Australian Journal of Basic and Applied Sciences, 1(1): 30-37, 2007
ISSN 1991-8178

Allozyme Variations of *Trichoderma harzianum* and its Taxonomic Implications

Shafiquzzaman Siddiquee, Faridah Abdullah, Tan Soon Guan and Leng Min See

Department of Biology, Faculty of Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

Abstract: Electrophoretic variation of nine allozyme systems encoded by 14 gene loci were studied on 47 isolates from 3 species of *Trichoderma* namely, *T. harzianum, T. aureoviride* and *T. longibrachiatum*. Polyacrylamide gel electrophoresis was used to investigate the taxonomic circumscription of *T. harzianum* populations and to evaluate the levels of genetic variations and the population structure., The Level of genetic variations in *T. harzianum* populations were moderately high (P= 57.10%, A= 0.7857, Ap= 0.60714 and H_E= 0.1542) compared to *T. aureoviride* and *T. longibrachiatum*. The genetic variation attributable to differences among populations was 7.857%. The mean gene flow among populations was Nm = 1.3351. Genetic identities (I) ranged from 0.9397 to 0.9642 with a mean of 0.94846. Outcrossing rates based on fixation indices average (t) was 0.2334. Nevertheless, the alleles for α-EST-b showed a very low frequency of 0.0400. The polymorphic locus of MDH1 was of the fast allele of *T. harzianum*, MD1-a, was prevalent in *T. aureoviride* and *T. longibrachiatum* populations. Using a UPGMA cluster analysis, *T. harzianum* and *T. longibrachiatum* populations were totally separated in these cluster except *T. aureoviride* populations. *T. harzianum* presents high levels of genetic diversity compared with other *Trichoderma* species.

Key word: Trichoderma, Polyacrylamide gel electrophoresis, Allozymes, Genetic variations, identification of fungi

INTRODUCTION

The genus *Trichoderma* has been extensively studied for their abilities to rapidly colonize substrates (Grondona *et al.*, 1997) for the induction acquired systemic resistance in plants (Enkerly *et al.*, 1999), for the growth inhibitory effect of their antibiotics on others pathogen (Keszler *et al.*, 2000) and for the production of cell wall-degrading enzymes against many plant pathogens (Lorito, 1998). Species of *Trichoderma* have been used as agents of biological control of plant diseases (Howell, 2003) whether in the form of whole cell or protein formulations and have been used for expressed genes in transgenic plants (Kubicek 2001).

Electrophoresis of allozyme was initiated in 1957, when Hunter and Markert (Hunter and Markert, 1957) exploited the idea of using the catalytic properties of enzymes to reveal their presence with histochemical methods. Thoroughly electrophoresis, it is now known that two properties have made protein-coding gene for geneticists; (a) an important proportion of these genes are polymorphic, that is they exist in the form of one or more alleles. This property, first shown by Lewontin and Hubby (1966) in *Drosophila* and by Harris in man, has since been found to exist in virtually all animals and plant species that have been studied (Pasteur 1974). (b) The alleles of protein-coding genes are generally codominant, i.e. both alleles are expressed in heterozygous organisms.

Electrophoretic technology facilitated the recognition that enzymes come in multiple forms. Markert and Moller (1959) proposed the term *isozyme* "to describe the different molecular forms in which proteins may exist with the same enzymatic specificity". This functional definition was intended to be broad, to cover all molecular forms of enzymes. Whitt (1981) noted "the use of *allozyme* data exploits only a portion of the genetic information available through electrophoresis". The codominant nature of expression of allozymes allowed the unambiguous identification of heterozygous and homozygous individuals. Interspecific comparisons of allozymes that result measured of "genetic relationships" entered the level of Mayr's (1969) taxonomy.

In the last decade, a wide range of molecular techniques has been introduced to obtain new characters for the classification of *Trichoderma*. These techniques include DNA sequencing (Appel and Gordon, 1996), random amplified polymorphic DNA (RAPD) (Kelly *et al*., 1994; Woo *et al.*, 1996), restriction fragment length

Corresponding Author: Shafiquzzaman Siddiquee, Mycology and Plant Pathology Laboratory Department of Biology, University of Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
E-mail: shafiqpab@yahoo.com

polymorphism (RFLP) (Rutherford *et al.*, 1995), and isozyme analysis (Ho *et al.*, 1985; Elias and Schneider, 1992).

Isozyme analysis has been used to differentiate closely related species within *Trichoderma* (Szekeres *et al.*, 2006). Electrophoresis of isozymes allows rapid and inexpensive analyses of a large number of isolates. Differences in amino acid sequences may lead to changes in the properties of the proteins and thus altered mobility in polyacrylamide gels (Rosendahl and Sen, 1992). Different enzymes can easily be visualized after completion of electrophoresis by using published staining recipes such as (Pasteur *et al.*, 1988; Harris and Hopkinson, 1976).

Stasz *et al.*(1988) evaluated 63 enzymes of *Trichoderma* by horizontal starch gel electrophoresis followed by a cladistic analysis for the evaluation relationships within the genus. Samuels *et al.*,(1994) showed that 23 enzyme analyses on starch gels were used to detect of *T. longibrachiatum*, *T. pseudokoningii* and *T. reesei*. Leuchtmann *et al.* (1996) studied the enzyme subgroups within *Trichoderma* section *Longibrachiatum*, using 78 strains and ten isozyme systems. Szekeres *et al.*, (2006) also found seven appropriate for identifying the species as *T. pseudokoningii, T. koningii* or *T. citrinoviride*. The results of this study supported Bissetts morphology based taxonomic scheme for this section (Bissett, 1992). Grondona *et al.* (1997) characterized 15 isolates of *T. harzianum* on the basis of morphological, physiological, molecular and biochemical features, including 99 isozyme bands revealed by PAGE based on six isozyme systems.

This article reports on the moderately high levels of genetic variation of *T. harzianum* populations. Allozyme loci were used to examine the population's genetic variations of the three known *Trichoderma* populations. The aims of this study were to (a) evaluate the levels of genetic variations and population's structure, and gene flow taking into explanation of all populations; (b) contrast the results and conclusions with other *Trichoderma* species; (c) examine the genetic differentiation by the cluster analyses based on allele frequencies of the gene loci.

MATERIAL AND METHODS

Isolates of Trichoderma Samples:

A total of forty- seven *Trichoderma* samples were used in the isozyme analysis of which 31 were from the Mycology laboratory, Department of Biology, UPM and 16 others were freshly isolated and identified by morphological means by this author (Table 1). The isolates were cultured in liquid basal medium, as that described by Nawawi and Ho (1990).

Table 1: List of species, code number, references and locality for *Trichoderma* strains analyzed in this study

Species name	code	Reference	Locality
T. harzianum	FA2, FA4, FA7, FA8, FA15, FA17, FA24, FA26, FA29, FA30, FA31, FA34, FA36, FA38, FA40, FA44,	this author	Sedenak Johor baru
T. harzianum	T32, T60, T66, T71, T79, T80, T100, T101, T102, T121, T124,	Illias, 2000	Negeri Sembilan
T. longibrachiatum	T28, T76, T82, T87, T90, T91, T99, T104, T118, T120,	Illias, 2000	Negeri Sembilan
T. aureoviride	T29, T45, T47, T55, T58, T65, T86, T106, T126, T127	Illias, 2000	Negeri Sembilan

Electrophoresis:

Approximately 100 mg of the ground mycelia powder was added 600 µl extraction buffer of centrifuge tubes by Manwell and Baker (1968) method. The samples were then centrifuged at 13,000 rpm for 10 minutes at 4 ^{0}C. The supernatants were used for further isozyme assay according to Lowry's methods (1951), which were stored on filter paper wicks at -20 ^{0}C. These isozyme extractions were prepared every one month until end in this experiment. Multilocus genotypes of each *Trichoderma* populations were obtained through horizontal gel electrophoresis (8 % w/v). For each individual population, allozymic variations were scored for 14 loci (Table 2). Electrophoresis was carried out at 4 ^{0}C for 3 h (constant current of 50 mA, and voltage of 250 V).

Table 2: Allozyme systems resolved in this study.

Allozyme	Abbreviation	EC no.	Buffer*
α-Esterase	α-EST	EC 3.1.1.1	TCB/P
Malate dehydrogenase	MDH	EC 1.1.1.37	TCB/P
Acid phosphatase	ACP	EC 3.1.3.2	TBE
Glucose-6-phosphate dehydrogenase	G6PDH	EC 1.1.1.49	TCB/P
General protein	GP	-	TBE
Superoxide dismutase	SOD	EC 1.15.1.1	TBE
Sorbitol dehydrogenase	SORDH	EC 1.1.1.14	TCE
Malic enzyme	ME	EC 1.1.1.40	TCB/P
Alcohol dehydrogenase	ADH	EC 1.1.1.1	TCB/P

Nomenclature and abbreviations follow Wendel and Weeden (1989), based on IUBNC (Nomenclature Committee of the International Union of Biochemists).
EC no. = Enzyme Commission number.
*Buffer
TCB/P = Tris- Citrate- Borate/ Poulik (pH 8.0), TBE= Tris-Borate- EDTA (pH 8.0), TCE= Tris- Citrate- EDTA (pH 6.90),

Gel Scoring and Analysis:

Loci were numbered consecutively and alleles at each locus were labeled alphabetically, beginning from the most anodal form in both cases. Allozyme frequencies at each locus were calculated for each population. Data were analysed using the POPGENE 32 (Yeh.and Yang, 1999) software based on the computer program. Percentage of polymorphic loci (P), mean number of alleles per locus (A) and mean number of alleles per polymorphic locus (A_P), effective number of alleles (A_E), observed heterozygosity (H_O), and expected mean heterozygosity (H_E) with respect to the Hardy–Weinberg equilibrium (Hedrick, 2000) were calculated. Loci were considered polymorphic if more than one allele was detected. Levels of genetic variation were calculated for individual populations and for all individuals pooled across all populations.

Fixation indices (F), reflecting deviations from Hardy–Weinberg equilibrium, were calculated and outcrossing rates (t) were estimated using t = (1 – F)/ (1 + F) (Weir, 1990). The partitioning of genetic diversity within and among all populations were analysed using F-statistics (Nei, 1973) according to the equations of Weir and Cockerham (1984).

The average gene flow among populations (Nm) was estimated from Fst values according equation such as Fst = 0.25(1 - Fst)/Fst (Nei, 1978). Nei's unbiased genetic distance and genetic identity value were calculated for pairwise comparisons of populations. A cluster analysis was performed using the unweighted pair-group method using arithmetic average (UPGMA) dendrogram (Sneath and Sokal, 1973), depicting the genetic relationships among the population of *Trichoderma* species. A dendrograms was constructed using the computer software package NTSYS-pc version 1.80 by Rohlf (1997).

RESULTS AND DISCUSSIONS

Results:

Loci and Alleles Scored:

Enzyme electrophoresis resulted in clear and consistent staining for 9 enzymes encoded by 14 putative loci: EST, MD1, MD2, AP, G6PD, GP1, GP2, GP3, SORD, SOD1, SOD2, SOD3, ME and AD1. All enzymes migrated anodally. Eleven loci were polymorphic in at least one population (78.37%, Table 3). A total of 28 alleles were detected in all populations of *Trichoderma* species. Nevertheless the allele lacking in these population EST-b was found of *T. harzianum* only with a very low frequency (0.0400) (Table 4). In the other polymorphic locus found (MDH1) the faster allele.

Table 3: Summery of genetic variation for 14 loci in three populations of *Trichoderma* species.

Population	P	A	Ap	A_O	A_E	H_O	H_E	F	t
T. harzianum	0.571	0.7857	0.60714	1.5714	1.2044	0.0350	0.1542	0.62141	0.2334
T. aureoviride	0.562	0.7857	0.57142	0.5136	1.2121	0.0666	0.1466	0.30976	0.5269
T. longibrachiatum	0.428	0.7500	0.50000	1.5000	1.2437	0.0957	0.1425	0.16495	0.7168

P= percentage of polymorphic loci; A = Mean number of alleles per locus; Ap = Mean number of alleles per polymorphic locus; A_O = Mean observed number of alleles; A_E = Mean effective number of alleles; Ho= Mean observed heterozygosity; He=Mean expected heterozygosity; F= fixation index; t= outcrossing rate.

Table 4: Allele frequencies of polymorphic loci in the three species of *Trichoderma*.

Locus	Allele	*T. harzianum*	*T. aureoviride*	*T. longibrachiatum*
EST	a	0.9600	1.0000	1.0000
b	**0.0400**	0.0000	0.0000	
MD1	a	**0.9615**	0.8889	0.9286
b	0.0385	0.1111	0.0714	
MD2	a	1.0000	0.9000	1.0000
c	0.0000	0.1000	0.0000	
AP	a	0.8519	0.6667	0.8333
b	0.0000	0.3333	0.1667	
c	0.1481	0.0000	0.0000	
G6PD	a	1.0000	1.0000	1.0000
GP1	a	1.0000	1.0000	0.2500
b	0.0000	0.0000	0.2500	
c	0.0000	0.0000	0.5000	
GP2	a	0.8519	0.8500	0.8333
b	0.1481	0.1500	0.1667	
GP3	a	0.8519	0.9500	0.7143
b	0.1481	0.0500	0.2857	
SORD	a	0.5000	0.9167	1.0000
b	0.3889	0.0833	0.0000	
c	0.1111	0.0000	0.0000	
SOD1	a	1.0000	1.0000	1.0000
SOD2	a	1.0000	0.6000	1.0000
c	0.0000	0.4000	0.0000	
SOD3	a	0.8800	1.0000	0.9375
b	0.1200	0.0000	0.0625	
ME	a	1.0000	0.9286	1.0000
b	0.0000	0.0714	0.0000	
AD1	a	1.0000	1.0000	1.0000

Genetic Variation within the Species:

Genetic variations were quantified with populations of three *Trichoderma* species (Table 3). Seven (50.00 %) of the 14 loci, 8 (57.10%) of the 14 loci and 6 (42.86%) of 14 loci (*P*) resolved for *T. harzianum, T aureoviride* and *T. longibrachiatum*, respectively. Mean number of alleles per locus (A) were 0.7857, 0.7857 and 0.7500, respectively and mean numbers of alleles per polymorphic locus (A_p) were 0.60714, 0.57142 and 0.50000, respectively. Observed mean heterozygosity were 0.0350, 0.0666 and 0.0957, respectively and expected mean heterozygosity were 0.1242, 0.1466 and 0.1425, respectively with all populations of Three *Trichoderma* species. Values of observed heterozygosity were lower than those of expected heterozygosity in all populations.

Fixation Indices and Outcrossing Rates:

Mean fixation indices (F) was ranged from 0.16495 to 0.62141 with a mean of 0.3654, indicating an overall conformance to Hardy-Weinberg equilibriums (table 1). F value was significantly greater than zero and positive, indicating excess of homozygotes. Twenty one of 42 fixation indices for individual loci (ranging from F= -0.0400 to F= 1.00) are significant. Outcrossing rates (t) based on fixation indics range from 0.2334 to 0.7168 (mean t=0.4923). Outcrossing rates (t) were low and far (mean t=0.4923) of all populations. The remaining value conformed to Hardy-Weinberg equilibrium.

Genetic Structure:

Genetic divergence among populations was qualified by computing the Fst parameter, which measures differentiation among populations (Table 5). The mean value of Fst was considerably low (Fst=0.1577; 95% confidence interval: 0.3472- 0.9636) with respect to the mean value of Fit (0.5469) but significantly different from zero (P<0.01). The Mean Fis, representing average deviation from Hardy-Weinberg expectations within populations to be positive and significantly different from zero (Fis=0.4620).

***Gene Flow*:**

Gene flow (Nm) for all loci indicated an average of 1.3351 among all populations of *Trichoderma* species. The lowest and highest Nm values were obtained from 0.2679 to 471.7541 of all populations (Table 5).

Table 5: Summary of F-statistics and gene flow for all loci among three populations of *Trichoderma* species (Nei, 1987)

Locus	Fis	Fit	Fst	Nm*
EST	-0.0417	-0.0135	0.0270	9.0000
MD1	-0.0937	-0.0795	0.0129	19.0888
MD2	1.0000	1.0000	0.0690	3.3750
AP	1.0000	1.0000	0.0854	2.6778
G6PD	-	-	0.0000	-
GP1	0.7333	0.8621	0.4828	0.2679
GP2	0.1934	0.1938	0.0005	471.7541
GP3	0.1114	0.1728	0.0691	3.3683
SORD	0.3236	0.4871	0.2417	0.7844
SOD1	-	-	0.0000	-
SOD2	1.0000	1.0000	0.3077	0.5625
SOD3	0.6194	0.6354	0.0420	5.6979
ME	-0.0769	-0.0244	0.0488	4.8750
AD1	-	-	0.0000	-
Mean	0.4620	0.5469	0.1577	1.3351

* Nm = Gene flow estimated from Fst = 0.25(1 - Fst)/Fst.

Genetic Distance:

The genetic similarity of 47 taxa examined using allozyme patterns and distance similarity equation was made for 3 groups of *Trichoderma* populations. The distance similarity coefficient of 3 species of *Trichoderma* were ranged from 0.9397 to 0.9642 (Table 6), with the lowest distance value obtained for *T. aureoviride* to *T. harzianum* population. *T. aureoviride* population*s* were shown the highest similarity value with of *T. harzianum* populations. The highest similarity value was 96.42%. In the species of *T. longibrachiatum* populations, a larger similarity coefficient was obtained from 93.97% % to 94.15% with *T. aureoviride* and *T. harzianum* populations.

Cluster Analysis:

Cluster analyses were valuable for determining relationships among 3 different species of *Trichoderma*. The phenetic analysis included all nine allozyme systems analyzed, thus giving much more completed

Table 6: Genetic identity (above diagonal) and genetic distance (below diagonal) value of 3 species of *Trichoderma* according to the index of Nei 1972.

Species of *Trichoderma*	*T. harzianum*	*T. aureoviride*	*T. longibrachiatum*
T. harzianum	0.0000	0.9642	0.9415
T. aureoviride	0.0358	0.0000	0.9397
T. longibrachiatum	0.0585	0.0603	0.0000

information than if each system was analyzed separately. Pairwise genetic similarity according to the distance similarity coefficient were calculated between taxa and used in the cluster analysis.

The dendrogram obtained by the UPGMA clustering method revealed the genetic relationship of 3 different species of *Trichoderma* from total 47 Tricho samples tested in this study (Figure 1). The genera examined were divided into two groups, A and B. According to the dendrogram the first major group was divided into two subgroups, A1 and A2. Whereas the second group was showed of *T. longibrachiatum* populations belonging to the genus of *Trichoderma*.

Figure 1. Showed two major groups and was in closely agreement with the cluster analysis. The members in the first major group were dispersed. They split into two distinct subgroups. The subgroups first and second were included *T. harzianum* and *T. aureoviride* from all Tricho populations. This sub group was clearly distinct from those of *T. harzianum* and *T. aureoviride* but the genetic distance similarity value was highly similar each other species (similarity value of 96.42%). Another major group was formed by the species of *T. longibrachiatum* populations.

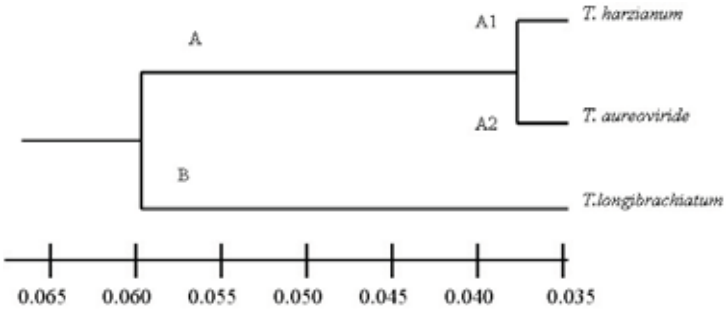

Fig. 1: Dendrogram based on UPGMA analysis of genetic similarity obtained from isozyme data, showing relationships among 3 species of *Trichoderma*.

Discussion:

There is a need for simple, rapid, inexpensive and reliable diagnostic methods for the identification of *Trichoderma* fungi. Identification of *Trichoderma* species on the basis of morphological and physiological characteristics are difficult, as these traits exhibit variations on a continuous scale that may overlap between the species. Although a detailed taxonomic key based on these characteristics is available for the identification of *Trichoderma* species (Bissett, 1984).

PAGE-mediated allozyme analysis seems to be an inexpensive and useful alternative for the identification of *Trichoderma* isolates. As there are many available enzyme systems for PAGE, it is highly probable that markers can be found for the differentiation and practical identification of other clinically relevant fungal species, as has been demonstrated for the genus of *Fusarium* (Laday and Szecsi, 2001). The best resolution of allozymes on pre-cast gels, a short run time (20 min) and minimal equipment requirements make PAGE a promising and inexpensive method for culture based diagnosis that can be applied for the identification of *Trichoderma* isolates.

Allozyme polymorphism has a simple genetic basis with the allozymes bands that denote polymorphism at a single locus often termed the allozymes (Prakash *et al*., 1969). Allozyme display the codominant expression so that heterozygous. The allozyme locus may be distinguished from either homozygote. When heterozygous at a locus for a monomeric enzyme, two allozyme bands will be produced if the enzyme is dimeric because the allelic product at demeric loci can associate at random producing both homodimer and heterodimer (Moore and Durham, 1996).

Allozyme polymorphism data obtained from this study provided good resolution to support the taxonomical identification. Although SOD are allozymes involving in the defense mechanism of mycelium (Weeden and Wendel, 1989), the enzymes levels and allozyme patterns are depending on various environmental factors

(Karpinska *et al.*, 2001). The two allozyme systems (GP and SOD) examined are more than suitable to assess genetic variability in *T. harzianum, T. aureoviride* and *T. longibrachiatum* populations. In these methods show different patterns which these patterns are useful for taxonomy identification. MDH did not reveal any variation between species but displayed variation between three species populations.

T. harzianum exhibits moderately high levels of genetic variation, especially at the taxon level. Standard measures of genetic variation are considerably higher (P=0.571, A=0.7857, H_E =0.1542) than the mean of *T. longibrachiatum* species in general (P=0.428, A=0.7500, H_E =0.1425) (Table 3). However, the population level of *T. aureoviride* (P=0.562, A=0.7857, HE =0.1466) are slightly difference when compared with the mean values of *T. harzianum* populations. The mean percentage of polymorphic loci in *T. harzianum* populations was slightly higher compare to other species of *T. aureoviride* (P=0.562) and *T. longibrachiatum* (42.80%) populations. The mean of expected heterozygosity within populations for *T. harzianum* was also slightly higher (H_E =0.1542) than those values reported for other populations of *T. aureoviride* and *T. longibrachiatum*. The moderately high levels of genetic variation reported here for *T. harzianum* may be closely related to the *Trichoderma* characteristic and reproductive system.

High levels of genetic diversity are not normally expected in a species that underwent founder events. For example, the dioecy in *D. angustifolium* may ameliorate the effects of local inbreeding. High genetic diversity has been reported in other cycads that have narrow distributions and relatively low population densities (cf. *Macrozamia riedlei,* (Byrne and James, 1991) and *D. edule*, Gonzalez-Astorga *et al.* (2003).

The morphological data and genetic polymorphisms are revealed by our allozyme analysis of the taxa studied corresponding very well of each other populations. The genetic similarity and cluster analysis among 47 taxa between three species of *Trichoderma* populations showed that *T. harzianum* populations are closer to *T. aureoviride* populations than to *T. longibrachiatum* populations *(*Table 6). *T. harzianum* and *T. aureoviride* populations show greater genetic similarity than *T. longibrachiatum* and *T. harzianum*. The dendrogram also displays two major groups (Fig. 1) the first major group contains *T. harzianum* populations and *T. aureoviride* populations, whereas *T. longibrachiatum* populations are in the second group. The results support the taxonomic character, which those *T. aureoviride* populations are closely related to *T. harzianum* populations.

Conclusion:

The allozyme technique has proved to be more successful in supporting the taxonomical identification and providing the cluster analysis of *T. harzianum* populations and related taxa. Levels of genetic variation are moderately high in *T. harzianum* species populations, especially considering differentiates status to other *Trichoderma* species populations. It appears to be a slightly difference character of *T. aureoviride* populations but *T. longibrachiatum* populations fully distinguished different character compare to *T. harzianum* populations. Whether, investigation of genomic DNA technique using *T. harzianum* populations such as allozyme and morphological data, should be reduced any confusion to identify of *T. harzianum* species populations.

ACKNOWLEDGMENTS

The author thanks Dr. Subah, Dr. Siti, Md. Nurul Amin, Ying-Ying and Mala for help with various aspects of this study. This study was carried out by Genetic laboratory, Department of Biology, UPM, Malaysia. This study was funded by the Ministy of Science, Technology and Innovation for the research grant from E-Science, Malaysia.

REFERENCE

Appel, D.J. and T.R. Gordon, 1996. Relationship among pathogenic and nonpathogenic isolates of *Fusarium oxysporum* based on the partial sequence of the intergenic spacer region of the ribosomal DNA. Molecular Plant-Microbe Interactions, 9: 125-139.

Bissett, J., 1984. A revision of the genus *Trichoderma.* I. Section *Longibrachiatum* sec. nov. Canadian Journal of Botany, 62: 924-931.

Bissett, J., 1992. *Trichoderma atroviride*. Canadian Journal of Botany, 70: 639-641.

Byrne, M. and S.H James, 1991. Genetic diversity in the cycad Macrozamia riedlei. Heredity, 67: 35-39.

Elias, S.K. and R.W. Schneider, 1992. Genetic diversity within and among races and vegetative compatibility groups of *Fusarium oxysporum* f. sp. *lycopersici* as determined by isozyme analysis. Phytopathology, 82: 1421-1427.

Enkerly, J., G. Felix and T. Boller, 1999. Elicitor activity of fungal xylanase does not depend on enzymatic activity. Journal of Plant Physiology, 121: 391-398.

Gonzalez-Astorga, J., Vovides, A.P., M.M. Ferrer and C. Iglesias, 2003a. Population genetics of Dioon edule Lindl. (Zamiaceae, Cycadales): biogeographical and evolutionary implications. Biological Journal of the Linnean Society, 80: 457-467.

Grondona, I., M.R. Hermosa, M. Tejada, M.D. Gomis, P.F. Mateos, P.D. Bridge, E. Monte and G.I. Acha, 1997. Physiological and biochemical characterization of *Trichoderma harzianum*, a biological control agent against soilborne fungal plant pathogens. Applied Environmental Microbiology, 63: 3189-3198.

Harris, H. and D.A. Hopkinson, 1976. *Handbook of Enzyme Electrophoresis in Human Genetics*. North-Holland Publishing Company.

Hedrick, P.W. 2000. Genetics of populations, 2nd edn. Sudbury, MA: Jones and Bartlett Publishers.

Ho, Y.W., G. Varghese and G.S. Taylor, 1985. Protein and esterase patterns of pathogenic *Fusarium oxysporum* f. sp. *elaeidis* and *F. oxysporum* var. *redolens* from Africa and non-pathogenic *F. oxysporum* from Malaysia. Phytopathology, 114: 217-222.

Howell, C.R., 2003. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. Plant Disease, 87: 4-10.

Hunter, H. and C.L. Markert, 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science, 125: 1294-1295.

Illias, G.N.M., 2000. Trichoderma Pers. Ex. Fr. and its efficacy as a biological control agent of basal stem rot of oil palms (Elaeis guineensis). Ph.D Thesis. University Putra Malaysia, Malaysia, pp283.

Karpinska, B., M. Karlsson, H. Schinkel, S. Streller, K. Suss, M. Melzer and G. Wingsle, 2001. A novel superoxide dismutase with a high isoelectric point in higher plant. Expression, regulation and protein localization. Plant Physiology, 126: 1668-1677.

Keszler, A., E. Forgacs, L. Kotali, J.A. Vizcaino, E. Monte, I. Garcia-Acha, 2000. Separation and identification of volatile components in the fermentation broth of *Trichoderma atroviride* by solid-phase extraction and gas chromatography–mass spectroscopy. Journal of Chromatograph Science, 38: 421-424.

Kelly, A., A.R. Alcala -Jimenez, B.W. Bainbridge, J.B. Heale, E. Perez-Artes and R.M. Jimenez-Diaz, 1994. Use of genetic fingerprinting and random amplified polymorphic DNA to characterize pathotypes of *Fusarium oxysporum* f. sp. *ciceris* infecting chickpea. Phytopathology, 84: 1293-1298.

Kubicek, C.P.R., L. Mach, C.K. Peterbauer and M. Lorito, 2001. "*Trichoderma*: from genes to biocontrol". Plant Pathology, 83: 11-24.

Laday, M. and A. Szecsi, 2001. Distinct electrophoretic isoenzyme profiles of *Fusarium graminearum* and closely related species. Systematic Applied Microbiology, 24: 67-75.

Leuchtmann, A., O. Petrini and G.J. Samuels, 1996. Isozyme subgroups in *Trichoderma* section *Longibrachiatum*. Mycologia, 88: 384-394.

Lewontin, R.C. and J.L. Hubby, 1966. A molecular approach to the study of genic heterozygosity in natural populations II. Genetics, 54: 595-609,.

Lorito, M., 1998. Chitinolytic enzymes and their genes. in: *Trichoderma* and *Gliocladium*. Vol. 2. G.E. Harman and C.P. Kubicek, eds. Taylor & Francis, London, pp: 73-99.

Lowry, O.H., N.K. Rosenbrough, A.L. Far and R.J. Randall, 1951. Protein measurement with the Folinphenol reagent. Biol. Chem, 193: 265-275.

Manwell, C. and C.M.A. Baker, 1968. Gentic variation of isocitrate, malate and 6-phosphogluconate dehydrogenase in snails of the genus *Cepaea* – introgressive hybridization, polymorphism and pollution? Comparative Biochemistry and physiology, 28: 195-209.

Markert, C.L. and F. Moller, 1959. Multiple forms of enzymes: tissue, ontogenetic and species specific patterns. Proceeding Natl. Academic Science, 45: 753-763.

Mayr, E., , 1969. *Principles of Systematic Zoology*. McGraw-Hill, New York.

Moore, G.A. and R.E. Durham, 1996. Molecular markers. Ins: Hammerschlag, F.A. Litz, R.E (eds), Biotechnology of Perennial Fruit Crops. University press, Cambridge: pp: 105-140.

Nawawi, A. and Y.W. Ho, 1990. Effects of temperature and pH on growth pattern of *Ganoderma boninense* from oil palm in Peninsular Malaysia. Pertanika, 13(3): 303-307.

Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, 89: 583-590.

Nei, M., 1973. Analysis of gene diversity in subdivided populations". Proceedings of the National Academy of Sciences of the USA, 70: 3321-3323.

Pasteur, N., G. Pasyeur, F. Bonhomme, J. Catalan and J. Britton-Davidian, 1988. *Genetic interpretation of gels*". In: *Practical Isozyme Genetics*. Johhn Wiley and Sons, Ellis Horwood, pp: 31-48.

Prakash, S., R.C. Lewontin and J.L. Hubby, 1969. A molecular approach to the study of genic heterozygosity in natural populations. IV. Patterns of genic variation in central, marginal and isolated populations of *Drosophila pseudoobscura*. Genetics, 61: 841-858.

Pasteur, G., 1974. Genetique biochemique et populations, ou: pourquoi sommes- nous polymorphes"? In Lamotte, L (ed) Lepolymphisme dans le regne animal. Societe zoologique de France Paris, pp: 473-531.

Rohlf, F.J. 1997. "*NTSYS-pc Numerical Taxonomy and Multivariate Analysis System*", Exeter software, Setauket, New York, USA.

Rosendahl, S. and R. Sen, 1992. Isozyme analysis of mycorrhizal fungal and their mycorrhizas". In *Methods in Microbiology* 24: *Techniques for the Study of Mycorrhizae* (ed. A. K. Varma, D. J. Read and J. R. Norris), pp: 169-194. Academic Press : London.

Rutherford, M.A., P.D. Bridge, R.R.M. Paterson, D. Brayford and V. Thomas, 1995. Development of *in vitro* identification techniques for special pathogenic forms of *Fusarium oxysporum*. Bulletin OEPP/EPPO Bulletin, 25: 137-142.

Samuels, G.J., O. Petrini and S. Manguin, 1994. Morphological and macromolecular characterization of Hypocrea schweinitzii and its *Trichoderma* anamorph. Mycologia, 86, 421-435.

Sneath, P.H.A. and R.R. Sokal, 1973. *Numerical taxonomy".* Freeman. San Francisco, pp 573.

Stasz, T.E., N.F. Weeden and G. Harman, 1988. Methods of isoenzyme electrophoresis for *Trichoderma* and *Gliocladium* species. Mycologia, 80: 870-874.

Szekeres, A., M. Laday, L. Kredics, J. Varga, Z. Antal, L. Hatvani, L. Manczinger, C. Vagvolgyi and E. Nagy, 2006. Rapid identification of clinical *Trichoderma longibrachiatum* isolates by cellulose-acetate electrophoresis-mediated isoenzyme analysis. The European Society of Clinical Microbiology and Infectious Diseases, 12: 369-375.

Weeden, N.F. and J.F. Wendel, 1989. *Genetic of plant isozymes*. In: Soltis, D.E., Soltis, P.S. (Eds.), Isozyme in Plant Biology. Dioscorides Press, Oregon, pp: 46–72.

Weir, B.S. and C.C. Cockerham, 1984. Estimating F-statistics for the analysis of populations structure. Evolution, 38: 1358-1370.

Weir, B.S., 1990. Genetic data analysis. Sunderland, MA: Sinauer Associates.

Whitte, G.S., 1981. Evolution of isozyme loci and their differential regulation. In: Scudder, G.G.E., Reveal, J.L. (Eds.), Evolution Today: Proc. 2nd Int. Congress of Systematic and Evolutionary Biology, Hunt Inst. Bot. Documentation, Carnegie-Mellon Univ., Pittsburgh, pp: 271-289.

Woo, S.L., A. Zoina, G. Del Sorbo, M. Lorito, B. Nanni, F. Scala and C. Noviello, 1996. Characterization of *Fusarium oxysporum* f. sp. *Phaseoli* by pathogenic races, VCGs, RFLPs and RAPD. Phytopathology, 86: 966-973.

Yeh, F.C. and R. Yang, 1999. "Microsoft Window-based Freeware for Population Genetic Analysis (POPGENE version 1.31)" . University of Alberta. Edmonton, AB Canada.