ORIGINAL RESEARCH

Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

JEB™

ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Effect of chickpea proteinase inhibitor on survival and parasitism of root-knot nematode, *Meloidogyne incognita*

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Key words

CaPI gene, Chickpea, *Meloidogyne incognita*, Proteinase inhibitor, Parasitism

Publication Info

Paper received :	07.12.2016
Revised received :	18.01.2017
Re-revised received :	23.01.2017
Accepted :	30.01.2017

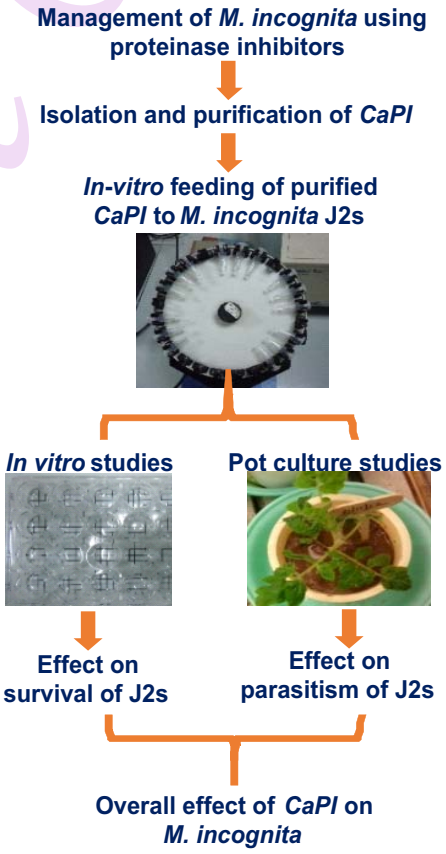
Abstract

Aim : Root-knot nematodes, *Meloidogyne incognita* are economically important plant parasitic nematodes of cultivated crops including vegetables. Eco-friendly and effective alternate methods are required for management of *M. incognita* to reduce use of chemical nematicides. Therefore, present study was conducted to assess the effect of *Cicer arietinum* proteinase inhibitor *(CaPI)* on survival and parasitism potential of *M. incognita* which can further be useful in developing management strategies against *M. incognita.*

Methodology : The *CaPI* gene was cloned in expression vector pET28a and pure *CaPI* protein was isolated and characterized. Purified protein was fed *(in vitro)* to second stage juveniles (J2s) of *M. incognita* to the study effect of PI on survival of J2s. The effect of *in vitro* feeding of *CaPI* on parasitism of *M. incognita* J2s was also studied by inoculation of protein fed J2s on tomato plants cv. Pusa Ruby. Tomato plants were observed after 35 days of inoculation to see the root galls and egg mass formation.

Results : The molecular weight of isolated and purified protein was ~30 kDa. *In vitro* studies revealed up to 27.73% mortality in *M. incognita* J2s. The parasitism potential of *CaPI* fed J2s was reduced thereby, root gall and egg mass production on tomato roots were reduced by 77.21 and 86.88% respectively, after 35 day of inoculation.

Interpretation : *In vitro* feeding of *CaPI* to the *M. incognita* J2s affected their survival and parasitism potential. Overall, *CaPI* has potential in management of *M. incognita.*



Introduction

Plant parasitic nematodes (PPN) are serious constraints to the world agriculture and affect the plant growth, yield and quality of crop produce. Worldwide losses due to PPN infection were estimated to be $173 billion annually (Elling, 2013). National loss due to PPN in twenty four different crops grown in India was estimated to the tune of 21 billion rupees (Jain *et al*., 2007). Among PPN, root-knot nematodes, *Meloidogyne* spp, including *Meloidogyne incognita,* are economically important nematodes. Root-knot nematodes (RKN) are obligate parasites with worldwide distribution and can attack roots of more than 3000 plant species (Abad *et al*., 2003). Infective second stage juveniles (J2s) penetrates the roots and forms permanent feeding site which consist of several giant cells functioning as a specialized sink to supply nutrients to the sedentary J2s. The parasitism by RKN causes the symptoms of severe root galling, yellowing and stunting of infected plant. The roots of infected plant often predisposed to secondary plant pathogens (Moens *et al*., 2009). Among RKN species, southern root-knot nematode, *Meloidogyne incognita*, is more serious pest in tropical and subtropical regions of the world and in India, it causes severe damage to vegetable crops like tomato, eggplant, okra, chilli and cucurbits cultivated in different regions (Khan *et al*., 2014).

Currently, management of PPN is achieved through cultural methods like crop rotation with non-host crop, resistant cultivars and chemical nematicides. However, management of RKN is a challenging task due to its wide host range, high reproductive potential and short life cycle (Trudgill and Blok, 2001). Cultural methods including soil solarization and crop rotation are effective but not feasible due to fear of loss of crop season (Papolu *et al*., 2016). Moreover, crop rotation becomes less effective for the management of nematodes having wide host range like *M. incognita* (Trudgill, 1997). Resistant cultivars are most preferred, eco-friendly and cost effective option however, very less number of resistant cultivars are available with farmers. The chemical control using nematicides becomes difficult due to continuous withdrawal of effective nematicides from pesticide markets due to groundwater contamination, high persistence and residue hazard to humans. Moreover, chemical pesticides are harmful and persistent in nature causing environmental pollution in all kind of ecosystems including aquatic ecosystems (Kandagal and Khetagoudar, 2013; de la Cruz *et al*., 2014). Therefore, an improved, ecofriendly and alternate methods are required for management of RKN to reduce the need of nematicides.

Proteinase inhibitors (PI) offer a great promise for the management of agricultural pests and are important element of the plant defense strategy against insect pests, as well as plant parasitic nematodes (Koiwa *et al*., 1997). The PI gene expression has been detected in leaves of several species following wounding, suggesting their role in protecting plants from injury due to insects (Lawrence and Koundal, 2002). PI affects PPN including root-knot nematodes through its anti-nematode feeding and anti-development strategies. Anti-nematode feeding strategy utilizes PI to block the activity of digestive proteases of nematodes (Atkinson *et al*., 1995; Fuller *et al*., 2008). PI based pest management has been achieved through two main approaches; one such approach is through transgenic expression of PI gene in important crop varieties against major insect pests (Stevens *et al*., 2012; Sharma, 2015) and nematodes (Fuller *et al*., 2008; Sirohi *et al*., 2010) while another approach is extraction of PI protein from seeds and use against major pests in conventional ways (Kansal *et al*., 2008; Nair *et al*., 2013). To date, number of transgenic plants have been developed using PI genes and reported to be effective against nematodes like *M. incognita* (Vain *et al*., 1998; Chan *et al.* , 2010; Papolu *et al*., 2016), *Globodera pallida* (Urwin *et al*., 1995) *Rotylenchulus reniformis* (Urwin *et al*., 2000) *Pratylenchus penetrans* (Vieira *et al*., 2015) etc. The nematode management using transgenic plants expressing PI genes reached the stage of field trials (Urwin *et al*., 2001; Tripathi *et al*., 2015) and studies on environmental biosafety of some these transgenics showed that PI are safe to non-target aerial insects, natural parasitoid enemies of insects as well as for the soil microbes (Fuller *et al*., 2008). Studies on the extraction of PI from seeds of *Cicer arietinum* and feeding of larvae on artificial diet containing isolated PI protein showed inhibition of gut protease activity of *Helicoverpa armigera* (Kansal *et al*., 2008), as well as decrease in weight and survival of 5th instar larva of *H. armigera* (Nair *et al*., 2013). Among PPN, *in vitro* feeding of PI isolated from *Vigna mungo* caused mortality in protein fed *M. incognita* J2s (Gawade *et al*., 2014). Therefore, PIs are effective for the management of PPN through its transgenic expression and *in vitro* feeding of isolated PI, however, information on *in vitro* feeding of PI from chickpea on survival and nematode parasitism is lacking. The present experiment was, therefore, conducted to observe the effect of *in vitro* feeding of PI on survival and parasitism of *M. incognita.* Attempts were made to clone and express *Cicer arietinum* proteinase inhibitor *(CaPI)* gene, a serine PI, in a bacterial expression system to obtain the desired protein and to test its efficacy against *M. incognita.*

Materials and Methods

***CaPI* gene and cloning :** The *CaPI* gene isolated from chickpea *(C. arietinum)* cv. Pusa 256 (Accession no. JN561785) was received from ICAR-National Research Centre for Plant Biotechnology (NRCPB), New Delhi, India. The full coding sequence of *CaPI* gene was amplified by polymerase chain reaction (PCR) using gene specific forward (5' CACATATGATGAAATCCATTGTA 3') and reverse primers (5' CAAAGCTTAACTGACGCATCAA 3') designed with *Nde*I and *Hind*III restriction endonuclease sites, respectively at 5' end. A 100 µl PCR reaction mixture contained 10 µl of 10X Taq buffer, 4 µl dNTPs mix (2.5 mM of each dNTP), 2µl of each forward and reverse primer (10 mM), 3 units of *Pfu* DNA polymerase and 100 ng plasmid DNA. The PCR conditions were as follows; initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 5 min. PCR product was loaded on 1% agarose gel with ethidium bromide and visualized

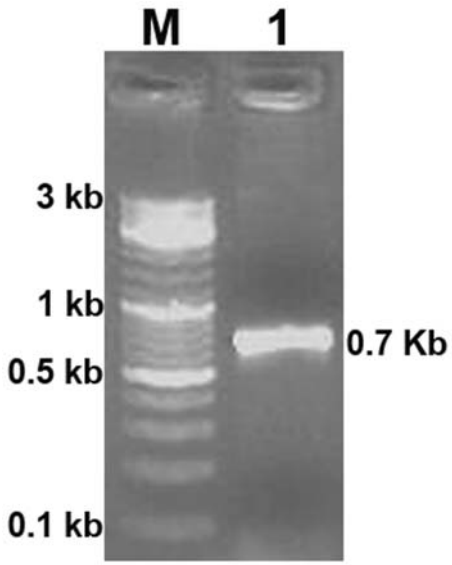


Fig. 1 : PCR amplification of *CaPI* gene. Lane M- 100 bp plus DNA ladder; Lane 1- PCR product

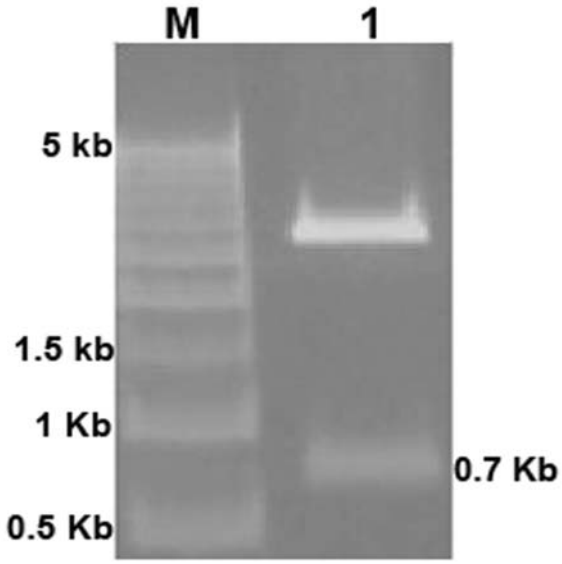


Fig. 2 : Confirmation of *CaPI* gene in pUC vector by restriction digestion with *NdeI* and *Hind*III restriction enzymes. Lane M- 500 bp DNA ladder; Lane 1- Positive pUC clone releasing an insert of ~700 bp

with an UV transilluminator. Purified full length *CaPI* gene was digested with *Nde*I and *Hind*III, cloned into pUC vector and positive clones were screened by restriction digestion and sequencing. Restriction digested gene insert was gel purified (MinElute Gel Extraction kit, Qiagen) and ligated into pET28a expression vector at *Nde*I and *Hind*III restriction sites to produce *CaPI*-His tag fusion protein. Transformed colonies of pET28a were screened using restriction digestion. The *CaPI*-pET28a plasmids were transformed into BL21(DE3) cells, positive colonies were used for protein induction using 1mM IPTG at 37°C for 2 hrs and checked on 12% SDS-PAGE.

Purification of recombinant protein : The pellet of BL21(DE3) harbouring *rCaPI* protein was suspended by gentle stirring in lysis buffer (1% Triton X100, 20mM Tris-HCl, 10mM EDTA, pH 7.5). The suspension was centrifuged for 20 min at 6000 × g. The protein was purified using Ni-NTA column (Qiagen) following manufacturer's protocol and checked on 12% SDS-PAGE.

J2s of *M. incognita* : Pure culture of *M. incognita* was raised and maintained on tomato cv. Pusa Ruby. Clean egg masses were collected from infested plants, surface sterilized with 0.1% $HgCl_2$ (1 min) and rinsed with sterile distilled water. Egg masses were placed at 28ºC and hatched juveniles were used for inoculation and *in vitro* studies.

Feeding of J2s with *CaPI* protein : Around 150 freshly hatched J2s of *M. incognita* were taken in 1.5 ml eppendorf tube and soaked in 100 µl PI solution containing *CaPI* protein of various concentrations (6, 8 12 and 16 µg ml^{-1}) and 50 mM octopamine to induce feeding in nematode outside the root system and 1 mg ml^{-1} fluorescein isothiocyanate (FITC) to trace the uptake of PI solution. There were five treatments including control (water), each replicated five times. The eppendorf tubes were rotated in dark for 18 hrs on rotospin (Tarsons) to induce feeding in J2s. The nematode mobility and mortality was observed under stereoscopic microscope at an interval of 24, 48, 72 and 96 hrs of treatment. Non-motile, straight J2s were counted as dead when unable to move even after mechanical prodding. After each time interval, all non-motile nematodes were transferred to water separately to observe the revival after 24, 48 and 72 hrs of transfer.

Infection studies on tomato : Three weeks old seedlings of tomato cv. Pusa Ruby were transplanted to pots of 4 inch diameter size containing 400cc of autoclaved soil and sand mixture (2:1 ratio). The experiment was arranged in completely randomized design with five replications in each treatment. The nematode J2s fed with *CaPI* protein (as per the method described for *in vitro* feeding) were inoculated at the rate of 2 J2s/cc of soil. For control, J2s were soaked in plain water. After 35 days of inoculation, plants were uprooted and number of galls and egg masses were counted under stereoscopic microscope.

Statistical analysis : All the data were subjected to analysis of variance (ANOVA) and means were separated by Duncan's multiple range test at $P < 0.05$ significance level using software, SPSS for Windows, Version 17.0. Chicago, SPSS Inc.

Results and Discussion

The *Cicer arietinum* PI protein was successfully isolated and characterized through bacterial expression method to study its

Table 1 : Effect of *in vitro* feeding of *CaPI* protein on survival of root-knot nematode *Meloidogyne incognita* J2s

	No. of dead J2s							
Protein fed in µg ml^{-1}	**24 hr**	**Percent Mortality**	**48 hr**	**Percent Mortality**	**74 hr**	**Percent Mortality**	**96 hr**	**Percent Mortality**
Control	0.8^{a}	0.53	1^{a}	0.66	1.4^{a}	0.93	2.2^{a}	1.46
6	4.6^{b}	3.06	7.2^{b}	4.80	8.8^{b}	5.86	14.4^{b}	9.60
8	6.6^{b}	4.40	13.8^{c}	9.20	17.4^{c}	11.60	20.8^{c}	13.86
12	9.6^{c}	6.40	21.2^{d}	14.13	26.2^{d}	17.46	37.2^{d}	24.80
16	11^{c}	7.33	24.2^{d}	16.13	31^{e}	20.66	41.6^{e}	27.73

Means in the same column followed by same letter are not significantly different at P=0.05, according to Duncan Multiple Range Test.

Table 2. Effect on root galling and egg mass production on tomato roots upon inoculation with *CaPI* protein fed (*in vitro*) J2s of root-knot nematode *Meloidogyne incognita*

Protein fed in µg ml^{-1}	**Root galls^{x} (±SD)**	**Percent reduction over control**	**Egg masses ^{x} (±SD)**	**Percent reduction over control**
Control	63.2±6.26^{a}	0	24.4±3.20^{a}	0
6	61.6±7.63^{a}	2.53	22.2±4.43^{a}	9.01
8	48.6±7.92^{b}	23.10	18.4±3.97^{b}	24.59
12	40.8±4.86^{c}	35.44	10.2±2.38^{c}	58.19
16	14.4±3.43^{d}	77.21	3.2±2.16^{d}	86.88

Means in the same column followed by same letter are not significantly different at P=0.05, according to Duncan Multiple Range Test. ^{x}Root galls and egg mass values are means of five replications.

efficacy against *M. incognita.* The cloning of *CaPI* PCR amplified gene (Fig. 1) in pUC vector was confirmed by restriction digestion (Fig. 2). The *CaPI* protein induced was purified and loaded on 12% SDS-PAGE for analysis. The molecular weight of the expressed protein was ~30 kDa, as evident from Fig. 3. The size of protein was comparable with *CaPI* which was earlier reported as effective in the management of *H. armigera* (Kansal *et al.*, 2008), suggesting wide range of *CaPI* activity against insect as well as nematodes.

The results summarized in Table 1 demonstrated that *CaPI* protein feeding affected the survival of *M. incognita* J2s over the factors of time of exposure and concentration of protein . The juvenile mortality increased with increase in protein concentration. Percent mortality was highest in 16 µg ml^{-1} treatment and lowest in 6 µg ml^{-1} treatment at each feeding time interval. Up to 27.73% juvenile mortality was observed after 96 hrs of feeding (Table 1). After 24 hrs of feeding, there was no significant difference between the treatments 6 µg ml^{-1} and 8 µg ml^{-1} while after 48 hrs of feeding, 12 µg ml^{-1} and 16 µg ml^{-1} were at par with each other. After 72 and 96 hrs of feeding, all the treatments varied significantly from each other. None of the non-motile nematode was able to revive when transferred to water after each time interval of exposure showing irreversible effect of *CaPI* on juvenile mortality. The results revealed that the *in vitro* feeding of *CaPI* protein caused mortality in the RKN J2s.

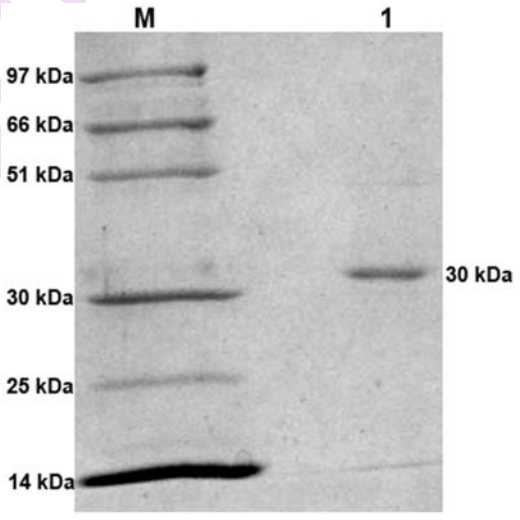


Fig. 3 : SDS-PAGE analysis of the purified expressed *CaPI* protein. Lane M- Protein molecular weight marker; Lane 1- Ni-NTA purified fusion protein

The data also revealed that *in vitro* feeding of J2s on *CaPI* resulted in reduced root gall development and reproduction

of *M. incognita* (Table 2). The number of root galls was maximum in 6 µg ml^{-1} treatment, while minimum in 16 µg ml^{-1} treatment. The treatment 16 µg ml^{-1} caused 77.21% reduction in galling when compared with control. There was no significant difference found between 6 µg ml^{-1} and control. The reduction in egg mass production was also observed upon feeding of *CaPI* protein. There was up to 86.88% reduction in egg mass production in 16 µg ml^{-1} treatment (Table 2). The treatment 6 µg ml^{-1} was least effective in which highest number of egg masses were formed with no significant difference with control. Since J2s in control treatment were able to develop numerous galls and egg masses, it indicated that J2s were viable and sufficient in number to initiate infection.

Proteinase inhibitors are natural antagonists of proteinases, known to inhibit protein digestion in wide range of insects and nematodes (Stevens *et al.*, 2012; Fuller *et al.*, 2008). In present study, *in vitro* feeding of *CaPI* caused mortality in *M. incognita* J2s with increase in time of exposure and concentration of PI. The findings corroborate with the studies of Gawade *et al.*, 2014, where PI from *Vigna mungo* caused mortality in *M. incognita* J2s. Similarly, PI from *C. arietinum* caused dose dependent mortality in the larvae of *H. armigera* and increase in dose or concentration of PI in artificial diet resulted in progressive death in larvae (Nair *et al.*, 2013). Heat stable nature of *CaPI* and activity over wide range of pH (Kansal *et al.*, 2008) could have positive effect in causing mortality in *M. incognita.* In insects, PIs inhibit the activity of proteinase enzymes and affects protein digestion. This also leads to hyper secretion of digestive proteases, as a result of this insect becomes weak, stunted and ultimately die due to deficiency of vital amino acids (Mendoza-Blanco and Casaretto, 2012; Sharma, 2015). Therefore, continuous inhibition of digestive proteinases, starvation and deprivation from important dietary amino acids might be the cause of mortality in *M. incognita* J2s.

PIs not only affect the survival of nematode but also hamper their growth and development. Feeding of *M. incognita* J2s on *CaPI* resulted in reduced number of root galls and egg masses on tomato roots, which indicated the effect of *CaPI* on nematode parasitism and fecundity. The effect of PI on target pest depends upon type of proteinase inhibited, degree of inhibition of target proteinase, concentration of PI expressed in feed/plant, pH of gut of pest etc. (Kansal *et al.*, 2008; Stevens *et al.*, 2012) and in nematodes, the loss of nematode cysteine proteinase activity of *M. incognita* was correlated with uptake of cystatin, a cysteine PI (Urwin *et al.*, 1997). Data mining of *M. incognita* degradome revealed that proteinases of various types are abundant in *M. incognita* genome including parasitism related serine proteinases (Castagnone-Sereno *et al.*, 2011). It was also suggested that, abundance of serine proteinases in pre-parasitic and parasitic stages of *R. reniformis* have important role in parasitism (Urwin *et al.,* 2000). Therefore, these studies relate to the reduction of parasitism by *M. incognita* and subsequent reduction in gall development and egg mass formation, as important parasitism related serine proteases might have been blocked due to *CaPI* feeding.

Transgenic plants expressing PI genes also showed detrimental effect of PI on nematode parasitizing these plants with respect to their growth and development. Feeding of RKN on cowpea trypsin inhibitor *(CpTI)* gene expressing transgenic potato reduced the fecundity of females (Urwin *et al.*, 1998). Sporamin, PI from sweet potato, expressed in transgenic sugar-beet hairy roots inhibited the growth and development of female cyst of beet cyst nematodes *H. schachtii* (Cai *et al.*, 2003). Another effective PI, OC-IΔD86 was found effective against diverse PPN including *M. incognita* (Fuller *et al.*, 2008, Chan *et al.*, 2010, Vieira *et al.*, 2015, Papolu *et al.*, 2016). OC-IΔD86 affected *M. incognita* in eggplant with 57.22% reduction in root gall formation and up to 55.7% reduction in fecundity of females (Papolu *et al.*, 2016). Egg production was severely hampered in females of *M. incognita* feeding on cystatin-expressing *Arabidopsis* plants (Urwin *et al.*, 1997) and higher expression of PI was correlated with reduced fecundity of *R. reniformis* feeding on *Arabidopsis* plants (Urwin *et al.*, 2000). These findings also corroborate with the results of the present study as root gall formation and egg mass production was significantly lowered in plants inoculated with *M. incognita* J2s fed on higher concentration of *CaPI*.

In conclusion, *CaPI* significantly affected the survival and parasitism potential of *M. incognita* and hence it has great potential for ecofriendly management of root knot nematodes. Further, it may provide environmentally safe method of management of insect pests, as well as plant parasitic nematodes.

Acknowledgments

First author acknowledges the Department of Science and Technology, Govt. of India for financial support (DST-INSPIRE fellowship: JRF-Professional) and ICAR-IARI for fellowship during early phase of research work. Authors are thankful to Head, Division of Nematology, ICAR-IARI, New Delhi for providing necessary facilities and resources for conducting experiments.

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