DOI : 10.5958/0974-0112.2019.00069.0

Indian J. Hort. 76(3), September 2019: 430-437

In Vitro screening technique and polymorphic DNA markers for introgression of root knot nematode resistance in tropical carrot

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ABSTRACT

The present study was aimed to develop an *In Vitro* procedure for screening carrot genotypes against root knot nematode (RKN), *Meloidogyne* spp and identify linked DNA markers.The RKN reaction of carrot genotypes was also confirmed in pot experiment and additionally, *In vitro* technique for screening carrot genotypes against RKN invasion by placing near 20 larvae J2s of *M. incognita* per root tip on pluronic gel media was effective in discriminating RKN susceptible and resistant genotypes. The technique revealed a wide variation in number of larvae that entered into roots, which was highest in black carrot Pusa Asita (12.5 larvae/root) and lowest in '6526B Sun2000' (1.0 J2s/root). The *Mj-1* resistance locus carrying European carrot lines ("6526 B Sun2000" and "8542B Vilmorin") have potential to introgress RKN resistance in susceptible varieties of carrot. For this, STS-SQ1 marker and *In vitro* screening will be helpful for handling segregating materials during RKN resistance breeding.

Key words: *Daucus carota*, molecular markers, pluronic gel, eel worm, asiatic carrot.

INTRODUCTION

Carrot (*Daucus carota* L.; Apiaceae) is a rich source of carotenoids and most popular root vegetable for its sweet taste and rich nutrient profile. In India, both temperate (European) and tropical (Asiatic) types of carrots are grown, but tropical type is more common because of its suitability for juice, porridge (*Halwa*), culinary items, particularly during the winter months (October to February). Carrot is a very remunerative crop and is being grown on 86, 000 ha with production of 13, 79, 000 MT (Anonymous, 2). In southern region of India, root knot nematode (RKN), *Meloidogyne* spp. is emerging as a major threat to carrot growers with yield losses of 45-50 per cent (Nisha *et al*., 13). Root knot nematode infection often causes substantial economic losses due to tap-root galling. Nematode damage at infection points on roots may induce secondary infections resulting in forked or ramified roots in carrot (Fig. 1). Sources of near-immunity resistance have been reported in carrot variety 'Brasilia' and inbred lines derived from this cultivar (Simon *et al*., 20). *M. incognita* and *M. javanica* are the two serious threats in the North Indian plains while *M. hapla* is common in hills. The RKN damage causes severe losses to marketable quality (Gugino *et al*., 9). *Meloidogyne. hapla* alone causes yield losses upto 19.1% and market losses upto 59.1% in Nilgiris and Kodaikanal hills of Tamil Nadu (Seenivasan, 16). Chemical nematicide based control measures are reported to be effective in reducing the nematode damage (Gugino *et al*., 9), but not sustainable for both economy and environment. However, resistant varieties are best option for managing RKN (Rhoades, 15) but most of the cultivar are susceptible in nature, hence, development resistant varieties is essential. Resistance to *M. javanica* in 'Brasilia' derived line 'Br1252' is reported to be governed by a single dominant locus '*Mj-1*' (Boiteux *et al*., 3; Simon *et al*., 20) indicating potential for use in hybrid breeding or introgression in elite varieties. However, breeding for RKN resistance in carrot is difficult primarily due to cumbersome procedure of phenotyping the reaction of breeding materials. It is a destructive and time consuming (50-60 days) practice. Besides, maintenance of desirable nematode population in test soil media in screening plots/pots is another challenge. Hence, two alternative options are available in other crops such as, *In vitro* pluronic gel screening technique and use of linked molecular markers. The first one has already shown promising results in crops like rice and tomato (Dutta *et al*., 7), brinjal (Shivakumara *et al*., 18) and wheat (Chen *et al*., 6). The Pluronic F-127 is important ingredient which is a stable, non-toxic to nematode or plant roots, copolymer being an excellent medium to study nematode behaviour (Ko and Van Gundy, 11; Shivakumara *et al*., 19). It forms a gel at room temperature at 20-30% concentration and facilitates

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Fig. 1. Carrot 'Pusa Vrishti' roots infested with root knot nematode.

second-stage juvenile larvaes of RKN to move freely and interact with target roots. However, no attempt has so far been made on standardizing the technique for phenotyping of carrot roots for nematode reaction to use in breeding programme.

Simon *et al*. (20) confirmed monogenic dominant locus *Mj-1* in 'Br1252' a line derived from variety 'Brasilia' which confers resistance against *M. javanica*, while Parsons *et al.* (14) identified five non-overlapping QTLs for resistance against *M. incognita* in carrot, one each on chromosomes 1, 2, 4, 8, and 9. Co-dominant sequence tagged sites (STS) flanking markers linked to RKN resistance locus have also been developed by Boiteux *et al*. (3). Ali *et al*. (1) mapped a single gene *Mj-2* with incomplete dominance in an Asiatic genotype PI 652188 which imparts resistance against *M. javanica*. Using molecular markers, they could reveal that the *Mj-1* and *Mj-2* are two different locations on chromosome 8. Parsons *et al*. (14) reported five non-overlapping QTLs for *M. incognita* resistance using three diverse resistance sources HM (from Syria), SFF (from Europe) and Br1091 (South America) one each on carrot chromosomes 1, 2, 4, 8, and 9. One QTL was present in all three populations, in the same region of *Mj-1*. Keeping in view the complexities of trait handling, the present study was designed to standardize the *In vitro* pluronic gel screening technique and to validate the available molecular markers for RKN resistance in carrot.

MATERIALS AND METHODS

Five tropical carrot varieties, namely Pusa Rudhira, Pusa Kesar, Pusa Vrishti (red), Pusa Meghali (orange) and Pusa Asita (black) and three temperate orange carrot exotic lines '6526B Sun2000', '8524' and '8542B Vilmorin' were used for the study. Genomic DNA was extracted from young leaf tissue of eight varieties/genotypes following the C-TAB procedure (Murray and Thompson, 12). DNA quality and quantity were assessed on a 1% (w/v) agarose gel stained with ethidium bromide (Sigma Aldrich Chemical Pvt. Ltd, Bangalore, India) and also by using a NanoDrop® ND-1000 spectrophotometer.

Sequences of various primers used in the study for detection of polymorphism for validation of markers for RKN resistance in carrot are given in Table 1. Polymerase chain reaction (PCR) analysis: All the markers were amplified by PCR (Thermocycler/2014Eppendorf Mastercycler ProS) in 15µl volume with 50ng genomic DNA, 1.0 U *Taq* DNA polymerase (Hi media Laboratories, Mumbai, India), 1.0 µM of each primer, 0.6 µl of 10 mM dNTP mix (Hi media Laboratories, Mumbai, India), and 1.5 µl of 10X PCR buffer having 17.5 mM $MgCl_2$ (Hi media Laboratories, Mumbai, India). Amplification conditions used for various genes were: one cycle of 94°C for 4 min; 35 cycles of 94 °C for 0.5 min, 55-65 °C (depending upon the Tm of each primer) for 1 min, and 72 °C for 1 min; and a final cycle of 72°C for 1 min. Amplified products were resolved on 3.0% agarose gel with TAE, stained with ethidium bromide, at a constant voltage of 60 V for 3 h using a horizontal gel electrophoresis system (BioRad, USA) and visualized and photographed under UV light in a gel documentation unit (AlphaImager EC/2011).

A pure culture of *M. incognita* was multiplied on eggplant (Pusa Purple Long) in a glasshouse. Egg masses were extracted from the roots of two month old plants and were hatched in a petri plate containing autoclaved spring water via modified Baermann assembly as per procedure described by Shivakumara *et al*. (19). Freshly hatched J2s were used for screening experiments.

In pot experiment screening, the small size plastic plug-trays (250g soil capacity) were filled with sterilized soil soil and the soilrite with the ratio of 3:1. The plug-trays were placed on field surface covered with black plastic mulch. The experiment was done during August to November months when mean temperature ranges from 33.6 to 28.2 °C. This period coincides with main growing period of tropical carrot in India and we could observe severe infection of RKN in carrot in research farm (Fig. 1). During November month, the pot area was covered with 200 micron UV stabilized plastic sheet in night hours to avoid low temperature effect. Five to ten seeds were sown per plug in alternate rows of plug-tray and after germination only one plant was retained per plug. The genotypes screened against *M. incognita* were commercial tropical varieties, namely Pusa Meghali, Pusa Kesar, Pusa Rudhira, Pusa

Table 1. DNA markers reported to be linked with root knot resistance (on chromosome 8) in carrot and used in present study.

Marker Name	Primer sequence (5' - 3')	Annealing temperature (°C)	Amplicon size (bp)	Source reference	Remark
SQ1	F- GGA cgA TGG CCA GGG AAA GC R- AACCAAGTCACGCCAACAGTAATT	59°C	700, 850	Boiteux *et al*. (2004)	Polymorphic
SQ6	F- GAGcgCCTTGATTGATGCTGTGTTGCC R-GAGcgCCTTGGCAGCATCGATTAGTAG	59°C	500	Boiteux *et al*. (2004)	Polymorphic
SCAR 21	F-TCCATACCGGCTTACAGTCC R-TTGGTCTAGCATGCTCCAAG	46°C	430, 530, 630	Ali *et al*. (2014)	Polymorphic
BSSR 132	F- GAAAGGAGGTGGCTGAAGAGTC R- AGCACAATACTAATAGGGAAATGCAA	55°C	220, 245	Cavagnaro *et al*. (2011)	Polymorphic
GSSR 44	AACTTCACCCCAGCTCACC CAAAGCAAGTAAAGAGACAGCG	54°C	209, 290, 320	Ali *et al*. (2014)	Monomorphic

Vrishti, Pusa Asita and the exotic donor genotypes '6526B Sun2000', '8542B Vilmorin', and '8524'. Five hundred second stage juveniles (J2) of *M. incognita* were placed in each plug 30 days after sowing. Standard crop practices were followed and plants were harvested at 60 days after inoculation (DAI) for observation. No chemical fertilizers were given to the plants. Sterilized water was used for irrigating the experimental pots. The nematode parasitic success on these carrot genotypes was observed in terms of number of galls and number of J3 and J4 larvae in roots as described by Shivakumara *et al*. (19). As per standard procedure, the gall index '1-5' scale was used for RKN reaction wherein, 1(highly resistant) = no gall or reproduction; 2 (resistant) = trace of galling/reproduction; 3 (light galling/ reproduction) = moderately resistant; 4 (moderate galling reproduction) = susceptible and 5 (highly susceptible) = abundant galling/reproduction as described by Kalloo (10).

In vitro pluronic gel screening for nematode reaction was carried out in the laboratory of Division of Nematology, ICAR- IARI, New Delhi. Carrot seeds (20-25 seeds/plate) were germinated in petriplates on double layered germination paper by incubating in seed germinators at 24 °C for 5 days. The procedure for preparation of pluronic gel and screening of genotypes was followed as described by Shivakumara *et al*. (18). Sterilized Petri plate (50 mm) were prepared for use in experiment by pouring 6 ml of 23% Pluronic F-127 (Sigma-Aldrich; remains in liquid state at or below 15° C) and allowed to solidify at room temperature. Ten thread-like healthy main roots of five day old seedlings of carrot genotypes were equidistantly placed in each petri dish. The trial was done in triplicate. The procedure for preparation of pluronic gel solution and screening of genotypes was followed as described by Shivakumara *et al*. (18, 19). The Petri plates with seedlings were incubated in a growth chamber at 28 °C for 2 hours to form root exudates concentration gradient. Twenty freshly hatched *M. incognita* J2s were placed 1cm posterior to the root tip and the plates were incubated in a growth chamber for 24hr with 16hr light and 8hr dark cycle photoperiod at 28 °C. Thereafter, infected roots were washed with autoclaved distilled water and stained with acid fuchsin. Stained roots were observed under a stereoscopic binocular (ZEISS Stereo Discovery.V20) in order to count the nematodes penetration ability on each carrot genotypes and also photographs were taken using Canon Power Shot G5 imaging system.

Data were analyzed using Microsoft Excel software for mean and range. The Pearson's correlation was calculated between results of *in vitro* Pluronic gel screening and pot experiment was analyzed using OPSTAT (http://hau.ernet.in).

RESULTS AND DISCUSSION

Resistance to *M. javanica* in carrot inbred line 'Brasilia-1252' has been reported to be governed by one or two duplicated linked dominant factors in the *Mj-1* locus (Simon *et al*., 20). For this, Boiteux *et al*. (3) reported RAPD markers (OP-$C21_{700}$, OP-$Q6_{500}$, OP-$U12_{700}$, and OP-$AL15_{500}$) and sequence-tagged site (STS) markers (SQ1 and SQ6) linked to *Mj-1* locus. Alleles $SQ1_{800R}$ and $SQ6_{500R}$ were identified in resistant parent whereas $SQ1_{700S}$ and $SQ6_{590S}$ in susceptible parent. Further, Ali *et al*. (1) reported incomplete dominance gene (*Mj-2*) for resistance to *M. javanica* in Asian carrot 'PI 652188' and suggested role of modifier genes. They mapped '*Mj-2*' locus

on chromosome 8 using SSRs (gSSR72, gSSR98, gSSR44, gSSR130, gSSR72 and gSSR107), sequence characterized amplified region (SCAR) marker (scar21A) and RAPD marker (Q1-850). A SSR marker BSSR132 linked to the *Mj-2* locus (Ali *et al*., 1) was also used in study. These earlier reported linked DNA markers were used in the present study (Table 1) and observed polymorphism among three resistant lines of European orange type and five varieties of tropical carrot. The results of these markers are presented in Fig. 2a-d. The gSSR44 produced three different bands of 209 bp, 290 bp and 320 bp but it could not distinguish genotypes and BSSR 132 was found monomorphic in all genotypes, hence these two were not taken for further analysis.

Comparison of phenotypic and genotypic status of genotypes with the markers from already mapped genes *Mj-1* and *Mj-2* revealed that all the genotypes amplified 500bp bands with SQ6 primer pair (Table 2) while Boiteux *et al*. (3) reported its presence only in resistant plants of F_2 population developed using 'Brasilia-1252'. The SQ1 primer pair amplified two alleles of sizes 700bp and 850 bp in the present study and they were polymorphic between susceptible and resistant parents. However, it was not polymorphic among all the genotypes because out of eight genotypes tested, four of them namely Pusa Vrishti, Pusa Asita and Pusa Meghali (RKN susceptible) and one '8524' (RKN resistant) showed the presence of resistant allele (850 bp). Two genotypes were in heterozygous state and had both 700 and 850 bp amplicons, whereas only one genotype (6526 B Sun2000) observed to be resistant but amplified a susceptible allele. The SQ1 did not amplify in Pusa Kesar, a root knot nematode susceptible commercial cultivar. Three amplicons of sizes 430, 530 and 630 bp each were observed from PCR analysis of genomic DNA with SCAR 21A marker. None of the genotype including all three resistant sources showed an allele of 430bp size in homozygous state which was linked to resistance locus as reported by Ali *et al*. (1). Pusa Kesar amplified this allele in heterozygous form along with an allele of 530 bp while Pusa Rudhira generated all three alleles (430bp, 530 bp and 630 bp). Two alleles of 220 and 245 bp sizes were observed with SSR marker BSSR 132 which have been shown to be linked to *Mj-2* by Ali *et al*., 1). Size of resistant or susceptible allele with marker BSSR132 has not been reported in any of the earlier study. In present investigation both 220 bp and 245 bp amplicons were observed in only two genotypes Pusa Virshti and Pusa Asita while Pusa Rudhira and Pusa Kesar had amplicon of 245 bp and Pusa Meghali generated 220 bp amplicon only (Table 2). This study revealed that (i) none of the already developed markers linked to *Mj-1* and *Mj-2* loci could correctly predict the phenotypic reaction (susceptible/resistant) of any of the genotype to root knot nematode (*M. javanica*) in tropical carrot materials, suggesting thereby that there is a need to develop new markers as also suggested

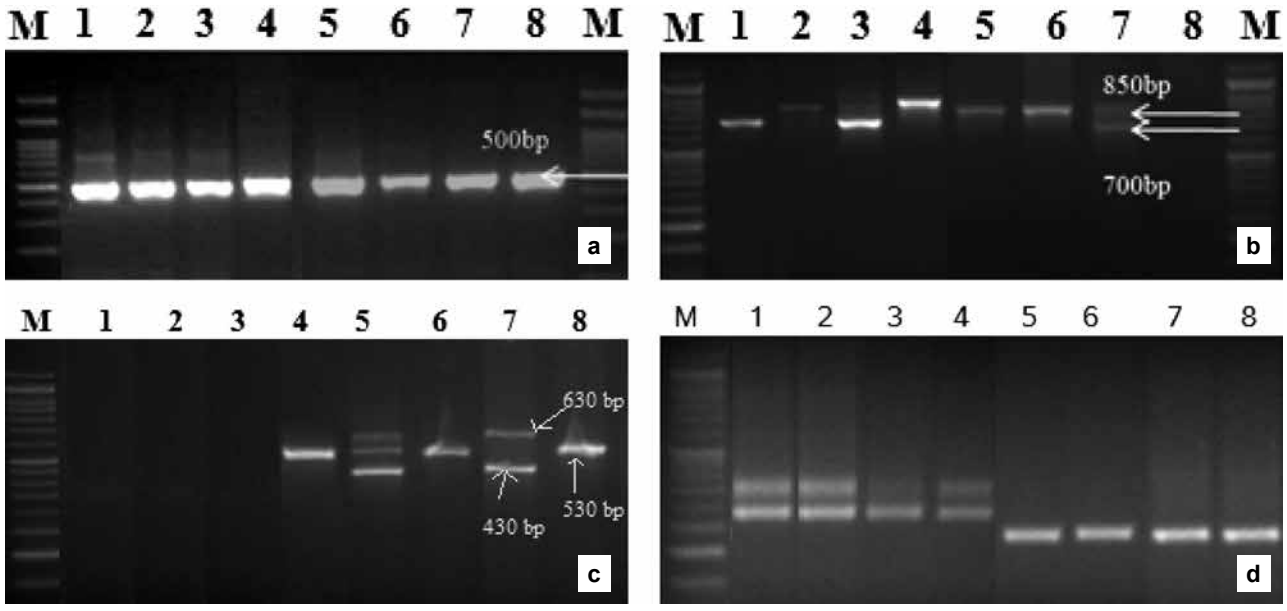

Fig. 2(a-d). Amplification of carrot resistant and susceptible lines with linked markers SQ6 (a), SQ1 (b), SCAR 21A (c) and gSSR 44 (d).

Lane M: 50 bp ladder, Lanes: 1-8: 6526 B Sun2000, 8542B Vilmorin, 8524, Pusa Asita, Pusa Vrishti, Pusa Meghali, Pusa Rudhira, Pusa Kesar

Table 2. Banding pattern observed in different carrot genotypes with markers linked to *Mj-1* and *Mj-2* loci.

Genotypes	Description	Reaction to *M. incognita**	SQ1		SQ6	SCAR21			BSSR 132	
			700 (S)	850 (R)	500 (R)	430 (R)	530 (S)	630	220	245
Pusa Vrishti	Tropical red	Highly susceptible	-	+	+	-	+	-	+	+
Pusa Rudhira	Tropical red	Highly susceptible	+	+	+	+	+	+	-	+
Pusa Asita	Tropical black	Susceptible	_	+	+	_	+	-	+	+
Pusa Meghali	Tropical orange	Susceptible	-	+	+	-	+	-	+	-
Pusa Kesar	Tropical red	susceptible	-	-	+	+	+	-	-	+
6526 B Sun2000	European orange	Resistant	+	-	+	-	-	-	-	-
8542B Vilmorin	European orange	Resistant	-	+	+	_	-	-	-	-
8524	European orange	Resistant	+	+	+	_	-	-	-	-

*Based on observations from pot experiment and *in vitro* pluronic gel screening technique; + allele present; - allele absent

by cavagnaro *et al.*, 5 to discriminate resistant and susceptible lines, (ii) Markers already used in earlier studies for mapping of the *Mj-1* and *Mj-2* are not conferring same polymorphism in nematode resistant and susceptible lines used by us, (iii) Some of the markers amplified new alleles in the present study indicating evolutionary differences between the genotypes used in earlier and present studies, and (iv) Additionally, there is also a possibility that the resistant sources used in the present study are harbouring a different type of resistance towards the nematode strain used in this study.

The carrot genotypes reaction to *M. incognita* was observed in terms of ability of the nematode to develop galls on roots of host plant in pot experiment. Gall index was taken as criteria because it is also effective to discriminate between resistant and susceptible genotypes and based on same disease scale as egg-mass index (Kalloo, 10). In potted experiment, abundance of galls was observed in fibrous roots of Pusa Vrishti and Pusa Rudhira followed by Pusa Asita, Pusa Meghali and Pusa Kesar showed moderate galling while '6526B Sun 2000' '8524' and '8542B Vilmorin' had trace or no galls on the roots (Table 3). Gall index is still in use and appears to be appropriate as Ali *et al*. (1) also scored segregating materials of carrot for galls in the range of 0 (no galls) to 8 (severely galled), using a modified version of the root-knot rating chart of Bridge and Page (4). Low variation within carrot lines (coefficient of variance = 0.68) for gall index of *M. incognita* was also reported by Seo *et al*. (17) in Korean carrot materials. According to gall index described by Kalloo (10), all the five commercial varieties of tropical carrot were found to be susceptible to *M. incognita* while exotic resistance sources '6526B Sun 2000' and '8524' proved to be resistant. This might be due to commonality in pedigree of Pusa Vrishti and Pusa Rudhira which have been derived from tropical red type carrot genetic material. Besides, Pusa Keshar (red) and Pusa Meghali (orange) are varieties derived from Local Red × Nantes and Pusa Keshar × Nantes, respectively (Gill, 8). Hence, the common susceptible reaction to RKN is due to genetic constitution of these varieties. The '6526B Sun 2000', '8524' '8542B Vilmorin' genotypes are non-Asiatic temperate type

Table 3. Response of carrot varieties/lines to *M. incognita* in pot experiment.

Variety	Range of galls present on roots	Mean ± SD	Galling incidence	No. of larvae recovered from roots				Host reaction
				J2 stage	J3 stage	J4 stage	Total	
Pusa Vrishti	55.0-69.0	60.6±7.3	Abundant	4.0	4.0	5.0	13.0	Highly susceptible
Pusa Rudhira	38.0-56.0	46.3±9.1	Abundant	3.0	3.0	2.0	8.0	Highly susceptible
Pusa Asita	37.0-44.0	40.3±3.5	Abundant	4.0	3.0	2.0	9.0	Highly susceptible
Pusa Meghali	32.0-42.0	37.0±5.0	Moderate	10.0	4.0	0.0	14.0	Susceptible
Pusa Kesar	22.0-28.0	25.0 ±3.0	Moderate	2.0	3.0	3.0	8.0	Highly susceptible
6526B Sun 2000	0.0-1.0	0.3±0.6	Trace	1.0	0.0	0.0	1.0	Resistant
8524	1.0-2.0	1.3±0.5	Trace	1.0	0.0	0.0	1.0	Resistant
8542B Vilmorin	1.0-1.5	1.1±0.2	Trace	0.0	1.0	0.0	1.0	Resistant

orange carrots which can be used as donors in carrot nematode resistance breeding programme. Further, we could count the number of the total number of larvae after 60 days of inoculation which were recorded to be highest in Pusa Meghali (14.0/g) followed by Pusa Vrishti (13/g) and Pusa Asita (Pusa Asita (9.0/g) (Table 3). Resistance sources (6526B Sun 2000, 8524 and 8542B Vilmorin) had negligible (1.0-2.0/g) larvae indicating their suitability for use as donors for root knot nematode resistance in tropical carrot varieties. During investigation, J3 and J4 stage larvae were also recovered from roots of Pusa Vrishti (4.0, 5.0), Pusa Rudhira (3.0, 2.0) and Pusa Asita (3.0, 2.0), however these could not be found in resistant lines. The inoculated larvae caused galls and presence of next stage (J3 and J4) larvae indicating suitability of host varieties for reproductive cycle of nematodes.

In pluronic gel technique, the entered nematode J2 larvae were visualized using staining method (Fig. 3a-h). Out of 20 J2 nematode larvae, 7.5 to 12.5 entered into roots of tropical type carrot varieties. The highest percentage of larvae entry was observed in Pusa Asita (62.5%) and Pusa Vrishti (47.5%), whereas it was least in '8542B Vilmorin (0.0) (Fig. 4). No larval observed in roots of carrot genotypes '6526B Sun 2000', '8542B Vilmorin' and '8524' which might be due to its suppressive effect of roots on larvae for penetration or unfavourable phtyochemical matrix inside roots. Resistance genotypes affect development of juvenile and adult stages, egg production and root-galling symptoms (Simon *et al*., 20; Boiteux *et al*., 3).

To facilitate fast and easy phenotyping of breeding materials for root knot nematode reaction in carrot, Pluronic gel *In vitro* technique was found as suitable option. The reaction of genotypes found to be in conformity from two experiments conducted through pot and *In vitro* pluronic gel technique. The DNA markers identified earlier for the root knot nematode resistance could not be validated hence; new linked markers need to be developed to be used in marker assisted selection for introgression of root knot nematode resistance in tropical carrots.

ACKNOWLEDGEMENTS

Authors acknowledge ICAR for financial assistance through CRP Molecular Breeding (Carrot) project and Head, Division of Vegetable Science, IARI for laboratory and field facilities. Authors also wishes to thank Dr. Phillip W. Simon, Research Geneticist & Professor of Horticulture, Department

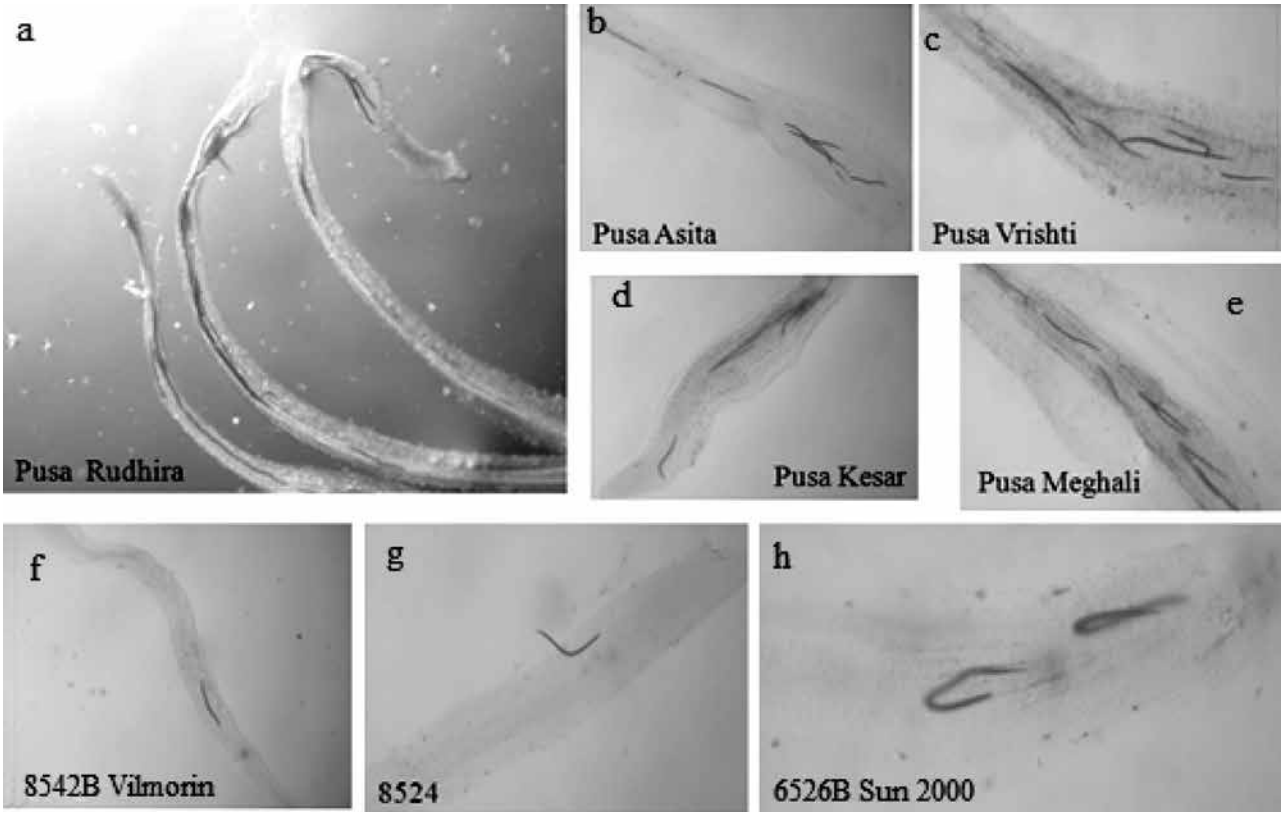

Fig. 3(a-h). Pluronic gel *In Vitro* screening for root knot nematode (*M. incognita*) in carrot varieties.

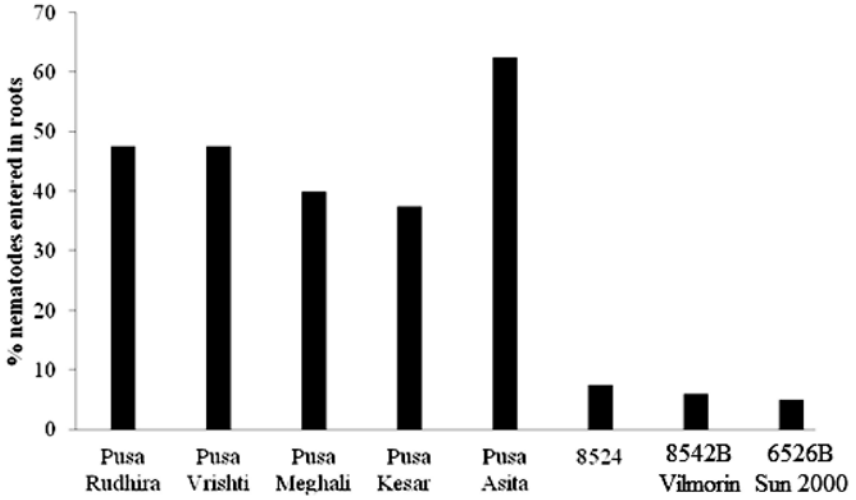

Fig. 4. RKN larvae entered into roots of carrot varieties during screening through *In vitro* Pluronic gel technique.

of Horticulture, 1575 Linden Drive, University of Wisconsin, Madison WI 53706, USA for his guidance for the present study.

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Received : June, 2019; Revised : August, 2019;
Accepted : August, 2019