parasitic nematodes. Several methods of nematode control are suggested so far. The most promising approach for nematode

management is combination of several control methods, such as nematode resistant host plants, cover crops, crop sequences, pre-plant solarisation of soil, least-toxic nematicidal material, organic amendments, biological control agents (Eissa and Abd-Elgawad, 2015).

A great deal of effort has been done to create biocontrol of nematode a most promising management method. Biocontrol method exploits natural enemies of nematode like predators, parasites and antagonistic organisms. These organisms include nematophagous fungi, bacteria, viruses, protozoans, mites etc.

2.3.1. Bacterial Biocontrol Agents

Bacterial biocontrol agents, used for the control of plant parasitic nematodes can be broadly grouped in to parasitic bacteria and non parasitic bacteria of nematodes (Siddiqui and Mahmood, 1999). Very precised classification of bacterial biocontrol agents based on their mode of action includes parasitic bacteria, opportunistic bacteria, rhizobacteria, Cry protein forming bacteria, endophytic bacteria and symbionts of entomopathogenic nematodes (Tian *et al*., 2007).

2.3.1.1. Parasitic Bacteria

Parasitic bacteria include *Pasteuria,* they are obligate, gram positive, mycelial, endospore forming bacteria. A number of bacterial species in this genus are biocontrol agents against plant parasitic nematodes. They occur worldwide and have been reported from at least 51 countries (Siddiqui and Mahmood, 1999). The main nematophagous species are *P. penetrans*, parasitizes root knot nematodes such as *Meloidogyne* spp., *P. thornei*, parasitizes root lesion nematodes such as *Pratylenchus* spp. and *P. nishizawae*, which occurs on cyst nematodes of the genera *Heterodera* and *Globodera* (Atibalentja *et al*., 2000). Two 'Candidatus' taxa, 'Candidatus Pasteuria usgae' and 'Candidatus Pasteuria aldrichii' were reported as parasites of sting nematode (*Belonolaimus longicaudatus* Rau) and the bacterivorous nematode *Bursilla* sp. respectively (Eissa and Abd-Elgawad, 2015).

In a pot culture study, *P. penetrans* were tested against root knot nematode infestation in chilli. When *P. penetrans* spores were applied to the soil

(@ 1×10^6 spores pan^{-1}) the nematode population was reduced by 74.00 per cent and egg mass production reduced by 91.00 per cent after 90 days of sowing (Swarnakumari and Sivakumar, 2012).

2.3.1.2. Opportunistic Parasitic Bacteria

Opportunistic parasitic bacteria usually live as saprophytes, and attack nematode as one of many possible nutrient resources. These groups of bacteria can parasitize their available nutrient resources. They penetrate through the nematode cuticle and kill them. They digest all the host (nematode) tissue as nutrients for growth. One of previously identified such bacteria is *Brevibacillus laterosporus* (Laubach) strain G4 (Tian *et al*., 2007).

2.3.1.3. Rhizobacteria

The term rhizobacteria was coined for bacteria which have the ability to colonize roots aggressively (Schroth and Hancock, 1982). There are rhizobacteria that are having nematicidal properties. They act against plant parasitic nematodes through different mechanisms *viz* direct antagonism, boosting of plant defense mechanism and competition. *Bacillus* spp. and *Pseudomonas* spp. are among the prevailing bacterial populations present in the rhizosphere that are able to antagonize plant parasitic nematodes (Tian *et al*., 2007). Rhizobacteria includes the broad range of bacterial biocontrol agents of phytonematodes.

Direct antagonism mainly occurs by synthesis of allelochemicals. Allelochemicals are secondary metabolites produced by the bacteria which kill or inhibit nematodes. The allelochemicals may be siderophores, antibiotics, biocidal volatiles, lytic enzymes etc. Antagonistic activity of *Pseudomonas fluorescens* (Flügge) Migula caused alteration of root exudates that influenced egg hatching, attraction and penetration behaviour of *Heterodera schachtii* Schmidt in sugar beet (Oostendorp and Sikora, 1989). According to Gallagher and Manoil (2001) hydrogen cyanide is the sole or primary toxic factor produced by *P. aeruginosa* PAO1 which rapidly paralyzed and killed the nematode *Caenorhabditis elegans* Maupas.

The antibiotics 2,4-DAPG (2,4-diacetylpholoroglucinol) and pyoluteorin produced by *P. fluorescens* CHA0 reduced egg hatch and caused substantial mortality of *M. javanica* juveniles. It act as the inducing agents of systemic resistance in tomato roots also (Siddiqui and Shaukat, 2003). Rhizobacterium, *B. subtilis* (isolate Bst) induces systemic resistance against *R. reniformis* in tomato (Niknam and Dhawan, 2003)

The cuticle degrading protease from *Bacillus* strains was found to be having nematicidal activity which inhibited root knot nematode *M. incognita in vitro* (Lian *et al.,* 2007). The isolate *Stenotrophomonas maltophilia* Palleroni and Bradbury strain G2 showed nematicidal activity by killing 90 per cent *Panagrellus redivivus* (Linnaeus) and 65 per cent *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle within 24 h. The crude extracellular protein extract from the bacterial culture caused its nematicidal activity and identified as a novel serine protease enzyme (Huang *et al.*, 2009). *B. megaterium* YMF3.25 produced nematicidal volatiles like benzeneacetaldehyde, 2- nonanone, decanal, 2-undecanone and dimethyl disulphide. These volatiles found active against both juveniles and eggs of *M. incognita* at the concentration of 0.5 mmol (Huang *et al.*, 2010).

Paenibacillus elgii strain HOA73 produced hydrolytic enzymes including gelatinase and chitinase. The bacterial cultural filtrate showed both egg hatching inhibition and juvenile mortality against root knot nematode. In pot culture study *P. elgii* strain HOA73 reduced root galling (62.10 per cent), number of egg masses (69.80 per cent), nematode population (53.00 per cent) and promoted plant growth in tomato (Nguyen *et al.*, 2013). Lee and Kim, (2016) reported that *B. pumilus* L1 produces both protease and chitinase against *M. arenaria*. It was found that 78.80 per cent inhibition of egg hatching after 5 days and 98.60 per cent juvenile (J_2) mortality after 3 days was observed when crude enzyme (0.07 mg mL^{-1}) produced by *B. pumilus* L1 was treated.

Many of the rhizobacteria can directly reduce the nematode population around host plants when they are applied to the soil. Some of them are even capable of improving growth parameters of the crop plants and are collectively known as plant

growth promoting rhizobacteria (PGPR). Jayakumar *et al*. (2004) isolated *P. fluorescens* (PF1) from rhizosphere of cotton. *P. fluorescens* (PF1) was tested against reniform nematode in cotton and observed reduced nematode population in root (70.40 per cent) and in soil (44.80 per cent). Nursery application of *B. macerans* @ 25g m^{-2} and drenching 7 days after sowing significantly reduced the population of *M. incognita* (75.00 per cent) resulting in increased yield (50.00 per cent) in brinjal (Sheela and Nisha, 2004).

P. fluorescens 10^{8}cfu g^{-1} was evaluated for its biocontrol potential against *M. incognita* on *Coleus forskohlii* Briq. Soil application of *P. fluorescens* @ 2.5 kg ha^{-1} showed increased plant growth and significant reduction in number of galls plant^{-1} (59.60 per cent), number of females (54.00 per cent) egg masses (68.40 per cent) and nematode population (74.40 per cent) (Senthamarai *et al*., 2006).

P. fluorescens was tested against *R. reniformis* in cotton. The maximum colonization of rhizobacterium and reduction in reniform nematode population (74.20 per cent) in roots was noticed when *P. fluorescens* was applied as combination of seed treatment with *P. fluorescens* 10g kg seed^{-1} (6×10^8 cfu g^{-1}) and split application of *P. fluorescens* @ 1, 0.75 and 0.75 kg ha^{-1} at sowing, 30 and 60 days after sowing respectively (Jayakumar *et al*., 2007).

Seedling root dip treatment of tomato seedlings in *P. fluorescens* aqueous formulation @ 2.00% (w v^{-1}) dilution for half an hour against *M. javanica* enhanced plant growth and showed 29.60 per cent reduction in galling 45 days after transplanting (Verma, 2009). Rhizome treatment with *P. fluorescens* at 3.00% w w^{-1} significantly improved the growth parameters and yield in kacholam by reducing the population of root knot nematodes by 78.00 per cent (Nisha and Sheela, 2012).

Six PGPR isolates, *Pseudomonas putida* Trevisan, *P. fluorescens, Serratia marcescens* Bizio*, Bacillus amyloliquefaciens* Priest *et al., B. subtilis* and *B. cereus* were found effective against root knot nematode in tomato. *S. marcescens* found to be most effective with lowest number of J$_2$ 10g soil^{-1} (78), galls root^{-1} (24.33) and egg masses root^{-1} (12.66) (Almaghrabi *et al*., 2013). Castillo *et al*. (2013) reported that cotton seed treatment with *B. firmus* (1.4×10^{7}cfu seed^{-1}) in non autoclaved field soil

infected with reniform nematode reduced vermiform life stages of *R. reniformis* by 37.00 per cent and eggs of *R. reniformis* by 21.00 per cent than the untreated seed control.

Nineteen bacterial strains were isolated in Yunnan from rhizosphere soils and plant tissues and found effective against *M. incognita* in tomato. *Bacillus methylotrophicus* strain R2-2 and *Lysobacter antibioticus* strain 13-6 exhibited the highest antagonistic activity against the tomato root knot nematode among 19 isolates. They recorded more than 99.00 per cent nematode mortality under *in vitro* condition and 68.00 per cent reduction in root knot disease incidence compared to control under field condition (Zhou *et al.*, 2016).

Five indigenous isolates of *Bacillus* spp. were tested against *M. incognita* on tomato under controlled conditions. Out of the five isolates, *B. weihenstephanensis* (TSB4) @ 5g plant^{-1} showed highest reduction in nematode population in soil (56.74 per cent) and root (67.76 per cent), number of egg masses (69.13 per cent), eggs egg mass^{-1} (55.25 per cent) and gall index (76.25 per cent) . It also increased fruit yield of tomato by 50.30 per cent over untreated control (Sarangi *et al.*, 2017).

Soft rot disease complex of carrot is caused by *M. incognita* and soft rot bacterium *Pectobacterium carotovorum* subsp. c*arotovorum*. Under *in vitro* condition *B. subtilis* IIHR BS-2 showed inhibition in hatching of eggs (94.65 per cent), juvenile mortality (91.26 per cent) and reduced growth of *P. carotovorum* (60.60 per cent). In field condition seed treatment with *B. subtilis* IIHR BS-2 (5 L ha^{-1}) and *B. subtilis* IIHR BS-2 enriched vermicompost (2 tons ha^{-1}) application recorded maximum increase in carrot yield (28.80 per cent), decrease in nematode population (69.30 per cent) and disease incidence (70.20 per cent) (Rao *et al.*, 2017).

The effect of four plant growth promoting rhizobacteria was evaluated on tomato as biological control of the root knot nematode *M. javanica*. PGPR include *P. fluorescens, P. striata, B. subtilis*, and *Paenibacillus polymyxa*. The reproductive factor of *M. javanica* was significantly reduced by *P. fluorescens* and *B. subtilis* (24.94 and 24.96) while control recorded a reproductive factor of 112.15 (Sohrabi *et al.*, 2018).

S. maltophilia strain W2-7 isolated from rhizosphere soil of pepper plants from Kerala. Soil drenching of *S. maltophilia* strain W2-7 (1×10^7 cfu mL^{-1}) @ 50mL pot^{-1} reduced *M. incognita* population in soil (72.69 per cent) and roots (82.23 per cent) in tomato under protected conditions (Vishnu, 2018).

2.3.1.4. Cry Protein Forming Bacteria

Bacillus thuringiensis Berliner (Bt) are known as Cry protein forming bacteria. The toxic proteins produced by *B. thuringiensis* are the most broadly used natural insecticides in agriculture. Cry protein are pore forming toxins. These crystals dissolve within the gut of the nematode and this is followed by proteolytic activation of protein. Eventually it leads to vacuole and pore formation and degradation of the intestine of host (Crickmore, 2005). Six Cry proteins (Cry5, Cry6, Cry12, Cry13, Cry14 and Cry21) are known to be toxic to juvenile of a number of free living or parasitic nematodes (Tian *et al*., 2007).

Muhammed *et al*. (2008) studied ten *B. thuringiensis* isolates against *M. incognita* in tomato. Out of the ten isolates Bt7N, BtDen, Bt18, BtK73, BtSoto and Bt7 showed highest mortality of juveniles (86.00 to 100.00 per cent). Also they studied the effect of culture fluid (CF), cell-free supernatant (CFS) and cell-pelleted residues (CP) of each isolates for nematicidal activity on tomato plant. The results showed that both crude suspension (CS) and cell-free supernatant (CFS) of isolate Bt7N was the best out of ten. It reduced the number of egg masses by 78.00 and 77.00 per cent, and number of eggs by 84.00 and 76.00 per cent respectively compared to control.

Zi-Quan *et al*. (2008) developed a bioassay method to use the parasporal crystal protein of *B. thuringiensis* against plant parasitic nematodes. Using this method, the parasporal crystal proteins of ten Bt strains tested against plant parasitic nematodes. The resulting LC50 values of strain YBT-021 against *M. hapla, Pratylenchus scribneri, Tylenchorhynchus* sp., *Ditylenchus destructor* Thorne and *Aphelenchoides* sp. were 35.62 mg mL^{-1}, 75.65 mg mL^{-1}, 94.31 mg mL^{-1}, 215.21 mg mL^{-1} and 128.76 mg mL^{-1} respectively.

Ten isolates of *B. thuringiensis* were isolated and evaluated for their nematicidal potential under laboratory and green house conditions. Out of this 10 isolates, isolate BT-64 showed 100 per cent juveniles mortality after 24 h under *in vitro* condition and maximum reduction in gall formation by 76.00 per cent in okra and 79.00 per cent in mung bean under greenhouse condition (Khan *et al.*, 2010).

B. thuringiensis strains ToIr65 and ToIr67 when tested against *M. javanica* showed 70.00 per cent nematicidal activity under *in vitro* condition. In pot experiments ToIr65 significantly decreased number of galls by 15.00 per cent than control also increased growth parameters on tomato (Ravari and Moghaddam, 2015).

2.3.1.5. Endophytic Bacteria

Endophytic bacteria were found internally in root tissue, where they persist in most plant species. They usually promote plant growth and suppress disease development by plant parasitic nematodes. Endophytic bacteria act against phytonematodes as they compete for ecological niche or a substrate, produce inhibitory chemicals against nematodes or induce systemic resistance in host plants.

Endophytic bacterial isolates of *B. subtilis* (EPB 5, 22, 31 and EPC 16) were isolated and prepared in talc-base. They were tested against *M. incognita, P. coffeae, R. similis* and *Helicotylenchus multicinctus* (Cobb) Golden on tissue cultured banana cv. Robusta (Musa AAA). The endophytes enhanced the activity of plant defense enzymes *viz* peroxidase, polyphenol oxidase and phenylalanine ammonia lyase in banana (Jonathan and Umamaheswari, 2006).

Endophytic bacteria were isolated from black pepper and screened against *R. similis* in different varieties of black pepper. Despite the black pepper variety, isolates TC 10 and BP 17 were found highly effective against *R. similis* and they are identified as *B. megaterium* and *Curtobacterium luteum* (Aravind *et al.*, 2010). *B. subtilis* endophytic strain BS5 with high surfactin and iturin activity suppressed hatching of eggs and killed second stage juveniles of *M. incognita* under *in vitro* condition (Kavitha *et al.,* 2012).

Four endophytic bacteria *viz Pantoea agglomerans* (Ewing and Fife) Gavini MK-29, *Cedecea davisae* MK-30, *Enterobacter* spp. MK-42 and *P. putida* MT-19 was studied against early root penetration and gall formation of *M. incognita* in tomato. The four selected endophytic bacteria significantly reduced early root penetration of *Meloidogyne* juveniles into tomato roots up to 56.00 per cent when applied as a root dipping and soil drench (Munif *et al.,* 2013).

2.3.1.6. Symbionts of Entomopathogenic Nematodes

Bacterial symbionts of entomopathogenic nematodes contribute to the symbiotic association by killing the insect and providing a suitable nutrient environment for nematode reproduction. Recent studies reveal that the symbiotic bacteria cause plant parasitic nematode suppression *via* production of defensive compounds. Three types of secondary metabolites have been identified as the nematicidal agent. They are ammonia, indole and stilbene derivative. These were found toxic to second stage juveniles of root knot nematode (*M. incognita*) and fourth stage juveniles and adults of pine wood nematode (*B. xylophilus*), and inhibited egg hatching of *M. incognita* (Hu *et al.,* 1999).

Pseudomonas oryzihabitans Kodoma from entomopathogenic nematode *Steinernema abbasi* and *Xenorhabdus nematophilus* from *S. carpocapsae* were tested against *M. javanica* under *in vitro* condition. The mortality of *Meloidogyne* juveniles was 100 per cent at dosages of 10^6 and 10^7 cells mL^{-1} at 24 h after exposure (Samaliev *et al.,* 2000). *P. oryzihabitans* poses antifungal and nematicidal activity. Under *in vitro* condition Cell-free culture filtrates of *P. oryzihabitans* at 0.10% dilution, showed strong antifungal and nematicidal properties. Fusarium wilt (*Fusarium oxysporum* f. sp. *lycopersici*) and root knot diseases (*Meloidogyne* spp.) were significantly reduced when soil was treated with 10 mL of a 10^4 cells mL^{-1} suspension of *P. oryzihabitans* (Vagelas and Gowen, 2012).

Materials and Methods

3. MATERIALS AND METHODS

The study "Management of reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira in vegetable cowpea using bacterial antagonists" was undertaken in the Department of Nematology, College of Agriculture Vellayani, during 2018-2020. The methods developed and materials used in the study are given below in detail.

3.1. ISOLATION OF INDIGENOUS BACTERIA

3.1.1. Survey

A survey was conducted in the cowpea growing fields from different locations of Thiruvananthapuram district, Kerala during 2018-19 (Plate 1).

3.1.1.1. Collection of Soil and Root Samples

Root and soil samples were collected from rhizosphere of cowpea plants which showed yellowing, stunting and wilting symptoms. Rhizospheric soil (200g) was collected from a depth of 10 to 20 cm in clean polypropylene bag using garden shovel. Root samples (5g) were collected separately into another polypropylene bag. Both the samples were tied tightly and labeled with required information *viz* farmer's name, location, place, date and previous crop.

3.1.2. Isolation and Maintenance of Bacterial Antagonists

Indigenous bacterial antagonists were isolated using standard techniques. Soil, root with egg masses collected from cowpea plants were used for isolation of bacterial antagonists.

3.1.2.1. From Soil

Serial dilution technique was used to isolate bacteria from soil. 10g soil was measured and mixed thoroughly with 90 mL distilled water in a conical flask under sterile condition. This suspension was labeled as stock solution with 10^{-1} dilution. Seven sterile test tubes were taken each with 9 mL of distilled water in a test tube stand, under sterile condition. One mL of previously prepared solution from conical

flask was measured using micropipette. This 1 mL solution was mixed with the sterile water taken in one of the test tubes and marked it as 10^{-2} dilution. The test tube then capped and agitated to mix the solution well. One mL of the solution from test tube with dilution 10^{-2} then transferred to another test tube with 9 mL distilled water, mixed thoroughly and marked it as 10^{-3} dilution. Likewise 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions were prepared. Nutrient agar was prepared, autoclaved and plated in sterile petri plates and allowed to solidify under sterile condition. 0.1 mL from each of 10^{-5}, 10^{-6}, 10^{-7} dilutions were plated separately in to nutrient agar plates using spread plate technique and incubated at 30°C for 48 h. The bacterial colonies formed in each plate were counted separately. The bacterial colonies showing colony characteristics similar to *Bacillus* spp. and *Pseudomonas* spp. were selected. These colonies were then sub cultured on nutrient agar plates for 48 h incubation at 30°C.

3.1.2.2. From Root

Cowpea roots collected were washed under running tap water to remove debris and then air dried. The air dried roots were rinsed with deionized water for three times. The roots were cut in to pieces of 2 to 3 cm and surface sterilized by immersing in 75% (v v^{-1}) ethanol for 2 min and 1% mercuric chloride for 1 min. Then roots were washed with sterile distilled water three times to remove surface sterilization agents. Surface sterilized roots (0.2g) were ground in a mortar and pestle with 10 mL sterile distilled water (Sheng *et al.*, 2008). Serial dilutions were prepared from the solution obtained and then plated on nutrient agar media by spread plate method. Plates were incubated for 48 h at 30°C. The bacterial isolates showing colony characters similar to *Bacillus* and *Pseudomonas* were sub cultured and stored under refrigerated condition.

3.1.2.3. From Egg Mass

Egg masses collected from feeder roots of *R. reniformis* infested cowpea plants were surface sterilized with 0.1% sodium hypochlorite (NaOCl) solution for one min. Surface sterilized egg masses were rinsed with sterile distilled water three times (Dhawan and Singh, 2010) and placed on nutrient agar medium in petri plates. The plates were incubated at 28±2°C for 48 h. The bacterial isolates obtained were sub cultured by streak plate method and stored in slants of nutrient agar under refrigerated
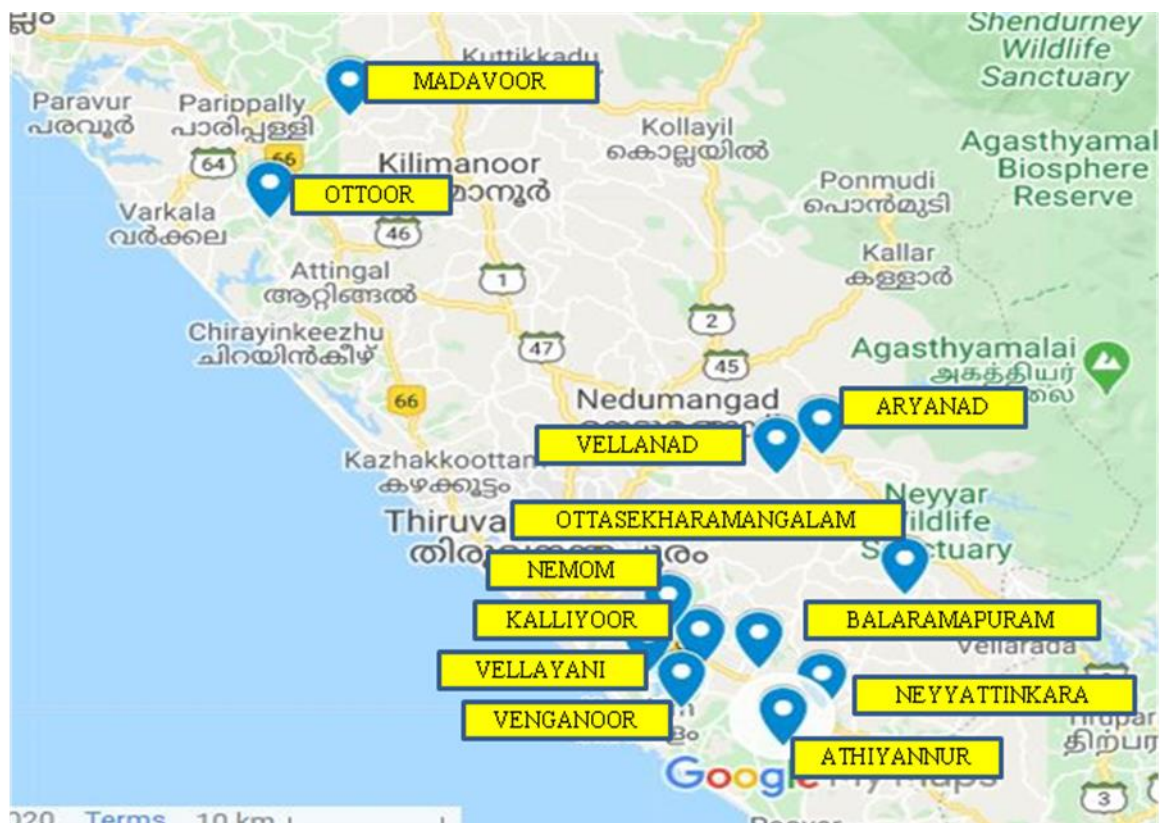
**Plate 1. Location selected for sample collection from cowpea in
Thiruvananthapuram district, Kerala**

condition.

The composition of culture media as follows

1. Nutrient agar media

Peptone	-5 g
Beef extract/Yeast extract	-3 g
Sodium chloride	-5 g
Agar	-15 g
Distilled water	-1000 mL

   pH adjusted to neutral (6.8) at 25 °C

2. Nutrient broth media

Peptone	-5 g
Beef extract/Yeast extract	-3 g
Sodium chloride	-5 g
Distilled water	-1000 mL

3.1.3. Maintenance of Reniform Nematode Culture

Reniform nematode culture was maintained by *in vivo* culturing technique in which the nematodes are maintained on live plants. Vellayani Geetika, the susceptible cultivar of cowpea plant was used to culture reniform nematode. Earthen pots of 5 kg capacity were used for planting. Potting mixture was prepared by mixing soil, compost and coir pith in 1:1:1 ratio. The potting mixture was solarized for 7 days, for sterilization. The pots were filled with sterilized potting mixture. Cowpea seedlings raised in pot trays filled with coir pith and compost mixture was transplanted to pots prepared. The reniform nematode egg masse were collected from naturally infested plants and transferred to culture plates containing sterile distilled water. This suspension was incubated for 1 week at 25±2°C for hatching (Niknam and Dhawan, 2002a). The juvenile suspension of *R. reniformis* was drenched in to the root zone of cowpea seedlings @ 5 mL pot^{-1}. Infestation of cowpea was confirmed by locating reniform female and eggs masses on cowpea roots (Plate 2a and b). The inoculated plants were maintained in glass house for collection of egg masses.

3.1.4. Bioassays for Nematicidal Activity

3.1.4.1. Preparation of Nematode Suspension

Feeder roots from *R. reniformis* infected plants were collected and washed gently to remove large debris. The roots were cut in to small pieces. To extract the eggs, root bits were shaken in 1% sodium hypochlorite solution for 4 min at 120 rpm (Castillo *et al*., 2013). The solution was sieved through 20 mesh sieve, to separate root bits, followed by 300 mesh sieve and collected the eggs. The eggs collected in the sieve were rinsed with water to remove the excess sodium hypochlorite solution. Then eggs were washed and collected in to a clean beaker. The suspension was incubated for 5 to 6 days at 28±2°C for egg hatching using modified Baermann's funnel technique (Schindler, 1961). The top of the wire mesh was covered with double layered tissue paper with the edges folded up to avoid water dripping off. The suspension with eggs was poured through the wire mesh with tissue paper so that the eggs were retained over the tissue. The bottom of Petri plate filled with water and water level was maintained 5 mm above the wire mesh. Nematode juveniles extracted in the petri plate were collected after 5 to 6 days.

3.1.4.2. Preparation of Cell Free Extracts (CFE) of Bacterial Isolates

A loop full of bacterial cells was inoculated in to 100 mL nutrient broth in 250 mL conical flask. The conical flask was incubated at 28±2°C for 48 h at 100 rpm in an incubator shaker. The nutrient broth culture of bacteria was centrifuged at 8000 rpm for 5 min. The supernatant after centrifugation was collected and filtered through bacteriological filters of 0.2 μm size aseptically in to sterile vials (Plate 3).

3.1.4.3. Testing Efficacy of Bacterial Isolates

All the bacterial isolates collected as mentioned in para 3.1.2 were tested for their efficacy on mortality of *R. reniformis* juveniles. CFE of bacterial isolates were prepared as mentioned in para 3.1.4.2. Fifty juveniles of *R. reniformis* were suspended in 5 mL of CFE of bacterial isolates. The mortality was recorded 24 hours after treatment (HAT). Isolates which showed more than 50 per cent juvenile mortality were selected for further study (Plate 4).

Plate 2a. Reniform nematode female on cowpea root

Plate 2b. Egg mass of reniform nematode on cowpea root

Plate 3. Cell free culture of bacterial isolates under study

Plate 4. Selected twenty effective isolates

3.1.4.4. Estimation of Colony Forming Units (cfu)

Number of colonies of bacterial isolates selected after testing their efficacy was counted using a plate counter. The number of colonies were counted and multiplied with the dilution factor to get the number of cfu.

3.1.5. Preliminary Screening of Selected Effective Bacterial Isolates

The bacterial isolates which showed more than 50 per cent mortality at 24 HAT from were selected for testing the mortality at different concentrations (S, S/2, S/3 and S/4).

3.1.5.1. Preparation of Different Concentrations of CFE

The different concentrations *viz* S (100 %), S/2 (50 %), S/3 (33.33 %) and S/4 (25%) were prepared as follows.

S   – 100% cell free extract (5 mL CFE)

S/2   – 50% cell free extract (2.5 mL CFE + 2.5 mL sterile water)

S/3   – 33.33% cell free extract (1.67 mL CFE + 3.33 mL sterile water)

S/4   – 25% cell free extract (1.25 mL CFE + 3.75 mL sterile water)

3.1.5.2. Testing Efficacy of Bacterial Isolates in vitro

The different concentrations of CFE of effective bacterial isolates were prepared as mentioned in 3.1.5.1. Fifty juveniles of *R. reniformis* were suspended in 5 mL of each concentration of selected bacterial isolate CFE in sterile vials. All the vials were kept in BOD incubator at 28°C. The number of dead and live juveniles was recorded at 6, 12 and 24 HAT. The per cent juvenile mortality was calculated from the data. Plain nutrient broth and sterile distilled water were taken as control.

Design – CRD   Treatment – 6   Replication – 4

T1 – CFE 100% concentration   T2 – CFE 50% concentration

T3 – CFE 33.33% concentration   T4 – CFE 25% concentration

T5 – Sterile water   T6 – Plain Broth

3.2. SCREENING OF BACTERIAL ISOLATES FOR THEIR NEMATICIDAL PROPERTY AGAINST *R. reniformis*

The isolates selected from 3.1.5. were tested for their ovicidal and larvicidal effect against *R. reniformis*. The different concentrations of CFE of the selected bacterial isolates were compared for their effect on egg hatch inhibition and juvenile mortality. The concentrations of CFE were prepared as mentioned in para 3.1.5.1.

3.2.1. Effect on Egg Hatching

The egg masses were collected from the reniform nematode infested cowpea roots. Equal sized egg masses were picked from roots using sterile needle. Surface sterilized egg masses were suspended in different concentrations of CFE of bacterial isolates. Surface sterilized egg masses placed in plain broth and sterile distilled water were maintained as control. The plates were incubated at 28°C and number of hatched and unhatched eggs was recorded at 3, 4, 5, 6, and 7 days after treatment (DAT).

Design – CRD   Treatment – 18   Replication – 4

T1 – CFE of isolate 1 (100%)   T2 – CFE of isolate 1 (50%)

T3 – CFE of isolate 1 (33.33%)   T4 – CFE of isolate 1 (25%)

T5 – CFE of isolate 2 (100%)   T6 – CFE of isolate 2 (50%)

T7 – CFE of isolate 2 (33.33%)   T8 – CFE of isolate 2 (25%)

T9 – CFE of isolate 3 (100%)   T10 – CFE of isolate 3 (50%)

T11 – CFE of isolate 3 (33.33%)   T12 – CFE of isolate 3 (25%)

T13 – CFE of isolate 4 (100%)   T14 – CFE of isolate 4 (50%)

T15 – CFE of isolate 4 (33.33%)   T16 – CFE of isolate 4 (25%)

T17 – Sterile water   T18 – Plain Broth

3.2.2. Effect on Juvenile Mortality

R. reniformis juvenile suspension was prepared as mentioned in para 3.1.4.1. Fifty juveniles each were suspended in four different concentrations (S, S/2, S/3 and S/4) of selected bacterial isolates in sterile vials. Fifty juveniles each in plain broth and sterile distilled water were kept as control. The vials were incubated at 28°C for 24 h and the mortality of juveniles were recorded at 6, 12 and 24 HAT.

Design – CRD   Treatments – 18   Replications – 4

T1 – CFE of isolate 1 (100%)	T2 – CFE of isolate 1 (50%)
T3 – CFE of isolate 1 (33.33%)	T4 – CFE of isolate 1 (25%)
T5 – CFE of isolate 2 (100%)	T6 – CFE of isolate 2 (50%)
T7 – CFE of isolate 2 (33.33%)	T8 – CFE of isolate 2 (25%)
T9 – CFE of isolate 3 (100%)	T10 – CFE of isolate 3 (50%)
T11 – CFE of isolate 3 (33.33%)	T12 – CFE of isolate 3 (25%)
T13 – CFE of isolate 4 (100%)	T14 – CFE of isolate 4 (50%)
T15 – CFE of isolate 4 (33.33%)	T16 – CFE of isolate 4 (25%)
T17 – Sterile water	T18 –Plain Broth

3.3. PATHOGENICITY TESTS OF BACTERIAL ISOLATES

Pathogenicity test of the selected isolates was done in the glass house of Department of Nematology, College of Agriculture, Vellayani. Potting mixture was prepared by mixing soil, compost and coir pith in 1:1:1 ratio. The potting mixture was sterilized by autoclaving at 15 lbs pressure and 120°C temperature for 15 to 20 min. Cups with 150 mL capacity were filled with 100g sterilized potting mixture. Cowpea seeds of variety Vellayani Jyothika were sown in the cups. Bacterial isolates were inoculated in five day old seedlings by three different methods. Expressions of pathogenicity were observed one and two weeks after inoculation of bacterial isolates.

a. Soil drenching method

The nutrient broth of selected bacterial isolates containing 1×10^7 cfu mL^{-1} were drenched in to the root zone of cowpea seedlings @ 2 mL cup^{-1}. Three holes were made around the plant in each pot and nutrient broth of selected bacterial isolates was poured in to the holes.

b. Pin-prick method

Bacterial isolates were inoculated on the lower surface of leaves by pricking with sterilized needle in multiple points. Cotton swabs soaked in nutrient broth of bacterial isolates (1×10^7 cfu mL^{-1}) were placed on the pricked area. Then the inoculated leaves were covered with plastic bags.

c. Spray Inoculation Method

Nutrient broth of bacterial isolates was sprayed on the leaves of cowpea plant with the help of a hand sprayer. Sprayed plants were incubated in humid condition.

Design – CRD   Treatment – 5   Replication – 4

T1 – Bacterial isolate 1

T2 – Bacterial isolate 2

T3 – Bacterial isolate 3

T4 – Bacterial isolate 4

T5 – Untreated check

3.4. MANAGEMENT OF RENIFORM NEMATODE

Pot culture study was conducted using selected bacterial isolates for testing their effect on multiplication of *R. reniformis* in vegetable cowpea. The study was conducted in glass house.

3.4.1. Preparation of Sterilized Potting Mixture and Sowing

Pots of 2 kg capacity were used for the study. Potting mixture was prepared by mixing soil, compost and coir pith at 1:1:1 ratio. The mixed potting mixture was then

packed in to polypropylene bags and autoclaved at 15 lbs pressure and 121°C temperature for 20 min. This potting mixture was filled in twenty plastic pots. Cowpea seeds of variety Vellayani Jyothika were sown @ 2 seeds pot^{-1} and at the seedling stage it was thinned to one seedling pot^{-1}.

3.4.2. Soil Drenching of Selected Bacterial Isolates

The nutrient broth of selected four bacterial isolates ($1×10^7$ cfu mL^{-1}) was drenched in the root zone of cowpea seedlings @ 2 mL 100g soil^{-1} 48 h before inoculation of *R. reniformis*.

3.4.3. Nematode Inoculation

The juvenile suspension of *R. reniformis* was prepared as mentioned in para 3.1.4.1. The juvenile suspension was drenched to the root zone of cowpea seedlings @ 2 juveniles g soil^{-1}.

Design – CRD   Treatment – 5   Replication – 4

T1 – Bacterial isolate 1

T2 – Bacterial isolate 2

T3 – Bacterial isolate 3

T4 – Bacterial isolate 4

T5 – Untreated check

3.4.6. Recording Observation

3.4.6.1. Estimation of Nematode Population in Soil

Soil samples (200cc) were collected from the rhizosphere of cowpea plants from each pot. The nematodes were extracted from soil by Cobb's sieving and decanting technique (Cobb, 1918) and modified Baermann's funnel technique (Schindler, 1961). Number of nematodes in soil was counted using counting dish and hand tally counter under a stereo microscope.

3.4.6.2. Number of Females in Root

Root samples (5g) were collected from the cowpea plants. The roots were cut in to 2 to 3 cm pieces and stained it with acid fuschsin-lactophenol dye. Acid fuschsin stock solution was prepared by dissolving 3.5g acid fuschsin in 250 mL of acetic acid and 750 mL of distilled water mixture. Lacto phenol was prepared by mixing liquid phenol (94 mL), lactic acid (83 mL), glycerin (160 mL) and distilled water (100 mL) (Van Bezooijen, 2006). The working solution of stain was prepared my mixing 1 mL of acid fuschsin stock solution to 100 mL of lactophenol solution. The stain was taken in a beaker and boiled it on a hot plate. The roots from each treatment was put in to the boiling stain for 1 min and washed in tap water. The roots were destained in lactophenol solution until the maximum contrast between the nematode and the root tissue was obtained. The processed roots were then observed under stereo microscope and counted females.

3.4.6.3. Number of Egg Masses in Root

The phloxine B solution was prepared by mixing 0.15g of phloxine B in 1L of water. Stained egg masses in roots were counted under a stereo zoom microscope.

3.4.6.4. Reproduction Factor

The reproduction factor of reniform nematode was calculated by the formula $Rf = Pf/Pi$ where Pf represent the final nematode population (nematodes in soil and females in root) and Pi represent the initial nematode population.

3.5. IDENTIFICATION OF BACTERIAL ISOLATES

3.5.1. Morphological and Cultural Characterization

3.5.1.1. Gram Staining

The most effective bacterial isolates from para 3.1.4.3 were gram stained. A smear of the bacterial isolates was made on a clean glass slide and heat fixed. A drop of crystal violet dye was added on the slide and washed under running water. Then a drop of mordant (Grams iodine) was added and washed under running water after 1 min. The smear then decolorized with ethanol. Then safranin was added as a counter

stain and washed under running water. Slides were then observed under stereo microscope. The gram positive cells were violet colored and gram negative cells were purple colored (Cyrabree and Hindshill, 1975).

3.5.2. Molecular Characterization

3.5.2.1. Genomic DNA Isolation from Bacteria

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

A part of culture is taken in a microcentrifuge tube. 180 µL of T1 buffer and 25 µL of proteinase K was added and incubated at 56°C in a water bath until it was completely lysed. After lysis, 5 µL of RNase A (100mg mL^{-1}) was added and incubated at room temperature for 5 minutes. 200 µL of B3 buffer was added and incubated at 70°C for 10 minutes. 210 µL of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 mL collection tube and centrifuged at 11000 x g for 1 min. The NucleoSpin® Tissue column was transferred to a new 2 mL tube and washed with 500 µL of BW buffer. Wash step was repeated using 600 µL of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5 mL tube and DNA was eluted out using 50 µL of BE buffer.

3.5.2.2. Agarose Gel Electrophoresis for DNA Quality and Quantity Check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µL of 6X gel-loading buffer (0.25 per cent bromophenol blue, 30 per cent sucrose in TE buffer pH 8.0) was added to 5µL of DNA. The samples were loaded to 0.80 per cent agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5µg mL^{-1} ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

PCR Analysis Mixture

2X Phire Master Mix	5μL
D/W	4μL
Forward Primer	0.25μL
Reverse Primer	0.25μL
DNA	1μL

Primers used

Target	Primer name	Direction	Sequence (5' → 3')
16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

PCR amplification profile conditions

16S rRNA

95°C - 5 min

95°C - 30 sec

60°C - 40 sec ⎤ 35 cycles

72°C - 60 sec

72°C - 7 min

4°C - ∞

3.5.2.3. Agarose Gel Electrophoresis of PCR Products

The PCR products were checked in 1.2 per cent agarose gels prepared in 0.5X TBE buffer containing 0.5μg mL^{-1} ethidium bromide. 1μL of 6X loading dye was mixed with 4μL of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 h, until

the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.5.2.4. ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 0.5µl of ExoSAP-IT and incubated at 37ºC for 15 min followed by enzyme inactivation at 85ºC for 5 min.

3.5.2.5. Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.

The Sequencing PCR mix consisted of the following components:

D/W	6.6µL
5X Sequencing Buffer	1.9µL
Forward Primer	0.3µL
Reverse Primer	0.3µL
Sequencing Mix	0.2µL
Exosap treated PCR product	1µL

PCR amplification profile

96ºC   -   2min

96ºC   -   30sec ⎫
50ºC   -   40sec ⎬ 30 cycles
60ºC   -   4min ⎭

4°C - ∞

3.5.2.6. Post Sequencing PCR Clean up

D/W	5 μL
3M Sodium Acetate	1 μL
EDTA	0.1 μL
100 per cent Ethanol	44 μL

1. Mix D/W, 125mM EDTA, 3M sodium acetate pH 4.6 and ethanol were prepared and were properly mixed.
2. 50 μL of mix was added to each well in the sequencing plate containing sequencing PCR product.
3. Vortex by Mixmate vortex and Incubated at room temperature for 30 min
4. Spun at 3700 rpm for 30 min
5. Decanted the supernatant and added 50 μl of 70 per cent ethanol to each well
6. Spun at 3700 rpm for 20 min.
7. Decanted the supernatant and repeated 70 per cent ethanol wash
8. Decanted the supernatant and air dried the pellet.

The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

3.5.2.7. Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems).

3.6. STATISTICAL ANALYSIS

The data from the experiment (3.1 to 3.4) were subjected to analysis of variance (ANOVA) test (Cochran and Cox, 1965). Those variables which did not satisfy the basic assumptions of ANOVA were subjected to angular or square root transformations.

Results

4. RESULTS

The results of the study "Management of reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira in vegetable cowpea using bacterial antagonists" are presented below.

4.1. ISOLATION OF INDIGENOUS BACTERIA

4.1.1. Survey

A random sampling was done in cowpea grown fields of Aryanad, Athiyannur, Balaramapuram, Kalliyoor, Madavoor, Nemom, Neyyattinkara, Ottasekharamangalam, Ottoor, Vellanad, Vellayani and Venganoor area of Thiruvananthapuram district and a total of sixty six each of soil and root samples were collected from the rhizosphere of cowpea. Isolation of bacteria was done and colonies showing characters similar to *Bacillus* (opaque or translucent colonies with white colour and wavy margins) and *Pseudomonas* (raised or convex, circular colonies with undulating or entire margin) were selected to test their efficacy against reniform nematode (Tables 1 to 3).

A total of eight samples were collected from rhizosphere of reniform nematode infected cowpea plants from Aryanad (Table 1). From eight samples two bacterial isolates were obtained, one from soil (isolate 17) and one from egg mass (isolate 16). Cell free extract (CFE) of isolate 16 obtained from eggmass of *R. reniformis* and isolate 17 from soil at 100 per cent concentration showed 70.50 and 49.50 per cent mortality of *R. reniformis* juveniles respectively. Isolates 32, 33, 34, 35 and 36 obtained from soil (4) and root (4) samples collected from cowpea grown areas of Athiyannur showed 45.00, 35.50, 43.50, 49.00 and 37.00 per cent mortality of *R. reniformis* juveniles respectively. Four samples (soil-2 and root-2) were collected from rhizosphere of cowpea plants in Balaramapuram area. Isolate 7 obtained from the root samples showed 43.50 per cent juvenile mortality. Isolates 1, 2, 3 and 4 obtained from soil (4) and root (4) samples collected from Kalliyoor showed 79.00, 36.00, 49.00 and 42.00 per cent juvenile mortality respectively under *in vitro* conditions (Table 1).

Table 1. Details of bacterial isolates collected from Aryanad, Athiyannur, Balaramapuram and Kalliyoor

Place	No. of samples collected		Disease symptoms observed in cowpea	Previous crops	Location coordinates	Mortality of *R. reniformis* juveniles		
	Soil (200cc)	Root (5g)				Isolates collected from		
						Soil	Root	Egg mass
Aryanad	4	4	Yellowing	Banana	8.569732° 77.080824°	Isolate 17 (49.50%)	-	-
			Rotting, Necrosis	Banana	8.569526° 77.093118°	-	-	Isolate 16 (70.50%)
Athiyannur	4	4	Yellowing	Vegetables	8.386071° 77.066711°	-	Isolate 34 (43.50%)	-
			Wilting	Banana	8.386528° 77.066169°	Isolate 33 (35.50%)	-	-
			Dead Plant	Banana vegetables	8.384968° 77.067413°	Isolate 36 (37.00%)	-	Isolate 32 (45.00%)
			Yellowing, Necrosis	Vegetables	8.384092° 77.067805°	-	-	Isolate 35 (49.00%)
Balaramapuram	2	2	Healthy	Banana	8.402943° 77.015254°	-	Isolate 7 (43.50%)	-
Kalliyoor	4	4	Root rotting, wilting	Cowpea vegetables	8.30168° 76.981530°	-	-	Isolate 1 (79.00%)
			Healthy	banana	8.33067° 77.013788°	-	Isolate 2 (36.00%)	-
			Wilting	banana	8.430624° 76.974524°	Isolate 4 (42.00%)	-	-
			Necrosis, Wilting	Banana vegetables	8.432846° 77.012450°	Isolate 3 (49.00%)	-	-

Figures in parenthesis are juvenile mortality recorded by respective isolates

From Madavoor, sixteen soil and root samples were collected from rhizosphere of cowpea plants which showed symptoms like root rotting, wilting, yellowing, rotting and necrosis. CFE of five bacterial isolates obtained were screened for juvenile mortality. Isolates 42, 43 and 45 were obtained from rhizosphere soil, isolate 41 from root and 44 from eggmass. They showed 40.00, 83.00, 33.00, 74.50 and 40.00 per cent mortality of *R. reniformis* juveniles respectively. Twelve samples were collected from rhizosphere of cowpea plant having symptoms like yellowing and wilting from Nemom. Isolate 5 and 6 obtained from soil showed 50.00 and 42.00 per cent juvenile mortality respectively under *in vitro* conditions. Three isolates (Isolate 22, 23 and 24) were obtained from the eight samples collected from rhizosphere of cowpea plants which showed reniform nematode infestation symptoms (yellowing and wilting) from Neyyattinkara. Isolate 24 was obtained from soil, isolate 22 from root and isolate 23 from eggmass. CFE of these three bacterial isolates showed 36.00, 47.50 and 47.50 per cent juvenile mortality respectively under *in vitro* conditions. Eight isolates were obtained from soil samples collected from rhizosphere of cowpea plants from Ottasekharamangalam. Isolate 8, 9, 11 and 15 were from soil, isolate 10 and 13 were from root and isolate 12 and 14 were isolated from eggmass of *R. reniformis*. CFE of these eight isolates showed 58.50, 64.50, 45.00, 99.50, 50.50, 42.00, 92.50 and 50.00 per cent mortality of *R. reniformis* juveniles respectively under *in vitro* conditions (Table 2).

CFE of Isolate 30 and 31 obtained from cowpea rhizosphere soil from Ottoor area showed 50.00 and 40.00 per cent *R. reniformis* juvenile mortality respectively. Isolate 18 (from eggmass), 19 (from soil), 20 (from root) and 21 (from soil) were obtained from cowpea grown area in Vellanad CFE of these four bacterial isolates showed 79.00, 60.00, 54.50 and 53.50 per cent juvenile mortality respectively. Four samples, two each of soil and roots were collected from the rhizosphere of cowpea grown in College of Agriculture, Vellayani Campus with natural infestation of reniform nematode. CFE of five isolates obtained, Isolate 25, 26, 27, 28 and 29 showed 98.50, 100.00, 63.50, 100.00 and 63.50 per cent juvenile mortality respectively. Eighteen samples were collected from rhizosphere of cowpea plant with symptoms like wilting, yellowing and root totting from Venganoor. CFE of isolate 37, 38, 39 and 40 showed

Table 2. Details of bacterial isolates collected from Madavoor, Nemom, Neyyattinkara and Ottasekharamangalam

Place	No. of samples collected		Disease symptoms observed in cowpea	Previous crops	Location coordinates	Mortality of *R. reniformis* juveniles		
						Isolates collected from		
	Soil (200cc)	Root (5g)				Soil	Root	Egg mass
Madavoor	8	8	Root rotting, Wilting	vegetables	8.810880° 76.823246°	Isolate 42 (83.00%)	-	Isolate 44 (74.50%)
			Yellowing, Wilting	vegetables	8.811071° 76.821282°	Isolate 45 (40.00%)	Isolate 41 (40.00%)	-
			Wilting	Tapioca, vegetables	8.812232° 76.824619°	Isolate 43 (33.00%)	-	-
Nemom	6	6	Wilting	Vegetables, banana	8.458368° 77.004586°	Isolate 5 (50.00%)	-	-
			Yellowing, Wilting	Banana	8.459403° 77.003175°	Isolate 6 (42.00%)	-	-
Neyyattinkara	4	4	Yellowing	Banana, vegetables	8.405681° 77.077153°	-	Isolate 22 (36.00%)	-
			Yellowing, Wilting	Banana, vegetables	8.404460° 77.078537°	Isolate 24 (47.50%)	-	Isolate 23 (47.50%)
Ottasekharaman galam	9	9	Yellowing	Banana	8.495111° 77.126371°	Isolate 8 (58.50%)	-	Isolate 12 (50.50%)
			Wilting	Vegetables, banana	8.489849° 77.125405°	Isolate 9 (64.50%)	Isolate 10 (45.00%)	-
			Root rotting, Wilting	Banana, tapioca	8.489932° 77.127553°	Isolate 11 (99.50%)	-	Isolate 14 (92.50%)
			Yellowing	Banana	8.488896° 77.127690°	Isolate 15 (50.00%)	Isolate 13 (42.00%)	-

Figures in parenthesis are juvenile mortality recorded by respective isolates

Table 3. Details of bacterial isolates collected from Ottoor, Vellanad, Vellayani and Venganoor

| Place | No. of samples collected | | Disease symptoms observed in cowpea | Previous crops | Location coordinates | Mortality of *R. reniformis* juveniles | | |
| | | | | | | Isolates collected from | | |
	Soil (200cc)	Root (5g)				Soil	Root	Egg mass
Ottoor	5	5	Yellowing, Wilting	Banana	8.736494° 76.775637°	Isolate 30 (50.00%)	-	-
			Healthy	Banana	8.735752° 76.775218°	Isolate 31 (40.00%)	-	-
Vellanad	6	6	Wilting	Cowpea, banana	8.551624° 77.056157°	-	-	Isolate 18 (79.00%)
			Necrosis, Wilting	Vegetables, banana	8.549979° 77.056641°	Isolate 19 (60.00%)	-	-
			Wilting	Banana	8.558614° 77.053802°	Isolate 21 (53.50%)	Isolate 20 (54.50%)	-
Vellayani	**2**	**2**	Yellowing, Wilting	Gourds, vegetables	8.429649° 76.990262°	Isolate 29 (63.50%)	-	Isolate 25 (98.50%)
								Isolate 26 (100.00%)
			Yellowing, Wilting, Root rotting	Bitter gourd, okra	8.428822° 76.990073°	Isolate 28 (100.00%)	-	Isolate 27 (63.50%)
Venganoor	**9**	**9**	Yellowing		8.401223° 76.993903°	Isolate 37 (65.50%)	Isolate 40 (39.50%)	Isolate 39 (65.00%)
			Root totting, Wilting		8.400953° 76.994295°	Isolate 38 (46.00%)	-	-

Figures in parenthesis are juvenile mortality recorded by respective isolates

65.50, 46.00, 65.00 and 39.50 per cent juvenile mortality under *in vitro* conditions (Table 3).

Out of forty five isolates selected, twenty most effective isolates with 50.50 to 100.00 per cent *R. reniformis* juvenile mortality were selected for preliminary screening. The selected isolates are isolate 26, 28, 25, 11, 14, 42, 1, 18, 44, 16, 37, 39, 9, 29, 27, 19, 8, 20, 21 and 12.

4.1.2. Colony Characters and cfu mL^{-1} of Selected Bacterial Isolates

The colony characters in nutrient agar plate (Plate 5) and cfu mL^{-1} of the selected bacterial isolates are presented in Table 4.

a. Isolate 26

White opaque characteristically large rhizoid colonies with filamentous margin, colony texture is dry, Gram staining positive with 2×10^8 cfu mL^{-1} was observed.

b. Isolate 28

Creamy-white to yellowish, opaque, circular and convex, medium sized colonies with entire margin, colony texture moist gram staining positive with 3×10^7 cfu mL^{-1} was observed.

c. Isolate 25

White small circular colonies with glossy surface slightly raised and entire margin, colony texture moist, gram staining positive with 4×10^7 cfu mL^{-1} was observed.

d. Isolate 11

Colonies are creamy white to white, circular medium sized, with coarsely wrinkled or folded margin, texture is moist, opaque, gram staining positive with 4×10^8 cfu mL^{-1} was observed.

e. Isolate 14

Colourless, translucent, circular colonies with smooth margin, texture mucoid, medium sized colonies, gram staining negative with 5×10^6 cfu mL^{-1} was observed.

f. Isolate 42

Whitish to creamy, moist, opaque, large colonies with smooth wavy margin, gram staining positive with 3×10^7 cfu mL^{-1} was observed.

Isolate 26	Isolate 28	Isolate 25	Isolate 11	Isolate 14
Isolate 42	Isolate 1	Isolate 18	Isolate 44	Isolate 16
Isolate 37	Isolate 39	Isolate 9	Isolate 29	Isolate 27
Isolate 19	Isolate 8	Isolate 20	Isolate 21	Isolate 12
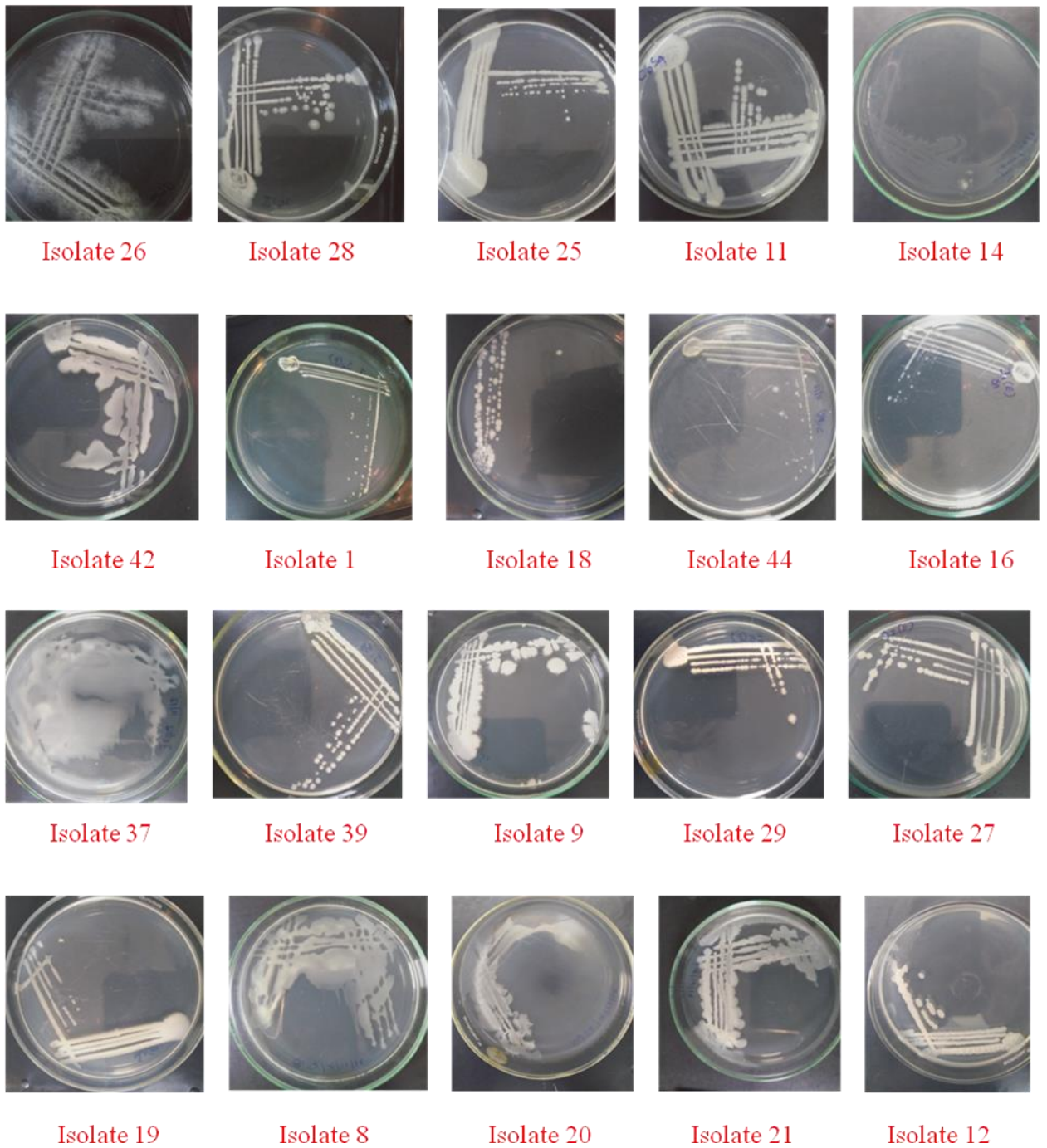
Plate 5. Growth of selected bacterial isolates on nutrient agar

Table 4. Morphological and cultural characteristics of bacterial isolates

Isolates	Colony shape	Colony colour	Colony size	Colony margin	Colony texture	Colony opacity	Gram staining	Cfu* mL^{-1}
Isolate 26	Rhizoid	White	Large	Filamentous	Dry	Opaque	positive	2×10^8
Isolate 28	Circular and convex	Creamy white to yellowish	Medium	Entire margin	Moist	Opaque	positive	3×10^7
Isolate 25	Circular slightly raised	White	Small	Smooth	Moist	Opaque	positive	4×10^7
Isolate 11	Circular	Creamy white to white	Medium	Undulating	Moist	Opaque	positive	4×10^8
Isolate 14	Circular	Colourless	Medium	Smooth	Mucoid	Translucent	negative	5×10^6
Isolate 42	Circular	Whitish to creamy	Large	Smooth wavy	Moist	Opaque	positive	3×10^7
Isolate 1	Pinpoint	whitish to creamy	Small	Smooth	Moist	Opaque	positive	6×10^7
Isolate 18	Circular	Whitish	Small	Smooth	Mucoid	Opaque	positive	2×10^8
Isolate 44	Pin point	Creamy white	Small	Smooth	Moist	Opaque	negative	4×10^7
Isolate 16	Circular	White	Small	Smooth	Viscid	Opaque	positive	3×10^6
Isolate 37	Circular	White	Large	Undulating	Moist	Opaque	positive	7×10^6
Isolate 39	Circular, convex	Creamy white	Small	Smooth	Dry	Opaque	positive	4×10^8
Isolate 9	Circular	White	Large	Wavy	Dry	Opaque	positive	1×10^8
Isolate 29	Circular	Off white	Small	Smooth	Moist	Opaque	positive	3×10^7
Isolate 27	Circular	White	Medium	Smooth	Moist	Opaque	positive	7×10^6
Isolate 19	Pin point	Off white	Small	Smooth	Viscid	Opaque	negative	2×10^6
Isolate 8	Circular	White	Medium	Smooth	Viscid	Opaque	positive	3×10^7
Isolate 20	Circular	White	Medium	Wavy	Moist	Opaque	positive	4×10^6
Isolate 21	Circular	Creamy white	Large	Entire margin	Dry	Opaque	positive	5×10^7
Isolate 12	Circular	Off white	Medium	Smooth	Dry	Opaque	positive	2×10^7

Cfu* Colony forming units

g. Isolate 1

Small, whitish to creamy, moist, opaque, pin point colonies, gram staining positive with 6×10^7 cfu mL^{-1} was observed.

h. Isolate 18

Circular, small, mucoid, opaque, whitish colonies with smooth margin, gram staining positive with 2×10^8 cfu mL^{-1} was observed.

i. Isolate 44

Small, opaque, moist, creamy white, pinpoint colonies with smooth margin, gram staining negative with 4×10^7 cfu mL^{-1} was observed.

j. Isolate 16

Circular, white, opaque, viscid, small colonies with smooth margin, gram staining positive with 3×10^6 cfu mL^{-1} was observed.

k. Isolate 37

White, large, circular, moist, opaque colonies with undulating margin, gram staining positive with 7×10^6 cfu mL^{-1} was observed.

l. Isolate 39

Creamy, small, circular raised or convex, opaque, dry textured colonies with smooth margin, gram staining positive with 4×10^8 cfu mL^{-1} was observed.

m. Isolate 9

White, large, circular, opaque, dry textured colonies with irregular wavy margin, gram staining positive with 1×10^8 cfu mL^{-1} was observed.

n. Isolate 29

Raised circular, small, off-white, moist, opaque colonies with smooth margin, gram staining positive with 3×10^7 cfu mL^{-1} was observed.

o. Isolate 27

White, opaque, moist, medium sized, circular colonies with smooth margin, gram staining positive with 7×10^6 cfu mL^{-1} was observed.

p. Isolate 19

Small pin point, viscid (stick to loop), opaque colonies with smooth margin and off-white colour, gram staining negative with 2×10^6 cfu mL^{-1} was observed.

q. Isolate 8

Medium sized, opaque, viscid, whitish colonies with smooth margin, gram staining positive with 3×10^7 cfu mL^{-1} was observed.

r. Isolate 20

Medium sized, moist, opaque, white colonies with wavy margin, gram staining positive with 4×10^6 cfu mL^{-1} was observed.

s. Isolate 21

Large circular, dry textured, opaque colonies with entire margin and creamy white colour, gram staining positive with 5×10^7 cfu mL^{-1} was observed.

t. Isolate 12

Off white, medium sized, circular, dry textured colonies with smooth margin, gram staining positive with 2×10^7 cfu mL^{-1} was observed.

4.1.3. Preliminary Screening of Bacterial Isolates for Nematicidal Property

Efficacy of twenty bacterial isolates was tested at 100, 50, 33.33 and 25% concentrations and observations were recorded 6, 12 and 24 hours after treatment. Results are given in Table 5 to 24.

4.1.3.1. Isolate 26

CFE of isolate 26 at four different concentrations (100, 50, 33.33 and 25%) showed statistically significant superiority over control (plain broth and sterile distilled water) at 6, 12 and 24 hours after treatment (HAT). Highest mortality of *R. reniformis* juveniles (72.50 per cent) was recorded by at 100% concentration at 6 HAT and it was significantly superior to all other concentrations. Lower concentrations (50, 33.33 and 25%) recorded 55.50, 50.50 and 36.00 per cent mortality of *R. reniformis* and effect of these three treatments was significantly different. At 12 HAT, the highest juvenile mortality (95.50 per cent) was recorded by 100% concentration of isolate 26 and it showed statistically significant variation from lower concentrations (50, 33.3 and 25%). 50% concentration recorded 75.50 per cent juvenile mortality and it was statistically on par with 33.33% concentration (63.00 per cent). CFE of isolate 26 at 25% concentration recorded 54.50 per cent juvenile mortality and it was statistically on par with 33.33% concentration. At 24 HAT, the

highest juvenile mortality was recorded at 100% concentration (100.00 per cent) and it was significantly different from lower concentrations (50, 33.3 and 25%). CFE of isolate 26 at 50% concentration recorded 90.00 per cent juvenile mortality and it was significantly different from 33.33 and 25% concentrations which recorded juvenile mortality of 79.00 and 73.50 per cent respectively. At 25% concentration recorded 73.50 per cent juvenile mortality and it was statistically on par with 33.33% concentration. No juvenile mortality was recorded in sterile water while percentage mortality of 3.50 and 5.50 per cent was observed in plain broth at 12 and 24 HAT respectively (Table 5).

4.1.3.2. Isolate 28

All the four concentrations (100, 50, 33.33 and 25%) of isolate 28 showed significant superiority over all other treatments at 6, 12 and 24 HAT. At 6 HAT, 100% concentration recorded highest juvenile mortality (65.50 per cent) and it was significantly different from all other treatments. CFE at 50% concentration recorded 48.00 per cent juvenile mortality and it was significantly different from 33.33 and 25% concentrations which showed mortality of 29.00 and 24.00 per cent respectively. At 12 HAT, highest mortality (91.50 per cent) was observed at 100% concentration of and it was significantly superior to all other treatments. The 50% concentration recorded 69.00 per cent juvenile mortality and it showed significant superiority to all other treatments. 25% concentration recorded 48.50 per cent juvenile mortality and it was statistically on par with 33.33% concentration (58.50 per cent). At 24 HAT, 100% concentration recorded 100.00 per cent juvenile mortality and was significantly different from 50, 33.33 and 25% concentrations which recorded 86.50, 78.00 and 69.50 per cent juvenile mortality respectively. No mortality was observed in sterile water while in plain broth juvenile mortality of 1.50 and 4.00 per cent was recorded at 12 and 24 HAT respectively (Table 6).

4.1.3.3. Isolate 25

All the concentrations (100, 50, 33.33 and 25%) showed statistically significant variation in mortality of *R. reniformis*. At 6 HAT highest juvenile mortality (64.50 per cent) was observed at 100% concentration of isolate 25 and it was found

Table 5. Effect of cell free extracts of isolate 26 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT [*]	12HAT	24HAT
100%	72.50 (58.43) [a]	95.50 (79.36) [a]	100.00 (89.59) [a]
50%	55.50 (48.16) [b]	75.50 (58.39) [b]	90.00 (71.84) [b]
33.33%	50.50 (45.29) [c]	63.00 (52.54) [bc]	79.00 (62.76) [c]
25%	36.00 (36.86) [d]	54.50 (47.59) [c]	73.50 (59.03) [c]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [e]	0.00 (0.40) [e]
plain broth	0.00 (0.40) [e]	3.50 (9.41) [d]	5.50 (13.07) [d]
CD (0.05)	(2.291)	(6.029)	(3.831)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

Table 6. Effect of cell free extracts of isolate 28 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT[*]	12HAT	24HAT
100%	65.50 (54.03) [a]	91.50 (73.86) [a]	100.00 (89.59) [a]
50%	48.00 (43.85) [b]	69.00 (56.27) [b]	86.50 (68.55) [b]
33.33%	29.00 (32.57) [c]	58.50 (49.90) [c]	78.00 (62.07) [c]
25%	24.00 (29.24) [d]	48.50 (44.14) [c]	69.50 (56.48) [d]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [d]	0.00 (0.40) [f]
plain broth	0.00 (0.40) [e]	1.50 (5.12) [d]	4.00 (11.35) [e]
CD (0.05)	(2.691)	(5.855)	(2.763)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

significantly superior to 50, 33.33 and 25% concentrations which recorded 50.00, 40.00 and 24.50 per cent juvenile mortality respectively. CFE of isolate 25 at 100% concentration showed 85.50 per cent juvenile mortality at 12 HAT which was significantly different from lower concentrations. Effect of isolate 25 at 50 and 33.33% concentrations was statistically on par in which 73.00 and 67.50 per cent mortality of *R. reniformis* juveniles was observed. CFE at 25% concentration recorded 49.00 per cent juvenile mortality and it was significantly inferior to all other treatments. At 24 HAT, highest juvenile mortality (98.50 per cent) was recorded by 100% concentration of isolate 25 which was significantly superior to all other concentrations. CFE of isolate 25 at 50, 33.33 and 25% concentrations recorded 89.00, 80.00 and 69.00 per cent juvenile mortality respectively and effect of these treatments was significantly different. Juvenile mortality of 3.50 per cent was observed in plain broth at 12 HAT while at 24HAT it was 8.50 per cent. No mortality was observed in sterile distilled water (Table 7).

4.1.3.4. Isolate 11

All the treatments with 100, 50, 33.33 and 25% concentrations of CFE of isolate 11 showed statistically significant superiority over control at 6, 12 and 24 HAT. At 6 HAT, 100% concentration recorded highest juvenile mortality (65.50 per cent) and was significantly superior over other treatments. The lowest (50, 33.33 and 25%) concentrations recorded 46.50, 38.00 and 25.50 per cent juvenile mortality respectively and were significantly different. At 12 HAT, 100% concentration recorded significantly superior juvenile mortality (84.50 per cent) over other treatments. Next best treatment, 50% concentration recorded juvenile mortality of 65.50 per cent and was significantly different. The lowest concentrations (33.33 and 25%) recorded 53.00 and 46.00 per cent juvenile mortality respectively and were significantly different. CFE of isolate 11 at 100% concentration recorded 99.50 per cent juvenile mortality at 24 HAT and was significantly superior to all other concentrations. The lowest concentrations (50, 33.33 and 25%) recorded 83.00, 72.00 and 65.50 per cent juvenile mortality respectively and were significantly different. No mortality was recorded in sterile water while plain broth recorded 3.50 and 8.50 per cent mortality at 12 and 24 HAT (Table 8).

Table 7. Effect of cell free extracts of isolate 25 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	64.50 (53.44) [a]	85.50 (67.67) [a]	98.50 (84.88) [a]
50%	50.00 (45.00) [b]	73.00 (58.90) [b]	89.00 (71.74) [b]
33.33%	40.00 (39.22) [c]	67.50 (55.39) [b]	80.00 (63.68) [c]
25%	24.50 (29.65) [d]	49.00 (44.43) [c]	69.00 (56.18) [d]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [e]	0.00 (0.40) [f]
plain broth	0.00 (0.40) [e]	3.50 (10.49) [d]	8.50 (16.87) [e]
CD (0.05)	(2.180)	(5.188)	(6.877)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

Table 8. Effect of cell free extracts of isolate 11 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	65.50 (54.05) [a]	84.50 (67.08) [a]	99.50 (87.66) [a]
50%	46.50 (42.99) [b]	65.50 (54.03) [b]	83.00 (65.74) [b]
33.33%	38.00 (38.05) [c]	53.00 (46.72) [c]	72.00 (58.12) [c]
25%	25.50 (30.31) [d]	46.00 (42.70) [d]	65.50 (54.04) [d]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [f]	0.00 (0.40) [f]
plain broth	0.00 (0.40) [e]	3.50 (10.49) [e]	8.50 (16.87) [e]
CD (0.05)	(2.010)	(3.764)	(3.859)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

4.1.3.5. Isolate 14

Isolate 14 at all concentrations (100, 50, 33.33 and 25%) showed statistically significant superiority over control at 6, 12 and 24 HAT. The highest juvenile mortality was recorded by 100% concentration (57.00 per cent) at 6 HAT and was significantly superior over other concentrations. Similar trend was observed at 12 and 24 HAT where 100% concentration recorded 74.50 and 92.50 per cent juvenile mortality respectively. At 6 HAT the 50, 33.33 and 25% concentrations recorded 42.00, 31.00 and 24.50 per cent juvenile mortality respectively and were significantly different. At 12 HAT the 50% concentration recorded 60.00 per cent juvenile mortality and showed significant variation from lower concentrations. The lowest (33.33 and 25%) concentrations recorded 49.50 and 41.50 per cent juvenile mortality and were significantly different. At 24 HAT the 50, 33.33 and 25% concentrations recorded 75.00, 65.50 and 56.50 per cent juvenile mortality respectively and were significantly different. The plain broth recorded 2.00 and 6.50 per cent juvenile mortality at 12 and 24 HAT while no juvenile mortality was recorded by sterile water (Table 9).

4.1.3.6. Isolate 42

CFE of isolate 42 at all concentrations (100, 50, 33.33 and 25%) showed statistically significant superiority over control at 6, 12 and 24 HAT. At 6 HAT, the highest juvenile mortality (52.50 per cent) was recorded by 100% concentration and it was significantly superior over other treatments. The 50, 33.33 and 25% concentrations recorded 42.00, 26.50 and 14.00 per cent juvenile mortality respectively and were significantly different. At 12 HAT, 100% concentration recorded significantly superior juvenile mortality (69.00 per cent) over other treatments. The 50, 33.33 and 25% concentrations recorded 60.50, 39.00 and 27.00 per cent juvenile mortality, respectively and were significantly different. At 24 HAT, 100% concentration recorded the highest juvenile mortality which is 83.00 per cent and was significantly superior over other treatments. The 50, 33.33 and 25% concentrations recorded 73.00, 57.50 and 49.50 per cent juvenile mortality respectively and were significantly different. Plain broth recorded juvenile mortality

Table 9. Effect of cell free extracts of isolate 14 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT[*]	12HAT	24HAT
100%	57.00 (49.03) [a]	74.50 (59.77) [a]	92.50 (74.75) [a]
50%	42.00 (40.39) [b]	60.00 (50.77) [b]	75.00 (60.07) [b]
33.33%	31.00 (33.79) [c]	49.50 (44.71) [c]	65.50 (54.03) [c]
25%	24.50 (29.58) [d]	41.50 (40.08) [d]	56.50 (48.74) [d]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [f]	0.00 (0.40) [f]
plain broth	0.00 (0.40) [e]	2.00 (7.05) [e]	6.50 (14.29) [e]
CD (0.05)	(3.169)	(4.414)	(4.737)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

Table 10. Effect of cell free extracts of isolate 42 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT[*]	12HAT	24HAT
100%	52.50 (46.44) [a]	69.00 (56.20) [a]	83.00 (65.69) [a]
50%	42.00 (40.39) [b]	60.50 (51.08) [b]	73.00 (58.79) [b]
33.33%	26.50 (30.96) [c]	39.00 (38.64) [c]	57.50 (49.32) [c]
25%	14.00 (21.85) [d]	27.00 (31.27) [d]	49.50 (44.71) [d]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [f]	0.00 (0.40) [f]
plain broth	0.00 (0.40) [e]	2.00 (7.05) [e]	7.00 (15.14) [e]
CD (0.05)	(2.663)	(4.025)	(3.633)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

of 2.00 and 7.00 per cent only while no mortality was recorded in sterile water (Table 10).

4.1.3.7. Isolate 1

All the concentrations (100, 50, 33.33 and 25%) of isolate 1 showed statistically significant superiority over control at 6, 12, 24 HAT. The highest juvenile mortalities were recorded by 100% concentration at 6, 12 and 24 HAT (45.50, 61.50 and 79.00 per cent respectively) and were significantly superior over other treatments. The 50% concentration at 6 HAT recorded 29.50 per cent juvenile mortality and was significantly different. The 25% concentration of CFE of isolate 1 recorded 18.50 per cent juvenile mortality and was statistically on par with that of 33.33% concentration which was 22.50 per cent. At 12 HAT, the 50% concentration recorded 51.50 per cent juvenile mortality and was significantly different. The 33.33% concentration recorded 32.00 per cent juvenile mortality and was statistically on par with that of 25% concentration which was 28.50 per cent. At 24 HAT the 50% concentration recorded 61.00 per cent juvenile mortality and showed significant variation over lower concentrations. The 25% concentration recorded 44.00 per cent juvenile mortality and was statistically on par with that of 33.33% concentration (48.50 per cent). Juvenile mortality of 0.50 and 5.00 were recorded by plain broth at 12 and 24 HAT while no mortality was recorded in sterile water (Table 11).

4.1.3.8. Isolate 18

CFE of isolate 18 at all concentrations (100, 50, 33.33 and 25%) showed statistically significant superiority over control at 6, 12 and 24 HAT. The highest juvenile mortality was recorded by 100% concentration at 6, 12 and 24 HAT (50.50 to 79.00 per cent) and it was significantly superior to other treatments. At 6 HAT the 50, 33.33 and 25% concentrations recorded 34.00, 18.50 and 14.00 per cent juvenile mortality respectively and were significantly different. At 12 HAT the 50% concentration recorded 54.00 per cent juvenile mortality and was significantly different. The 25% concentration recorded 30.00 per cent juvenile mortality and was statistically on par with that of 33.33% concentration (37.00 per cent). At 24 HAT, 50, 33.33 and 25% concentrations recorded 67.00, 53.00 and 41.50 per cent juvenile

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Table 11. Effect of cell free extracts of isolate 1 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	45.50 (42.41) [a]	61.50 (51.66) [a]	79.00 (62.85) [a]
50%	29.50 (32.87) [b]	51.50 (45.86) [b]	61.00 (51.36) [b]
33.33%	22.50 (28.24) [c]	32.00 (34.40) [c]	48.50 (44.14) [c]
25%	18.50 (25.40) [c]	28.50 (32.22) [c]	44.00 (41.55) [c]
sterile water	0.00 (0.40) [d]	0.00 (0.40) [d]	0.00 (0.40) [e]
plain broth	0.00 (0.40) [d]	0.50 (2.34) [d]	5.00 (12.57) [d]
CD (0.05)	(2.931)	(4.120)	(3.726)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

Table 12. Effect of cell free extracts of isolate 18 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	50.50 (45.29) [a]	69.50 (56.50) [a]	79.00 (62.76) [a]
50%	34.00 (35.65) [b]	54.00 (47.30) [b]	67.00 (54.95) [b]
33.33%	18.50 (25.40) [c]	37.00 (37.44) [c]	53.00 (46.72) [c]
25%	14.00 (21.95) [d]	30.00 (33.20) [c]	41.50 (40.10) [d]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [e]	0.00 (0.40) [f]
plain broth	0.50 (2.34) [e]	2.00 (5.78) [d]	4.00 (11.06) [e]
CD (0.05)	(3.182)	(4.796)	(3.510)

Figures in parenthesis are angular transformed values   * Hours After Treatment
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mortality, respectively and were significantly different. No juvenile mortality was observed in sterile water. Plain broth recorded 0.50, 2.00 and 4.00 per cent juvenile mortality at 6, 12 and 24 HAT, respectively (Table 12).

4.1.3.9. Isolate 44

CFE of isolate 44 at all four concentrations (100, 50, 33.33 and 25%) showed statistically significant superiority over control at 6, 12 and 24 HAT. The highest juvenile mortality at 6 HAT was recorded by 100% concentration which was 40.50 per cent and was significantly superior over other treatments. The 50, 33.33 and 25% concentrations of CFE recorded 27.00, 16.00 and 7.50 per cent juvenile mortality respectively and were significantly different. At 12 HAT, highest mortality (57.50 per cent) was recorded by 100% CFE concentration and it was significantly superior over other treatments. The 50% concentration recorded 38.50 per cent juvenile mortality and was significantly different from lower concentrations. The 33.33 and 25% concentrations recorded 18.50 and 26.00 per cent juvenile mortality and were statistically on par. At 24 HAT highest juvenile mortality was recorded by 100% concentration (74.50 per cent) and was significantly superior over other treatments. The 50% CFE concentration recorded 47.00 per cent juvenile mortality and was statistically on par with that of 33.33% concentration. The CFE at 33.33 and 25% concentrations recorded 39.50 and 33.50 per cent juvenile mortality respectively and was statistically on par. Juvenile mortality of 0.50 was recorded by sterile water at 24 HAT. Plain broth recorded 4.50 and 8.50 per cent juvenile mortality at 12 and 24 HAT (Table 13).

4.1.3.10. Isolate 16

All the treatments with four concentrations (100, 50, 33.33 and 25%) of CFE of isolate 16 showed statistically significant superiority over control in mortality of *R. reniformis* juveniles at 6, 12 and 24 HAT. Highest juvenile mortality (37.00 per cent) was observed in 100% CFE concentration at 6 HAT and was significantly superior over other treatments. The lower CFE concentrations (50, 33.33 and 25%) recorded 23.50, 14.50 and 9.50 per cent juvenile mortality respectively and were significantly different. At 12 HAT, 100% concentration recorded highest juvenile

Table 13. Effect of cell free extracts of isolate 44 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	40.50 (39.52) [a]	57.50 (49.34) [a]	74.50 (59.81) [a]
50%	27.00 (31.27) [b]	38.50 (38.34) [b]	47.00 (43.28) [b]
33.33%	16.00 (23.48) [c]	26.00 (30.39) [c]	39.50 (38.93) [bc]
25%	7.50 (15.71) [d]	18.50 (25.40) [c]	33.50 (35.25) [c]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [e]	0.50 (2.34) [e]
plain broth	0.00 (0.40) [e]	4.50 (11.72) [d]	8.50 (16.67) [d]
CD (0.05)	(2.929)	(5.215)	(5.364)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

Table 14. Effect of cell free extracts of isolate 16 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	37.00 (37.44) [a]	55.50 (48.16) [a]	70.50 (57.19) [a]
50%	23.50 (28.95) [b]	32.00 (34.42) [b]	51.50 (45.86) [b]
33.33%	14.50 (22.27) [c]	24.00 (29.31) [c]	40.50 (39.51) [c]
25%	9.50 (17.55) [d]	22.00 (27.94) [c]	40.50 (39.52) [c]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [e]	0.00 (0.40) [e]
plain broth	0.00 (0.40) [e]	1.00 (4.27) [d]	3.50 (9.22) [d]
CD (0.05)	(3.885)	(3.778)	(5.003)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

mortality which is 55.50 per cent and was significantly superior. CFE at 50% concentration recorded 32.00 per cent juvenile mortality and was significant different. The lowest (25%) concentration recorded 22.00 per cent juvenile mortality and was statistically on par with that of 33.33% concentration (24.00 per cent). At 24 HAT, highest juvenile mortality (70.50 per cent) was recorded by 100% concentration and was significantly superior to all other concentrations. The 50% concentration recorded 51.50 per cent juvenile mortality and was significantly different from lower concentrations. The lower concentrations (25 and 33.33%) of CFE recorded 40.50 and 40.50 per cent juvenile mortality respectively and were statistically on par. No mortality was observed in sterile water while plain broth recorded juvenile mortality of 1.00 and 3.50 per cent at 12 and 24 HAT (Table 14).

4.1.3.11. Isolate 37

All the CFE concentrations (100, 50, 33.33 and 25%) of isolate 37 showed statistically significant superiority over control at 6, 12 and 24 HAT. At 6 HAT highest juvenile mortality (31.00 per cent) was recorded by 100% concentration and was significantly superior over all other treatments. The lower concentrations (50, 33.33 and 25%) of CFE recorded 17.50, 11.50 and 7.00 per cent juvenile mortality respectively and were significantly different. Highest juvenile mortality (51.00 per cent) was recorded at 12 HAT was by 100% CFE concentration and was significantly superior to other concentrations. The 50% concentration recorded 31.00 per cent juvenile mortality and was statistically on par with that of 33.33% concentration. The lower CFE concentrations (33.33 and 25%) recorded 28.00 and 22.50 per cent juvenile mortality respectively and were statistically on par. At 24 HAT, the highest juvenile mortality (65.50 per cent) was recorded by 100% concentration and was significantly superior over lower concentrations. The 50 and 33.33% concentrations recorded 49.00 and 41.00 per cent juvenile mortality respectively and were statistically on par. The 25% concentration recorded 43.00 per cent juvenile mortality and was statistically on par with that of 33.33% concentration. Plain broth recorded a juvenile mortality of 0.50 and 3.50 per cent at 12 and 24 HAT respectively while no mortality was observed in sterile water (Table 15).

4.1.3.12. Isolate 39

The data on mortality of *R. reniformis* juveniles at four concentrations (100, 50, 33.33 and 25%) of isolate 39 showed statistically significant superiority over control at 6, 12 and 24 HAT. Highest juvenile mortality (39.50 per cent) was recorded at 6 HAT by 100% concentration of CFE and it was significantly superior over all other treatments. The 50, 33.33 and 25% concentrations recorded 24.00, 11.50 and 7.00 per cent juvenile mortality respectively and were significantly different. At 12 HAT, 100% concentration recorded significantly superior juvenile mortality (50.50 per cent). The 50% concentration recorded 36.50 per cent juvenile mortality and was significantly different from other treatments. The 33.33% concentration recorded 22.50 per cent juvenile mortality and was statistically on par with that of 25% concentration which was 17.50 per cent. At 24 HAT, 100% concentration recorded highest juvenile mortality (65.00 per cent) and was significantly superior over other treatments. The 50% concentration recorded 55.50 per cent juvenile mortality and was significantly different. The lower (33.33 and 25%) concentrations recorded 40.50 and 35.00 per cent juvenile mortality and were statistically on par. No mortality was recorded by sterile water. Plain broth recorded 3.00 and 7.50 per cent juvenile mortality at 12 and 24 HAT (Table 16).

4.1.3.13. Isolate 9

All the treatments with four concentrations (100, 50, 33.33 and 25%) of CFE of isolate 9 showed statistically significant superiority over control at 6, 12 and 24 HAT. At 6 HAT, the highest juvenile mortality (36.00 per cent) was recorded by 100% concentration and was significantly superior to all treatments. Lower concentrations (50, 33.33 and 25%) recorded 25.00, 18.00 and 12.00 per cent juvenile mortality, respectively and were significantly different. Highest juvenile mortality (49.50 per cent) was recorded by 100% concentration at 12 HAT and was significantly superior to other concentrations. The 50% concentration recorded 34.50 per cent juvenile mortality and was statistically on par with that of 33.33% concentration (33.00 per cent). The lowest concentration (25%) recorded 24.00 per cent juvenile mortality and was significantly inferior to other concentrations. At 24 HAT, highest

Table 15. Effect of cell free extracts of isolate 37 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	31.00 (33.78) [a]	51.00 (45.57) [a]	65.50 (54.07) [a]
50%	17.50 (24.66) [b]	31.00 (33.79) [b]	49.00 (44.42) [b]
33.33%	11.50 (19.60) [c]	28.00 (31.91) [bc]	41.00 (39.81) [bc]
25%	7.00 (15.14) [d]	22.50 (28.30) [c]	43.00 (40.97) [c]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [d]	0.00 (0.40) [e]
plain broth	0.00 (0.40) [e]	0.50 (2.34) [d]	3.50 (10.49) [d]
CD (0.05)	(3.850)	(3.668)	(3.553)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

Table 16. Effect of cell free extracts of isolate 39 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	39.50 (38.93) [a]	50.50 (45.29) [a]	65.00 (53.80) [a]
50%	24.00 (29.19) [b]	36.50 (37.15) [b]	55.50 (48.18) [b]
33.33%	11.50 (19.56) [c]	22.50 (28.25) [c]	40.50 (39.51) [c]
25%	7.00 (15.14) [d]	17.50 (24.71) [c]	35.00 (36.26) [c]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [e]	0.00 (0.40) [e]
plain broth	0.00 (0.40) [e]	3.00 (9.64) [d]	7.50 (15.81) [d]
CD (0.05)	(4.182)	(3.546)	(4.163)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

juvenile mortality (64.50 per cent) was recorded by 100% concentration and it showed significant superiority over other treatments. The 50% concentration recorded 47.00 per cent juvenile mortality and was statistically on par with 33.33% concentration. The 33.33 and 25% concentrations recorded 43.00 and 38.00 per cent juvenile mortality respectively and were statistically on par. No mortality was observed in sterile water while plain broth recorded juvenile mortality of 2.50 and 6.00 per cent at 12 and 24 HAT respectively (Table 17).

4.1.3.14. Isolate 29

CFE of isolate 29 at all the concentrations (100, 50, 33.33 and 25%) recorded statistically significant superiority over the control at 6, 12 and 24 HAT. At 6 HAT, highest juvenile mortality (50.50 per cent) recorded by 100% concentration and was significantly superior to other treatments. Lower concentrations (50, 33.33 and 25%) of CFE recorded 34.00, 18.50 and 14.00 per cent juvenile mortality respectively and were significantly different. Highest juvenile mortality recoded at 12 HAT was 69.50 per cent by 100% concentration and it was significantly superior to other treatments. The 50% concentration recorded 54.00 per cent juvenile mortality and was significantly different. The 33.33% concentration recorded 37.00 per cent juvenile mortality and was statistically on par with that of 25% concentration (30.00 per cent). At 24 HAT, highest juvenile mortality (79.00 per cent) was recorded by 100% concentration which was significantly superior to other treatments. The 50% concentration recorded 67.00 per cent juvenile mortality and was significantly different. The lower concentrations (33.33 and 25%) recorded 53.00 and 41.50 per cent juvenile mortality, respectively and were significantly different. Plain broth recorded 0.50, 2.00 and 4.00 per cent juvenile mortality at 6, 12 and 24 HAT respectively while no mortality was observed in sterile water (Table 18).

4.1.3.15. Isolate 27

All the concentrations (100, 50, 33.33 and 25%) of isolate 27 showed statistically significant superiority over control at 6, 12 and 24 HAT. At 6 HAT, the highest juvenile mortality (35.50 per cent) was recorded by 100% concentration and was significantly superior to other treatments. The 50 and 33.33% concentrations

Table 17. Effect of cell free extracts of isolate 9 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	36.00 (36.86) [a]	49.50 (44.72) [a]	64.50 (53.48) [a]
50%	25.00 (29.98) [b]	34.50 (35.95) [b]	47.00 (43.28) [b]
33.33%	18.00 (25.09) [c]	33.00 (35.05) [b]	43.00 (40.97) [bc]
25%	12.00 (20.24) [d]	24.00 (29.29) [c]	38.00 (38.04) [c]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [e]	0.00 (0.40) [e]
plain broth	0.00 (0.40) [e]	2.50 (7.90) [d]	6.00 (14.08) [d]
CD (0.05)	(1.852)	(4.417)	(3.199)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

Table 18. Effect of cell free extracts of isolate 29 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	50.50 (45.29) [a]	69.50 (56.50) [a]	79.00 (62.76) [a]
50%	34.00 (35.65) [b]	54.00 (47.30) [b]	67.00 (54.95) [b]
33.33%	18.50 (25.40) [c]	37.00 (37.44) [c]	53.00 (46.72) [c]
25%	14.00 (21.95) [d]	30.00 (33.20) [c]	41.50 (40.10) [d]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [e]	0.00 (0.40) [e]
plain broth	0.50 (2.34) [e]	2.00 (5.78) [d]	4.00 (11.06) [e]
CD (0.05)	(3.182)	(4.796)	(3.510)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

recorded 28.50 and 24.50 per cent juvenile mortality respectively and were statistically on par. The 25% concentration recorded 14.00 per cent juvenile mortality and was significantly inferior to other concentrations. At 12 HAT, highest juvenile mortality (50.50 per cent) was observed at 100% concentration and was statistically on par with that of 50% concentration (43.00 per cent). The 33.33% concentration recorded 37.00 per cent juvenile mortality and was statistically on par with that of 50% concentration. The juvenile mortality recorded by 33.33 and 25% (31.00 per cent) concentrations was statistically on par. At 24 HAT, the highest juvenile mortality (63.50 per cent) was observed at 100% concentration. The 50% concentration recorded 59.00 per cent juvenile mortality and was statistically on par with that of 100% concentration. The 33.33 and 25% concentrations recorded 51.50 and 46.00 per cent juvenile mortality respectively and were statistically on par. Juvenile mortality of 0.50 per cent was observed at 24 HAT in sterile water. Plain broth recorded 0.50, 2.50 and 6.50 per cent juvenile mortality at 6, 12 and 24 HAT respectively (Table 19).

4.1.3.16. Isolate 19

CFE of isolate 19 at four concentrations (100, 50, 33.33 and 25%) showed statistically significant superiority over control at 6, 12 and 24 HAT. Highest juvenile mortality (37.50 per cent) was recorded at 100% concentration at 6 HAT and it was significantly superior to other treatments. The lower concentrations (50, 33.33 and 25%) recorded 26.50, 18.50 and 14.00 per cent juvenile mortality, respectively and were significantly different. At 12 HAT, the highest juvenile mortality (47.00 per cent) was recorded by 100% concentration and it showed significant superiority over other treatments. The 50% concentration recorded 35.50 per cent juvenile mortality and was significantly different. The 33.33% concentration recorded 27.00 per cent juvenile mortality and was statistically on par with that of 25% concentration (25.50 per cent). At 24 HAT, highest juvenile mortality (60.00 per cent) was recorded by 100% concentration and it was significantly superior to other treatments. The 50% concentration recorded 52.00 per cent juvenile mortality and was statistically on par with that of 33.33% concentration (47.00 per cent). CFE of isolate 19 at 25% concentration recorded 39.00 per cent juvenile mortality and was significantly

Table 19. Effect of cell free extracts of isolate 27 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT[*]	12HAT	24HAT
100%	35.50 (36.57) [a]	50.50 (45.29) [a]	63.50 (52.85) [a]
50%	28.50 (32.24) [b]	43.00 (40.97) [ab]	59.00 (50.19) [a]
33.33%	24.50 (29.60) [b]	37.00 (37.46) [bc]	51.50 (45.86) [b]
25%	14.00 (21.90) [c]	31.00 (33.82) [c]	46.00 (42.70) [b]
sterile water	0.00 (0.40) [d]	0.00 (0.40) [e]	0.50 (2.34) [d]
plain broth	0.50 (2.34) [d]	2.50 (6.63) [d]	6.50 (14.29) [c]
CD (0.05)	(3.521)	(4.846)	(4.307)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

Table 20. Effect of cell free extracts of isolate 19 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT[*]	12HAT	24HAT
100%	37.50 (37.76) [a]	47.00 (43.28) [a]	60.00 (50.78) [a]
50%	26.50 (30.90) [b]	35.50 (36.53) [b]	52.00 (46.15) [b]
33.33%	18.50 (25.36) [c]	27.00 (31.29) [c]	47.00 (43.28) [b]
25%	14.00 (21.90) [d]	25.50 (30.31) [c]	39.00 (38.64) [c]
sterile water	0.00 (0.40) [e]	0.00 (0.40) [e]	0.00 (0.40) [e]
plain broth	0.00 (0.40) [e]	3.00 (8.56) [d]	8.00 (16.10) [d]
CD (0.05)	(3.202)	(4.615)	(3.277)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

different. No mortality was observed in sterile water while 3.00 and 8.00 per cent juvenile mortality was recorded by plain broth at 12 and 24 HAT (Table 20).

4.1.3.17. Isolate 8

All the CFE concentrations (100, 50, 33.33 and 25%) of isolate 8 showed significant superiority over the control at 6, 12 and 24 HAT. 100% concentration recorded highest mortality (37.00 per cent) of *R. reniformis* juvenile at 6 HAT and it was significantly superior to other treatments. Lower concentrations (50, 33.33 and 25%) recorded 32.50, 26.00 and 18.00 per cent juvenile mortality respectively and were significantly different. At 12 HAT, 100% concentration recorded 46.00 per cent juvenile mortality and was statistically on par with that of 50% concentration (42.00 per cent). The 33.33% concentration of isolate 8 recorded 37.00 per cent juvenile mortality and was statistically on par with that of 50% concentration. The 25% concentration recorded 29.00 per cent juvenile mortality and was significantly inferior to other concentrations. At 24 HAT, highest juvenile mortality (58.50 per cent) was observed at 100% concentration and it was statistically on par with that of 50% concentration (53.00 per cent). The 33.33% concentration recorded 47.00 per cent juvenile mortality and it was statistically on par with 50% concentration. The 25% concentration recorded 38.00 per cent juvenile mortality and was significantly inferior to other concentrations. Sterile water recorded 0.50, 0.50 and 0.50 per cent juvenile mortality at 6, 12 and 24 HAT respectively. Juvenile mortality of 2.50 and 6.00 was recorded by plain broth at 12 and 24 HAT respectively (Table 21).

4.1.3.18. Isolate 20

All the concentrations (100, 50, 33.33 and 25%) of CFE of isolate 20 showed statistically significant superiority over control at 6, 12, and 24 HAT. At 6 HAT, highest juvenile mortality (9.00 per cent) was recorded by 100% concentration and it was statistically on par with 50% concentration (6.00 per cent). The 33.33% concentration recorded 4.50 per cent juvenile mortality and it was statistically on par with 50% concentration. The 25% concentration recorded 3.50 per cent juvenile mortality and was significantly inferior to other concentrations. At 12 HAT 100% concentration recorded highest juvenile mortality (28.00 per cent) and it showed

Table 21. Effect of cell free extracts of isolate 8 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	37.00 (37.46) [a]	46.00 (42.70) [a]	58.50 (49.90) [a]
50%	32.50 (34.75) [b]	42.00 (40.38) [ab]	53.00 (46.73) [ab]
33.33%	26.00 (30.62) [c]	37.00 (37.46) [b]	47.00 (43.28) [b]
25%	18.00 (25.02) [d]	29.00 (32.57) [c]	38.00 (38.05) [c]
sterile water	0.50 (0.40) [e]	0.50 (2.34) [e]	0.50 (2.34) [e]
plain broth	0.00 (0.40) [e]	2.50 (8.98) [d]	6.00 (13.92) [d]
CD (0.05)	(2.496)	(3.692)	(4.248)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

Table 22. Effect of cell free extracts of isolate 20 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	9.00 (17.09) [a]	28.00 (31.88) [a]	54.50 (47.59) [a]
50%	6.00 (14.08) [ab]	22.00 (27.94) [b]	37.50 (37.74) [b]
33.33%	4.50 (12.01) [b]	16.50 (23.88) [c]	29.00 (32.57) [c]
25%	3.50 (10.49) [b]	11.50 (19.70) [d]	23.50 (28.93) [c]
sterile water	0.00 (0.40) [c]	0.00 (0.40) [e]	0.50 (2.34) [e]
plain broth	0.00 (0.40) [c]	0.00 (0.40) [e]	3.00 (9.83) [d]
CD (0.05)	(3.827)	(3.295)	(3.881)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

significantly superiority to other treatments. Lower concentrations (50, 33.33 and 25%) recorded 22.00, 16.50 and 11.50 per cent juvenile mortality respectively and were significantly different. At 24 HAT, highest juvenile mortality (54.50 per cent) was recorded by 100% concentration and was significantly superior to other concentrations (50, 33.33 and 25%). The 50% concentration recorded 37.50 per cent juvenile mortality and was significantly different. The 33.33 and 25% concentrations recorded 29.00 and 23.50 per cent juvenile mortality respectively and were statistically on par. Sterile water recorded 0.50 per cent juvenile mortality at 24 HAT while plain broth recorded 3.00 per cent juvenile mortality at 24 HAT (Table 22).

4.1.3.19. Isolate 21

The four CFE concentrations (100, 50, 33.33 and 25%) of isolate 21 showed statistically significant superiority over control at 6, 12 and 24 HAT. At 6 HAT, 100% concentration recorded highest juvenile mortality (23.50 per cent) and was statistically on par with that of 50% concentration (20.00 per cent). The 33.33% concentration recorded 16.50 per cent juvenile mortality and was statistically on par with that of 50% concentration. The 25% concentration recorded 13.00 per cent juvenile mortality and was statistically on par with that of 33.33% concentration. At 12 HAT, highest juvenile mortality (37.50 per cent) was recorded by 100% concentration and was significantly superior over other treatments. The 50% concentration recorded 30.00 per cent juvenile mortality and was statistically on par with that of 33.33% concentration (27.00 per cent). The 25% concentration recorded 26.00 per cent juvenile mortality and was statistically on par with that of both 50 and 33.33% concentrations. At 24 HAT, 100% concentration recorded highest juvenile mortality (53.50 per cent) and was significantly superior to other concentrations. The 50% concentration recorded 41.00 per cent juvenile mortality. The 33.33% concentration recorded 37.00 per cent juvenile mortality and was statistically on par with that of 50% concentration. The 25% concentration recorded 34.00 per cent juvenile mortality and was statistically on par with that of 33.33% concentration. No mortality was observed in sterile water while plain broth recorded a mortality of 2.00 and 5.00 per cent at 12 and 24 HAT respectively (Table 23).

4.1.3.20. Isolate 12

All the concentrations of isolate 12 showed statistically significant superiority over control at 6, 12 and 24 HAT. Highest juvenile mortality (14.50 per cent) at 6 HAT was recorded by 100% concentration and was statistically on par with that of 50% concentration (11.50 per cent). The 33.33% concentration recorded 3.50 per cent juvenile mortality and was significantly different. The 25% concentration recorded 1.00 per cent juvenile mortality and was significantly inferior to other concentrations. At 12 HAT, highest juvenile mortality (33.00 per cent) was recorded by 100% concentration and was statistically on par with that of 50% concentration (27.00 per cent). The 33.33% concentration recorded 8.00 per cent juvenile mortality and was statistically on par with that of 25% concentration (4.50 per cent). At 24 HAT, highest juvenile mortality (50.50 per cent) was recorded by 100% concentration and it showed significant superiority over other treatments. The lower concentrations (50, 33.33 and 25%) of isolate 12 recorded 37.50, 21.50 and 13.00 per cent juvenile mortality respectively and were significantly different. Plain broth recorded 0.50 and 2.50 per cent juvenile mortality at 12 and 24 HAT while no mortality was observed in sterile water (Table 24).

From the preliminary screening results four most effective bacterial isolates were selected. The isolate 26, 28, 25 and 11 at 100% concentration showed 100.00, 100.00, 98.50 and 99.50 per cent mortality of *R. reniformis* juveniles respectively at 24 HAT. Isolate 26, 28, 25 and 11 at lower concentration of CFE (25%) recorded 73.50, 69.50, 69.00 and 65.50 per cent mortality of *R. reniformis* juveniles at 24 HAT.

4.3 SCREENING OF BACTERIAL ISOLATES FOR THEIR NEMATICIDAL PROPERTY AGAINST *R. reniformis*

The effect of cell free extract of selected four bacterial isolates (isolate 26, 28, 25 and 11) at 100, 50, 33.33 and 25% concentrations were tested for egg hatch inhibition and juvenile mortality of *R. reniformis* under *in vitro*. The results are shown in Table 25 and 26.

Table 23. Effect of cell free extracts of isolate 21 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	23.50 (28.92) [a]	37.50 (37.75) [a]	53.50 (47.01) [a]
50%	20.00 (26.52) [ab]	30.00 (33.20) [b]	41.00 (39.81) [b]
33.33%	16.50 (23.80) [bc]	27.00 (31.29) [b]	37.00 (37.46) [bc]
25%	13.00 (20.55) [c]	26.00 (30.65) [b]	34.00 (35.66) [c]
sterile water	0.00 (0.40) [d]	0.00 (0.40) [d]	0.00 (0.40) [e]
plain broth	0.00 (0.40) [d]	2.00 (7.05) [c]	5.00 (12.57) [d]
CD (0.05)	(4.871)	(3.289)	(3.138)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

Table 24. Effect of cell free extracts of isolate 12 on mortality of *R. reniformis* juveniles *in vitro*

Cell free extract concentrations	Mortality (%)		
	6HAT*	12HAT	24HAT
100%	14.50 (22.30) [a]	33.00 (35.02) [a]	50.50 (45.29) [a]
50%	11.50 (19.49) [a]	27.00 (31.16) [a]	37.50 (37.74) [b]
33.33%	3.50 (10.68) [b]	8.00 (16.21) [b]	21.50 (27.57) [c]
25%	1.00 (4.26) [c]	4.50 (12.01) [b]	13.00 (21.06) [d]
sterile water	0.00 (0.40) [c]	0.00 (0.40) [c]	0.00 (0.40) [f]
plain broth	0.00 (0.40) [c]	0.50 (2.34) [c]	2.50 (7.71) [e]
CD (0.05)	(4.254)	(4.814)	(4.740)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

4.3.1. Effect on Egg Hatching

All concentrations of CFE of isolate 26, 28, 25 and 11 (100, 50, 33.33 and 25%) showed statistically significant difference on egg hatching at three to seven days. No hatching of eggs was observed in first two days (Table 25).

On third day after treatment (DAT) lowest egg hatching of *R. reniformis* (1.67 per cent) was recorded by 100% concentration of isolate 26. The egg hatching recorded by 100% CFE concentration of four isolates was statistically on par. Effect of 100, 50, 33.33 and 25% concentrations of isolate 26 was statistically on par giving 1.67 to 2.50 per cent egg hatching. Isolate 26 at its lowest concentration (25%) recorded 2.29 per cent egg hatching and it was as effective as higher concentrations (100 and 50% concentration) of isolate 28 and 25 and 100, 50 and 33.33% concentration of isolate 11. Egg hatching per cent in these treatments ranged from 2.29 to 3.33. The second lowest (2.08 per cent) egg hatching was recorded by 100% concentration of both isolate 28 and 25. Their effect was statistically on par with that of isolate 26 (100, 50, 33.33 and 25% concentration) and isolate 11 (100, 50 and 33.33% concentration). Isolate 25 at 25% concentration recorded highest egg hatching among isolates (7.50 per cent) and was significantly different. The effect of isolate 28 (33.33% concentration), isolate 25 (50% concentration) and isolate 11 (50, 33.33 and 25% concentration) was statistically on par with 2.92 to 3.96 per cent egg hatching. Sterile water recorded the highest egg hatching (19.58 per cent) and was statistically on par with egg hatching (18.96 per cent) recorded by plain broth.

On fourth DAT minimum egg hatching (3.12 per cent) was observed in 100% CFE concentration of isolate 26. The effect of 100% concentrations of isolate 26, 28, 25 and 11 was statistically on par with 3.12 to 4.17 per cent egg hatching. Isolate 26 at 100, 50 and 33.33% concentrations were equally effective with 3.12 to 4.38 per cent egg hatching. The effect of lowest concentrations of isolate 26 (33.33 and 25%) was statistically on par with that of higher (100 and 50%) concentrations of isolate 28 and isolate 11 giving 3.96 to 5.42 per cent egg hatching. The 33.33 and 25% concentrations of isolate 25 recorded highest egg hatching (8.96 and 10.83 per cent) and were found to be equally effective but significantly different from other isolates.

Table 25. Effect of cell free extracts of bacterial isolates on egg hatching of *R. reniformis* under *in vitro* condition

Isolate	Treatments	Egg Hatching (%)				
		3 DAT *	4 DAT	5 DAT	6 DAT	7 DAT
ISOLATE 26	100%	1.67 (7.29) [h]	3.12 (10.12) [h]	3.33 (10.48) [j]	3.33 (10.48) [l]	3.33 (10.48) [k]
	50%	2.50 (9.03) [fgh]	3.54 (10.72) [h]	5.00 (12.90) [i]	5.42 (13.45) [k]	5.62 (13.71) [j]
	33.33%	2.29 (8.61) [fgh]	4.38 (12.07) [gh]	5.62 (13.67) [hi]	6.88 (15.15) [jk]	7.71 (16.10) [i]
	25%	2.29 (8.61) [fgh]	5.42 (13.41) [fg]	7.91 (16.30) [fg]	9.17 (17.58) [hi]	9.58 (17.99) [hi]
ISOLATE 28	100%	2.08 (8.26) [gh]	3.96 (11.34) [gh]	5.83 (13.89) [hi]	7.71 (16.10) [ij]	8.96 (17.38) [i]
	50%	2.50 (9.03) [fgh]	5.00 (12.90) [fg]	8.96 (17.35) [ef]	11.46 (19.72) [fg]	12.92 (21.01) [fg]
	33.33%	3.96 (11.43) [de]	7.50 (15.83) [cde]	12.08 (20.31) [cd]	15.42 (23.08) [de]	17.92 (25.01) [cd]
	25%	5.62 (13.66) [c]	8.54 (16.92) [cd]	12.08 (20.29) [cd]	16.46 (23.91) [de]	20.21 (26.70) [bc]
ISOLATE 25	100%	2.08 (8.26) [gh]	3.33 (10.48) [h]	6.46 (14.70) [ghi]	9.58 (18.01) [gh]	11.67 (19.97) [g]
	50%	3.33 (10.47) [def]	6.25 (14.44) [ef]	8.96 (17.40) [ef]	12.29 (20.52) [f]	14.58 (22.44) [ef]
	33.33%	4.58 (12.30) [cd]	8.96 (17.40) [bc]	12.71 (20.86) [c]	17.71 (24.88) [cd]	19.79 (26.41) [bc]
	25%	7.50 (15.88) [b]	10.83 (19.20) [b]	15.42 (23.11) [b]	19.17 (25.95) [c]	21.04 (27.30) [b]
ISOLATE 11	100%	2.29 (8.61) [fgh]	4.17 (11.66) [gh]	6.04 (14.15) [hi]	7.71 (16.10) [ij]	8.12 (16.55) [i]
	50%	2.92 (9.81) [efg]	4.38 (12.03) [gh]	6.67 (14.92) [gh]	10.42 (18.81) [fgh]	11.25 (19.56) [gh]
	33.33%	3.12 (10.16) [defg]	6.04 (14.22) [ef]	9.58 (18.01) [ef]	12.29 (20.50) [f]	13.33 (21.40) [fg]
	25%	3.96 (11.40) [de]	6.67 (14.92) [def]	10.21 (18.60) [de]	14.58 (22.43) [e]	16.25 (23.76) [de]
sterile water		19.58 (26.20) [a]	41.88 (40.31) [a]	64.79 (53.61) [a]	82.5 (65.29) [a]	94.17 (76.14) [a]
plain broth		18.96 (25.74) [a]	40.62 (39.58) [a]	67.71 (55.38) [a]	79.79 (63.34) [b]	93.96 (75.94) [a]
CD (0.05)		(2.207)	(2.127)	(1.976)	(1.880)	(1.942)

Figures in parenthesis are angular transformed values   * Days After Treatment

Numerical values followed by different alphabet are significantly different from each other

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Isolate 28 (33.33 and 25% concentration) and isolate 25 (33.33% concentration) at their lower concentrations found to be statistically on par. Highest egg hatching (41.88 per cent) at 4 DAT was recorded by sterile water and was statistically on par with that of plain broth (40.62 per cent).

On fifth DAT lowest egg hatching percentage (3.33 per cent) was recorded by isolate 26 CFE at 100% concentration and it was significantly different from other treatments. Isolate 26 at its lower concentrations (50 and 33.33%) and isolate 28, 25 and 11 at their higher concentration (100%) were equally effective giving 5.00 to 6.46 per cent egg hatching. 100% concentration of isolate 25 recorded 6.46 per cent egg hatching and was found to be statistically on par with that of lower concentrations of isolate 26 (25%) and isolate 11 (33.33%). The effect of isolate 26 (33.33% concentration), isolate 28 (50% concentration), isolate 25 (50% concentration) and isolate 11 (33.33% concentration) were statistically on par giving 7.91 to 9.58 per cent egg hatching. The 25% concentration of isolate 25 recorded 15.42 per cent egg hatching and was significantly inferior to other isolates. Lower concentrations of isolate 28 (33.33 and25%) and isolate 11(25%) recorded 10.21 to 12.08 per cent egg hatching and was equally effective. Plain broth recorded highest egg hatching (67.71 per cent) and was statistically on par with that of sterile water (64.79 per cent).

On sixth DAT egg hatching percentage recorded by 100% CFE concentration of isolate 26 remained the same as previous day (3.33 per cent) and was significantly superior to other treatments. Isolate 26 at 50 and 33.33% concentrations recorded 5.42 and 6.88 per cent egg hatching respectively and found to be equally effective. CFE of isolate 28 and 11 at 100% concentration recorded 7.71 per cent egg hatching and were statistically on par with that of lower concentrations of isolate 26 (33.33 and 25%). Isolate 25 at 100% concentration recorded 9.58 per cent egg hatching and found to be statistically on par with isolate 26 (25% concentration) and isolate 11 (50% concentration). 50% concentration of isolate 28, 25 and 11 were equally effective giving egg hatching of 10.42 to 12.29 per cent. Lower concentration (25%) of isolate 11 recorded 14.58 per cent egg hatching and was as effective as isolate 28 at 33.33 and 25% concentration which recorded 15.42 and 16.46 egg hatching respectively. Isolate 25 at 33.33 and 25% concentrations recorded 17.71 and 19.17 per cent egg

hatching respectively and were statistically on par. Isolate 25 (33.33% concentration) and isolate 28 (33.33 and 25% concentration) were statistically on par and egg hatching in these treatments ranged from 15.42 to 17.71 per cent. Highest egg hatching (82.50 per cent) was recorded by sterile water followed by plain broth (79.79 per cent) and effect of these two was significantly different.

On seventh DAT CFE of isolate 26 at 100% concentrations recorded 3.33 per cent egg hatching and it was significantly superior to other treatments. Half concentration (50%) of CFE of isolate 26 recorded 5.62 per cent egg hatching and was significantly different from other treatments. Isolate 28 and isolate 11 at their higher concentration (100%) were statistically on par with lower concentrations of isolate 26 (33.33 and 25%) giving 7.71 to 9.58 per cent egg hatching. The 50% concentration of isolate 11 and 25% concentration of isolate 26 were equally effective with 11.25 and 9.58 per cent egg hatching respectively. CFE of isolate 25 at 100% concentration recorded 11.67 per cent egg hatching and was statistically on par with that of lower concentrations of isolate 28 (50%) and isolate 11 (50 and 33.33%). CFE of isolate 11 at 25% concentration and isolate 28 at 33.33% concentration recorded 16.25 and 17.92 per cent egg hatching respectively and effect of these treatments was statistically on par. Egg hatching recorded by sterile water (94.17 per cent) and plain broth (93.96 per cent) was the highest of all treatments and were statistically on par.

4.3.2. Effect on Juvenile Mortality

The juvenile mortality of *R. reniformis* recorded by the four concentrations of CFE of isolates 26, 28, 25 and 11 showed statistically significant superiority over control (sterile water and plain broth) at 6, 12 and 24 HAT. The results are given in Table. 26.

At 6 HAT 100% CFE concentration of isolate 26 recorded the highest mortality (72.50 per cent) of *R. reniformis* juveniles and it was significantly superior to other treatments. The higher (100%) concentration of isolates 28, 25 and 11 recorded 65.50, 64.50 and 65.50 per cent juvenile mortality respectively and were statistically on par. The 50% concentration of isolate 26 recorded 55.50 per cent juvenile mortality and was inferior to 100% concentration of isolate 26. The 33.33%

Table 26. Effect of cell free extracts of bacterial isolates on mortality of *R. reniformis* juvenile under *in vitro* condition

Isolate	Treatments	Juvenile Mortality (%)		
		6HAT[*]	12HAT	24HAT
ISOLATE 26	100%	72.50 (58.43) [a]	95.50 (79.36) [a]	100.00 (89.59) [a]
	50%	55.50 (48.16) [c]	72.50 (58.39) [d]	90.00 (71.84) [b]
	33.33%	50.50 (45.29) [d]	63.00(52.54) [efg]	79.00 (62.76) [def]
	25%	36.00 (36.87) [e]	54.50 (47.59) [ghi]	73.50 (59.03) [efg]
ISOLATE 28	100%	65.50 (54.03) [b]	91.50 (73.86) [b]	100.00 (89.59) [a]
	50%	48.00 (43.85) [d]	69.00 (56.27) [de]	86.50 (68.55) [bc]
	33.33%	29.00 (32.57) [f]	58.50 (49.90) [fgh]	78.00 (62.07) [def]
	25%	24.00 (29.24) [g]	48.50 (44.14) [i]	69.50 (56.48) [gh]
ISOLATE 25	100%	64.50 (53.44) [b]	85.50 (67.67) [c]	98.50 (84.88) [a]
	50%	50.00 (45.00) [d]	73.00 (58.90) [d]	89.00 (71.74) [b]
	33.33%	40.00 (39.22) [e]	67.50 (55.39) [de]	80.00 (63.68) [de]
	25%	24.50 (29.65) [g]	49.00 (44.43) [i]	69.00 (56.18) [gh]
ISOLATE 11	100%	65.50 (54.05) [b]	84.50 (67.08) [c]	99.50 (87.66) [a]
	50%	46.50 (42.99) [d]	65.50 (54.03) [def]	83.00 (65.74) [cd]
	33.33%	38.00 (38.05) [e]	53.00 (46.72) [hi]	72.00 (58.12) [fgh]
	25%	25.50 (30.31) [fg]	46.00 (42.70) [i]	65.50 (54.04) [h]
sterile water		0.00 (0.40) [h]	0.00 (0.40) [k]	0.00 (0.40) [j]
plain broth		0.00 (0.40) [h]	4.00 (11.35) [j]	7.50 (15.81) [i]
CD (0.05)		(2.542)	(4.995)	(4.733)

Figures in parenthesis are angular transformed values   * Hours After Treatment
Numerical values followed by different alphabet are significantly different from each other

concentration of isolate 26 recorded 50.50 per cent juvenile mortality and was statistically on par with that of 50% concentration of isolates 28, 25 and 11. The 25% concentration of isolate 26 recorded 36.00 per cent juvenile mortality and was statistically on par with juvenile mortality recorded by 33.33% concentration of isolate 25 (40.00 per cent) and 11 (38.00 per cent). CFE of isolate 28 at 33.33% concentration recorded 29.00 per cent juvenile mortality and it was statistically on par with that of 25% concentration of isolate 11. The juvenile mortality recorded by 25% concentration of isolates 28, 25 and 11 was statistically on par giving mortality of 24.00 to 25.50 per cent. No mortality of juveniles was recorded in plain broth and sterile water.

At 12 HAT highest mortality of *R. reniformis* juveniles was recorded by 100% concentration of isolate 26 which is 95.50 per cent and was significantly superior to other treatments. The second highest juvenile mortality percentage was recorded by 100%concentration of isolate 28 (91.50 per cent) and was significantly different from other treatments. The 100% concentrations of isolate 25 and 11 recorded 85.50 and 84.50 per cent juvenile mortality respectively and were statistically on par. The 50% concentration of isolate 26 recorded 72.50 per cent juvenile mortality and was statistically on par with that of 50% concentration of isolate 28 (69.00 per cent), 50 and 33.33% concentrations of isolate 25 (73.00 and 67.50 per cent) and 50% concentration of isolate 11 (65.50 per cent). The 33.33% concentration of isolate 26 recorded 63.00 per cent juvenile mortality and was statistically on par with 50% concentration of isolate 28, 33.33% concentration of isolate 25 and 50% concentration of isolate 11. Isolate 28 at 33.33% concentration recorded 58.50 per cent juvenile mortality at 12 HAT and was statistically on par with juvenile mortality recorded by 33.33% concentration of isolate 26 and 50% concentration of isolate 11. The lowest CFE concentration (25%) of isolate 26 recorded 54.50 per cent juvenile mortality and was statistically on par with that of 33.33% concentrations of isolate 26 and 28. The 33.33% concentration of isolate 11 recorded 53.00 per cent juvenile mortality and was statistically on par with that of 33.33% concentration of CFE of isolate 28 and 25% concentration of isolate 26. The lowest concentrations (25%) of isolate 28, 25 and 11 recorded 48.50, 49.00 and 46.00 per cent juvenile mortality respectively and were

statistically on par. Effect of these isolates at 25% concentration was found statistically on par with juvenile mortality recorded by 33.33% concentration of isolate 11 and 25% concentration of isolate 26. No mortality was observed in sterile water while plain broth recorded 4.00 per cent juvenile mortality.

At 24 HAT, 100% concentration of isolate 26, 28, 25 and 11 recorded 98.50 to 100.00 per cent *R. reniformis* juvenile mortality respectively and were statistically on par. Half concentration (50%) of CFE of isolate 26, 28 and 25 recorded 86.50 to 90.00 per cent juvenile mortality and were statistically on par. The 50% concentration of isolate 11 recorded 83.00 per cent juvenile mortality and was statistically on part with juvenile mortality recorded by 33.33% concentration of isolate 26, 28 and 25 giving mortality of 79.00, 78.00 and 80.00 per cent respectively. CFE of isolate 28 at 25% concentration recorded 73.50 per cent juvenile mortality and it was equally effective with isolate 26 and25 (25% concentration) and isolate 11 (33.33% concentration). Isolate 11 at 33.33% concentration recorded 72.00 per cent juvenile mortality and was statistically on par with isolate 28 (33.33% concentration) and isolate 26 (33.33 and 25% concentration). Isolate 11 at 33.33% concentration was statistically on par with 25% concentration of isolate 26, 28 and 25 giving 69.00 to 73.50 per cent juvenile mortality. Lowest juvenile mortality (65.50 per cent) was recorded by 25% concentration of isolate 11 and was statistically on par with 33.33% concentration of isolate 11 and 25% concentration of isolate 28 and 25. No mortality of juveniles was observed in sterile water while plain broth recorded 7.50 per cent juvenile mortality.

4.4. PATHOGENICITY TEST OF BACTERIAL ISOLATES

The four bacterial isolates (isolate 26, 28, 25 and 11) were tested for their pathogenic reaction towards cowpea plant. Bacterial isolates were inoculated by three different methods *viz* soil drenching, pin prick method and spray inoculation method. No observable symptoms (spots, lesions, necrosis, wilting, blight etc) were found on the plants (Plate 6). Plants found to be healthy even 2 weeks after bacterial isolates inoculation.

One week after inoculation of bacterial isolates

Two weeks after inoculation of bacterial isolates

Plate 6. Pathogenic reaction of selected bacterial isolates on cowpea

4.5. MANAGEMENT OF RENIFORM NEMATODE

The results of pot culture study revealed that the four isolates had significant effect in reducing the population and reproduction of *R. reniformis* on cowpea plants. The initial nematode population was 400 nematodes 200cc soil^{-1}.

4.5.1. Nematode Population in Soil

The data on the effect of bacterial isolates on population build up of nematodes in the rhizosphere of cowpea plants estimated 45 days after planting was presented in Table 28. The four bacterial isolates showed statistically significant reduction in nematode population when compared with control (925.00 nematodes 200cc soil^{-1}).Soil application of bacterial isolate 26 (1×10^7 cfu mL^{-1}) @ 2 mL 100g soil^{-1} recorded lowest nematode population (76.00 nematodes 200cc soil^{-1}) and it was significantly superior to isolate 2825 and 11. The second most effective isolate was isolate 11 (1×10^7 cfu mL^{-1}) which recoded 83.75 nematodes 200cc soil^{-1} and it showed significant superiority over isolate 28 and 25. Soil drenching of isolate 28 and 25 (1×10^7 cfu mL^{-1}) @ 2 mL 100g soil^{-1} recorded 96.75 and 107.25 nematodes 200cc soil^{-1} respectively and effect of these two treatments was significantly different (Table 27).

4.5.2. Number of Females

All the treatments showed statistically significant variation in reducing the number of females in 5g root (Table 27). The mean number of reniform nematode females ranged from 6.25 to 14.50 in treatments with four bacterial isolates while in control it was 37.50 females 5g root^{-1}. Among the bacterial isolates, isolate 26 (1×10^7 cfu mL^{-1}) @ 2 mL 100g soil^{-1} recorded lowest mean number of females (6.25 females 5g root^{-1}) and it showed statistically significant superiority over other isolates (Plate 7). Isolate 28 (1×10^7 cfu mL^{-1}) @ 2 mL 100g soil^{-1} recorded 10.50 females 5g root^{-1} and was statistically on par with isolate 11 (1×10^7 cfu mL^{-1}) treated plants which recorded 12.00 females 5g root^{-1}. The isolate 25 (1×10^7 cfu mL^{-1}) @ 2 mL 100g soil^{-1} recorded 14.50 females 5g root^{-1} which was inferior to isolate 26, 28 and 11.

Table 27. Effect of bacterial isolates on multiplication of *R. reniformis* in cowpea

Treatments	Final nematode population (200cc soil)	No. of females (5g root)	Total no. of nematodes (soil+root)	Reproduction factor (Rf=Pf/Pi)[*]	No. of egg masses (5g root)
Isolate 26	76.00 (8.77) [e]	6.25 (2.68) [d]	82.25 (9.12) [e]	0.21 (1.10) [e]	1.75 (1.65) [d]
Isolate 28	96.75 (9.88) [c]	10.50 (3.38) [c]	107.25 (10.40) [c]	0.27 (1.13) [c]	4.50 (2.33) [c]
Isolate 25	107.25 (10.40) [b]	14.50 (3.93) [b]	121.75 (11.07) [b]	0.30 (1.14) [b]	7.00 (2.81) [b]
Isolate 11	83.75 (9.20) [d]	12.00 (3.59) [c]	95.75 (9.83) [d]	0.24 (1.11) [d]	5.00 (2.43) [bc]
Control	925.00 (30.43) [a]	37.50 (6.20) [a]	962.50 (31.04) [a]	2.41 (1.85) [a]	16.00 (4.11) [a]
CD (0.05)	(0.300)	(0.329)	(0.283)	(0.007)	(0.422)

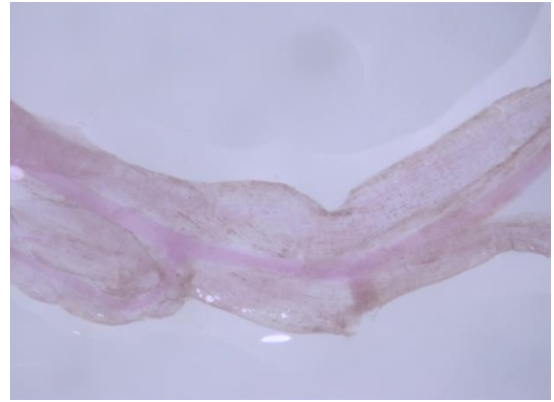[*] Pf-Final population of nematodes   Pi-Initial population of nematodes
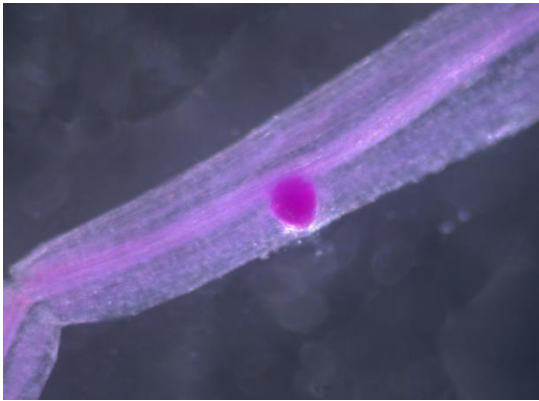Figures in parenthesis are square root transformed values
Numerical values followed by different alphabet are significantly different from each other
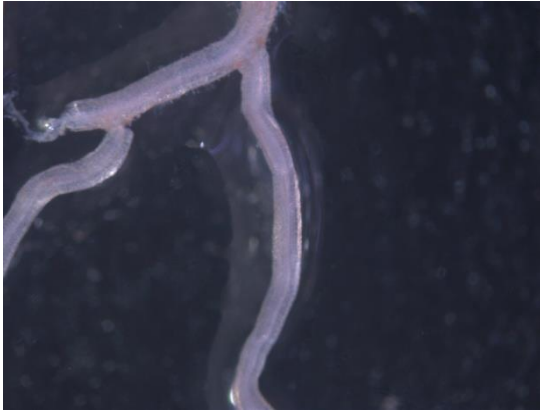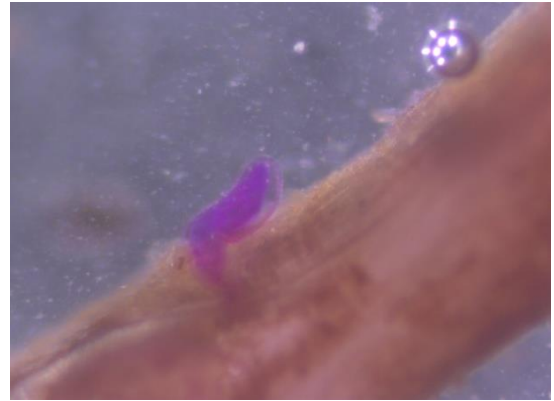
ISOLATE 26

ISOLATE 28

ISOLATE 11

ISOLATE 25

CONTROL

Plate 7. Female reniform nematode root penetration in treated and untreated cowpea plants

4.5.3. Reproduction factor

All the four isolates showed statistically significant superiority over control in reducing total number of nematode in soil and root (Table 27). Highest reduction in total number of nematodes (82.25) was recorded by isolate 26 (1×10^7 cfu mL^{-1}) @ 2 mL 10 soil^{-1} and was significantly superior over other isolates. Minimum reproduction factor (0.21) was obtained in Isolate 26 treated plants. Isolate 11 treated plants recorded 95.75 nematodes in soil and root followed by isolate 28 (107.25) and these two isolates showed significant superiority over isolate 25 which recorded 121.75 nematodes in soil and root. The reproduction factor recorded in isolate 11, 28 and 25 treated plants was 0.24, 0.27, 0.30 and respectively and were significantly different. Reproduction factor in control plants was 2.41.

4.5.4. Number of Egg Masses in Root

All the treatments significantly reduced the number of egg masses in root (5g) (Table 27). Isolate 26 (1×10^7 cfu mL^{-1}) @ 2 mL 100g soil^{-1} recorded lowest mean number of egg masses in 5g root (1.75) and it showed statistically significant superiority over isolate 28, 25 and 11. The isolate 28 (1×10^7 cfu mL^{-1}) @ 2 mL 100g soil^{-1} recorded an average of 4.50 egg masses 5g root^{-1} and it was statistically on par with isolate 11 which recorded 5.00 egg masses 5g root^{-1}. Plants treated with isolate 25 (1×10^7 cfu mL^{-1}) @ 2 mL 100g soil^{-1} (7.00 egg masses 5g root^{-1}) found to be statistically on par with isolate 11. Highest number of egg masses 5g root^{-1} (16.00) was recorded in control plants.

4.6. IDENTIFICATION OF BACTERIAL ISOLATES

4.6.1. Morphological and Cultural Characterization

Colony of isolate 26 was white opaque characteristically large rhizoid colonies with filamentous margin. Dry textured and gram staining positive. Isolate 28 exhibited creamy-white to yellowish, opaque, circular and convex, medium sized colonies with entire margin, colony texture moist and gram staining positive. Isolate 25 was with white small circular colonies with glossy surface slightly raised and entire margin, colony texture was moist and gram staining positive. Colonies of Isolate 11 was

creamy white to white, circular medium sized, with coarsely wrinkled or folded margin, moist texture, opaque and gram staining positive.

4.6.2. Molecular Characterization

The 16Sr RNA sequences of selected bacterial isolates (isolate 26, 28, 25 and 11) are exhibited in Table 29 and 30 (Plate 8). The result of BLAST analysis showed the highest match for the sequence of bacterial isolates given Table 30. The analysis of the sequence similarity of 16S rDNA gene sequence of bacterial isolates with that on Genbank database revealed the similarity of isolates. Isolate 26 showed 99.88 per cent similarity with *Lysinibacillus capsici* strain PB300. Isolate 28 showed 99.49 per cent similarity with *Bacillus paramycoides* strain NH24A2. 99.74 per cent similarity was shown by isolate 25 with *Bacillus thuringiensis* strain ATCC 10792. Isolate 11 have shown 99.02 per cent similarity with *Bacillus luti* strain FJ, *Bacillus albus* strain DLOU-Yingkou, *Bacillus wiedmannii* strain SR52, *Bacillus mobilis* strain ML-A2C4 and *Bacillus pseudomycoides* DSM 12442.

The 16S rRNA sequence of the four bacterial isolates was deposited in the NCBI GenBank data base as new strains of bacteria. The details and accession number of bacterial isolates in NCBI data base are given in Table 31. The four best bacterial isolates were identified as *Lysinibacillus capsici* strain NSK-KAU, *Bacillus paramycoides* strain NSK-KAU, *Bacillus thuringiensis* strain NSK-KAU and *Bacillus* sp. strain NSK-KAU.

Table 28. Analysis of sequence of isolate 26 and isolate 28

Sl.No	Isolates	16Sr RNA sequence
1	Isolate 26	TTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAA CCTACCCTATAGTTTGGGATAACTCCGGGAAACCGGGGCTA ATACCGAATAATCTCTTTTGCTTCATGGTGAAAGACTGAAA GACGGTTTCGGCTGTCGCTATAGGATGGGCCCGCGGCGCAT TAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATG CGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA TCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCG TGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAG GGAAGAACAAGTACAGTAGTAACTGGCTGTACCTTGACGG TACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCC GCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTG GGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTG AAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG GGGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGT AGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGC GAAGGCGACTTTCTGGTCTGTAACTGACGCTGAGGCGCGA AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC ACGCCGTAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCC CCTTAGTGCTGCAGCTAACGCATTAAGCACTCCG
2	Isolate 28	ATGAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACC TGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAA TACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAG GCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATT AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGC GTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGA GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT CTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGT GAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGG GAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGG TACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC CGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTG GGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGA AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGG AGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTA GCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCG AAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAA AGCGTGGGGAGCAAACAGGATTAGATACCCTGGGTAGTCC ACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGGTTTCC GGCCCTTTAGTGCTGAAGTTAACGCA

Table 29. Analysis of sequence of isolate 25 and isolate 11

Sl.No	Isolates	16Sr RNA Sequence
1	Isolate 25	TTATGAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGG
2	Isolate 11	CTTATGAGGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGCGCTGCATGGCGCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGTGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCCTGGGGAGTACG
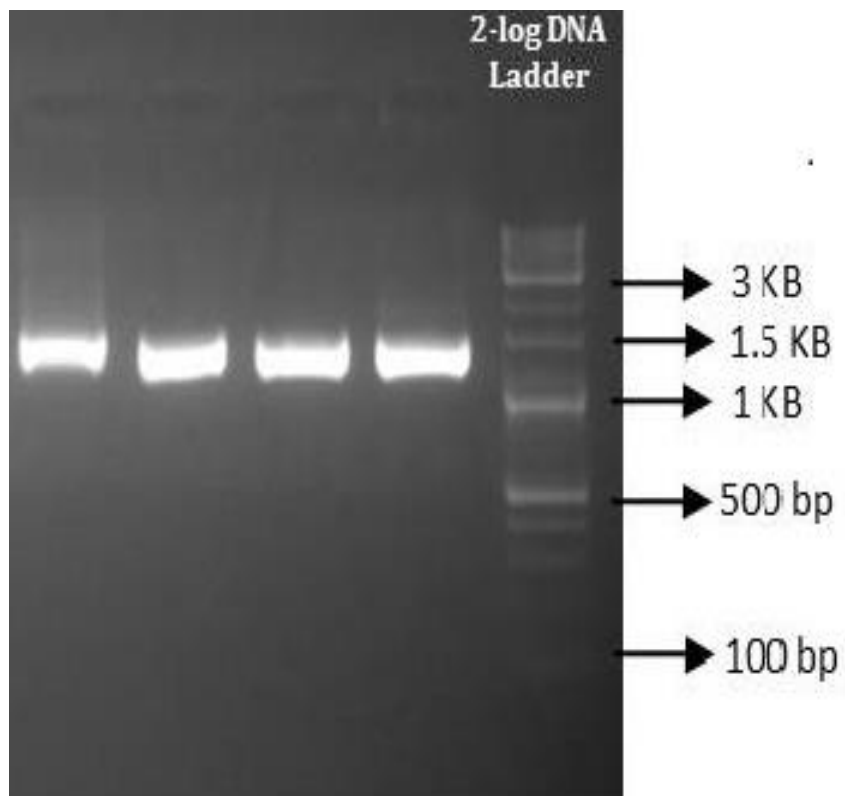
Plate 8. Gel profile of the PCR products

Table 30. Blast details of the DNA of rRNA sequence of isolate 26, 28, 25 and 11 in NCBI data base.

Isolates	Description	Max score	Total score	Query cover	E value	Identity	Accession number
26	*Lysinibacillus capsici* strain PB300	1472	1472	100%	0.0	99.88%	NZ_PXXX0 1000046.1
28	*Bacillus paramycoides* strain NH24A2	1437	1437	100%	0.0	99.49%	NZ_MAO10 1000012.1
25	*Bacillus thuringiensis* strain ATCC 10792	1395	15243	99%	0.0	99.74%	NZ_CP0210 61.1
11	*Bacillus luti* strain FJ	1465	20472	100%	0.0	99.02%	NZ_CP0403 36.1
	Bacillus albus strain DLOU-Yingkou	1465	20456	100%	0.0	99.02%	NZ_CP0403 44.1
	Bacillus wiedmannii strain SR52	1465	20369	100%	0.0	99.02%	NZ_CP0323 65.1
	Bacillus mobilis strain ML-A2C4	1465	20343	100%	0.0	99.02%	NZ_CP0314 43.1
	Bacillus pseudomycoides DSM 12442	1465	1465	100%	0.0	99.02%	NZ_CM0007 45.1

Table 31. Accession number of selected bacterial isolates

Sl. No.	Isolate	Scientific name	Accession number
1	Isolate 26	*Lysinibacillus capsici* strain NSK-KAU	MT509533
2	Isolate 28	*Bacillus paramycoides* strain NSK-KAU	MT510176
3	Isolate 25	*Bacillus thuringiensis* strain NSK-KAU	MT509428
4	Isolate 11	*Bacillus* sp. strain NSK-KAU	MT510172

Discussion

5. DISCUSSION

Vegetable cowpea (*Vigna unguiculata* (L.) Walp.) is an important crop grown in Kerala. It provides proteins, calories, minerals and vitamins. Estimated annual yield loss in cowpea due to phytonematodes is 15.1 per cent (Ravichandra, 2014). Reniform nematode, *Rotylenchulus reniformis* and root knot nematode, *Meloidogyne* spp. are the major constraints to cowpea cultivation in India (Sikora *et al.*, 2005). *R. reniformis* is a sedentary semi-endoparasite which infests the feeder roots and act as predisposing agent in developing bacterial and fungal diseases. Recently, occurrence of reniform nematode was observed in *Fusarium* wilt affected cowpea plants in many farmers' fields of Kerala and the nematode cause for yield loss in cowpea directly or indirectly. Therefore the management of reniform nematode in cowpea and other crops are mandatory.

Management of the nematodes are difficult since most plant parasitic nematodes spend their lives either in the soil or within plant roots. However environment safety and health hazard problem retarded the use of chemical nematicides. No registered chemical nematicides are available recently in Kerala. Hence use of the biocontrol agents to replace these chemicals is gaining importance. The best method to manage reniform nematode is the use of natural biocontrol agents which keep the nematode population below economic injury level. Variety of micro organisms and natural enemies antagonistic to nematodes are present in soil. Exploitation of such microbial antagonists for the management of nematodes gains much importance. In this context, the present study is undertaken to isolate and identify indigenous antagonistic bacteria for managing reniform nematode in vegetable cowpea.

5.1. ISOLATION OF INDIGENOUS BACTERIA

A random sampling was done during 2018-19 in the cowpea grown fields of Aryanad, Athiyannur, Balaramapuram, Kalliyoor, Madavoor, Nemom, Neyyattinkara, Ottasekharamangalam, Ottoor, Vellanad, Vellayani and Venganoor area of Thiruvananthapuram district. A total of sixty six soil and sixty six root samples were collected from the rhizosphere of cowpea. The bacterial isolates with colony

characters similar to that of *Bacillus* and *Pseudomonas* were selected and made into pure culture by streak plate method. Forty five such bacterial isolates were collected with larvicidal activity against *R. reniformis*. Out of forty five bacterial isolates, twenty three were isolated from soil, nine from root and thirteen from egg masses.

Two isolates obtained from Aryanad showed 49.50 and 70.50 per cent mortality of *R. reniformis* juveniles. Five isolates collected from samples from Athiyannur showed 35.50 to 49.00 per cent juvenile mortality. One bacterial isolate obtained from roots of healthy cowpea plants in Balaramapuram showed 43.50 per cent mortality. From Kalliyoor four isolates with 36.00 to 79.00 per cent mortality of *R. reniformis* juveniles were obtained. Five isolates obtained from Madavoor showed 33.00 to 83.00 per cent mortality of *R. reniformis* juveniles. Two isolates with 42.00 and 50.00 per cent mortality were obtained from Nemom. From Neyyattinkara three isolates with 36.00 to 47.50 per cent juvenile mortality were obtained. Eight isolates with 42.00 to 99.50 per cent mortality of *R. reniformis* juveniles were obtained from Ottasekharamangalam. From Ottoor, two isolates with 40.00 and 50.00 per cent juvenile mortality were isolated from rhizosphere soil of cowpea. Four isolates with 53.50 to 79.00 per cent mortality were obtained from Vellanad. From Vellayani five isolates with 63.50 to 100.00 per cent juvenile mortality was isolated. Four isolates obtained from Venganoor showed 39.50 to 65.50 per cent mortality of *R. reniformis* juveniles. Twenty most effective isolates which showed 50.50 to 100.00 per cent *R. reniformis* juvenile mortality were selected for preliminary screening. The morphological and cultural characteristics and colony forming units of the twenty isolates were studied.

The selected bacterial isolates were subjected to preliminary screening for their larvicidal ability against reniform nematode. The four concentrations (100, 50, 33.33 and 25%) of cell free extract (CFE) of bacterial isolates were tested for mortality of *R. reniformis* juveniles at 6, 12 and 24 hours after treatment (HAT). All the bacterial isolates at their four different concentrations of cell free extract recorded significant superiority over control. The twenty isolates at their different concentrations of CFE recorded 1.00 to 100.00 per cent juvenile mortality at 6, 12 and 24 HAT. Out of these twenty isolates four isolates showed 64.50 to 100.00 per cent juvenile mortality at

their 100% CFE concentration. The effect of these isolates was significantly superior to all other sixteen isolates. The four isolates (26, 28, 25 and 11) at their lowest concentration (25%) recorded 65.50 to 73.50 per cent juvenile mortality at 24 HAT (Fig.1). CFE of these four isolates at higher concentration (100%) exhibited higher juvenile mortality (84.50 to 95.50 per cent) even at 12 HAT (Fig.2). Isolate 26 and 25 were isolated from egg masses of *R. reniformis* collected from infected roots of cowpea from Vellayani area. Isolate 28 was collected from rhizosphere soil of cowpea from Vellayani. Isolate 11 was isolated from rhizosphere soil of cowpea from Ottasekharamangalam area of Thiruvananthapuram district.

5.2. SCREENING OF BACTERIAL ISOLATES FOR THEIR NEMATICIDAL PROPERTY AGAINST *R. reniformis*

CFE of four bacterial isolates (26, 28, 25 and 11) selected were screened for egg hatch inhibition and juvenile mortality at four different concentrations under *in vitro.* All the concentrations (100, 50, 33.33 and 25%) of CFE of four isolates showed significant superiority in egg hatch inhibition (78.96 to 96.67 per cent) for three to seven days of exposure in comparison with plain broth and sterile distilled water (Fig.4). The 100% CFE concentration of isolate 26 recorded 1.67 to 3.33 per cent egg hatching which is the minimum egg hatching recorded at three to seven days of treatment (Fig.3). Lower concentrations of isolate 26 (33.33 and 25%) showed 2.29 to 9.58 per cent egg hatching and was equally effective with higher concentration (100%) of isolate 28 and 11. Highest reduction in egg hatching (90.42 to 96.67 per cent) among the four isolates was recorded by isolate 26, at all concentrations, followed by isolate 11 (83.75 to 91.88 per cent) within seven days of exposure (Fig.4). Isolate 28 and 25 also reduced egg hatching considerably (78.96 to 91.04 per cent) within 7 days of exposure.

The egg hatching percentage was inversely proportional to the CFE concentrations of isolates and directly proportional to the period of exposure. The lower concentrations of isolates recorded more egg hatching than higher concentrations as the dilution decreases toxicity of the cell free extract. The 25% concentration of isolate 26, 28, 25 and 11 recorded 2.29 to 21.04 per cent egg hatching

at three to seven days of treatment. While the 100% concentration of four isolates recorded 1.67 to 11.67 per cent egg hatching. Similar trend was observed in many studies. *Bacillus subtilis* strain BS$_t$ at 100% (S) cell free filtrate concentration recorded 99.00 per cent egg hatch inhibition of *R. reniformis* at 48 h after exposure while lower concentrations (S/4, S/8, S/16 and S/20) recorded 96.30, 97.50, 93.10 and 94.10 per cent inhibition (Niknam and Dhawan, 2002a). Undiluted standard filtrate (S) of *Pseudomonas fluorescens* (Isolate Pf$_1$) recorded 100 per cent egg hatch inhibition of *R. reniformis,* while the dilutions (S/4, S/8, S/16 and S/20) recorded 92.60, 86.10, 84.80 and 86.50 per cent inhibition respectively after 72 h of exposure (Niknam and Dhawan, 2002b). *B. subtilis* strain IIHR BS-2 inhibited hatching of eggs (94.65 per cent) and juvenile mortality (91.26 per cent) of *M. incognita* in carrot under *in vitro* condition (Rao *et al.*, 2017). Vishnu (2018) reported that cell free extract of *Stenotrophomonas maltophilia* strain W2-7 and *B. thuringiensis* strain a57 reduced hatching of eggs of *M. incognita* .These two isolates at 25% concentration recorded 74.55 to 76.45 per cent egg hatch inhibition at three to eight days after treatment.

The above results are the evidence of presence of inhibitory compound in the cell free extracts of bacterial isolates causing egg hatch inhibition. Nematode eggs are composed of three membranes *viz* external protein layer, middle chitinous layer and inner lipid layer. Results of many studies revealed that bacterial antagonists of nematodes have appreciable effect in reducing egg hatching. *S. maltophilia* and *Chromobacterium* sp. inhibited egg hatch of the potato cyst nematode up to 70.00 per cent when compared with an untreated control by producing chitinase enzyme (Cronin *et al.*, 1997). The culture filtrate of bacterium isolated from the rhizosphere of tomato, *P. fluorescens* strain CHA0 inhibited egg hatching and caused mortality of *M. javanica* juveniles *in vitro* (Siddiqui *et al.*, 2006). Endophytic strain of *B. subtilis* (BS5) with high surfactin and iturin activity suppressed *M. incognita* egg hatching under *in vitro* condition (Kavitha *et al.,* 2012). The bacterium *Bacillus* sp. strain D2 isolated from the rhizosphere of potato produced extra cellular chitinase which degraded the eggshell and inhibited hatching of potato cyst nematode *Globodera rostochinensis* (Margino *et al.*, 2012). Extracellular gelatinase and chitinase enzymes produced by *Paenibacillus elgii* strain HOA73 inhibited hatching of *M. incognita*
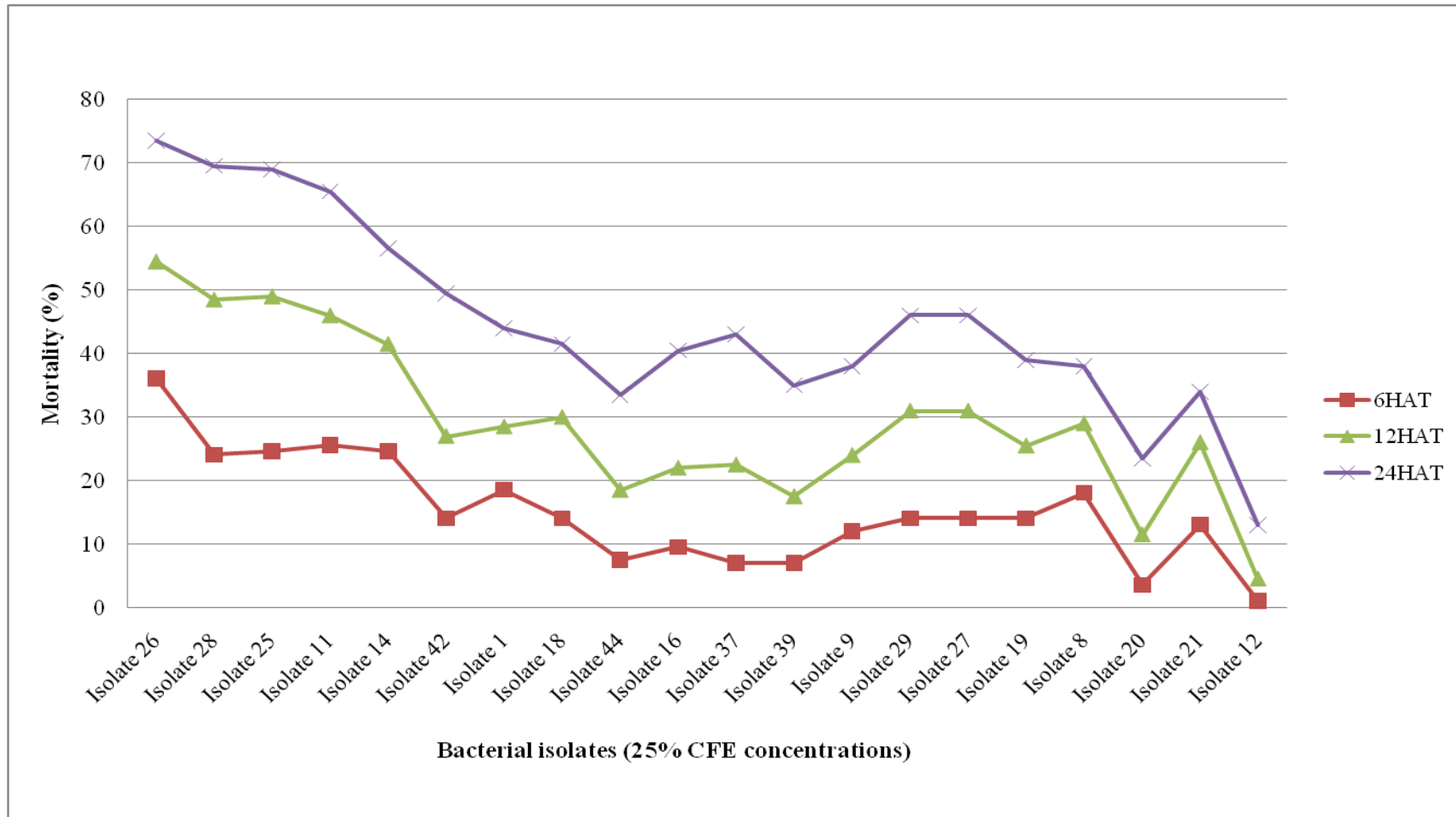
Fig 1. Effect of indigenous bacterial isolates on juvenile mortality of *R. reniformis* at different time of exposure under *in vitro* condition (25% CFE)
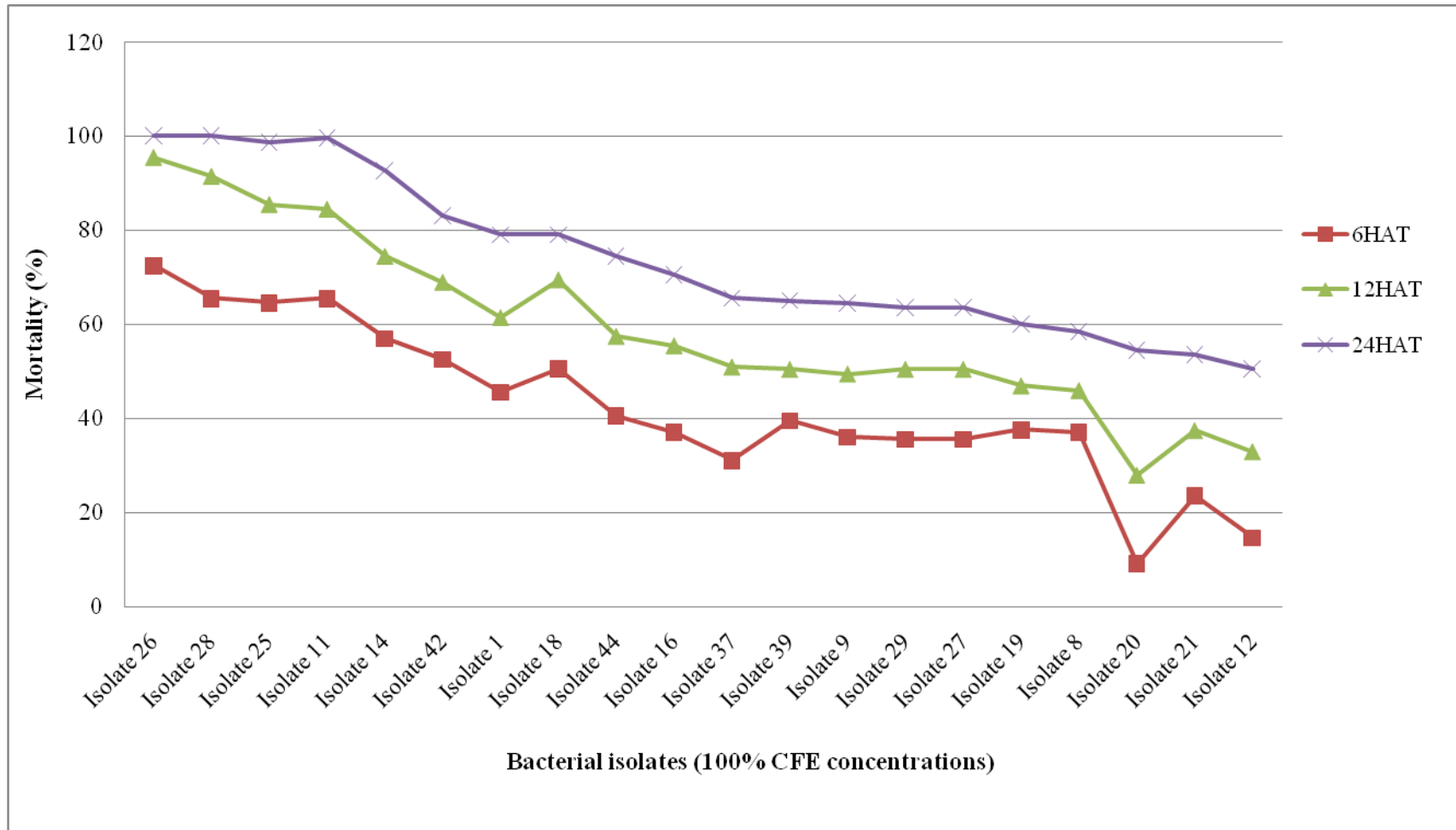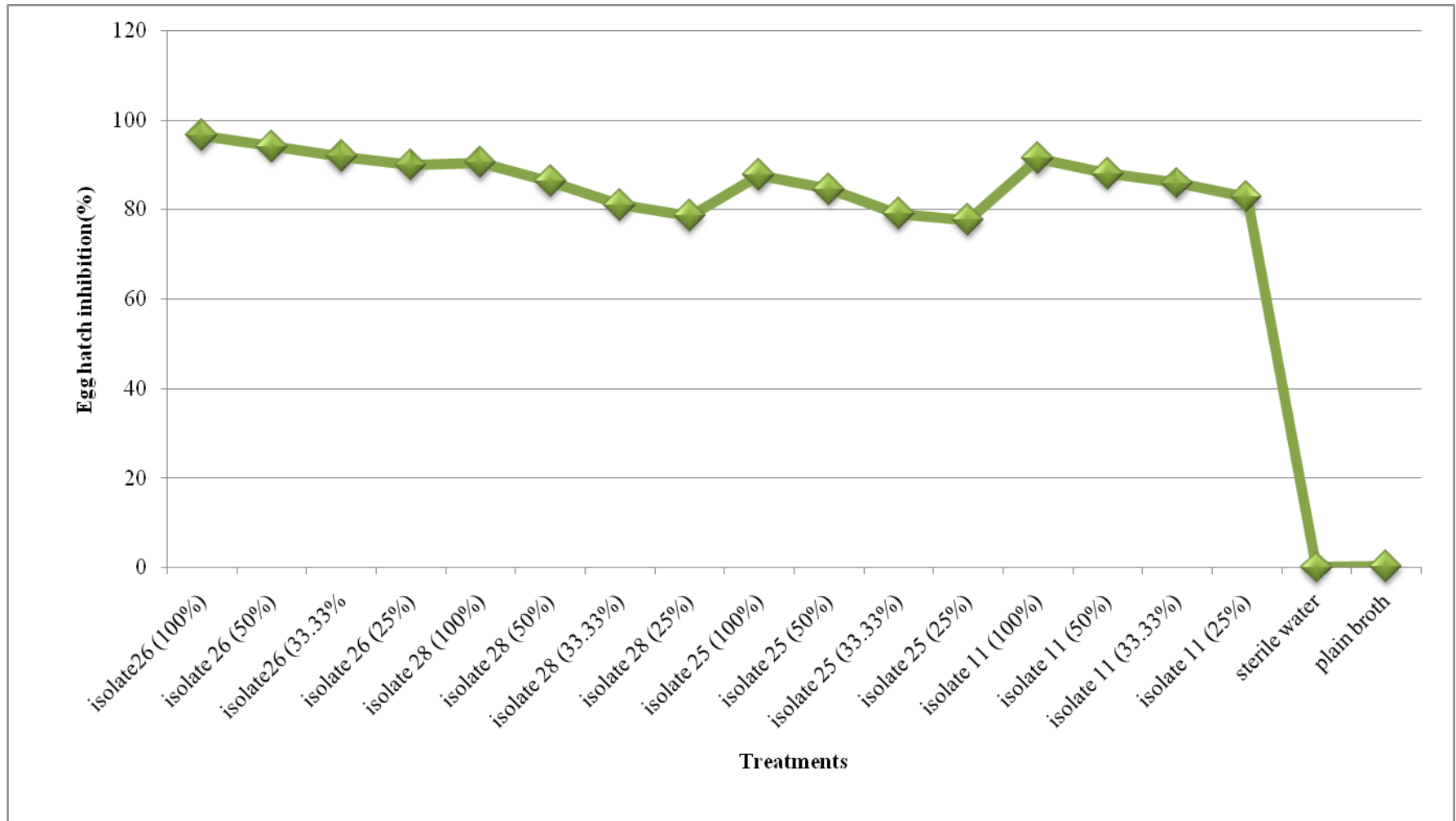
Fig 2. Effect of indigenous bacterial isolates on juvenile mortality of *R. reniformis* at different time of exposure under *in vitro* condition (100% CFE)
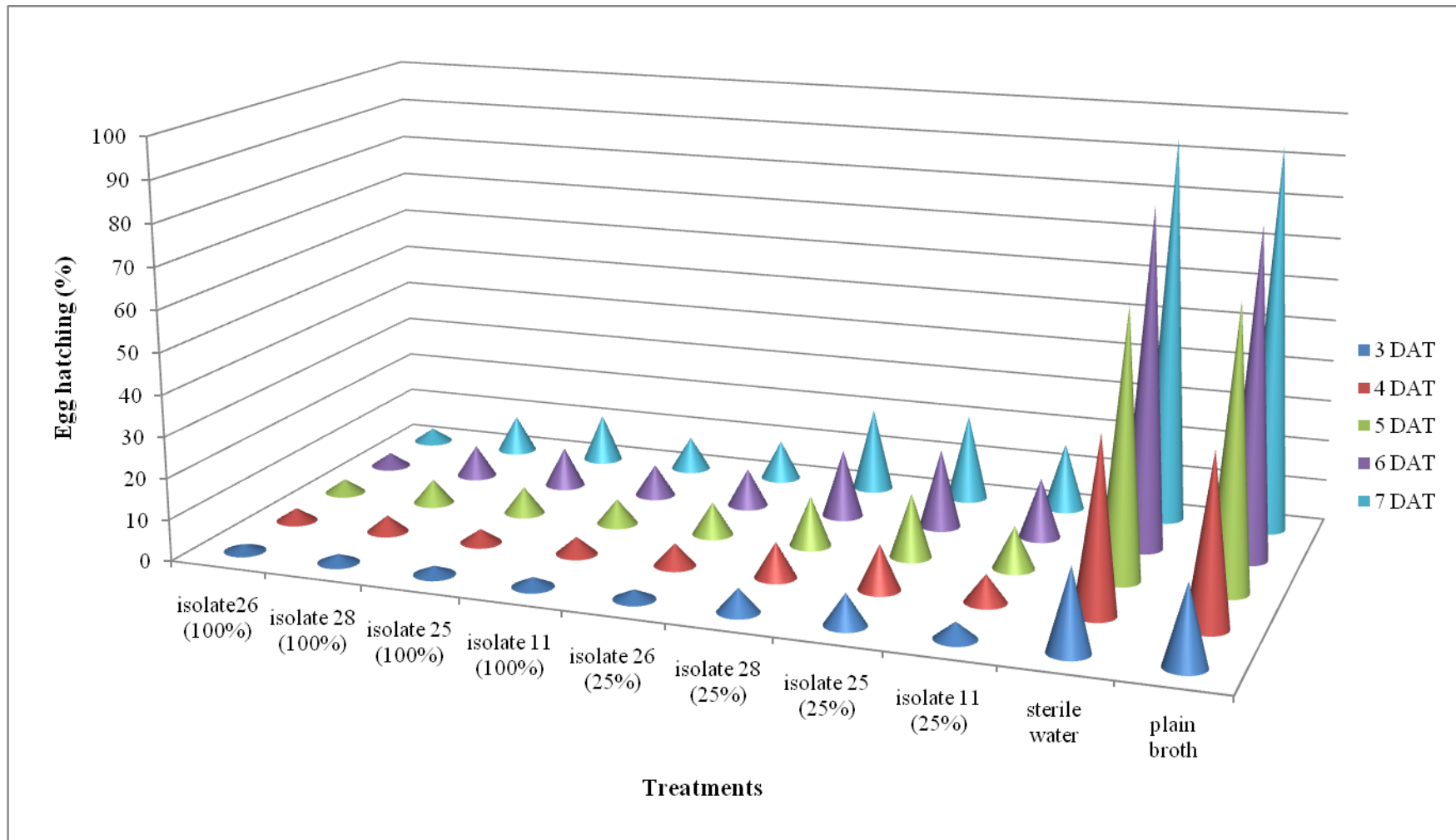
Fig 3. Effect of cell free extracts of bacterial isolates on egg hatching of *R. reniformis*

Fig 4. Effect of cell free extracts of selected bacterial isolates in inhibiting egg hatching of *R. reniformis*

eggs (Nguyen *et al.,* 2013). According to Soilman (2019), the extracellular chitinases and proteases produced by bacteria damage the egg shell and reduce egg hatching. The egg hatch inhibition exhibited by isolate 26, 28, 25 and 11 may be due to the presence of toxic metabolites present in the cell free extract of the bacteria.

Selected four isolates at all concentrations (100, 50, 33.33 and 25%) showed statistically significant superiority in mortality of *R. reniformis* juveniles over control at 6, 12 and 24 hours after treatment. Highest mortality was recorded by isolate 26 and 28 (100% CFE) at 24 HAT (Fig.5). Both recorded 100.00 per cent mortality and was statistically on par with that of 100% of isolate 25 and 11 (98.50 and 99.50 per cent). The trend of decreasing efficacy with dilution was prominent in case of juvenile mortality also. Even though lower concentrations recorded considerable increase in juvenile mortality with prolonged exposure time. The lowest (25%) CFE concentrations of the isolates recorded 65.50 to 73.50 per cent juvenile mortality within 24 HAT while it was 24.00 to 36.00 per cent at 6 HAT. In the study done by Niknam and Dhawan (2002a) 100% (S) cell-free filtrate concentration of *B. subtilis* strain BS_t recorded 88.10 per cent juvenile mortality of *R. reniformis* at 72 HAT while diluted concentrations (S/4, S/8, S/16 and S/20) recorded 42.30 to 14.50 per cent mortality. *P. fluorescens* (Isolate Pf_1) at undiluted standard filtrate (S) concentration recorded 80.10 per cent *R. reniformis* juvenile mortality at 72 HAT while its dilute concentrations recorded (S/4, S/8, S/16 and S/20) 67.40 to 30.00 per cent mortality (Niknam and Dhawan, 2002b). The juvenile mortality was found to be directly proportional with time. At 6 HAT the four isolates recorded 24.00 to 72.50 per cent mortality, while it increased to 65.50 to 100.00 per cent at 24 HAT. Similar trend was observed in different studies. The culture filtrate (CF) of *P. fluorescens* recorded 60.50 to 99.00 per cent mortality of *R. reniformis* at 24 to 72 HAT (Subramanian and Senthamizh, 2006). Devapriyanga *et al.,* (2012) tested *Pseudomonas* and *Bacillus* isolates against root knot nematode. They found that juvenile mortality increased from 42.50 per cent to 91.25 per cent when treated with *Pseudomonas* isolate and 40.75 to 88.00 per cent when treated with *Bacillus* at 24 to 72 HAT. Cell free extract of *B. thuringiensis* strain a57 and *S. maltophilia* strain W2-7 recorded 91.00 to 94.00 per cent mortality of *M. incognita* juveniles 72 HAT (Vishnu, 2018). The juvenile

mortality recorded by the isolates in this study clearly highlights the presence of nematicidal compounds present in the cell free extracts of indigenous bacteria.

Bacterial biocontrol agents inhibit nematodes either by parasitism or by antagonism where they produce variety of antagonistic chemicals against nematodes. The above observations clearly depict the chance of presence of nematicidal compound in the cell free extract of the selected four bacterial isolates. According to Gallagher and Manoil (2001) hydrogen cyanide is the sole or primary toxic factor produced by *P. aeruginosa* PAO1 which rapidly paralyzed and killed the nematode *Caenorhabditis elegans* (Maupas). The biochemical composition of nematode body wall structure includes collagens, lipids etc. during the mobile stages, as well as chitin, protein, and lipids in the sedentary stages such as eggs, mature females etc. Here comes the importance of bacterial biocontrol agents which produces hydrolytic enzymes like protease, chitinase etc which can degrade nematode body wall. The antibiotics 2,4-DAPG (2,4-diacetylpholoroglucinol) and pyoluteorin from *P. fluorescens* CHA0 reduced egg hatch and caused substantial mortality of *M. javanica* juveniles also. It acts as the inducing agents of systemic resistance in tomato roots (Siddiqui and Shaukat, 2003). The cuticle degrading protease from *Bacillus* strains was found to be having nematicidal activity which inhibited root knot nematode *M. incognita in vitro* (Lian *et al.*, 2007). *S. maltophilia* G2 showed significant nematicidal activity by producing extracellular protease against *Panagrellus redivivus* (Linnaeus) and *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle which degraded their body wall within 48 h under *in vitro* condition (Huang *et al.*, 2009). *B. pumilus* L1 found to produce both protease and chitinase against *M. arenaria* (Lee and Kim, 2016).

Results of the *in vitro* study clearly highlighted that bacterial isolates 26, 28, 25 and 11 possess ovicidal and larvicidal properties against *R. reniformis*. Hence these four isolates were evaluated under pot culture condition on cowpea to confirm the biocontrol potential against reniform nematode.
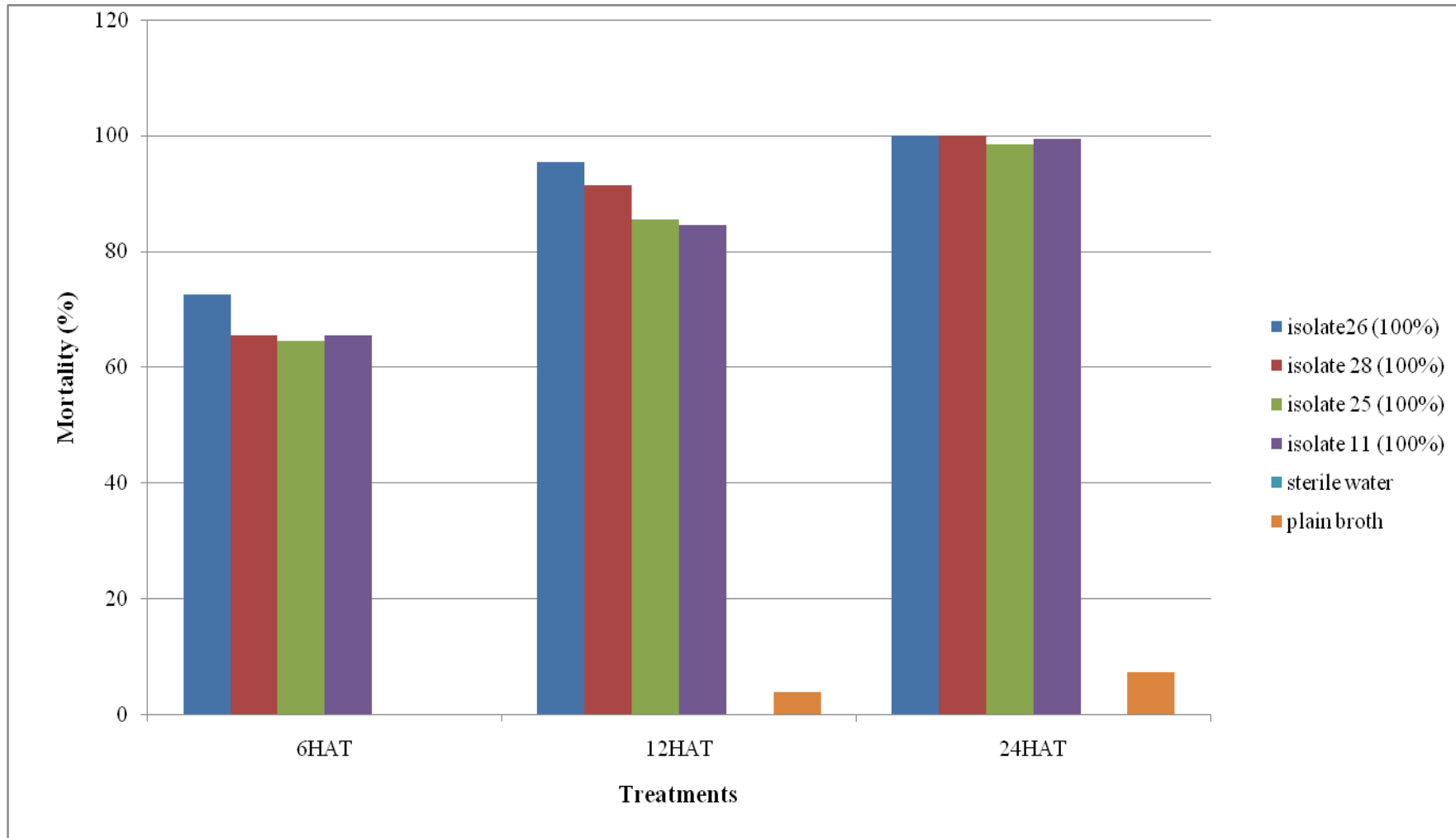
Fig 5. Effect of cell free extracts of bacterial isolates on juvenile mortality of *R. reniformis* under *in vitro* condition (100%)

5.3. MANAGEMENT OF RENIFORM NEMATODE

Results presented in para 4.5. revealed that all the isolates showed significant reduction in multiplication of reniform nematode in the rhizosphere of vegetable cowpea compared to control. Maximum reduction (91.45 per cent reduction over control) in total nematode population (final nematode population in soil and root) was observed in plants drenched with isolate 26 (1×10^7 cfu mL^{-1}) @ 2 mL 100g soil^{-1} followed by isolate 11 (1×10^7 cfu mL^{-1}) @ 2 mL 100g soil^{-1} (90.05 per cent reduction over control). The isolate 28 and 25 (1×10^7 cfu mL^{-1}) @ 2 mL 100g soil^{-1} recorded 88.86 and 87.35 per cent reduction in total nematode population over control respectively (Fig.6). The application of bacterial isolates reduced the number of female penetrated which was reflected in the number of egg masses. The isolate 26 treated plants recorded 1.75 egg masses in 5g root while in control plants it was 16.00 egg masses 5g root^{-1}. Isolate 28, 25 and 11 also showed statistically significant superiority in reducing number of egg masses over control. Reproduction factor is a nematode reproduction assessment tool. Isolates 26, 28, 25 and 11 recorded reproduction factor of 0.21, 0.27, 0.30 and 0.24 respectively while in control plants it was 2.41. Effect of soil application of bacterial bio control agents in reducing population of *R. reniformis* and *M. incognita* was already reported by many authors. Jayakumar *et al.* (2004) reported application of *P. fluorescens* (PF1) reduced *R. reniformis* population in soil (44.80 per cent) and root (70.40 per cent) of cotton. Rhizome treatment with *P. fluorescens* (@ 3% w w^{-1}) significantly improved the growth parameters and yield in kacholam by reducing the total population of root knot nematodes by 78.00 per cent (Nisha and Sheela, 2012). Castillo *et al.* (2013) reported that, cotton seed treatment with *B. firmus* (1.4×10^7cfu seed^{-1}) in non autoclaved field soil infected with reniform nematode reduced vermiform life stages of *R. reniformis* by 37.00 per cent and eggs of *R. reniformis* by 21.00 per cent than the untreated seed control. Soil drenching of *S. maltophilia* Palleroni and Bradbury strain W2-7 (1×10^7 cfu mL^{-1}) @ 50 mL pot^{-1} reduced root knot nematode population in soil (72.69 per cent) and in root (82.23 per cent) and increased yield in tomato (Vishnu, 2018).

From this study it was concluded that the selected four isolates have significant biocontrol potential as it reduced the nematode population and egg mass production

significantly which is reflected in the reproduction factor. Hence these four isolates were identified by molecular characterization.

5.4. IDENTIFICATION OF BACTERIAL ISOLATES

Colonies of isolate 26 was white opaque large rhizoid colonies with filamentous margin. The colony texture was dry and gram staining positive. Isolate 28 was exhibited as Creamy-white to yellowish, opaque, circular and convex, medium sized colonies with entire margin, colony texture was moist and gram staining positive. Isolate 25 were White small circular colonies with glossy surface slightly raised and entire margin, colony texture was moist and gram staining positive. Colonies of Isolate 11 are Colonies are creamy white to white, circular medium sized, with coarsely wrinkled or folded margin, texture was moist, opaque and gram staining positive.

The analysis of the sequence similarity of 16S rDNA gene sequence of bacterial isolates with that on Genbank database revealed the similarity of isolates. Isolate 26 showed 99.88 per cent similarity with *Lysinibacillus capsici* strain PB300. *L. capsici* is a novel species proposed by Burkett-Cadena *et al.* (2019) with type strain PB300. This is a gram-positive, strictly aerobic, motile, rod-shaped, endospore forming bacterium. It was originally isolated from rhizospheric soil of a pepper plant (*Capsicum annuum* L) when screening and bioprospecting for plant beneficial microorganisms were done in USA. So the biocontrol potential of *L. capsici* against *R. reniformis* is reported first time in this study. Other species from genus *Lysinibacillus* have shown biocontrol potential against nematodes. *L. mangiferahumi*, novel species with the type strain M-GX18T was isolated from the rhizosphere soil of mango, China. The isolate produced nematicidal volatile compounds with activities against the root knot nematode *M. incognita* (Yang *et al.*, 2012). *L. sphaericus* isolated from soil were found to reduce root knot nematode juveniles (J_2) in soil in tomato under greenhouse condition (Colagiero *et al.*, 2017). *L. sphaericus* recorded 84.69 per cent *M. incognita* juvenile mortality after 24 h of exposure *in vitro* condition. *L. sphaericus* produced chitinase against nematode (Abdel-Salam *et al.*, 2018).
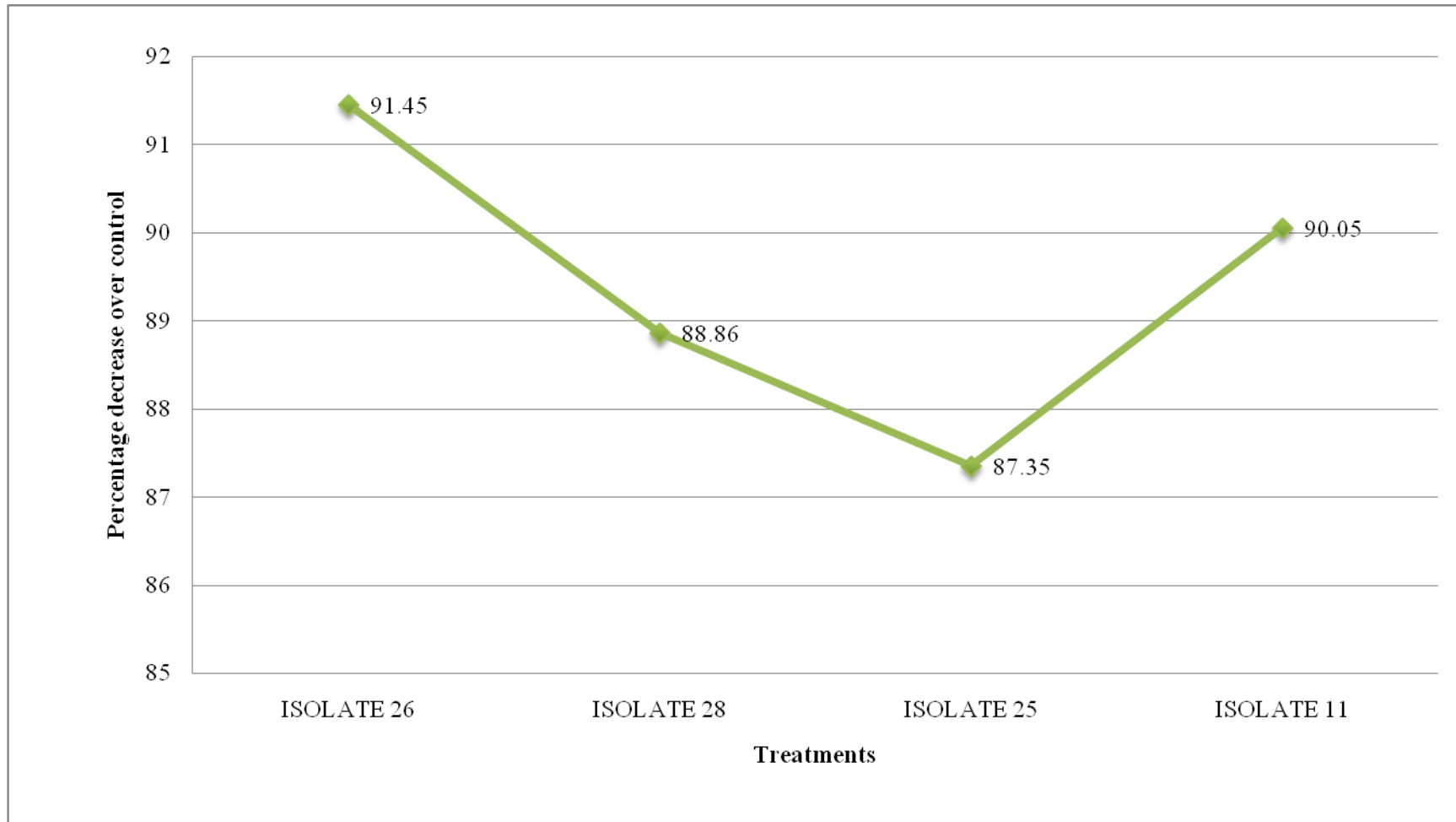
Fig 6. Effect of bacterial isolates on total nematode population in cowpea

Isolate 28 showed 99.49 per cent similarity with *Bacillus paramycoides* strain NH24A2. *B. paramycoides* is a novel species with type strain, NH24A2 reported by Liu *et al.* (2017) from sediment of the South China Sea. They described it as gram-stain-positive, facultatively anaerobic, non motile, rod-shaped bacterium with waxy, circular, non-translucent colonies. Rashid *et al.* (2020) reported that indigenous strain of *B. paramycoides* from Pakistan is capable to bioremediate domestic, hospital, textile, pharmaceutical and mixed wastewaters under optimal conditions. Rhizobacterial isolates from different crops in China were evaluated for their growth promoting capacity on pea plant (*Pisum sativum* L.). One among the isolates was *B. paramycoides* and it showed improved growth character in pea plant. They also found to be capable of siderophore production, phosphate solubilisation and IAA production (Osman and Yin, 2018). Nematode antagonistic property of *B. paramycoides* is reported first time in this study. This bacterium can be exploited as a bio control agent as well as growth promoter.

Isolate 25 showed 99.74 per cent similarity with *Bacillus thuringiensis* strain ATCC 10792. *B. thuringiensis* is known for effective control of insect pests in agriculture. They produce pore forming toxins called Cry protein which cause degradation and gaps in the cell membrane of gut epithelial cells of host. Out of the different cry proteins identified from the *B. thuringiensis* six Cry proteins (Cry5, Cry6, Cry12, Cry13, Cry14, Cry21) are so far known to be toxic to juveniles of nematodes (Tian *et al.*, 2007). Muhammed *et al.* (2008) studied ten *B. thuringiensis* isolates against *M. incognita* in tomato. Out of the ten isolates cry proteins of isolates Bt7N, BtDen, Bt18, BtK73, BtSoto and Bt7 showed highest mortality of juveniles (86.00 to 100.00 per cent). They studied the effect of culture fluid (CF), cell-free supernatant (CFS) and cell-pelleted residues (CP) of each isolates for nematicidal activity on tomato plant. The results showed that both crude suspension (CS) and cell free supernatant (CFS) of isolate Bt7N was the best out of ten. It reduced the number of egg masses by 78.00 and 77.00 per cent, and number of eggs by 84.00 per cent and 76.00 per cent, respectively compared to control. Results of the present study revealed that *B. thuringiensis* exhibited 98.50 percent juvenile mortality under *in vitro* condition and under pot culture condition 88.41 and 61.33 per cent reduction in

nematode population was observed. Hence this bacterium can be exploited as a potential biocontrol agent against *R. reniformis.*

The isolate 11 showed 99.02 per cent similarity with *Bacillus luti* strain FJ*, Bacillus albus* strain DLOU-Yingkou*, Bacillus wiedmannii* strain SR52*, Bacillus mobilis* strain ML-A2C4 and *Bacillus pseudomycoides* DSM 12442.

Bacillus albus, *B. luti* and *B. mobilis* are novel species reported by Liu *et al.* (2017). *B. albus* is gram-stain-positive, facultatively anaerobic, non-motile, rod-shaped bacterium with white, circular, non-translucent colonies. It was isolated from sediment of the South China Sea with type strain, N35-10-2T. *B. mobilis* is gram-stain-positive, facultatively anaerobic, motile by means of peritrichous flagella, rod-shaped bacterium with milk white, circular, nontranslucent colonies. It was isolated from sediment of the Indian Ocean with type strain, 0711P9-1T. *B. luti* is gram-stain-positive, facultatively anaerobic, motile by means of peritrichous flagella, rod-shaped bacterium with white, circular, non-translucent colonies. It was isolated from sediment of the Pacific Ocean with type strain, TD41T.

Bacillus wiedmannii, was one among the root-associated bacterial endophytes isolated and studied by Khaskheli *et al.*, (2020). It was found to be antagonistic against rice (*Oryza sativa* L.) fungal pathogens. *B. wiedmannii* strain MP1a isolated from rice rhizosphere showed 65.00 per cent rice blast disease inhibition (Netravathi, 2018). *B. wiedmannii* strain IPR7a was found to be potent antagonist against *Sclerotium rolfsii* in tomato (Sahu, 2018).

An antimicrobially active substance was found to be produced by *B. pseudomycoides* known as Pseudomycoicidin. It is a Class II Lantibiotic. Lantibiotics are ribosomally synthesized antimicrobial peptides (Basi-Chipalu *et al.*, 2015).

The sequences of the four bacterial isolates were uploaded in the NCBI data base as new strains of bacteria. The isolates were identified as *Lysinibacillus capsici* strain NSK-KAU, *Bacillus paramycoides* strain NSK-KAU, *Bacillus thuringiensis* strain NSK-KAU and *Bacillus* sp. strain NSK-KAU. The results from this study

indicate that the four bacterial isolates are potent biocontrol agents for reniform nematode. The isolate can be further studied and biocontrol potential of bacterial isolates can be exploited by isolation and characterization of nematicidal toxic principles from its secondary metabolites. The possibilities to prepare formulations and field use of these isolates can also be exploited.

Summary

6. SUMMARY

The study entitled "Management of reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira in vegetable cowpea using bacterial antagonists" was done in the Department of Nematology, College of Agriculture Vellayani, during 2018-2020. Main objective of the study were to isolate indigenous bacterial antagonists and evaluate their biocontrol potential against *R. reniformis* in vegetable cowpea.

A survey was conducted in Thiruvananthapuram district during 2018-19. Sixty six samples of rhizosphere soil and roots were collected from cowpea grown fields of Aryanad, Athiyannur, Balaramapuram, Kalliyoor, Madavoor, Nemom, Neyyattinkara, Ottasekharamangalam, Ottoor, Vellanad, Vellayani and Venganoor areas. Samples were collected from plants showing symptoms (yellowing, wilting, necrosis and root rotting) of reniform nematode infestation and healthy plants. The samples (soil, root and egg masses from infected roots) were processed for isolation of indigenous bacterial antagonists of reniform nematode. Bacterial colonies showing colony character similar to *Pseudomonas* and *Bacillus* were isolated and purified.

The collected bacterial isolates were tested for their efficacy against *R. reniformis* juveniles. A total of forty five bacterial isolates which showed 33.00 to 100.00 per cent mortality of *R. reniformis* juveniles were selected for further study. From theses forty five isolates twenty isolates showed more than 50.00 per cent mortality of *R. reniformis* juveniles. Morphological and cultural characteristics of these twenty isolates were studied and colony forming units were also estimated.

A preliminary screening was done among the selected twenty isolates. In preliminary screening the different concentration (S, S/2, S/3 and S/4) of cell free extracts (CFE) of bacterial isolates were tested for their effect on *R. reniformis* juvenile mortality. The twenty isolates at their different concentrations of CFE recorded varying degree of juvenile mortality (1.00 to 100.00 per cent) at 6, 12 and 24 HAT. Isolate 26, 28, 25 and 11 at 100% CFE concentration showed 98.50 to 100.00 per cent mortality of *R. reniformis* juveniles at 24 HAT. These four isolates at lowest

concentration (25%) of CFE recorded 65.50 to 73.50 per cent juvenile mortality at 24 HAT.

In vitro studies were conducted to evaluate the effect of different concentrations (100, 50, 33.33 and 25%) of selected bacterial isolates (isolate 26, 28, 25 and 11) on egg hatch inhibition and juvenile mortality. All concentrations of isolate 26, 28, 25 and 11 showed statistically significant superiority over control (sterile water and plain broth) in egg hatch inhibition. Isolate 26 at different concentrations (100, 50, 33.33 and 25%) recorded 1.67 to 9.58 per cent egg hatching at an exposure period of 3 to 7 days followed by isolate 11 which showed 2.92 to 16.25 per cent egg hatching. Minimum egg hatching (1.67 to 3.33 per cent) was recorded by isolate 26 at 100% concentration on 3rd, 4th and 5th, 6th and 7th day after treatment (DAT). Egg hatching recorded by isolate 26 at lower concentration (25%) found to be statistically on par with 50% concentration of isolate 28 and 25 giving 2.50 to 3.33 per cent hatching of eggs on 3DAT.

Regarding juvenile mortality of *R. reniformis,* all concentrations of isolate 26, 28, 25 and 11 showed significant effect compared to control. Effect of CFE of four isolates at 100% concentration was statistically on par with 98.50 to 100.00 per cent juvenile mortality at 24 HAT. The effect decreased with increase in dilution of CFE of isolates where higher concentration (100%) of four isolates recorded 84.50 to 95.50 per cent mortality while lower concentration (25%) recorded 46.00 to 54.50 per cent at 12 HAT. Lower concentration (25%) of the four isolates recorded more than 65.00 per cent mortality of *R. reniformis* juveniles at 24 HAT.

A study was conducted in glass house condition to find out any pathogenic reaction of these four isolates on cowpea. The bacterial isolates were inoculation by different means (soil drenching, pin prick method and spray inoculation method). No pathogenic reactions of bacterial isolates were found on cowpea plants. The cowpea plants were found healthy even after 2 weeks of inoculation.

Pot culture study was conducted to evaluate the efficacy of four indigenous isolates (isolate 26, 28, 25 and 11) on reproduction and multiplication of *R. reniformis* on vegetable cowpea. The results were assessed in terms of nematode population

characteristics (number of nematodes in soil, number of female in root, number of egg masses in root, reproduction factor). The four bacterial isolates were able to bring down the population of reniform nematode to below economic threshold level within the time period of one full life cycle of reniform nematode. Soil application of the bacterial isolates (1×10^7 cfu mL^{-1}) @ 2 mL 100g soil^{-1} significantly reduced nematode population in soil (88.41 to 91.78 per cent reduction over control) and root (61.33 to 83.33 per cent reduction over control). Regarding number of egg masses 5g root^{-1} also the four isolates recorded significant reduction compared to control (56.25 to 89.06 per cent). Isolate 26, 28, 25 and 11 recorded a reproduction factor less than 0.50 while in control it was 2.41. These findings strongly indicate that these four indigenous bacterial isolates have excellent biocontrol potential against *R. reniformis*.

Morphological and cultural characteristics of the four isolates (isolate 26, 28, 25 and 11) were studied. Colonies of isolate 26 was white opaque characteristically large rhizoid with filamentous margin. The colony texture was dry and gram staining positive. Isolate 28 was exhibited as Creamy-white to yellowish, opaque, circular and convex, medium sized colonies with entire margin, colony texture was moist and gram staining positive. Isolate 25 were white small circular colonies with glossy surface slightly raised and entire margin, colony texture was moist and gram staining positive. Colonies of Isolate 11 was creamy white to white, circular medium sized, with coarsely wrinkled or folded margin. Colony texture was moist, opaque and gram staining positive.

Molecular characterization was done for the identification of bacterial isolates. The BLAST result of amplified 16S rRNA of bacterial isolates in the NCBI GenBank data base revealed the similarity of bacterial isolates. Isolate 26 showed 99.88 per cent similarity with *Lysinibacillus capsici* strain PB300. Isolate 28 showed 99.49 per cent similarity with *Bacillus paramycoides* strain NH24A2. Isolate 25 showed 99.74 per cent similarity with *Bacillus thuringiensis* strain ATCC 10792. The isolate 11 showed 99.02 per cent similarity with *Bacillus luti* strain FJ, *Bacillus albus* strain DLOU-Yingkou, *Bacillus wiedmannii* strain SR52, *Bacillus mobilis* strain ML-A2C4 and *Bacillus pseudomycoides* DSM 12442. Based on the BLAST analysis, the isolated bacterial sequences were identified as *Lysinibacillus capsici* strain NSK-KAU,

Bacillus paramycoides strain NSK-KAU, *Bacillus thuringiensis* strain NSK-KAU and *Bacillus* sp. strain NSK-KAU.

Based on these results, *L. capsici, B. paramycoides, B. thuringiensis* and *Bacillus* sp. which showed high egg hatch inhibition and juvenile mortality under *in vitro* condition and low reproduction factor and egg masses under *in vivo* condition are potential bacterial antagonists against *R. reniformis* in vegetable cowpea. The nematicidal properties of these bacterial isolates are being reported for the first time from Kerala. These bacterial antagonists can be exploited as successful biocontrol agents for the management of reniform nematode in cowpea.

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MANAGEMENT OF RENIFORM NEMATODE,
Rotylenchulus reniformis LINFORD AND OLIVEIRA
IN VEGETABLE COWPEA USING BACTERIAL
ANTAGONISTS

by

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ABSTRACT

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ABSTRACT

The research entitled "Management of reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira in vegetable cowpea using bacterial antagonists" was under taken in the Department of Nematology, College of Agriculture Vellayani, during 2018-2020. The objective was to isolate indigenous bacterial antagonists and to evaluate their biocontrol potential against *R. reniformis* in vegetable cowpea.

A random sampling was done during 2018-19 in the cowpea grown fields of Aryanad, Athiyannur, Balaramapuram, Kalliyoor, Madavoor, Nemom, Neyyattinkara, Ottasekharamangalam, Ottoor, Vellanad, Vellayani and Venganoor areas of Thiruvananthapuram district. A total of sixty six soil and root samples were collected from rhizosphere of vegetable cowpea for isolation of indigenous bacterial antagonists against reniform nematode by serial dilution technique. The bacterial colonies having characters similar to *Bacillus* and *Pseudomonas* were made into pure culture by streak plate method.

Cell free extracts (CFE) of forty five bacterial isolates obtained from soil, root and egg masses of *R. reniformis* were screened for juvenile mortality against *R. reniformis* and isolates which showed more than 50 per cent juvenile mortality were selected for preliminary screening under *in vitro* condition. CFE of twenty bacterial isolates at 100% concentration showed 50.50 to 100.00 per cent juvenile mortality 24 hours after treatment (HAT). The morphological and cultural characteristics and colony forming units of the twenty isolates were studied. CFE of four isolates (Isolate 26, 28, 25 and 11) at lowest concentration (25%) showed 65.50 to 73.50 per cent mortality of R. *reniformis* juveniles at 24 HAT while at highest concentration (100%) it was 98.50 to 100.00 per cent.

The selected bacterial isolates were tested for effect on egg hatching and juvenile mortality of *R. reniformis*. Experiment was laid out in CRD with 100, 50, 33.3 and 25% concentration of four selected isolates, plain broth and sterile distilled water as treatments and four replications. Isolate 26, 28, 25 and 11 at 100% concentration

recorded 3.33, 8.96, 11.67, 8.12 per cent egg hatching respectively at 7 days after treatment which was significantly superior to plain broth (93.96) and sterile distilled (94.17). CFE of Isolate 26, 28, 25 and 11 at 100% recorded 100, 100, 98.5 and 99.5 per cent juvenile mortality respectively 24 HAT and it was statistically on par. CFE of Isolate 26 at 100% concentration recorded 95.50 per cent juvenile mortality at 12 HAT.

The four best isolates were tested for pathogenic reaction towards cowpea plant and none of them were pathogenic. Pot culture study was laid out in CRD to find out the biocontrol potential of the four isolates on *R. reniformis* in vegetable cowpea with five treatments and four replications. Effect of the four indigenous isolates were significantly superior to the untreated in reducing the nematode population in soil (87.35 to 91.45 per cent reduction over untreated) and number of egg masses in root (56.25 to 89.06 per cent reduction over untreated). The reproduction factor was also found reduced by the bacterial isolate application. The reproduction factor recorded by isolate 26, 28, 25 and 11 was 0.21, 0.27, 0.30 and 0.24 respectively while in control it was 2.41.

The molecular characterization was done for identification of the bacterial isolates. Internal transcribed regions of DNA of 16SrRNA of bacterial isolates were amplified by PCR using CAGGCCTAACACATGCAAGTC as forward primer and GGGCGGWGTGTACAAGGC as reverse primer. The blast search of amplified DNA in NCBI data revealed the identity of bacterial isolates. The Isolate 26, 28, 25 and 11 were identified as *Lysinibacillus capsici* strain NSK-KAU, *Bacillus paramycoides* strain NSK-KAU, *Bacillus thuringiensis* strain NSK-KAU, and *Bacillus* sp. strain NSK-KAU respectively.

L. capsici, B. paramycoides, B. thuringiensis and *Bacillus* sp. showed high egg hatch inhibition and juvenile mortality under *in vitro* condition and low reproduction factor and egg masses under *in vivo* condition. This was reported first time from Kerala. From this study, it could be concluded that these bacterial antagonists can be exploited as successful biocontrol agents for the management of reniform nematode in cowpea.