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RESEARCH ARTICLE

Marker-assisted identification of *Meloidogyne incognita* and *Phytophthora infestans* resistant elite indeterminate tomato lines for protected cultivation

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Abstract

To mitigate the effect of root-knot nematode and late blight disease on tomato yield, multiple disease-resistant indeterminate lines were developed and evaluated for resistance to root-knot nematode, Phytophthora late blight and horticultural traits. Among the total thirty-three lines developed, eight lines (LBNR 6-5-1, LBNR 52-5-5, LBNR 77-1-1, LBNR 229-6-4, LBNR 300-1-9, LBNR 300-7-1LBNR 311-2-8 and LBNR 447-2-2) were found promising for both the pathogens, during screening at nursery stage. These eight lines were then evaluated for horticultural traits in root-knot nematode-infested polyhouse. The resistance of these selected lines was again confirmed conventionally as well as using molecular markers specific to *Mi* and *Ph* genes. Six lines *viz.*, LBNR 6-5-1, LBNR 52-5-5, LBNR 77-1-1, LBNR 229-6-4, LBNR 300-1-9 and LBNR 300-7-1 confirmed resistance against nematode and possessed *Mi 1.2* gene. The nematode-resistant lines also caused 24.78-43.43 per cent reduction in soil nematode population during crop season, over the initial soil nematode population as compared to susceptible lines. For late blight disease, three lines, LBNR 6-5-1, LBNR 52-5-5 and LBNR 229-6-4 exhibited high level of resistance and also possessed both *Ph2* and *Ph3* genes while two lines, LBNR 77-1-1 and LBNR 300-7-1 showed a resistant reaction. Three lines, LBNR 6-5-1, LBNR 229-6-4 and LBNR 52-5-5 were identified with multiple disease resistance along with superior horticultural traits. The resistant tomato lines with better horticultural traits identified in the current study could be further exploited for commercial purposes in nematode-infested soils or can be used as a parent in tomato improvement programme.

Keywords: Late blight, *Mi* gene, *Ph* gene, Root-knot nematode, *Solanum lycopersicum*.

Introduction

Tomato (*Solanum lycopersicum* L.) is the most important vegetable crop being grown worldwide next to potato (Costa et al. 2018). It is being cultivated both under open as well as under protected cultivation systems occupying an area of 5 million ha with a production of 181 million tons globally. India is the second-largest producer of tomato contributing 19 million tons next to China (62.8 million tons) (Anonymous 2021a). In Punjab state, tomato is cultivated over an area of 10.28 thousand hectares with a production of 266.91 thousand tons (Anonymous 2021b). Cultivation of indeterminate type tomato under polyhouse is gaining popularity in the state to harvest the benefit of off-season crop production, better quality, early maturity, year-round cultivation and higher productivity with minimal use of chemicals and water resources. But, controlled environmental conditions of the protected structures with high temperature and humidity also favoured the attack of pests and diseases (Sharma et al. 2009). The successful production of tomato crop under polyhouse is threatened by root-knot nematodes of *Meloidogyne* spp. and late blight disease of tomato caused by *Phytophthora infestans* under Punjab conditions.

Amid more than a hundred characterized species of *Meloidogyne*, *M. incognita*, *M. javanica* and *M. arenaria* are the most prevalent species. These nematodes are polyphagous in nature and infect all the crops being grown under protected cultivation (Sharma et al. 2007; Kaur et al.

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2010; Patil et al. 2017; Murungi et al. 2018). *M. incognita* alone accounts for 23 percent yield loss in tomato under open field conditions. Under protected cultivation, an annual yield loss up to 60% has been reported in major horticultural crops due to nematodes (Gowda et al. 2017). The nematode population build-up to the damaging limits over 3-4 crops under protected cultivation because of continuous host availability, favourable environment, high fecundity rate and shortening of life cycle of the nematode due to a higher temperature (Desaeger and Csinos 2006; Sharma et al. 2009). Due to their short life cycle (25-30 days), they can complete a greater number of generations in a short period of time under the optimum moisture and temperature of polyhouse compared to the open field conditions. These nematodes also predispose the plants to other soil-borne bacterial and fungal pathogens, forming disease complexes which are more difficult to manage.

Late blight is another major devastating disease of tomato causing significant yield losses worldwide (Fry and Goodwin 1997; Ohlson and Foolad 2016) The disease requires humid, cool and moist environment for rapid development and can cause crop loss upto 100% in tomato under open fields or greenhouses (Nowicki et al. 2012; Chowdappa et al. 2013). The diseased plants show brownish-black lesions on the leaves as well as on the stem. These lesions initially appear water soaked with chlorotic borders, expanding in size and becoming necrotic. The infected tomato fruits show dark brown firm lesions that enlarge in later stage and destroy the entire fruit. Development and growing resistant cultivars are one of the most economical and eco-friendly strategies to manage these diseases without incurring any extra cost to the farmers. Further, use of host resistance is the most important component of the integrated disease management programme.

In tomato, resistance to *Meloidogyne* spp. (*M. incognita, M. javanica* and *M. arenaria*) is governed by dominant *Mi* genes (*Mi-1* to *Mi-9*) which were transferred from the wild tomato relative, *Solanum peruvianum* L. to the cultivated tomato, *S. lycopersicum* (Cap et al. 1993; Williamson 1998; Ammiraju et al. 2003). Among these, *Mi-1* has been widely utilized for imparting resistance against these nematodes in cultivated tomato (Williamson 1998; Wang et al. 2001). For late blight resistance, five resistance genes (*Ph1*, *Ph2*, *Ph3*, *Ph4*, and *Ph5)* have been identified in wild tomato species *S. pimpinellifolium* and introgressed into the cultivated tomato. *Ph2* and *Ph3* genes are being used commercially to improve resistance in tomato cultivars as molecular markers linked to *Ph2* and *Ph3* genes are available (Nowic Ki et al. 2012; Zhang et al. 2014). To increase the productivity of tomato crop, it is very important to develop superior cultivars suitable for protected cultivation possessing resistance to prevailing pathogens with good horticultural traits. Keeping this in mind, the indeterminate tomato lines were developed by crossing late blight resistant and root-knot nematode resistant parents. The BC_1F_4 lines were then screened against root-knot nematode, *M. incognita* and *Phytopathora infestans* under artificial conditions and eight elite lines possessing resistance against root-knot nematode and late blight were selected and evaluated for horticultural performance as well as disease resistance under polyhouse conditions.

Materials and methods

The current research work was conducted at Vegetable Research Farm and Molecular Breeding Laboratory in the Department of Vegetable Science, Punjab Agricultural University Ludhiana, India. The indeterminate tomato lines possessing resistance to root-knot nematode (RKN) and late blight disease were developed through hybridization between late blight resistant (LBR-10) and root-knot nematode-resistant (NR-14) parents. The late blight resistant parent, LBR-10, possesses both *Ph2* and *Ph3* genes and shows a highly resistant reaction against the disease. Resistance to root-knot nematode in parent NR-14 is monogenic dominant. The F_1 plants generated from cro sses between late blight resistant (LBR-10) and root-knot nematode resistant (NR-14) parents were then crossed with local indeterminate tomato cultivar 'Punjab Sartaj'. The resulting three-way F_1 plants were then backcrossed with Punjab Sartaj to obtain the BC_1F_1 population. The BC_1F_2 - BC_1F_4 populations were then screened phenotypically and genotypically for root-knot nematode and late blight resistance. The marker assisted selection was performed on a population consisting of 210 individuals from which 33 individuals were selected and advanced to BC_1F_4. The homozygous plants for the target genes (*Mi* gene and *Ph2*/*Ph3* gene) were selected with a marker-assisted screening of the population. A schematic description of marker-assisted selection for the development of root-knot nematode and blight-resistant tomato lines is given in Fig. 1. The eight stable indeterminate tomato lines were selected and evaluated for yield parameters and resistance against root-knot nematodes and late blight under root-knot nematode infested polyhouse.

Screening of BC_1F_4 lines against root-knot nematode and late blight at nursery level

Total 33 BC_1F_4 lines, resistant parents (LNR-10, NR-14), and susceptible check Punjab Chhuhara were screened artificially against root-knot nematode, *M. incognita* and *Phytophathora infestans* artificially. For root-knot nematode screening, seeds of all the lines were sown directly in *M. incognita* infested sick plot (initial nematode population 300 J_2 per 250 g soil) maintained at Vegetable Farm, Department of Vegetable Science, PAU, Ludhiana. In two replications, about 100 seeds of each line and parents were sown on raised nursey beds (1 m wide and 15 cm high). The susceptible cultivar Punjab Chhuhara was repeated after every five lines

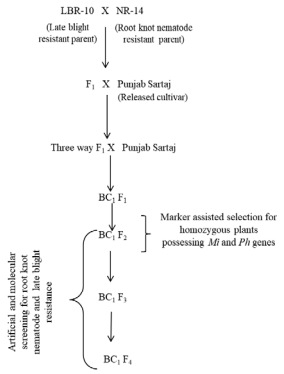

Fig. 1. Schematic presentation of developed resistant lines in tomato

so as to access the pathogenicity of the inoculum. After 60 days of sowing about 40 seedlings were uprooted randomly from each replication, gently washed under tap water and observations were recorded on root galling index (RGI) and egg mass index (EMI) as per 0-5 scale given by Taylor and Sasser 1978. Where, 0 = no galls/egg mass per root system; 1= 1-2 galls/egg mass per root system; 2=3-10 galls/egg mass per root system; 3 = 11-30 galls/egg mass per root system; 4=31-75 galls/egg mass per root system; 5 = more than 75 galls/egg mass per root system. Each line was then classified resistant or susceptible as follows; RGI 0.0 = Immune (I); 0.1-1.0 = highly resistant (HR); 1.1-2.0 = resistant (R); 2.1-3.0 = moderately susceptible (MS); 3.1- 4.0 = susceptible (S) and 4.1 and above = highly susceptible (HS).

The artificial screening for late blight disease was performed using the detached leaf method. The pathogen, *Phytophthora infestans* was maintained on late blight susceptible tomato leaflets, which were placed abaxial side up in the Petri dishes, containing a thin layer of water agar to maintain high relative humidity (RH). The infected leaflets were incubated at 16 ± 2°C and 100% RH on a 12-hour photoperiod for 7 to 10 days. The inoculum (sporangial suspension) was prepared by submerging these leaflets in distilled water for about 1 hour and the concentration was adjusted to approximately 2×10^4 spores/ml using a hemocytometer (Ohlson et al. 2018). For screening, young leaves of all the lines along with resistant check (LBR-10) and susceptible check varieties (Punjab Sartaj and Punjab Chuhhara) were taken from the nursery plants followed by thoroughly washing with tap-water and air-drying. The leaves were then placed upside down in opaque plastic trays lined with moistened paper towels. The leaves were placed with an adaxial surface facing upwards so as to facilitate the penetration of pathogen spores. The sporangial suspension of *Phytophthora infestans* (approximately 2x104 spores/mL) was evenly misted upon the abaxial surface of the leaves. Trays were then incubated in a growth chamber at 18 ± 2°C. These were covered with black polythene sheets for the first 24 hours of incubation and later were subjected to 12 hours of photoperiod (Foolad et al. 2015). The disease scoring was done after 7 days of inoculation using the 0-6 scale by Chen et al. (2008) and using these scores, percent disease index was calculated using the following formula:

$$\text{PDI (\%)} = \frac{\text{Sum of all individual disease ratings}}{\text{Total number of plants observed x Maximum Grade}}$$

Soil nematode population analysis of polyhouse

For evaluation of tomato lines, a polyhouse situated at Vegetable Research Farm, Department of Vegetable Science was selected in which vegetable crops are being grown for the past decade and is highly infested with root-knot nematode, *Meloidogyne incognita*. To work out the initial soil nematode population of the polyhouse ten random soil samples were drawn using a soil sampling auger (45 × 2.5 cm, Passey) up to the depth of 20 cm from each plot and mixed thoroughly to draw a working sample of 250g (Coyne et al. 2014). The soil samples were then brought to the laboratory and processed as per Cobb's sieving technique for nematode extraction and population assessment (Whitehead and Hemming 1965).

Evaluation of selected lines for horticultural traits and resistance under polyhouse

Based on nursery screening, eight lines showing resistance to both *M. incognita* and *Phytophthora infestans* were selected and were evaluated in root-knot nematode, *M. incognita* infested polyhouse (initial soil nematode population 266.33J_2/250cc soil) situated at Vegetable Research Farm, Department of Vegetable Science, PAU, Ludhiana for horticultural traits for two seasons. The selected tomato lines along with parents, NR-14 (RKN resistant parent), LBR-10 (Late blight-resistant parent) and check varieties Punjab Chhuhara and Punjab Sartaj were transplanted on the raised beds (2.5 x 3.0m) during the month of September in 2019-20 and 2020-21. Three replications of each line were maintained in a randomized block design and each replication accommodated six plants. After transplanting, necessary training and pruning of the plants were done. The standard package and practice for cultivating tomato crop under polyhouse as given by Punjab Agricultural University, Ludhiana was followed for proper crop maintenance (Anonymous, 2021b). The data was recorded on yield per plant, fruit traits *viz*., fruit colour, fresh fruit weight, and fruit shape, number of locules/fruit, pericarp thickness and TSS.

The selected lines were again screened for late blight resistance, for this, young leaves of all the eight lines along with resistant check (LBR-10) and susceptible check varieties (Punjab Sartaj & Punjab Chuhhara) were taken from the plants growing in the polyhouse followed by screening against late blight by detached leaf method as described earlier.

Table 1. Molecular markers used for the targeted resistance genes

S. no.	Marker name	Type of DNA marker	Targeted gene	Chr. no.	Restriction enzyme	Expected product size (S, R) ~bp	Forward primer sequence 5`–3`	Reverse primer sequence 5`–3`	Reference
1.	*Mi* 23	SCAR	*Mi* 1.2	6	-	420, 380	TGGAAAAATGTGAATTTCTTTTG	GCATACTATAGGCTTGTTTACCC	Seah et al. 2007
2.	dTG422	CAPS	*Ph2*	10	HinfI	245, 275	Dr. Martha Mutschler personal communication, College of Agriculture and Life Sciences, Cornell University, USA		
3.	TG328	CAPS	*Ph3*	9	ApoI	274, 243			

CAPS= Cleaved Amplified Polymorphic Sequence, SCAR= Sequence Characterized Amplified Region

At termination of trial, reaction against root-knot nematode was recorded by uprooting the individual plants gently and indexing them on (0-10) rating chart for easy and accurate field assessment of damage by *Meloidogyne* spp. (Bridge and Page 1980). Where, 0 = Healthy root system; 1 = Few galls upon close examination; 2 = Small galls detected easily; 3 = Numerous small galls; 4 = Numerous small galls and a few big ones; 5 = 25% of the root system severely galled; 6 = 50% of the root system severely galled; 7=75% of the root system severely galled; 8 = No healthy root but the plant is still green; 9 = Completely galled root system and plant dying; 10= Plants and roots dead. The lines were then categorised resistant or susceptible based on RGI as follows; RGI 0=immune (I); 0.1-2.0= resistant (R); 2.1-5.0= moderately resistant (MR); 5.1-6.0= moderately susceptible (MS); 6.1-8.0 = susceptible (S) and 8.1-10.0 highly susceptible (HS). Soil nematode population was analysed by extracting nematodes from soil using the modified Cobb's sieving and decanting technique (Whitehead and Hemming 1965). The number of nematodes extracted after 24 hrs were counted under a compound microscope for soil population assessment. The soil nematode population build-up in the root zone of each tomato line was estimated as reproduction factor (Rf = Pf/Pi) where Pf was the final nematode population recorded at end of the crop and Pi was the initial nematode population at the start of the experiment. Rf>1 indicated multiplication of RKN in the treatment.

Genotypic screening of the selected lines for the presence of Mi and Ph genes

The selected lines were further confirmed for disease resistance using molecular markers specific to *Mi* and *Ph* genes. The genomic DNA (deoxyribonucleic acid) was extracted from all the genotypes by taking young leaves during the active growing stage of the crop as per the protocol given by Doyle and Doyle 1987. The extracted DNA was quantified using a NanoDrop (Thermo Scientific NanoDropTM 1000 Spectrophotometer) and subjected to PCR assay using molecular markers for *Mi* gene imparting resistance to *Meloidogyne* spp. and *Ph2* and *Ph3* markers imparting resistance against Phytophthora late blight. The PCR reactions were performed by making 10 µL reaction using PCR components viz., DNA (200 ng/µL) 1.0 µL + water 4.3 µL (double distilled autoclaved) + 2X Premix PCR master mix (EmeraldAmp GT) 3.5 µL + Forward and reverse primers (20pico mole) 0.6 + 0.6 µL}. The DNA from tomato lines was analyzed using gene (*Mi*, *Ph2* and *Ph3*) specific markers (Table 1). The amplified PCR products of the CAPS (Cleaved Amplified Polymorphic Sequence) markers were digested using a restriction enzyme mixture (0.2 enzyme+ 0.3 buffer + 1.5 µL dH_2O) @ 2 µL per tube. The fragments obtained after restriction were separated in 2% agarose gel prepared using 1X TAE (Tris-acetate-EDTA) buffer and stained with ethidium bromide (5.0 µL/100 mL). A DNA ladder was used for analysing the bands in the gel. The gel photographs were captured with the help of ultra violet (UV) light through Alphamager® HP system.

Statistical analysis

The data recorded during screening against root knot neatode and late blight at nursey stage were analysed by comparing means against check by Dunnet test ($p < 0.05$) using SPSS software. The data recorded on horticultural traits and disease parameters in polyhouse trial were subjected analysis of variance for randomised block desing (RBD).

Results

Screening of BC_1F_4 lines for resistance to root-knot nematode and late blight

Among the 33 BC_1F_4 lines, screened in nursery trial for resistance to root-knot nematode, *M. incognita* two lines viz., LBNR-52-5-5 (RGI 1.0; EMI=0.03) and LBNR-300-1-9 (RGI=0.86; EMI =0.05) showed highly resistant reaction while four lines, LBNR-77-1-1, LBNR-299-6-4, LBNR- 6-5-1, LBNR-300-7-1 exhibited resistant reaction with EMI ranging from 1.0 to 1.86 and RGI ranging from 1.50 to 2.0. Five lines, LBNR-52-4-3, LBNR-97-1-3, LBNR-72-163-1, LBNR-311-2-8 and LBNR-447-2-2 exhibited moderately susceptible reaction (EMI = 2.15 to 2.45; RGI= 2.11 to 2.86), while remaining lines, susceptible checks (Punjab Chuhhara & Punjab Sartaj) and late blight resistant parent (LBR-10) showed susceptible reaction against root-knot nematode. But, the root-knot nematode resistant parent, NR-14 showed highly resistant reaction to root-knot nematode (Table 2).

For late blight disease, three lines viz., LBNR-6-5-1, LBNR-299-6-4 and LBNR-52-5-5 showed highly resistant reaction with PDI ranging from 3.33-6.67, while nine lines, LBNR-8-5-2, LBRR-8-6-3, LBNR-6-2-1, LBNR-6-3-1, LBNR-6-4-2, LBNR-18-3-1, LBNR-77-1-1, LBNR-97-1-3, LBNR-300-7-1 showed resistant

Table 2. Screening of BC_1F_4 lines for resistance to root-knot nematode and late blight at nursery stage

S. No.	Tomato Lines	Root-knot nematode				Late blight
		Egg mass index (EMI) (0-5) scale	Root galling Index (RGI) 0-5 scale	Disease Reaction	PDI	Disease Reaction
1	LBNR-8-2-1	3.50^{ns} ± 0.17	4.00^{ns} ± 0.13	S	60.00^{ns}	HS
2	LBNR-8-5-2	3.00* ± 0.20	3.33^{ns} ± 0.07	S	12.80*	R
3	LBRR-8-6-3	3.20^{ns} ± 0.40	3.60^{ns} ± 0.17	S	15.50*	R
4	LBNR-8-4-1	3.00* ± 0.40	3.26^{ns} ± 0.12	S	26.67*	MR
5	LBNR-8-1-1	3.33^{ns} ± 0.16	3.33^{ns} ± 0.23	S	30.00*	MR
6	LBNR-8-3-3	3.50^{ns} ± 0.30	4.00^{ns} ± 0.08	S	33.33*	MR
7	LBNR-6-2-1	2.80* ± 0.29	3.56^{ns} ± 0.26	S	16.67*	R
8	LBNR-6-3-1	3.00* ± 0.20	3.36^{ns} ± 0.11	S	10.00*	R
9	LBNR-6-4-2	3.00* ± 0.20	3.26^{ns} ± 0.32	S	16.60*	R
10	LBNR-6-5-1	1.00* ± 0.05	1.86* ± 0.53	R	3.33*	HR
11	LBNR-6-6-4	3.56^{ns} ± 0.08	3.33* ± 0.33	S	33.33*	MR
12	LBNR-6-2-1	4.20^{ns} ± 0.21	4.00^{ns} ± 0.22	S	46.67*	S
13	LBNR-18-3-1	4.00^{ns} ± 0.38	4.00^{ns} ± 0.35	S	10.00*	R
14	LBNR-52-3-1	3.33^{ns} ± 0.03	3.66^{ns} ± 0.23	S	48.00*	S
15	LBNR-52-1-2	3.00* ± 0.08	3.55^{ns} ± 0.16	S	30.00*	MR
16	LBNR-52-2-3	3.00* ± 0.32	3.20^{ns} ± 0.07	S	46.67*	S
17	LBNR-52-3-9	2.88* ± 0.18	3.11^{ns} ± 0.03	S	53.33^{ns}	S
18	LBNR-52-4-3	2.30 ± 0.09	2.60* ± 0.24	MS	42.80*	S
19	LBNR-52-5-5	0.03* ± 0.00	1.00* ± 0.10	HR	6.67*	HR
20	LBNR-52-6-5	4.00^{ns} ± 0.17	3.66^{ns} ± 0.31	S	25.00*	MR
21	LBNR-64-3-1	3.33^{ns} ± 0.33	3.33^{ns} ± 0.20	S	26.67*	MR
22	LBNR-77-1-1	1.33* ± 0.18	1.50* ± 0.34	R	13.33*	R
23	LBNR-79-3-7	4.00^{ns} ± 0.27	3.86^{ns} ± 0.33	S	56.67^{ns}	S
24	LBNR-93-1-1	4.00^{ns} ± 0.23	4.00^{ns} ± 0.38	S	50.00^{ns}	S
25	LBNR-97-1-3	2.35* ± 0.26	2.33* ± 0.23	MS	16.67*	R
26	LBNR-72-163-1	2.15* ± 0.18	2.11* ± 0.08	MS	66.67^{ns}	HS
27	LBNR-299-6-4	1.00* ± 0.13	1.36* ± 0.24	R	3.33*	HR
28	LBNR-71-300-3	3.86^{ns} ± 0.12	3.60^{ns} ± 0.28	S	26.67*	MR
29	LBNR-300-1-9	0.05* ± 0.00	0.86* ± 0.10	HR	31.23*	MR
30	LBNR-300-4-1	3.66^{ns} ± 0.20	4.00^{ns} ± 0.11	S	33.33*	MR
31	LBNR-300-7-1	1.56* ± 0.12	2.00* ± 0.40	R	14.66*	R
32	LBNR-311-2-8	2.33* ± 0.36	2.60* ± 0.12	MS	25.00*	MR
33	LBNR-447-2-2	2.45* ± 0.19	2.86* ± 0.13	MS	26.67*	MR
34	NR-14 (PKN resistant parent)	0.02* ± 0.00	0.05* ± 0.00	HR	50.00^{ns}	S
35	Punjab Sartaj	3.20^{ns} ± 0.36	3.51^{ns} ± 0.19	S	63.33^{ns}	HS
36	LBR-10 (Late blight resistant parent)	3.67^{ns} ± 0.12	3.85^{ns} ± 0.42	S	2.66*	HR
37	Punjab Chuhhara (Susceptible check)	4.00 ± 0.17	4.00 ± 0.14	S	66.67	HS

The data represents mean of two replications ± SD. Values having `*' are significantly different from control (Susceptible check, Punjab Chuhhara) and `ns' indicates non-significance according to Dunnett test (p=0.5%). R = Resistant; HR = Highly Resistant; MR = Moderately resistant; MS = Moderately susceptible; S = Susceptible; HS = Highly susceptible and PDI = Per cent disease index

reaction with PDI ranging from 10.0 to 16.67. Among others, ten lines (LBNR-8-4-1, LBNR-8-1-1, LBNR-8-3-3, LBNR-52-1-2, LBNR-52-6-5, LBNR-64-3-1, LBNR-6-6-4, LBNR-300-1-9, LBNR-311-2-8, LBNR-447-2-2) exhibited moderately resistant reaction (PDI=25.00 - 33.33), remaining all lines including NR-14 (nematode-resistant parent) exhibited susceptible reaction with PDI ranging from 42.80 – 56.67. Line LBNR-8-2-1, LBNR-72-163-1, Punjab Chuhhara and Punjab Sartaj showed highly susceptible reaction (PDI= 60.00 - 66.67). The late blight-resistant parent LBR-10 showed a highly resistant reaction with PDI 2.66 (Table 2).

Among these 33 lines, eight lines viz., LBNR-6-5-1, LBNR-52-5-5, LBNR-77-1-1, LBNR-299-6-4, LBNR-300-1-9, LBNR-300-7-1, LBNR-311-2-8 and LBNR-447-2-2 exhibited varying levels of resistance against both root-knot nematode and late blight disease. Thus, these eight lines with multiple disease resistance were selected for further study of yield parameters and other horticultural traits.

Evaluation of tomato lines for horticultural traits under polyhouse

All the selected tomato lines exhibited significant variations in the data recorded on the horticultural traits (Table 3). The fresh fruit weight varied significantly among the lines. The mean fruit weight of lines varied from 55.33g in LBNR-300-7-1 to 135.65 g in LBNR-300-1, with an overall mean of 83.26 g. The fruit weight is the most important trait as it contributes to total plant yield. Among the lines under study, lines LBNR 6-5-1, LBNR 300-1-9 and LBNR 447-2-2 performed better for fruit weight. The mean yield of the lines under study varied from 4.06 kg in LBNR 447-2-2 to 5.17 kg in LBNR 300-1-9, with a mean of 4.52 kg per plant. Higher total fruit yield is the basic objective of any crop breeding programme and is important for selection of a genotype. The maximum yield was recorded in line LBNR 300-1-9 (5.17 kg) followed by LBNR 229-6-4 (4.98 kg) and line LBNR 52-5-5 (4.58 kg). The lines LBNR 300-1-9 and LBNR 229-6-4 recorded significantly higher yield than check Punjab Sartaj.

Apart from yield and fruit weight, fruit firmness and pericarp thickness are the other important traits which contribute to shelf life of tomato fruits. The firmness of the fruit is inversely correlated with number of locules per fruit. Lower the number of locules per fruit, more firm is the fruit. The locules per fruit varied from 3.0 in LBNR 229-6-4 to 4.61in LBNR 6-5-1, with average of 3.44 locules per fruit. The line LBNR 6-5-1 exhibited a greater number of locules/ fruit followed by line LBNR 447-2-2 and LBNR 300-1-9. The overall mean pericarp thickness of the tomato lines was 6.80 mm with maximum pericarp thickness (7.73 mm) in line LBNR 229-6-4 and minimum in line LBNR 77-1-1. The lines LBNR 229-6-4, LBNR 52-5-5 and LBNR 300-1-9 recorded more pericarp thickness. The fruits with thicker pericarp have improved shelf-life and reduced post-harvest losses. Total soluble solids (TSS), i.e., relative sugars and acids concentration in the fruit determines the flavor of tomato. The TSS of the lines varied from 4.01°Brix (LBNR 77-1-1) to 5.05°Brix (LBNR 311-2-8) with overall mean value of 4.69°Brix. The lines, LBNR 311-2-8, LBNR 52-5-5 and LBNR

Table 3. Performance of multiple disease resistant tomato lines for horticultural traits in polyhouse

S. No.	Genotype	Fresh Fruit weight (g)	Fruit Yield (kg/plant)	Fruit colour	Number of locules/ fruit	Pericarp thickness (mm)	TSS (oBrix)	Polar diameter (cm)	Equatorial width (cm)	P/E ratio	Fruit shape
1	LBNR 6-5-1	135.65	4.43	Red	4.61	5.75	4.70	5.28	6.89	0.77	Flat
2	LBNR 52-5-5	62.11	4.58	Red	3.03	7.73	4.82	4.44	4.91	0.90	Round
3	LBNR 77-1-1	83.84	4.56	Red	3.40	5.33	4.01	3.86	3.56	1.08	Round
4	LBNR 229-6-4	57.94	4.98	Red	3.00	7.83	4.60	4.68	4.52	1.04	Round
5	LBNR 300-1-9	93.22	5.17	Red	3.50	7.18	4.80	4.89	5.25	0.93	Round
6	LBNR 300-7-1	55.33	4.13	Red	3.01	6.58	4.72	4.72	4.63	1.02	Round
7	LBNR 311-2-8	85.67	4.29	Red	3.20	6.46	5.05	6.40	4.89	1.31	Pear
8	LBNR 447-2-2	92.33	4.06	Red	3.80	7.52	4.82	4.84	5.34	0.91	Round
9	NR-14 (RKN resistant parent)	31.00	0.67	Red	3.40	2.35	3.44	3.70	2.50	1.48	Pear
10	LBR-10 (Late blight resistant parent	108.23	1.55	Red	3.00	5.68	4.48	4.70	5.32	0.88	Flat
11	Punjab Sartaj (Check)	86.63	4.50	Red	3.60	8.80	5.01	5.03	4.98	1.01	Round
12	Punjab Chhuhara (Check)	28.33	0.55	Red	3.00	2.35	3.40	3.70	2.50	1.48	Pear
Range		55.33-135.65	4.06-5.17 4.52		3.0-4.61 3.44	5.33-7.83 6.80	4.01-5.05	3.86-6.40 4.89	3.56-6.89 5.02	0.77-1.31	
Mean		83.26	0.42		0.42	0.22	4.69	0.18	0.33	0.99	
CD at 5%		3.81					0.28			0.14	

300-1-9 showed higher TSS. However, only line LBNR-311-2-8 recorded significantly higher TSS as compared to check Punjab Sartaj. The ratio of polar to equatorial diameter indicates the fruit shape index i.e. whether the fruit is round or oblong. The fruits of most lines were round in shape with a P/E ratio ranging from 0.90 to 1.08. The fruits of line LBNR 72-6-5 were flat in shape with P/E ratio 0.77, while fruits of line LBNR 72-311-2 were pear-shaped with P/E ratio 1.31. The P/E ratio varied from 0.77 to 1.31 with overall mean of 0.99.

Evaluation of lines for root-knot nematode and late blight disease under polyhouse

The artificial screening of the lines against Phytophthora late blight (Table 4) using the detached leaf method revealed that three lines viz., LBNR 6-5-1, LBNR 52-5-5 and LBNR 229-6-4 along with parent LBR-10 showed highly resistant reaction against late blight disease with PDI ranging from 2.33 to 5.66. Two lines (LBNR 77-1-1 and LBNR 300-7-1) exhibited resistant reactions with PDI 18.0 and 13.67, respectively. The lines, LBNR 300-1-9, LBNR 311-2-8 and LBNR 447-2-2 recorded moderately resistant reaction (PDI= 33.00 - 38.66) while parent NR-14 showed susceptible reaction to late blight (PDI=53.0). Both the check varieties, Punjab Sartaj and Punjab Chuhhara exhibited highly susceptible reaction (PDI 65.33 & 62.66) under artificial screening.

As per the observations recorded on root galling index (RGI) (Table 4) by uprooting the individual plant of each line at the termination the of trial, the two lines (LBNR 6-5-1 and LBNR 52-5-5) and nematode-resistant parent NR-14 exhibited immune reaction against root-knot nematode with no galls on the roots. The lines viz., LBNR 77-1-1, LBNR 229-6-4, LBNR 300-1-9 and LBNR 300-7-1 showed resistant reaction with RGI ranging from (0.11-1.44). In contrast, line LBNR 311-2-8 (RGI=5.14) and LBNR 447-2-2 (RGI=5.38) showed moderately susceptible reaction, parent LBR-10 (RGI=6.78) and Punjab Sartaj (RGI=6.47) recorded susceptible reaction. In contrast, variety Punjab Chuhhara recorded highly susceptible reaction (RGI=7.50) against root-knot nematode at end of the crop season. Fig. 2 shows the sympoms produced on root knt nematode resistant and susceptible

Table 4. Phenotypic and genotypic screening of multiple disease resistant tomato lines against root-knot nematode and late blight in polyhouse

S. No.	Genotype	Screening against root-knot nematode					Screening against late blight			
		Phenotypic				Genotypic	Phenotypic		Genotypic	
		RGI (0-10) scale	Soil nematode population/250g soil	Rf= Pf/Pi	Disease Reaction	*Mi* Gene	PDI	Disease Reaction	*Ph2* Gene	*Ph3* Gene
1	LBNR 6-5-1	0.00(1.00)*	150.67	0.56	I	+	3.67	HR	+	+
2	LBNR 52-5-5	0.00 (1.00)	186.67	0.70	I	+	3.56	HR	+	+
3	LBNR 77-1-1	0.11 (1.05)	167.33	0.63	R	+	18.00	R	-	+
4	LBNR 229-6-4	1.44 (1.56)	165.33	0.62	R	+	5.66	HR	+	+
5	LBNR 300-1-9	0.61 (1.26)	180.67	0.68	R	+	33.67	MR	+	-
6	LBNR 300-7-1	0.78 (1.33)	200.33	0.75	R	+	13.67	R	-	+
7	LBNR 311-2-8	5.14 (2.47)	333.33	1.25	MS	-	38.66	MR	-	+
8	LBNR 447-2-2	5.38 (2.52)	350.00	1.31	MS	-	33.00	MR	-	-
9	NR-14 (RKN Resistant Parent)	0.00 (1.00)	166.67	0.63	I	+	53.00	S	-	-
10	LBR-10 (Late blight resistant parent)	6.78 (2.78)	586.00	2.20	S	-	2.33	HR	+	+
11	Punjab Sartaj (Susceptible Check)	6.47 (2.73)	550.00	2.07	S	-	65.33	HS	-	-
12	Punjab Chhuhara (Susceptible Check)	7.50 (2.91)	600.00	2.25	S	-	62.66	HS	-	-
	CD at 5%	0.15	57.71							

*Square root transformed values; Initial nematode population (Pi) = 266.33 J_2/250 g soil ;Category index: RGI 0 = iImune(I), 0.1-2.0 = Resistant (R); 2.1-5.0 = Moderately resistant (MR); 5.1-6.0 = Moderately susceptible (MS); 6.1-8.0 = Susceptible (S) and 8.1-10.0 Highly susceptible (HS)., Rf = Reproduction factor, Pf = Final population, Pi = Initial population, I = Immune, HR = Highly resistant, MR = Moderately Resistant, MS = Moderately susceptible, S = Susceptible, HS = Highly susceptible and PDI = Per cent disease index

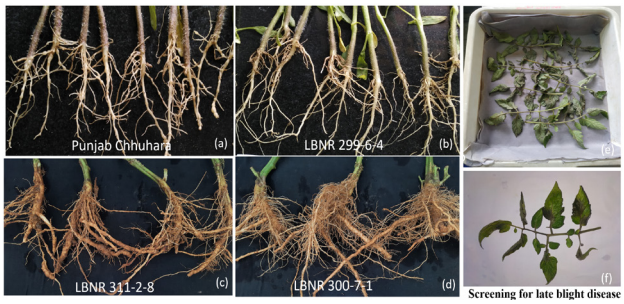

Fig. 2. Charactersitic symptoms of root knot nematode infestation and late blight disease produced on tomato plants. (a) Small bead like galls produced on roots of nursery plants of susceptible variety Punjab Chhuhara; (b) Resistant line (LBNR 299-6-4) with clear roots; (c) Roots of mature plants of susceptible line LBNR 311-2-8 with multiple galls; (d) Roots of resistant line LBNR 300-7-1 with clear roots; (e and f) = Typical small, water-soaked areas on leaves of susceptible lines during artificial screening for late blight disease

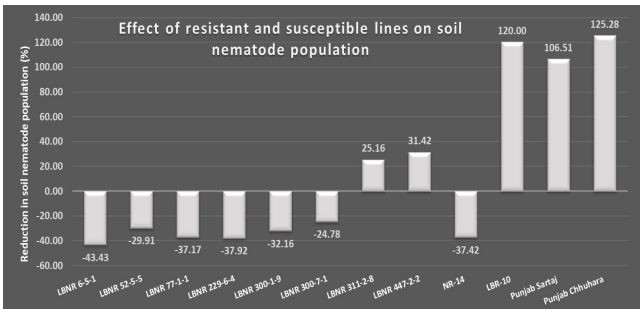

Fig. 3. Effect of resistant and susceptible tomato lines on soil nematode population over the initial nematode population in the polyhouse.

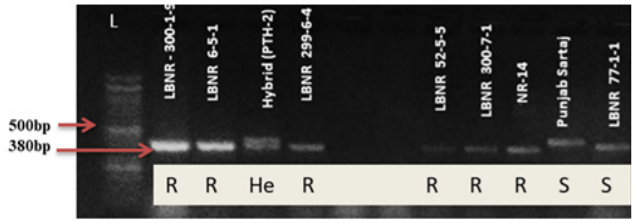

Fig. 4. Agarose gel (1.5%) showing resistant lines and resistant parent, NR-14 exhibiting 380bp band; F1 hybrid (PTH-2) exhibiting both 380 and 420bp band (not included in the present study); susceptible line and susceptible check Punjab Sartaj exhibiting 420bp band with SCAR marker Mi23. (R = Resistant; He = Heterozygous; S = Susceptible; L = DNA ladder 100bp)

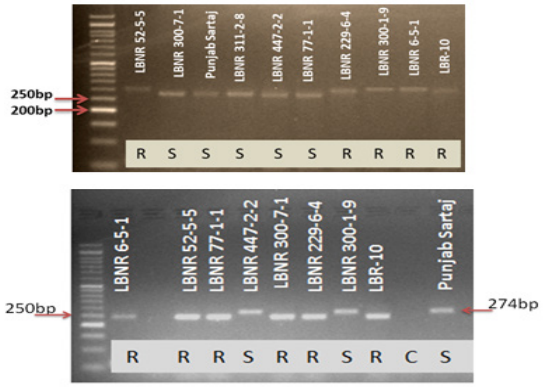

Fig. 5. (A) Agarose gel (1.5%) showing resistant lines along with resistant parent, LBR-10 exhibiting 275bp band; susceptible line and susceptible check, Punjab Sartaj exhibiting 245bp band with CAPS markerdTG422. (B) Agarose gel (1.5%) showing resistant lines along with resistant parent, LBR-10 exhibiting 245bp band; susceptible line and susceptible check, Punjab Sartaj exhibiting 274bp band with CAPS marker TG328. (R = Resistant; S = Susceptible; C=Control; L=- DNA ladder 50bp)

genotypes (at nursey level and at maturity of plants) and typical symptoms of late blight under artificial screening.

The soil nematode population analysis conducted at end of trial revealed that the immune (LBNR 6-5-1, LBNR 52-5-5 and parent NR-14) and resistant (LBNR 77-1-1, LBNR 229-6-4, LBNR 300-1-9 and LBNR 300-7-1) tomato lines caused 24.78 to 43.42 per cent reduction in soil nematode population over the initial soil nematode population (266.33 J_2/250g soil) with reproduction factor (Rf) < 1.0 (Rf ranging from 0.56-0.75) (Fig. 3; Table 4). Whereas, lines showing moderately susceptible (LBR 447-2-2 and LBNR 311-2-8) and check varieties viz., LBR-10, Punjab Sartaj and Punjab Chuhhara resulted in 106 to 125 per cent increase in soil nematode population over the initial soil population at the end of the cropping season with Rf >1 in these lines.

Genotypic screening of the selected lines for presence of Mi and Ph genes

All the lines along with resistant and susceptible parents were subjected to marker-assisted screening for presence of *Mi1.2* gene imparting resistance to *Meloidogyne* spp. and *Ph2* & *Ph3* gene imparting resistance against late blight using specific markers. Six lines, LBNR 6-5-1, LBNR 52-5-5, LBNR 77-1-1, LBNR 229-6-4, LBNR 300-1-9 and LBNR 300-7-1 and parent, NR-14 showed presence of *Mi* gene while the gene was absent in two lines namely LBNR 447-2-2 and LBNR 311-2-8 as well in susceptible check varieties (Table 4; Fig. 4). Similarly, for late blight resistance, the lines exhibiting highly resistant reaction (LBNR 6-5-1, LBNR 52-5-5 and LBNR 229-6-4) showed the presence of both *Ph2* and *Ph3* genes while lines with resistant and moderately resistant reaction showed the presence of either *Ph2* or *Ph3* gene. The line LBNR 447-2-2 and check varieties showed the absence of both the *Ph* genes (Table 4; Fig. 5A & B).

Discussion

Growing resistant cultivars is a key component of integrated disease management programme and one of the most economical and eco-friendly strategies to manage plant diseases. For the past many years, various fungicides have been extensively used to manage late blight disease (Fry 2016; Small et al. 2015) which led to the emergence of some fungicide-resistant *P. infestans* strains (Hu et al. 2012) making it more difficult to control. The development of new cultivars

with genetic resistance to *P. infestans* is the most effective way to protect tomato and minimize crop losses caused by late blight. In the case of root-knot nematodes, the use of host resistance is a more attractive option because it not only protects the crop yields from nematode damage but also hampers nematode development and reproduction (Ogallo et al. 1999; Roberts 2002), thus protecting the subsequent susceptible crops by suppressing nematode population densities. Being soil-borne in nature, it's very difficult to manage root-knot nematode populations in protected cultivation once they are introduced. They multiply quickly because of the favourable microenvironment of the protected structures and the continuous availability of host crops (Sharma et al. 2009). As most of the vegetable crops being grown under protected cultivation are susceptible to these nematodes, it is very important to grow resistant cultivars so as to bring down the nematode population to sustain the production of subsequent susceptible crops.

In this study, thirty-three indeterminate tomato lines of BC_1F_4 population developed by using late blight resistant (LBR-10) and root-knot nematode-resistant (NR-14) parents were screened at nursery stage. Among these, six lines were found resistant to root-knot nematode while 22 lines (3 highly resistant, 9 resistant and 10 moderately resistant) showed resistance against late blight disease. Eight elite indeterminate tomato lines selected from these lines were then evaluated for resistance against root-knot nematodes, late blight disease and for better horticultural traits in nematode infested polyhouse. All the selected lines exhibited significant variability in horticultural traits related to yield and shelf life of tomato fruits like in earlier studies conducted on tomato genotypes for these traits (Kaushik et al. 2011; Olakojo and Adetula 2014; Kumar et al. 2019; Singh et al. 2020). The lines, LBNR 300-1-9, LBNR 229-6-4 and LBNR 52-5-5 performed better for yield and other important fruit traits. Three lines LBNR 6-5-1, LBNR 52-5-5 and LBNR 229-6-4 exhibited resistance against both root-knot nematodes and Phytophthora late blight.

Lines showing resistance to root-knot nematode also exhibited reduction in soil nematode population at end of the crop season and thus decreased the initial inoculum load for the next susceptible crop. Earlier studies also reported that growing resistant cultivars interferes with nematode development and multiplication (Ogallo et al. 1999; Roberts 2002), which results in decreased soil nematode population. The resistant lines also showed the presence of *Mi* 1.2 gene with SCAR marker *Mi* 23 conferring resistance against root-knot nematodes and *Ph2* & *Ph3* gene with CAPS marker dTG422 & TG328 conferring resistance against Phytophthora late blight. The SCAR marker *Mi* 23 is tightly linked with the *Mi* 1.2 gene and is widely used for marker-assisted screening and a selection of resistant plants possessing *Mi* gene in tomato (Kaur et al. 2014; Reddy et al. 2016; Kumar et al. 2019). For late blight resistance, the lines showing highly resistant reaction against late blight (LBNR 6-5-1, LBNR 52-5-5 and LBNR 229-6-4) amplified both *Ph2* and *Ph3* gene whereas, lines exhibiting resistant and moderately resistant reaction showed presence of either *Ph2* or *Ph3* gene. The *Ph2* gene has been reported to confer an incomplete dominant or partial resistance to some *P. infestans* isolates (Moreau et al. 1998) while Ph-3 gene imparts partial-dominant resistance to different *P. infestans* tomato races that overcome *Ph-1* and *Ph2* genes (Chunwongse et al. 2002). The genes, *Ph-2* and *Ph3* are complementary to each other and together confer resistance to a broader range of pathogen isolates compared to either gene alone (Chen et al. 2008). The earlier studies also documented, that tomato genotypes that possesses both *Ph2* and *Ph3* genes exhibited highly resistant reaction during conventional screening (Hanson et al. 2016; Arafa et al. 2017). Kumar et al. 2019 also revealed that the tomato lines that carried *Ph2* gene showed moderate resistance against late blight during phenotypic screening. The multiple disease-resistant lines with good horticultural traits identified in the present study can be further exploited at a commercial level and /or they can be used as a parent in developing high-yielding hybrids possessing multiple disease resistance.

Authors' contribution

Conceptualization of research (SKJ, AS, SK); Designing of the experiments (SK, SKJ, AS, RG); Contribution of experimental materials (SKJ, AS); Execution of field/lab experiments and data collection (SKJ, SK, AS, RG); Analysis of data and interpretation (SK, SKJ, AS, RG); Preparation of manuscript (SK, SKJ, AS, RG).

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