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Cultural and Physiological Characterization of *Phytophthora parasitica* causing Foot Rot of Betelvine (*Piper betle* L.)

Y.P. Prasad*, M.P. Basavarajappa, Y.S. Mahesh, R.K. Mesta, D.L. Rudresh and Shankaragouda Patil

Harosagara Marabanahalli Post Channagiri tq Davanagere Dist. 577551, Karnataka, India
**Corresponding author*

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A B S T R A C T

Betelvine *(Piper betel* L.) is an important horticulture crop of commercial values and belongs to family Piperaceae. Betelvine is mainly grown under moist and humid shade conditions, which makes the crop more prone to many diseases. Among them foot rot caused by *Phytophthora parasitica* is a major one and leads to total crop loss. The mycelia of the fungus grew satisfactorily on the entire agar media used. Maximum radial growth of the pathogen was recorded on czapeck-dox agar after eight days of incubation. The optimum temperature for the mycelial growth of the fungus was observed at 20 ^{o}C followed by 25 ^{o}C and the least growth at 40 ^{o}C. The maximum mycelial dry weight of the pathogen was recorded at a pH 8 followed by pH 7. The result with respect to effect of relative humidity showed that maximum mycelial dry weight of *P. parasitica* at 90% followed by 85% and the least was noticed at 60%. Amongst the carbon sources supplemented, L-asparagine, was best utilized with the highest mycelia dry weight and the least was recorded in calcium carbonate. Whereas with respect to nitrogen sources the maximum mycelial dry weight was recorded in potassium nitrate and the least in ammonium phosphate.

Introduction

Foot rot disease is an important disease in betelvine causing a yield loss of 44-86 % and even in certain cases, entire plantations were being destroyed by the pathogen (Singh and Chand 1971). For the first time Dastur (1926) reported the occurrence of foot-rot was caused by *Phytophthora parasitica*.

The attack of *P. parasitica* on the roots reduces significantly the number of functional roots which leads to wilting of the plant and finally reduction in yield. *Phytophthora* species generally composed of tube-like (coenocytic), in contrast to the septate hyphae of fungi (Singh *et al.*, 2006).

The fungus seems to have a poor competitive saprophytic ability and this contributes to the lack of success in isolating and growing of *P. parasitica* in an artificial medium. In order to culture the fungi in the laboratory, it is necessary to supplement suitable medium, temperature, pH and essential elements (carbon and nitrogen) need for growth and other metabolic processes. Hence, different media, temperature, pH, relative humidity, carbon and nitrogen sources were tried in the

investigation to select the best medium and other suitable condition for growth of pathogen.

Material and Methods

Isolation and pure culture of the Foot rot associated pathogen

Direct tissue isolation technique was employed for isolation *Ph. parasitica* on V8 media supplemented with antibiotics such as pimaricin (100 µg/ml), penicillin (50 µg/ml), polymixin B (50 µg/ml) and rifampicin (10 µg/ml) as described by Drenth and Sendall (2001). Stem/collar portion showing actively progressing lesions were used for isolation of pathogen. The infected stem portions were washed thoroughly in tap water. The samples were then cut into small pieces having infected and healthy portion. Surface sterilised with 0.1 per cent sodium hypochlorite solution for 60 seconds and then washed thoroughly for three to four times in sterile distilled water under aseptic condition. The samples were then placed aseptically in sterile Petri plates containing medium and incubated at 25 ± 2 ^{o}C for 8-10 days. Pure cultures of the fungi were obtained by hyphal tip isolation method.

Effect of different solid media on the growth of *Phytophthora parasitica*

Growth characters of *Phytophthora parasitica* on ten different media *viz.*, Potato dextrose agar, V-8 Juice agar, Oat meal agar, Malt extract agar, Corn meal agar, Carrot agar, Sabouraud's agar, Richards's agar, Czapek's Dox agar and Glucose aspergine agar. All the media were sterilized in autoclaved. To carry out the study, 15 ml of each of the medium was poured in 90 mm Petri plates. Such Petri plates were inoculated with 5 mm disc cut from periphery of actively growing cultures and incubated at 23±1^{o}C. Each treatment was replicated thrice. Observations like colony size, mycelial color and substrate color, margin of the colony and topography of the colony were recorded at eight days after inoculation. The effect of media on radial growth was analyzed statistically. The best performing media was used in further experiments.

Studies on growth rate of isolated pathogen in liquid media

From the previous experiment, the solid media that support the good growth of the pathogen was selected for growth studies, but as broth. Twenty five ml of respective liquid media was pipetted into each of the 100 ml conical flasks. The broths were subjected to Autoclaving. The mycelial bits from the pure culture were inoculated to the conical flask containing the broth and incubated up to 20 days at 23±1^{0}C. A set of three flasks were harvested at every 2 days interval to know the growth curve. Cultures were filtered through Whatman No. 1 filter paper. The mycelial mat on the filter paper was thoroughly washed with distilled water to leach out any salts associated with the mycelium. Subsequently, the filter papers along with mycelial mat were dried in hot air oven at a temperature of 45± 5 ^{o}C and dry mycelial weight was recorded.

Effect of temperature on growth of *Phytophthora parasitica*

Temperature range *i.e*. 15, 20, 25, 30, 35 and 40 ^{o}C were tried to see the effect on the growth of the pathogen. 25 ml of sterilized medium was dispensed in 100 ml conical flask and inoculated aseptically with 5 mm disc of the pathogens from a seven days old culture. Conical flasks were incubated at different temperature and each treatment was replicated four times. At the end of eight days of incubation, the mycelia of the pathogens were harvested and dried in hot air oven as

explained earlier. Finally dried mycelium weight was recorded.

Effect of pH on the growth of pathogen

Different media pH of 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 were used. The culture was inoculated to each of 100 ml flask containing 25 ml of basal medium. Inoculated flasks were incubated at 23±1 ^{o}C for eight days, four replications were maintained for each treatment. Dry mycelial weight of the fungus was recorded.

Effect of relative humidity on the growth of pathogen

Different levels of relative humidity *viz.*, 60, 65, 70, 75, 80, 85 and 90 per cent were tried against the growth of pathogen by dissolving 38, 36, 33, 30, 27, 23 and 18 per cent concentrated sulphuric acid (H_2SO_4) respectively in the desiccators containing distilled water. Twenty five ml of sterilized broth medium was dispensed in 100 ml conical flask and inoculated aseptically with 5 mm disc of the pathogen from a seven days old culture. The conical flasks were kept in the desiccators and were incubated at ambient temperature. Three replications were maintained for each treatment. At the end of eight days of incubation, the mycelia were harvested as explained earlier and data were recorded.

Effect of different carbon sources on the growth of *Phytophthora parasitica*

The quantity of each carbon compound to be added was determined on the basis of their molecular weight, so as to provide equivalent amount of carbon as that of carbon compound present in the basal medium. The carbon compounds used were glucose, fructose, xylose, sodium carbonate, L-asparagine, calcium carbonate and sucrose. 25 ml of each media were poured into 100 ml flasks and autoclaved. Each treatment was replicated thrice. All the flasks were aseptically inoculated with 5 mm mycelial disc of seven days old culture and incubated at 23± 1 ^{o}C for eight days. At the end of eight days of incubation, the mycelia were harvested as explained earlier and recorded the dry mycelial weight.

Effect of different nitrogen sources on the growth of *Phytophthora parasitica*

The quantity of each nitrogen compound to be added was determined on the basis of their molecular weight, so as to provide equivalent amount of nitrogen as that of nitrogen compound present in the basal medium. The nitrogen compounds used were ammonium phosphate, calcium nitrate, L-glutamic acid, L-arginine, potassium nitrate, ammonium sulphate and sodium nitrate. Twenty five ml of each media were poured into 100 ml flasks and autoclaved at 1.05 kg cm^{-2} pressure with a temperature of 121.6 ^{o}C for 15 min. Each of the treatment was replicated thrice. All the flasks were aseptically inoculated with 5 mm mycelial disc of seven days old culture and incubated at 23± 1 ^{o}C for eight days. At the end of eight days of incubation, the mycelia were harvested as explained earlier and recorded dry mycelial weight.

Statistical analysis

The results of experiments were analyzed by using the methods as described by Fischer and Yates (1963).

Result and Discussion

In the present study culture of *P. parasitica* showed moderately compact, sparse to densely raised colonies, daisy, porcelain to white mycelial growth with uniform to wavy margin when grown on different medium (Table 1 and Plate 1). Morphometric studies revealed that, the size of sporangium varies

from 10.80-32.38 µm × 7.14-24.04 µm arising from the sporangiophore (19.23 µm to 46.45 µm in length). Aerial hyphae were long, sparingly branched (6.76 µm to 68.52 µm distance between the branches), uniform in diameter (2.90 µm). Sporangial shapes were pear, ovoid and spherical in different isolates and they were papillate. On the basis of study of cultural and morphometric characteristics, it was concluded that the species closely resembled *P. parasitica* as characterised by Ribeiro (1978).

Among 10 solid media evaluated against *P. parasitica*, the maximum growth of fungus was observed in czapeck dox agar (86.50 mm) at eight days of incubation followed by Sabouraud's agar (85.30 mm). Whereas the minimum growth of the pathogen was recorded on malt extract agar with a colony diameter of 41.70 mm (Table 1 and Plate 1). These results are in contradiction to findings of Rao *et al.,* (1962) who recorded the maximum growth of *P. parasitica* Dast. var. *macrospora* Ashby on rice meal agar (85 mm) followed by oat meal (76.55 mm) and minimum mycelium growth in czapek's dox agar (10 mm). Whereas Padmaja, *et al.,* (2015) obtained the best growth of *P. colocasiae* on carrot agar (86 mm), followed by papaya sucrose agar (80.6mm) and less growth of the pathogen was recorded on PDA (22 mm).

Growth phase study was conducted to know the progress of pathogen growth on liquid media over a time. For this experiment solid media that supported the good growth of *P. parasitica* was selected as liquid media to record the dry mycelial weight of the pathogen. Czapeck dox agar that supported the good growth of the pathogen, so this medium selected to assess the dry mycelial weight of the pathogen. Maximum dry weight (307.13 mg) of *P. parasitica* was recorded at 8th day after incubation at 23±1 ^{o}C (Table 2). *Phytophthora parasitica* grow at wide range of temperature ranging from 15-40 ^{o}C. But the maximum mean dry mycelium weight (330.38 mg) was observed at 20 ^{o}C after eight days of incubation, which is considered to be optimum temperature for the better growth of the pathogen, followed by 25 ^{o}C (250.05 mg) (Table 3 and Plate 2). The least growth of the pathogen was observed at 40 °C (102.98 mg). These results were in conformity with the findings of Mounde *et al.,* (2012) who reported that the colony diameter of 55 mm and 0 mm for *P. citrophthora* was observed at 24 ^{o}C and 35 ^{o}C respectively. Mbong *et al.,* (2015) reported that optimum temperature for maximum growth of pathogen was 24 ^{o}C (72.30 mm).

Maximum growth of the *P. parasitica* was recorded at pH 8 (377.00 mg) which was significantly superior to all other pH range followed by pH 7 (243.38 mg) and least growth of the fungus was observed at pH 4.0 (136.13 mg) (Table 4 and Plate 3).

The results of the present study are in accordance with the earlier findings of Gaston *et al.,* (2014) who observed that the pH ranging from 6 to 9 support better radial growth of *P. colocasiae*. Mbong *et al.,* (2015) reported that among the different pH levels, ranging from 6 to 8 supported the good growth of the *P. colocasiae* but the maximum growth was observed at pH 7 (85.30 mm) and least growth was recorded at pH 4 (66.00 mm).

Better growth of *P. parasitica* was observed at 90 per cent RH with 177.40 mg of mycelial dry weight followed by 85 per cent (166.13 mg) and least growth was at 60 per cent (119.23 mg) (Table 5 and Plate 4). These results were in line with the findings of Naik *et al.,* (2016) that *R. solani* flourished well at 80 and 90 per cent RH with colony diameter of 89.27 mm and 89.20 mm and least growth of the pathogen was noticed at 60 per cent (80.31 mm).

Table.1 Radial growth and Morphological characteristics of *Phytophthora parasitica* on different media

Sl. No.	Medium	Mean mycelial diameter (cm) *Phytophthora parasitica*	Colony margin/edge	Pigmentation	Colony color	Aerial mycelium
1	Carrot agar	8.15	Wavy	No	White	Undulated / raised
2	Corn meal agar	7.77	Smooth / uniform	No	Daisy	Sparse
3	Czapek dox agar	8.65	Wavy	No	White	Raised
4	Glucose aspergine agar	4.18	Uniform	No	Porcelain	Sparsely raised
5	Malt extract agar	4.17	Uniform / smooth	Short bread	Porcelain	Sparse
6	Oat meal agar	8.18	Uniform / smooth	Tuscan sun	White	Densely raised
7	Potato dextrose agar	5.45	Uniform / smooth	Dandelion	White	Sparsely raised
8	Richrd's agar	7.40	Wavy	No	Cotton	Densely raised
9	Sabouraud dextrose agar	8.58	Wavy	Tiger	Porcelain	Sparse
10	V8 juice agar	6.78	Uniform	No	White	Cottony cushion densely raised
	S. Em ±	**0.13**				
	CD (0.01)	**0.53**				
	CV (%)	**3.31**				

Table.2 Growth rate of *Phytophthora parasitica* on czapek dox broth

Sl. No.	Days after incubation	Mean mycelial dry weight (mg)
1	02	81.57
2	04	90.27
3	06	96.83
4	08	307.13
5	10	173.25
6	12	153.80
7	14	130.00
8	16	201.30
9	18	189.33
10	20	264.80
	S. Em ±	**3.18**
	CD (0.01)	**12.80**
	CV (%)	**3.26**

Table.3 Effect of different temperature on the growth of *Phytophthora parasitica*

Sl. No.	Temperature (^{o}C)	Mean mycelial dry weight (mg)
1	15	227.78
2	20	330.38
3	25	250.05
4	30	156.88
5	35	142.65
6	40	102.98
	S. Em ±	**2.37**
	CD (0.01)	**9.65**
	CV (%)	**2.34**

Table.4 Effect of different pH levels on the growth of *Phytophthora parasitica*

Sl. No.	pH levels	Mean mycelial dry weight (mg)
1	4	136.13
2	5	203.13
3	6	205.23
4	7	243.38
5	8	377.00
6	9	219.35
	S. Em ±	**1.92**
	CD (0.01)	**5.21**
	CV (%)	**1.11**

Table.5 Effect of relative humidity on growth of *Phytophthora parasitica*

Sl. No.	Relative humidity (%)	Mean mycelial dry weight (mg)
1	60	119.23
2	65	128.50
3	70	138.83
4	75	144.73
5	80	149.50
6	85	166.13
7	90	177.40
	S. Em ±	**2.18**
	CD (0.01)	**9.19**
	CV (%)	**2.58**

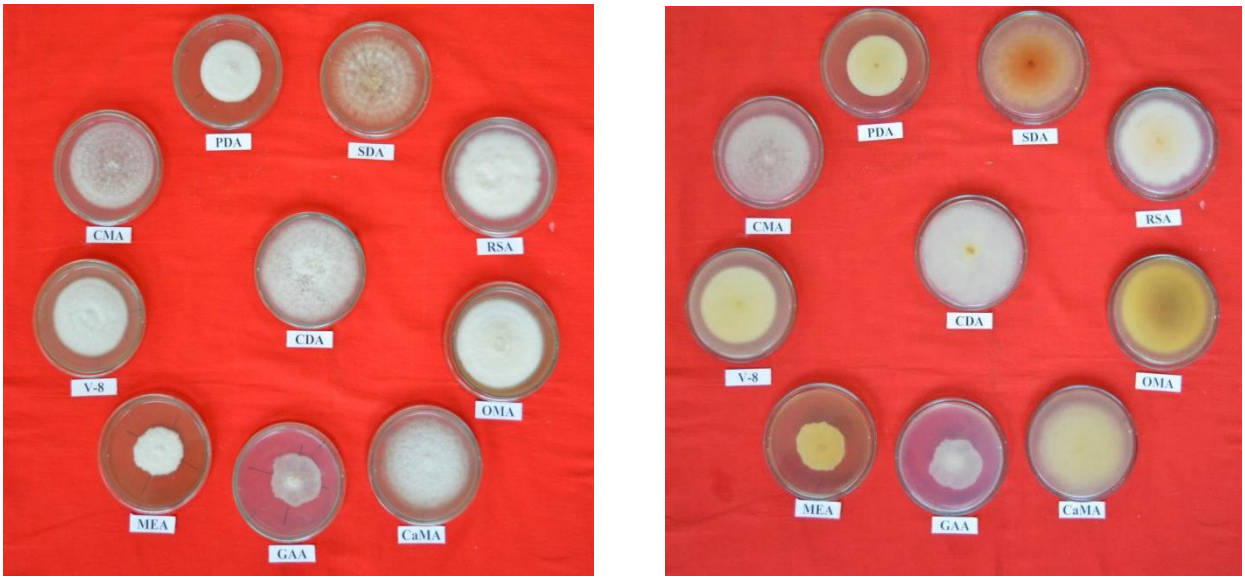
Table.6 Effect of different carbon sources on growth of *Phytophthora parasitica*

Sl. No.	Carbon source	Mean mycelial dry weight (mg)
1	Calcium carbonate	0.00
2	Fructose	318.73
3	Glucose	339.13
4	L- asparagine	481.70
5	Sodium carbonate	442.03
6	Sucrose	396.10
7	Xylose	286.80
	S. Em ±	**4.67**
	CD (0.01)	**19.67**
	CV (%)	**2.50**

Table.7 Effect of different nitrogen sources on growth of *Phytophthora parasitica*

Sl. No.	Nitrogen source	Mean mycelial dry weight (mg)
1	Ammonium phosphate	132.27
2	Ammonium Sulphate	206.07
3	Calcium nitrate	282.70
4	L- arginine	165.87
5	L- glutamic acid	169.03
6	Potassium nitrate	349.17
7	Sodium nitrate	199.07
	S. Em ±	**2.24**
	CD (0.01)	**9.43**
	CV (%)	**1.80**

Plate.1 Growth of *Phytophthora parasitica* on different media



A. Mycelial growth **B. Pigmentation**

Plate.2 Growth of *Phytophthora parasitica* at different temperature

Plate.3 Growth of *Phytophthora parasitica* at different hydrogen ion concentration (pH)

Plate.4 Growth of *Phytophthora parasitica* at different levels of relative humidity (RH)

Plate.5 Growth of *Phytophthora parasitica* on different carbon sources

Plate.6 Growth of *Phytophthora parasitica* on different nitrogen sources

Among different carbon sources evaluated, better growth of *Phytophthora parasitica* was recorded on L-asparagine (481.70 mg), followed by sodium carbonate (442.03 mg) and no growth was recorded in calcium carbonate (0.00 mg) eight days after incubation (Table 6 and Plate 5). This result was contradictory with the findings of Shepherd and Pratt (1973) who found out of 37 carbon sources on growth of *P. nicotianae* var. *nicotianae*. The result revealed that maximum dry weight of mycelium was supported by sucrose (42.70 mg) followed by raffinose (42.2 mg), melezitose (42.00 mg), amylose (40.50 mg) and maltose (36.80 mg). Whereas, the least growth of pathogen was recorded in cellulose (1.5 mg).

Among seven nitrogen sources tested in the present investigation, maximum mean dry mycelial weight of *P. parasitica* was observed in potassium nitrate (349.17 mg) followed by calcium nitrate (282.70 mg). Whereas, least mean dry mycelial weight was recorded in ammonium phosphate (132.27 mg) after eight days of incubation (Table 7 and Plate 6). These results are in contradiction to findings of Peries *et al.,* (1979) who reported that DL-asparagine (89 mg dry mycelium weight), DL- alanine (100 mg), L (+) glutamic acid (83 mg), L (-) histidine (85 mg), L (-) proline (111 mg) and L (-) serine (89 mg) were the best nitrogen sources for the growth of the *P. meadii*. But for *P. palmivora* DL- asparagine (90 mg), L (+) aspartic acid (92 mg), L (-) arginine (95 mg) and L (-) proline (96 mg of dry mycelium weight) supported the growth of the pathogen. These findings revealed that the nitrogen requirement vary from species to species.

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