S. L. J. Tea Sci. 74(2), 52 - 61, 2009, Printed in Sri Lanka

Genetic and Morphological Variation of Tea (*Camellia sinensis*) Blister Blight Pathogen (*Exobasidium vexans*) in Southern India revealed by RAPD Markers and Spore Morphology

Sarvottam D Joshi, A Balamurgan, P R Rahul, A K A Mandal, R Raj kumar, U I Baby, R Premkumar and N Muraleedharan

(UPASI Tea Research Institute, Nirar Dam BPO., Valparai 642 127, Coimbatore District, Tamil Nadu, India)

ABSTRACT

Exobasidium vexans is an obligate fungal pathogen, which causes blister blight disease in tea plant (*Camellia sinensis*). This disease cause severe crop loss during monsoon in southern India. As it has been noticed that the clones resistant to blister blight in one region are susceptible in other regions, a study was carried out to investigate the morphological and molecular diversity of *E. vexans* within and between six different tea growing regions in southern India. Microscopic study and DNA finger printing analysis using RAPD have revealed high degree of genetic diversity among the samples of the *E. vexans*. Microscopic observation of spores of the fungus revealed wide variations in spore size, but not their shape and colour. DNA fingerprinting was carried out with four RAPD primers and a total of 37 clear and reproducible polymorphic bands were scored and used for analysis. Molecular data and spore morphology both indicated a greater genetic and morphological variation among isolates while a greater distribution of genetic and morphological variation was evident within regions than between regions suggesting no or low population differentiation between regions within *E. vexans*.

Key words: *Camellia sinensis*, *Exobasidium vexans*, genetic diversity, RAPDs, spore morphology.

INTRODUCTION

Blister blight is an important foliar disease of tea (*Camellia sinensis*) caused by fungal pathogen *Exobasidium vexans* Massee (class Basidiomycetes). The pathogen is an obligate parasite, which infects young harvestable shoots. The spores of the fungus germinate on the young leaves of tea shoots (first or second leaf). The first visible symptom of the disease develops as an oily spot within six to nine days after infection while the characteristic white blisters appear within 14 - 18 days.

Blister blight disease occurs in almost all the tea growing countries of the world. In southern India, the disease attains an epidemic proportion during June to December (monsoon period), leading to severe crop loss. For control of the disease, fungicides are applied at a short interval of seven to ten days, however, repeated applications of fungicides is not advisable from the environmental and economic points of view. Tea plantations of south India consist of a variety of cultivars some of which are resistant to blister blight. However, it has been noticed that clones resistant to blister blight in one region are susceptible in other region. This may be due to difference in virulence of the pathogen or presence of different biotypes of the pathogen in different tea growing regions of southern India.

As the pathogen *E. vexans* is an obligate parasite, attempt to culture the fungus on artificial medium was unsuccessful, and hence, distinguishing different biotypes on the morphological characters is not possible. In such cases, DNA fingerprinting will be helpful to understand the genetic polymorphism. To visualize the DNA polymorphism in fungus, several methods like RFLP (Kim *et al.*, 1992) and respective genomic sequences (Kistler *et al.*, 1991), large subunit r-RNA sequencing (Gudadet *et al.*, 1989) have been applied. In many cases random amplified polymorphic DNA (RAPD) technique has been used for identification of individuals in different populations and for identifying and distinguishing isolates of various fungi (Welsh and Mecllland, 1990; Williams *et al.*, 1990, Guthrie *et al.*, 1992; Manulis *et al.*, 1994; Shi *et al.*, 1996; Vakalounakis and Fragkiadakis, 1999; Doherty *et al.*, 2003; Moore *et al.*, 2001; Chadha and Gopalakrishna, 2005). Among the different molecular techniques, RAPD has been widely used for estimating genetic diversity in natural populations (Annamalai *et al.*, 1995) because the technique is simple, inexpensive and does not require any sequence information. Therefore, in the present study an attempt was made to investigate the genetic relationships among different *E. vexans* isolates from different tea growing regions of southern India and within and between region variation of *E. vexans* using RAPDs and spore morphology.

MATERIALS AND METHODS

Spore collection and microscopic study

Fifteen infected tea shoots with mature blister lesions were collected from 17 tea estates of six different tea growing regions of southern India (Table 1). The shoots were thoroughly washed in sterile distilled water and the cut ends of the shoots were kept in small vial containing 1% glucose solution. A sterile beaker containing sterilized, 1% glucose solution (1.5 ml) was covered with the leaf of the shoot having a well developed sporulating lesion in such a way that the lesion is inside the cavity of the beaker. A small glass plate was kept on the leaf and a weight was kept on it for support. The entire setup was kept inside a moistened belljar so as to maintain 100 % relative humidity overnight for the collection of spores. The spores released from the lesion were collected in the glucose solution in the beaker. The spore suspension thus obtained was kept under refrigeration till use. Spore

Table 1.List of blister blight samples studied, their places of collection and variation in spore size

S. No.	Sample Code	Region of collection	Spore size (μ) (Length X Width)
1	BB1	Highwavys	12.9 X 4.1
2	BB2	Highwavys	14.5 X 4.8
3	BB3	Vandiperiyar	15.0 X 4.4
4	BB4	Gudalure	15.2 X 4.8
5	BB5	Gudalure	14.7 X 4.5
6	BB6	Wayanad	15.1 X 4.2
7	BB7	Wayanad	15.0 X 4.5
8	BB8	Munnar	16.1 X 4.7
9	BB9	Munnar	14.8 X 4.0
10	BB10	Munnar	14.4 X 4.2
11	BB11	Munnar	14.3 X 4.2
12	BB12	Anamallais	14.7 X 4.4
13	BB13	Anamallais	16.1 X 4.9
14	BB14	Anamallais	15.3 X 4.7
15	BB15	Anamallais	14.8 X 4.3
16	BB16	Anamallais	14.1 X 3.9
17	BB17	Anamallais	13.2 X 3.7

suspension (5 ml) was spotted on clean slide and observed under microscope. At least one hundred spores were measured with the help of ocular micrometer from ten randomly selected microscopic fields. Experiment was repeated five times.

DNA extraction from spores

The collected spores were sedimented by adding 0.5 % (final concentration) sodium dodecyl sulphate (SDS) to the beaker and placing at 65 °C for 10 min. (Joshi *et al.*, 2004). Entire solution was transferred to 1.5 ml eppendorf tube and then centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was discarded. Spore pellet was treated with 100 μl of solution A (glucose 50 mM, Tris-HCL 20 mM, and EDTA 10 mM) to maintain osmoticum before lysis followed by 150 μl of alkaline lysis solution (1M sodium chloride and 4 % SDS), homogenized by vortexing (for 5min) and incubated for 10 min at 65 °C. Then 100μl of sodium acetate (3 M at pH 5.4) was added and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was transferred to another fresh 1.5 ml eppendorf tube. The DNA was precipitated by adding 0.7 vol of iso-propanol and DNA pellet was collected by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatant was discarded and the DNA pellet was re-suspended by adding 200 μl of TE buffer (10 mMTris-HCl and 1 mM EDTA, pH 8) followed by treating with 5 μl of RNase-A (10 g dm^{-3}) and incubated at 37°C

for one hour. Then equal volume of phenol: chloroform: iso-amyl alcohol (25:24:1) mixture was added, mixed gently by inverting tube and centrifuged at 10,000 rpm for 10 min at 4°C. The aqueous phase was transferred to new eppendorf tube, and DNA was precipitated by adding 2.5 volumes of chilled absolute ethanol and incubating at –20 °C for 30 min. The DNA pellet was collected by centrifugation at 12,000 rpm for 10 min at 4 °C. The DNA pellet was washed two times with 500 µl of 70 % ethanol and air-dried. The pellet was re-suspended in 50 µl of sterile distilled water and stored at –20 °C till use. Quality and the quantity of the DNA obtained were determined by subjecting it to gel electrophoresis (0.9 % agarose) and using UV-VIS spectrophotometry respectively.

PCR and agarose gel electrophoresis

Polymerase chain reaction was carried out in peltier thermal cycler (PTC-200, MJ Research, Inc.,USA). Each 25 µl reaction mixture contained 1 unit of *Taq* DNA polymerase, 0.2 mM each d*NTP*, 1X reaction buffer, 3 mM $MgCl_2$ (Bangalore Genei Pvt. Ltd. India), 10 µmole of primer (Table 2, OPERON-Qiagen) and approximately 50 ng of template genomic DNA. PCR conditions were as follows: initial denaturation at 94 °C for 4 min, followed by 45 cycles of denaturation at 92 °C for 30 seconds, annealing at 36 °C for 60 seconds and extension at 72 °C for 120 seconds followed by final extension at 72 °C for 10 min. The amplified products were separated on 2 % agarose gel using 0.5X TBE buffer. Gels were stained with ethidium bromide prior to photography.

Scoring and data analysis

Bands were scored as present (1) or absent (0) in all the sample, only reproducible bands were scored. Molecular weight of each band was estimated using 1 kb DNA ladder (Fermentas) as a standard. Similarity coefficient matrix was constructed by calculating Jaccard's similarity coefficient values for each pair wise comparison between samples (Jaccard, 1908). A dendrogram was generated from this matrix following unweighted pair group method with arithmetic average analysis (UPGMA).

RESULTS AND DISCUSSION

Genetic variability study was carried out to identify polymorphic bands using decamer random primers. A total of 10 RAPD primers (Table 2) were used for screening the samples. Of the ten primers used, four primers gave highly reproducible and polymorphic bands in all 17 samples. Remaining primers that gave either sub-optimal or indistinct amplification were not considered for further studies. The RAPD profile generated with primer OPA-01 is presented in Figure 1. The size of the amplification products obtained from all the primers varied from 0.23 to 4.8 kb. Interestingly all amplified bands were found polymorphic (Table 2), which indicates that genetic base of the samples are broad. A wide range (0.113 to 0.605) of Jaccard's similarity coefficient was noticed among the different samples (Table 3).

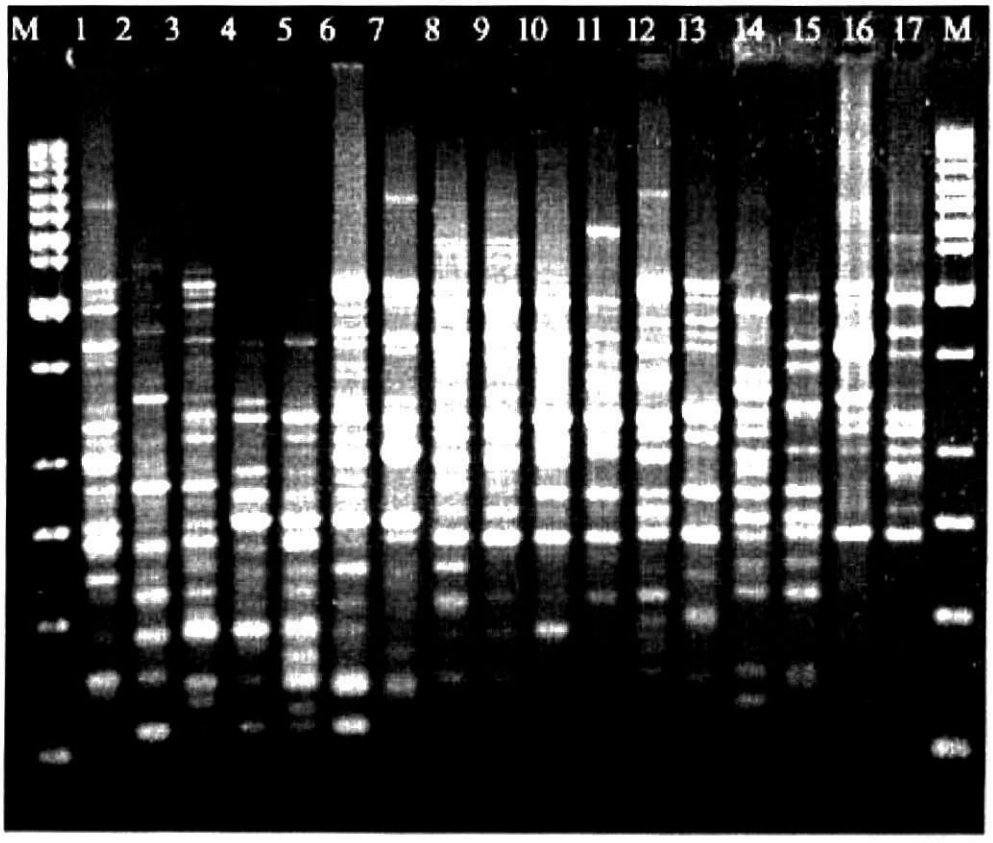
Table 2.Primers used in the study along with their sequences and some characteristics of the amplification products in 17 samples of blister blight fungus

SL. No.	Primer	Sequence	No. of polymorphic bands	Mol. wt. range of RAPD product (kb)
1	OPA-01	CAGGCCCTTC	13	0.29 to 4.86
2	OPA-02	TGCCGAGCTG	7	0.24 to 2.0
3	OPA-04	AGTCAGCCAC	9	0.32 to 2.96
4	OPA-06	AATCGGGCTG	8	0.23 to 3.58

Table 3.Jaccard's similarity coefficient matrix of 17 blister blight samples collected from six different tea growing regions of South India

BB1	1.000															
BB2	0.358	1.000														
BB3	0.397	0.605	1.000													
BB4	0.262	0.226	0.233	1.000												
BB5	0.250	0.308	0.351	0.460	1.000											
BB6	0.274	0.196	0.267	0.309	0.389	1.000										
BB7	0.404	0.220	0.296	0.320	0.255	0.333	1.000									
BB8	0.345	0.158	0.271	0.203	0.233	0.377	0.367	1.000								
BB9	0.180	0.250	0.232	0.226	0.236	0.241	0.271	0.245	1.000							
BB10	0.298	0.216	0.268	0.314	0.273	0.302	0.400	0.283	0.319	1.000						
BB11	0.333	0.304	0.278	0.226	0.236	0.367	0.356	0.320	0.250	0.348	1.000					
BB12	0.226	0.143	0.197	0.169	0.263	0.246	0.300	0.296	0.185	0.375	0.255	1.000				
BB13	0.339	0.235	0.241	0.236	0.268	0.273	0.306	0.255	0.189	0.300	0.286	0.367	1.000			
BB14	0.293	0.235	0.263	0.193	0.291	0.250	0.208	0.232	0.212	0.354	0.235	0.314	0.347	1.000		
BB15	0.232	0.213	0.222	0.192	0.250	0.280	0.289	0.235	0.239	0.311	0.326	0.356	0.364	0.538	1.000	
BB16	0.203	0.113	0.153	0.123	0.155	0.158	0.154	0.140	0.204	0.196	0.180	0.313	0.265	0.192	0.217	1.000
BB17	0.273	0.200	0.246	0.222	0.250	0.197	0.237	0.345	0.309	0.213	0.220	0.246	0.210	0.230	0.190	0.315	1.000
	BB1	**BB2**	**BB3**	**BB4**	**BB5**	**BB6**	**BB7**	**BB8**	**BB9**	**B10**	**BB11**	**BB12**	**BB13**	**BB14**	**BB15**	**BB16**	**BB17**

From this study, overall a high degree of genetic diversity was noticed among samples of blister blight fungus collected from different tea growing regions of South India. Similar observations of wide genetic diversity have been reported in many other fungi. Li *et al.*, (2001) have studied western gall rust fungus (*Endocronartium harknesii*) collected across western and central Canada from two host pine species. Most of the genetic variation was found between the two host species, but within hosts there was more variation among geographically distant locations than within locations. A considerable genetic variation among isolates of symbiotic fungus *Leucoagaricus gongylophorus* has been reported by Doherty *et al.* (2003) using RAPD markers. High degree of genetic diversity among the



M: 1kb Marker (Fermantas) and from 1 to 17 (BB1, BB2, BB3, BB4, BB5, BB6, BB7, BB8, BB9, BB10, BB11, BB2, BB13, BB14, BB15, BB16 and BB17)

Figure 1. RAPD profile of 17 blister blight samples generated by primer OPA-01

Indian isolates of rice blast pathogen (*Magnaportha grisea*) has been observed from RAPD markers study (Chadha and Gopalakrishna 2005).

The UPGMA based dendrogram is presented in Figure 2. It separated all the samples into three clusters (CL-1, CL-2 and CL-3 with less than 0.3 similarity value). The CL-1 comprised of ten samples where highest similarity (0.5) was observed between BB14 and BB15 (collected from Anamallais region). Out of the six samples collected from Anamallais region, four (BB12, BB13, BB14 and BB15) were grouped in this cluster indicating genetic similarity among them. Among other samples BB6 and BB7 of Wayanad and BB8, BB10 and BB11 of Munnar were also included in this CL-1. The remaining one sample of CL-1 came from Highwavys region.

CL-2 also comprised of four isolates from three different regions; One from Highwavys, one from Vandiperiyar and two from Gudalur. Highest degree of similarity (> 0.60) was

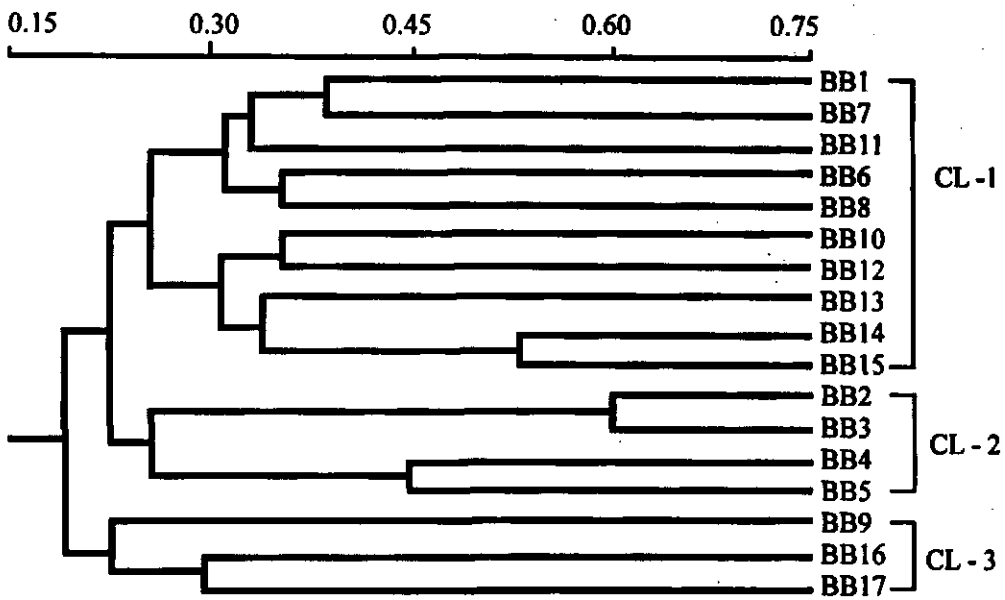


Figure 2. Dendrogram generated using UPGMA analysis demonstrating the relationship among 17 samples of blister blight fungus based on RAPD data

seen between BB2 (from Highwavys) and BB3 (from Vandiperiyar). CL-3 comprised of three samples, two (BB16 and BB17) from Anamallais and another one (BB9) from Munnar.

When compared region wise, two samples from Highwavys (BB1 and BB2) were grouped in two different clusters (CL-1 and CL-2). The sample BB1 (from Highwavys) showed close similarity with sample BB7 (from Wayanad). Sample BB2 (Highways) showed a very close similarity (0.6) with BB3 (from Vandiperiyar). It can be noted that Highwavys and Wayanad are distantly located in two different states, while Highwavys and Vandiperiyar are close to each other.

Two samples BB4 and BB5 (from Gudalur) also showed similarity among them and grouped in CL-2. Another two samples BB6 and BB7 (from Wayanad) also showed similarity among them and grouped in the same cluster (CL-1). Among the four samples BB8, BB9, BB10 and BB11 (from Munnar), three were grouped into same cluster (CL-1) indicating similarity among them while one (BB9) is placed in another cluster (CL-3), where sample BB16 and BB17 (both from Anamallais) were placed. It should be noted that Anamallais and Munnar are closely situated. Out of six samples (BB12, BB13, BB14, BB15, BB16 and BB17) from Anamallais four were grouped in one cluster (CL-1) indicating similarity among them while remaining two were placed in another cluster (CL-3).

This genetic diversity may have developed due to simple mutation, and/or genetic recombination during reproduction. Other mechanism like horizontal gene transfer between

Exobasidium vexans and its host and evolution resulting from natural and stress induced transposition may also be possible. Similar mechanism had been suggested for genetic diversity of other pathogenic fungus also (Yamasaki and Niizeki, 1965; Zeigler, 1998; Ikeda *et al.*, 2001; Chadha and Gopalakrishna, 2005).

Experiment carried out to identify morphological and genetic variation among the spores of *Exobasidium vexans* isolates, collected from different regions of southern India revealed a wide variation between the samples in their size. There was no variation in their colour and shape. Immature spores were hyaline, elliptical and single celled whereas they became two celled in attaining maturity. All were smooth walled, the only exception was BB8 where spore wall has ornamentation. Most of the spores (BB2, BB5, BB9, BB10, BB11, BB12, BB15 and BB16) were of 14.5 X 4.4 µ size. Spores of two samples BB1 and BB17 (12.9 X 4.1 µ and 13.2 X 3.7 µ respectively) were comparatively smaller, whereas two others, BB8 and BB13 (16.1 X 4.7 µ and 16.1 X 4.9 µ respectively) were larger in size. The remaining five samples BB3, BB4, BB6, BB7 and BB14 were intermediate in size (Table 1).

On the basis of the present study, it is concluded that population of blister blight fungus present in the south Indian tea growing regions are genetically heterogeneous with broad genetic base, high genetic variation among samples and no difference between regions. To our knowledge, this is the first report of genetic diversity study of *E. vexans* present in the southern Indian tea growing regions.

ACKNOWLEDGEMENTS

The financial assistance given by the Tea board, Kolkatta under tenth five year plan is gratefully acknowledged. The authors are grateful to the Director, UPASI-TRF for his constant support throughout the study. We are also very thankful to all the Advisory officer of UPASI-TRF for their kind help during spore collection from different regions of southern India.

REFERENCES

Annamalai P, Ishii H, lalithakumari, D and Revathi R 1995 Polymerase chain reaction and its applications in fungal disease diagnosis. *J. Plant Disease Prot.* 102, 91 - 104.

Chadha S and Gopalakrishna T 2005 Genetic diversity of Indian isolates of rice blast pathogen (*Manguaporthe grisea*) using molecular markers. *Cur. Sci.* 88,1466 - 1469.

Doherty K R, Zweifel E W, Elde N C, McKone M J and Zweifel S G 2003 Random amplified polymorphic DNA markers reveal genetic variation in the symbiotic fungus of leaf-cutting ants. *Mycologia.* 95, 19 - 23.

Gudadet J, Julien J, Lafay J F and Brygoo Y 1989 Phylogeny of some *Fusarium* species as determined by large sub-unit rRNA sequence comparison. *Mol. Biol. Eval.* 6, 227 - 242.

Guthrie P A I, Mgill C W, Fredriksen R A and Odrody G N 1992 System for identifying and differentiating isolates of *Colletotrichum graminicola*. *Phytopathology*. 82, 832 - 835.

Ikeda K, Nakayasiiki H, Takagi M, Tosa Y and Mayama S 2001 Heat shock, copper sulphate and oxidative stress activate the retrotransposon MAGGY resident in the plant pathogenic fungus *M. grisea*. *Mol. Genet. Genomics*. 266, 318 - 325.

Jaccard P 1908 Nouvelles recherches sur la distribution florale. *Bulletin de la Societe Vaudoise des Science Naturelles*. 44, 223 - 270.

Joshi S D, Haridas V, Thomas J, Rahul P R and Raj Kumar R 2004 Isolation of spores of *Exobasidium vexans* of tea and extraction of genomic DNA. *J. Plantation Crops*. 32 (suppl.), 351 - 353.

Kim D H, Martyn R D and Magill C W 1992 Restriction fragment length polymorphism groups and physical map of mitochondrial DNA from *Fusarium oxysporum, f. sp. Niveum*. *Phytopathology*. 82, 346 - 353.

Kistler H C, Momol E A and Benny U 1991 Repetative genomic sequences for determining relatedness among strains of *Fusarium oxysporum*. *Phytopathology*. 81, 331 - 336.

Li C, Yeh F C and Hiratsuka Y 2001 Random amplified polymorphic DNA variability among geographic isolates of western gall rust fungus in Canada. *Can. J. Forest Res.* 31, 1304 - 1311.

Manulis S, Kogan N, Reuven M and Ben-Yephet Y 1994 Use of RAPD technique for identification of *Fusarium oxysporum, f. sp. Dianthi* from carnation. *Phytopathology*. 84, 98 - 101.

Moore A, Challen M P, Warner P and Elliott T Y 2001 RAPD discrimination of *Agaricus bisporus* mushroom cultivars. *Appl. Microbial Biotechnol.* 55, 742 - 749.

Shi Y L, Loomis P, Christain D, Carries L M and Leung H 1996 Analysis of the genetic relationship among wheat bunt fungi using RAPD and ribosomal DNA markers. *Phytopathology*. 86, 311 - 318.

Vakalounakis D J and Fragkiadakis G A 1999 Genetic diversity of *Fusarium oxysporum* isolates from cucumber Differentiation by vegetative compatibility, pathogenecity and RAPD fingerprinting. *Phytopathology.* 89, 161 - 168.

Welsh J and Mecllland M 1990 Fingerprinting genomes using PCR with arbitrary primers. *Nucl. Acids Res.* 18, 7213 - 7218.

Williams J G K, Kubelik A R, Livak K J, Rafalski J A and Tingey S V 1990 DNA polymorphism amplified by arbitrary primers are useful as genetic marker. *Nucl. Acids Res.* 18, 6531 - 6535.

Yamasaki Y and Niizeki H 1965 Studies on variation of the rice blast fungus *Pyricularia oryzae* Car. 1. Karyological and genetical studies on variation. *Bull. Natl. Inst. Agric. Sci.* 13, 231 - 274.

Zeigler R S 1998 Recombination in *Manguaporthe grisea.* Annu. Rev. *Phytopathology.* 36, 249 - 274.