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Evaluation of field pea accessions for root-knot nematode resistance and possible role of NADP dependent malic enzyme gene in host resistance

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Abstract

The present study was undertaken to evaluate resistance of different field pea accessions to RKN, *Meloidogyne javanica* under microplots and pot culture conditions. Three hundred accessions of field pea were screened initially using sick soil and evaluated after 2 months of sowing using root gall index of 1-5 scale. Based on galling index eleven accessions showing resistant reaction were further screened under controlled condition. All eleven acessions were consistent in their response to RKN infection under pot culture experiment. The partial sequence of NADP-dependent malic enzyme (ME) gene was PCR amplified, sequenced and used to screen field pea accessions showing resistant and susceptible reaction against RKN using PCR amplification. PCR screening using ME specific primers showed PCR amplification only in accessions showing resistant reaction. There was no amplification of *ME* gene from susceptible field pea accessions. The partial sequence of putative NADP dependent Malic enzyme gene from resistant accessions of field pea has been cloned and characterized. The results indicate possible link between *ME* and resistance against RKN in field pea. Role of malate metabolism in generating resistance reaction against RKN is discussed.

Key words: Field pea, malic enzyme, root-knot nematode, *Meloidogyne javanica*, *Pisum sativum*, resistance

Introduction

Field pea (*Pisum sativum* L.) belonging to the family Fabaceae is cool season crop and one of the important leguminous crops of the world. In India, it is grown mostly in *rabi* season in high altitudes as well as plains due to low temperature. The yield of the crop is limited by various biotic factors of which root-knot nematode is one of the limiting factors causing up to 33% losses (Upadhyay and Dwivedi, 1987). Root-knot nematodes (RKN) belonging to the genus *Meloidogyne* are polyphagous and sedentary endo-parasites infecting various crop plants. These obligate parasites are distributed across the globe under different geographical situations, prevalent in tropical and sub-tropical climatic zones and are reported to infest more than 3000 wild and cultivated plant species (Hussey and Janssen, 2002; Rich et al. 2008). Their global distribution coupled with extensive host range and involvement with fungal, bacterial and viral pathogens place them among the major pathogens affecting global food supply. RKN are drastically affecting global food production and bringing enormous losses to farmers (Williamson and Hussey, 1996). There are approximately 97 described species of root-knot nematodes, but four *Meloidogyne* species *viz*., *M. incognita, M. javanica, M. arenaria*, and *M. hapla* account for the major crop losses (Hunt and Handoo 2009). The nematode infection initiates root gall formation in the invaded roots. The root knot differs in size and number according to RKN species and host. Diseased plants show symptoms which appear like day time wilting or deficiency of minerals and/or nutrients due to hindrance in water and nutrients uptake and translocation.

The management of RKN can be achieved through chemical nematicides. The chemical methods including fumigant and non-fumigant chemicals are effective but unsafe, costly and responsible for environmental pollution. The resistant varieties are one of the options for management of plant parasitic nematodes as these are cheap, safe and ecofriendly.

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The evaluation of available germplasm for identification of resistant accessions is one of the important activities. The resistant accessions are ready to use source of resistant genes and can be utilized in breeding nematode resistant lines/varieties. However, sources of host plant resistance against root-knot nematode are very limited. Recently, researchers have identified some resistance source to root-knot nematodes in legumes (Gautam et al. 2013; Khan et al. 2017). Moreover, sustenance of long time durable resistance in developed varieties poses a major challenge to crop breeders. (McDonald and Linde, 2002; Castagnone-Sereno, 2006). Nematode resistance gene actions are complex and durable one are generally governed by polygenes. Hence, it is necessary to identify and pool different sources of resistance and introgress them into popular varieties to achieve durable and broad resistance against root-knot nematodes (Castagnone-Sereno 2002, 2006), McDonald and Linde, 2002).

Alterations in malate levels and activity of malate-transforming enzyme in response to biotic and abiotic stress suggest a role of malate in plant defense (Levitt, 1980, Schaaf et al. 1995). It has been shown that malic acid (MA) is secreted from roots of *Arabidopsis thaliana* in response to attack by pathogen *Pseudomonas syringae* and selectively signals and recruits the beneficial rhizobacterium. The elevated levels of MA promote biofilm formation on *Arabidopsis* roots (Rudrappa et al. 2008). In chickpea, acidic exudates from trichomes in which oxalic and malic acid are major acid constituents on the surface of chickpea plants has been correlated with reduced pod damage (Rembold, 1981; Rembold and Winter, 1982; Srivastava and Srivastava, 1989; Rembold et al. 1990a,b). Malate is involved in several metabolic pathways and one of the important malate metabolizing enzymes is NADP-malic enzyme (NADP-ME). NADP-ME is known to have role in different pathways in plants, other than C4 photosynthesis. This enzyme is implicated in defense-related deposition of lignin by providing NADPH for the two NADPH-dependent reductive steps in monolignol biosynthesis (Casati et al. 1999). Role of NADP-ME in plant pathogenic nematodes resistance is largely unexplored.

In present work, evaluation of field pea accessions was performed against RKN, *M. javanica* to identify RKN resistant accessions. Attempts were also made to isolate and sequence NADP dependent malic enzyme gene from field pea.

Materials and methods

Pea germplasm

A total of 300 accessions of pea germplasm procured from Project Coordinator Unit, ICAR-Indian Institute of Pulses Research (IIPR), Kanpur, India were evaluated for their host status to the root-knot nematode, *M. javanica*.

Root-knot nematode inoculums

Root-knot nematode, *M. javanica* multiplied on black gram (*Vigna mungo*) plants grown in microplots was used for preliminary screening. The population density second stage Juvenilers (J2s) of *M. javanica* J2s was determined before sowing of seeds in microplots. Infected soil was thoroughly mixed and J2s were extracted from 10 subsamples of 200-cc soil using sieving and decantation method. The average of all the subsamples was used for calculation of population density. Pure culture of *M. javanica* was raised using single egg mass collected from the infected roots of black gram. Egg masses were kept in distilled water for hatching at room temperature and freshly hatched juveniles were inoculated on tomato plants grown in sterilized soil in earthen pots for further multiplication. This pure culture of *M. javanica* maintained on tomato plants was used for pot culture experiments. Root-knot nematode species, *M. javanica* was identified on the basis of perineal pattern characteristic of female nematodes.

Evaluation against root-knot nematode in microplots

The preliminary evaluation of 300 accessions was carried out during rabi season of 2014-15 in the microplots infested with RKN, *Meloidogyne javanica.* At the time of sowing of field pea, the average nematode population was about three J2s per cc soil. The seeds were sown in row with five replications of each germplasm and all the accessions were randomized. Plants were irrigated whenever required. After 60 days of sowing, four plants of each accession in each replication were uprooted. The root system was washed gently with tap water and observations were recorded for each plant on number of galls per root system. Based on galling index (GI) of 1-5 scale, the accessions were categorized as 0=no gall, 1=1-2, 2=3-10, 3=11-30, 4= 31-100, 5>100 galls per root system (Taylor and Sasser 1978). Host responses of field pea germplasm were determined using GI to be designated as Immune (I) when GI=0.0, resistant (R) GI $\leq$ 2.0 and susceptible (S) >2.0 (Sasser et al. 1984).

Evaluation against root-knot nematode under pot conditions

The accessions showing resistant reaction under microplot conditions were sown again for second year during *rabi* 2015-16 in plastic pots of 10-cm diameter filled with 500-cc steam sterilized field soil. Each accession was replicated five times. Three seeds of each selected accession were sown in respective pots, after germination, one plant was maintained in each pot. The freshly hatched 1000 J_{2s} of *M. javanica* suspension in 10 ml water were dispensed around the exposed roots of each pot with the help of pipette, when the plants were at two-leaf stage and pots were watered whenever required. The plants were uprooted after 45 days of nematode inoculation and root system was cleaned from the soil, washed under tap water before recording the observations on the root gall index in the same manner as evaluated under microplots. The reactions of accessions under pot conditions were taken into consideration and further molecular basis of resistance was investigated.

Amplification and detection of ME gene

The seeds of eleven resistant accessions (with less than 10 root galls), three accessions showing moderately resistant reaction (11-15 root galls) and sixteen susceptible accessions showing different galling scale were used. The pedigree status of genotypes is given in Table 1. The seeds of *P. sativum* were sown, leaf samples were collected after 7 days of nematode inoculation and DNA was isolated using CTAB method (Murry and Thompson, 1980) with minor modifications. Primers were designed for NADP-dependent malic enzyme gene using a pea EST (http://primer3.ut.ee/) (Forward primer-CTCGCCAACAACCGGAGTAT and reverse primer-TTGCGTGGCCTCTTACCTCC). The *Me* gene was amplified with PCR conditions: 94^{o}C for 3 min, 32 cycles at 94^{o}C for 1 min, 60^{o}C for 30 sec and 72^{o}C for 1.5 min and a final extension at 72^{o}C for 7 min. The PCR product was loaded on 1.5% agarose gel to determine the size of gene and quality of amplified DNA. The samples found positive for *ME* gene were purified and sequenced.

Prediction of ME protein structure

The isolated and sequenced *Me* gene fragments were used for prediction of protein. The nucleotide sequences were translated into amino acids sequences by using Translate tool of ExPASy (http://web.expasy.org/translate/). Protein structure studies involving prediction of functional domains and 3D structures was performed using SWISS-MODEL automated protein structure homology-modelling server (swissmodel.expasy.org/) (Arnold et al. 2006).

Table 1. Pedigree of the field pea accessions used for evaluation against root-knot nematode, *Meloidogyne javanica*

S.No.	Accessions	Pedigree
1	IPFD 11-10	IPF 99-25 X DDR 27
2	IPFD 99-13	HFD 4 X LFP 80
3	NDP 1200	-
4	RFP 2009-4	Rachna X EC 334160-1
5	KPF 1028	HFP 9907B X EC 499997
6	RFP 72	RPG 3X Rachna
7	HFP 8909	EC 109185 X HFP 4
8	HFP 715	DMR 50 X HFP 9948
9	Pant P 244	Pant P 13 X VL 45
10	KPF 101	Jayantix EC 398602) x (Uttra x EC 502159)
11	Pant P-243	Pant P 14 X Pant P 41
12	RFP 2009-3	Rachna X EC 334160-1
13	KFD 12-04	HFP 4 X IPFD 99-13
14	IPFD 13-2	IPFD 1-10 X DDR 23
15	IPFD 13-14	EC 53804 X IPF 27
16	IPF 12-17	IPF 27 X EC 538004
17	Pant P-42	(HUDP 7 X HFP 4) X EC-1
18	IPFD 11-5	(DDR 16 X HUDP 7) X DDR 16
19	IPFD 13-4	IPFD1-10 X IPFD 99-13
20	IPF 2-17	-
21	IPFD 99-25	PDPD 8 X Pant P 5
22	IPFD 12-8	IPFD 1-10 X DDR 27
23	IPFD 12-2	HUDP 15 X EC 342002
24	IPF 11-15	IPF 99-25 X EC 501259
25	IPF 13-13	EC 32413 X IPFD 1-10
26	NDP 12-102	HUDP 15 X P 150
27	Pant P-195	Pant P 14 X IPFD 1-10
28	IPFD 5-19	KPMR 144-1 X EC 8495
29	IPFD 1-10	-
30	IC219002	Germplasm

Results and discussion

Microplot studies

In preliminary evaluation of 300 accessions of field pea were used for resistance to *M. javanica*. The

uprooted plants were observed for presence of root galls and based on number of root galls induced divided into different categories. Out of 300, eleven (11) accessions (Table 1) were found resistant as these have shown less than 10 root-galls per root system. All other remaining accessions considered to be susceptible because large number of root galls were formed with GI > 2.

Evaluation under pot conditions

Eleven accessions of field pea on which less than 10 root galls per root system observed in preliminary evaluation were selected for further evaluation in pots with artificial inoculation in steam sterilized soil to reconfirm their host status against *M. javanica*. All eleven accessions shown consistent performance for resistance against *M. javanica*, as nematode induced less than 10 root galls per plant root system and were designated as a resistant. The resistant genotype HFP 8909 was the best accession with 97.5% reduction over susceptible accession Pant P-195 (Fig. 1).

Isolation of gene encoding NADP-malic enzyme

In order to isolate malic enzyme gene, a local BLAST search was performed using NADP-malic enzyme CDS sequences from soybean as query against total ESTs of field pea (downloaded from dbEST, NCBI). The best BLAST hit EST which showed significant similarity with NADP-malic enzyme of soybean and chickpea were used to design primers for PCR amplification of NADP-ME from field pea. A PCR using these primers resulted in amplification of single band of ~500bp. These primers were used to screen 30 field pea accessions (resistant, moderately resistant and susceptible). As shown in the Fig. 2, PCR amplification was observed only in resistant and moderately resistant cultivars, which produced less than 15 root galls per root system. To validate, if the amplicon is indeed from NADP-ME, the PCR product was purified and subjected for DNA sequencing. The DNA sequencing of the amplicon confirms its amplification from NADP-ME. The partial sequence NADP-ME with 517 and 484bp long have been deposited to Genbank (Accession no. KX347451, KX231805). *P. sativum* malic enzyme gene showed 90% sequence identity with *Cicer arietinum* NADP-dependent malic enzyme (LOC101510396)

In silico analysis of NADP-ME gene

Structural and functional characterization of proteins coded by resistance genes revealed many interesting insights on mechanism of resistance in host plants against nematodes (Vos et al. 1998; Milligan et al. 1998). In this study, partial NADP-ME (Malic Enzyme) gene sequences have been identified from field pea. The partial gene harbors three exons, which code for 71 amino acid long ME protein sequence (AOX47693.1). *ME* encoded protein structural analysis revealed presence of, malic enzyme, N-terminal domain (malic super family, acc. no. Cl27704, e-value-2.18e-37). Multiple sequence alignment of the filed pea ME protein sequence to that of ME/ME like protein from other plants shows high sequence identity at the conserved N-terminal domain (Fig. 3). The 3-D structure of NADP-ME is built using homology modeling shows monomer of N-terminal domain of malic enzyme (Fig. 4).

Localization of malic enzyme

Amino acid sequence of the partial gene was used to predict possible sub-cellular localization of identified ME. Gene functional annotation tool QuickGO (http://www.ebi.ac.uk/QuickGO/GTerm?id=GO:0005829) and subcellular protein target location tool (TargetP http://www.cbs.dtu.dk/services/TargetP/) revealed its cytosolic presence. Similar reports of *Me* 2, a chloroplast peptide and another isoform of *Me* 1, was expressed in leaves and roots of C3 dicot plant *F. bidenvis* and observed to be cytosolic in origin (Walter *et al*., 1990; Drincovich et al., 2001). Role of cytosolic Me 2 in plant defense responses was also reported (Schaaf et al. 1995; Laporte et al. 2002).

Natural resistance in plants is one of the preferred option or strategy for controlling nematode infestation due to environmental and health issues related with chemical control methods. In the present study, 300 accessions of field pea were evaluated for the resistance to *M. javanica*, out of that 11 accessions were found resistant to nematode infection, showing fewer gall formations (gall index <GI> less than 2.0). Reported microscopic studies provide some explanation on how the host plant manages resistance (Dropkin, 1969; Paulson and Webster, 1972; Ho et al. 1992; Moon et al. 2010). Parasites invade both susceptible and resistant plants alike and migrate to the vascular tissues of the root for feeding. In a resistant plant, interestingly, spread of the infection is restricted by localized cell death (necrosis) or hypersensitive response. Egg-laying is restricted and the nematodes eventually die (Williamson and Hussey, 1996). It has been reported that plants utilize same type of R genes based resistance to protect

Fig. 1. Field pea roots infected with root-knot nematode, *M. javanica* A) Highly susceptible to RKN (Pant P-195 showing heavy root galls); B) Resistant accession (HFP 8909) with a few root galls

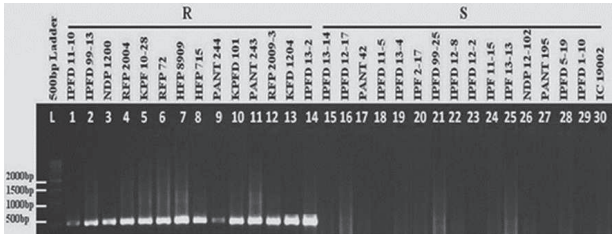

Fig. 2. PCR amplification of ME gene in different field pea accessions. R and S indicate accession with resistant and susceptible reaction, respectively

```
P.sativum:AOX47693.1            FLRGLLPPVVSSQQLQLQEKKLMHNIRQVEVPLQKYMAMMDLQQERNERLFYKLLIDNVK
M.truncatula:XP_003630727.1     YLRGLLPPTVSSQQ--LQEKKLMHNIRQVEVPLQKYVAMMDLQ-ERNERLFYKLLIDNVE
C.arietinum:XP_004503480.1      YLRGLLPPSCSTQQ--LQEKKLMNNIRQVEVPLQKYMAMMDLQ-ERNERLFYKLLIDNVE
B.distachyon:XP_003568785.1     YLRGLLPPVIASQE--LQEKKLMHNLRQYTVPLQRYIAMMDLQ-ERNERLFYKLLIDNVE
S.lycopersicum:XP_010325075.1   YLRGLLPPTVSSQQ--LQEKKVMHNLRQVQVPLQRYMAMMDLQ-ERNERLFYKLLIDNVE
S.tuberosum:XP_006364722.1      YLRGLLPPSVSSQQ--LQEKKVMHNLRQVQVPLQRYMAMMDLQ-ERNERLFYKLLIDNVE
S.indicum:XP_011080458.1        FLRGLLPPVVISQQ--LQVKKMMHNMRQYQVPLQRYMAMMDLQ-ERNERLFYKLLIDNVE
                                :*******   :*:  ** **:*:*:*** ****:*:****** **************:

P.sativum:AOX47693.1            ELLPIVYTPVV
M.truncatula:XP_003630727.1     ELLPIVYTPVV
C.arietinum:XP_004503480.1      ELLPIVYTPVV
B.distachyon:XP_003568785.1     ELLPVVYTPVV
S.lycopersicum:XP_010325075.1   ELLPVVYTPTV
S.tuberosum:XP_006364722.1      ELLPVVYTPTV
S.indicum:XP_011080458.1        ELLPVVYTPTV
                                ****:****_*
```

Fig. 3. Multiple sequence alignment of putative field pea (*Pisum sativum*) ME with ME/ME like proteins from other plants (*Medicago truncatula, Solanum lycopersicum, Solanum tuberosum, Cicer arietinum, Brachypodium distachyon* and *Sesamum indicum*)

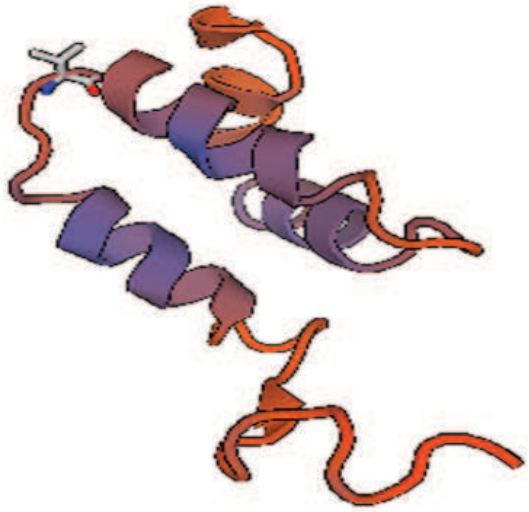

Fig. 4. Structure of 71 amino acid stretch of ME protein showing monomer of Malic enzyme, N-terminal domain

themselves against nematodes. Plant R genes which encode surveillance proteins help in the detection of effector molecules from pathogens directly or initiate effective defense responses.

Malate concentrations are subject to considerable changes in plant cells under physical (heat, cold, and drought), chemical (pesticides, herbicides, pollutants) and pathogen caused stresses (Levitt 1980; Lance and Rustin 1984). In present study, NADP-dependent malic enzyme gene was PCR amplified, sequenced and used to screen field pea accessions showing resistant and susceptible reaction against RKN. PCR amplification of partial gene encoding malic enzyme from resistant and moderately resistant field pea accessions and not from susceptible accessions (Fig. 2) indicates possible variation in gene at sequence level. Since, malic enzyme is a house keeping gene it is unlikely to be absent in the susceptible field pea accessions, however, there could be some sequence variation that might be a reason for no amplification. As there was no PCR amplification in susceptible field pea accessions, we could not identify possible

Table 2: Reaction of field pea accessions to *Meloidogyne javanica* based on the root galling index.

Genotype	A.v. no. of galls/ root system	Percent reduction over control[a]	Gall index	Host status[b]
IPFD 11-10	6.6	95.40	2	R
IPFD99-13	8.2	94.28	2	R
NDP 1200	8.2	94.28	2	R
RFP 2009-4	5.8	95.96	2	R
KPF 1028	4.4	96.93	2	R
RFP 72	4.6	96.79	2	R
HFP 8909	3.6	97.49	2	R
HFP 715	7.0	95.12	2	R
Pant P 244	8.2	94.28	2	R
KPF 101	4.2	97.07	2	R
Pant P-243	5.4	96.23	2	R
Pant P-195	143.6	0	5	S

[a]percent reduction of gall formation in resistant accessions as compared to most susceptible accession (Pant P-195); [b]Host status of *Pisum sativum* accessions was determined using root gall index (GI) as; GI $\geq$ 1=Highly resistant (HR); GI $\geq$ 2 resistant (R); and GI < 2= susceptible (Sasser et al. 1984)

sequence variation/allelic variation between resistant and susceptible accessions. Whether, any such possible variations in gene sequence between resistant and susceptible accessions is responsible for variation in observed resistance in field pea accessions against RKN need further investigation.

Yoshida et al. (1997) studied the role of oxalic and malic acids in exudates of chickpea trichomes and observed that malic acid inhibits growth of nematode larvae and thus reduces the damage caused. An increase in the levels of NADP-ME could provide energy and building blocks for synthesis of defense related compounds suggesting a role of metabolism of malate in plant defense (Casati et al. 1999). NAD-dependent isoforms (NADP-ME) function predominantly in mitochondria to produce pyruvate required for oxidation in the TCA (tricarboxylic acid) cycle (Artus and Edwards, 1985), while NADP dependent MEs (NADP-ME) are found in plastids as well as in cytosol (Edwards and Andreo, 1992; Drincovich et al. 2001). Cytosolic isoforms have been linked with plant defense reactions (Schaaf et al. 1995) In silico investigations on sub cellular location of ME protein employing bioinformatics tools in the present studies also support its cytosolic presence in supporting the involvement of ME proteins in host defense. In bean (*Phaseolus vulgaris*), promoter of malic enzyme gene harbors a cis-regulatory elements which is possibly involved in fungal elicitors and UV induced gene expression (Walter et al. 1994). Analysis of promoter of this gene fused to the â-glucoronidase reporter gene in tobacco plants showed the activation of promoter by different effectors related to plant defense (Schaaf et al. 1995). Further, UV-B induced expression of NADP-ME was also observed in bean (Pinto et al. 1999). These studies clearly suggest that cytosolic enzyme is involved in generation of plant defense responses, possibly by providing NADPH for the biosynthesis of lignin and flavonoids (Drincovich et al. 2001).

Results from this study and number of reports as seen in literature survey with respect to role of ME in producing defense responses, indicate a possible role of ME in producing resistance against RKN. However, further research would be required to establish how ME plays a definitive role in nematode resistance in plants.

Authors' contribution

Conceptualization of research (NKG, SSM); Designing of the experiments (NKG, SSM); Contribution of experimental materials (ZK, BS); Execution of field/ lab experiments and data collection (NKG, SSM, NM); Analysis of data and interpretation (DPW, BHG); Preparation of manuscript (NKG, SSM, ZK, DPW).

Declaration

The authors declare no conflict of interest.

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