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Development of a promising interspecific hybrid in black pepper (*Piper nigrum* L.) for *Phytophthora* foot rot resistance

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Abstract For the first time in the history of black pepper cultivation, a partly fertile interspecific hybrid having partial resistance to the dreaded disease *Phytophthora* foot rot was developed through hybridizing *Piper nigrum* with the wild species *Piper colubrinnum*. Hybridity of interspecific progenies was established through morphology, anatomy, cytology, and molecular studies. The hybrid, whose chromosome number is 2n = 39, is a triploid hybrid between a tetraploid and diploid species. The hybrid designated as Culture P5PC-1 exhibited distinct anatomical and morphological feature with a large number of long spikes with reduced setting percentage. The RAPD primers OPE 07 and OPG 08 were identified as hybrid specific molecular markers. Functional evaluation revealed partial introgression of genes—responsible for *Phytophthora* foot rot resistance—into the hybrid. This hybrid is considered as a successful breakthrough for introgression of resistance to the cultivated species *Piper nigrum* from the wild species *Piper colubrinnum*.

Keywords *Piper nigrum* · *Piper colubrinnum* · *Phytophthora capsici* · Interspecific hybridization · Amphidiploid

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Introduction

Black pepper, the King of spices, is a major revenue earning spice crop for many countries. *Piper nigrum* belonging to the family Piperaceae is a perennial climber. Cultivated *P. nigrum* is monoecious, having hermaphrodite and protogynous flowers, and predominantly self-pollinated. *P. nigrum* is a tetraploid species with chromosome number 2n = 52 having basic chromosome number $x = 13$ (Jose and Sharma 1984). Foot rot disease of black pepper caused by the pathogen *Phytophthora capsici* is a major constriction in black pepper production. All *Piper nigrum* cultivars are susceptible to this menace and have to be chemically protected. The high cost incurred in this kind of disease control, harmful effect of extensive and frequent use of fungicide, and the demand for 'clean spices' free from chemical residues, have led to an intensive search for a host-plant resistance. Further, resistance genes in crop plants are considered highly important because of pollution and residue problems. Interspecific hybridization has contributed significantly to the genetic enhancement of many crops. In developing synthetic amphidiploids, interspecific hybridization is a useful tool. This tool is useful for introducing alien variation and introgressing desirable genes across species (Roy 1984), generating morpho-physiological variation (Prakash 1973), and creating genetic diversity (Choudhary and Joshi 2001). Successful interspecific hybridization, to varying extents, was reported in many crops. In some cases the hybrids were completely sterile while in other cases partly fertile. Sasikumar et al. (1999) reported the success of interspecific hybridization between *Piper nigrum* and *Piper attenuatum*.

The only species resistant to *Phytophthora* foot rot disease is the wild species *P. colubrinum*. *P. colubrinum* has originated from Brazil and hence commonly known as Brazilian thippali due to the resemblance of its fruit with thippali (*P. longum*). It is a marshy weed, having profuse adventitious root habit. The potential resistance source for *Phytophthora capsici* in *P. colubrinum* is not exploited so far. The peculiarity of black pepper is that it is blessed with the twin advantage of vegetative propagation and viable sexual reproduction, which offers immense scope for exploiting hybrid vigor as well as selection breeding. In this study, an attempt was made to transfer resistance from *P. colubrinum* to *P. nigrum*, through interspecific hybridization. This attempt has been succeeded for the first time. This could be a turning point in the cultivation and production of black pepper, resistant to *Phytophthora* foot rot disease.

Materials and methods

Interspecific hybridization between *P. nigrum* (the cultivated species of black pepper which is susceptible to *Phytophthora* foot rot disease) and *P. colubrinum* (the wild species which is resistant to *Phytophthora* foot rot) was carried out during the rainy season of the year 2000. Different varieties of *P. nigrum* viz., Panniyur-1, Panniyur-2, Panniyur-3, Panniyur-4, Panniyur-5, Panniyur-6, Panniyur-7 and Karimunda were taken as female parent and *P. colubrinum* was taken as male parent. The pollen grains collected from *P. colubrinum* suspended in distilled water, repeatedly brushed on emasculated female spikes, as there is a chronological maturity of flowers in spike. The pollination of a spike took 10–14 days varying with varieties. Each day after pollination the spike was bagged to eliminate foreign pollen. The hybrid seeds were harvested during February to March and sown in potting mixture in poly bags. The germinated and survived seedlings were planted in the field during 2002. During 2004 and 2005 the interspecific hybridization was continued. Hybridity of interspecific progenies was confirmed based on morphology, anatomy, cytology, molecular studies, and functionally for reaction to *Phytophthora capsici*.

Morphology

Morphological characters such as plant growth and adventitious root habit, leaf length, leaf width, leaf margin, leaf tip shape, petiole length, internode length, shoot tip color, leaf shape and leaf texture of parents, and the putative hybrid were recorded. Inflorescence and fruit characters like spike orientation, spike shape, spike length, peduncle length, and average number of berries per spike were also recorded.

Anatomy

Transverse section of stem of putative hybrid and parents was studied. Stem cuttings of 1 cm size was fixed in formalin–acetic acid–alcohol mixture. The materials were processed for microtomy as per standard procedures (Johansen 1940). In an electric sledge microtome, sections were cut at 5-µm thickness and stained with toludine blue for histological studies (Krishnamoorthy 1988).

Molecular analysis

RAPD analysis of putative hybrid and parents was carried out using 33 random primers. DNA was extracted from young leaves, using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). Polymerase Chain Reaction (PCR) was carried out in a 25-µl reaction mix containing 5 pico mol of 10-mer random primers (Operon technologies, Inc., Alameda, CA, USA), and 25 ng of genomic DNA. PCR amplification conditions were 94°C for 3 min followed by 35 cycles of 95°C for 5 s, 37°C for 30 s, 72°C for 60 s, and a final extension at 72°C for 3 min. The RAPD fragments were separated by electrophoresis on 1.2% agarose gel and visualized with ethidium bromide.

Cytology

Actively growing root tips were collected from cuttings of the putative hybrids and the parent species between 11.00 and 12.00 h and treated with 1% solution of α-bromonaphthalene at 4–5°C for 4 h. The materials were then washed thoroughly in distilled water and fixed in 3:1 mixture of ethanol, acetic acid and chloroform for 24 h. The fixed root tips were hydrolyzed with 1N HCl at 60°C for 15–20 min and stained in 2:1 lactopropionic orcein for 4 h and squashed in 45% propionic acid. Five well spread mitotic metaphase plates, each from two slides, was used for counting chromosome number (Sasikumar et al. 1999).

Pollen fertility

Apparent fertility of pollen grains was studied by staining the contents of pollen grains in 1% acetic carmine and glycerine in the ratio 1:1 (Choudhary and Choudhary 1989). The filled pollen grains took up the stain where as the empty pollen grains did not.

Functional evaluation

To test the transfer of *Phytophthora* foot rot resistance from *P. colubrinum* to *P. nigrum* natural screening of the rooted cuttings was done by keeping the cuttings in a sick nursery having sufficient inoculum load of the pathogen to infect all the susceptible plants of the host population. Artificial screening was done placing *Phytophthora* disc on actively growing leaf and stem and also through spraying zoospore suspension as per standard procedures (Sarma and Premkumar 1988). Twenty-five rooted cuttings of 6 months old were used for screening purpose.

Results and discussion

Interspecific hybrid in black pepper, with tolerance to *Phytophthora* foot rot disease, between the species *P. nigrum* and *P. colubrinum* has been produced for the first time. As *P. nigrum* is tetraploid and *P. colubrinum* is diploid, the probability of success of inerspecific hybridization is very meager. In the present study, a constant and repeated attempt of crossing between these two different species having different ploidy level ended up in a fruitful result, which is a break through in the area of *Phytophthora* resistance breeding in black pepper. The percentage of F_1 hybrid seeds obtained after interspecific hybridization was very low (9.7%) compared to intervarietal hybridization (22%). The germination of interspecific hybrid seeds was only 53% and survival percentage of hybrid seedlings was 52% of the germinated hybrids showing reduced seed set and seedling vitality. Out of 12 hybrid seedlings planted during 2002, only two hybrids viz., *P. nigrum* (vty Panniyur 5) × *P. colubrinum* and *P. nigrum* (vty Panniyur 3) × *P. colubrinum* survived in the field. These hybrids were further multiplied vegetatively. Out of these two interspecific hybrids, the growth performance of *P. nigrum* (vty Panniyur 5) × *P. colubrinum* (Culture PSPC-1) was found to be promising having vigorous vegetative growth and potential to produce large number of long spikes (Fig. 1). This culture flowered for the first time during 2003 with very less number of spikes. During 2005 it produced large number of long spikes. But the berry setting percentage was very low (21.4 per spike) showing that the hybrid is partially sterile. Interspecific hybridization was continued during 2004 and 2005 and as a result 33 seedlings of hybrid between different varieties of *P. nigrum* and *P. colubrinum* were planted in the field during the kharif season of 2006.

The results of morphological, anatomical, cytological, molecular, and functional evaluation of the interspecific hybrid are discussed below.

Morphology

Morphological characters of hybrid and parents are depicted in Table 1. Seedling of the interspecific hybrid showed the adventitious root habit and robust stem nature of *P. colubrinum* (Fig. 2). The adventitious root habit was not prominent when the hybrid had grown up. The setting percentage in spikes was very low because of the triploid nature of the hybrid but there was large number of spikes per vine (56 Spikes/m^2). Average spike length was12.8 cm. The hybrid is intermediate between the parental species for the characters adventitious root production, spike length, and peduncle length. Internode length and berries per spike were lower, leaf width and petiole length was higher than parental species. The hybrid resembled the female parent *P. nigrum* for the characters plant growth habit, leaf texture, spike orientation, and spike shape. In general appearance, the hybrid is more inclined to the cultivated species *P. nigrum.* At the same time the hybrid inherited the characters leaf length, leaf shape, leaf tip shape, leaf margin, and shoot tip color from the male parent *P. colubrinum*.

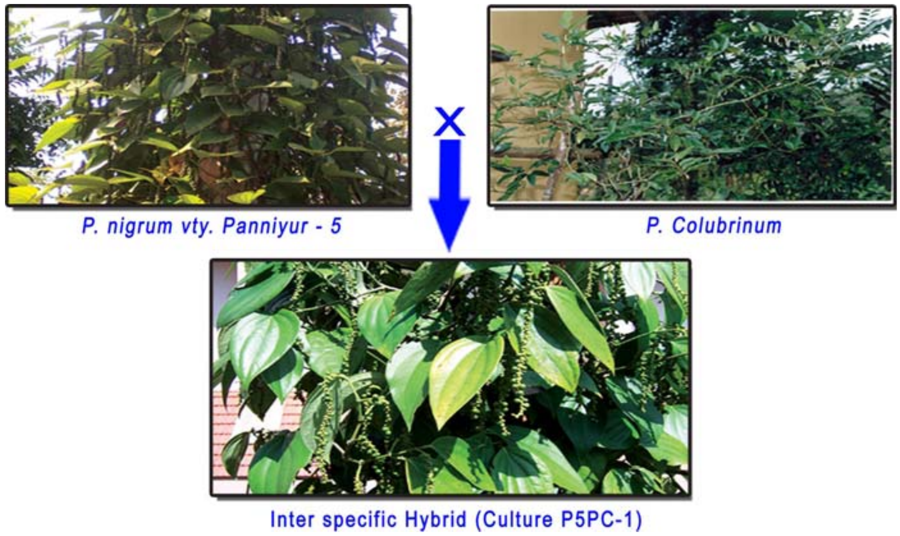

Fig. 1 First interspecific hybrid between *Piper nigrum* and *Piper colubrinnum*

Table 1 Morphological characters of *Piper* species and interspecific hybrid

Characters	Species/hybrid		
	P. nigrum (vty Panniyur 5)	*P. colubrinum*	Hybrid (culture P5PC-1)
Vegetative characters			
Plant growth habit	Climbing	Trailing	Climbing
Adventitious root production	Nil	Many	Few at seedling stage
Leaf margin	Even	Slight wavy	Slight wavy
Leaf length (cm)	15.0	13.7	13.7
Leaf width (cm)	8.3	6.2	8.4
Leaf shape	Ovate lanceolate	Ovate eliptic	Ovate elliptic
Leaf texture	Glabrous	Smooth and shining	Glabrous
Leaf tip	Acuminate	Acute	Acute
Petiole length (cm)	1.5	1.4	1.7
Internode length (cm)	5.6	5.5	5.1
Shoot tip color	Light purple	Light green	Light green
Inflorescence and fruit characters			
Spike orientation	Prostrate	Erect	Prostrate
Spike shape	Filiform	Cylindrical	Filiform
Spike length (cm)	13.1	5.1	12.8
Peduncle length (cm)	1.76	1.09	1.33
Av. no. of berries/spike	51	301.6	21.4

Anatomy

The dimensional variation for anatomical traits in *Piper* species and interspecific hybrid is described in Table 2. Stem anatomy of the species and hybrids showed structural and dimensional variations. Murty (1959), Pal (1961), Ravindran (1990) and Menancherry (1993) studied anatomy of *Piper* species. Ravindran (2000) reported the anatomy of *P. colubrinum*. These workers reported the presence of central and peripheral mucilage canals in *P. nigrum* and absence of mucilage canal in *P. colubrinum*. The present study also confirmed this anatomy. The hybrid *P. nigrum* × *P. colubrinum* was found to be intermediate between the parent species

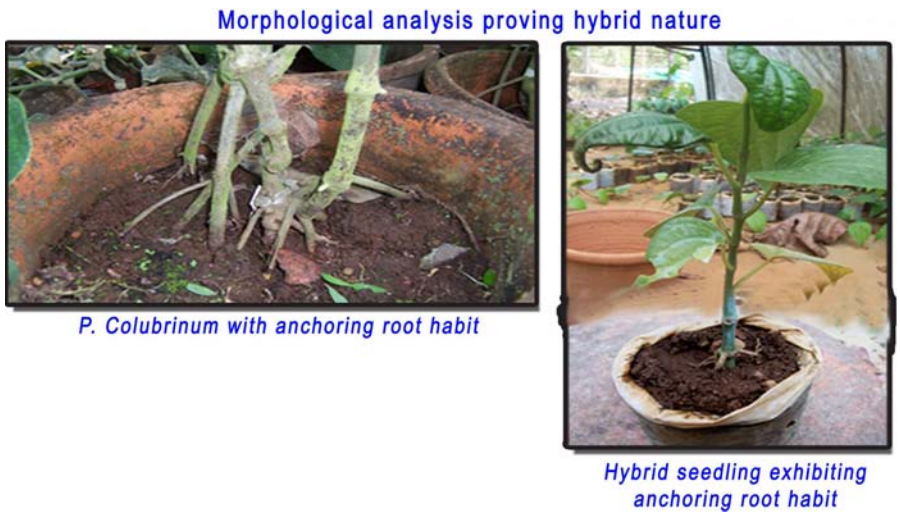

Fig. 2 Morphological analysis proving hybrid nature

Table 2 Dimensional variation for anatomical traits in *Piper* species and interspecific hybrids

Anatomical characters	*P. nigrum* (vty Panniyur 5)	*P. colubrinum*	*P. nigrum* × *P. colubrinum*
No. of peripheral vascular bundles	21	29	22
No. of medullary vascular bundles	6	7	6
No. of mucilage canals	5	0	3
Presence of medullary rays	Absent	Present	Present
Shape of outer vascular bundles	Conical shape	Cylindrical shape	Both conical and cylindrical shaped
Arrangement of tracheids in outer/ medullary vascular bundles	At the base of the vascular bundles	Along the entire length of the vascular bundles	Conical shaped ones with basally arranged tracheids and cylindrical shaped ones with vascular bundles arranged all along the length
Presence of sclerenchyma layer beneath the epidermis	A continuous sclerenchyma layer is present	Sclerenchyma layer is absent	A broken layer of sclerenchyma layer is present

having less number of mucilage canals than that of maternal parent (Table 2 and Fig. 3). The peripheral vascular bundle of *P. nigrum* was conical in shape while that of *P. colubrinum* was cylindrical. The putative hybrid exhibited both types of peripheral vascular bundles. Peripheral vascular bundles were found in large numbers in *P. colubrinum* than in *P. nigrum*. The hybrid was having intermediate number of peripheral vascular bundles. In the case of medullary vascular bundles, the hybrid akin to the maternal parent *P. nigrum*. In the male parent *P. colubrinum*, medullary rays were present in between the peripheral vascular bundles, which were absent in the female parent *P. nigrum*. In the hybrid, the medullary rays were seen present. There was a continuous sclerenchyma layer beneath the epidermis in *P. nigrum* but seen absent in *P. colubrinum*. The hybrid exhibited a broken layer of sclerenchyma. Sasikumar et al. (1999) reported the intermediate behavior of stem anatomy of the interspecific hybrid of *P. nigrum* with *P. attenuatum* and *P. nigrum* with *P. barberi*.

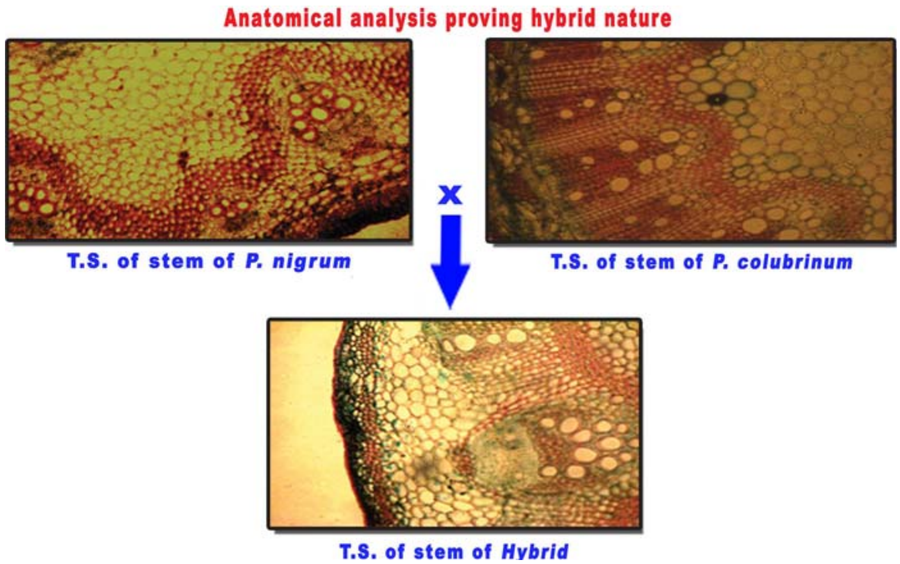

Fig. 3 Anatomical analysis proving hybrid nature

Molecular analysis

RAPD analysis with the Operon primers OPE 07 and OPG 08 (2nd and 3rd sets in Fig. 4) proved the hybrid nature of the interspecific hybrid. In the DNA profile of parents and hybrid with the primer OPE 07, the hybrid inherited all three amplicons of the female parent *P. nigrum*, of which two thin amplicons which were present in the male parent (1,000 and 500 bp) got intensified in the hybrid. In the case of the primer OPG 08, the DNA profiles of the hybrid and that of male parent *P. colubrinum* shared a common male specific thick amplicon of size 600 bp.

Cytology

The chromosome distribution of parent species and putative hybrid in the mitotic stage of growing root tip is depicted in Fig. 5. As reported earlier (Mathew 1958; Anand et al. 2000) the somatic chromosome number of *P. nigrum* was found to be 2n = 52 and that of *Piper colubrinum* 2n = 26. The somatic chromosome number of the hybrid was found to be 2n = 39. In this case, hybridization between a tetraploid and a diploid species resulted in the production of a triploid hybrid. Triploids may be either completely sterile or partly fertile. In the present study, the hybrid was partly fertile which reflected in the poor setting of spikes. The basic chromosome number of *P. nigrum* and *P. colubrinum* may be same ($n = 13$) but differ in ploidy level. Partial sterility may be due to irregularities in pairing of homologous chromosomes. Partial success of the tetraploid × diploid species, which gave small and full seeds with good germination, was earlier reported in brassica by Howards (1939) and Nishiyama et al. (1991) in *Solanum demissum* (2n = 6× = 72) crossed with diploid *S. chacoense*, *S. saltense*, and *S. rybinii.* Matsubayashi (1962) studied a rare occurrence of two kinds of hybrids with 2n = 48 (4×) and 60 chromosomes (5×). Success of production of inter generic hybrids between *Triticum aestivum* (2n = 42) and *Agropyron fragile* (2n = 28) was reported by Ahamad and Comeau (1991). As the newly developed triploid hybrid is partially sterile and trapped only partial resistance from *P. colubrinum*, at the present stature the hybrid will not be preferable to farmers but will form a bridge hybrid for the development of fully fertile high yielding black pepper variety having resistance to *Phytophthora* foot rot disease.

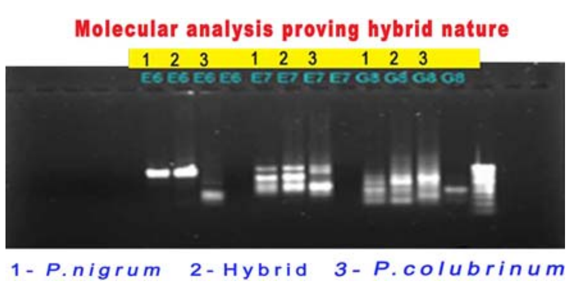

Fig. 4 Molecular analysis proving hybrid nature

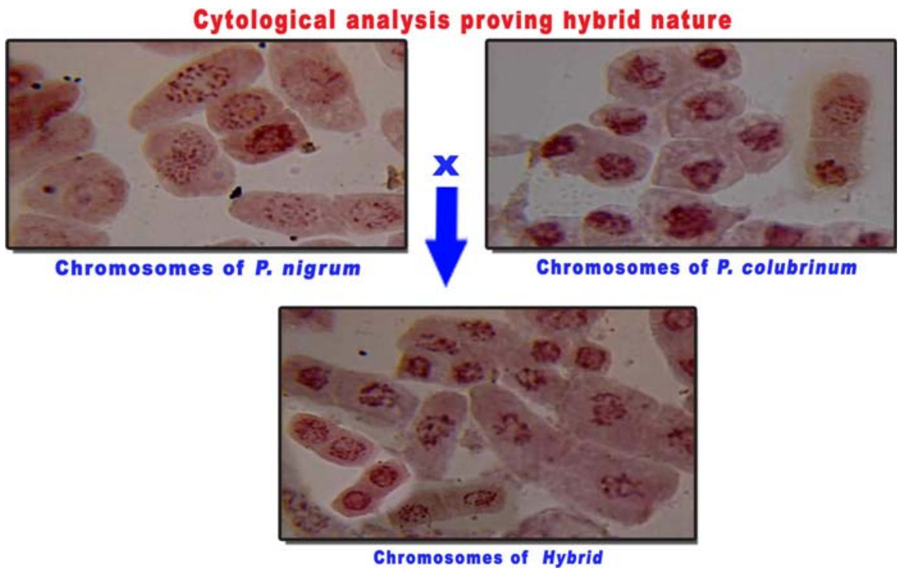

Fig. 5 Cytological analysis proving hybrid nature

Pollen fertility

The pollen fertility of *Piper nigrum* was 54% and that of *P. colubrinum* was 78%. The pollen fertility of the putative hybrid was found to be 7.7%. Irregularities in the pairing of homologous chromosomes would be the reason for low pollen fertility and thereby poor setting percentage in the large number of spikes in F_1 hybrids.

Functional evaluation

When rooted cuttings of the putative hybrid (Culture P5PC-1) was naturally screened for *Phytophthora* foot rot by keeping the rooted cuttings in the sick nursery along with susceptible variety, the entire rooted cuttings of susceptible varieties died, but all the cuttings of the putative hybrid survived with local cell death having yellow halo (Table 3). This may be cell death for hypersensitive response stimulating the cells around the infection site, which all react to pathogen attack and produce signals to activate defense responses (Gross et al. 1993).

The result of artificial screening by placing *Phytophthora* disc is given in Table 4. When there was continuous spread of disease in susceptible variety, in the putative hybrid even though the inoculated leaves had taken infection, there was no further spread of disease in the cuttings. When the artificial screening was done through zoospore spray, the rooted cuttings of susceptible variety died due to leaf, collar, and root infection within 13 days after inoculation (Table 5). At the same time, by 20 days after inoculation, there was no collar or root infection for the hybrid cuttings even though the leaves shed away due to infection. Further, the cuttings of the hybrid later rejuvenated.

Table 3 Reaction of *Piper* spp. and interspecific hybrids to *Phytophthora capsici* when naturally screened

Species/hybrid	Observation on infection rate at fortnight interval						
	After 7 days in sick nursery		After 21 days in sick nursery		After 35 days in sick nursery		Remarks
	% of cuttings wilted	% of infected leaves in live cuttings	% of cuttings wilted	% of infected leaves in live cuttings	% of cuttings wilted	% of infected leaves in live cuttings	
P. nigrum	100	NA					All cuttings wilted within 1 week
P. colubrinum	0	0	0	0	0	0	No infection
P. nigrum × *P. colubrinum*	0	0	0	0	0	0	No infection

Table 4 Reaction of *Piper* spp. and interspecific hybrids to *Phytophthora capsici* when artificially screened through placing Phytophthora disc on actively growing leaf and stem

Species/hybrid	Observation on leaf infection rate				
	3 DAI	6 DAI	9 DAI	12 DAI	15 DAI
P. nigrum					
Percentage of leaf infection	33	50	75	80	All infected leaves shed
Lesion size	1 cm	1.5 cm	4.5 cm	6.7 cm	
P. colubrinum	No infection	No infection	No infection	No infection	No infection
P. nigrum × *P. colubrinum*					
Percentage of leaf infection	17	17	Infected leaves shed and no further infection to adjacent leaves	No further spread of infection, the rooted cuttings remained healthy	No further spread of infection, the rooted cuttings remained healthy
Lesion size	1 cm	2.3 cm			

DAI, Days after inoculation

Table 5 Reaction of *Piper* spp. and interspecific hybrids to *Phytophthora capsici* when artificially screened through zoospore spray

Species/hybrid	Observation on infection rate				
	4 DAI	7 DAI	10DAI	13DAI	15 DAI
P. nigrum					
Percentage of leaf infection	25	50	100	Entire cuttings dead due to leaf infection and collar infection	
Lesion size	0.5 cm	1 cm	4.2 cm		
Collar infection	Nil	Nil	Present		
P. colubrinum	No infection	No infection	No infection	No infection	No infection
P. nigrum × *P. colubrinum*					
Percentage of leaf infection	Nil	25	78	93	All leaves shed
Lesion size	NA	2.3 cm	3.5 cm	4.5 cm	NA
Collar infection	Nil	Nil	Nil	Nil	Nil

DAI, Days after inoculation

Screening result for *Phytophthora* foot rot resistance showed that the hybrid partially inherited the resistance mechanism from the resistant male parent. More than a single gene may be involved in determining the resistance mechanism and they may be having partial dominance. Polygenic nature of *Phytophthora* foot rot resistance in black pepper was previously reported (Annual report 2004–2005 of IISR). Hence back crossing of this partially fertile interspecific hybrid having partially introgressed resistance genes, with both parents and selection from second filial generation and again back crossing have to be carried out to get a fully fertile *Phytophthora* foot rot resistant variety.

Conclusion

A partly fertile interspecifc hybrid in black pepper tolerant to *Phytophthora* foot rot disease was developed for the first time through interspecific hybridization between the cultivated species *P. nigrum* and the wild species *P. colubrinum*. This hybrid has large number of spikes/unit area and long spike but low berry setting. One of the bottlenecks in the exploitation of alien variation for crop improvement is the lack of expression of the alien genes. However, the results of the present study showed partial expression of the alien gene(s) for resistance to *Phytophthora capsici*. This will be the bridge hybrid from which completely resistant fully fertile cultivated *Piper nigrum* species can be developed through back crossing with either parents or selection from advanced filial generations and then back crossing with recurrent parent. This hybrid will be the source material for production of NILs leading to tagging and cloning of genes responsible for resistance to *Phytophthora* foot rot. Chromosomes of *P. nigrum* will have a tendency to pair preferably with their own homologous rather than with homoeologous chromosomes of *P. colubrinum.* Hence, as pointed out by Kimber (1984), gene transfer by spontaneous recombination per se would have low probability of success in the new hybrid combination due to the low level of chromosome pairing. Hence, alternate approaches would have to be utilized to achieve gene(s) transfer. Towards this goal, amphidiploid seeds obtained from the F_1 hybrid could be the best starting material for the production of alien addition and alien substitution lines, and to facilitate a range of other studies for the genetic improvement of black pepper.

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