Combined Approach of Morphological and Molecular Diagnosis of *Fusaria* spp. Causing Diseases in Crop Plants

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Most plant pathologists, at some time in their career, must identify a culture of a *Fusarium* species. The complexity of the problem varies, depending on the host from which the culture originated and the degree of resolution required in the identification. *Fusarium* species cause a huge range of diseases on an extraordinary range of host plants. The fungus can be soilborne, airborne, or carried in plant residue and can be recovered from any part of a plant from the deepest root to the highest flower. In addition, *Fusarium* taxonomy has been plagued by changing species concepts, with as few as nine or well over 1,000 species being recognized by various taxonomists during the past 100 years, depending on the species concept employed. The taxonomy of *Fusarium* spp. is based primarily on the morphology and development of conidia and conidiophores and, to a lesser degree, on host plant association and colony morphology. However, if symptoms are novel or unusual for the diseased host, as the characters are continuously variable and there is no way of knowing the degree of

variation tolerable within the individual species, the information provided by this morphological approach alone may not be particularly valuable.

Recently, molecular approaches have been used to study the phylogeny of a wide range of phytopathogenic fungi (Paavanen-Huhtala et al. 1999; Jasalavich et al. 2000; Yli-Mattila et al. 2002). Phylogenetic species recognition, based on DNA sequence data from multiple loci, allows greater numbers of species to be distinguished than in the exclusive use of morphological features (Taylor et al. 2000). The nuclear ribosomal repeat includes both highly conserved genes and more variable spacer regions (Taylor et al. 2000). The intergenic spacer region (IGS), which separates rDNA repeat units, appears to be more rapidly evolving than any region in the rDNA repeat units (Hillis and Dixon 1991). Closely related species may show considerable divergence in IGS, often reflecting both length and sequence variation (Hillis and Dixon 1991); length and restriction site variation may even occur within the rDNA of an individual in some fungi (Martin 1990). However, the multiple copies of IGS do not evolve independently (Martin 1990). Therefore, sequences of the IGS and internal transcribed spacer (ITS1 and ITS2) regions have been used for RFLP analysis of *Fusarium oxysporum* (Appel and Gordon 1995;

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Paavanen-Huhtala et al. 1999), *Fusarium avenaceum* (Paavanen-Huhtala 2000), and different species of *Fusarium* section *Sporotrichiella* (Bulat et al. 1997).

Our objective in this chapter is to outline a practical approach for authentic identification of *Fusarium* species by using the combination of morphological and molecular methods.

3.1 Overall Identification Strategy

The first step in the identification process is to clearly describe the plant disease and the symptoms observed on the diseased plant and to note the weather conditions under which the disease occurred. By using the following methods, the isolation and purification of causal agent are done and identified by using morphological and molecular criteria. These steps are evaluated in more detail in the text that follows.

3.2 Diseases Symptoms and Distribution

Strains of *Fusarium* species can cause an extraordinarily broad range of plant diseases. The most important are the crown and root rots, stalk rots, head and grain blights, and vascular wilt diseases that are well known to most pathologists (Nelson et al. 1981; Summerell et al. 2001), but lesser known diseases such as malformation disease in mango (Ploetz 2001) and bakane disease in rice can have important local economic impact. The nature of the disease provides important clues as to the species that will be recovered and often limits the range of species that need to be distinguished (Table 3.1).

Fusarium species recovered from both natural and agricultural ecosystems have distinct climatic

Table 3.1 *Fusarium* species commonly recovered as causes of diseases from economically important host plants

Plant species	*Fusarium* species
Banana	*F. oxysporum* f. sp. *cubense*
Cotton	*F. oxysporum* f. sp. *vasinfectum*
Legumes	*F. avenaceum* and *formae speciales* of *F. oxysporum* and *F. solani*
Maize	*F. graminearum*, *F. proliferatum*, *F. oxysporum subglutinans*, *F. verticillioides*
Rice	*F. fujikuroi*
Sorghum	*F. andiyazi*, *F. proliferatum*, and *F. thapsinum*
Vegetables	*Formae speciales* of *F. oxysporum* and *F. solani*
Wheat/barley/oats	*F. culmorum*, *F. graminearum*, and *F. pseudograminearum*

Table 3.2 Species of *Fusarium* regularly recovered from various parts of diseased plants as saprophytes

Plant part	*Fusarium* species
Roots and stem bases	*F. acuminatum*, *F. avenaceum*, *F. compactum*, *F. equiseti*, *F. proliferatum*, *F. oxysporum*, *F. solani*
Leaves and aerial parts	*F. proliferatum*, *F. semitectum*
Flowers	*F. semitectum*
Seed and grain	*F. chlamydosporum*, *F. equiseti*, *F. poae*, *F. semitectum*

Symptoms caused by *Fusaria* on different hosts

preferences (Backhouse and Burgess 2002; Backhouse et al. 2001; Burgess and Summerell 1992). The climate, and even local variations in weather, can limit the range of species observed, even if several are present, and influence their relative frequency. In broad terms, there are species that prefer tropical climates, hot arid climates, or temperate climates. Some *Fusarium* spp. have a cosmopolitan range (Table 3.2).

3.2.1   Symptoms caused by *Fusaria* on different hosts
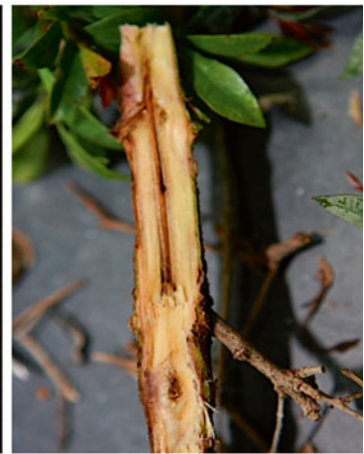

Banana wilt

wilt of cotton

Tomato wilt

Mango Malformation

Cob rot of maize

Guava wilt

Bakanae disease of rice

3.3   Isolation and Preservation

3.3.1   Isolation Techniques

There are many techniques to isolate soil fungi. The soil dilution plate technique was first developed for the isolation of bacteria, but it has been successfully applied on soil fungi which give quantitative results (Warcup 1955; Garrett 1981). Similarly, suspension-plating method is used for estimation of *F. oxysporum f. melonis* population in soils (Wensly and McKeen 1962). The screened immersion-plate technique gives a wider range and variety of fungal species isolated from soils (Chesters and Thornton 1956). On the other hand, direct soil plating method gives an advantage of detecting low fungal population in soils (Reinking and Wollenweber 1927; Warcup 1950). Moreover, *Fusarium* species could also be isolated by using living root or sterile straw baiting techniques, e.g., peas, flax, grass, banana tissue, and wheat straw (Burgess et al. 1994).

However, plating of soil dilutions or individual soil particles spread onto nutrient agar is performed by many researchers in general (McMullen and Stack 1983). Comparatively, debris isolation technique gives a higher diversity of *Fusarium* species recovered (McMullen and Stack 1983). The use of modified Nash and Snyder's medium (MNSM = PPA) is effective to determine the population of *F. solani* f. sp. *glycines* in soybean soils, while Komada's medium is selective for *F. oxysporum* (Komada 1975).

3.3.2   Preservation

There are several techniques to preserve *Fusarium* cultures into a collection. Sterilized carnation leaf pieces are good substrates for long-term preserving cultures of *Fusarium* species that was kept at −30 °C (Fisher et al. 1982). A spore suspension in sterilized 15 % glycerol kept in deep freezer at −70 °C has also been used for preservation (Leslie and Summerell 2006). The isolates that are preserved by using this method could remain viable up to 10 years. However, lyophilization preservation technique could maintain the viable cells for an extended period of time for more than 20 years. Lyophilization preservation technique is done by freeze-drying the culture with a colonized leaf piece (Tio et al. 1977). Another method used to preserve the cultures is soil preservation (Leslie and Summerell 2006). The soil must be sterilized completely in order to preserve the *Fusarium* species. This method is also considered as a long-term preservation technique.

3.4   Morphological Identification

Fusarium species are of frequent occurrence in most soils and because of their competitive ability can be easily isolated in the presence of phycomycetes, dry-spored molds, actinomycetes, or bacteria. *Fusarium sporodochia* found on woody stems or other plant tissue should be moistened, and a dilute spore suspension should be made from the mass of spores. *Fusarium* species are identified on the basis of cultural characters *viz.,* growth rate, culture pigmentation; microscopic characters *viz.,* phialides, conidia (microconidia and macroconidia) and chlamydospores.

1. *Fusarium oxysporum* **Schlecht**
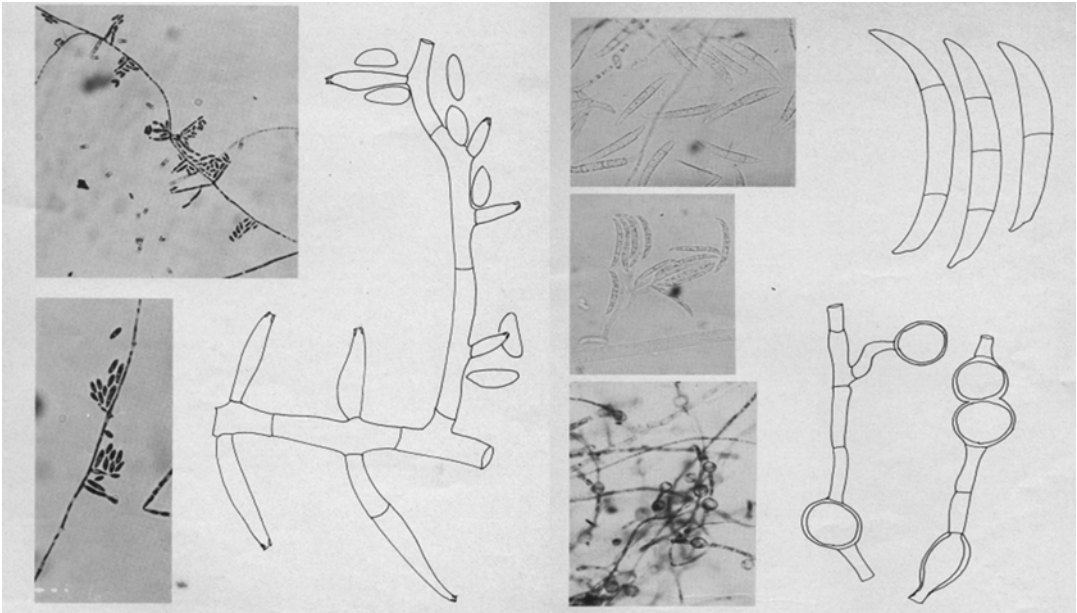
Growth rate: 4.5 cm

*Culture pigmentation*: white, peach, salmon, vinaceous gray to purple, violet

Microconidia: oval–ellipsoidal, cylindrical, straight or curved, 5–12 × 2.2–3.5 μ, produced from simple, short, lateral phialides often grouped forming *Tubercularia*-like sporodochia

Macroconidia: generally 3–5 septate, 27–60 × 3–5 μ, thin walled, fusoid

Chlamydospores: globose, formed singly or in pairs, intercalary or on short lateral branches

Diagnostic characters: the short simple phialides producing the microconidia together with the presence of chlamydospores

2. *Fusarium solani* **(Mart.) Sacc**
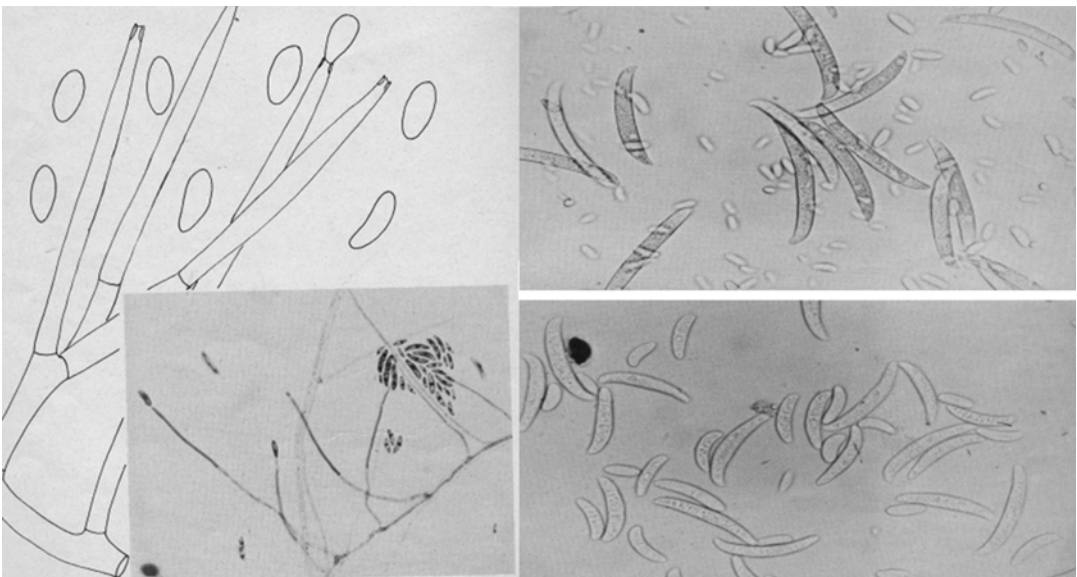
Growth rate: 3.2 cm

*Culture pigmentation*: grayish-white to white, light brown

Microconidia: 8–16 × 2–4 μ, cylindrical to oval and may become 1 septate, produced from long slender, lateral phialides 45–80 × 2.5–3.0 μ, laterally borne or on branched conidiophores

Macroconidia: generally 3–5 septate, 27–60 × 3–5 μ

Chlamydospores: globose, formed singly or in pairs, intercalary or on short lateral branches

Diagnostic characters: the short simple phialides producing the microconidia together with the presence of chlamydospores

3. *Fusarium moniliforme* **Sheldon**
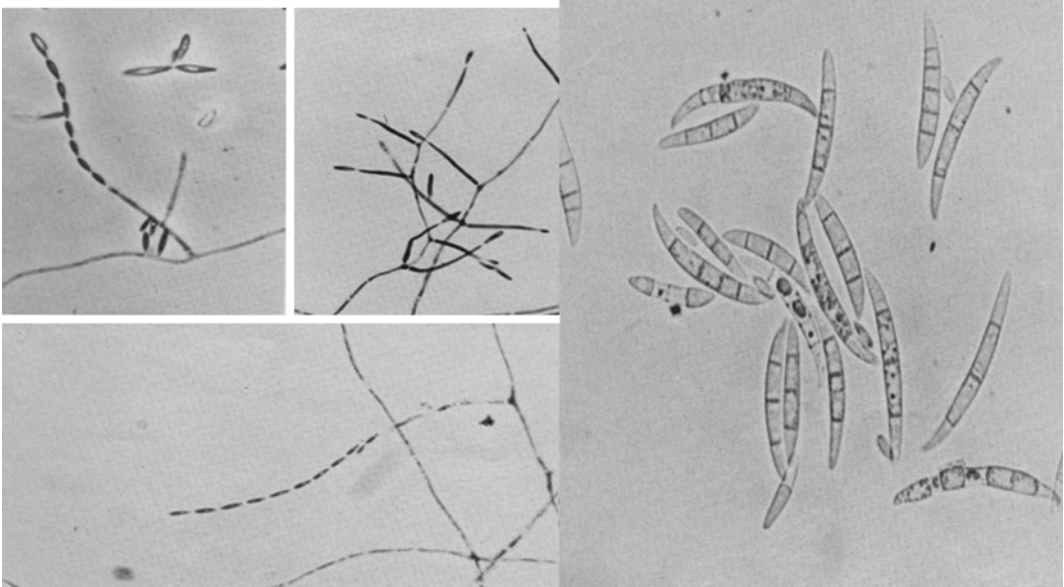
Growth rate: 4.6 cm.

Culture pigmentation: peach salmon, vinaceous purple to violet.

Microconidia: fusoid to clavate, 5–12 × 1.5–2.5 μ, occasionally becoming 1 septate and produced in chains from subulate lateral phialides, 20–30 × 2.0–3.0 μ, at the base.

Macroconidia: Some strains do not readily form macroconidia, but when present they are in equilaterally fusoid, thin walled, 3–7 septate, 25–60 × 2.5–4.0 μ.

Chlamydospores: absent but globose stromatic initial cells may be present in some cultures.

Diagnostic characters: the presence of the chains of microconidia which can be best observed in situ and the absence of chlamydospores.

4. *Fusarium decemcellulare* **Brick**
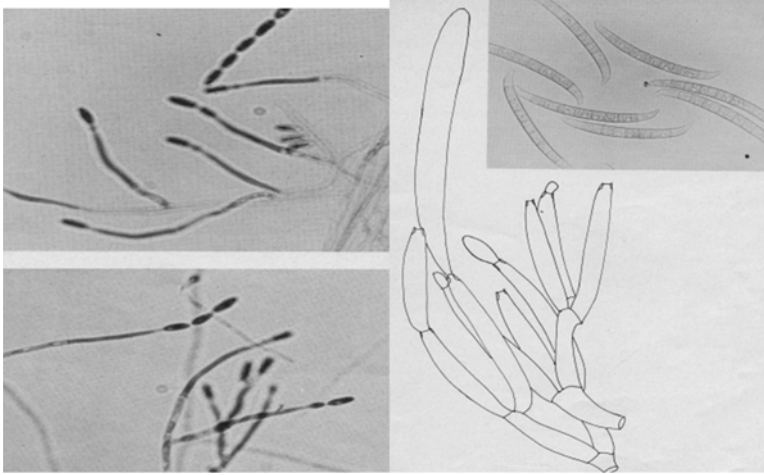
Growth rate: 3.2 cm.

Culture pigmentation: rose darkening to red, aerial mycelium white but pustules of macroconidia cream to yellow.

Microconidia: formed in chains from well-developed phialides. They are oval, aseptate, to 1 septate, 10–15×3.0–5.0 µ.

Macroconidia: formed on sporodochia from well-developed phialides. They are 7–10 septate, 55–130×6–10 µ.

Chlamydospores: absent.

Diagnostic characters: The presence of the chains of microconidia, distinct spore shape and size, pigmentation.

5. *Fusarium equiseti* (**Corda**) **Sacc**
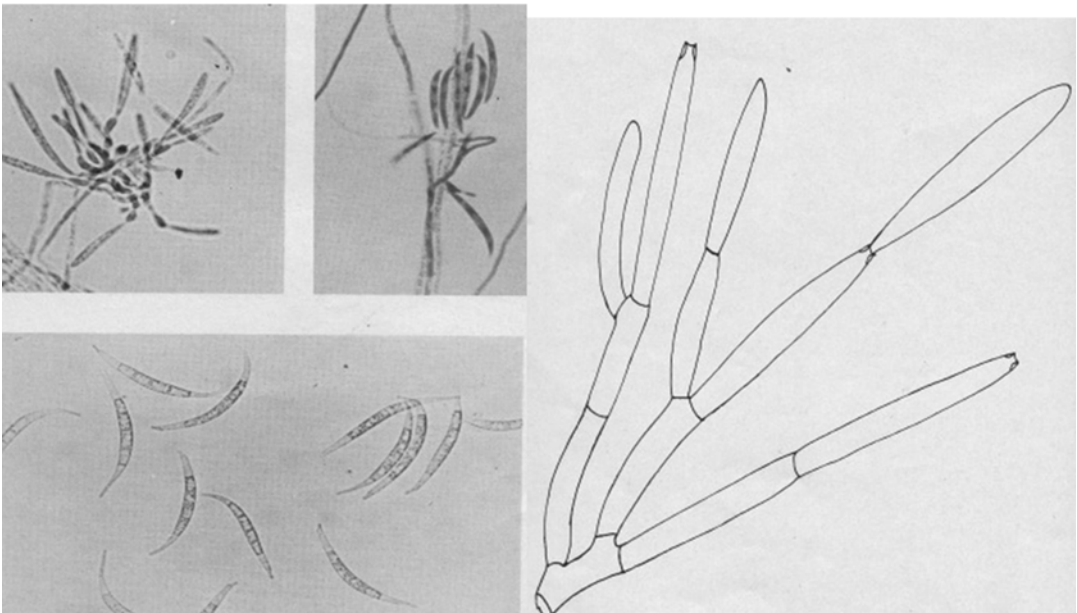
Growth rate: 5.9 cm

Culture pigmentation: peach usually changing to avellaneous and finally becoming buff brown

Microconidia: absent

Macroconidia: only are produced and these may be variable in size and are produced from single solitary or grouped phialides; conidia 4–7 septate, 22–60 × 3.5–9.0 μ

Chlamydospores: globose, 7–9 μ d, intercalary, solitary, in chains or clumps

Diagnostic characters: the absence of microconidia and pigmentation

6. *Fusarium acuminatum* **Ellis and Everhart**

Growth rate: 4.5 cm.

*Culture pigmentation*: saffron to bay to carmine red

Microconidia: absent

Macroconidia: only are produced and these may be variable in different isolates, 3–7 septate, 30–70 × 3.5–5.0 μ often with an incurved elongation of the apical cell and are produced from phialides

Chlamydospores: intercalary in knots or in chains

Diagnostic characters: spore shape and carmine-red pigmentation

7. *Fusarium udum* **Butler**
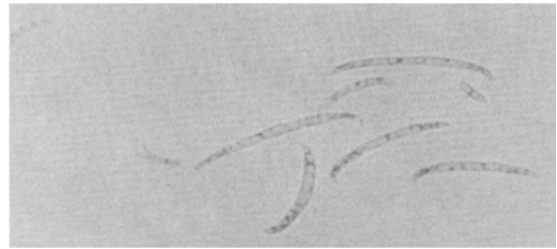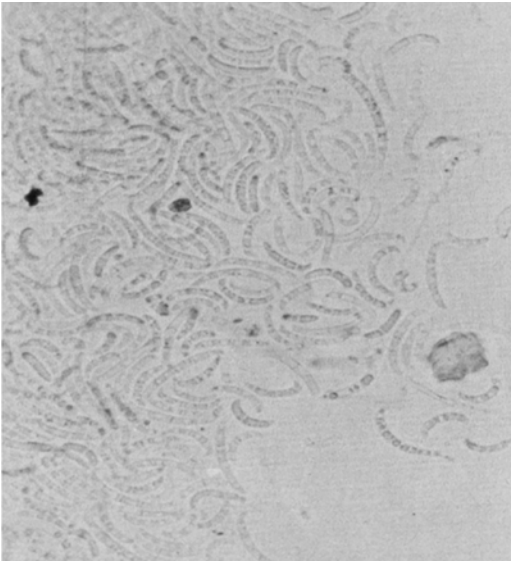
Growth rate: 4.2 cm

Culture pigmentation: Pale sulfurous to rose-buff becoming salmon-orange with production of conidia, occasional strains have purple pigmentation

*Conidia*: no clear distinction between microconidia and macroconidia. Conidia variable with a strongly curved or hooked apex, 6–8 × 3–3.5 μ and 30–40 × 3–3.5 μ

Chlamydospores: sparse, oval to globose, 8–11 × 8–12 μ

Diagnostic characters: extremely variable conidia with strongly curved apex and limited host range on *Cajanus* and *Crotalaria*

8. *Fusarium pallidoroseum* **Berk and Rav**
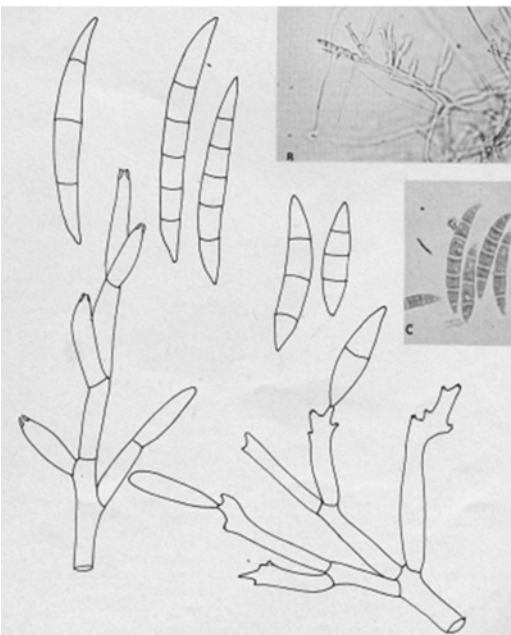
Growth rate: 6.1 cm

Culture pigmentation: peach changing to avella-neous and finally becoming buff-brown

Microconidia: absent

*Macroconidia*: of two types, primary and secondary

Primary macroconidia: with wedge-shape foot cell, 0–5 septate, 7.5–35 × 2.5–4.0 μ, formed as blastospores from polyblastic sympodial cells, up to five separate spores formed by each cell

Secondary macroconidia: with typical heeled foot cell, 3–7 septate, 20–46 × 3.0–5.5 μ, formed from phialides usually grouped in sporodochia

Chlamydospores: often sparse, globose, 10–12 μ d, becoming brown, intercalary, single, or in chains

Diagnostic characters: the presence of primary and secondary macroconidia, pigmentation, and spore form and presence of chlamydospores

9. *Fusarium graminearum* Schwabe
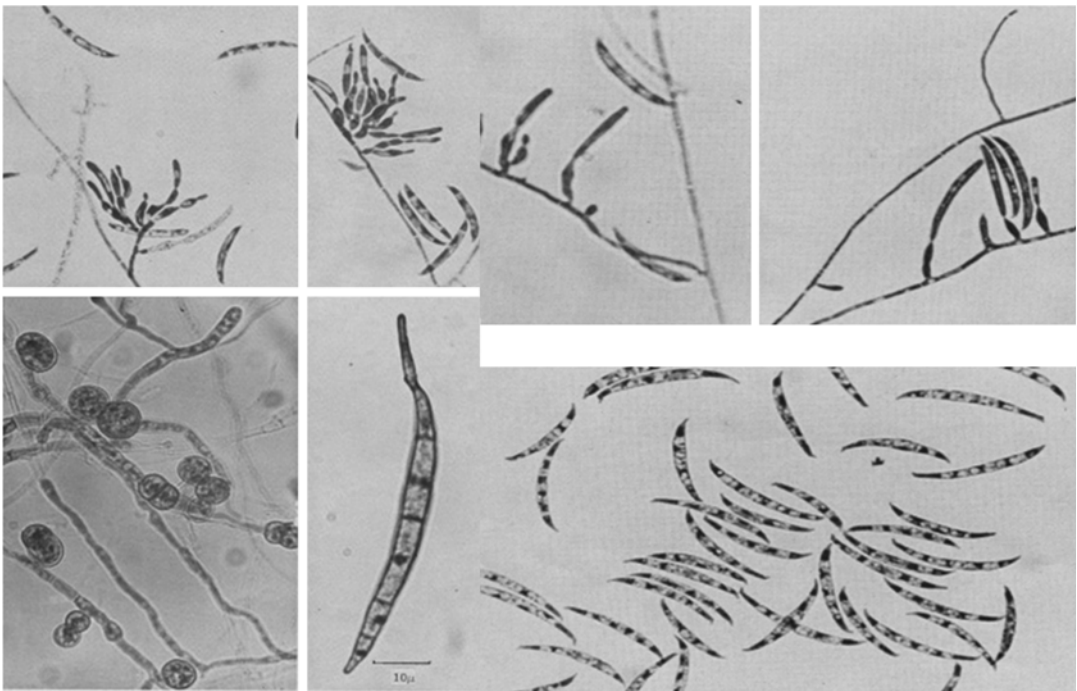
Growth rate: 8.9 cm.

Culture pigmentation: rose, coral becoming vina-ceous with a brown tinge.

Microconidia: absent.

*Macroconidia:* only are produced from simple lateral phialides which may or may not

become grouped on branched conidiophores. Macroconidia falcate generally with an elon-gated epical cell narrowing gradually to a point, 3 septate, 30–50 × 3.5–4.0 μ, 5–7 septate, 36 × 3.5–5.0 μ.

Chlamydospores: absent or rare. If present, intercalary, 10–12 μ d in knots or in chains.

Diagnostic characters: The long falcate macroconidia often formed sparsely in many strains are characteristic. Many isolates of this species with floccose aerial mycelium and rose to coral pigmentation produce neither macroconidia nor chlamydospores until surface of colony is washed clean of mycelium and culture reincubated.

10. *Fusarium dimerum* **Penz**
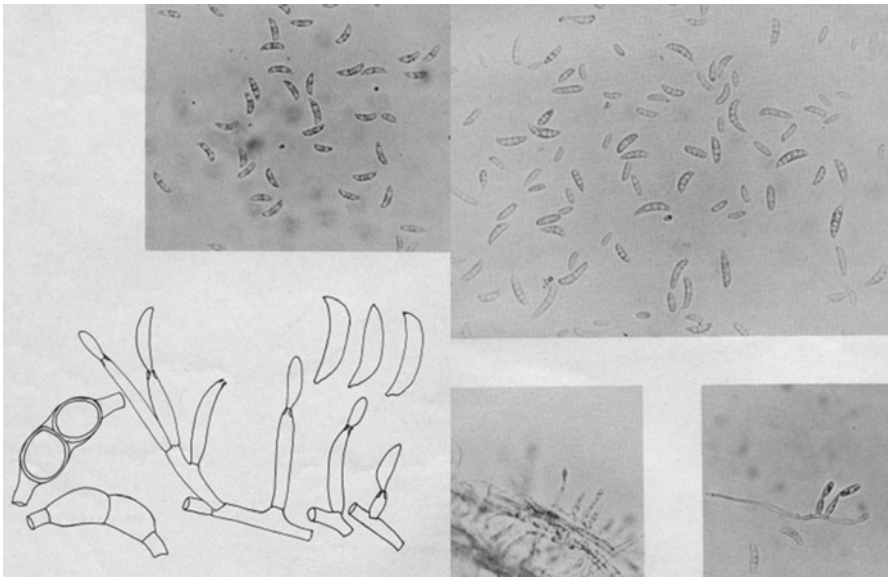
Growth rate: 2.7 cm.

Culture pigmentation: orange-beige to apricot.

Conidia: Somewhat heterogeneous probably representing primary and secondary conidia as occasional phialides develop, 0 septate, 6.5–10.5×2.3–2.5 μ, 1–2 septate, 10–12×3.0–3.5 μ.

Chlamydospores: globose, oval to smooth, 8–12 μ d, intercalary, formed singly or in chains.

Diagnostic characters: Conidial form and presence of chlamydospores separate it from the related species.

11. *Fusarium aquaeductuum* **Lagerh**
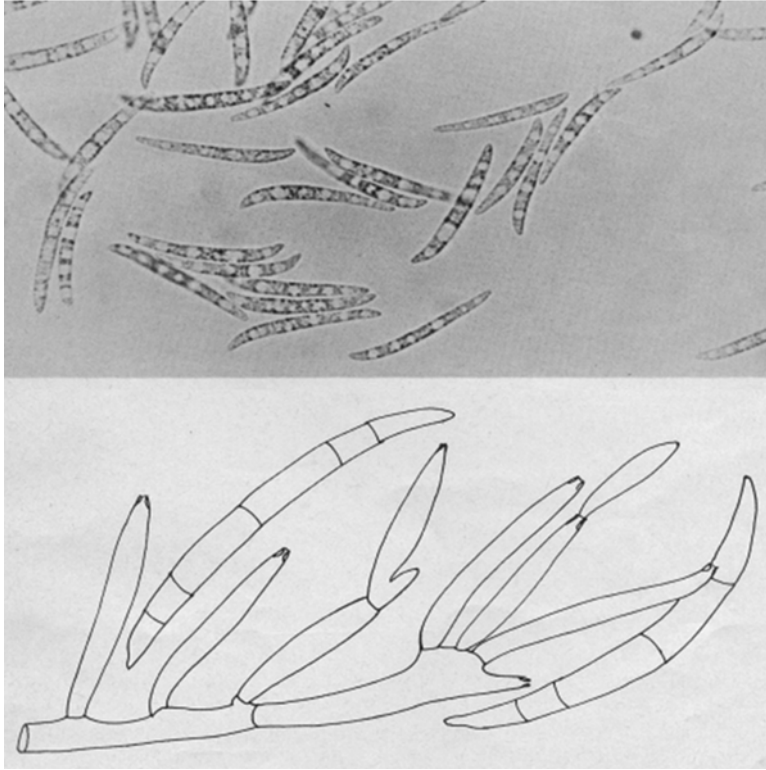
Growth rate: 0.5 cm.

Culture pigmentation: pale cream becoming orange or salmon-pink with convoluted, mermismoid or fibrillose surface appearance.

Microconidia: Absent.

Macroconidia: only are formed from pionnotes sporodochia. indistinctly 1–5 septate, variable in length, 15–65 × 2.5–4.0 μ.

Chlamydospores: absent.

Diagnostic characters: slow growth, macroconidia shape and size, absence of chlamydospores. Most occur in sewage or polluted water and also occur as parasites on sphaeriaceous fungi.

3.5   Molecular Identification

Conventional characterization of toxigenic *Fusarium* species has been based mainly on morphological methods (Leslie et al. 2001), which are the most routinely performed. Nevertheless, recognition by morphological characters sometimes is not enough for accurate identification of fungal isolates at the species level. Furthermore, morphological characterization is time-consuming and requires considerable expertise in *Fusarium* taxonomy and physiology (Leslie and Summerell 2006). As identification of

Fusarium species is critical to predict the potential mycotoxigenic risk of the isolates, there is a need for accurate and complementary tools which permit a rapid, sensitive, and reliable specific diagnosis of *Fusarium* species.

The concerns indicated above may be overcome by appropriate DNA sequencing and species-specific PCR assays (Jurado et al. 2006a, b). Various PCR assays have been developed for the identification of toxigenic species of *Fusarium*. Some of them are based on single-copy genes directly involved in mycotoxin biosynthesis while others are species specific (Gonzalez Jaén et al. 2004; Mulé et al. 2005). The last ones often amplify multicopy target sequences, such as IGS or ITS regions (intergenic spacer and internal transcribed spacer of rDNA units, respectively), which increase the sensitivity of the assay in comparison with PCR assays based on single-copy sequences. The use of these PCR approaches has been already useful in epidemiological analyses (Jurado et al. 2004, 2006a; b; Sreenivasa et al., 2008). Nevertheless, the worldwide distribution of toxigenic *Fusarium* species is a challenge to the universality of any species-specific PCR assay, and therefore, specificity should be confirmed in strains from various crops and/or geographic locations. Regarding DNA sequencing, the translation elongation factor 1-α (*TEF1-α*) gene appears to occur consistently as a single copy in *Fusarium* and shows a high level of sequence polymorphism among closely related species (Geiser et al. 2004), even when compared with the intron-rich portions of protein-coding genes such as calmodulin, β-tubulin, and histone H3 (Rahjoo et al. 2008). For these reasons, *TEF1-α* has become the marker of choice as a single-locus identification tool in *Fusarium*.

3.5.1   Different Techniques

3.5.1.1 RAPD–PCR Technique

El-Fadly et al. (2008) explored the possible utilization of random amplified polymorphic DNA (RAPD–PCR) technique for identifying *Fusarium* spp., either alternatively or complementary to those based upon morphological and pathological characteristics.

Random amplified polymorphic DNA (RAPD–PCR) was used to identify some *Fusarium* isolates. Seven *Fusarium* isolates which were identified by their morphological and pathological characteristics as *F. semitectum*, *F. culmorum*, *F. moniliforme*, *F. solani*, *F. graminearum*, *F. oxysporum* f. sp. *lycopersici*, and *F. oxysporum* f. sp. *vasinfectum* were used in this study. RAPD analysis was carried out using eight random primers; each of them consisted of ten base pairs. Genetic variability among such species and *formae speciales* under study were recorded. Six out of the eight primers were differentiated between some of the tested *Fusarium* species, since 100 % similarity was recorded between two or three different species of the fungus, while the rest of the two primers clearly distinguished each of all studied *Fusarium* spp. including the two *formae speciales*.

In conclusion, RAPD–PCR technique is a useful tool for differentiating between species and *formae speciales* of the genus *Fusarium* either alternatively or complementary to methods based upon morphological and pathological characteristics.

3.5.1.2 Amplified Translation Elongation Factor 1-α Gene Fragment

Nitschke et al. (2009) used this tool for reliable identification based on sequence information of the translation elongation factor 1-α (*TEF1-α*) gene for the numerous *Fusarium* spp. being isolated from sugar beets. In all, 65 isolates from different species (*Fusarium avenaceum*, *F. cerealis*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. proliferatum*, *F. redolens*, *F. solani*, *F. tricinctum*, and *F. venenatum*) were obtained from sugar beet at different developmental stages from locations worldwide. Database sequences for additional species (*F. sporotrichioides*, *F. poae*, *F. torulosum*, *F. hostae*, *F. sambucinum*, *F. subglutinans*, and *F. verticillioides*), isolated from sugar beets in previous studies, were included in the analysis. Molecular sequence analysis of the partial *TEF1-α* gene fragment revealed sufficient variability to differentiate between the *Fusarium* spp., resulting in species-dependent separation of the isolates analyzed. This interspecific divergence could be translated into a polymerase chain reaction restriction fragment length polymorphism assay using only two subsequent restriction digests for the differentiation of 17 of 18 species.

3.5.1.3 IGS–RFLP Analysis

The intergenic spacer (IGS) regions of the rDNA of several *Fusarium* spp. strains obtained from the collaborative researchers were amplified by polymerase chain reaction (PCR), and an IGS–RFLP analysis was performed by Konstantinova and Yli-Mattila (2004). Restriction digestion with AluI, MspI, and PstI allowed differentiation

between the related *Fusarium poae* and *Fusarium kyushuense* species. *Fusarium langsethiae* was also separated from *Fusarium sporotrichioides* (including var. minus) on the basis of the banding patterns after MspI digestion, while specific XhoI, AluI, and MspI restriction patterns were found in the IGS amplicons of *F. sporotrichioides* var. minus. According to the phylogenetic analysis of IGS–RFLP patterns, *F. langsethiae* (except for one strain), *F. sporotrichioides*, *F. poae*, and *F. kyushuense* strains formed four well-supported clades with high-bootstrap values. Based on the sequence differences in the IGS region, species-specific primers were designed for the *F. langsethiae*/*F. sporotrichioides* group and for *F. poae*. The specificity and sensitivity of the primers were tested on various *Fusarium* species and isolates and on several other important fungal genera associated with cereals. The *F. poae-specific* primers, designed in this study, showed the same specificity as primers Fp82f/Fp82r developed previously. The two phylogenetic subgroups *of F. langsethiae*, found by IGS sequencing analysis, were separated on the basis of size differences of the amplification products with primers CNL12/PulvIGSr specific for the *F. langsethiae*/*F. sporotrichioides* group.

RFLP analysis of the amplified IGS region is a useful molecular assay for characterization and a phylogenetic study of several related *Fusarium* species—*F. langsethiae*, *F. sporotrichioides*, *F. sporotrichioides* var. *minus*, *F. poae*, and *F. kyushuense*. The primers designed in this study were highly specific and allowed identification of *F. poae* and the *F. langsethiae*/*F. sporotrichioides* group.

3.5.1.4 Real-Time PCR Assay

Real-time PCR assays allow species-specific quantification of *Fusarium* biomass. A real-time PCR technique was applied for the quantification of trichothecene-producing *Fusarium* species as well as the highly toxigenic *Fusarium gra-minearum* present in barley grain and malt (Sarlin et al. 2006). PCR results were compared to the amounts of trichothecenes detected in the samples to find out if the PCR assays can be used for trichothecene screening instead of expensive and laborious chemical analyses.

3.5.1.5 Analysis of the ITS rRNA Region

Accurate morphological identification of *Fusarium* spp. beyond the genus is time-consuming and insensitive. Young Mi et al. (2000) examined the usefulness of the nuclear ribosomal RNA (rRNA) internal transcribed spacer regions (ITS1 and ITS4) to detect and differentiate *Fusarium* spp.

To investigate the genetic relationship among 12 species belonging to the *Fusarium* section *Martiella*, *Dlaminia*, *Gibbosum*, *Arthrosporiella*, *Liseola*, and *Elegans*, the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) were amplified with primer pITS1 and pITS4 using the polymerase chain reaction (PCR). After the amplified products were digested with seven restriction enzymes, restriction fragment length polymorphism (RFLP) patterns were analyzed. The partial nucleotide sequences of the ITS region were determined and compared. Little variation was observed in the size of the amplified product having sizes of 550 bp or 570 bp. Based on the RFLP analysis, the 12 species studied were divided into five RFLP types. In particular, strains belonging to the section *Martiella* were separated into three RFLP types. Interestingly, the RFLP type of *F. solani* f. sp. *piperis* was identical with that of isolates belonging to the section *Elegans*. In the dendrogram derived from RFLP analysis of the ITS region, the *Fusarium* spp. examined were divided into two major groups. In general, section *Martiella* excluding *F. solani* f. sp. *piperis* showed relatively low similarity with the other section. The dendrogram based on the sequencing analysis of the ITS2 region also gave the same results as that of the RFLP analysis. As expected, 5.8S, a coding region, was highly conserved, whereas the ITS2 region was more variable and informative. The difference in the ITS2 region between the length of *F. solani* and its *formae speciales* excluding *F. solani* f. sp. *piperis* and that of other species was caused by the insertion/deletion of nucleotides in positions 143–148 and 179–192.

3.5.1.6 Combined Approach
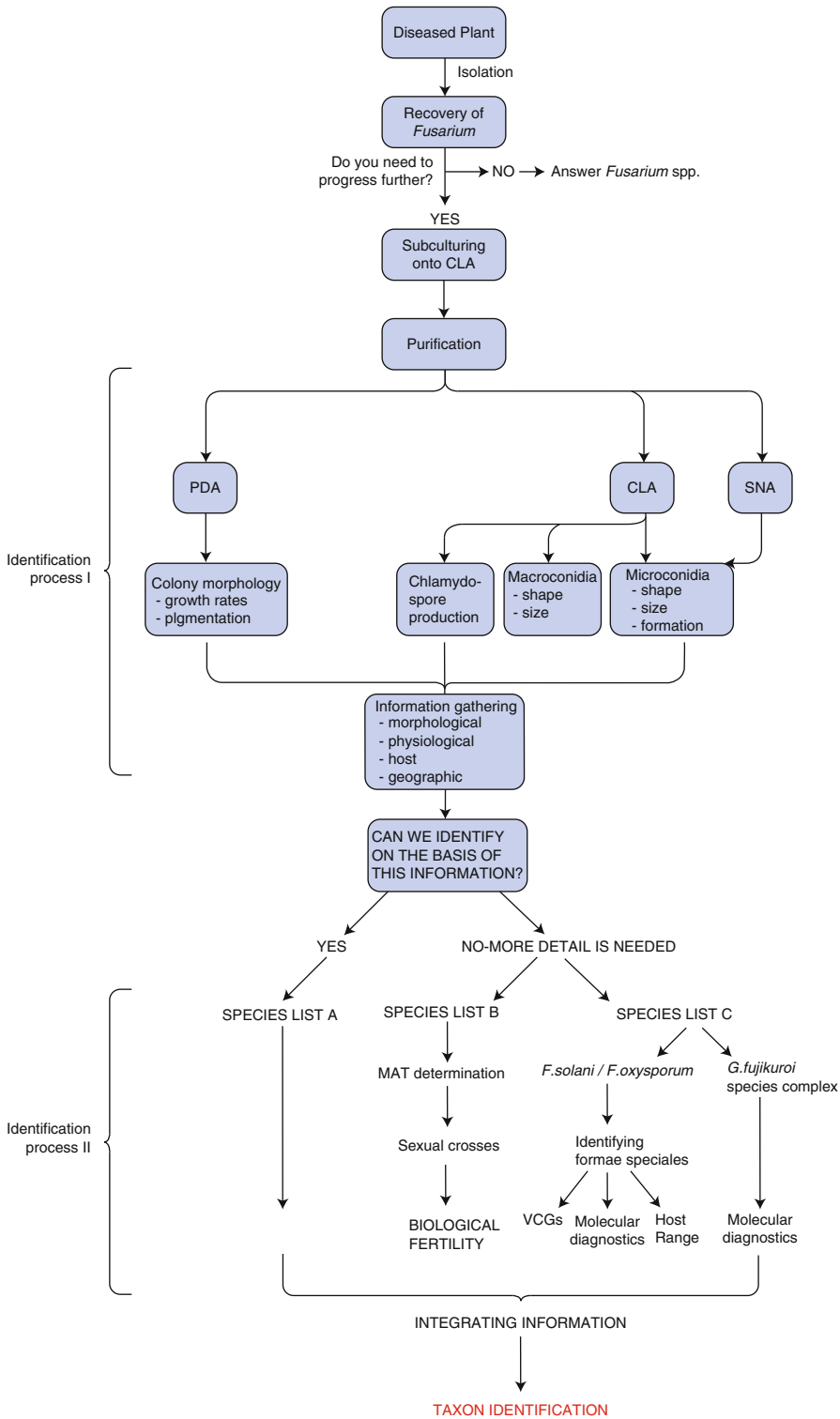
3.6 Conclusion

Different concepts have been used to define the fungal species. Morphological identification of plant pathogenic fungi is the first and the most difficult step in the identification process. This is especially true for *Fusarium* species. Although morphological observations may not suffice for complete identification, a great deal of information is usually obtained on the culture at this stage. But these approaches for identifying fungi are laborious and time-consuming and provide insufficient taxonomic resolution. The major disadvantages are that all the assays based on phenotypes are too sensitive to growth conditions and depend on gene expression. For species that cannot be reliably identified in this way, especially for members of the *G. fujikuro* complex, additional analysis such as DNA sequencing and species-specific PCR assays must be conducted. The integration of morphology and molecular-based techniques gave fair solution in easing some of the complex problems that are associated with either morphology or molecular techniques. Therefore, in this chapter, the combined morphological and molecular approaches are described and incorporated for authentic identification of *Fusarium spp*.

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