# MORPHOLOGICAL, PATHOGENIC, GENETIC AND MOLECULAR VARIABILITIES OF *Fusarium* spp., THE PATHOGENS OF ASPARAGUS CROWN AND ROOT ROT IN MALAYSIA AND BRUNEI DARUSSALAM

by

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## List of Abbreviations

| | |
|---|---|
| µg | Microgram ($10^{-3}$ gram) |
| µl | Micro liter ($10^{-3}$ ml) |
| µM | Micromolar |
| A | Asparagus |
| ANOVA | Analysis of Variance |
| B | Selangor State |
| BEA | Beauvericin |
| BM | Basal Medium |
| bp | Base Pair |
| C | Cytosine |
| cfu | Colony Forming Unit |
| CLA | Carnation Leaf-piece Agar |
| cm | Centimeter |
| CRD | Complete Randomized Design |
| dd$H_2O$ | Dionized Distilled Water |
| DOA | Department of Agriculture |
| DMRT | Duncan's Multiple Range Test |
| DNA | Deoxyribonucleic Acid |
| dNTPs | Deoxyribonucleotide Triphosphates |
| DSI | Disease Severity Index |
| EDTA | Ethylene Diaminetetraacetic Acid |
| EtBr | Ethidium Bromide |
| f. sp. | Forma Specialis |
| f.spp. | Formae Speciales |
| FA | Fusaric Acid |
| $FB_1$ | Fumonisin $B_1$ |
| Fo | *Fusarium oxysporum* |
| Fp | *Fusarium proliferatum* |
| FUP | Fusaproliferin |
| G | Guanine |
| g | Gram |
| hr | Hour |
| ha | Hectare |
| HX | Hypoxanthine Medium |
| Kb | Kilobase |
| Kg | Kilogram ($10^3$ gram) |
| L | Liter |
| M | Molar |
| M | Melaka State |
| MARDI | Malaysia Agriculture Research and Development Institute |
| min | Minute |
| ml | Milliliter |
| mm | Millimeter |
| mM | Milimolar |
| MMC | Minimal agar Medium with Chlorate |
| MON | Moniliformin |

| | |
|---|---|
| ng | Nanogram |
| $NH_4$ | Ammonium Medium |
| $NO_2$ | Nitrite Medium |
| $NO_3$ | Nitrate Medium |
| NPK | Nitrogen, Phosphorous, Potassium |
| NTSYS-pc | Numerical Taxonomy and Multivariate Analysis System |
| °C | Degree Centigrade |
| OPA | Operon Technologies Primer Series A |
| P | Probability |
| p.s.i | Per Square Inch |
| PCR | Polymerase Chain Reaction |
| PDA | Potato Dextrose Agar |
| PDAC | Potato Dextrose Agar Medium with Chlorate |
| PPA | Peptone Pentachloronitrobenzene Agar |
| PSA | Potato Sucrose Agar |
| R | Perlis State |
| RAPD | Random Amplified Polymorphic DNA |
| RDI | Root lesions with vascular Discoloration in crown and root Index |
| RFLP | Restriction Fragment Length Polymorphism |
| rpm | Revolution per min |
| S | Sabah State |
| SA | Soil Agar |
| SMC | Simple Matching Coefficient |
| spp | Species |
| SPSS | Statistical Package for Social Science |
| TBE | Tris-Borate-EDTA |
| TE | Tris-EDTA |
| U | Unit |
| UPGMA | Un weighted Paired Group Matching Analysis |
| USM | Universiti Sains Malaysia |
| UV | Ultraviolet Light |
| V | Volt |
| v/v | Volume/Volume |
| VCGs | Vegetative Compatibility Groups |
| W | Watt |
| WA | Water Agar |

**KEVARIABELAN MORFOLOGI, PATOGENIK, GENETIK DAN MOLEKULAR *Fusarium* spp., PATOGEN PENYAKIT REPUT PANGKAL BATANG DAN AKAR ASPARAGUS DI MALAYSIA DAN BRUNEI DARUSSALAM**

## ABSTRAK

Asparagus (*Asparagus officinalis*) menjadi semakin penting di Asia Tenggara (SEA) dan dalam waktu yang singkat menjadi sayuran pilihan. Semua varieti yang ditanam di seluruh SEA menghadapi ancaman serangan penyakit yang paling banyak menimbulkan kerosakan, iaitu penyakit reput pangkal batang dan akar. Sasaran utama tesis ini adalah mengumpul dan memencilkan *Fusarium* spp. daripada asparagus yang menunjukkan gejala reput pangkal batang dan akar dan juga daripada tanah di Malaysia dan Brunei Darussalam. Objektif lain adalah untuk menilai kepatogenan dan kepelbagaian genetik *Fusarium* spp. yang dipencilkan serta kevariabelannya menggunakan RAPD berasaskan PCR.

Sejumlah 110 pencilan yang terdiri daripada lima spesies *Fusarium* telah diperolehi daripada enam kawasan pensampelan di Malaysia dan satu di Brunei Darussalam. Lima spesies yang telah dikenalpasti ialah *F. proliferatum, F. oxysporum, F. solani, F. semitectum* dan *F. longipes*, berdasarkan ciri morfologinya. *F. proliferatum* dan *F. oxysporum* merupakan spesies yang terbanyak dipencilkan (83%). Ujian kepatogenan pencilan *F. proliferatum* dan *F. oxysporum* yang dilakukan dengan menginokulat anak benih asparagus varieti UC157 di rumah tanaman mengesahkan bahawa kesemua pencilan adalah patogeni. Pada awalnya, gejala penyakit yang diperhatikan adalah kekuningan di bahagian daun dan cabang. Pokok yang terjangkit menjadi terencat dengan akar menjadi perang-kemerahan dan mengecut. Belahan

batang dan pangkal tisu yang terjangkit jelas menunjukkan warna perang-kemerahan. Tanaman yang parah terjangkit akhirnya mati.

Tujuh puluh lima pencilan *F. proliferatum* dan 16 pencilan *F. oxysporum* telah digunakan untuk menghasilkan mutan reduksi nitrat (*nit*) sebagai sektor rintang klorat di atas media agar-agar kentang dekstrosa (PDAC) dan media minimum (MMC), yang ditambah dengan 2.0% dan 3% $KClO_3$. Frekuensi purata sektor, mutant *nit*-1 dan *nit*-3 di atas PDAC didapati lebih tinggi dan menunjukkan perbezaan yang bererti ($P \leq 0.05$) berbanding di atas MMC bagi kedua-dua spesies. Frekuensi purata Nit-M setiap koloni di atas MMC lebih tinggi dan menunjukkan perbezaan yang bererti ($P \leq 0.05$) berbanding di atas PDAC bagi kedua-dua spesies. Kemudiannya, mutan *nit* yang dijana telah digunakan dalam ujian komplementasi untuk mengetahui keserasian vegetatifnya. Sebanyak 23 kumpulan keserasian vegetatif (VCGs) talah dikenalpasti untuk *F. proliferatum* dan enam untuk *F. oxysporum*.

DNA bagi 50 pencilan yang mewakili dua spesies *Fusarium* tersebut dianalisis menggunakan empat primer RAPD iaitu OPA-02, OPA-03, OPA-04 dan OPA-10. Primer dipilih berdasarkan keupayaan mereka menghasilkan jalur yang jelas. Keputusan daripada analisis RAPD berupaya menunjukkan kevariabelan di kalangan dan di antara kedua-dua spesies *Fusarium*. Analisis kluster menggunakan UPGMA berdasarkan Simple Matching Coefficient (SMC) menunjukkan pencilan-pencilan tersebut tergolong di dalam dua kluster utama, iaitu spesies dan lokasi yang sama tergolong dalam kluster yang sama.

Keseluruhan kajian menunjukkan bahawa kompleks penyakit reput pangkal batang dan akar sangat penting pada semua varieti asparagus di Malaysia dan Brunei Darussalam. Patogennya telah dikenalpasti sebagai *F.*

*proliferatum* dan *F. oxysporum* berdasarkan kepada ciri morfologi, genetik (VCG) dan teknik molekul.

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## ABSTRACT

Asparagus (*Asparagus officinalis*) is becoming more important in South East Asia (SEA) and quickly becoming a preferred vegetable. All varieties planted throughout SEA have been and now are still facing the most destructive disease i.e. asparagus crown and root rot. The main aim of the thesis was to collect and isolate *Fusarium* spp. from asparagus plants showing crown and root rot symptoms and their soils in Malaysia and Brunei Darussalam. The other objectives were to evaluate pathogenicity and genetic diversity within the *Fusarium* spp. and their variability using PCR-based RAPD.

A total of 110 isolates comprising five species of *Fusarium* have been isolated from six sampling areas in Malaysia and one in Brunei Darussalam. The five species identified were *F. proliferatum*, *F. oxysporum*, *F. solani*, *F. semitectum* and *F. longipes,* based on morphological characteristics. *F. proliferatum* and *F. oxysporum* represented the highest percentage (83%). Pathogenicity tests of *F. proliferatum* and *F. oxysporum* isolates by inoculating healthy asparagus seedlings var. UC157 in the greenhouse confirmed that all isolates tested were pathogenic. The typical symptoms were initially observed as yellowing of leaves and branches. Infected plants were stunted with reddish-brown discoloration and shrivelled roots. Sliced crowns and stems clearly showed reddish-brown discolorations of the infected tissues. Heavily infected plants collapsed and died.

Seventy five isolates of *F. proliferatum* and 16 isolates of *F. oxysporum* were used to generate nitrate reduction (*nit*) mutants as chlorate resistant sectors on two media i.e. potato dextrose agar (PDAC) and minimal medium (MMC), each amended with 2.0% and 3.0% $KClO_3$. Mean frequencies of sectors, *nit*-1 and *nit*-3 mutants on PDAC were significantly higher ($P \leq 0.05$) than those on MMC for the two species. Mean frequencies of Nit-M per colony on MMC was significantly higher ($P \leq 0.05$) than those on PDAC for the two species. Later, recovered *nit*-mutants were used in complementation tests for vegetative compatibility. Twenty three and six vegetative compatibility groups (VCGs) were identified from *F. proliferatum* and *F. oxysporum*, respectively.

DNA of 50 isolates representing the two *Fusarium* species were analysed by using four RAPD primers i.e. OPA-02, OPA-03, OPA-04 and OPA-10. The primers were chosen based on their ability to produce well-defined and reproducible banding patterns. Results of the RAPD analyses were able to show variabilities within and between the two species of *Fusarium*. Cluster analysis with UPGMA by using Simple Matching Coefficient (SMC) showed that the isolates were clustered into two main groups i.e. the same species and location were found to group together in the same cluster.

The whole experiments showed that crown and root rot disease complex was the most important disease on all varieties of asparagus in Malaysia and Brunei Darussalam. The most prevalent pathogens were identified as *F. proliferatum* and *F. oxysporum* by using morphological, genetical (VCG) and molecular techniques.

# CHAPTER 1

## INTRODUCTION

Asparagus (*Asparagus officinalis* L.) is a hardy perennial vegetable, native to the coastal region of Europe and eastern Asia, where it has been cultivated for over 2,000 years (Sandsted *et al.*, 2001). It was a well-known and valued vegetable to both the Greeks and Romans (Sandsted *et al.*, 2001). The word asparagus comes from the Greek *asparagos*, meaning shoot or sprout (Sandsted *et al.*, 2001). The genus *Asparagus* belongs to the lily Family i.e. *Liliaceae* and includes over 25 cultivated species, but only *A. officinalis*, the garden asparagus, is grown for food (Bailey and Bailey, 1976). Asparagus is produced in temperate and tropical regions that span over 50 countries. Fields are established with seeds, transplants or crowns, and the marketable spears are cut when they are 18 - 22 cm long after the second or third year. Growers expect their asparagus fields to remain profitable for 10 - 15 years with most fields reaching peak production after 5 - 8 years. In 1997, asparagus was grown on over 215,000 ha world wide. When compared to 1992, this acreage represented a 27% increase in land cropped with asparagus (Nigh, 1999).

Nutritionally, asparagus has a low content of both calorie and sodium, yet it provides significant amount of vitamins A and C in the diet. It also provides the vitamins such as riboflavin, niacin, and thiamine and the minerals such as iron, phosphorus, and potassium (Sandsted *et al.*, 2001). Asparagus is becoming more important in South East Asia (SEA). Most of the varieties cultivated in this region were introduced from temperate or semi-temperate countries into SEA (Salleh *et al.*, 1996). It is believed that the first asparagus

seeds were introduced to Malaysia from Taiwan in the 1950s. Although it is a fairly new crop, asparagus has become a preferred vegetable especially by Malaysian in the higher income groups (Salleh *et al.*, 1996).

During recent and continuous disease surveys on 24 farms in Malaysia, Indonesia, Thailand and Brunei Darussalam carried out by Salleh (1990) and Salleh *et al.* (2004), the most destructive disease was found to be crown and root rot (*Fusarium proliferatum*), followed by wilts (*F. oxysporum* f. sp. *asparagi*), anthracnose (*Colletotrichum gloesporioides*), brown spot (*Curvularia* spp.) (Salleh *et al.*, 1996), Phomopsis blight (*Phomopsis asparagi*), stem canker (*Fusarium* spp.), Phytophthora rot (*Phytophthora megasperma*), rust (*Puccinia asparagi*), crown spot and shoot die-back (*Alternaria tenuissima*), gray mold (shoot blight) (*Botrytis cinerea*), purple spot (*Stemphylium vesicarium*), Cercospora blight (*Cercospora asparagi*), and several viral and bacterial diseases.

Elsewhere in the temperate countries, over 12 species of *Fusarium* are found colonizing crown tissues of asparagus. However, only three diseases are recognized i.e. Fusarium crown and root rot, dead stem and spear spot and spear rot (Blok and Bollen, 1995; Schreuder *et al.*, 1995 Elmer *et al.*, 1996). It seems that Fusarium crown and root rot of asparagus is the most economically important disease of asparagus all over the world. The disease has been described under several names; these include dwarf asparagus (Cooke, 1923), wilt and root rot (Cohen and Heald, 1941), seedlings blight (Graham, 1955), foot rot (van Bakel and Kertsen, 1970), crown rot complex (Endo and Burkholder, 1971), and stem and crown rot (Johnston *et al.*, 1979). It was also cited as the primary disease associated with asparagus decline and replants

problems (Grogan and Kimble, 1959; Elmer *et al.*, 1996), and replant bound early decline (Blok and Bollen, 1995). The plethora of common names reflects the wide range of opinions as to the manifestation of the symptoms of this disease at different stages. Asparagus decline was defined by Grogan and Kimble (1959) as a "slow decline in the productively old asparagus plantings, to the point where the plantings become unprofitable to maintain". Damage from asparagus decline includes a reduction in spear size and number, and eventual death of the crown. Additional loss is incurred if abandoned asparagus fields are replanted with asparagus. In these plantings, stunting, chlorosis, wilt and death appear to prevent stand establishment (Grogan and Kimble, 1959). The replant problem in asparagus has many similarities to asparagus decline. Grogan and Kimble (1959) defined the replant problem as "the inability to establish productive planting in the field where planting have declined". Overall, one of the most devastating diseases of asparagus is crown and root rot caused by *F. proliferatum* and *F. oxysporum* in United States of America, Europe, Africa and Canada (Blok and Bollen, 1995; Schreuder *et al.,* 1995; Elmer *et al.,* 1996; Elmer, 2001; Yergeau *et al.,* 2005). The disease can be devastating to seedlings and young plants. Symptoms noted were extensive rotting of feeder and storage roots. Vascular discoloration is also observed in the crown and base of infected stems, followed by fern chlorosis, wilt and death. Reddish brown lesions were also present on the exterior of stems and roots (Schreuder *et al.*, 1995).

In Malaysia, the species of *Fusarium* associated with asparagus crown and root rot was identified as *F. proliferatum* by Salleh (1990), and later the causal organisms of the disease complex were identified as *F. nygamai*, *F.*

*oxysporum* and *F. proliferatum* by Sapumohotti (1992), based on morphological characteristics. The main characteristics used were the shape of macroconida, presence or absence of microconidia, shape and mode of formation of microconidia, nature of the conidiogenous cells bearing microconidia, presence or absence of chlamydospores as described by Nelson *et al.* (1983), Burgess and Liddell (1983), Burgess and Trimboli (1986), Singh *et al.* (1991), and Burgess *et al.* (1994).

Visual assessment of the disease caused by *Fusarium* species is often insufficient to diagnose the causal agents of the disease particularly where several organisms induced similar symptoms within a disease niche. Conventional methods for the identification of *Fusarium* species in plant tissues involve isolation of the fungus into axenic cultures. The isolated organisms are generally identified on the basis of morphological characteristics of the colony, conidia and conidiogenous cells. Reliable identification to the species level requires considerable expertise and is greatly complicated by plasticity and instability of *Fusarium* species in culture. These features of *Fusarium* species have resulted in several classification systems, with widely differing in species concepts, and were proposed by Wollenweber and Reinking (1935), Raillo (1935; 1950), Snyder and Hansen (1940; 1941; 1945), Gordon (1952), Bilai (1955; 1970), Snyder *et al.* (1957), Messian and Cassini (1968; 1981), Booth (1971), Matuo (1972), Joffe (1974), Gerlach and Nirenberg (1982), Nelson *et al.* (1983) and Brayford (1993). The morphological and physiological methods used for identification and classification of *Fusarium* species have proved problematic (Nelson, 1991; Summerell *et al.,* 2003). Therefore recent use of molecular markers has revolutionized the analysis of

identification and population biology of plant pathogens (Milgroom and Fry, 1997).

The development of random amplified polymorphic DNA (RAPD) markers (Welsh and McClelland, 1990; William *et al.*, 1990) has provided a powerful technique for investigating intra-specific and genetic variations in fungi. RAPD analysis has been particularly useful for studies of *Fusarium* spp., and it has provided genetic markers that facilitate population studies and identification of species such as *F. oxysporum* (Assigbetse *et al.*, 1994; Crowhurst *et al.*, 1995) and *F. proliferatum* (Nicholson, 2001). PCR (polymerase chain reaction)-based markers, especially RAPD have become more popular because of their technical simplicity, and potential for rapid screening of large numbers of individuals using minimal amount of DNA. This technique has been successfully used to assess genetic variability within many plant pathogenic fungi (Goodwin and Annis, 1991; Jones and Dunkle, 1993; Huff *et al.*, 1994; Kelly *et* al., 1994) including *Fusarium* spp. in the Section Liseola (Amoah *et al.,* 1995; 1996; Voigt *et al.*, 1995; MacDonald and Chapman, 1997). In these studies, isolates from different countries were surveyed and RAPD techniques were successfully used to distinguish between mating populations of *Fusarium* spp. in the Section Liseola, the most difficult group to be confidently identified by using morphological characteristics.

Vegetative compatibility group (VCG) is another aspect that can be used as a new approach to detect genetic lines within the population of various species, particularly in asexual fungi. VCG are ideal markers for population studies because they occur naturally and are easy to score using spontaneous *nit*-mutants (Puhalla, 1985; Sidhu, 1986; Correll *et al.*, 1986b; 1987a; Bosland

and Williams, 1987; Jacobson and Gordon, 1988). Today, most population genetic studies on *Fusarium* spp. such as those in the Section Liseola have been conducted using the VCG as a marker for genotyping other fungal isolates (Farrokh-Nejad and Leslie, 1990; Campbell *et al.*, 1992; Kedera *et al.*, 1994). Strains that are vegetatively compatible i.e. belong to the same VCG, can form a stable heterokaryon, and share an identical set of alleles at about 10 *vic* loci (Leslie, 1993). The VCG technique is particularly suitable for population genetic studies, of especially on *Fusarium* spp. in the Section Liseola, because field isolates of this fungus normally belong to many VCGs (Leslie *et al.,* 1992).

The occurrence and distribution of the most important disease i.e. crown and root rot in South East Asia (SEA), its epidemiological factors and the magnitude of losses inflicted upon asparagus were reported by Salleh (1990) and Salleh *et al.* (2004). The disease situation seems to be aggravated by the fact that many asparagus farmers in SEA are now actively engaged in production of asparagus. The meager knowledge of this disease in SEA (especially in Malaysia and Brunei Darussalam) coupled with the potential major constraints to asparagus production necessitate a full attention. The present study is therefore, intended:

1. To isolate and identify *Fusarium* spp. from soils, debris and infected asparagus plants from different locations in Malaysia and Brunei Darussalam.
2. To reconfirm *F. oxysporum* and *F. proliferatum* as the causal agents of asparagus crown and root rot (Koch's Postulate).

3. To evaluate the genetic diversity within the two *Fusarium* species that caused crown and root rot of asparagus and to investigate the correlation between VCG and geographic distribution.
4. To study the molecular characteristics by Random Amplified Polymorphic DNA (RAPD) of the two *Fusarium* species and to determine their genetic relatedness.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Taxonomy and Classification of *Fusarium* spp.

The taxonomy of *Fusarium* began with the description of the genus by Link in 1809 based on the presence of fusiform non-septate spores, borne on a stroma. The publication of Die Fusarien by Wollenweber and Reinking (1935) became the foundation of the present system of classification. Later, Booth (1971) introduced a system of classification based on morphology of the conidiogenous cells and conidium ontogeny. However, many mycologists found that the two classification systems were too detailed and difficult to be followed. Thus, efforts are being made by *Fusarium* taxonomists all over the world to simplify the classification systems. This undoubtedly has led to the existence of several different systems of classification which regretfully, are still not satisfactory for the identification of all *Fusarium* species. The most acceptable part of the two classification systems has been the separation of the genus into Sections or groups which were defined by Booth (1971) as "aggregations of related species". Later, Nelson *et al.* (1983) separated each Section based on 1) presence or absence of microconidia, 2) shape of the microconidia, 3) presence or absence of chlamydospores, 4) location of chlamydospores; intercalary or terminal, 5) shape of macroconidia, and 6) shape of basal cells or foot cells of macroconidia. One of the Sections recognised by all *Fusarium* taxonomists is Liseola.

Wollenweber and Reinking (1935) included three species and three varieties in the Section Liseola. Snyder and Hansen (1945), however, reduced the number to a single species i.e. *F. moniliforme* Sheldon amended Snyder and Hansen. Booth (1971) recognised one species i.e. *F. moniliforme* with one variety i.e. *subglutinans.* According to Booth (1971), the characteristics of the species in this Section are based on 1) microconidia formed in chains or false heads, 2) microconidia spindle to ovoid in shape, 3) macroconidia slender with constricted apical cell and pedicellate basal cell, 4) chlamydospores absent, and 5) cultures brownish white to orange cinnamon. Later, Gerlach and Nirenberg (1982) increased the number to nine species and five varieties. Nelson *et al.* (1983), however, recognised only six species.

An ideal taxonomic system should reflect the genetic relatedness of taxa. It should also recognise, at an appropriate level, taxa which are distinguished by practical and significant aspects of their pathogenicity, myocotoxicology or ecology (Burgess *et al.,* 1997). The history of *Fusarium* systematics has shown marked swings between excessively narrow species concepts and those which are so broad that practical information such as pathogenicity and toxigenicity has been lost. Recent studies on biodiversity in *Fusarium* are based on the examination of large population of isolates in which traditional morphological criteria are integrated with detailed data on pathogenic specialization, toxin production and ecology, and more recently with information derived from molecular taxonomic studies (Burgess *et al.*, 1997). During the first decades of taxonomic research, many scientists contributed to describe over 1000 species, varieties and forms of *Fusarium.* Appel and Wollenweber (1910) and Wollenweber (1913) published a series of important studies on this

unique genus. On this basis, the modern concept of the genus *Fusarium* was created in Eastern Europe (Wollenweber and Reinking, 1935). The authors of this monograph reduced over 1100 species of *Fusarium* to 65 species and 22 forms and varieties. However, a much simpler system with only nine species was published by Snyder and Hansen (1940; 1941; 1945) in the USA. Later, several classification systems were developed by Messiaen and Cassini (1968; 1981), Gerlach and Nirenberg (1982), Nelson *et al.* (1983). One of the most used systems by Booth (1971) was based on descriptions of 12 Sections, 44 species and 7 varieties of *Fusarium.* Recently, Brayford (1993), a successor of Dr. Colin Booth at the Commonwealth Mycological Institute, considered 12 Sections with 52 species and 4 varieties. This classification and its phylogenetic relationships were varified by molecular and genetic criteria (Logrieco *et al.,* 1997).

Taxonomically, the genus *Fusarium* is classified in the class *Hyphomycetes*, belonging to the Sub-division *Deuteromycotina*. Teleomorphs of *Fusarium* spp. have been placed in the genera *Nectria* and *Gibberella*, order Hyphocreals (Ascomycetes). Until today, the taxonomy of the genus *Fusarium* is not settled and the number of species and Sections varies (Zema'nkova and Lebeda, 2001).

### 2.1.1 Morphological Characteristics

The genus *Fusarium* is characterized by usually fast growing, pale or bright–coloured colonies with a felty aerial mycelium and diffused or sporodochial sporulation. *Fusarium* spp. produce fusiform, curved, multiseptate

macroconidia with a pointed apical cell and a pointed basal cell that has the appearance of a foot, hence called foot cell. In some species, smaller 0 – 1 septate microconidia are formed. Thick-walled chlamydospores may be present, depending on the species (Booth, 1971).

Most of the *Fusarium* species isolated from nature produce their macroconidia on sporodochia. These sporodochial types often mutate in culture, especially on rich media. Mutations however, may rarely occur in nature. The mutants mostly show loss of pathogenicity and toxigenicity (Nelson *et al.,* 1983). Two major types of mutants arise from the sporodochial type are the pionnotal type and the mycelial type. The pionnotal type produces little or no aerial mycelium, mass of macroconidia on the surface of the colony and more intense pigmentation of colonies than the sporodochial type. The characteristics of the mycelial type are the production of abundant aerial mycelium with very few or no macroconidia and frequently a lack of sporodochia and pigmentation in culture (Nelson *et al.*, 1994).

Studies on morphological characteristics were used to determine whether phenotypic characters could be found and used to differentiate sub-species categories e.g. Group I and Group 2 strains of *F. graminearum* (Aoki and O'Donnell, 1999). Morphological species under Linnaean definitions are delimited with two primary criteria. These are: (i) within-species (morphological consistency, and (ii) sharp breaks in consistency between species (Mayr, 1963). For several purposes, morphologically-based species concepts and taxonomies are useful tools for (at least) initial classification of biodiversity. As noted by Taylor *et al.* (2000), the greatest strengths of the morphological species concepts for fungi are its general applicability to any fungal taxon and

its widespread and historical use. The Gerlach and Nirenberg (1982) and Nelson *et al.* (1983) taxonomies are both morphological in nature and phylogenetic species concepts are being tested and into which new species are being grafted.

Both physical and physiological characters have been used a morphological characters to distinguish *Fusarium* species. The shape of the macroconidia often is giving the greatest weighting when defining species, but differences in macroconidial shape and size can be confusing, subjective, and dependent upon the environment in which they are produced. Other spores, e.g., microconidial and chlamydospores, also are important in morphological species.

The value of physiological characters including growth rates, mycotoxins production, and secondary metabolites produced in different media varies. At present, growth rates, most commonly at 25°C, sometimes are used by some researchers to separate closely related species, but this character is never the primary character for a species definition of *Fusarium*. The production of secondary metabolites, including mycotoxins, also may be used as an important character in *Fusarium* taxonomy (Thrane, 2001), but is technically difficult and requires equipment and chemical expertise that many mycologists and plant pathologists lack. It is be used to define species, even though the ability of a species to produce a particular secondary metabolite (s) often is a character of critical ecological and economic importance.

### 2.1.2 Molecular and Genetic Characteristics

Besides using morphological and physiological characteristics, molecular and genetic characteristics are probably more reliable in species delimitation of *Fusarium* because these characteristics can be used directly to assess genetic variability at DNA level.

#### 2.1.2.1 Genetic Variation

Genetic variation forms the basis of species and ecosystem diversity and basic component of biodiversity, since species are composed of population that exist somewhat independently of each other. Genetic diversity exists both within and among species in any population. Direct analysis of DNA polymorphisms is a more general approach in establishing genetic variation in organisms. Vegetative compatibility groups (VCGs) are naturally occurring and may be used as genetic markers in fungi. They could provide means of identifying and characterizing the various sub-populations of different species (Anderson, 1982; Puhalla and Hummel, 1983; Sidhu, 1986;; Bentley *et al.*, 1998). Therefore, VCGs serve as a marker for genotyping fungal isolates (Farrokhi–Nejad and Leslie, 1990; Campbell *et al.*, 1992; Kedera *et al.*, 1994) from the view point of population biology. VCG analysis assay involves only one marker, while additional markers are needed for detailed population studies, e.g. determining the extent of genetic variation and its distribution in a pathogen population.

### 2.1.2.2 Vegetative Compatibility Groups (VCGs)

Hyphae of vegetatively compatible fungal strains can fuse during growth, and the fusion cell survives and may grow in some species. Isolates that are vegetatively compatible belong to a common vegetative compatibility group (VCG). However, if hyphae of the two strains do not fuse, or if one or both of the fused cells die, creating a "barrage" zone between the two mycelia, then the strains are considered to be vegetatively incompatible (Puhalla, 1979). Thus, strains within a species may be classified according to their vegetative reaction with other isolates from the same species. Vegetative compatibility is controlled by the action of a set of vegetative incompatibility (*vic* or *v-c*) or heterokaryon incompatibility (*het)* loci in Ascomycetes (Glass and Kuldau, 1992), where the sexual stages of *Fusarium* spp. belong. Incompatibility reaction may be homogenic or allelic, in which a stable heterokaryon is formed only when the two interacting strains carry the same alleles at all *vic* loci, or heterogenic, in which the alleles at one locus interact with alleles of other loci (Puhalla, 1981). The types of interaction demonstrated for *Fusarium* are of the homogenic type. Two strains in the same VCG carry the same alleles at all *vic* loci. Studies conducted on the genetic control of vegetative compatibility in *Fusarium* estimated that 10 or more unlinked *vic* loci control vegetative compatibility in *F. moniliforme* (Puhalla and Spieth, 1983; Puhalla and Speith, 1985; Sidhu, 1986).

The formation of a heterokaryon between two genetically different haploid strains is an essential part of the life cycle in most heterothallic fungi. Heterokaryon formation between different fungal individuals is an important

component in many fungi life cycles. Complementation or heterokaryosis may differ from it constituents in aggressiveness or host range; some of these aspects have been previously reviewed (Christensen and De Vay. 1955; Parmeter *et al.*, 1963; Isaac, 1967; Thinline and McNeil, 1969; Boone, 1971; Webster, 1974; Ogoshi, 1987; Rayner, 1991; Glass and Kuldau, 1992). In most cases, vegetative compatibility is homogenic that is, two fungi are vegetative compatibility if the alleles at each of their corresponding *vic* loci are identical.

Sexual and vegetative compatibility or heterokaryons are quite distinct from one another in many fungi. Strains capable of forming a successful sexual heterokaryon may be unable to form a successful vegetative heterokaryon and *vice versa*. Strains that are vegetatively compatible with one another are frequently described as members of the same vegetative compatibility or VCG. Sexual compatibility is usually governed by one or more mating–type loci that may have two or more alleles (Fincham *et al.,* 1979; Glass and Kuldau, 1992).

In Ascomycetous fungi such as *Aspergillus*, *Cryphonectria*, *Fusarium* and *Neurospora*, vegetative compatibility reactions were extensively studied (Leslie, 1993). The fungi served as models for the basic study of the genetic mechanisms controlling vegetative compatibility and they can be used to illustrate some of the ways in which vegetative compatibility may be used in the study of fungal populations (Christensen and De Vay, 1955; Brasier, 1983).

Classification by means of VCGs, does, however, have limitations. Firstly generation of *nit* -mutants necessary for VCG assignment, is laborious and time consuming for some isolates (Correll *et al.,* 1987a). Generation of Nit-M or *nit-3* mutants is very difficult, if not impossible in some isolates. Secondly, self-incompatibility can make the VCG assignment of an isolate impossible

(Jacobson and Gordon, 1988). Certain isolates have been found that they are not vegetatively compatible with themselves or other isolates, and thus can not be placed into the VCG. This phenomenon of vegetative self–incompatibility must be taken into account when examining the population biology of *Fusarium* species. This phenomenon has been found among certain isolates of *F. oxysporum* (Puhalla, 1984a; Correll *et al.,* 1987a; Bosland and Williams, 1987; Jacobson and Gordon, 1988).

In a sexually reproducing fungi, vegetatively compatible strains are more likely to be genetically similar than vegetatively incompatible strains. For example, strains of fungus those are vegetatively compatible are quite similar with respect to traits colony size (Croft and Jinks 1977; Correll *et al.,* 1986b), antibiotic production (Croft and Jinks, 1977), sanguinarine sensitivity (Puhalla and Hummel, 1983), virulence (Correll *et al.,* 1985; 1986b; Gordon *et al.,* 1986) and isozyme patterns (Bosland and Williams, 1987).

Vegetative compatibility systems generally act to restrict the transfer of nuclear and cytoplasmic elements during growth. *Nit* mutants were used as forcing markers for heterokaryon tests and VCG served as a natural means to analyze fungal populations (Leslie, 1993). In the fungal population, strains can be classified into different vegetative compatibility groups (VCGs) based on their ability to form heterokaryons with one another (Puhalla and Spieth, 1985). The allelic vegetative compatibility reaction has been described in different ascomycetous fungi, such as *Aspergillus*, *Cryphonectria*, *Fusarium* and *Neurospora*. At least 10 different vegetative incompatible (*vic*) loci (termed *het* loci) have been identified and five have been mapped in *Neurospora* (Mylyk, 1975; Perkins, 1975) while eight *vic* loci are known in *Aspergillus nidulans*

(Croft and Jinks, 1977). There also is evidence for genetic segregation of *vic* loci in both *F. moniliforme* (perfect stage *Gibberella fujikuroi*) (Puhalla and Spieth, 1983) and *F. graminearum* (perfect stage *Gibberella zeae)* (Bowden and Leslie, 1992). At least 10 *vic* loci are expected and one *vic* locus (*vic* 1) has been mapped in *F. moniliforme*. To be in the same VCG, two strains must be identical of each other at of least 10 different *vic* loci. Differences at a single *vic* locus are sufficient to block the formation of a stable heterokaryon (Leslie *et al.*, 1992).

Vegetative incompatibility serves to regulate genetic variability by controlling heterokaryosis and parasexual recombination (Leslie *et al.*, 1993). It has generally been assumed by analogy with sexual incompatibility systems. This vegetative incompatibility will markedly reduce the spread of suppressive cytoplasmic genetic elements, including, viruses, and from strain to strain in nature. Caten (1971) have suggested that vegetative incompatibility might serve to protect mycelia from invasion by suppressive cytoplasmic determinants following hyphal anastomosis, and that its role is therefore one of cellular defence against genetic infection.

Using VCGs to determine the identity of a distinct population may provide valuable insight into relationship between new and established infestation, and patterns of disease spread. Although virulence has been an extremely useful characteristic for differentiating isolates within species such as *Fusarium*, it is still only a single trait. Moreover, virulence has been shown to be influenced by a number of variables including temperature (Pound and Fowler, 1953), method of inoculation and (Kraft and Hagland, 1978) and host age (Hart and Endo, 1981) and

At the present time, most population genetic studies of *Fusarium* spp. such as *F. moniliforme* and *F. oxysporum* have been conducted using the vegetative compatibility group (VCGs) as a marker for genotyping fungal isolates (Farrokhi–Nejad and Leslie, 1990; Campbell *et al.,* 1992; Kedera *et al.,* 1994). Strains that are vegetative compatible, i.e. belong to the same VCG, can form a stable heterokaryon, and share an identical set of alleles at about 10 *vic* loci (Leslie, 1993). The VCG technique is particularly suitable for population genetic studies of *Fusarium* such as *F. moniliforme*, because field isolates of this fungus belong to many VCGs (Leslie *et al.,* 1992). Isolates of *F. moniliforme* the belonging to the same VCG are presumed to be clones, and VCG analysis might therefore be used for strain identification (Kedera *et al.*, 1994).

Most fungi can utilize nitrate as a nitrogen source by reducing it to ammonium via nitrate reductase and nitrite reductase (Garraway and Evans, 1984), but the higher Basidiomycetes, the Saprolegniaceae, and the Blastocladiales apparently can not synthesize nitrate reductase (van Alfen, 1982). The reduction of chlorate to chlorite by nitrate reductase can presumably results in chlorate toxicity in these organisms. In general, the growth of chlorate sensitive strains is restricted by the chlorate resistant strains that either do not take up chlorate or are unable to reduce chlorate to chlorite. Nitrate non-utilizing mutants *(nit* mutants) are usually unable to reduce chlorate to chlorite because of a lesion of one or more of the loci that control nitrate reductase, thus rendering them chlorate resistant.

*Nit* mutant have also been recovered from a number of other fungi, including *N. crassa* (Marzluf *et al.* 1985), *F. graminearum* (Puhalla, 1985;

Puhalla and Spieth, 1985), *F. oxysporum* (Puhalla, 1985; Correll, *et al.,* 1987a), *Verticillum albo-atrum* (Gordon *et al.,* 1986), *A. flavus* (Papa, 1986), *F. moniliforme* (Kistler *et al.*, 1987; Bowden and Leslie, 1992) and *F. poae* (Liu and Sundheim, 1996).

#### 2.1.2.2a Using VCGs to Assess Life Cycle

Many Ascomycetes are capable of performing both sexual and asexual reproduction. The lack of VCG diversity within a population can be due to the lack of sexual recombination or the selection of a particularly fit genotype, as in the asexual proliferation of a given genotype during an epidemic (Klein and Correll, 2001). Consequently, the extent of VCG diversity in a population may serve as an indicator of the relative frequencies of sexual and asexual reproduction in the population. For example, in a field study where asymptomatic corn was sampled, but 100 VCGs were identified in a population of *F. moniliforme* with very few (3%) of the VCGs represented more than once (Klein *et al.,* 1995) suggesting that sexual reproduction may be important in shaping population structure. In contrast, a population study of *F. proliferatum* from asparagus found 20 VCGs among a sample of 110 isolates, but most of the isolates (88 out of 110) belong to one of three common VCGs (Elmer, 1991). These examples indicate that, unless only certain VCGs are being selected, asexual reproduction may be the predominant factor affecting population structure.

### 2.1.2.2b VCG Diversity in Epidemics: Formae Speciales (f. spp.)

Many investigators assume that a forma specialis (f. sp.) implied some degree of genetic or evolutionary relationship among isolates within a group. Puhalla (1985) used *nit* mutants to examine vegetative compatibility within and between several f. spp. of *F. oxysporum*. Studies followed evaluating vegetative compatibility as a tool for assessing genetic diversity within population of a given f. sp. or the relatively large non–pathogenic portion of a population and its utility for pathogen and race identification (Correll *et al.*, 1986a; Correll *et al.*, 1986b; Bosland and Williams, 1987). Vegetative compatibility tests have been widely used to characterize genetic diversity in *F. oxysporum* and often provide the framework for studies on host specificity, molecular phylogenetic relationship, and population diversity. VCG diversity has been examined in over 30 f. spp. of *F. oxysporum* and a systematic numbering system was established (Kistler *et al.*, 1998), although studies vary in the extent of geographic diversity of the samples and utilization of other biochemical and molecular markers. Molecular markers and VCG usually are not independently associated. These results are interpreted to mean VCG in *F. oxysporum* represent clones, or closely strains descended of a common ancestor (Anderson and Kohn, 1995; Gordon and Martyn, 1997). The relationships between VCGs, races, and molecular haplotypes in various f. spp. has been examined (Gordon and Martyn, 1997). There are a few examples in which a f. sp. contains only one VCG, and isolates belong to a single race e. g. f. sp. *lactucum* and *albedinis* (Hubbard and Gerik, 1993; Tantaoui *et al.,* 1996).

In *F. oxysporum*, Puhalla (1984b; Puhalla, 1985) showed that the vegetative compatibility group (VCG) may be a handy tool for differentiating f. sp. of *F. oxysporum*. This study indicated that vegetative compatibility grouping can be, at least, used to substitutive the mean for pathogenicity tests. As the perfect stage of *F. oxysporum* is not known (Snyder and Toussoun, 1965), vegetative compatibility is the only mean to show the exchange of genetic information between two strains of *F. oxysporum* (Correll, 1986a; Correll *et al.*, 1986b; Correll *et al.*, 1987a). All strains within a sub-group (VCG) readily formed heterokaryon with each other, whereas strains from different groups would not. It was demonstrated that strains that were vegetatively compatible were much more likely to be genetically similar than vegetatively incompatible strains. The merger has been exploited in studies of *F. oxysporum* to determine the genetic relatedness of isolates which belong to the same race or f. sp. (Puhalla, 1985; Correll *et al.*, 1986a; Correll *et al.*, 1987a; Jacobsen and Gordon, 1988; Katan and Katan, 1988; Elmer and Stephens, 1989).

Heterokaryosis has been recognized in several species of the genus *Fusarium*, including *F. subglutinans* and *F. sporotrichioides* (Cullen *et al.*, 1983), *F. moniliforme* (Puhalla and Spieth, 1983), *F. oxysporum* (Puhalla, 1985; Correll *et al.*, 1987a), *F. graminearum* (Adams *et al.*, 1987; Bowden and Leslie, 1992), *F. poae* (Liu and Sundheim, 1996) and *F. proliferatum* (Elmer *et al.*, 1999).

### 2.1.2.2c Application of VCG Technique in Fungi

Vegetative or heterokaryon compatibility has been recognized in several species of the genus *Fusarium*, including *F. oxysporum* (Sidhu and Webster, 1979; Puhalla, 1984a; 1985; Correll *et al.,* 1986a; Correll *et al.,* 1986b; Correll *et al.,* 1987a; Bosland and Williams, 1987; Ploetz and Correll, 1988; Elmer and Stephens, 1989; La Mondia and Elmer, 1989; Katan *et al.,* 1989; Jacobson and Gordon, 1990; Manicom *et al.,* 1990; Molnar *et al.,* 1990; Correll, 1991; Katan *et al.*, 1991; Elias *et al.,* 1991; Elias and Schneider, 1992; Gordon and Okamoto, 1992; Venter *et al.,* 1992; Sapumohotti and Salleh, 1993; Fernandez *et al.,* 1994; Katan *et al.*, 1994; Fiely *et al.,* 1995; Woudt *et al.,* 1995; Katan *et al.,* 1996; Blok and Bollen, 1997; Ahn *et al.*, 1998; Ooi and Salleh, 1999; Ogiso *et al.*, 2002), *F. graminearum* (Leslie, 1987), *F. moniliforme* (Klittich and Leslie, 1988; Correll *et al.,* 1989; La Mondia and Elmer, 1989; Huang *et al.,* 1997; Giovanni and Caterina, 1998), *F. proliferatum* (Elmer, 1991; Giovanni and Caterina, 1998; Elmer, 1999), *F. poae* (Liu and Sundheim, 1996; Kerenyi *et al.,* 1997).

The VCG technique has been used widely in vegetative compatibility studies within many plant pathogenic fungi and has been proven to be a powerful tool in examining genetic diversity among their natural population for e.g. *Verticillium dahliae* (Puhalla, 1979; Puhalla and Hummel, 1983; Joaquim and Rowe, 1991; Chen, 1994, Korolev *et al.*, 2000), *Aspergillus flavus* (Papa, 1986), *Cryphonectria parasitica* (Anagnostakis *et al.,* 1986) *Verticillium albo-atrum* (Correll *et al.,* 1988), *Leucostoma persoonii* (Adams *et al.,* 1990) and *Colletotrichum gloeosporioides* (Brooker *et al.,* 1991).

### 2.1.2.3 Polymerase Chain Reaction (PCR) Technique

Several molecular techniques are available for investigating genetic variability within plant pathogenic fungi population. These include RFLP (Restriction Fragment Length Polymorphism) analysis of both nuclear and mitochondrial DNA (McDonald and Martinez, 1990; Kim *et al.,* 1992), and Random Amplified Microsatellites (RAMS). In recent years, PCR (Polymerase Chain Reaction)–based markers, especially RAPD (Random Amplified Polymorphic DNA) have become popular because of their technical simplicity, and potential for rapid screening of large numbers of individuals using minimal a mount of DNA (Huang *et al.,* 1997).

Newton and Graham (1997) defined PCR as an enzymatic DNA amplification method that comprises multiple rounds of primer extension by cycling template DNA and primers between temperature that allow repeated DNA denaturation, primer annealing and primer extension. PCR involves the enzymatic amplification of target DNA sequence by a thermostable DNA polymerase such as *taq* from *Thermus aquaticus* (Nicholson, 2001). PCR generally involves the annealing of two oligonucleotide primers to denatured DNA strands flanking the region to be amplified. The primers are extended by the DNA polymerase across the region to be amplified and so generate a copy of the target region. DNA melting is repeated up to 50 times, so producing many millions of copies of the target region (Henson and French, 1993). The sensitivity of the PCR process lies in the amplification while the level of specificity is determined by choice of primers used in the reaction. PCR has found widespread use in plant pathology and a number of PCR–based assays

have been developed for use in the identification of *Fusarium* species. Recently, PCR–based techniques have been developed to assess DNA polymorphisms in a wide variety of organisms including fungi, human and plants (Welsh and McClelland, 1990; Williams *et al.,* 1990). Polymorphisms between individuals are detected following electrophoresis of the PCR product amplified using arbitrary sequence primers (Crowhurst *et al.,* 1995). Molecular analysis might provide the additional, useful information required for a more profound understanding of the pathogens. Molecular methods involving the use of the polymerase chain reaction (PCR) have been recently described to resolve genetic variation between strains (Choi *et al.,* 1997).

**2.1.2.3a Random Amplified Polymorphic DNA (RAPD) Marker**

The molecular techniques called random amplified polymorphic DNA (RAPD) have recently been used to create genomic finger prints from species of which little is known about the target to be amplified. RAPD technique was developed by Williams *et al.* (1990). RAPD has several advantages over other polymorphic DNA-detecting technique, including RFLP. These advantages include quickness; small amount of template DNA needed and has very low reproducibility (Williams *et al.,* 1993). However, RAPD technique is very sensitive to slight changes in reaction mixtures used and contamination (Williams *et al.,* 1993). Furthermore, there is no need for a species-specific gene library or any form of clone required as probe. All that is required is one or several suitable 10-mer primers (Foster *et al.,* 1993; Grajal-Martin *et al.* 1993). RAPD analysis involves PCR using a single oligonucleotide primer (generally

10 bases) of arbitrary sequence. This analysis does not require any prior knowledge of DNA sequence of the target organism and can be carried out on a large number of isolates without the need for copious quantities of high quality DNA. These factors have led to the widespread use of this technique in studies of variability within and between species, including many *Fusarium* spp. (Bently *et al.,* 1995; Amoah *et al.,* 1996). In recent years PCR based markers especially RAPD have become popular because of their technical simplicity and the potential for rapid screening of large numbers of individuals using minimal amount of DNA. RAPD markers are especially suitable for haploid plant pathogenic fungi. This technique has been successfully used to assess genetic variability within many plant pathogenic fungi (Goodwin and Annis, 1991; Jones and Dunkle, 1993; Huff *et al.,* 1994; Kelly *et al.,* 1994), including *Fusarium* in the Section Liseola (Voigt *et al.,* 1995; Amoah *et al.,* 1995; 1996; MacDonald and Chapman, 1997).

**2.1.2.3b Application of RAPD Markers in Fungi**

Currently, molecular characterisations are increasingly being used as additional taxonomic criteria in classification or to resolve controversies in the taxonomic position of fungal taxa. RAPD markers are well suited for genetic mapping, for plant and animal breeding application and for DNA finger printing, with particular utility for studies of population genetics. RAPD markers can also provide efficient assay polymorphisms, which should allow rapid identification and isolation of chromosome-specific DNA fragments (Williams *et al.,* 1990). The development of RAPD markers has provided a powerful technique for

investigation of intra-specific genetic variation in fungi (Welsh and McClelland, 1990; Williams *et al.,* 1990). RAPD offers several advantages that may be useful in studying formae speciales and races of phytopathogenic fungi such as *Fusarium* isolates (Coddington *et al.,* 1987; Grajal-Martin *et al.,* 1993; Bently *et al.,* 1995). RAPD reduces the time needed for race identification in diseased plants, and provides genetic information on isolates studied, allowing for finger-printing of isolates (Welsh and McClelland, 1990; Grajal-Martin *et al.* 1993). RAPD analysis has been particularly useful for studies of *Fusarium* spp. and it has provided genetic markers that facilitate population studies of species such as *F. oxysporum* (Assigbetse *et al.,* 1994; Crowhurst *et al.,* 1995), *F. avenaceum* (Yli-Mattila *et al.,* 1996), *F. solani* (Achenbach *et al.,* 1996), *F. graminearum* (Schilling *et al.,* 1996), *F. poae* (Kerenyi *et al.,* 1997), *F. moniliforme* (Huang *et al.,* 1997; Kini *et al.,* 2002), *F. lateritium* (Wook Hyun and Clark, 1998), *F. proliferatum* (Nicholson, 2001), *F. subglutinans* (Zheng and Ploetz, 2002), Also RAPD has been used for detection of genetic variability for many fungi such as anthracnose pathogen of sorghum (*Colletotrichum graminicola*) (Guthrie *et al.,* 1992), dry rot fungus causing rapid timber decay in buildings and mines (*Serpula lacrymans*) (Theodore *et al.,* 1995), rust fungus *Uromyces appendiculatus* (Maclean *et al.,* 1995), dollar spot of turf grass pathogen (*Sclerotinia homoeocarpa*) (Raina *et al.,* 1997), powdery mildew *Uncinula necator* (Delye *et al.,* 1997), an entomopathogen *Paecilomyces farinosus* (Chew *et al.,* 1998), early blight pathogens (*Alternaria solani* and *Alternaria alternata*) of potato and tomato (Weir *et al.,* 1998), *Alternaria* spp. (Cooke *et al.,* 1998), ectomycorrhizal fungus *Pisolithus tinctorius* (Junghans *et*