Journal of Spices and Aromatic Crops
Vol. 15 (2) : 81–87 (2006)

Indian Society for Spices

Variability in *Phytophthora capsici* (Leonian) isolates of black pepper (*Piper nigrum* L.) based on nutritional preferences and isozyme profiles

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Received 29 December 2005; Revised 31 May 2006; Accepted 07 July 2006

Abstract

Experiments were conducted to study the intraspecific variability among five isolates of black pepper (*Piper nigrum*) foot rot pathogen, *Phytophthora capsici*, in its nutritional preferences and isozyme profiles. The carbon sources most suited for the vegetative growth of the isolates were sucrose and glucose, followed by starch, fructose, cellulose and maltose. Among the nitrogen sources, the L-isomers of the amino acids, glutamate, proline, aspartate, asparagine, histidine, serine, arginine and glycine, and also the organic sources of nitrogen, urea, yeast extract, casein hydrolysate, peptone and tryptone supported good growth. Among the vitamins studied, only thiamine was essential for vegetative growth of *P. capsici*. A considerable degree of variation was observed among the five isolates in their preferences for nutrient sources for their optimal growth. From the isozyme profiles of esterase, acid phosphatase, superoxide dismutase and catalase systems, the similarity between the isolates ranged between 25.0%–44.7%.

Keywords: black pepper, intraspecific variation, isozymes, *Phytophthora capsici*, *Piper nigrum*.

Introduction

Phytophthora capsici (Leonian) is one among the 43 species known to exist in this remarkable genus (Waterhouse *et al.* 1983). *P. capsici*, the pathogen of black pepper (*Piper nigrum* L.), had previously been described as *P. palmivora* 'MF 4'. Based on extensive studies on comparative morphology and physiology, it was proposed to merge the two species and redesignate it as *P. capsici* Leonian emend, Alizadeh and Tsao (Tsao & Alizadeh 1988). *P. capsici* is the causative agent of foot rot, the most destructive of the diseases reported on black pepper (Sarma *et al.* 1991).

Intraspecies variability is common in *Phytophthora* spp. Oudemans & Coffey (1991) used isozyme analysis to examine intraspecific diversity in isolates of *P. capsici*, including those previously identified as *P. palmivora* 'MF4', and separated these into three subgroups, namely, CAP1, CAP2 and CAP3. The subgroup species of CAP1 were widely distributed geographically on a range of hosts including *Capsicum* spp., tomato, cucurbits, cocoa and black pepper and contained the greatest amount of diversity. CAP2 isolates were found primarily on black pepper and CAP3 isolates were the most geographically restricted. This study attempts to understand the variability among five isolates of *P. capsici*,

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isolated from black pepper plants, based on their nutrient preferences and isozyme polymorphism.

Materials and methods

Intraspecies variability based on nutrient preferences

Five isolates of *P. capsici* were selected from the National Repository of *Phytophthora* maintained at the Indian Institute of Spices Research (ICAR), Calicut (Table 1).

Table 1. Source of *Phytophthora capsici* isolates

Isolate	Source of the isolate	Place of collection
P128	Rhizosphere	Kodagu, Karnataka
P57	Root	Wayanad, Kerala
P148	Collar	Ponnumbeta, Karnataka
P158	Stem	Sirsi, Karnataka
P200	Leaf	Bhagya, Karnataka

All the isolates were tested for pathogenicity on black pepper by artificial inoculation and found virulent, with marginal variation in virulence, purified on selective antibiotic PVPH medium (Tsao 1970) and maintained on 20% carrot agar medium (Ribeiro 1978).

Two discs of 5 mm diameter, obtained from margins of 72 h old cultures were incubated at 25°C in 100 ml conical flasks containing 25 ml of Bartnicki-Garcia liquid medium (Bartnicki-Garcia 1966). The carbon and nitrogen sources and vitamins of the original media were substituted by the nutrient sources under study. The pH of the medium was adjusted to 6.5 using 6N KOH, for all treatments. The media were sterilized in 20 ml volumes at 121°C and 1.5 kg cm^{-2} pressure for 20 min. Later 5 ml of sterilized 10 x glucose solution was measured into each flask aseptically. The glucose solution was autoclaved separately since toxic substances have been reported to be formed when glucose and any one of several amino acids including asparagine are autoclaved together (McKeen 1956). Four replications were maintained for each treatment. After 10 days of incubation, the dry weight of mycelia was recorded.

The carbon sources tested were, the pentoses: D-arabinose, D-ribose and D-xylose; the hexoses: D-fructose, D-glucose and D-galactose; the disaccharides: sucrose, maltose and lactose; the polysaccarides: starch, cellulose and pectin; the carboxylic acids: glycerol, D-mannitol and D-sorbitol; the organic acids: lactic acid, malonic acid, fumaric acid, succinic acid and L (+) tartaric acid. All these carbon sources were used in amounts that contained 8 g of carbon, equivalent to the concentration in the original Bartnicki-Garcia media, which contains 20 g l^{-1} of glucose. All the treatments were compared against a control lacking carbon source.

The nitrogen sources tested were, L- stereoisomers of the amino acids: alanine, aspartic acid, arginine, asparagine, cysteine, cystine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan and valine; and the organic sources: urea, casein hydrolysate, peptone, tryptone and yeast extract. The inorganic sources tested were, ammonium chloride, ammonium nitrate, ammonium phosphate, ammonium sulfate, calcium nitrate, potassium nitrate and sodium nitrate. All the nitrogen sources were used in amounts equivalent to nitrogen in 2 g of asparagine; the polymers casein hydrolysate, peptone, tryptone and yeast extract were used at 2 g l^{-1}. The dry weight of mycelia of the treatments was compared with a control lacking nitrogen sources.

The B vitamins tested were, thiamine, riboflavin, nicotinic acid, pyridoxine and inositol. A combination of the above vitamins, all at 1 mg l^{-1} concentration were tried, namely, all the above five vitamins; all but thiamine; all but riboflavin; all but nicotinic acid; all but pyridoxine and all but inositol. All glassware were washed with chromic acid and rinsed with distilled water before use to check contamination with traces of vitamins. All the above treatments were compared against a control lacking vitamins.

Intraspecific variability based on isozyme profile

Five discs of 10 mm each, of the five isolates of *P. capsici*, maintained on 20% carrot agar and cut from the margins of a 72 h old culture, were incubated in 100 ml volumes of Bartnicki-Garcia medium for 7 days. The resulting mycelial mat (3 g) was homogenized in 5 ml of 0.05M Tris. HCl buffer at pH 7.4, containing cysteine. HCl 0.1%, ascorbic acid 0.1% and sucrose 17%. The homogenate was centrifuged at 26,000 x g for 30 min. The above extraction was done at 4°C and the supernatant used for isozyme studies.

Polyacrylamide gel electrophoresis (PAGE) was performed in the mini-dual model of the Genei vertical slab gel electrophoresis system (Genei, India). The dimensions of the gel were 8 x 7 x 0.1 cm. The separations were performed on a 2.5% stacking gel consisting of 0.5M Tris. HCl, pH 6.8 buffer and 7.5% resolving gel consisting of 3M Tris. HCl, pH 8.8 buffer. The reservoir buffer contained 0.025M Tris and 0.19M glycine, pH 8.3, according to the method of Hames (1994). The extract (50 µl) was loaded and separation was carried out at a constant 70 v until proper stacking was achieved and then at 150 v, until the tracking dye reached the end of the gel. The staining procedures were those of Harris & Hopkinson (1976) for catalase (EC 1.11.1.6) and esterase (EC 3.1.1.2); of Ravindranath & Fridovich (1975) for superoxide dismutase (SOD) (EC 1.15.1.1); and of Sadasivam & Manikam (1992) for acid phosphatase (EC 3.1.3.2). After staining, the gels were fixed in acetic acid 7% and the bands marked, Em values calculated and zymograms constructed from the average of three replications for each isolate. The paired affinity indices (PAI) for the five isolates were calculated from the ratio between the number of shared bands between two isolates and the total number of bands (Payan & Dickson 1990). The per cent similarity between the isolates based on all the four isozymes was calculated from the per cent of the sum of individual PAI values.

Results and discussion

Nutrient preferences

P. capsici isolates preferred the carbon sources sucrose, glucose, fructose, starch, cellulose and maltose for their vegetative growth; xylose was less effectively used while other carbon sources like sodium citrate did not support growth at all. The preference for source of carbon of the five isolates, in descending order was:

P128: Sucrose, glucose, starch, fructose, xylose, maltose, cellulose.

P57: Sucrose, starch, fructose, glucose, cellulose, maltose, xylose.

P148: Sucrose, glucose, starch, maltose, fructose, cellulose, xylose.

P158: Sucrose and fructose, starch, glucose, maltose, cellulose, xylose.

P200: Sucrose, glucose, fructose, starch, cellulose, maltose, xylose.

P128 used sucrose, glucose, starch and fructose equally well. P128 and P57 exhibited vegetative growth ~12 times greater in sucrose compared to control. P148 and P158 grew about 10 times more in sucrose. In sodium citrate, these isolates grew by less than half compared to control. Compared to glucose, sucrose supported over 6%–11% more mycelial growth. P200 used sucrose about 5% better than either glucose or fructose and 13 times greater growth than in control. Growth of P200 in sucrose, glucose, fructose and starch were on par.

There was variability between the isolates in their preference for nitrogen sources as well. Glutamate, proline, aspartate, asparagine, histidine, serine, arginine and glycine were able to serve as sole nitrogen sources. Glutamine, phenylalanine, threonine and valine were less effective. Cysteine, Lys, Leu and Trp were the least effective. Among the inorganic sources of nitrogen, the nitrates of sodium, calcium and potassium were the best, the rest being less effective. All the polymers, urea, yeast extract, peptone, tryptone and casein hydrolysate were also good nitrogen sources. P200 exhibited good growth in the media containing threonine, while other isolates did not prefer this amino acid. P57 grew equally well in Pro, urea, Asp and Glu, dis-

playing over 13 times as much growth as in control; it did not prefer calcium nitrate, potassium nitrate, yeast extract and threonine, while the other three isolates did not prefer threonine alone for their growth. P128 and P148 grew the most in the presence of Glu and Pro, over 12–17 times that of control. P158 grew least on Trp.

Among the B vitamins, only thiamine was found essential for vegetative growth of *P. capsici* isolates and growth of all the isolates were equally poor in both control and the treatment containing all vitamins except thiamine. Vitamin requirement did not vary much among isolates (Table 2).

In summarising this study, xylose scored better than the one reported by Roncadori (1965), where it supported only traces of growth in different species of *Phytophthora*. This study is largely in accordance with the review by Hohl (1983) on the nutrition of *Phytophthora*, where the best carbon sources have been identified as sucrose and glucose, followed by fructose, maltose and starch and that hexoses were generally preferred; the best nitrogen sources were the L- isomers of asparagine, arginine, proline, alanine, histidine, serine, glycine, threonine, glutamate, aspartate and glutamine and of the inorganic sources - nitrates. The ability to better utilize the oxidized form of nitrogen, as seen in our isolates, compared to the reduced form in ammonium salts, is considered a primitive trait by Cantino & Turian (1959). That only thiamine is required of the B vitamins is supported by the report of Cameron (1966) for the genus *Phytophthora*.

Isozyme profile

Isozymes separated by native discontinuous gel electrophoresis gave distinct patterns for esterase (Fig. 1) and acid phosphatase (Fig. 2). A total of 15 distinguishing bands were present in the acid phosphatase zymogram and the esterase zymogram had six distinguishing bands. Catalase (Fig. 3) and superoxide dismutase (Fig. 4) zymograms grouped the five isolates into two–one containing P128 and 200 and the other of P57, 148 and 158. In the SOD zymogram, the isolates P57, 148 and 158 were distinguished by the presence of an additional anodic band of Em 0.618. The paired affinity indices (per cent similarity) were worked out for the five isolates, based on the above isozyme phenotypes (Table 3). It varied between a low of 25.0% among the isolates P128 and 158 and 26.2% between P128 and 148, to a high of 44.7% between P148 and 158 and 44.4% between P57 and 148. The similarity between the other pairs of isolates were of intermediate values.

Table 2. Effect of combination of vitamins on vegetative growth of isolates of *Phytophthora capsici*

Treatment	Isolate					Mean
	P128	P57	P148	P158	P200	
Control (no vitamins)	0.024	0.029	0.022	0.024	0.023	0.024
All 5 vitamins	0.191	0.202	0.234	0.195	0.192	0.203
All but thiamine	0.024	0.025	0.026	0.023	0.023	0.024
All but riboflavin	0.214	0.200	0.240	0.221	0.169	0.209
All but nicotinic acid	0.181	0.198	0.238	0.220	0.192	0.206
All but pyridoxine	0.182	0.191	0.242	0.210	0.191	0.203
All but inositol	0.152	0.195	0.237	0.213	0.185	0.196
Mean	0.138	0.149	0.177	0.158	0.139	
CD (P=0.05) Within isolates	0.007	0.012	0.003	0.007	0.011	

CD (P=0.05) Between isolates=0.003, p<0.0000; Between treatments=0.003, p<0.0000
Values indicate dry weight of mycelium in g

Table 3. Paired affinity indices (% similarity) between five isolates of *Phytophthora capsici* based on acid phosphatase, esterase, catalase and SOD isozyme profiles

Isolate	P128	P57	P148	P158	P200
P128	-	16/47(34.0%)	11/42(26.2%)	10/40(25.0%)	12/42(28.6%)
P57		-	20/45(44.4%)	17/43(39.5%)	14/44(31.8%)
P148			-	17/38(44.7%)	13/39(33.3%)
P158				-	13/37(35.1%)
P200					-

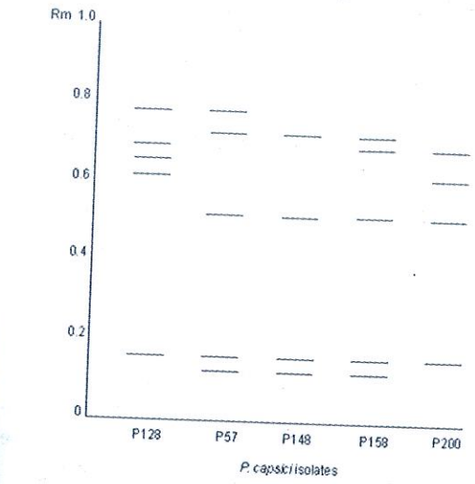

Fig. 1. Esterase zymogram of *Phytophthora capsici* isolates

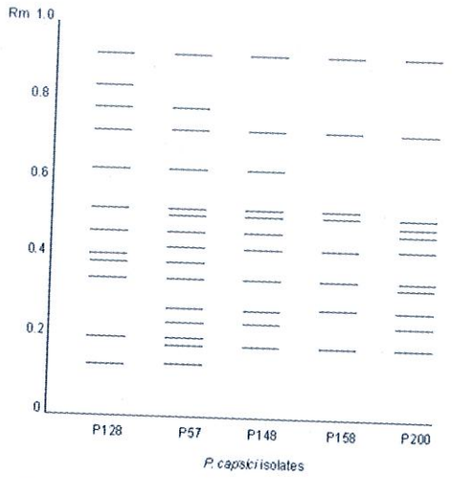

Fig. 2. Acid phosphatase zymogram of *Phytophthora capsici* isolates

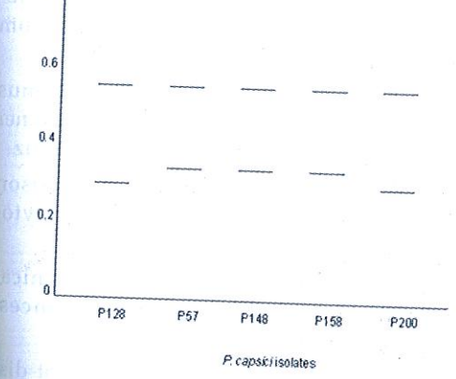

Fig. 3. Catalase zymogram of *Phytophthora capsici* isolates

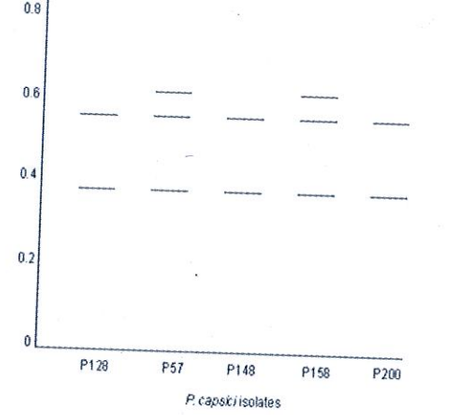

Fig. 4. Superoxide dismutase zymogram of *Phytophthora capsici* isolates

Intraspecific variability between *P. capsici* isolates could be quantified with greater accuracy using the isozyme profiles. Sarma & Nambiar (1982) have already described the morphology of *P. capsici*. Though this study comprised of only a small population size, the objective was to differentiate between isolates from different sources. The reasonable degree of variability noticed among the five isolates based on their isozyme profiles of four enzyme systems, helped confirm those based on their nitrogen nutrient preferences. The uniqueness of isolate P200 from the rest of the isolates based on the nutrition studies, was in conformity with the results from the isozyme profiles, as it showed only low to moderate similarity with the remaining isolates. Several workers have conducted simi-

lar studies on *Phytophthora* spp. variability based on isozyme polymorphism (Nwaga *et al.* 1990; Oudemans & Coffey 1991; Mosa *et al.* 1993).

According to Roncadori (1965), it is doubtful whether preferences for carbon sources can be used for species separation since variation was noticed between both isolates of same species and occasionally among replicates of an isolate. Although the present study has demonstrated variability among *P. capsici* isolates, these isolates did not differ in their virulence on black pepper. Probably more hosts and cultivars should be tested for their reaction to these isolates to find out possible variability in their virulence also.

Acknowledgments

The authors are grateful to the University Grants Commission for funding this programme and to the Indian Institute of Spices Research, Calicut, for providing the facilities.

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