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Analysis of diversity of *Phytophthora* species prevalent on some common economically important crops through morphological and molecular methods

***SANJOY GUHA ROY[1], SAMIR KUMAR MUKHERJEE[1] AND SOMNATH BHATTACHARYYA[2]**
[1]*Department of Microbiology, University of Kalyani, Kalyani 741235, W. B.*
[2]*Crop Research Unit, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur 741252, Nadia*

Phytophthora, oömycetous organism with about, 70 recognised species is one of the most destructive of plant pathogens affecting a wide host range consisting of economically important crops having a widespread occurrence in the wet tropical regions; and as a result, almost a third of the *Phytophthora* species reported worldwide are from India. Identification and taxonomy of *Phytophthora* is still acknowledged to be 'difficult' primarily due to its morphological plasticity.

An attempt was made, therefore, to identify and characterise the *Phytophthora* population prevalent on some of the common high value economically important crops grown in this region; brinjal, betel vine, guava, sesame, roselle, chilli, black pepper, pointed gourd and taro both through molecular methods like Restriction Fragment Length Polymorphism (RFLP) of the internal Transcriber Spacer (ITS) region of rDNA, sequencing of the ITS region and also on morphological parameters. The *Phytophthora* species prevalent on these crops, which were identified by the above mentioned molecular methods are *P. melonis*, *P. nicotianae*, *P. colocasiae* and *P. capsici*. Multiple occurrences of different *Phytophthora* species on a single crop were also observed. Data on all of the above-mentioned aspects of the isolates, twenty-six in number, under accession at World *Phytophthora* Collection, (WPC), USA were discussed.

Key words : *Phytophthora*, population diversity, high value crops, rDNA ITS sequencing, *P. nicotianae*, *P. capsici*, *P. colocasiae*, *P. melonis*. fungicide sensitivity, cluster analysis

INTRODUCTION

Phytophthora, the stramenopile, oomycetous 'plant destroyer', is one of the most destructive genera of plant pathogens in temperate and tropical regions, causing annual damages of billions of dollars (Drenth and Guest, 2004a). There are more than 70 species in the genus *Phytophthora*; all of them plant pathogenic, causing diseases on thousands of plant species in a wide range of ecological niches (Erwin and Riberio, 1996). While about a third of the total established species of *Phytophthora* known from earlier morphological descriptions have been reported from India alone, yet no proper survey had been undertaken till date to assess the diversity of these pathogens, which is desirable, if there is to be an effective management of these diseases.

With this objective in view a survey of *Phytophthora* diseases has been undertaken affecting some common high value crops, namely Betelvine, Brinjal, Guava, Sesame, Roselle, Chilli, Black pepper, Pointed gourd and Taro growing in the lower gangetic plains of Bengal which is a major vegetable growing region of the country. Identification and assessment of diversity is done on morphological, cultural and physiological parameters. As identification of *Phytophthora* has been acknowledged to be taxonomically difficult (Brasier *et al.*, 1981; Erwin, 1983; Brasier, 1991), the identification based on morphological criteria are corroborated by using an rRNA ITS (Internal

Present Address : * *Department of Botany, Ramananda College, Bishnupur 722122, Bankura, W. B. India.*
* *(Corresponding author: s_guharoy@yahoo.com)*

Transcriber Spacer) region phylogenetic system (Cooke and Duncan 1997; Cooke *et al.*, 2000a) which is the most comprehensive till date (Martin and Tooley, 2003) and done as before and described elsewhere (Guha Roy *et al.*, 2006a).

Further more with the objective of providing a basis for effective control of the polyphagous species *P. nicotianae*, which is the most prolific in this region as, evidenced by this study in contrast to SE Asia, where *P. palmivora* is the most recorded pathogen (Drenth and Guest, 2004b); *in vitro* sensitivity of *P. nicotianae* isolates from different hosts to different fungicides are also assessed in continuance to an earlier study. (Guha Roy *et al.*, 2003).

In order to assess stable and more reliable morphometric parameters for species delineation, a cluster analysis of sporangial characters of the *Phytophthora* species under study has been attempted. Intra specific distance of the most abundant species, *P. nicotianae* has also been assessed for possible correlation with host and geographic origin.

MATERIALS AND METHODS

Collection, isolation, morphological parameters and identification of pathogen isolates

Phytophthora isolates were collected from farmer's field in the different agro climatic zones of this region through two cropping seasons. Isolation was done by plating on a modified P_{10}ARP, V_8JA medium (Guha Roy *et al.*, 2006a) and incubated in dark with cellophane overlays for seven days at 26 ±1ºC. Sporangium morphology was checked for by 'agar-disk-in-water' technique (Erwin and Riberio, 1996) and thereafter pure cultures were routinely maintained on V_8JA medium (Riberio, 1978).

Identification of isolates was done following key of Stamps *et al.* (1990). All cultures in this study were accessioned at the International repository for *Phytophthora* cultures the World Phytophthora Collection, USA and some additionally at Virginia State University, USA. Morphological parameters of sporangial length, breadth, length / breadth ratio (Table 1a) and colony characteristics on V_8JA (Fig. 3) were also assessed.

Physiological and interaction parameters

Physiological parameters of growth were measured as colony diameter on two different media; V_8juice (Campbell Soup Co., USA) agar medium and PDA (HiMedia, India) after 72 hours of growth. Other interaction parameters with a biocontrol agent were also assessed using a novel indigenous *Pseudomonas aeruginosa* strain (Sinha and Mukherjee, 2005) having antagonistic property against *Phytophthora sp.* (Guha Roy *et al.*, 2006b). Interaction with live bacteria in dual culture (Jin and Hee, 1989) and inhibition zones of different isolates were measured after 72 hours of growth in the dark at 26±1°C. Final observations from all the replicates were converted to percentage inhibition with respect to the control set (Table1a).

DNA isolation, ITS amplification and molecular identification of pathogen isolates

DNA extraction from the fungal mycelium was done according to Cooke *et al.* (2000a) with minor modifications. DNA extraction, PCR amplification of genomic DNA with primers ITS4 (5' TCCTCCGCTTATTGATATGC 3') and ITS6 (5' GAAGGTGAAGTCGTAACAAGG 3'), visuali-zation and checking of the amplicon as single band of ~900 bp. on 1% agarose gels, restriction digestion of the amplicon with *AluI* & *MspI* to produce a characteristic genus specific profile (Cooke *et al.*, 2000b) were all done as described previously (Guha Roy *et al.*, 2006a).

Sequencing of ITS 1 & ITS 2 regions with primers in reverse ITS 7 (AGCGTTCTTCATCGATGTT GC) and in forward ITS 8 (GCACATCGATGAAG AACGCT) which were located in the 5.8S gene (Cooke, 2000a) were used and the amplicon(s) were sequenced commercially (Sanmar Speciality Chemicals, India) as before (Guha Roy *et al.*, 2006a). The sequences were compared with published GenBank sequences, analysed using BLAST algorithm and accordingly species identification was done. Consequently the ITS1 & ITS2 sequences, twenty in number, were deposited and accessioned in GenBank (Table1b).

Fungicide sensitivity assays

Sensitivity to the protectant fungicides, mancozeb 75% WP (Dithane M-45, DE-NOCIL) and chlorothalonil 75% WP (Kavach, Syngenta); contact fungicides, copper hydroxide 77% WP (Kocide101, Dupont)), copper oxychloride 50% WP (Blitox 50WP, Rallis)) and the organic heterocyclic compound captan 50% WP (Captaf 50WP, Rallis)

were determined *in vitro* by 'poison-agar' technique. *P. nicotianae* isolates were choosen from among different hosts under the present study. Fungicides as formulated commercial products were suspended in sterile water to make suspensions ranging from 50μg a.i./ml to 300μg a.i./ml. These suspensions were then added to V_8JA medium at 4 concentrations, V_8JA medium without fungicide being used as the control. V_8 J agar discs (9 mm in diameter) containing mycelium of different *P. nicotianae* isolates were obtained from the perimeter of an actively growing colony and were placed centrally in a 9 cm petri plate containing V_8 J agar with or without fungicide, with three replications. Growth was measured as colony diameter less the diameter of inoculum discs after 72 hours of incubation at 26 ±1°C. Data were obtained as the proportion (percent) of growth on fungicide amended medium relative to growth on the no-fungicide control. EC_{50} was estimated for each isolate from a regression of relative mycelial growth versus fungicide concentration (Table 2).

Statistical and cluster analysis

All data were analyzed using analysis of variance. For the morphological and physiological parameters a one-way ANOVA was performed (Table 4) and for fungicide sensitivity assays a comparative two factor ANOVA was performed (Table 3).

Sporangial characters (length, breadth, length/ breadth ratio) were taken for cluster analysis (Fig. 1). Five morpho-physio-cultural characteristics (Table 1) were used for a hierarchical cluster analysis within the *P. nicotianae* species from different hosts and geographical origins. Dendrograms were constructed using complete linkage, furthest neighbour technique using Euclidean distances (Fig. 2).

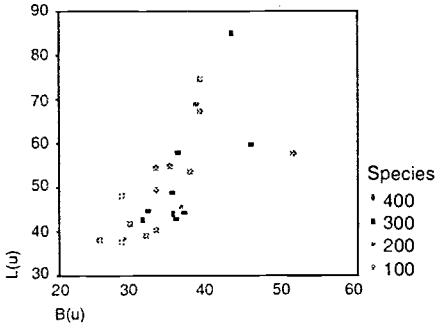

Fig. 1 : Inter species cluster analysis of sporangial characters showing positive correlation for species delimitation among the 4 *Phytophthora sps.* of lower gangetic plains of Bengal.

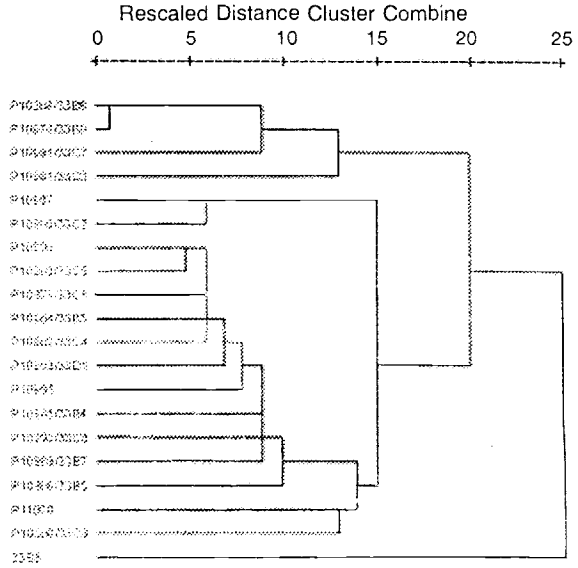

Fig. 2 : Inter specific distance of *P. nicotianae* species isolated from different hosts and geographical regions of lower gangetic plains of Bengal based on morho-physio-cultural parameters.

RESULTS AND DISCUSSION

Identification of pathogens is the first crucial key step in any disease control strategy. Identification of *Phytophthora* species solely on morphological criteria often leads to misidentification, with morphological variants of existing taxa incorrectly assigned as new disease threats and conversely new species being wrongly assigned to current taxa (Chowdappa *et al.*, 2003; Mirabolfathy *et al.*, 2001; Guha Roy *et al.*, 2006a). Identification of species in this study was therefore done by both morphological and molecular methods for purposes of clear scientific communication and practical control.

It was found that the polyphagous species *P. nicotianae* is the most widespread in this region attacking most of the high value crops. (Table 1). *P. nicotianae* affecting *Piper nigrum* (Bell pepper), *P. capsici* on *Capsicum annuum* (chilli) and *P. capsici* affecting *Piper betel* (Betelvine) is a new record for the Eastern region of the country and is being reported here for the first time. The fruit and vine rot disease of pointed gourd caused by *P. melonis* has also just been reported as a first report from India (Guha Roy *et al.*, 2006a) and as regards host range a first report globally.

It is interesting to note that there are multiple occurrences of *Phytophthora* sps. i.e. *P. capsici* and *P. nicotianae* on the betelvine host. (Table 1a)

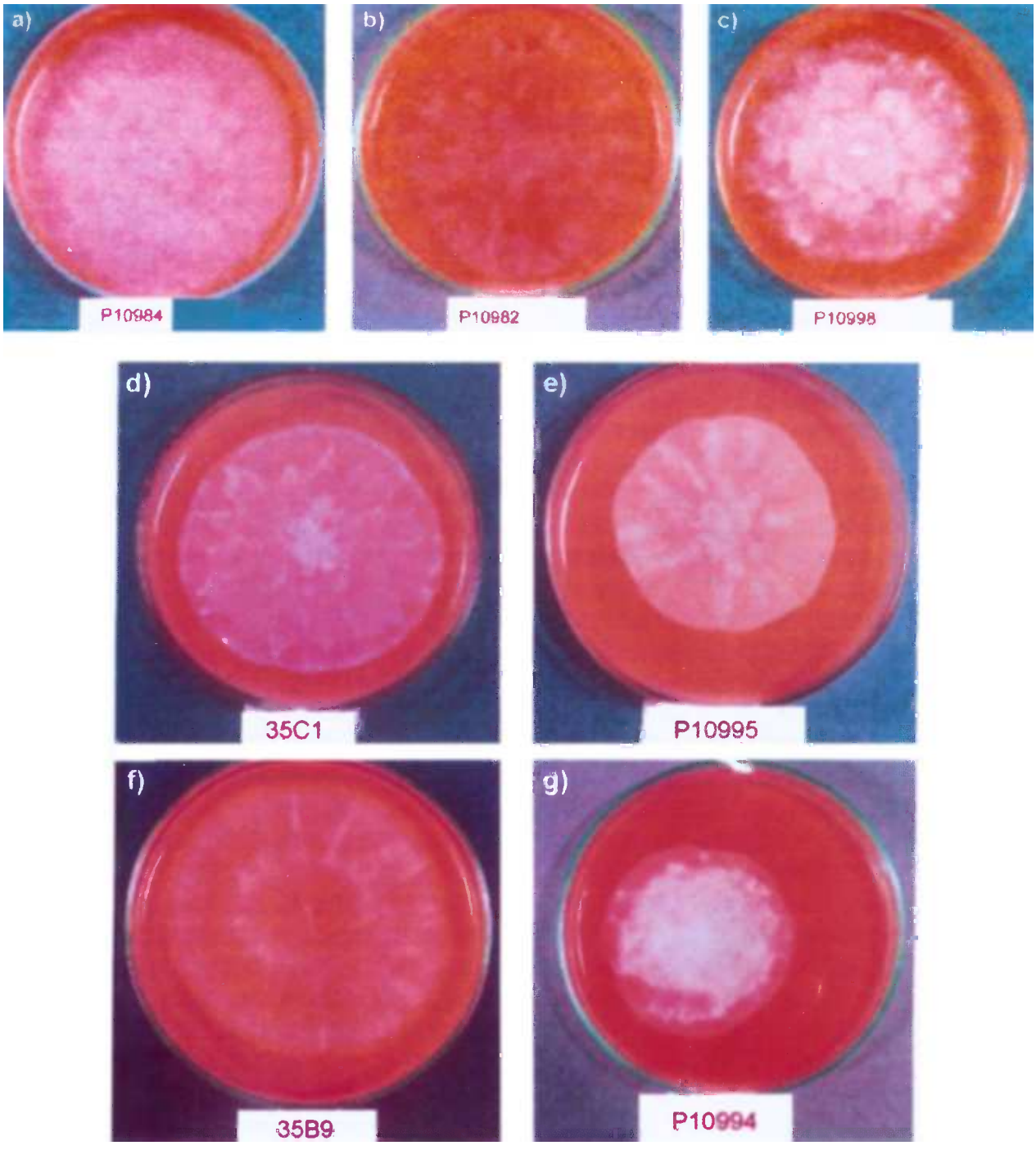

Fig. 3 : Inter and intra-specific variation in colony pattern among the *Phytophthora* species.

having identical symptoms. This has wide ramifications for control of the disease on betelvine.

Colony characteristics ranged from no discernible pattern/ diffuse growth to cottony with slightly striated/ petalloid in case of *P. nicotianae*, predominantly stellate in *P. capsici*, dense cottony/ no pattern to slightly rosaceous in *P. melonis* and stellate with a different kind of architecture than *P. capsici* in *P. colocasiae* (Fig. 3). Intra specific diversity of colony morphology was higher with six types [cottony mycelium no pattern (P10979/33C1), dense cottony mycelium no pattern (P10985/33B4), fluffy cottony mycelium no pattern (P10992/33C8),

Table 1a : Morpho-physio-cultural characteristics of *Phytophthora* isolates from lower gangetic plains of Bengal.

ACCN. NOS. [a]	HOST	ORGANISM	SPORANGIAL DIMENSIONS L (μ) [b]	B (μ) [c]	L/B [d]	GROWTH (cm) [e] V8JA	PDA	PERCENT [f] INHBITION
P10984, 33B3	Brinjal	*P.nicotianae*	44.80±4.10	31.8±2.62	1.41	3.11±0.05	1.00±0.00	51.19±0.69
P10985, 33B4	Brinjal	*P.nicotianae*	49.50±1.50	33.00±0.00	1.50	2.71±0.12	0.73±0.15	44.44±0.00
P10986, 33B5	Brinjal	*P.nicotianae*	38.15±1.25	25.30±2.28	1.53	2.56±0.02	0.50±0.00	47.06±0.00
P10987	Brinjal	*P.nicotianae*	42.91±1.45	35.60±1.78	1.21	2.69±0.18	0.90±0.06	37.72±2.53
P10988, 33B6	Brinjal	*P.nicotianae*	59.90±4.80	45.57±4.28	1.36	2.48±0.00	0.93±0.07	47.49±0.07
P10989, 33B7	Brinjal	*P.nicotianae*	38.44±1.28	28.67±0.33	1.34	2.60±0.00	0.63±0.03	40.63±1.80
33B8	Brinjal	*P.nicotianae*	44.29±1.17	36.64±1.07	1.21	2.49±0.01	0.50±0.00	16.23±0.25
P10993, 33D1	Sesame	*P.nicotianae*	40.49±0.77	32.97±1.90	1.24	2.34±0.01	0.60±0.00	45.22±4.46
P10999	Sesame	*P.nicotianae*	40.49±0.77	32.97±1.90	1.24	2.36±0.14	0.53±0.03	51.18±2.20
P11000	Pepper	*P.nicotianae*	53.72±1.14	37.50±1.61	1.44	3.14±0.09	0.87±0.07	62.50±0.00
P10998	Roselle	*P.nicotianae*	39.12±0.40	31.56±1.14	1.24	2.17±0.08	0.70±0.00	45.22±4.46
P10990, 33C6	Guava	*P.nicotianae*	54.50±2.36	32.89±3.31	1.69	2.43±0.23	1.13±0.06	52.17±6.35
P10991, 33C7	Guava	*P.nicotianae*	55.00±9.00	34.72±2.71	1.63	2.38±0.06	0.54±0.04	45.56±1.04
P10992, 33C8	Guava	*P.nicotianae*	42.50±0.25	31.25±0.66	1.36	2.39±0.15	0.43±0.03	58.82±0.00
P10978, 33B9	Betelvine	*P.nicotianae*	69.00±0.58	38.33±1.45	1.81	3.06±0.15	0.67±0.03	51.18±2.20
P10979, 33C2	Betelvine	*P.nicotianae*	37.75±4.12	28.25±4.25	1.36	2.59±0.06	0.67±0.07	50.00±0.00
P10980, 33C2	Betelvine	*P.nicotianae*	41.63±1.56	29.43±0.79	1.42	2.17±0.16	0.90±0.15	33.33±0.00
P10981, 33C3	Betelvine	*P.nicotianae*	58.00±5.29	36.00±3.46	1.61	3.13±0.02	0.77±0.03	55.00±0.00
P10982, 33C4	Betelvine	*P.nicotianae*	45.67±3.76	36.33±2.02	1.25	2.72±0.02	0.93±0.07	52.94±0.00
P10983, 33C5	Betelvine	*P.nicotianae*	44.00±0.58	35.28±1.69	1.25	2.70±0.35	0.93±0.02	48.53±0.85
35C1	Betelvine	*P.capsici*	74.53±3.38	38.80±0.61	1.92	3.25±0.18	2.10±0.05	35.42±1.20
P10995	Chilli	*P.capsici*	85.17±3.05	43.08±0.93	1.96	2.12±0.12	1.78±0.30	62.50±0.00
P10996	Chili	*P.capsici*	67.16±5.06	38.92±2.60	1.74	2.74±0.15	1.55±0.03	58.33±0.00
P10997	Chilli	*P.capsici*	57.78±1.16	51.43±1.53	1.13	2.98±0.06	1.67±0.03	62.50±0.00
P10994, 33C9	Pointed Gourd	*P.melonis*	48.00±0.00	28.33±0.88	1.70	2.32±0.04	0.73±0.13	66.00±1.55
35B9	Colocasia	*P.colocasiae*	48.86±3.46	35.03±5.05	1.43	2.03±0.07	1.61±0.05	57.14±0.00

[a] Accession numbers at World Phytophthora Collection, USA (prefix P) and Virginia State University, USA (prefix 33), others in house nos..
[b] Sporangial length [c] Sporangial breadth [d] Length/breadth ratio [e] Radial growth of mycelium [f] Growth inhibition by biocontrol antagonist.
Data are the mean, ± SE for all isolates (25 sporangia for sporangial dimensions, 3 replications for growth and percentage inhibition studies per isolate)

Table 1b : Molecular identification of *Phytophthora* isolates of the lower gangetic plains of Bengal

ISOLATE ACCN. NOS[a].	HOST	ORGANISMS	GENBANK ACCN. NOS[b].
P10986, 33B5	Brinjal	*P. nicotianae*	DQ075224, DQ075225} = AH015040
P10979, 33C1	Betelvine	*P. nicotianae*	DQ075222, DQ075223} = AH015042
P10981, 33C3	Betelvine	*P. nicotianae*	DQ124717, DQ124716} =AH015112
P10991, 33C7	Guava	*P. nicotianae*	DQ075218, DQ075219} =AH 015040
P10993, 33D1	Sesame	*P. nicotianae*	DQ075220, DQ075221} =AH015041
35C1	Betelvine	*P.capsici*	DQ124718, DQ124719} =AH015113
P10995	Chilli	*P.capsici*	DQ124721, DQ124720} =AH015114
P10997	Chilli	*P.capsici*	DQ124723, DQ124722} =AH015115
35B9	Taro	*P.colocasiae*	DQ075214, DQ075215} =AH015038
P10994	Pointed gourd	*P.melonis*	DQ075216, DQ075217} =AH015039

[a] Accession numbers at World Phytophthora Collection, USA (prefix P) and Virginia State University, USA (prefix 33), others in house nos..
[b] rDNA ITS 1 & ITS 2 sequences accessioned at NCBI GenBank between the period April 2005-September 2005.

Table 2 : EC_{50}s (μg a.i./ml) of test commercial fungicide formulations against *Phytophthora nicotianae* isolates from different hosts grown on amended agar medium

ISOLATE	HOST	BLITOX 50WP	KOCIDE101®	CAPTAF 50WP	KAVACH	DITHANE M 45®
P10979	Betel vine	94.98	72.27	29.37	1.07	11.20
P10993	Sesame	122.68	153.31	28.5	9.84	5.38
P11000	Pepper	136.46	154.57	58.05	68.86	31.21
P10986	Brinjal	71.16	74.82	82.49	242.69	19.59
P10998	Roselle	105.82	130.87	3.21	2.87	0.33
P10990	Guava	119.94	126.11	3.61	131.65	6.25

fluffy cottony mycelium and cottony with slightly striated pattern (P10981/33C3), cottony with slightly pettaloid pattern (P11000), cottony mycelium with slightly stellate pattern (P10984/33B3)] in case of *P. nicotianae* but patterns could not consistently be correlated to host/or geographical origin unlike Appiah *et al.*,(2003).

Significant intra specific differences of *P. nicotianae* in responses to the commonly used fungicides (Table 2 and 3) against *Phytophthora* diseases point to the fact that there has been a different history of exposure levels of each isolate to the different fungicides in field conditions. This information would be useful to formulate strategies for effective fungicide selection and usage against *P. nicotianae* in this region.

The isolate from Betelvine (P10979) has earlier had the least and the isolate from Brinjal (P10986) has had more exposure respectively to the fungicides under study. This study also indicates that the commercial formulation of Dithane M-45 (mean EC_{50} value 12.327) was the most effective while Kocide 101(mean EC_{50} value 118.659) and Blitox 50W(mean EC_{50} value 108.505) little or least effective (Table 2 & 3). Trends for individual recommendations of commercial formulations for the control of *P. nicotianae* isolates can therefore be as (i) Betelvine isolates -chlorothalonil 75% WP (ii) Black pepper isolates - mancozeb 75% WP (iii) Brinjal isolates- mancozeb 75% WP (iv)· Guava isolates- captan 50% WP (v) Roselle isolates- mancozeb 75% WP (v) Sesame isolates- mancozeb 75% WP.

Table 3 : Analysis of variance for interaction among isolates and sensitivity to test commercial fungicide formulations against *Phytophthora nicotianae* isolates from different hosts grown on amended agar medium.

Source	df [a]	MS [a]	*F* [a]	P value
Replication	2	13.4313	0.526	0.1
Isolate	5	7597.01	297.597	0.01
Chemicals	4	38230.8	1497.615	0.01
I X Ch	20	7285.48	285.39	0.01
Error	58	25.527		
Total	89			

[a] df, MS and *F* = degrees of freedom, mean squares and *F* statistic respectively

The cluster analysis of sporangial characters of the species under this study showed there was a strong positive correlation between sporangial length and breadth, (Fig. 1 & Table 4) suggesting that the relationship may be genetically governed (Appiah *et al.*, 2003), such that despite the considerable variation in length and breadth of individual sporangia, their ratio is characteristic of the species, making it an important diagnostic parameter (Erwin and Riberio, 1996) and hence can be used for preliminary diagnosis.

Table 4 : Analysis of variance of the morpho-cultural characteristics

Univariate Tests

Dependent Variable		Sum of Squares	df	Mean Squares	F	Sig.
PDA	Contrast	6.710	3	2.237	41.794	.000
	Error	2.569	48	5.352E-02		
V8JA	Contrast	1.109	3	.370	2.872	.046
	Error	6.179	48	.129		
L	Contrast	1606.684	3	535.561	3.800	.016
	Error	6764.901	48	140.935		
LB	Contrast	.161	3	5.354E-02	.693	.561
	Error	3.710	48	7.730E-02		
B	Contrast	640.556	3	213.519	6.722	
	Error	1524.590	48	31.762		

The F tests the effect of SPECIES. This fest is based on the linearly independent pairwise comparisons among the estimated marginal means.

Intra specific distance analysis of *P. nicotianae* species from different hosts in this region (Fig. 2) showed that they did not cluster according to host or geographical origin prompting to conclude that it may have grouped according to other (undetermined) factors.

The results presented here have highlighted the aspects of identification on both morphological and molecular grounds, leading to three new reports for the Eastern region, intra and inter specific relationships and also certain disease control recommendations for *Phytophthora* species affecting this region.

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