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Identification of *Phytophthora* and nematode-resistant source-from opens pollinated progenies of black pepper (*Piper nigrum*) using a modified protocol

R SUSEELA BHAI [1], S J EAPEN[2], M ANANDARAJ[3] and K V SAJI[4]

Indian Institute of Spices Research, Calicut, Kerala 673 012

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ABSTRACT

Foot rot disease caused by *Phytophthora capsici* and slow decline disease caused by plant parasitic nematodes are serious diseases of black pepper (*Piper nigrum* L.). In this study open-pollinated seedling progenies of black pepper were screened during 2004–06 to identify sources of resistance against *P. capsici* and nematodes. A total of 11 632 seedlings raised from 30 cultivars, 42 hybrids and 1-open pollinated selection were subjected for preliminary screening. Forty progenies (21 Karimunda progenies, 10 progenies from other cultivars, 8 progenies from hybrids and 1 progeny from the selection 'IISR Shakti') that took no infection in the preliminary screening against *P. capsici* were multiplied vegetatively and subjected to second round of screening adopting root inoculations. They were assessed for disease severity after 100 days of inoculation adopting a new protocol for the second and the final round of screening. The progenies were rated initially based on the time taken for mortality and then assayed for aerial infection and final score was based on average disease severity index which was not followed in the earlier screenings. Of the 11 632 progenies screened, 1 progeny, viz '04-P24-1' showed resistant reaction and 1 hybrid progeny, viz 04-HP-400-1 showed moderate resistant reaction towards *P. capsici*.

Besides the progeny 04-HP 1533 (2) showed resistant reaction towards the root-knot nematode *M. incognita*. However, all short listed progenies were found susceptible to *R. similis.* The initially shortlisted phytophthora '04-P24-1' consistently showed resistant reaction even after repeated inoculations and is under field evaluation since 2006 onwards. This is the first report of identifying a *Phytophthora*-resistant source in black pepper (*Piper nigrum* L.) and this modified protocol can be used for screening open-pollinated progenies of black pepper.

Key words: Black pepper, *Meloidogyne incognita*, *Phytophthora capsici*, *Radopholus similis*, Resistance, Screening

Foot rot caused by *Phytophthora capsici* (Leonian) and slow decline by plant parasitic nematodes *Radopholus similis* (cobb) Thorney and *Meloidogyne incognita* (Kofold and White) along with *P. capsici* are serious diseases of black pepper (*Piper nigrum* L.) prevalent in all pepper- growing countries (Anandaraj 2000). Several technologies are available to manage the above diseases through chemical, biological and integrated means (Ramachandran *et al*. 1991, Anandaraj and Sarma 1995). However, these control measures often do not yield the desired results as the root damage caused by the above organisms is manifested very late under field conditions. Therefore, in an integrated disease management strategy against these diseases-resistant lines should play a major role. Earlier workers have screened around 1 million seedlings and selected 1 progeny from the cultivar 'Perambramundi' as moderately resistant to *P. capsici* infection. Another cultivar 'Ottaplackal' was found to be resistant to *M. incognita* and was released as 'Pournami' (Ravindran *et al*. 1992). But it becomes imperative to extend the study further to identify more resistant sources having resistance to both the pathogens through extensive screening of the rich diversity available in black pepper.

Indian Institute of Spices Research, Calicut, has got the largest collection of black pepper germplasm including wild accessions and cultivars. In addition, a sizable number of hybrids are also generated. Many of these collections and hybrids are promising with regard to yield and other qualitative traits. Studies are underway to locate resistant sources from these collections both by conventional (Suseela Bhai *et al*. 2007) and molecular approaches (Anandaraj *et al.* 2008). Besides, there is considerable variability in seedling progenies of black pepper. So the present study was taken up to exploit the high degree of variability in open-pollinated seedlings of high-yielding and promising black pepper cultivars to locate sources of resistance to both *P. capsici* and nematodes. A new protocol for rating of open-pollinated

[1,2,4]Senior Scientist (e mail: suseela@spices.res.in), [2]Senior Scientist (e mail: sjeapen@spices.res.in), [3]Project coordinator (e mail: anandaraj@spices.res.in), [4]Senior Scientist (e mail: saji@spices.res.in)

progenies by giving due importance to both underground and aerial infections is also proposed in this paper.

MATERIALS AND METHODS

Ripened berries collected from 30 germplasm accessions, 42 hybrids and 1 *Phytophthora* moderately resistant line 'IISR Shakti' maintained at IISR Experimental Farm, Peruvannamuzhi were used as the source material. Seeds were extracted, washed in running tap water and sown in sterilized nursery mixture (soil, sand and farmyard manure 1 : 1 : 1) in polyethylene basins of 30 cm diameter.

The preliminary screening was carried out during 2004–05 season. Three-month-old seedlings (3–4 leaf stage) were inoculated around the roots by drenching with a suspension of *P. capsici* zoospores. The suspension was added @ 100 ml/basin (having a spore load of 1×10^6/ml and a germination percentage of more than 75%). The seedlings which did not succumb to infection till 4 months of inoculation (termed 'survivals') were selected for the second round screening after vegetative multiplication.

The seedlings which escaped infection in the preliminary screening were selected and multiplied by serpentine method in polyethylene bags containing sterilized nursery mixture (Thankamani *et al*. 2007). Single node cuttings were produced in sufficient numbers and grown for 3 months. These rooted cuttings were used in the second round of screening during June–August 2005 where all of them were simultaneously subjected to root inoculations.

The 3-month-old rooted cuttings in polyethylene bags were inoculated with 72 hr old culture of *P. capsici* in the form of mycelial discs. Five mycelial discs of 10 mm diameter were incorporated into the root zone of plants and observed for infection/mortality. For each line 10 plants were inoculated. Root/collar infection is manifested as decay of the collar portion or root which extends upwards resulting in the total collapse of the plant. Here the mortality of the plant was taken as the measure of disease severity. The presence of the inoculum in the soil was determined by baiting method (Anandaraj and Sarma 1991). The plants were rated based on the number of days taken for a mortality (Eikemo *et al.* 2001, 2003; Gevens *et al.* 2006). Those plants that showed up to 30% mortality in > 21 days were tested for aerial infection.

For stem inoculation, a slight pinprick injury was made on the third internode on the stem from the top of the plants and a mycelial plug (5 mm size) of *P. capsici* cut from the edge of a 72 hr-old actively growing colony was placed over it (Sarma *et al*. 1994). The inoculated portion was covered with wet cotton wad to keep the inoculum continuously wet and tied with polythene strip. Simultaneous with the stem inoculation, the third or fourth leaf from the top was also inoculated but without making any injury. The inoculated plants were incubated for 72 hr at a temperature of 24–25°C with relative humidity 80–90%. There were 10 plants/progeny.

After 72 hr, leaf lesion was scored on a 0–4 scale as 0 for no lesion, 1 for 1–5 mm lesion, 2 for 6–10 mm, 3 for 11–15 mm and 4 for >15 mm lesion size. Simultaneously the external stem lesion length was also measured and scored on a 0–4 scale as 0 for no lesion, 1 for 1–5 mm lesion, 2 for 6–20 mm, 3 for 21–30 mm and 4 for >30 mm lesion length. The lesion diameter was also analyzed statistically. From the lesion scorings, the disease severity index (DSI) was calculated for stem and leaf using the formula of Kim *et al.* (2000).

The initial selection was made based on the root infection assessed by the mortality of plants due to *Phytophthora* infection. The time taken for mortality was taken as criteria for short listing the progenies. Those plants that took > 21 days for mortality were assessed for their reaction to aerial infection and rated as average DSI < 30% as resistant, 31–40% as moderately resistant and > 40% as susceptible.

To study the reaction of short listed plants against plant parasitic nematodes, the shortlisted plants for *P. capsici* were multiplied vegetatively and 10 plants each were planted in sick beds infested with either root-knot nematode *M. inocognita* or *R. similis*. The root galling and root decay were recoded after 4 months of planting (Ramana and Mohandas 1989).

RESULTS AND DISCUSSION

Initial screening

Out of 11 632 seedlings inoculated with *P. capsici*, 40 seedlings escaped infection in the first round of screening. This included 21 Karimunda seedlings, 5 seedlings of 'HP 1533' hybrid (13.8% survival), 4 seedlings each of the cultivars '5097' (7.8%) and '4095' (3.73%), 2 seedlings of 'HP 1263' hybrid (11.11%) and 1 seedling each of 'IISR Shakti' (0.12%), cultivars 'C 1422' (3.85%), 'C 4098' (5.71%) and hybrid 'HP 400' (5%). The percentage survival of plants after inoculation varied from 0 to 13.8%. Among the survived progenies, the maximum survival was observed in 'HP 1533' (13.8%) and minimum in 'IISR Shakti' (0.12%).

Second round screening

Root inoculation: Forty progenies shortlisted in the first round of screening were multiplied and screened by root inoculation with mycelial discs. Certain progenies showed mortality in 7 days, whereas certain other progenies took 65 days for the same. Of the 21 progenies obtained from cultivar 'Karimunda,' 16 progenies showed 100% mortality in 7–28 days. Among the 19 progenies obtained from hybrids/cultivars/selections, 6 progenies ('04-P24-1', '04-C 4095-3', '04-C 1422-1', '04-C 4098-1' and '04-HP-1533-5)' took >21 days for infection of which progeny '04-P24-1' showed no mortality even after 100 days. Progenies of '04-HP-400-1', '04-K17', '04–HP 1533-2' and 04-HP 1533-3 showed only 10–30% mortality in 38–41 days. Some progenies took 15–30 days, whereas some others took only <15 days for 100% mortality. The susceptible check, Subhakara showed 100%

mortality in 21 days (Table 1). Hence, based on the time taken for mortality, the progenies were classified into 2 groups. The group 1 took only < 21 days, whereas the group 2 took > 21 days for mortality (Table 1).

Table 1 Classification of progenies based on time taken for root infection and mortality

Progeny	Days taken for mortality	Root infection in 100 DAI (%)	Per cent mortality within 21 days	Per cent mortality in > 21 days
04-P24-1	0	0	0	0
04-C 5097-1	12	100	100	
04-C 5097-2	11–13	100	100	
04-C 5097-3	11–13	50	50	
04-C 5097-4	11	70	70	
04-C 4095-1	7–24	50		50
04-C 4095-2	24–65	60		60
04-C 4095-3	24–65	100		100
04-C 4095-4	24–31	50		50
04-C 1422-1	9	40	40	
04-C 4098-1	11	40	40	
04-HP1533-1	90	60		60
04-HP1533-2	39–41	20		20
04-HP1533-3	39–40	30		30
04-HP1533-4	9–14	70	70	
04-HP1533-5	7	40	40	
04-HP–400-1	38	10		10
04-HP 1263-1	9	80	80	
04-HP 1263-2	3–9	80	80	
04-K-1	5–16	100	100	
04-K-2	5–12	100	100	
04-K-3	5–13	100	100	
04-K-4	5–13	100	100	
04-K-6	11–21	100	100	
04-K-7	11	100	100	
04-K-8	20	100	100	
04-K-9	18–20	100	100	
04-K-10	18–31	60	60	
04-K-12	9–12	100	100	
04-K-13	28	100		100
04-K-14	18	80	80	
04-K-15	28	40		40
04-K-16	18	100	100	
04-K-17	39	10		10
04-K-18	7	100	100	
04-K-19	9–39	60		60
04-K-20	7	100	100	
04-K-21	7	100	100	
04-K-28	11	100	100	
04-K-29	11	100	100	
'Subhakara' (Check)	16–21	100	100	

DAI, Days after infection

Aerial inoculation (stem and leaf inoculation)

The rooted cuttings when subjected to infection by stem and leaf inoculation methods, showed varying levels of reaction towards *P.capsici* (Table 2). The leaf lesion size varied from 2.8 to 36.3 mm and disease severity index due to leaf infection varied from 7.5 to 100% (Table 2). Among the progenies, '04-P24-1' showed the smallest leaf lesion size (2.8 mm) and disease severity index (7.5%), followed by '04-HP-400-1' (leaf lesion size 11.2 mm and disease severity index 37.50%).

Similarly, the stem lesion size varied from 5.2 mm to 51.22 mm and disease severity index (DSI) due to stem infection varied from 27.5 to 100% (Table 2). Here also among the progenies, 04-P24-1 showed the smallest stem lesion size (5.2 mm) and DSI (27.5%). This is followed by 04-HP-400-1 (stem lesion size 4.9 mm and DSI 37.5%).

The overall DSI of the progenies due to aerial infection varied from 17.5 to 100%. Among the progenies screened 04-P24-1 showed the lowest DSI (17.5%), followed by '04-HP-400-1' (37.5%). Rest of the progenies showed more than 75% DSI (Table 2). Finally the assessment was made in such a way that the progenies that took > 21 days for root infection with < 30% DSI as resistant, 31–40% as moderately resistant and > 40% as susceptible (Table 3).

Screening for nematode resistance

The open-pollinated progenies short listed for resistance to *P. capsici* in the initial level of screening were subjected to nematode screening tests in individual beds infested with *R. similis* and *M. incognita* for 3 months. All the seedlings were found susceptible to *R. similis* as indicated by the lesions on the roots. Evaluation against *M.incognita* showed that all the progenies except '04-HP 1533' (2) is susceptible. No root galling or knots were observed in these plants even after 4 months of incubation (Table 4)

Ranking of progenies for Phytophthora infection

Based on the average DSI of aerial infections, the open pollinated progeny 04-P24-1 was found as resistant showing <30%, DSI and progeny 04-HP-400 as moderately resistant with DSI between 31 and 40%. All other progenies had more than 75% DSI (Table 3).

Evaluating germplasm accessions, hybrids or selections from open-pollinated progenies by artificial inoculation is the conventional practice followed for locating source of resistance against *P. capsici*. Techniques of mass screening of open-pollinated seedling progenies or rooted cuttings to assess the relative degree of tolerance/resistance to *P. capsici* have been developed (Sarma and Nambiar 1979, Sarma *et al.* 1990). Using these methods, an open-pollinated seedling progeny from a cultivar of Perambramundi, was released as moderately resistant *Phytophthora* line ('IISR Shakti'). In the present study, seedlings raised from 11 632 progenies were screened, of which 1 progeny from 'IISR Shakti' was

Table 2 Rate of aerial infection (leaf and stem) and disease severity index (DSI) of progenies

Progeny	Leaf lesion (mm)	Stem lesion (mm)	DSI due to leaf infection	DSI due to stem lesion	Average DSI
04-P24-1	2.80 a	5.20 a	7.50	27.50	17.50
04-C 5097-1	11.00 bc	17.00 cdef	22.50	50.00	36.25
04-C 5097-2	10.92 bc	17.00 abcd	52.50	66.67	59.60
04-C 5097-3	10.00 bc	8.67 ab	63.90	41.67	52.79
04-C 5097-4	27.60 g	24.88 def	100.00	75.00	87.50
04-C 4095-1	10.88 bc	12.60 abc	12.50	42.50	27.50
04-C 4095-2	14.25 bcd	17.80 bcde	70.00	80.00	75.00
04-C 4095-3	16.45 bcd	16.33 bcde	83.33	75.00	79.17
04-C 4095-4	25.40 fg	10.45 abcd	97.50	61.36	79.43
04-C 1422-1	24.80 fg	37.91 fg	72.50	90.90	81.70
04-C 4098-1	18.00 de	6.27 ab	92.50	29.54	61.02
04-HP1533-1	13.55 d	12.45 abcd	60.00	65.90	62.95
04-HP1533-2	36.33 h	32.80 efg	100.00	100.00	100.00
04-HP1533-3	29.60 g	37.72 fg	100.00	100.00	100.00
04-HP1533-4	35.60 h	33.09 ef	100.00	100.00	100.00
04-HP1533-5	26.80 g	31.70 ef	100.00	100.00	100.00
04-HP-400-1	11.20 bc	4.90 ab	37.50	37.50	37.50
04-Hp 1263-1	24.20 fg	48.20 g	100.00	100.00	100.00
04-Hp 1263-2	20.70 ef	20.10 bcde	97.50	77.50	87.50
04-K-1	4.2 ab	8.70 ab	7.50	52.50	30.00
04-K-2	3.6 a	6.30 a	2.50	27.50	15.00
04-K-3	10.3 bcd	20.80 abcde	25.00	65.00	45.00
04-K-4	19.16 fg	22.50 bcdef	70.00	67.50	68.75
04-K-6	16.80 def	23.70 bcdef	82.50	77.50	80.00
04-K-7	9.20 bcd	31.30 efg	47.50	77.50	62.50
04-K-8	12.50 cde	18.90 defg	72.50	77.50	75.00
04-K-9	15.91 def	32.40 bcdef	75.00	92.50	83.75
04-K-10	17.38 ef	19.90 defg	62.50	72.50	67.50
04-K-12	13.56 cdef	41.00 abcde	100.00	100.00	100.00
04-K-13	27.7 hi	11.10 efg	47.50	57.50	52.50
04-K-14	27.9 hi	37.30 gh	100.00	92.50	96.25
04-K-15	8.70 abc	51.22 abc	100.00	100.00	100.00
04-K-16	30.78 ij	32.50 fgh	100.00	100.00	100.00
04-K-17	31.33 ij	31.80 h	97.50	80.00	88.75
04-K-18	35.70 j	26.20 defg	100.00	100.0	100.00
04-K-19	25.90 hi	33.40 defg	95.00	100.00	97.50
04-K-20	29.0 hi	26.60 cdefg	100.00	87.50	93.75
04-K-21	23.75 gh	15.60 efg	97.50	80.00	88.75
04-K-28	27.90 hi	31.60 cdefg	100.00	80.00	90.00
04-K-29	23.50 gh	34.20 abcd	100.00	100.00	100.00
'Subhakara' (Check)	10.80 bc	16.60 abc	62.50	100.00	81.25

Figures followed by the same letters are not statistically different

found as resistant to *Phytophthora* infection based on several methods and rounds of screening. Sarma *et al.* (1984) adopted zoospore drenching method for short listing seedling progenies resistant to *P. capsici* infection and the selected ones were further screened by field testing. In this study also, preliminary selection was made by zoospore drenching method. In the persent study, the seedlings were retained in the inoculum till 5 months and the one which did not succumb to infection were transplanted, multiplied in sufficient numbers and were further subjected to second round of screening using stem and leaf inoculation. In both the types of secondary screening the DSI was calculated and disease reaction were rated and the average DSI of the aerial infection was taken for assessing the progenies. This method of assessment was not followed in the earlier screening experiments. Screening for *Phytophthora* resistance in black pepper, Mammootty *et al.* also used mortality of the plants due to root infection as the criteria for assessing cultivars or accessions (Mammootty *et al.* 2008).

Though the aim of the experiment was to identify source

Table 3 Short listing of progenies based on aerial infection and mortality

Progeny	Av DSI (%) due to aerial infection	Mortality (%)	Time taken for mortality	Reaction
04-P24-1	17.50	0		R
04-HP1533-2	100.00	20	41	S
04-HP1533-3	100.00	30	40	S
04-HP-400-1	37.50	10	38	MR
04-K-17	88.75	10	39	S
'Subhakara' (Check)	8 81.25	100	21	S

Table 4 Reaction of shortlisted lines to plant parasitic nematodes

Progeny	*R. similis*	*M. incognita*
04-P24-1	+	+
04-HP 1533-2	+	Resistant
04-HP 1533-3	+	+
04-HP-400-1	+	+
04-K-17	+	+
'Subhakara' (Check)	+	+

of multiple resistance, none of the lines showed multiple resistance. The line which was found resistant to *Phytophthora* was found susceptible to both the nematodes.

Accordingly in the present study, 1 open-pollinated progeny '04-P24-1' showed resistance, while hybrid progeny '04-HP 400-1' showed moderate resistance towards *P. capsici*. The short listed progenies when screened for nematode resistance (against *R. similis* and *M.incognita*), (1) progeny '04-HP 1533 (2)' showed resistance to *M. incognita*, whereas all others are found susceptible. Thus in the present study, the modified method was found as a suitable protocol for identifying resistant sources in black pepper.

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