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Screening of betel vine inter-specific hybrids for resistance to *Phytophthora nicotianae*

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ABSTRACT: An experiment was conducted to standardize the isolation and screening protocol *Phytophthora nicotianae* on betel vine to identify the resistance source. The artificial inoculation was carried out by detached leaf assay (pin prick method) and drenching with spore suspension for the root. The result showed that carrot agar was suitable for active culture subsistence, and the pin prick method was the best method for inoculation and screening. Among the inter-specific hybrids used in the present investigation, IIHRPBIH9 recorded significant resistance to Phytophthora foot rot compared to the susceptible check IIHRBV170. Thus, screening of inter-specific hybrids for this disease through artificial inoculation helps to identify resistance sources for the breeding programme, thereby meeting the demand for superior quality, disease-free planting materials and effective disease management. This is the first report for identifying the resistance source in betel vine against *P. nicotianae*.

Keywords: Betel vine, Inter-specific hybrids, *Phytophthora*, Resistance, Screening

INTRODUCTION

Betel vine, *Piper betle* L. (F: Piperaceae), is a dioecious, perennial creeper that originated from Central and Eastern Malaysia. In India, it is considered an important cash crop and stands top in the production of betel leaves in the world (Arulmozhiyan *et al.,* 2005), with an estimated area and production of 53,539 ha and 57,530 MT, respectively (Anon., 2019). Overall the country has exported 6158.39 MT of betel vine leaves worth 26.18 crores (Anon., 2020). Betel vine is considered a highly labour-intensive crop estimated to employ about 20.80 lakh people in the country throughout the year (Das *et al.,* 2017). The presence of eugenol, chavibetol, caryophyllene, and methyl eugenol gives the leaves their antioxidant, anti-apoptotic, anti-inflammatory, anti-cancerous, and anti-microbial characteristics, in addition to their chewing benefits.

The crop is highly susceptible to diseases and pests (Rahman *et al.,* 2020; Javaregowda, 2006). Foot rot is the major disease caused by *Phytophthora nicotianae*, which results in the complete death of the plant. The pathogen attacks the plant at the collar region and below the soil. The characteristic symptom of the disease is the disappearance of the lustre of leaves, followed by wilting and drooping of the vine. Meanwhile, underground parts of the plant rot completely. The extent of losses may vary from 5 to 90 per cent due to foot rot (Dasgupta *et al.,* 2008) and 20 to 40 per cent in case of leaf rot, leading to total crop failure (Dasgupta *et al.,* 2000).

Betel vine is a vegetatively propagated crop; hence it is easy to fix a favourable combination of important traits, unique chemotypes and morphotypes (Glemin *et al.,* 2006). Unfortunately, only some countable efforts have been made till now in identifying resistant sources or developing resistant hybrids. *Piper colubrinum* is a distant relative of the cultivated betel vine, showing high resistance to Phytophthora foot rot. At ICAR-Indian Institute of Horticultural Research, interspecific hybrids have been developed by crossing Phytophthora resistant species *Piper colubrinum* and *Piper betle* to develop resistant sources for this devastating disease (Turner, 1969; Purseglove, 1981). The standardization of *in-vitro* screening methods and protocols for foot rot (*Phytophthora nicotianae*) is important to understand the resistance mechanism and host-pathogen interaction, which aids in the identification of resistant sources for future crop development and disease management. In this view, the present study was focused on standardizing the protocol and screening of 13 interspecific hybrids through artificial inoculation for resistance to foot rot in betel vine.

MATERIALS AND METHODS

Plant material

Interspecific hybrids (Table 1) developed at the Division of Flower, and Medicinal Crops, ICAR-IIHR, Bengaluru, were maintained under polyhouse. The betel

vine stem cuttings collected from the selected mother vine were prepared by giving a slant cut at the proximal end and a horizontal cut at the distal end. A duly punched nursery polybags were filled with soil, sand and FYM in a 2:1:1 ratio, respectively. The cuttings were planted in nursery polybags of 200 gauge and size 4" × 6" for effective growth of cuttings. Stem cuttings with 3-5 nodes were used for propagation and planted so that one to two nodes were buried in nursery media and one node was kept above nursery media. The bags were then kept in polytunnels for 15 days to induce sprouting. After 30-45 days, rooted plants were shifted to pots filled with nursery mixture. Nursery management practices, *viz.,* regular watering, weeding and plant protection, were taken up periodically.

Table 1. List of Inter-specific hybrids used for screening of Phytophthora foot rot disease Reaction in betel vine

IIHR/Acc. No	Inter-specific hybrids
IIHRPBIH1	IIHRBV53/ *Piper colubrinum*
IIHRPBIH2	IIHRBV53/ *Piper colubrinum*
IIHRPBIH3	IIHRBV53/ *Piper colubrinum*
IIHRPBIH4	IIHRBV53/ *Piper colubrinum*
IIHRPBIH5	IIHRBV53/ *Piper colubrinum*
IIHRPBIH6	IIHRBV53/ *Piper colubrinum*
IIHRPBIH8	IIHRBV42/ *Piper colubrinum*
IIHRPBIH9	IIHRBV42/ *Piper colubrinum*
IIHRPBIH13	IIHRBV53/ *Piper colubrinum*
IIHRPBIH18	IIHRBV42/ *Piper colubrinum*
IIHRPBIH19	IIHRBV53/ *Piper colubrinum*
IIHRPBIH21	IIHRBV96/ *Piper colubrinum*
IIHRPBIH22	IIHRBV68/ *Piper colubrinum*
IIHRBV170*	Meetapan

*Check variety

Standardization of different media

Growth characters of *Phytophthora nicotianae* were studied on three semi-synthetic solid media *viz*., potato dextrose agar (PDA), V-8 Juice agar and carrot agar (CA). All the media were sterilized for 15 minutes at 121.6 ^{0}C of pressure 1.05 kg cm^{-2}. Further, growth was recorded on the Petri plate.

Fungal isolate for inoculation

Direct tissue isolation and isolation from leaf samples technique was employed to isolate wilt associated pathogens from betel vine showing typical symptoms of foot rot. In direct tissue isolation, the samples were collected from the field where the incidence of foot rot disease was high. Subsequently, the root samples were washed and treated with 0.1 per cent sodium hypochlorite, followed by drying, sectioning of root samples and plating onto PDA, CA, and V-8 juice agar medium and then incubated at 24 ± 2 0C for 07-10 days. In case of isolation from leaf samples, the infected leaf portion showing actively progressing lesions was cut into small pieces and sterilized with 0.1 per cent sodium hypochlorite, washed with distilled water and placed aseptically in sterile Petri plates containing medium and incubated at 24 ± 2 0C for 07-10 days.

Identification and maintenance of pure culture

Phytophthora culture was identified based on spore morphology and colony characters, referring to the description by Drenth and Sendall (2001). *Phytophthora nicotianae* was maintained on CA medium in the Petri plates by culturing a single bit of previously grown culture by hyphal tip isolation method to obtain a pure culture of a pathogen. The culture plates with pathogens were covered properly and kept at a low temperature (4 0C) to arrest further growth. The pathogen was sub cultured periodically for 10 to 15 days during the investigation.

Molecular identification of the pathogen

The fungal DNA was extracted from five days old cultures grown in culture plates by CTAB method. The mycelium from the culture plate was scraped out with the help of a sterile spatula. Around 0.1 g of sample was placed in a mortar and homogenized with 1 ml of extraction buffer and the homogenate was transferred to a 2 ml-microfuge tube. An equal volume of Phenol: Chloroform: Isoamlyalcohol (25:24:1) was added to the tubes and mixed well by gently shaking the tubes. The tubes were centrifuged at room temperature for 15 minutes at 14,000 rpm. The upper aqueous phase was transferred to a new tube and an equal volume of Chloroform: Isoamlyalcohol (24:1) was added and mixed. The resulting mycelium tissue homogenate was centrifuge at 14,000 rpm for 10 min and supernatant was transferred to a fresh tube. Add 0.1 volume of 3 M Sodium acetate (pH 7.0) and 0.7 volume of Isopropanol to precipitate the DNA from the solution. The tubes were centrifuged at 4 ^{0}C for 15 minutes at 14,000 rpm, after keeping them for incubation at room temperature for 15 minutes. The DNA pellet was washed twice using 70 per cent ethanol and then using 100 per cent ethanol and air

Table 2. Details of primers used for amplification

Oligo name	Sequence (5`and 3`)	Tm (^{0}C)	GC- Content (%)
ITS Forward	TCCGTAGGTGAACCTGCGG	57	63.15
ITS Reverse	TCCTCCGCTTATTGATATGC	53	45.00

Table 3. Percentage disease Index (PDI) of interspecific hybrids

Accession No.	Time Intervals			
	3rd dpi	5th dpi	7th dpi	Mean
IIHRPBIH1	24.45 (29.63)	44.43 (41.80)	71.20 (57.55)	46.70 (42.99)
IIHRPBIH2	28.84 (32.48)	42.25 (40.54)	67.13 (55.02)	46.07 (42.68)
IIHRPBIH3	26.67 (31.09)	54.88 (47.80)	73.23 (58.84)	51.59 (45.91)
IIHRPBIH4	22.23 (28.13)	48.89 (44.35)	57.44 (49.28)	42.85 (40.59)
IIHRPBIH5	28.87 (32.50)	55.47 (48.14)	77.83 (61.91)	54.06 (47.52)
IIHRPBIH6	24.41 (29.60)	57.66 (49.41)	73.40 (58.95)	51.82 (45.99)
IIHRPBIH8	15.59 (23.26)	37.87 (37.98)	53.25 (46.86)	35.57 (36.03)
IIHRPBIH9	0.00 (2.87)	2.25 (8.62)	11.14 (19.50)	4.46 (10.33)
IIHRPBIH13	20.08 (26.62)	42.27 (40.55)	64.43 (53.39)	42.26 (40.19)
IIHRPBIH18	13.15 (21.26)	31.16 (33.93)	42.10 (40.45)	28.80 (31.88)
IIHRPBIH19	22.28 (28.16)	33.28 (35.23)	55.56 (48.21)	37.04 (37.20)
IIHRPBIH21	24.45 (29.63)	46.67 (43.09)	66.26 (54.49)	45.79 (42.41)
IIHRPBIH22	17.79 (24.94)	40.00 (39.23)	62.50 (52.24)	40.10 (38.81)
IIHRBV170	40.00 (39.23)	71.35 (57.64)	97.75 (81.44)	69.70 (59.45)
Mean	22.06 (27.10)	43.46 (40.59)	62.37 (52.73)	
For comparison of mean	**S. Em ±**	**CD @ 5 %**	**CV (%)**	
Treatment (T)	0.62	1.78		
Condition (C)	0.29	0.82	4.46	
Interaction (T × C)	1.09	3.08		

*****Mean of three replication; Figures in the parenthesis are arc sine transformed values

Table 4. Per cent disease infection in interspecific hybrids

Accession No.	Time Intervals*			
	3rd dpi	5th dpi	7th dpi	Mean
IIHRPBIH1	9.85 (18.29)	24.76 (29.84)	16.25 (23.77)	16.95 (23.96)
IIHRPBIH2	1.24 (6.39)	3.93 (11.43)	19.92 (26.50)	8.37 (14.78)
IIHRPBIH3	9.03 (17.49)	26.02 (30.67)	21.19 (27.40)	18.75 (25.19)
IIHRPBIH4	4.29 (11.95)	8.08 (16.52)	18.39 (25.39)	10.25 (17.95)
IIHRPBIH5	7.13 (15.49)	21.91 (27.90)	37.73 (37.89)	22.26 (27.09)
IIHRPBIH6	8.80 (17.26)	28.33 (32.15)	22.22 (28.12)	19.78 (25.85)
IIHRPBIH8	2.11 (8.34)	3.53 (10.81)	18.61 (25.55)	8.08 (14.90)
IIHRPBIH9	0.00 (2.87)	0.16 (2.23)	0.68 (4.69)	0.28 (3.26)
IIHRPBIH13	1.95 (8.01)	4.92 (12.81)	20.59 (26.98)	9.15 (15.94)
IIHRPBIH18	3.25 (10.37)	3.90 (11.39)	10.24 (18.66)	5.80 (13.48)
IIHRPBIH19	3.40 (10.62)	10.64 (19.03)	19.95 (26.53)	11.33 (18.73)
IIHRPBIH21	1.87 (7.87)	7.68 (16.09)	27.94 (31.91)	12.50 (18.62)
IIHRPBIH22	2.47 (9.04)	9.62 (18.07)	24.72 (29.81)	12.27 (18.97)
IIHRBV170	9.32 (17.78)	28.31 (32.14)	64.57 (53.47)	34.07 (34.47)
Mean	4.62 (11.55)	12.99 (19.36)	23.07 (27.62)	
For comparison of mean	**S. Em ±**	**CD @ 5 %**	**CV (%)**	
Treatment (T)	0.19	0.54		
Condition (C)	0.08	0.25	4.24	
Interaction (T × C)	0.33	0.93		

*Mean of three replication; Figures in the parenthesis are arc sine transformed values

dried. The DNA was dissolved in TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM). To remove RNA, 5 µl of DNAse free RNAse (10 mg/ml) was added to the DNA.

Quantity and quality determination

The quantity of the extracted DNA was determined by measuring the absorbance at 260 nm using a spectrophotometer (Thermo Scientific NanoDrop 1000). The quality of DNA (2 µl) was accessed by subject to 1.5 per cent agarose gel electrophoresis. The DNA band was observed under the UV Trans illuminator gel documentation system. PCR amplification was performed using ITS1 and ITS4 primers to amplify the Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA in a thermal cycler (Table 2). The temperature profiles of 94^{0}C for 3 minutes of initial denaturation, followed by 30 cycles of denaturation at 94 ^{0}C for 3 min, with constant annealing at 50 ^{0}C for 1 minute and extension at 72 ^{0}C for 2 minutes with a final extension at 72 ^{0}C for 7 min (Source: GenBank ON358198).

Experimental design

The pathogenicity test was carried out in the division of crop protection, ICAR-IIHR, Bengaluru. For inoculation of a pathogen, betel vine leaves were used, and leaves were carefully washed with adequate tap water to remove

excess adhering materials before surface sterilization and wiped with 70 per cent ethanol, followed by air drying on the sterile filter papers. After the drying process, the leaves were inoculated with two different inoculation methods, *viz*., detached leaf (pin prick at the centre of the leaf) and spore suspension method (soil drenching and zoospore suspension spray on leaf), and observed for their effectiveness for screening using *Phytophthora nicotianae* with 13 interspecific hybrids of betel vine.

Detached leaf method of inoculation

Mycelium plugs of 8 mm in diameter from 7-day-old culture cultivated at 24± 2 0C were taken from the edge of an actively growing colony and placed on the detached leaf with the mycelial side facing down at the center of the leaves. While performing the pin prick method before inoculation, the leaves were washed with sterilized water and air-dried. The sharp, sterilized stainless steel paper pins were used to prick the leaves. The plug was gently pressed to ensure good contact with the leaf surface. Inoculated leaves were placed on moist blotting paper towels in transparent polystyrene plastic boxes to maintain humidity and incubated at 24± 2 0C. The uninoculated leaves were maintained as a control for comparison with the inoculated leaves. After 3 days of inoculation, disease symptoms were observed, and lesion size was measured at 3, 5 and 7-day intervals.

Spore suspension method

One to two-month-old betel vine plants were used for inoculating pathogens using the spore suspension method. Spores were harvested from the 7-day-old culture of the pathogen. Induction of zoospore release was achieved by flooding the colonies with 10 mL of sterile distilled water, then incubating at 4 ^{0}C for 15 minutes, followed by 30 minutes at room temperature. After chilling, 1 µL drops were viewed under a microscope to count the average zoospore with a hemocytometer. Finally, a concentration of 1.0×10^4 zoospores per mL was obtained, and 10 mL of this suspension was used for soil drenching. Spores were sprayed uniformly on the betel vine leaves using a sprayer, and plants were covered with polythene covers (above 51 microns) to generate humidity which helps with spore germination and infection. After 14 days of inoculation, symptoms were observed and recorded.

After symptom development, re-isolation of the pathogens was done from the artificially infected plants. The inoculated betel vine leaf with typical symptoms was selected for isolation. Thus obtained fungi culture was compared with the original culture for confirmation. These cultures were subsequently used for further investigations and were maintained in slants at 4 ^{0}C.

Microscopic observation and disease assessment

The plate containing culture was incubated under light for 24 to 48 h. The mycelial was taken off, and slides were prepared for microscopic observation for the presence of sporangia. The lesion size development was observed regularly on the detached leaf, inoculated with *Phytophthora nicotianae*. The disease severity was calculated on the seventh day by measuring the area covered by the pathogen by using the 0 to 5 scale as described by (Goswami *et al.,* 1993): 0= infection free or healthy leaf; 1= leaf area covered up to 5 per cent; 2= 6 to 15 per cent leaf area covered; 3= 16 to 30 per cent leaf area covered; 4= 31 to 50 per cent leaf area covered; 5= leaf area covered above 50 per cent. Disease severity scale (0-5) for foot rot disease of betel vine using detached leaf assay method. Thus the individual leaf ratings were recorded, the per cent disease index was calculated using the formula (Wheeler, 1969), and analysis was carried out using WASP-2.

RESULTS AND DISCUSSION

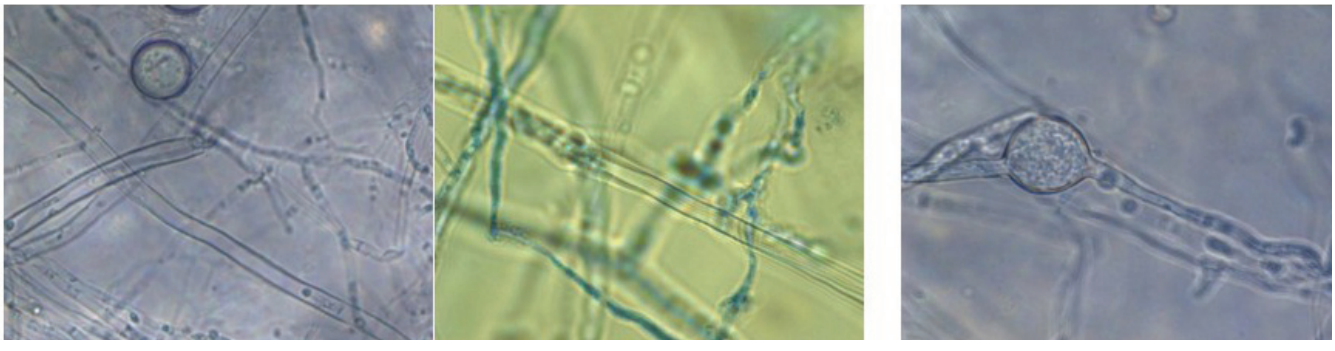
$$PDI = \frac{\text{Sun of all disease scores}}{\text{Number of leave observed} \times \text{Maximum disease score}} \times 100$$

Effect of media and inoculation methods

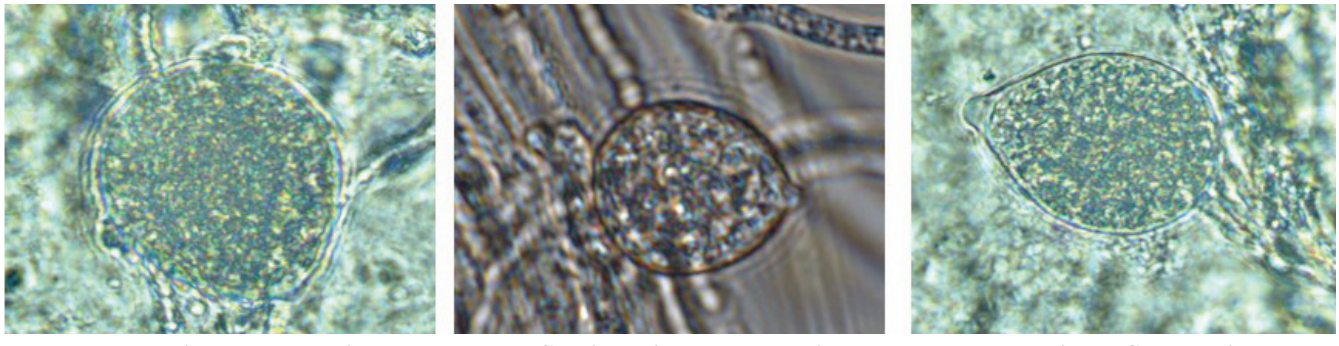
`In the present study, carrot agar was found to be suitable for the active subsistence of culture. The pin prick method was found to be the best method of detached leaf assay for inoculation and screening of Phytophthora foot rot disease compared to the spore suspension method. The disease system appeared on betel vine leaves 3 days after inoculation with the pin prick method at 24± 2 ^{0}C. However, the disease system appeared on roots by the spore suspension inoculation method only after 14 days. As the spore suspension method is laborious and time-consuming, the pin prick method was considered the best method for screening Phytophthora in betel vine as it is simple and rapid. Phytophthora disease development was observed on a leaf at the 3rd dpi, 5th dpi and 7th dpi. Leaf was incubated in water after 7th dpi, and microscopic observation was recorded accordingly to confirm spore development (Fig 1).

Percentage disease index (PDI)

Among the interspecific hybrids used, IIHRPBIH9 showed significant resistance to Phytophthora with a mean value of 4.46 per cent (Table 3), as IIHRPBIH9 at initial days doesn't develop any symptoms. But on 7 dpi, there was a trace amount of symptoms which was noticed to be 11.14 per cent. The key effectors triggering the resistance were hypersensitive response (HR) and



Aseptate mycelium | Chlamydospore



Non-papillate sporangia | Semi-papillate sporangia | Papillate Sporangia

Fig 1. Morphometric observation of *Phytophthora nicotianae*

phenolic component. And it was seen that the necrosis was at a trance amount under hypersensitive defense responses compared to the susceptible check variety (IIHRBV170). The mean value of susceptible check was significantly higher at 69.70 per cent, and it was noticed that there was a gradual increase in the trend of disease development as compared to IIHRPBIH9 interspecific hybrid.

Assessment of disease progress of interspecific hybrids

The detached leaf assay was carried out with artificial inoculation under control conditions in order to check the disease progression. The total infected area of 3rd dpi, 5th dpi and 7th dpi was recorded manually and it was found that the inter-specific hybrid IIHRPBIH9 shows less of severity and the mean area of infection was found to be 0.28 per cent, ranging from 0.00 to 0.68 percent at different days interval (Table 4). Whereas the susceptible check variety (IIHRBV170), the mean area of infection was around 34.07 per cent, ranging from 9.32 to 64.57 per cent. This shows that if the infection is observed at early stages, rotting of leaves is observed and ultimately leads to the death of the plant.

CONCLUSION

The present investigation, inferred that IIHRPBIH9 shows the resistance against Phytophthora foot rot infection as the disease progression was found to be comparatively lesser than the susceptible check IIHRBV170. Screening of inter-specific hybrids for Phytophthora foot rot through artificial inoculation helps to identify resistance sources for breeding programme thereby meeting the demand of superior quality, disease free planting materials and effective management of the disease.

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