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RESEARCH

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Nematode egg parasitic fungus, *Purpureocillium lilacinum*: efficacy of indigenous strains for the management of *Meloidogyne incognita* in chickpea

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Abstract

Background Nematode egg parasitic fungus, *Purpureocillium lilacinum* is the most effective biocontrol agent and has been widely used commercially in many countries for the management of root-knot nematode, *Meloidogyne incognita*. Availability of indigenous potential strains specific to an agro ecosystem is very crucial for their successful commercial exploitation for suppression of nematode population. Hence, an attempt was made to isolate, characterize, evaluate and identify potential indigenous strains of *P. lilacinum* from pulse ecosystem for root-knot nematode management in chickpea.

Results The fungal colony was initially white and when spore was formed it turned into pink colour in 72 to 96 h. Hyphae was hyaline and septate, conidiophore was blunt, and phialides were with wide base and long neck bearing round to oval conidia in chains. Molecular identification of the species, *P. lilacinum* was carried out based on ITS1-5.8S-ITS2 region of the genomic DNA. In vitro bioassay of cultural filtrates on juvenile mortality revealed that maximum percentage of mortality was observed in IIPR-Pl-11 (88.36%). Spectrophotometric assay on chitinolytic activity showed that the strain IIPR-Pl-11 produced significantly high chitinolytic activity, chitinase enzyme and total protein content (0.139, 51.1 and173.75 µg/ml at 5days); (0.245, 90.1 and 272.67 µg/ml at 10 days) and (0.273, 100.4 and 306.25 µg/ml), respectively, at 15 days of culturing in colloidal chitin-enriched medium C. 2D gel electrophoresis of the crude chitinase suspension showed the presence of chitinase (32, 46 kDa size) in the sample from chitinase-induced medium C. In vitro bioassay of the cultural filtrates of the fungus grown in chitin-enriched medium C on inhibition on egg hatching revealed that the highest percent inhibition on egg hatching showed by IIPR-Pl-8 strain at 5 days of inoculation (42.6%) and IIPR-Pl-11 at 10 and 20 days of inoculation (62.80 and 93.50%), respectively. In vivo pot experiment revealed that among all strains, IIPR-Pl-11 was efficient in promoting plant growth very effectively by reducing gall number (41.3 per plant), egg mass (28.3 per plant) and soil population (284.3 per 200cc of soil) compared to control.

Conclusion *P. lilacinum* strain IIPR-Pl-11 was the highest potential strain from pulse rhizosphere for the management of root-knot nematode, *M. incognita* in chickpea.

Keywords *Purpureocillium lilacinum*, ITS1-5.8S-ITS2, *Meloidogyne incognita*, Management, Chickpea

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Background

Root-knot diseases caused by parasitic nematodes, *Meloidogyne* spp. are one of the key limiting factors of Chickpea, *Cicer arietinum* L. production throughout the world. Global losses due to root-knot nematode (RKN) infection in chickpea have been approximated at 13.7% which translated to loss of billions of rupees annually. In India, Gaur et al. (2001) estimated the avoidable yield losses due to RKN infection in pulses ranged from 20 to 35%. The nematode enters the root, migrates intercellularly and starts feeding on root cambium cells and transforms them to nutrient sink "Giant cell" for continuous supply of nutrients, typically called as sedentary endo-parasitism. The various physiological reactions due to nematode infection in the host cells culminated into the development of typical "gall or knot" on the root, which hinders the translocation of water and nutrients. Intensive agriculture system favours rapid built up of nematode population in a season, because of its high fecundity and relatively short life cycle of 25 days. So, once the field is infected with the nematode, it is extremely difficult to eliminate completely. Although chemicals are highly effective, nowadays, their usage has been discouraged, because of health hazard, soil and water contamination. Utilization of antagonistic microorganisms in rhizosphere, *i.e.* biocontrol agents is a promising alternative to chemicals and capable of providing environment friendly and sustainable solution to nematode problems.

The fungus genus *Paecilomyces* was first described by Bainer et al. (1907), and it was redescribed by Samson (1974). Currently, there are 145 species that belong to the genus *Paecilomyces* in fungal taxonomy database "The Fungorum". With the advancement of the latest bio-technological tool in taxonomical classification, *Paecilomyces lilacinus* (Thom) Samson was given the new genus name, *Purpureocillium lilacinum* by Luangsa-Ard et al (2011). *P. lilacinum* can parasitize different stages of nematode development, *i.e.* egg, young or adult female. Thus, it has been widely used as an important biocontrol agent in different formulations around the world. The efficacy of *P. lilacinum* for the management of *Meloidogyne* spp. in different crops has been demonstrated well (Abd-Elgawad and Askary 2018).

Non-availability of well-characterized high potential strains of *P. lilacinum* for pulse ecosystem, which is mostly rainfed, limits the utilization of its full potential for pulse nematode management in India. The aim of present study was: (1) to isolate and characterize *P. lilacinum* strains from pulse rhizosphere and (2) to identify potential strain for the management of RKN, *M. incognita* in chickpea.

Methods

Collection of soil sample

Surveys were carried out in four agro climatic zones, viz. eastern plains (Varanasi, Uttar Pradesh), central plains (Sitapur, Uttar Pradesh), north eastern plains (Deoria & Kushinagar, Uttar Pradesh) and southern plateau and hill zone (Salem, Tamil Nadu) in India during 2017–19 and collected soil and plant samples from the root-knot nematode infected pulses rhizosphere for isolation of *P. lilacinum*. Random sampling method was followed, at each site, weights of 200 to 300g of soil and root samples from rhizosphere were collected and packed well in polyethylene bag with proper labelling. Information on crop history, soil type and other relevant data were also collected for further reference. The samples were stored in refrigerator at 4 °C for future processing.

Isolation, identification of *P. lilacinum*

Three methods were employed for isolation: (1) Dilution plating method of infected soil: serial dilutions of 1g soil up to 10^{-5} level were carried out using autoclaved double distilled water (ADDW). A total of 100µl of suspension from 10^{-5} dilution was spread in a Petri dish containing PDA medium and incubated at 26 ± 2 °C for 72 h. (2) Direct inoculation of egg masses: infected roots were washed thrice with running water to remove adhering soil particles. Egg masses from the roots were removed and surface-sterilized with 0.4% sodium hypochlorite solution (NaOCl). Surface-sterilized egg masses were washed three times in autoclaved double distilled water and kept on PDA medium. The plates were incubated at 26 ± 2 °C for 72 h. (3) Dilution plating method of infected root: washed infected roots were chopped into small pieces and ground in blender. The crude material was filtered to remove root debris and the filtrate was used for dilution plating on PDA, following previously described procedure. The suspected colony of *P. lilacinum* from the plates was sub cultured and used to study various morphological features for identification of fungus (Samson 1974) by slide culture method with the help of *Leica* compound microscope.

Electron microscopy

Samples were prepared for electron microscopy by fixation in glutaraldehyde formaldehyde in 0.1M phosphate buffer (pH 7.2) and then in 1% osmium tetroxide. The buffers were removed by repeated washing and the samples were dehydrated through different ethanol concentrations (10 to100% ethanol). Samples were mounted for scanning electron microscopy and coated with 20nm of gold. Fungal specimens were observed for their various

structures with the help of Scanning Electron Microscope (Cambridge 250 Mark 3). The morphometrics were carried out by image software.

Single spore culture

Spore suspension: 1 ml of ADDW was applied on the surface of fully grown *P. lilacinum* colony on PDA. The Petri dishes were rotated both clockwise and anticlockwise for complete washing off conidial spores, and they were collected in Eppendorf tube. The spore suspension was stored at 4 °C till further use. Serial dilutions of this spore suspension were done up to 10^{-7} dilution as per the previously described procedure to reduce the density of spores in the suspension. A total of 50 µl of dilution 10^{7} suspension was inoculated on PDA plates through spread plate technique. The emerging colony from individual spore was cork bored and transferred to new PDA plates; thus, single spore culture was developed for different isolates. This pure culture was stored at 4 °C and utilized for all further studies (Bin Liu et al. 2020).

Genomic DNA isolation

The fungus was grown in potato dextrose broth (PDB) at 26 ± 2 °C for 10 days in shaker incubator. After well growth, the mycelial mat was separated by filtering the cultural filtrates through Whatman No.1 filter paper. The genomic DNA was extracted from fungal mat by using nucleopore bacteria/fungal genomic kit, following the manufacturer protocol. The DNA was checked with 0.8% agarose GEL and concentration, and purity of DNA was ascertained by using NanoDrop (Baron NC et al. 2020).

PCR amplification and sequencing

The ITS1-5.8S-ITS2 region was amplified using universal ITS primers ITS1 5´TCCGTAGGTGAACCTGCG G 3´ and ITS4 5´TCCTCCGCTTATTGATATGC3´ (White et al. 1990). The reaction mixture was prepared for 25 µl: 2 µl DNA template, 0.8 µl Primer both forward and reverse, 12 µl PCR master mix and 9.4 µl DNAse free water. The thermal cycler was programmed for 30 cycles with the following timings: 4 min at 94 °C for initial denaturation, 30 s at 94 °C for denaturation, 30 s at 55 °C for annealing, 60 s at 72 °C for elongation, 4 min at 72 °C for final elongation. The PCR product was checked in 1.6% agarose gel and processed further with nucleopore gel extraction kit following manufacturers' protocol for sequencing (Baron NC et al. 2020).

Maintenance of pure culture of *M. incognita*

Pure culture of *Meloidogyne* sp. was developed from single egg mass culture. Individual egg masses along with adult females were collected from infected roots and kept individually in 12 well tissue culture plates containing 1ml of ADDW for hatching. The species, *M. incognita* was identified based on perineal pattern of the female. The freshly hatched juveniles from *M. incognita* were inoculated on susceptible host tomato cv. NS-585 in 15-cm-diameter pots for multiplication. The egg masses and juveniles from the pure culture were used for further study (Hanan et al. 2013).

Colloidal chitin preparation

A total of one hundred grams of chitin flakes was added slowly to 500 ml concentrated HCl and mixed gently for 3 h on a magnetic stirrer. This solution was then filtered through muslin clothes sandwiched by glass wool to 5 l of pre-chilled, distilled water with constant mixing and allowed to settle at 4 °C overnight. A dense white precipitate formed was separated from supernatant and then centrifuged at 10,000 rpm for 10 min at 4 °C, washed in cold, distilled water repeatedly until the pH of the wash reached 5.5. The supernatant was discarded, and the remaining colloidal chitin pellets were stored in a refrigerator at 4 °C for future use.

Chitinolytic activity

The chitinolytic activity of *P. lilacinum* was evaluated by using fungal cultural filtrates obtained after 10 days grown in chitin-enriched PDB (5g colloidal chitin/l of medium) as crude chitinase solution and colloidal chitin suspension as substrate. These two components were mixed in 1:1 ratio and incubated at 37 °C for 24 h. Spectrophotometric OD value of the solution was recorded at 510 nm. The standard curve of N-acetylglucosamine was used to calculate chitinase enzyme. The total protein content was estimated by using Lowry's method with Bovine Serum Albumin (BSA) as a standard (Lowry et al. 1951).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of extracellular proteins of *P. lilacinum* grown on chitin-enriched medium C was carried out by the procedure given by Shanmugam et al. (2001). Seven-day-old culture discs of *P. lilacinum* IIPR-Pl-11 grown on PDA were inoculated separately into three 250-ml flask containing 150 ml chitin-enriched medium C and incubated at 28 ± 2 °C in a shaker at 120 rpm. After 5, 10 and 15 days, the suspension was filtered through a sterile Whatman no. 1 filter paper and collected in a sterile flask. The protein was precipitated by adding highly charged Ammonium sulphate ions to 80% saturation (52.3 g/100 ml) and allowed overnight at 4 °C, then, centrifuged at 10,000 rpm for 15 min at 4 °C. The protein pellets were dissolved in 20mM Tris Hcl buffer, pH 7.0, and dialysed against the same overnight at 4 °C. The dialysate was

concentrated with the help of sucrose, and the concentrated protein was kept in − 20 °C for future use.

The sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins was performed in polyacrylamide slab gels consisting of 5% stacking gel and 12% separating gel using cavoy mini-P-4 vertical electrophoresis unit with gel in dimensions of 8.6 × 7.7 × 0.15 cm (width x length x thickness) following the method suggested by Laemmli (1970). The protein content of the sample was estimated by the Bradford method (Bradford 1976) and the sample was loaded at the rate of 50µg per well. The electrophoresis was carried out at 60 V initially, when dye front moved to the resolving gel, the voltage was increased to 100 V and the gel was run until the bromophenol blue reached the bottom of resolving gel. Molecular weights were determined based on the RF value of the protein bands.

In vitro bioassay on egg hatching and juvenile mortality

Cultural filtrates

The fungus was grown in potato dextrose broth (PDB) and colloidal chitin-enriched medium C at 26 ± 2 °C for 10 days in shaker incubator. Cultural filtrate was separated from mycelial mat by filtering Whatman No.1 filter paper and collected in 25-ml centrifuge tube. The fungal mycelium was further removed by centrifugation at 8000*g* for 5 min. The supernatant was further filtered through 0.22-µm bacterial filter and used for in vitro bioassay. Extraction of eggs: surface-sterilized egg masses of *M. incognita* were kept in (50 × 15 mm) Petri dishes containing 0.5% NaOH for 2.0 min. Frequent gentle stirring was given to fasten the dissolution of gelatinous matrix and maximum eggs release. Eggs were collected using 500 mesh sieve and washed three times with autoclaved double distillation water. The number of eggs per 1 ml suspension was counted by WUR counting dish and standardized inoculum density at 100eggs/50µl with autoclaved double distilled water. Extraction of juveniles: Cell strainer (Hi-Media) was kept inside 25 × 15 mm petri dish and double distilled water filled half of the petri dish and cell strainer. The surface-sterilized egg masses were kept in water present the cell strainer. The freshly hatched juveniles were migrated into the Petri dish bottom and the nematode inoculum was standardized as 100 J_2/50 µl for experiment. Cultural filtrates of all the strains and distilled water as a control with three replications were taken in 12 well tissue culture plates. Two sets were maintained for carrying out bioassay on juvenile mortality and egg hatching. In first set, a total of 100 freshly hatched juveniles was inoculated in each strains as treatment, only cultural filtrates as untreated control and distilled. After 48 h the dead nematodes were counted and kept separately in water to confirm whether juveniles were dead or nematostatic. Similarly in the second set, after 48 h the number of unhatched eggs was counted. Percentage of juvenile mortality and inhibition on egg hatching were calculated by using Abbot's formula (Abbott WS 1925).

$$\text{Juvenile mortality percentage} = [(M_t - M_c)/(100 - M_c)] \times 100$$

Mt—juvenile mortality in treatment; M_c—juvenile mortality in control

$$\text{Egg Inhibition percentage} = [(E_t - E_c)/(100 - E_c)] \times 100$$

E_t—unhatched egg in treatment; E_c—unhatched egg in control.

In vivo greenhouse experiment

Preparation of stock solution

One ml of sterilized double distilled water was added on fully grown fresh mother culture of *P. lilacinum* and then scraped with a spade to produce slurry and then transferred to 99 ml of distilled water to prepare a suspension that was referred as stock solution. From this stock solution, a total of 10 ml suspension was transferred into 90 ml distilled water that was referred as 2nd dilution suspension. Subsequent dilutions were made repeating the same process till 6th dilution suspension, *i.e.* spore load of 2×10^6 CFU/ml, was obtained.

Treatments and experimental layout

The experiment was laid out in a Complete Randomized Design with 12 treatments, viz. ten strains of *P. lilacinum* at 5ml/kg soil, carbofuran at 3g a.i/ha (treated control) and distilled water 5 ml/kg (untreated control). Each treatment was replicated three times. Soil mixture was sterilized in an autoclave for 2 h at 15 psi and 121.6 °C temperature. Sterilized soil was left in cage house for at least 10 days for maturation. After 10 days, *P. lilacinum* at 5ml/kg soil was added to all pots and allowed it for 15 days for its establishment. On 25th day, three chickpea seeds (DCP 92–3) were sown in each pot. After 10 days of germination, thinning was done to allow single plant per pot and inoculated with 1000 juveniles of *M. incognita* at ETL level (1 nematodes per g of soil) to all pots. The experiment was completed, 60 days after nematode inoculation. Observations on various plant growth parameters, viz. shoot and root weight, shoot and root length, were recorded. Observations on nematode reproduction, viz. number of galls/plant, number of egg masses/plant and final nematode population/200 cc soil, were also recorded. For studying the nematode infection, the roots were stained with 0.1% acid fuchsin lacto-phenol at 80 °C for 2–3 min (Byrd et al. 1983).

Data analysis

The experimental data were analysed using Minitab USA software [Minitab® 21.1 (64-bit)]. Analysis of variance (ANOVA) was performed, and grouping was done through Tukey pairwise comparison to 95% confidence interval.

Results

Isolation

Totally, 77 RKN infected soil and root samples were collected from four agro-climatic zones. From these samples, ten isolates of *P. lilacinum* were isolated through dilution plating method of infected roots and single spore culture of all isolates was developed for characterization (Table 1 and Fig. 1).

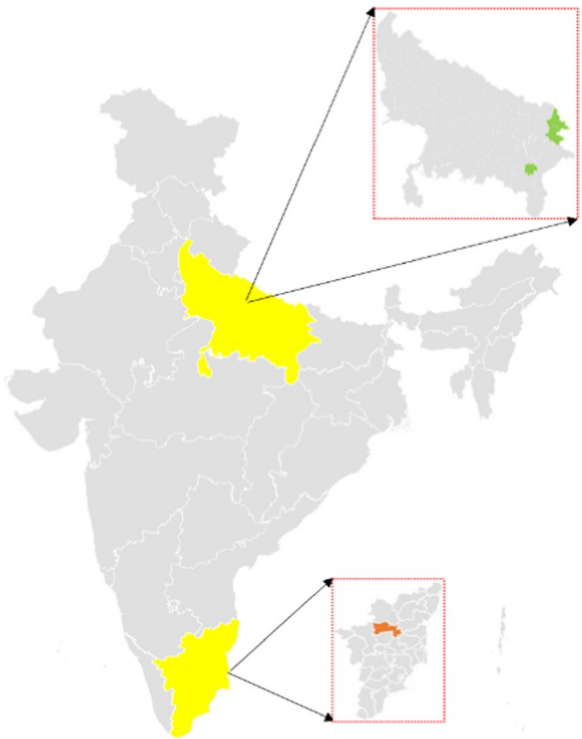


Fig. 1 Sampling agroclimatic zones

Morphometrics

Colony growth on PDA was relatively quick and attained 3.0 to 3.5 cm diameter in 7 days. Colony growth formed a basal felt with floccose aerial mycelium giving rise to conidiophores, phialides and conidia. Initially, the colony was white colour and turn into pinkish to lilac colour after 3rd day when sporulation started (Fig. 2a). The hyphae were hyaline (24–48 h., Fig. 2b), septate raise into conidiophore which beard flask-shaped phialides (48–72 h.; Fig. 2c). Conidia that arose from phialides were round to ellipsoidal in shape and form diverged chains (after 72h; Fig. 2d). Measurements of various structures were taken in SEM images by using ImageJ software. Vegetative hyphae are 1.2 µm wide, conidiophore 3 to 4 µm in length, phialides 8.8 µm long, consisting of a swollen basal part, about 1 µm wide. Conidium is 1.9×1.3 µm. (Fig. 3a–d).

Molecular characterization

PCR reaction with ITS1 and ITS 4 primers yielded amplified DNA product size of 600 bp (Fig. 4). The Sanger sequence data of the amplicon were trimmed by using software Clustal W and compared to other homologous sequences through NCBI blast for identification of species. The sequences showed 98% similarity with *Purpureocillium lilacinum*. The sequences were submitted to NCBI and accession numbers were received (Table 2).

In vitro bioassay on juvenile mortality

Cultural filtrates of *P. lilacinum* strain IIPR-Pl-11 were causing the highest juvenile mortality (88.36%) among all followed by IIPR-Pl-9 (74.31%) compared to control (Table 3 and Fig. 5).

Table 1 Details of survey, sampling sites and isolates

Agro climatic zone	State, District	Villages	Samples	No of isolates
Eastern plains (AZ34)	Uttar Pradesh, Varanasi,	Adalpura, sultanpur, aradili, maharaccha, mahagaon, jakkhini, shakhansapur, chandapur, jachahari	30	4
Central plains (AZ31)	Uttar Pradesh, Sitapur	Gharkatara, Mallapura, Mahanandpur, Kultazpur, Navinagar, Katru mohalla, Acharyapura, Bhawanipur	35	4
North eastern plains (AZ32)	Uttar Pradesh, Deoria, kushinagar	khampar, jasui, bakhri and tapura villages in Bhatpar Rani Tehsil of Deoria district and sargatiya, munnu chawan, doriacot, baruvatola villages in Tamkuhi Raj Tehsil of Kushinagar district	29	0
Southern Plateau and Hills (AZ122)	Tamil Nadu, Salem, Attur	Karumandhurai, Gengavelli	12	2

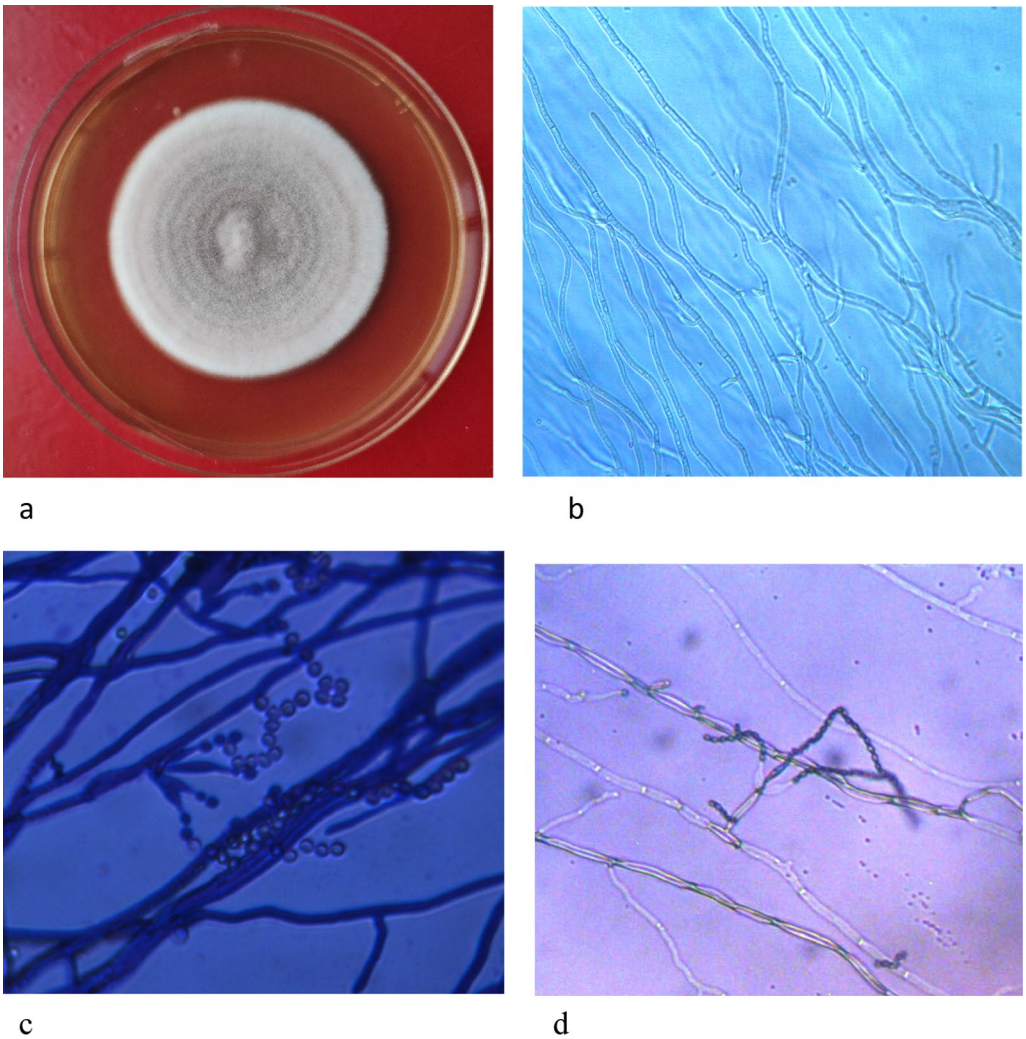


Fig. 2 Light microscopic study of morphology of *Purpureocillium lilacinum*(IIPR-PI-11). **a** Colony of *Purpureocillium lilacinum* on PDA (at 7 days); **b** septate Hyphae with conidiophore (24–48 h); **c** phialides with conidia (48–72 h); **d** typical identification structures: conidiophore, phialides and conidia chain (after 72 h)

Chitinase production

It was observed in chitinase plate assay that the colour of the media was changed from yellowish orange to pink colour as the fungus grow, it indicated that all the strains of *P. lilacinum* were having the ability to produce chitinase enzyme. Based on the intensity of colour changes of media, the strains were categorized as high chitinase producing strains (IIPR-Pl-1, 4, 7, 9, 10 and 11) and moderate chitinase production strains (IIPR-Pl-3, 5,6 and 8) (Fig. 6).

Chitinolytic activity

Enzymatic action of chitinase on substrate chitin produces monomers N-acetylglucosamine, which was reflected through absorbance value at OD_{510} nm at 5, 10 and 15 days of growth was recorded and chitinase enzyme and total protein concentrations were calculated and analysed. Maximum absorbance was recorded with IIPR-Pl-11 at 5 days (0.139), 10 days (0.245) and 15 days (0.273) and minimum absorbance was recorded in IIPR-Pl-2 (0.082) at 5 days, IIPR-Pl-6 (0.148) at 10 days and IIPR-Pl-1 (0.139) at 15 days (Table 4). Maximum chitinase enzyme was observed with IIPR-Pl-11 at 5 days (51.1 µg/ml), 10 days (90.1 µg/ml) and 15 days (100.4 µg/ml) and minimum chitinase enzyme was observed in IIPR-Pl-2 (30.1 µg/ml) at 5 days, IIPR-Pl-6 (54.4 µg/ml) at 10 days and IIPR-Pl-1 (51.1 µg/ml) at 15 days (Table 5). Total protein estimated was the highest with IIPR-Pl-11 at 5 days (173.75 µg/ml), 10 days (272.67 µg/ml) and 15 days (306.25 µg/ml) and the lowest with IIPR-Pl-2 (102.5 µg/ml) at 5 days, IIPR-Pl-5

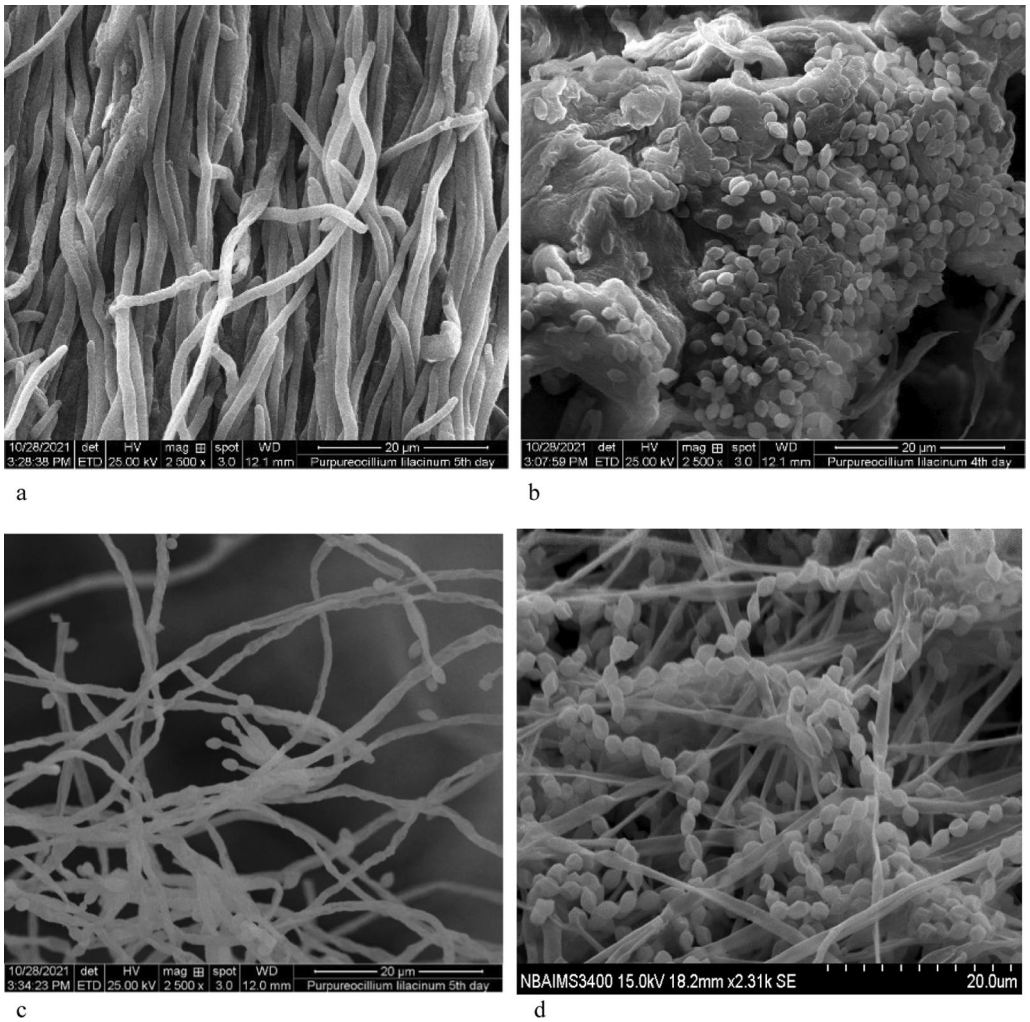


Fig. 3 Scanning electron microscopy study on morphological features of *Purpureocillium lilacinum* (IIPR-PI-11); **a** hyphae of *P. lilacinum;* **b** conidia of *P. lilacinum;* **c** phialides with conidia; **d** typical identification structures: conidia chain

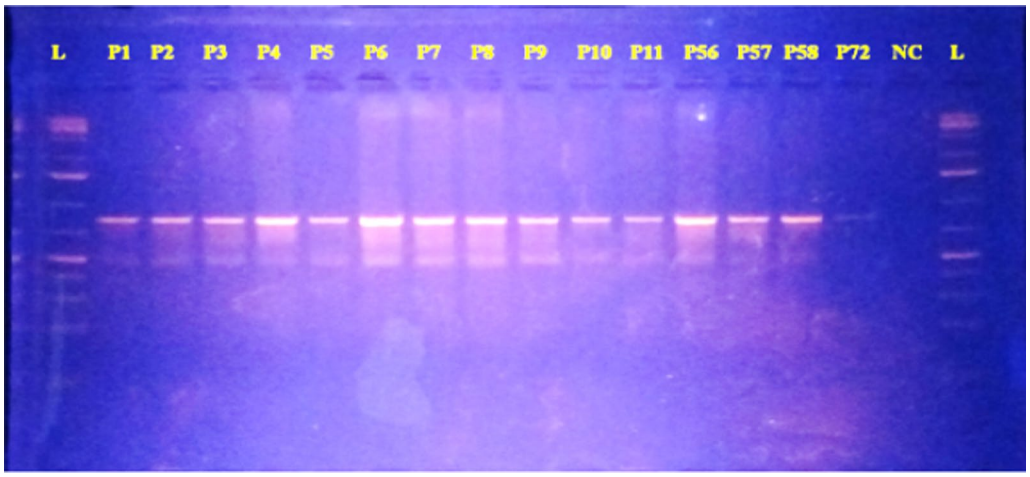


Fig. 4 ITS-5.8-ITS2 region amplification of 600 bp L: ladder; P1 to P11 *Purpureocillium lilacinum* Strains; PL56, PL57, PL58, PL72: positive control; NC: negative control

Table 2 List of submitted *Purpureocillium lilacinum* strains and their NCBI accession number

Sl. No	Species	Strains	Target region	NCBI accession	NAIMCC, Mau accession number
1	*P. lilacinum*	IIPR-Pl-1	ITS1-5.8S-ITS2	MK696570	NAIMCC-F-04225
2	*P. lilacinum*	IIPR-Pl-2	ITS1-5.8S-ITS2	MK713625	NAIMCC-F-04226
3	*P. lilacinum*	IIPR-Pl-3	ITS1-5.8S-ITS2	MK713626	NAIMCC-F-04227
4	*P. lilacinum*	IIPR-Pl-4	ITS1-5.8S-ITS2	MK713627	NAIMCC-F-04228
5	*P. lilacinum*	IIPR-Pl-5	ITS1-5.8S-ITS2	MK713628	NAIMCC-F-04229
6	*P. lilacinum*	IIPR-Pl-6	ITS1-5.8S-ITS2	MK713629	NAIMCC-F-04230
7	*P. lilacinum*	IIPR-Pl-7	ITS1-5.8S-ITS2	MK713630	NAIMCC-F-04231
8	*P. lilacinum*	IIPR-Pl-8	ITS1-5.8S-ITS2	MK713631	NAIMCC-F-04232
9	*P. lilacinum*	IIPR-Pl-9	ITS1-5.8S-ITS2	MK713632	NAIMCC-F-04233
10	*P. lilacinum*	IIPR-Pl-11	ITS1-5.8S-ITS2	MK713634	NAIMCC-F-04234

Table 3 In vitro bioassay on juvenile mortality

***Purpureocillium lilacinum* strains**	**No of dead Juvenile**	**% juvenile mortality**
IIPR-Pl-1	54.67 ± 10.0^{cd}	53.42
IIPR-Pl-2	36.67 ± 5.1^{ef}	34.93
IIPR-Pl-3	$48.00 \pm 3.0d^{ef}$	46.57
IIPR-Pl-4	31.67 ± 3.0^{f}	29.79
IIPR-Pl-5	32.33 ± 7.2^{f}	30.48
IIPR-Pl-6	66.00 ± 4.5^{bc}	65.07
IIPR-Pl-7	58.67 ± 7.6^{bcd}	57.53
IIPR-Pl-8	52.67 ± 7.0^{cde}	51.37
IIPR-Pl-9	75.00 ± 7.9^{ab}	74.31
IIPR-Pl-11	88.67 ± 5.1^{a}	88.36
Broth	3.00 ± 1.5^{g}	0.34
Control	2.67 ± 1.0^{g}	0.00
Pooled S.D	5.91	
P (α = 0.05)	0.001	

The value of no of dead juveniles is the mean of three replications. Different case letters in a column represent statistically differences of mean according to Tukey pairwise comparisons (grouping information using the Tukey method and 95% confidence); % juvenile mortality calculated by Abbott's formula

(156.6 µg/ml) at 10 days and IIPR-Pl-6 (185.0 µg/ml) at 15 days (Table 6). Time series analysis revealed that the strains showed differences in their chitinolytic activity over a period of time. Six strains were showing increasing trend (IIPR-Pl-3,4,6,7,9 and 11), and others were decreasing trend (IIPR-Pl-1,2,5 and 8) indicating the efficiency of strains on chitinase enzyme production (Fig. 7). SDS PAGE revealed the presence of two bands with the size of 32 kDa and 46 kDa (Fig. 8).

In vitro bioassay on egg hatching

Crude chitinase suspensions of all strains were negatively influenced on egg hatching significantly at all the growth period over control. At 5 days, strain IIPR-Pl-8 inhibited maximum percentage (42.6%) and IIPR-Pl-5 exhibited minimum inhibition of egg hatching (19.4%), at 10 days, strain IIPR-Pl-11 exhibited maximum inhibition (62.8%) and minimum inhibition (29.6%) was observed in IIPR-Pl-3 and at 15 days, maximum inhibition (93.5%) was observed in IIPR-Pl-11, and minimum inhibition (73.5%) was observed in IIPR-Pl-3 (Table 7 and Fig. 9).

Green house experiment

Plant growth

The fungal strains and carbofuran were significantly promoting overall plant growth compared to control. Among the strains, IIPR-Pl-11 was efficient in increasing shoot length (35.87cm), shoot weight (5.7g), root length (19.9cm) and root weight (2.69g), which were statistically at par with carbofuran. Minimum shoot length (22.37cm), shoot weight (3.2g), root length (13.8cm) and root weight (1.42g) were observed in IPR-Pl-5, IIPR-Pl-5, IIPR-Pl-2 and IIPR-Pl-6, respectively (Table 8, Figs. 10).

Nematode reproduction: Both fungal strains and carbofuran had reduced nematode reproduction significantly over the control. Among strains, IIPR-Pl-11 was very effective in reducing gall number (41.3 per plant), egg mass (28.3 per plant) and soil population (284.3 per 200cc of soil) than the control (Table 9 and Fig. 11).

Discussion

In this study, isolation of *P. lilacinum* was successful with one of the three methods followed, i.e. serial dilution plating method of infected root suspension. Since the fungus exhibited ability of acquiring nutrients on various substrates like soil, decaying debris, nematode eggs and roots, it is very important to try all the possible methods for getting success. Light microscopic and SEM study on the shape and size of hyphae, conidiophore, phialides and

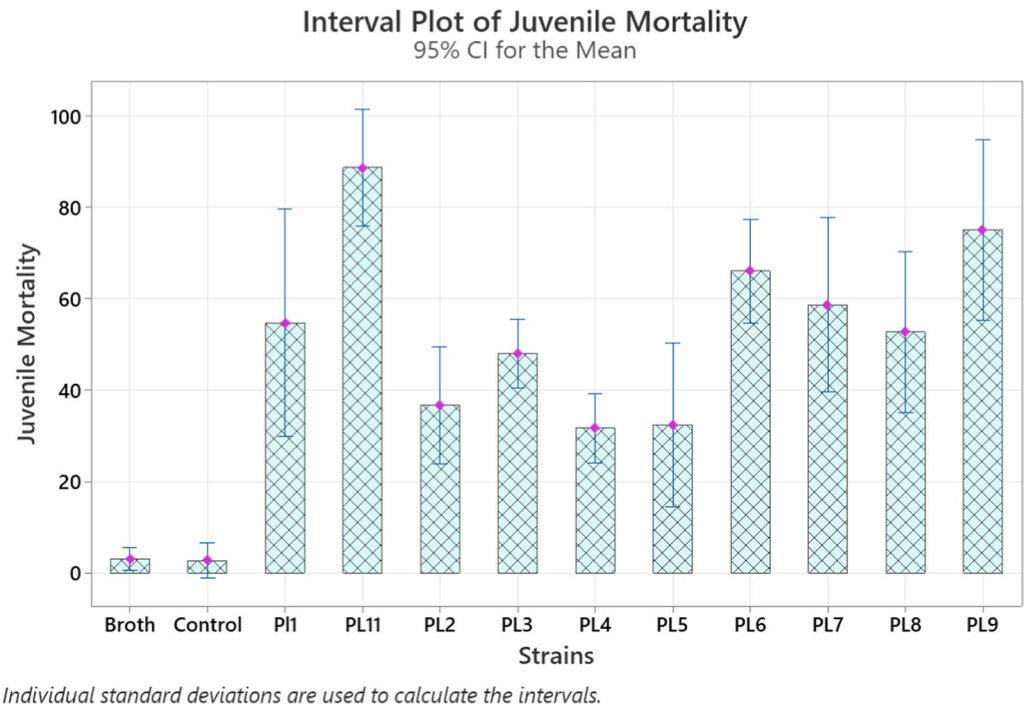


Fig. 5 In *vitro* bioassay on juvenile mortality

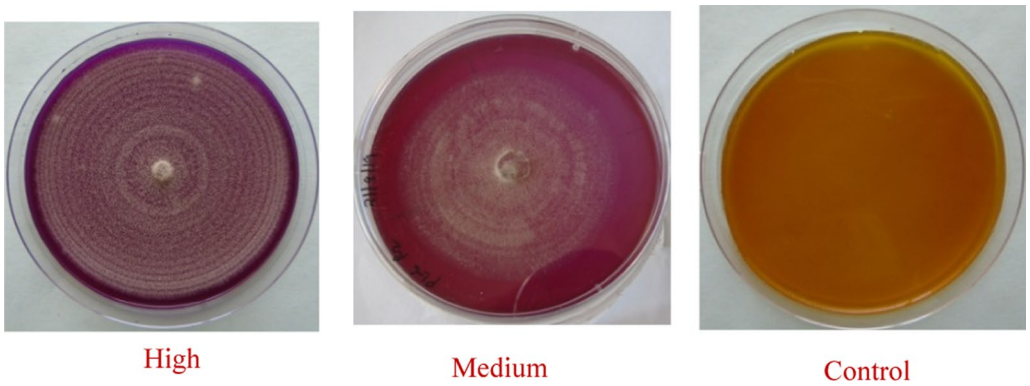


Fig. 6 Chitinase plate assay

conidia confirmed the description of the species made by Samson (1974). The typical flask shapes characteristics of phialides with chain of conidia which differentiates *Purpureocillium* from close related genus *Penicillin* were observed and used for species identification as described in various studies (Luangsa-ard et al. 2011).

Classical taxonomical study was substantiated with more precise way of determination of species, i.e. by molecular markers. The ITS region of the rDNA exhibits hyper-variability and used to differentiate the strain both at species level and at low levels, has been recognized as the official region of DNA barcode for fungi (Badotti et al. 2017). In the present work, the sequencing of the ITS regions of the rDNA and NCBI Blast confirmed the taxonomic identity of ten strains of *P. lilacinum*.

Screening assay on chitinase production through chitinase plate assay revealed the inter strain variations present among the strains. Chitinase produced by growing fungal strains degraded colloidal chitin present in the medium and released N-acetyl glucosamine (NAGA) which influenced the pH of the medium. The dye bromocresol purple (BCP) (5′,5″-dibromo-o-cresolsulfophthalein, pKa 6.3) was added in growth medium to detect pH change with the colour change from yellow (at low

Table 4 Chitinolytic activity of *Purpureocillium lilacinum* strains at different duration of growth

***Purpureocillium lilacinum* strains**	**Absorbance (OD) at 510 nm**		
	5 days	**10 days**	**15 days**
IIPR-Pl-1	0.102 ± 0.001[d]	0.149 ± 0.048[c]	0.139 ± 0.005[f]
IIPR-Pl-2	0.082 ± 0.010[e]	0.206 ± 0.015[ab]	0.178 ± 0.004[cd]
IIPR-Pl-3	0.114 ± 0.004[bcd]	0.162 ± 0.014[bc]	0.202 ± 0.005[b]
IIPR-Pl-4	0.108 ± 0.010cd	0.158 ± 0.007[bc]	0.167 ± 0.015[de]
IIPR-Pl-5	0.104 ± 0.003[d]	0.163 ± 0.008[bc]	0.157 ± 0.002[ef]
IIPR-Pl-6	0.113 ± 0.002[bcd]	0.148 ± 0.002[c]	0.157 ± 0.002[ef]
IIPR-Pl-7	0.103 ± 0.004[d]	0.156 ± 0.005[bc]	0.192 ± 0.006[bc]
IIPR-Pl-8	0.126 ± 0.006[ab]	0.217 ± 0.004[a]	0.183 ± 0.000[bcd]
IIPR-Pl-9	0.123 ± 0.001[bc]	0.152 ± 0.006[c]	0.170 ± 0.002[de]
IIPR-Pl-11	0.139 ± 0.002[a]	0.245 ± 0.009[a]	0.273 ± 0.007[a]
Pooled S.D	0.005	0.017	0.006
P (= 0.05)	< 0.001	< 0.001	< 0.001

The value of absorbance at different duration of growth is the mean of three replications. Different case letters in a column represent statistically differences of mean according to Tukey pairwise comparisons (grouping information using the Tukey Method and 95% Confidence)

Table 5 Chitinase enzyme production of *Purpureocillium lilacinum* strains at different duration of growth

***Purpureocillium lilacinum* strains**	**Chitinase enzyme (µg/ml)**		
	5 days	**10 days**	**15 days**
IIPR-Pl-1	37.5 ± 0.36[d]	54.8 ± 17.8[c]	51.1 ± 2.02[f]
IIPR-Pl-2	30.1 ± 3.60[e]	75.7 ± 5.51[ab]	65.4 ± 1.65[cd]
IIPR-Pl-3	41.9 ± 1.65[bcd]	59.6 ± 5.15[bc]	74.3 ± 2.02[a]
IIPR-Pl-4	39.7 ± 3.68[cd]	58.1 ± 2.57[bc]	61.4 ± 5.70[de]
IIPR-Pl-5	38.2 ± 1.12[d]	59.9 ± 3.13[bc]	57.7 ± 0.92[ef]
IIPR-Pl-6	41.5 ± 0.92[bcd]	54.4 ± 0.92[c]	57.7 ± 0.73[ef]
IIPR-Pl-7	37.9 ± 1.65[d]	57.4 ± 1.84[bc]	70.6 ± 2.39[bc]
IIPR-Pl-8	46.3 ± 2.21[ab]	79.8 ± 1.65[a]	67.3 ± 0.21[bcd]
IIPR-Pl-9	45.2 ± 0.36[bc]	55.9 ± 2.39[c]	62.5 ± 0.73[de]
IIPR-Pl-11	51.1 ± 0.73[a]	90.1 ± 3.31[a]	100.4 ± 2.76[a]
Pooled S.D	2.00	6.44	2.42
P (= 0.05)	< 0.001	< 0.001	< 0.001

The value of chitinase enzyme production at different growth period is the mean of three replications. Different case letters in a column represent statistically differences of mean according to Tukey pairwise comparisons (grouping information using the Tukey method and 95% confidence)

pH 5.2) to violet (above pH 6.8) of the substrate visually. (Kamala and Indira 2012).

In addition to the plate assay, the released N-acetylglucosamine in chitin-enriched medium C was more precisely measured spectrophotometrically. The strains showed striking differences in their maximum efficiency/persistence of chitinase production in different incubation period. In this study, at 10 days of incubation the strains IIPR-Pl-2, 8 and 11 were showing high chitinase activity, but at 15-day incubation only IIPR-Pl-11 was showing high chitinase activity and rest two were showing less chitinase activity than at 10 days. The strain IIPR-Pl-11 showing more chitinolytic activity at 15 days due to higher competitive, aggressive and virulent nature than those strains which were producing maximum activity at 10 days. This character is very significant for sustainable nematode egg parasitism and was used to identify the potential nematicidal strain. Homthong et al. (2016) previously studied chitinolytic activity of *P. lilacinus* strains in two liquid media (medium C and basal medium) supplemented with colloidal chitin and found greater activity in medium C than basal medium. Similarly, Alamgir Khan et al. (2003), studied chitinolytic activity of *P. lilacinus* with minimal medium supplemented with chitin at 0.4% (w/v). Castellanos–Moguel et al. (2008) evaluated chitinase, protease activities and virulence of 18 strains; *P. fumosoroseus* grown in synthetic liquid medium supplemented with remazol bright blue stained colloidal chitin. The SDS-PAGE analysis from the culture filtrate of colloidal chitin-enriched medium C showed two bands with molecular masses of about 32 kDa and 46 kDa which could be chitinase isomers of chi32 and chi46. This corroborative inference was based on the results of the previous study by Nguyen et al. (2008) who purified and characterized two monomers of chitinase with an apparent mass of 32 kDa for chi32 and 46 kDa for chi46 from *Paecilomyces variotii* parasitizing *Meloidogyne* eggs.

Table 6 Total protein content of *Purpureocillium lilacinum* strains at different duration of growth

***Purpureocillium lilacinum* strains**	**Total protein (µg/ml)**		
	5 days	**10 days**	**15 days**
IIPR-Pl-1	127.50 ± 1.25[d]	158.67 ± 60.60[c]	186.67 ± 6.88[f]
IIPR-Pl-2	102.50 ± 12.5[e]	177.67 ± 18.80[ab]	257.50 ± 5.64[cd]
IIPR-Pl-3	142.08 ± 5.64[bcd]	201.67 ± 17.50[bc]	202.08 ± 6.88[b]
IIPR-Pl-4	135.00 ± 12.5[cd]	166.67 ± 8.75[bc]	197.50 ± 19.4[de]
IIPR-Pl-5	129.58 ± 3.82[d]	156.67 ± 10.63[bc]	204.17 ± 3.15[ef]
IIPR-Pl-6	140.83 ± 3.15[bcd]	157.00 ± 3.15[c]	185.42 ± 2.50[ef]
IIPR-Pl-7	128.33 ± 5.64[d]	191.67 ± 6.25[bc]	195.00 ± 8.13[bc]
IIPR-Pl-8	157.50 ± 7.50[ab]	182.67 ± 5.64[a]	270.83 ± 0.72[bcd]
IIPR-Pl-9	153.75 ± 1.25[bc]	170.00 ± 8.13[c]	189.58 ± 2.50[de]
IIPR-Pl-11	173.75 ± 2.50[a]	272.67 ± 11.25[a]	306.25 ± 9.38[a]
Pooled S.D	6.82	21.90	8.24
P (= 0.05)	< 0.001	< 0.001	< 0.001

The value of total protein content at different growth period is the mean of three replications. Different case letters in a column represent statistical differences of mean according to Tukey pairwise comparisons (grouping information using the Tukey method and 95% confidence)

The juvenile mortality by the cultural filtrates of the strains may be due to the toxic secondary metabolites released during the growth of the fungus. The secondary

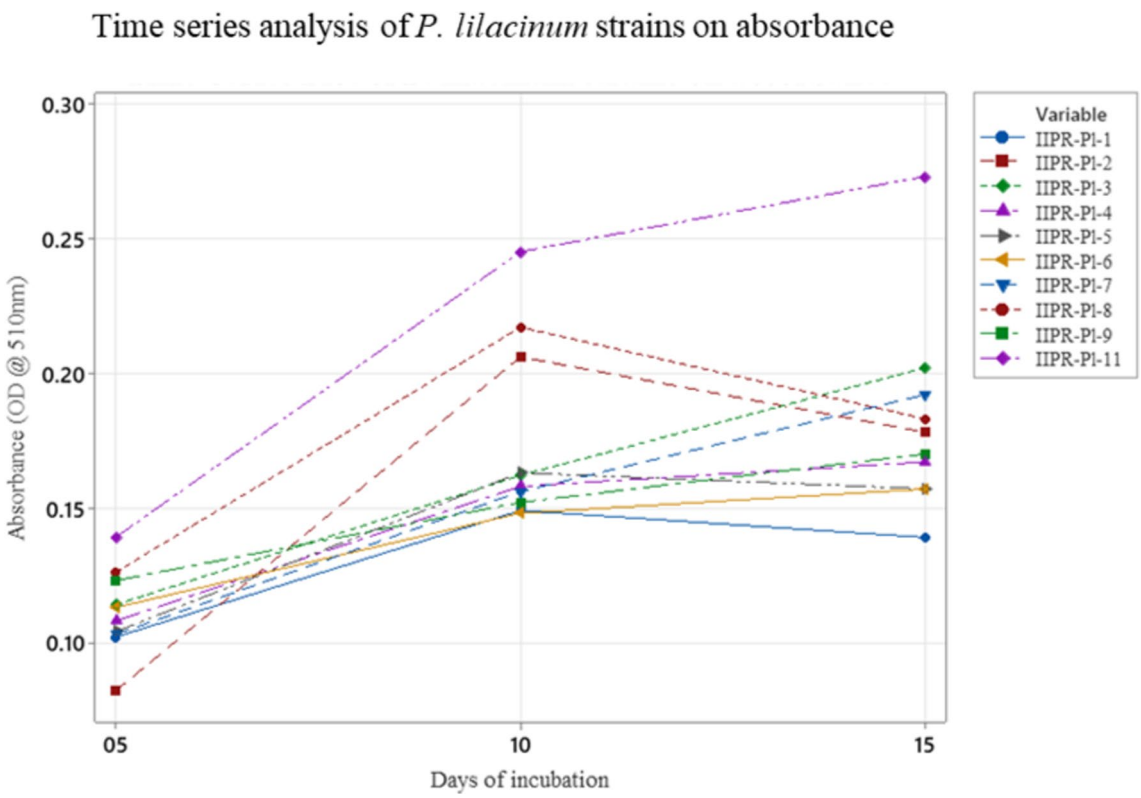


Fig. 7 Time series plot *Purpureocillium lilacinum* on chitinolytic activity

metabolites from 13 known species of *Paecilomyces* were identified as polyketide, terpenoid, peptide, alkaloid, quinone, pyrone, sterol, fatty acid having important biological properties, viz. antimicrobial, antitumor, insecticidal, herbicidal, antimalarial, enzyme-inhibiting and nematicidal (Dai et al. 2020). The findings of the present study are consistent with the numerous previous studies on in vitro bioassay which were also concluded that the cultural filtrates caused significant mortality of juveniles of *Meloidogyne* spp. in 24 to 48 h. (Kumar and Dara 2021).

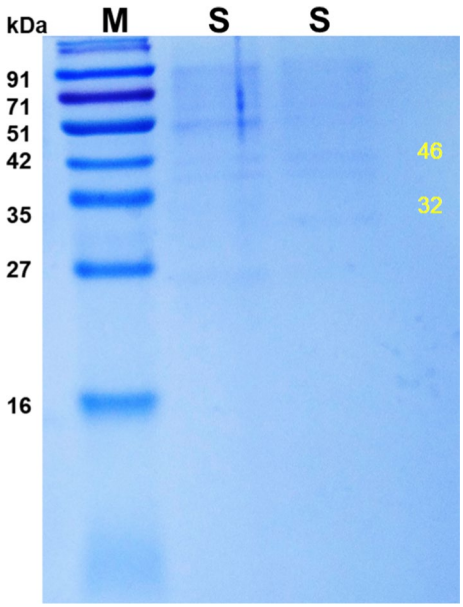


Fig. 8 SDS-PAGE of chitinase solution (40 µl each) loaded in each lane M: ladder; S: sample

The suppression of egg hatching observed in in vitro bioassay was due to degradation of chitin layer present in the egg shell by chitinase enzyme which allows other hydrolytic enzymes and secondary metabolites acting on young juveniles and kills them. Previous studies on the exposure of nematode eggs to chitinase also provided the similar results and suppressed hatching. The purified chitinase LPCHI1 from *Lecanicillium psalliotae* and YS1215 *Lysobacter capsici* destroyed the chitin layer and significantly reduced the hatching of *M. incognita* (Lee et al. 2014). Chitinases produced from nematoparasitic fungi *Verticillium chlamydosporium* and *V. suchlasporium* damaged egg cuticle of *Globodera pallida* and suppressed hatching (Tikhonov et al. 2002). Studies concerning the inhibition of egg hatching and degradation of nematodes' eggshells showed that the application of chitinase in the biological control of plant nematode is the potential

Table 7 Effect of cultural filtrates on inhibition of egg hatching

***Purpureocillium lilacinum* strains**	**No. of unhatched eggs**		
	5 days after incubation	**10 days after incubation**	**15 days after incubation**
IIPR-PI-1	22.33 ± 6.43^{b} (21.8)	38.67 ± 4 .93^{bc}(37.8)	90.00 ± 1.73^{ab}(89.8)
IIPR-PI-2	21.33 ± 5.86^{b} (20.8)	31.67 ± 5.51^{c} (30.7)	76.33 ± 8.50^{bc} (75.8)
IIPR-PI-3	22.67 ± 3.06^{b} (22.1)	30.00 ± 7.21^{c} (29.6)	74.00 ± 0.00^{b} (73.5)
IIPR-PI-4	22.67 ± 2.52^{b} (22.1)	37.33 ± 4.16^{bc} (36.4)	84.67 ± 6.11^{bc} (84.3)
IIPR-PI-5	20.00 ± 4.00^{bc} (19.4)	31.67 ± 3.51^{c} (30.7)	76.33 ± 7.02^{bc} (75.8)
IIPR-PI-6	25.00 ± 1.00^{b} (24.5)	34.67 ± 6.03^{c} (33.7)	78.00 ± 1.0^{bc} (77.5)
IIPR-PI-7	24.00 ± 4.36^{b} (23.5)	35.33 ± 3.79^{c} (34.5)	83.67 ± 7.64^{bc} (83.3)
IIPR-PI-8	43.00 ± 4.58^{a} (42.6)	60.00 ± 3.21^{bc} (59.5)	81.33 ± 7.64^{bc} (80.9)
IIPR-PI-9	30.00 ± 11.53^{ab} (29.5)	61.00 ± 5.57^{ab} (60.5)	86.33 ± 14.3^{bc} (86.0)
IIPR-PI-11	36.33 ± 11.93^{ab} (35.9)	63.33 ± 13.45^{a} (62.8)	93.67 ± 3.21^{a} (93.5)
Broth (non-chitin)	3.67 ± 1.52^{cd} (3.0)	3.00 ± 1.00^{d} (1.7)	3.33 ± 1.52^{c} (1.3)
Control	0.67 ± 0.57^{d} (0.0)	1.33 ± 0.57^{d} (0.0)	2.00 ± 1.00^{c} (0.0)
Pooled S.D	5.96	5.84	6.72
P (= 0.05)	< 0.001	< 0.001	< 0.001

The value of inhibition on egg hatching is the mean of three replications. Different case letters in a column represent statistical differences of mean according to Tukey pairwise comparisons (grouping information using the Tukey method and 95% confidence interval); values in parenthesis are percent inhibition on egg hatching calculated by Abbott's formula

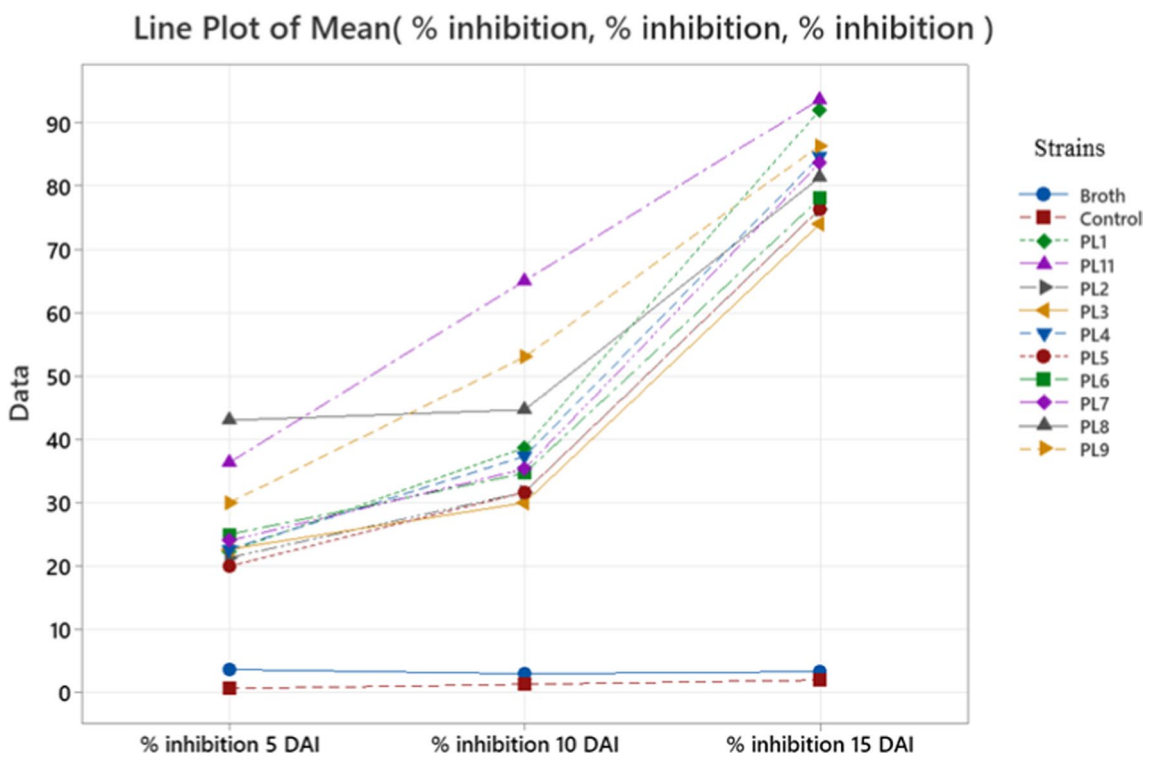


Fig. 9 Percentage inhibition on egg hatching

Table 8 Effect of *Purpureocillium lilacinum* strains on plant growth parameters of chickpea

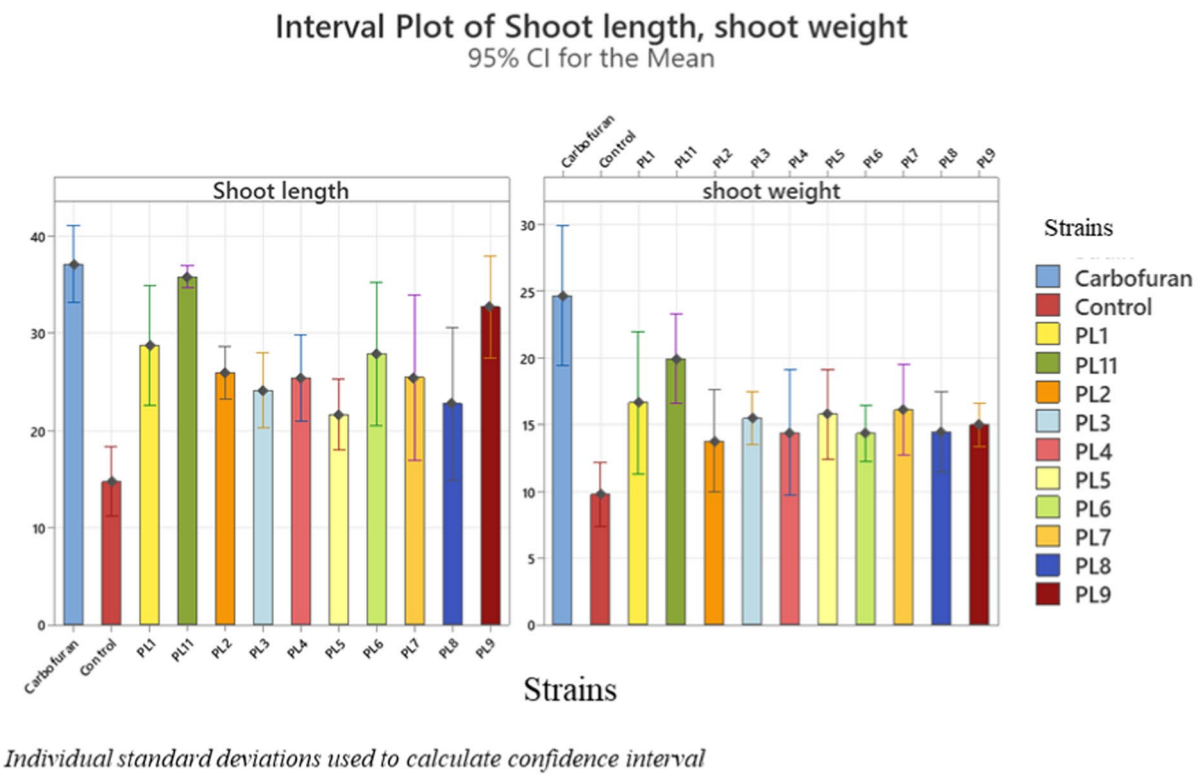
***Purpureocillium lilacinum* strains**	**Shoot length (cm)**	**Shoot weight (g)**	**Root length (cm)**	**Root weight (g)**
IIPR-Pl-1	28.77 ± 2.47^{bc} (46.36)	4.6 ± 0.43^{bcde} (69.57)	16.67 ± 2.14^{bc} (32.98)	2.44 ± 0.09^{abc} (83.20)
IIPR-Pl-2	25.37 ± 1.90^{c} (39.17)	4.8 ± 0.97^{bc} (70.83)	13.80 ± 1.53^{cd} (19.06)	2.38 ± 0.05^{bc} (82.77)
IIPR-Pl-3	24.13 ± 1.55^{c} (36.06)	4.7 ± 0.15^{bcd} (70.21)	15.50 ± 0.80^{cd} (27.94)	2.20 ± 0.04^{bcd} (81.36)
IIPR-Pl-4	25.40 ± 1.80^{c} (39.25)	3.3 ± 0.43^{de} (57.58)	14.43 ± 1.90^{cd} (22.61)	2.10 ± 0.11^{bcd} (80.48)
IIPR-Pl-5	22.37 ± 2.65^{c} (31.01)	3.2 ± 0.17^{e} (56.25)	15.80 ± 1.34^{cd} (29.30)	1.73 ± 0.05^{cd} (76.30)
IIPR-Pl-6	27.90 ± 2.95^{bc} (44.70)	3.4 ± 0.66^{de} (58.82)	14.37 ± 0.85^{cd} (22.25)	1.42 ± 0.21^{d} (71.13)
IIPR-Pl-7	25.47 ± 3.42^{c} (39.41)	3.7 ± 0.20^{cde} (62.16)	16.13 ± 1.35^{bc} (30.76)	1.77 ± 0.11^{bcd} (76.84)
IIPR-Pl-8	22.77 ± 3.18^{c} (32.23)	4.7 ± 0.58^{bcd} (70.21)	14.50 ± 1.20^{cd} (22.97)	1.93 ± 0.53^{bcd} (78.76)
IIPR-Pl-9	32.77 ± 2.12^{ab} (52.91)	3.6 ± 0.58^{cde} (61.11)	15.00 ± 0.65^{cd} (25.53)	1.83 ± 0.69^{bcd} (77.60)
IIPR-Pl-11	35.87 ± 0.47^{a} (56.98)	5.7 ± 0.11^{ab} (75.44)	19.97 ± 1.36^{b} (44.06)	2.69 ± 0.17^{ab} (84.76)
Carbofuran	37.17 ± 1.15^{a} (58.48)	6.8 ± 0.45^{a} (79.41)	24.70 ± 2.12^{a} (44.06)	3.33 ± 0.58^{a} (87.69)
Control	15.43 ± 1.15^{d} (0.0)	1.4 ± 0.32^{f} (0.0)	11.67 ± 0.47^{e} (0.0)	0.41 ± 0.05^{e} (0.0)
Pooled S.D	2.26	0.20	1.41	0.32
P ($\alpha = 0.05$)	0.001	0.001	0.001	0.001

The value of different growth parameters is the mean of three replications. Different case letters in a column represent statistical differences of mean according to Tukey pairwise comparisons (grouping information using the Tukey method and 95% confidence interval); values in parenthesis are percent increase over control

option. Green house experiment results revealed that *P. lilacinum* assisted in increasing over all plant growth and reduced nematode reproduction. The increase in plant growth may be due to production of Indole Acetic Acid (IAA) by *P. lilacinum* which increased radical surfaces of roots and ammonification which results in enhanced nutrient uptake. The reduction of nematode reproduction may be due to production wide range of hydrolytic enzymes like protease, lipase, chitinase and other volatile organic compounds (VOC); secondary metabolites which is nematicidal in nature and egg parasitism. This observation was confirming the study by Cavello et al., (2015) who reported that *P. lilacinum* LPSC #876 was able to promote plant growth by producing physiologically active auxins, indoleacetic acid (IAA) and also produces proteases, keratinases, laminarases, and chitinases enzymes simultaneously which turn *P. lilacinum* into a promising candidate for the development of an organic biofertilizer with biological control. Dahlin et al., (2019) also revealed that the soil application of *P. lilacinum* strain 251 along with chemical nematicides can suppress the population of RKNs and root damage in tomato. Winarto and Liswarni (2018) observed wide variation in the pathogenicity of different strains of *P. lilacinum* against *Meloidogyne* spp. The variation in the virulence was attributed to the physical, chemical and biological conditions of the ecosystem from which the strains were originated. The well-characterized strain with high virulence has the potential to be developed as bio pesticide for nematode management.

Conclusion

Ten strains of *P. lilacinum* were isolated, identified morphologically and confirmed with ITS of rDNA. Based on the evaluation of all the strains on their chitinolytic activity, in vitro bioassay on inhibition of egg hatching and green house experiment on nematode management, it was found that *P. lilacinum* strain IIPR-Pl-11 was promising and can be used for RKN management, *M. incognita* in chickpea.



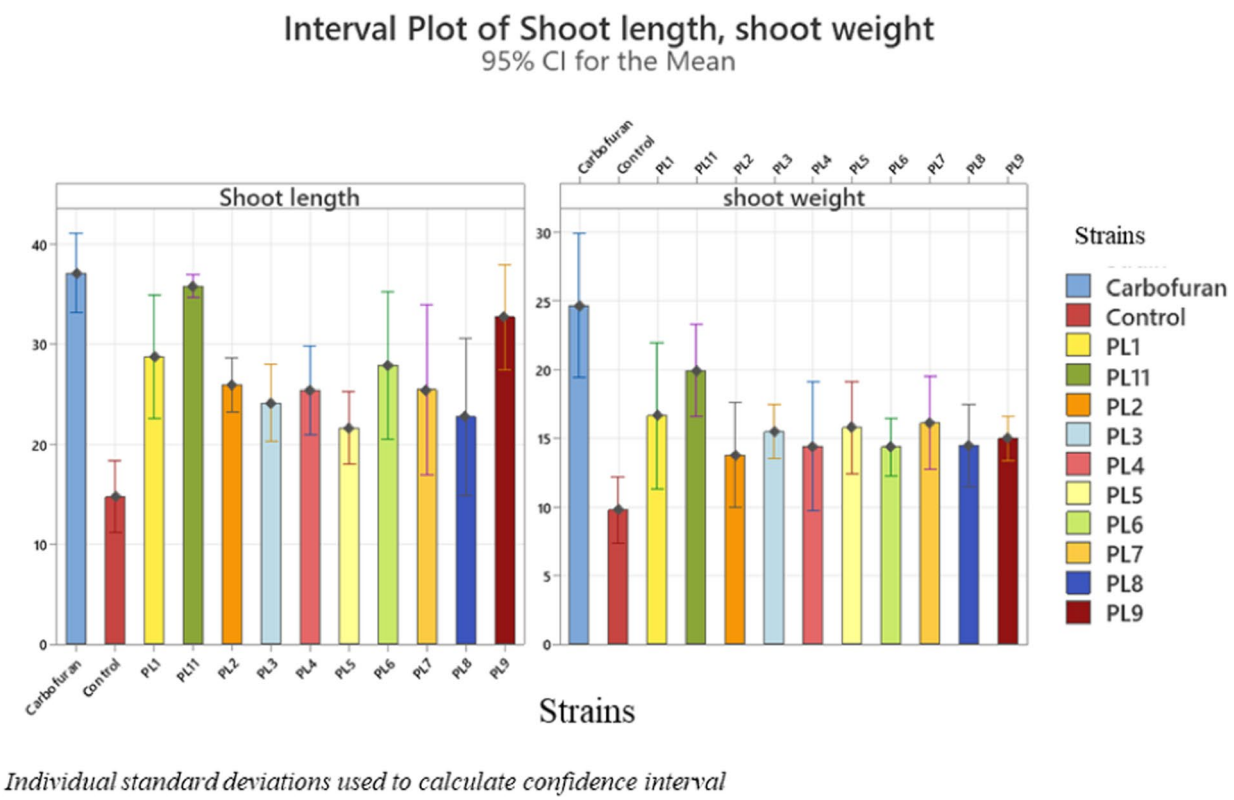


Fig. 10 Efficacy of *Purpureocillium lilacinum* strains on plant growth parameters of chickpea

Table 9 Effect of *Purpureocillium lilacinum* strains on nematode reproduction parameters

***Purpureocillium lilacinum* strains**	**No. of Galls**	**No. of Egg mass**	**Soil population**
IIPR-PI-1	51.0 ± 1.73^{bcd} (32.00)	34.7 ± 3.51^{b} (45.18)	444.0 ± 68.5^{b} (34.42)
IIPR-PI-2	60.0 ± 7.94^{b} (20.0)	39.3 ± 4.51^{b} (37.91)	431.3 ± 36.7^{b} (36.29)
IIPR-PI-3	46.3 ± 2.52^{bcde} (38.27)	38.3 ± 4.04^{b} (39.49)	444.3 ± 70.5^{b} (34.37)
IIPR-PI-4	45.7 ± 4.73^{bcde} (39.07)	33.3 ± 4.16^{bc} (47.39)	421.0 ± 33.0^{b} (37.81)
IIPR-PI-5	48.7 ± 5.51^{bcd} (35.07)	41.0 ± 5.20^{b} (35.23)	430.7 ± 22.5^{b} (36.38)
IIPR-PI-6	55.3 ± 2.52^{bc} (26.27)	32.0 ± 3.61^{b} (49.45)	362.3 ± 66.5^{bc} (46.48)
IIPR-PI-7	49.0 ± 5.57^{bcd} (34.67)	32.7 ± 1.52^{bc} (48.34)	366.0 ± 37.2^{bc} (45.94)
IIPR-PI-8	48.7 ± 8.14^{bcd} (37.33)	32.0 ± 4.58^{bc} (49.45)	359.0 ± 7.00^{bc} (46.97)
IIPR-PI-9	44.0 ± 3.00^{cde} (35.07)	38.7 ± 4.04^{bc} (38.86)	369.7 ± 56.2^{bc} (45.39)
IIPR-PI-11	41.3 ± 3.21^{de} (44.93)	28.3 ± 1.52^{bc} (55.29)	284.3 ± 15.1^{c} (58.01)
Carbofuran	32.7 ± 5.03^{e} (56.40)	21.7 ± 3.51^{c} (65.72)	276.3 ± 21.0^{c} (59.19)
Control	75.0 ± 4.36^{a} (0.0)	63.3 ± 8.02^{a} (0.0)	677.0 ± 18.2a (0.0)
Pooled S.D	4.93	4.33	43.41
P (α = 0.05)	0.001	0.001	0.001

The value of different growth parameters is the mean of three replications. Different case letters in a column represent statistical differences of mean according to Tukey pairwise comparisons (grouping information using the Tukey method and 95% confidence interval); values in parenthesis are percent decrease over control

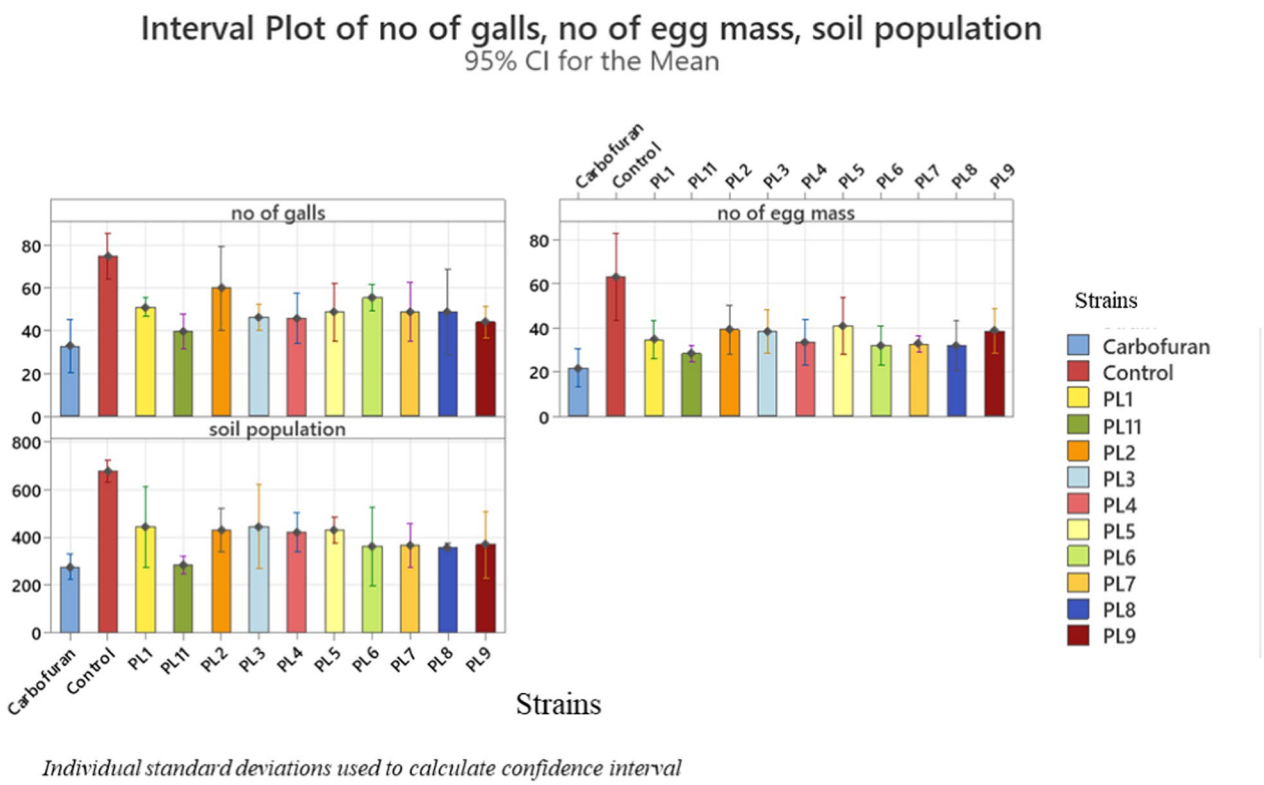


Fig. 11 Efficacy of *Purpureocillium lilacinum* on reproduction of *M. incognita*

Abbreviations

ICAR	Indian Council of Agricultural Research
ITS	Internal transcribed spacer
PDA	Potato dextrose agar
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
IIPR	Indian Institute of Pulses Research

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Author contributions
JR was involved in the conceptualization, design of the work, manuscript drafting, and data analysis. JD carried out all the experiments and data collection. VK contributed to the spectrophotometric assay and 2D gel electrophoresis. GKS contributed to the 2D gel electrophoresis study, critical revision and editing of the manuscript.

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Declarations

Ethics approval and consent to participate
Not Applicable.

Consent for publication
All the authors give their consent to publish the submitted manuscript as "Original paper" in EJBPC.

Competing interests
The authors whose names are listed immediately below certify that they have NO affiliation with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript. The authors certified that there is no conflict of interest in the research work and the article.

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