The *Fusarium* Laboratory Manual

John F. Leslie
and
Brett A. Summerell

Photographs by
Suzanne Bullock

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Blackwell Publishing Professional
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Tel.: +61 (0)3 8359 1011

Produced directly from camera-ready copy provided by the authors.

Contribution no. 06-163-B from the Kansas Agricultural Experiment Station, Manhattan.

First edition, 2006

Library of Congress Cataloging-in-Publication Data

Leslie, John F., 1953-
Fusarium laboratory manual / John F. Leslie and Brett A. Summerell.
p. cm.
ISBN-13: 978-0-8138-1919-8 (alk. paper)
ISBN-10: 0-8138-1919-9 (alk. paper)
1. Fusarium-Laboratory manuals. I. Summerell, Brett A. II. Title.

SB741.F9L47 2006
632'.4677-dc22
2006004759

The last digit is the print number: 9 8 7 6 5 4 3 2 1

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Foreword

In 1753, Linnaeus placed all the fungi in the genus *Chaos*. In the case of the genus *Fusarium* things remained more or less chaotic until 1935 when Wollenweber & Reinking created some order in their monumental monograph, *Die Fusarien* (2359). The morphological differences that they used to differentiate between 65 species, 55 varieties and 22 forms were sometimes very small and variable. Subsequently two schools of *Fusarium* taxonomists developed – the "splitters" mainly in Europe and the "lumpers" in the USA where Snyder and Hansen reduced the genus to nine species in 1945. During the 1980s, collaboration between *Fusarium* taxonomists from Europe, the USA, Australia and South Africa resulted in a remarkable level of agreement between the taxonomic treatments of Gerlach & Nirenberg in Germany, Nelson, Toussoun & Marasas in the USA, and Burgess & Summerell in Australia. This relative uniformity shifted drastically again during the 1990s with the application of the phylogenetic species concept to DNA sequences of diagnostic genes. The resulting plethora of new species of *Fusarium* often cannot be distinguished morphologically and the genus *Fusarium* might appear to some to be heading for chaos again.

Thus, the publication of this *Fusarium Laboratory Manual* by John Leslie and Brett Summerell is most timely because it integrates the morphological, biological and phylogenetic species concepts. *The Fusarium Laboratory Manual* includes detailed chapters on Techniques and Methods as well as on approaches to Taxonomy and Identification of *Fusarium* followed by Species Descriptions of 70 *Fusarium* species, arranged alphabetically from *F. acuminatum* to *F. verticillioides.* Each species description contains photographs and descriptions of key morphological characters, together with information on sexual stage, taxonomy, pathology, ecology, genetics and molecular biology. A comprehensive literature review is given for each species, including references to the most recent publications. This is a formidable undertaking for 70 species, considering that over 19,000 abstracts are available in *Biological Abstracts* for *Fusarium* with between 5000 and 6000 for *F. oxysporum* alone. This kind of information is provided for the first time for many of the 70 *Fusarium* species, particularly those described after 1983.

Many mycologists and plant pathologists who must identify isolates of *Fusarium* are extremely concerned about the proliferation of *Fusarium* species that are difficult, if not impossible, to identify morphologically. On the one hand they realize that many *Fusarium* taxa are heterogeneous and that in many cases the apparent limits of morphology have been reached for separating species. In short we have run out of morphological characters before we have run out of species that need to be separated. On the other hand they are alarmed that new species are being described based on molecular data from one or no more than a few isolates without due regard for the biology, pathology, toxicology and ecology of the taxa. The success or failure of implementing this "new" *Fusarium* taxonomy will depend on the successful integration of morphological characters, measures of cross fertility and DNA sequence data into a species concept based on the whole fungus. The methods and descriptions in this book provide a firm foundation from which such discussions and descriptions can begin.

The Fusarium Laboratory Manual is a milestone in the study of the genus *Fusarium* and will help bridge the gap between morphological and phylogenetic taxonomy. It will be used by everybody dealing with *Fusarium* in the Third Millennium.

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Preface

Laboratory workshops, in which researchers spend a week with experts in the field to learn to identify various *Fusarium* species, are an established part of the *Fusarium* community. The best known of these were run by the late Dr. Paul Nelson at Pennsylvania State University in the 1980s and 1990s. It was at one of these workshops where one of us (jfl) was first introduced not only to *Fusarium* as a taxonomic object, but also to a cohort of other beginning *Fusarium* researchers, *e.g.*, Tom Gordon, Anne Desjardins, Jim Correll, and Marian Beremand, who along with the instructors of the class, T. A. Toussoun, W. F. O. Marasas, Carol Windels, Lester Burgess and Paul Nelson, have influenced much of the research in *Fusarium* over the last 20 years.

This manual springs from the tradition of *Fusarium* Laboratory Workshops and is in some ways a successor to both the manual of Nelson *et al.* (1551) and that of Burgess *et al.* (279), which had their roots in these teaching and outreach efforts. However, our goal was to take this tradition beyond morphological species descriptions to include additional techniques and identification processes that are widely used in the *Fusarium* research community. We also wanted to include some of the evolutionary biology and population genetic thinking that has begun to inform the understanding of agriculturally important fungal pathogens. The need for as many different techniques to be used when examining these fungi has never been clearer. In combination with the two recent volumes from APS Press (468, 2083), this volume presents a relatively current introduction to the genus *Fusarium*, the toxins these fungi produce and the diseases they can cause.

Much of the material in this manual has been used by participants who attended workshops held at Kansas State University (2000, 2001, 2003 and 2005), the University of Sydney (2002), and the University of Pretoria (2004). The material included in this manual is meant to serve as the basis for future workshops, as well as a guide to the field for those who are looking for a reference, a description of a common species, or a technique. The content also has been influenced by the presentations at these workshops of the other instructors – Lester Burgess, David Geiser, Antonio Logrieco, W. F. O. Marasas, John Rheeder, Baharuddin Salleh, Keith Seifert, Carol Windels, Brenda Wingfield, and Kurt A. Zeller. Their thoughts on what is important, and what is not, were passed through our filters as we developed this manual.

Thanks are due to many for their efforts both with the running of the workshops and the materials that have gone into this volume. First and foremost of these is Suzanne Bullock, who is responsible for the numerous photomicrographs in this manual and whose efforts should make the morphological species descriptions accessible even to those who have never before worked with these fungi. Second to Kurt Zeller, Amgad Saleh, Jim Jurgenson, and Brook van Scoyoc, for their help in developing many of the protocols included in this volume and distilling them to a form that is (hopefully) easily understood by those who have not used them before. Finally to all of those who have helped run the various workshops, but especially Amy Beyers, Anita Kesler, Ingelin Leslie, Brook van Scoyoc, and Celest McGowan, who have developed the protocols and the timing for both the scientific, fiscal, and social aspects of the workshops that the rest of us have followed with such great success.

Portions of this book were written while one of us (jfl) was on sabbatical leave at the University of Sydney and the Royal Botanic Gardens and Domain Trust (RBGDT) and sponsored by the Australian-American Fulbright Foundation and the RBGDT. We thank the American Phytopathological Society for permission to reprint Table 11-1 and Figures 4-2 and 11-1, the American Society for Microbiology for permission to reprint Figure 7-2, and the Genetics Society of America for permission to reprint Figure 7-3. Portions of Chapter 9 are based on Leslie *et al.* (1235), and portions of Chapter 11 are based on Summerell *et al.* (2087). Both the workshops and the manual have benefited from financial support from both our home institutions, and from INTSORMIL (the International Sorghum and Millet Collaborative Research Support Program, based at the University of Nebraska, Lincoln, Nebraska), IITA (the International Institute of Tropical Agriculture, Ibadan, Nigeria), and ABRS (the Australian Biological Resource Study).

Much of the book has been evaluated by others including Walter A. J. de Milliano, Anne Desjardins, Stanley Freeman, David Geiser, Cristiano Lima, Antonio Logrieco, W. F. O. Marasas, Randy Ploetz, Amgad Saleh, Baharuddin Salleh, Brenda Wingfield, and Kurt Zeller. Their eyes have found errors and omissions that we would have missed and their suggestions have only improved the book's accuracy, utility and readability. Errors that remain, however, are solely our own, and corrections and suggestions for topics to be considered for inclusion in future versions will be gratefully received.

A final, and perhaps the greatest note of gratitude is to our wives, Ingelin and Liz, and families for their encouragement, for sharing their homes and hospitality as early and final versions of the manuscript were first put to paper and then finally put to rest, and for tolerating our interest in this group of fungi, when they might have had other places they would like for us to have focused instead. They know better than we do that the idea of a book is grand and the finished product is even better, but that the time in between is not always one of great joy.

July 2005

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1

Introduction

The genus *Fusarium* was introduced by Link in 1809 (1263), and is now approaching its third century as a genus that contains many plant-pathogenic fungi. The members of this genus can incite directly diseases in plants, humans, and domesticated animals, *e.g.*, Boonpasart *et al.* (221), Goldschmied *et al.* (718), Krcmery *et al.* (1136), Martino *et al.* (1373), Rabodonirina *et al.* (1760), Rebell (1783) and Vismer *et al.* (2263). The mortality rate for human patients with systemic *Fusarium* infections is > 70% (1136), and HIV-infected patients are susceptible to such *Fusarium* infections as well (562, 778, 1486). In addition, *Fusarium* spp. produce an intriguing array of secondary metabolites that are associated with plant disease, as well as with cancer and other growth defects in humans and domesticated animals. Some of these secondary metabolites are used commercially either directly or as the starting material for chemical syntheses of plant and animal growth promoters in both first world and third world settings (858, 1976, 2157). Allegations of the use of mycotoxins produced by some of these fungi as biological weapons also have been made (855, 1454, 1833). Two of these compounds, T-2 and diacetoxyscirpenol (DAS) are on the USDA/CDC select agent list and require special permits for study in the United States (www.cdc.gov/od/sap/docs/saist.pdf). Naturally occurring outbreaks of *Fusarium* toxicoses directly affecting humans have occurred historically, *e.g.*, Athens in the 5th century B.C. (1922), and in the Soviet Union during World War II (666, 982). Thus, *Fusarium* has always been a visible genus with many strains, species and metabolites of an importance that transcends just science or agriculture.

As a social phenomenon, *Fusarium* plant diseases have had several major impacts. One was the near devastation of the commercial banana industry in the 1960s by panama wilt caused by *Fusarium oxysporum* f. sp. *cubense* (1706). The recent losses of several billion dollars (2342) by many wheat and barley farmers to Fusarium head scab in the upper Midwest of the United States has shifted cropping strategies and bankrupted farmers in the region. At the same time, the causal agent of Fusarium head scab can be used in commercial fermentations to produce a precursor for one of the most widely used commercial cattle growth promotants (858). Recent problems caused by strains of *Fusarium* which may have originated from endophytes or pathogens of native *Gossypium* species (2286), are threatening the future of the cotton industry in Australia while simultaneously demonstrating the relatedness of native and agricultural populations and suggesting new avenues for understanding how these fungi evolve.

Many plants have at least one *Fusarium*-associated disease. A recent perusal of the plant disease list maintained by the American Phytopathological Society (www.apsnet.org/online/common/search.asp) revealed that over 81 of the 101 economically important plants on the list had at least one associated *Fusarium* disease. As these fungi also may grow as apparently symptomless endophytes under many conditions, the claim that, “If it is green, there is some *Fusarium* that can grow on it, in it, or with it” probably is not too far removed from the truth. The types of diseases induced are quite varied as is their severity, and may include root or stem rots, cankers, wilts, fruit or seed rots, and leaf diseases. Thus identifying the *Fusarium* strain present in a diseased plant sample, usually to species and sometimes further, has been and remains an important task in many plant diagnostic laboratories.

The need to identify strains and to attach names to them is as strong, or stronger, in *Fusarium* than it is in any fungal genus. That these names have a value that transcends the science from which they spring means that these names need to be assigned and changed with care and caution. Depending on the era and the identification scheme being followed (see Chapter 8), the number of *Fusarium* species could range from as few as nine to well over a thousand. Since the 1980s the number of recognized species has increased gradually, with the number of recognized species now > 80, of which 70 are described and illustrated in this text. If preliminary work from cur-

rent molecular studies stands, then this number should increase, perhaps dramatically, during the coming years. As a genus, *Fusarium* lacks a large number of morphological characters that can be used to easily differentiate species, and it is not uncommon to "run out of characters before you run out of species." This problem has left numerous species definitions less-than-well defined, has complicated problems of identifying the "real" *Fusarium xxx*, and has resulted in spirited debates between individuals with quite different views of what a species is or should be. Although many morphological species concepts have changed, many have remained stable, even when challenged with genetic and molecular criteria.

The relatively large amount of work done on the morphological taxonomy of these fungi means that as a genus, *Fusarium* often has served as testing ground for new speciation concepts in fungi. The use of molecular approaches to differentiate species has been tried with a number of strains usually considered problematic, *i.e.* not clearly fitting within a given species, but not clearly distinguishable from it either. Studies of phylogenetic lineages based on multiple-gene genealogies, and the grouping patterns resulting from studies with amplified fragment length polymorphisms (AFLPs) both provide new means of evaluating relatedness. As these techniques are independent of one another, each can be used to test the hypothesis of distinctness generated by the other, to look for continuous gradations between representative types, and to identify putative inter-specific hybrids. Studies based on biological species concepts also have been used to delimit some species, most commonly within the *Gibberella fujikuroi* species complex, while also testing hypotheses of sameness/distinctness based on both molecular and morphological characters. Subspecific differences could be indicative of "evolution in action", suggesting that these fungi are not evolutionary fossils, but entities that continue to change in response to both manmade and naturally occurring selective pressures and for the elucidation of evolutionary mechanisms. Thus *Fusarium* has been and remains an important genus for the testing and development of species concepts in fungi.

For a researcher or diagnostician not interested in state-of-the-art taxonomy and wanting primarily to attach a name to a culture, the apparent nomenclatural turmoil can be confusing and perhaps even threatening. Fortunately, many of the most important pathogens can be diagnosed, with some experience, on the basis of morphological characters. Even isolates not identified to a single species can be assigned to a group of usually closely related species. Accurate morphological characterizations require that the fungus be subcultured and handled with at least some care. Growing the fungus on media appropriate for the morphological features to develop takes time, sometimes several weeks, and means that a careful diagnosis often is a slow process. As molecular studies progress and the definition of common and important species solidify, the development of molecular diagnostics for many species also should be possible. These diagnostics should be much faster than the present morphological diagnoses, as at most limited culturing of the fungus is required. The molecular diagnostics will need to be carefully evaluated on a broad range of species as well as strains within the species to accurately define their diagnostic abilities and limitations. For laboratories that currently lack and are unable to acquire molecular expertise, morphological species definitions will remain the rule. Such scientists should seek partnerships with researchers with access to molecular technologies to confirm critical findings and conclusions, and to ensure that morphological similarities are not disguising significant genetic differences.

This book is designed as a laboratory manual and guide to techniques and species likely to be encountered. It is neither a mycological monograph (a number of species are missing) nor a comprehensive summary of work that has been done with various *Fusarium* spp. (there are > 19,000 citations to *Fusarium* in *Biological Abstracts* alone). It does contain recipes for many commonly used media, techniques that will work for common field and molecular applications, species descriptions of the most common species, and our thoughts on what a *Fusarium* species is and how the field might be evolving. We hope that you will find this manual a useful place to begin a serious study of a particular species or a *Fusarium*-induced disease, or to find a quick answer regarding these most interesting and important fungi.

Techniques and Methods

2

Media – Recipes and Preparation

2.1 Media for Growing and Identifying *Fusarium*

Various agar media have been used as standards on which to grow cultures for the identification of *Fusarium* species. *Fusarium* species have notoriously variable phenotypes when cultured on different agar media, *e.g.*, Burgess *et al.* (280). Morphological features that are common in cultures grown on some media may be absent or altered when the same strain is cultured on another medium. The basic principle is to use the same medium as that used by the authors of the identification guide to which comparisons are being made. Different media may have the same common name, *e.g.*, V-8 juice agar or potato dextrose agar, but vary in their composition. Thus, a standard recipe needs to be followed in preparing media to be used for species characterization. Adding a detergent to the media, *e.g.*, Triton X-100 or tergitol, can restrict colony size and make processes such as colony enumeration or replica plating simpler (1746, 2230). Carnation Leaf-piece Agar (CLA), Spezieller Nährstoffarmer Agar (SNA), and Potato Dextrose Agar (PDA) are the standard media used in the identification of *Fusarium* species in this volume.

Media usually are dispensed into Petri dishes or slants, sometimes termed an agar slope, in a test tube for media with agar, or in flasks for media without agar. Media for use in Petri dishes are made, autoclaved, and then dispensed into sterile Petri dishes after autoclaving. Media containing agar should be made in a flask that is twice the volume of the medium being made, *e.g.*, one liter of medium in a 2-L flask, with a 2-L flask generally the largest convenient size. Cooling the agar to ~50°C before it is dispensed is essential if a heat-sensitive solution, *e.g.*, an antibiotic, is to be added, and reduces the amount of bubbles on the medium surface and the condensate on the dish lid in all cases. The most commonly used Petri dishes are those with a 100 mm or 60 mm nominal diameter. One liter of medium usually suffices to fill 40-50 of the larger dishes (20-25 ml medium/dish), or 80-100 of the smaller dishes (10-12 ml medium/dish). If the medium is for sexual crosses, then more medium is dispensed per dish than if the medium is for a vegetative analysis (see section 2.6).

The same media used in Petri dishes also can be used in agar slants, but media that are viscous, *e.g.*, carrot agar (see section 2.6) or oatmeal agar, generally cannot be used in slants since even the slowest decompression cycle of most autoclaves results in the plugs capping the tubes being blown off or in the media boiling over the tops of the tubes. A medium to be dispensed into slants is usually made up, melted, and dispensed into the slants with a repeating syringe before the medium is autoclaved. It is very important to ensure good mixing and

complete melting of the agar before dispensing the medium; otherwise, some slants may receive too much agar and others too little, which can make the slants difficult to work with. The most commonly used test tube sizes for slants are 10×75, 13×100, and 16×150 mm nominal sizes, which receive 1.25, 2.5 and 6.0 ml of medium/slant respectively. After the medium has been added, the tubes are stoppered with cotton or foam plugs, or covered with commercially available plastic or metal caps. When autoclaving slants it is very important to use a slow decompression cycle to prevent the medium from boiling, as plugs wetted by boiling media render those cultures more susceptible to contamination. In practice, plugs wetted by media should be discarded and replaced with dry sterile plugs (made by autoclaving some tubes that contain no medium). After autoclaving, the tubes must be placed at a slant before they harden to provide additional surface area for culture growth. To get the media at a slant, the 10×75 and 13×100 mm tubes usually are left in the racks in which they were autoclaved, and the entire rack of tubes is slanted at one time. The larger slants, 16×150 mm size, may be slanted in the rack, but this often results in variation in the amount of slanting that actually occurs. A more uniform slant for these tubes is obtained by placing them individually on a slanting board to harden.

Carnation Leaf-Piece Agar (CLA). CLA is a natural substrate medium (625, 2017) prepared by aseptically placing sterile carnation leaf pieces, 3-5 mm², into a Petri dish and adding sterile 2% water agar (20 g agar in 1 L of H_2O). Usually one carnation leaf piece is added per 2 ml of medium. In 60 mm diameter Petri dishes, 5-6 pieces per dish and in 100 mm diameter dishes, 10-12.

The carnation leaf pieces are prepared from fresh carnation leaves free from fungicide or insecticide residue. Immediately after collection, the leaves are cut into 5-8 mm² pieces (they shrink when they are dried) and dried in a forced-air oven (~70°C) for 3-4 hours until brittle. Leaf pieces also can be dried in a microwave oven; the time required varies by oven. The dried leaf pieces are packaged in aluminum or polycarbonate containers and sterilized by gamma irradiation (2.5 megarads). Sterilized leaf pieces can be stored dry at room temperature for up to 12 months before use.

Most species of *Fusarium* sporulate on CLA in 6-10 days. Cultures grown on CLA produce macroconidia that are more uniform in size and form, than do cultures grown on carbohydrate-rich media such as PDA or Czapek-Dox. Macroconidia form primarily in sporodochia, which usually develop on the leaf pieces. Macroconidia formed in sporodochia are preferred for identification as they are more consistent in shape and length than are macroconidia formed from more isolated phialides. Microconidia are more common on hyphae growing on the agar, often away from the leaf pieces. The mode of formation of microconidia, *i.e.* monophialides or polyphialides, the presence of false heads or chains of microconidia, and the presence of chlamydospores can be determined by direct examination with a compound microscope (100×) when strains are grown on small (60 mm diameter) plates of CLA. CLA also is suitable for the production of large numbers of conidia for experimental work, although many researchers also use the modified Czapek-Dox medium (see section 2.5), and can be prepared for this purpose in large flat bottles or larger Petri plates, usually 150 mm nominal diameter.

Gibberella zeae (anamorph – *Fusarium graminearum*), homothallic strains of *Haemanectria haematococca* (anamorph – *Fusarium solani*), and appropriately mixed cultures of heterothallic *Fusarium* species form perithecia on CLA if incubated under light (see section 6.5). Older carnation leaves may give better results than younger carnation leaves for perithecia formation (2154). For genetic investigations, most researchers presently use carrot agar or V-8 juice agar (see section 2.6) to produce perithecia from which ascospores are collected.

Spezieller Nährstoffarmer Agar (SNA). SNA is a weak nutrient agar used for the identification and maintenance of strains of *Fusarium* and *Cylindrocarpon* (1566). SNA is prepared by autoclaving, in 1 L of distilled H_2O:

KH_2PO_4	1 g
KNO_3	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
Glucose	0.2 g
Sucrose	0.2 g
Agar	20 g

Placing 1-2 pieces of sterile filter paper (Whatman № 1), approximately 1 cm², on the agar surface after the medium has gelled can increase sporulation.

Culture degeneration, which is common on many synthetic media, usually does not occur on SNA. The medium promotes sporulation and good conidiogeneous cell development; however, because sporodochia formation is limited, macroconidial morphology is not as uniform or as reliable as seen with CLA. Cultures grown on SNA often are of value for examining microconidia, as this medium supports the formation of a range of different microconidia. In some cases microconidia formed on SNA will differ morphologically from those seen on CLA. Many strains also form chlamydospores more readily on SNA than they do on CLA, so for species in which

microconidial morphology or the presence of chlamydospores are important identification criteria, cultures from both CLA and SNA should be examined. SNA is transparent, so cultures can be viewed directly with a compound microscope (up to 100×) or small agar blocks can be mounted on a slide with a drop of water and covered with a cover slip for observation at higher magnifications.

Potato Dextrose Agar (PDA). PDA is a carbohydrate-rich medium that contains 20 g dextrose, 20 g agar, and the broth from 250 g white potatoes made up to 1 L with tap water. The potatoes are unpeeled, washed, diced and boiled until soft (actual time varies with the size of the potato). Filter the boiled potatoes through a single layer of cheesecloth, which leaves some sediment in the broth. Commercially available preparations of PDA, *e.g.*, Difco, BBL, *etc.*, are often more convenient and usually are as suitable for identification purposes as home-made preparations.

Conidia formed on PDA are not as consistent in either size or shape as those formed on CLA or SNA, and thus are much less reliable for use for identification purposes. However, colony morphology, pigmentation and growth rates of cultures of most *Fusarium* species on PDA are reasonably consistent if the medium is prepared in a consistent manner, and if the cultures are initiated from standard inocula and incubated under standard conditions. These colony characteristics often are useful secondary criteria for identification.

PDA is used by some researchers for the isolation of *Fusarium* species. We do not recommend this medium for this purpose, as many saprophytic fungi and bacteria also can grow on the medium and interfere with the recovery of the *Fusarium* present. If PDA is used for the recovery of fungi from plant material, then the concentration of potato and dextrose should be reduced by 50-75%, and broad-spectrum antibiotics (see PPA Medium in section 2.3) included to inhibit bacterial growth.

2.2 Supplementary Identification Media

Water Agar (WA). WA (2%) consists of 20 g agar in 1 L of distilled H_2O. This medium is recommended for germinating conidia used to initiate *Fusarium* cultures (see sections 4.1-4.3). As hyphal growth is sparse on this medium, it is suitable for growing cultures from which individual hyphal tips are taken to initiate new colonies (see section 4.3). In some instances, the sparse growth on WA facilitates the isolation of *Fusarium* species from plant material, particularly roots.

WA (0.05%) consists of 0.5 g agar in 1 L of distilled H_2O. WA (0.5%) is used in the preparation of soil dilution series. The small amount of agar retards the sedimentation rates of fungal propagules. The water is heated until the agar dissolves, and the heated medium distributed into flasks, or, more commonly, McCartney bottles. Bottles are capped loosely during sterilization and then tightened after sterilization and cooling to room temperature.

Soil Agar (SA). Chlamydospore formation is enhanced on SA (1094), making this medium useful for the identification of some species of *Fusarium*. SA is prepared by placing 250-500 g of sieved dry soil into a flask and bringing the total volume to 1 L with tap water and autoclaving for 15 min. After the first autoclaving, add 15 g of agar and autoclave for a second 15 min. The amount of soil used varies with soil type. Abundant chlamydospore formation by various species has been observed on SA prepared with 250 g black clay soil, but other soil types also have been used successfully. While pouring plates, the autoclaved medium should be regularly mixed or swirled to ensure even distribution of the solids to all plates.

KCl Agar. When 4-8 g/L KCl is added to WA or to CLA the fungal cultures produce more and longer microconidial chains (626). The chains also are easier to see since there is less moisture on the agar surface and fewer moisture droplets in the aerial mycelium.

2.3 Media for Isolating *Fusarium*

A number of media have been developed for the specific isolation of *Fusarium* spp. Those with the longest history of use are PPA and Komada's, with Komada's usually preferred when the target is *F. oxysporum*, and PPA preferred for most other uses. MGA and RbGu media are variations on PPA that have been widely used by some researchers. MGA may become more prominent as the availability of PCNB decreases. New media are being continuously developed to meet particular needs, *e.g.*, SSM and SFA, or with formulations that are purportedly more selective for *Fusarium* species than were earlier media, *e.g.*, the medium of Vujanovic *et al.* (2271) which contains myclobutanil as the selective agent.

Peptone PCNB Agar (PPA or Nash-Snyder Medium). The original medium was described by Nash & Snyder (1534), with modifications by Nelson *et al.* (1551). PPA is composed of a sugar-free basal medium supplemented with antibiotics and fungicides. The base medium contains:

Peptone	15 g
KH_2PO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g

PCNB (Pentachloronitrobenzene)	750	mg
Agar	20	g
H_2O	to 1	L

PCNB is usually added as 1 g of Terrachlor, which contains 75% PCNB (w/w). The pH should be adjusted to 5.5-6.5, if necessary. The streptomycin stock solution is 5 g of streptomycin in 100 ml distilled H_2O, and is used at the rate of 20 ml/L of medium. The neomycin stock solution is 1 g of neomycin sulfate in 100 ml distilled H_2O, and is used at the rate of 12 ml/L of PPA. Streptomycin is effective against Gram-negative bacteria, and neomycin against Gram-positive bacteria.

The medium may be poured into plates onto which soil or diseased plant material is placed, or dispensed into vials into which the material is placed directly. Vials can be particularly useful if samples are taken immediately from field material and then returned to the lab for processing, and as a means to avoid problems associated with the importation of diseased plant material that are usually not encountered with cultures. If plates are being used in soil dilutions (see section 3.3), then it is best if they are allowed to sit for several days to dry before use. This drying time allows the water in the soil suspensions to be absorbed more quickly into the agar gel.

PPA is highly inhibitory to most other fungi and bacteria but allows slow growth of *Fusarium*. It enables the selective isolation of *Fusarium* species from soil dilutions and from rotting plant material that often is infested with other fast-growing microbes. Most species of *Fusarium* do not form distinctive colonies on PPA. Often, sporulation is poor and conidial morphology abnormal. This means that colonies usually must be subcultured before a definitive identification can be made. Although the sporulation is poor, there may be enough spores so that a subculture originating from a single spore can be generated from colonies growing on PPA. Cultures should not be left on PPA for more than 2-4 weeks. The sole nutrient source in the medium is peptone, and its breakdown generates relatively high levels of ammonia that eventually kill the colonies.

Komada's Medium. Komada's medium was developed for the selective isolation of *F. oxysporum* from soil (1115). The basal medium contains:

D-Galactose	20	g
L-Asparagine	2	g
KH_2PO_4	1	g
KCl	0.5	g
$MgSO_4 \cdot 7H_2O$	0.5	g
PCNB (Pentachloronitrobenzene)	750	mg
Fe_3Na EDTA	10	mg
Distilled H_2O	to 1	L

PCNB is usually added as 1 g of Terrachlor, which contains 75% PCNB (w/w). The pH is adjusted to 3.8 ± 0.2 with 10% phosphoric acid, if necessary, prior to autoclaving. The basal medium is autoclaved and cooled to ~50°C before adding filter-sterilized supplemental stock solutions. The streptomycin stock solution is 5 g of streptomycin in 100 ml distilled H_2O, and is used at the rate of 6 ml/L of medium. The Oxgall stock solution contains 5 g Oxgall and 10 g $Na_2B_4O_7 \cdot 10H_2O$ in distilled H_2O, and is used at the rate of 10 ml/L of medium.

Colonies of *F. oxysporum* are distinctly pigmented on this medium, and usually separable from other *Fusarium* species on this basis (272). However, there is enough overlap with some related species, *e.g.*, those in the *G. fujikuroi* species complex, that simple counts of colonies with different morphologies need not be an accurate measure of the *F. oxysporum* levels in the sample. Growth of other *Fusarium* species may be suppressed by the medium, and this medium often is not a good choice for the recovery of *Fusarium* communities that contain species other than *F. oxysporum.*

Malachite Green Agar (MGA). MGA was developed in 1997 by Castellá *et al.* (326) as an alternative to PPA or Komada's medium, and has performed well in some comparison studies with other media (232). This medium contains:

Peptone	15	g
KH_2PO_4	1	g
$MgSO_4 \cdot 7H_2O$	0.5	g
Malachite green oxalate	2.5	mg
Agar	20	g
H_2O	to 1	L

After autoclaving, streptomycin and chloramphenicol are added as for PPA. This medium has not been used as widely as has PPA or Komada's. The only difference between this medium and PPA is that the PCNB is replaced by 2.5 ppm malachite green. This medium probably will become more important as the availability of PCNB (due to its carcinogenicity) declines. MGA also is reported to be more inhibitory of common contaminants, *e.g.*, *Aspergillus* spp. and *Penicillium* spp., without reducing the number of colonies of *Fusarium* spp. recovered than is PPA. The malachite green level in the medium is important, as the level of malachite green (15-50 ppm) used in the Singh-Nene medium (1991), from which this formulation was derived, prevents germination of many *Fusarium* spores.

Another variant on PPA (712) is to replace the PCNB with 1 g/L 2-benzoxazolinone, an antimicrobial compound produced by many maize lines. This medium is particularly effective for selecting for maize pathogens

such as *F. graminearum*, *F. subglutinans*, and *F. verticillioides*.

Selective Fusarium Agar (SFA). SFA was developed for the selective isolation of *Fusarium* species from soil debris. SFA is a modified Czapek-Dox medium (see section 2.5) that contains antimicrobial agents (2151).

Glucose (Dextrose)	20	g
KH_2PO_4	0.5	g
$NaNO_3$	2	g
$MgSO_4 \cdot 7H_2O$	0.5	g
Yeast extract	1	g
1% $FeSO_4 \cdot 7H_2O$ (aqueous)	1	ml
Agar	20	g
H_2O	to 1	L

The basal medium is autoclaved and allowed to cool to ~50°C before adding filter-sterilized antibiotic stock solutions. The streptomycin and neomycin stocks are the same as those for PPA, and are used at the same rate – streptomycin stock is 5 g of streptomycin in 100 ml distilled H_2O, and used at the rate of 20 ml/L of SFA, and the neomycin stock is 1 g of neomycin sulfate in 100 ml distilled H_2O, and used at the rate of 12 ml/L of SFA. Dichloran (2,6-dichloro-4-nitroanaline) is prepared as 50 mg of dichloran (or 100 mg Allisan or Botran) in 100 ml ethanol, and used at the rate of 13 ml/L SFA. If dichloran is not available, then PCNB, as used in PPA – 750 mg or 1 g Terrachlor – can be incorporated into the medium prior to autoclaving.

SFA permits the slow growth of *Fusarium* species from plant roots and soil debris, and is less inhibitory than PPA to most fungi. Colonies of different species developing from a single root fragment or piece of debris are easier to differentiate on SFA than on PPA. Although SFA was developed for isolation of *Fusarium* from soil debris, it is not suitable for the isolation of *Fusarium* species from soil dilutions.

Rose Bengal-Glycerine-Urea Medium (RbGU). This medium was developed by van Wyk *et al.* (2224) for isolations from both soil and plant debris.

Glycerol	10	g
Urea	1	g
L-Alanine	0.5	g
PCNB (Pentachloronitrobenzene)	1	g
Rose Bengal	0.5	g
Agar	15	g
H_2O	to 1	L

The basal medium is autoclaved and allowed to cool to ~50°C before adding a filter-sterilized streptomycin solution that is the same as that used for PPA – 5 g of streptomycin in 100 ml distilled H_2O. The medium is similar in selectivity to both PPA and Komada's medium but with the advantage that colony morphology is somewhat easier to distinguish on this medium.

Specific Screening Media (SSM). SSM was developed for the selective isolation of *Fusarium pseudograminearum* and other fungal pathogens from the crown region of wheat plants (2077). SSM contains 10 g dextrose, 20 g agar, and the broth from 125 g white potatoes made up to 1 L with tap water. The potatoes are unpeeled, washed, diced, and boiled until soft. Filter the boiled potatoes through a single layer of cheesecloth, which leaves some sediment in the broth. Commercially available preparations of PDA, *e.g.*, Difco, BBL, *etc.*, may be used at half of the recommended strength, but must be supplemented with an additional 10 g/L agar. After the basal medium has been autoclaved and cooled to ~50°C supplemental stock solutions are added. The streptomycin stock solution is 5 g of streptomycin in 100 ml distilled H_2O, and is used at the rate of 3 ml/L of medium. The neomycin stock solution is 1 g of neomycin sulfate in 100 ml distilled H_2O, and is used at the rate of 6 ml/L of SSM. Dichloran is prepared as 50 mg of dichloran (or 100 mg Allisan or Botran) in 100 ml ethanol, and used at the rate of 13 ml/L of SSM.

SSM permits the formation of distinctive colonies of *F. pseudograminearum*, and suppresses the growth of mucoraceous fungi and *Trichoderma* species. This medium is particularly well-suited for studies of crown rot of wheat in which *F. pseudograminearum* is known to form a morphologically distinguishable colony on the medium. In such studies, *e.g.*, Burgess *et al.* (268), the medium is prepared in large trays to enable a very large number of samples to be processed rapidly.

2.4 Media for the Preparation of Natural Inocula

Chaff-Grain Medium. Inoculum suitable for addition to soil in pathogenicity tests can be prepared by using colonized chaff-grain as a substrate (we have successfully used wheat, barley and oats, but expect that maize, sorghum and rice also would work). Cereal chaff and grain are mixed together in an approximately 5:1 ratio (often a commercial horse feed will suffice).

To a 2-L beaker, add approximately 500 ml of the chaff-grain mixture, and then add tap water to approximately the 1-L level. Mix thoroughly to release any air bubbles and to wet the entire mixture; add additional water to bring the total volume back to 1 L, if necessary. Place the beaker at 5°C for at least overnight, but not more than 24 hours, to leach phenolic compounds. After

leaching is complete, cover the mouth of the beaker with a layer of cheesecloth or Miracloth, and invert the beaker on a drainboard to drain the water. After 5-10 min, wrap the chaff-grain mixture in the cheesecloth and squeeze until no more water can be released.

The drained mixture is distributed into glass jars or Erlenmeyer flasks, which are filled to a depth of approximately 5 cm. The containers are sealed with a large cotton wool plug and autoclaved for 15 minutes on each of two successive days. Containers are inoculated with a conidial or mycelial suspension ($\geq 10^5$ cfu/ml) at the rate of 2 ml of the fungal suspension per 250 ml chaff-grain mixture.

The inoculated material is shaken vigorously to evenly distribute the inoculum throughout the medium. Inoculated material is incubated at 25°C until the material is completely colonized. The length of the incubation depends on the rate of growth for the fungus. Usually 7-14 days of incubation is sufficient. These cultures should not be maintained for more than 21 days, as viability begins to decrease. Cultures should be shaken daily for the first 3-4 days to encourage more rapid and uniform colonization of the substrate. Once the substrate is completely colonized, it is removed from the container and air dried at room temperature (20-25°C) overnight (perhaps somewhat longer if the relative humidity is high). When dry, then material is crushed to the required size (usually particles pass through a 2 mm aperture sieve) for addition to soil. The dried crushed substrate may be stored for up to 12 months at 2-5°C.

In pot cultures or disease nurseries, the inoculum often is mixed with soil at a rate of 1-2% of the final total volume before planting. In other cases, *e.g.*, Liddell *et al.* (1255), the inoculum is placed as a layer above the seed and then covered with soil, and the seedlings grow through the inoculum layer. Infection may occur at this time, or it may be delayed by withholding moisture from the layer that contains the inoculum.

This inoculum is particularly appropriate for species that do not form chlamydospores, but that persist in soil as hyphae in plant residues. This procedure has been used with numerous *Fusarium* species, *e.g.*, Liddell (1253) and Liddell *et al.* (1255), including *F. crookwellense*, *F. culmorum*, *F. oxysporum*, *F. pseudograminearum*, *F. solani*, and *F. verticillioides*. It also is suitable for studies with a number of fungal genera, *e.g.*, Summerell *et al.* (2081), including *Cylindrocarpon*, *Pythium*, *Rhizoctonia*, and *Sclerotium*.

2.5 Synthetic and Semi-synthetic Media

Synthetic and semi-synthetic media often are used for studying physiological characters, for isolating DNA, and for genetic studies. The media in this section are all based on the Czapek-Dox medium recipe, as modified by Correll *et al.* (402). The basal medium contains:

KH_2PO_4	1	g
$MgSO_4 \cdot 7\ H_2O$	0.5	g
KCl	0.5	g
Agar (Bacto)	20	g
Trace element solution	0.2	ml
Distilled H_2O	to 1	L

It is critical to use distilled water in this medium to prevent unintentional contamination of the medium with salts that may adversely affect growth characteristics or trace element levels. Agar should be at least of bacteriological grade. Higher levels of agar purity occasionally are warranted, *e.g.*, see nitrite medium below, but are not necessary for routine work. Lower grades of agar may have trace amounts of vitamins, nucleic acids, and/or amino acids that may make it difficult to score auxotrophic mutations clearly. For work with liquid cultures, the agar is omitted completely.

The trace element solution supplies critical nutrients required for some enzyme activities, *e.g.*, Mo is essential for nitrate reductase activity, and should not be omitted. Note that all *Fusarium* species require iron, zinc, manganese, copper, molybdenum and boron for proper vegetative growth and sporulation, but that excessive amounts of these trace elements may be inhibitory (1310). The trace element solution contains:

Citric acid	5	g
$ZnSO_4 \cdot 6H_2O$	5	g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	1	g
$CuSO_4 \cdot 5H_2O$	250	mg
$MnSO_4$	50	mg
H_3BO_3 (Boric Acid)	50	mg
$Na_2MoO_4 \cdot 2H_2O$	50	mg
Distilled water	95	ml

This recipe makes a nominal 100 ml. The citric acid should be added to the water first and dissolved completely to prevent other salts from precipitating. The solution may be filter-sterilized, although this is not necessary. More commonly this solution is stored in a capped bottle unsterilized at 4°C indefinitely following the addition of approximately 1 ml of $CHCl_3$ to the stock solution. The $CHCl_3$ forms a small ball at the bottom of the container and it should be possible to detect its presence by smelling the solution in the bottle. If during storage the smell disappears or the ball of $CHCl_3$ shrinks signifi-

cantly or is no longer visible, then the stock solution is probably no longer saturated with $CHCl_3$, and additional $CHCl_3$ should be added. A monthly check usually suffices.

Additional ingredients are added to the basal medium (see below), depending upon the experimental protocol, prior to autoclaving and use. Adding a drop or two of vegetable-dye based food coloring, available at the local grocery store, to media before autoclaving allows media types to be distinguished based on color rather than by relying on stripes on the side of a plate or differences in the plugs for slants to make these distinctions. The food coloring does not affect the morphology or growth rates of *Fusarium* spp.

Minimal Medium (MM). This medium is the standard Czapek-Dox formulation as modified by Correll *et al.* (402). This medium is made by adding 2 g/L $NaNO_3$ and 30 g/L sucrose to the basal medium prior to autoclaving. Most *Fusarium* species grow well vegetatively on this medium, but sporulation characters vary, which makes it unsuitable for routine taxonomic studies. As with PDA, the high level of sugar can cause problems with character degeneration in some strains. In studies of vegetative compatibility, this medium functions as the NO_3 test medium. This medium can be used to identify strains with nutritional auxotrophies (such strains will grow poorly if at all), and is suitable as a liquid medium for studies of enzyme activity and for the isolation of nucleic acids.

Complete medium (CM). This medium is a semi-synthetic version of the Czapek's-Dox medium that we routinely use in our laboratories. To the basal medium the following additions are made prior to autoclaving:

Sucrose	30	g
$NaNO_3$	2	g
N-Z Amine	2.5	g
Yeast extract	1	g
Vitamin stock solution	10	ml

N-Z Amine is an amino acid digest that is used as a source of amino acids and fixed nitrogen. Other hydrolytic protein digests can be substituted for N-Z Amine at a similar level of supplementation. We routinely use Bacto (Difco, Detroit, Michigan) Yeast Extract, but other yeast extracts should perform similarly. Yeast extracts vary somewhat from lot to lot. This variation can be particularly important for methionine and adenine auxotrophs, and samples from several lots should be tested to make sure that an adequate level of the supplement is present before embarking on extensive studies of such mutants with this medium. The vitamin stock solution is composed of:

Inositol	4	g
Ca pantothenate	200	mg
Choline•Cl	200	mg
Thiamine	100	mg
Pyridoxine	75	mg
Nicotinamide	75	mg
Ascorbic acid	50	mg
Riboflavin	30	mg
p-aminobenzoic acid	5	mg
Folic acid	5	mg
Biotin	5	mg
50:50 ethanol:H_2O	to 1	L

The vitamin solution may be filter-sterilized, although this is not necessary. More commonly though, this solution is stored indefinitely in the dark (dark bottle or bottle wrapped in aluminum foil) in a tightly capped bottle unsterilized at 4°C following the addition of 1-2 ml of $CHCl_3$ to the stock solution. The $CHCl_3$ forms a small ball at the bottom of the container and it should be possible to detect its presence by smelling the solution in the bottle. If during storage the smell disappears or the ball of $CHCl_3$ shrinks significantly or is no longer visible, then additional $CHCl_3$ should be added to maintain a saturated solution. A monthly check usually suffices. Storage in the dark is important as some of the vitamins decompose when exposed to light. Choline and pantothentic acid must be added as salts to maintain pH and to increase solubility. Nicotinic acid is added as nicotinamide for the same reason. Some researchers add additional vitamins to this mixture, but we have not observed any cases in which such additions have significantly affected experimental results.

Virtually all *Fusarium* species grow well vegetatively on CM, but sporulation characters vary, which makes it unsuitable for growing cultures for routine taxonomic studies. As with PDA, the high level of sugar can cause problems with character degeneration in some strains. The availability of fixed nitrogen means that *nit* mutants (see section 5.3) can revert without a noticeable change in phenotype. *nit* mutants isolated from sectors on chlorate-containing medium usually must be grown on CM for preservation or to obtain large numbers of spores, but in general their culture on this medium should be as limited as possible. Auxotrophic mutants that require amino acids not represented in casein usually grow poorly on CM, as they rely solely on the amino acids in the yeast extract for growth. Arginine is almost always limiting, and, if required, must be added as a separate supplement. Virtually all other *Fusarium* strains, both prototrophs and auxotrophs, grow well on this medium.

Chlorate Medium. This medium was designed for the recovery of nitrate non-utilizing (*nit*) mutants for use in

vegetative compatibility tests (402, 1745). To the basal medium the following additions are made prior to autoclaving:

Sucrose	30 g
$NaNO_3$	2 g
L-asparagine	1.6 g
$KClO_3$	15 g

A common level of chlorate in the medium is 1.5% (the level reflected in the recipe). This level is usually the minimum chlorate level that is effective for generating *nit* mutant sectors. This recipe has numerous variants. If too few NitM sectors are recovered, then changing the fixed nitrogen source from asparagine to proline may help (1090). If colonies seem to grow slowly across the plate without generating sectors, then omitting the asparagine often helps to restrict colony growth sufficiently to permit the identification of *nit* sectors. The chlorate level also can be increased, with 2-3% chlorate commonly used in many labs. The maximum chlorate level is 6% (60 g $KClO_3$/L). Plates with high levels of chlorate dry easily and have a feathery pattern of crystal formation in the medium. Such plates should be discarded. A final alternative is to drop the pH of the medium, but not so far as to prevent the agar from gelling. Conditions developed for generating *nit* mutants in *Fusarium* often, but not always, work with other ascomycete fungi *e.g.*, *Colletotrichum* (250), *Aspergillus* (156, 557), and *Verticillium* (1126). The media developed for studies in *Aspergillus* can be particularly useful for strains that sector relatively poorly on the chlorate media described here.

Phenotyping Medium. Phenotyping medium was developed by Correll *et al.* (402) to distinguish the mutations that affect different portions of the nitrate assimilation pathway (see section 5.3). There are three forms of phenotyping medium that are named according to the nitrogen source that is added to basal medium before autoclaving: ammonium (1.6 g/L ammonium tartrate), hypoxanthine (0.2 g/L hypoxanthine), and nitrite (0.5 g/L $NaNO_2$). All contain 30 g/L sucrose as the carbon source. The ammonium medium is the control. Strains that will not grow on this medium have another nutritional requirement that needs to be diagnosed. Hypoxanthine medium is used to differentiate NitM mutants, as these mutants are unable to utilize hypoxanthine as a sole nitrogen source due to a defective molybdenum co-factor that is shared by nitrate reductase and purine dehydrogenase (1376). Nitrite medium can be problematic. Nitrite is potentially toxic to the cells. If they are supplied with too much NO_2 they will not grow due to NO_2 toxicity, but if there is not enough NO_2 then they will not grow due to N insufficiency. In some cases, replacing the bacteriological agar in nitrite medium with Noble agar will make it easier to score the growth on this medium.

Media to Restrict Radial Growth of Colonies. Minimal medium + tergitol and sorbose (MMTS) and Complete medium + tergitol and sorbose (CMTS) were modified by Bowden & Leslie (230) from the original recipe of Puhalla & Spieth (1746) to restrict radial growth of colonies on agar plates, and to enable the isolation of numerous colonies from a single plate following a selective treatment, *e.g.*, a mutagenesis experiment. These media are made by substituting 30 g/L sorbose for the 30 g/L sucrose in the minimal or complete recipes given above, and adding 0.5 ml/L tergitol (type NP-10). If colonies in a more "pelleted" form are desired from a liquid culture, then similar changes can be made to the liquid media (1216). The sorbose level used can range from 2-6% (20-60 g/L), and tergitol from 0.05-0.2% (0.5-2 ml/L). Either compound can be used alone, but a higher concentration of the single compound is required to obtain the same net effect on colony diameter. In some cases a small amount of other sugars, *e.g.*, 0.2% dextrose and 0.2% fructose, also may be added to the medium to support initial growth and spore germination. Both interspecific and intra-specific variation for sensitivity to tergitol and sorbose are known, and the level of sorbose and/or tergitol used in the medium may need to be adjusted to reflect this variation.

2.6 Media for Sexual Crosses

Obtaining a sexual stage is essential for analyses of biological species in some heterothallic species. Many species will form perithecia and mature ascospores on CLA. Some investigators also have used cultures grown on various natural substrates, *e.g.*, rice straw (905) or mulberry twigs (469) on water agar or PDA, to obtain the sexual stage. For studies of development or for genetic studies, however, more perithecia with a larger number of ascospores usually are required. Two media commonly are used for this purpose, carrot agar and V-8 juice agar. Fertility on either medium occasionally can be increased by placing a piece of sterile filter paper (Whatman №. 1) over a portion of the plate prior to inoculation, and perhaps impregenating it with a drop or two of linoleic acid (535).

Carrot Agar. Carrot agar was developed for fertility studies of *Fusarium* species with a *Gibberella* sexual stage (1090). The medium is prepared by washing, peeling and dicing 400 g of fresh carrots. Place the carrots in a flask in 400 ml of water and autoclave for 20 minutes. Blend the carrots in a blender until the mixture appears smooth and with no large lumps. Add an additional 500

ml of H_2O, using some of the water to rinse the blender, and 20 g of agar. Autoclave for 30 minutes. Both autoclaving steps and the length of the second one are important to avoid contamination of plates by soilborne bacteria with heat-resistant spores. Plates should be poured thick (15-17 ml per 60 mm diameter dish), as they may be incubated for up to 6 weeks. Plates that are a normal thickness usually dry, the agar curls, and meaningless, if any, results are obtained. Some species, *e.g.*, *G. nygamai* (1085), are reported to be more fertile on a half strength medium (200 g carrots) than on the full strength medium, but there has been no systematic testing of this effect for most of the species with known sexual stages.

V-8 Juice Agar. This medium commonly is used to elicit the formation of the sexual stage of many *Fusarium* species. In some cases this medium also is used for routine maintenance of vegetative cultures. The medium is commonly made by mixing 300 ml of V-8 juice (Cambell Soup Co., Camden, NJ) with 700 ml water, and 20 g of agar. Before addition of the agar, the pH of the juice should be adjusted to 5.5-6.5 using 1 M NaOH. There are many variants of this basic formula, see Tuite (2189) for numerous examples. These variations usually have to do with whether (and how much) $CaCO_3$ is added to the medium, and whether and how the pH is adjusted. We have found that for fertile crosses, V-8 juice agar usually gives good results, but for crosses that are less-fertile, carrot agar usually is more consistent and more productive.

2.7 Sterilization of Media and Materials

Sterilization of media and utensils is essential to destroy or kill all the living organisms. The method of sterilization depends primarily on the physical nature of the material to be sterilized.

Heat Sterilization. The temperature and time required for killing are inversely related, *i.e.* the higher the temperature the shorter the period of time for which the material must be exposed to it. Both the temperature and the length of the exposure (Table 2-1) also depend on the nature of the heat applied – moist, *e.g.*, with an autoclave, or dry, *e.g.*, in an oven. Exposing materials to these conditions is not a guarantee of sterility, but as a general guideline moist heat, applied in an autoclave at 121°C for 15 minutes, or dry heat, applied in an oven at 160°C for one hour, usually suffices. More heat-resistant microbes, however, may require longer exposures before they are killed. Such microbes usually are rich in organic sub stances, with fat composition especially important in resisting killing due to moist heat.

Table 2-1. Times of exposure to moist and dry heat that result in sterilization of media and equipment.

Temperature °C	Moist Heat	Dry Heat
100	20 hr	
110	150 min	
115	50 min	
121	15 min	8 hr
125	6.5 min	
130	2.5 min	
140		150 min
160		1 hr
170		40 min
180		20 min

Moist Heat. Moist heat generally is applied along with an increase in pressure to keep liquid materials from boiling and is usually administered in a pressure cooker, autoclave, or other containment vessel that can withstand the additional pressure generated. Moist heat kills microorganisms by coagulating and denaturing enzymes and structural proteins, and requires that the steam contact the microorganism. If the microbes are protected from the steam by a layer of grease or oil, then a longer exposure is required for effective sterilization. All culture media are sterilized with moist heat, with those containing large amounts of lipids usually being autoclaved for a more extended period of time. Items exposed to saturated steam usually reach the equilibrium temperature much more quickly than do those exposed to dry heat. Although 121°C for fifteen minutes in the autoclave is the most common treatment, some materials are not amenable to the temperature, the pressure, or the setting, *e.g.*, large lots of soil or plant material, and often can be effectively sterilized following exposure to a normal steam for 30-45 min on each of several (usually at least three) successive days.

Autoclaves must be adjusted to prevent the chamber pressure from falling too rapidly as this will result in media boiling and wetting the plugs (and to contamination of the media before or during its use). Leaving media in the autoclave for 5-10 min after it has returned to atmospheric pressure is usually a good safety precaution, as superheated liquids can boil over causing both burns and large messes when disturbed. Materials should not be left in an autoclave for an extended period following the completion of the cycle, however, because a vacuum usually builds up that both makes the autoclave difficult to open and leads to loss of the liquid volume. Sterilizing large and small volumes of media in the same run also

should be avoided wherever possible, as the extra time required for a large volume of liquid to reach the sterilization temperature may result in too long of an exposure for some of the media present only in a small volume.

pH also is an important variable when moist heat is applied since the resistance of bacterial spores is highest at pH 7.0 and decreases as either the acidity or alkalinity increases. On a practical level, this means that metal instruments boiled in water containing 2% Na_2CO_3 for 10 min. have been as effectively treated as those boiled in plain water for several hours.

Dry Heat. Dry heat kills microbes by oxidation and is preferred for dry glassware such as test tubes, Petri dishes, flasks, pipettes, syringes and instruments such as forceps, scalpels, and scissors. Dry heat also is used to sterilize dry materials in sealed containers and powders, fats, oils and greases that are impermeable to moisture. These materials may exhibit significant mass transfer effects with respect to heat transfer, *i.e.*, it may take a relatively long time for all of the material to reach the ambient temperature, and they are, therefore, best sterilized as several smaller amounts, rather than as one large one. For example, an oven packed with glassware may require 2-3 hours to reach 160°C, and this length of time must be added to the time required for sterilization.

Sterilization by Filtration. Microbes also can be removed from liquids by filters with very small pores that trap contaminating yeasts, bacteria and spores. This method is used to sterilize items such antibiotic and vitamin solutions that are heat labile. Most filter sterilization is done with membrane filters. Membrane filters are thin, porous sheets of cellulose esters or similar polymeric materials. All particles exceeding the pore size, which range from 0.22-14 µm depending on the type of filter purchased, are retained by the filter. The fibers defining the pores generally are resistant to water, dilute acids and alkalis, aliphatic or aromatic hydrocarbons, and non-polar liquids of interest to most microbiologists.

Normally a 0.22 µm membrane filter should be used for filter sterilization, as these filters will retain all known bacteria. Reusable membranes that are sterilized, usually by autoclaving after mounting in an appropriate holder, may be purchased; however, disposable units that come sterile and ready to use are common and convenient. The liquid to be sterilized usually is allowed to flow through the filter by gravity, or by positive pressure, *e.g.*, when a solution is forced through a filter that is mounted on a syringe. If the suspension being sterilized contains a great deal of suspended particulate matter, then filtration through glass wool or some other prefilter may be necessary.

Sterilization of Instruments During Use. Forceps, inoculating needles and other instruments must be sterilized before and after contact with a culture to avoid cross-contamination. Inoculating needles are readily sterilized by heating to red hot in a flame. The needle must be cooled to room temperature before being used to make a transfer. A needle that is too hot is one of the most common reasons for unsuccessful subculturing. Forceps and scalpels are sterilized by standing them in a beaker or similar container of alcohol. Before use, the alcohol is burnt off by passing the instrument through a flame and igniting the alcohol. The instrument should not be held in the flame immediately before use as this will heat it up too much. Do not place a hot or flaming instrument in or near the alcohol, since it is possible to ignite all of the alcohol in the container. As a safety measure, have a watch glass or other item near at hand that can be used to cover the mouth of the container containing the alcohol in case the alcohol catches fire.

Sterilizing Work Surfaces. Trays, benches and other surfaces may be sterilized with any commercially available liquid disinfectant. Alcohol also is commonly used and works best as a sterilant when mixed with water; a solution of 70% ethanol is suitable. Hypochlorite (bleach) solutions, *e.g.* 1% sodium hypochlorite, also are very effective, but may have an offensive odor or leave a solid residue and are harmful to clothing and many other common laboratory materials.

3

Techniques for Recovering *Fusarium*

Fusarium is common in both agricultural and native environments, but before strains can be identified, characterized and studied, they must be recovered from the environment and subcultured in such a manner that other microbes that might accompany them have been eliminated. Following that, a single-spore subculture (section 4.3) is needed to ensure that only a single strain is being examined, as many natural sources of *Fusarium* contain genetically distinct strains of the same and different species. The collection strategy and recovery techniques used depend on the question(s) being asked. A strategy suitable for identifying the causal agent of a plant disease could be quite different from one in which the goal was an inventory of the *Fusarium* species present on a native grassland or determining if there were *Fusarium* strains associated with a "sick building." This chapter provides a few general guidelines towards the design of collecting strategies, but is neither a complete nor all-inclusive guide to these strategies, which are heavily dependent upon the question being addressed.

3.1 Collecting Strategy

This section presumes a sampling strategy in which a number of samples are needed from the same location for an evaluation of some sort. The most important properties of such a sample usually are its size and its representtativeness of the *Fusarium* species/strains present in the original source.

Sample size is an important character. Normally the sample should be large enough to ensure that all of the most common species/strains were recovered with some statistically significant degree of confidence. Assuming a random distribution of the species/strains within the sample, *i.e.*, that the sample is a representative one, these values are calculated from a binomial/multinomial equation.

If a particular strain is present at frequency a in the sample, then the probability that this strain would be selected on any individual draw from the sample is a, and the probability that it would not be selected is $1 - a$. If x draws are made from the sample, *i.e.*, the sample size, then $(1 - a)^x$ is the probability that none of the draws will be of the strain in question. Thus, the lower the value for a the larger the sample will need to be to be certain that a strain representing the group has been recovered. Statistically significant frequencies for items in a sample often are considered to be either 5% or 1%. The certainty of recovery, $1 - (1 - a)^x$, usually is set at 95% or 99%. Given a value for a or x, the equation can be easily solved for the other variable. To be 95% certain of seeing a strain present at a frequency of 5%, the sample size should be 59, and to be 99% certain the sample size should be 90. To be 95% certain of seeing a strain present at a frequency of 1%, the sample size should be 299, and to be 99% certain the sample size should be 459. The application of these numbers requires some discretion. For example in a sample of 59, which has 95% certainty of identifying all species/strains present at the 5% level, the probability of missing at least one of the species present at the 1% level is 50%.

The target of the sampling also helps determine the sample size. For example, suppose the goal of the sampling is to obtain 59 cultures of a particular species. If that species is the only one present, then a sample size of 66 should suffice for analysis at the 5% level with 95% confidence, assuming that 90% of the items sampled will yield a fungal culture. If cultures of the target species can be recovered from only 50% of the items sampled, however, then the sample size will need to be proportionately increased to ensure that enough cultures of the target fungus have been obtained for the results to be statistically meaningful.

Comparing samples from different locations often is a goal of a project that requires sampling. In such cases the samples are most useful if they are taken in a hierarchical manner. The smallest unit usually is a single plant or a single lesion on a plant. From there the progression usually is to a "field", to a town, village or county, to a state or other larger district, etc. The number of layers in the hierarchy usually is determined by the known or suspected distribution of the organism of interest and the practical limitations on the total number of samples that can be analyzed. As a general rule, more samples are generally preferred to fewer, but once a sample is "large enough", increasing it further may add little additional statistical power to the argument. If only a relatively few samples can be analyzed, then the locations to be sampled need to be chosen with special care to ensure that the results obtained can support the inevitably broad inferences that will be made from them. Unless the questions asked include, "Is there more than one species/strain per plant?", we recommend that only a single sample be taken per plant. Many plants are infected with multiple strains of *Fusarium* from the same or different species, *e.g.*, Kedera *et al.* (1029), and Leslie *et al.* (1226). In such cases if a second recovery is made of the same strain/species its independence from the first recovery cannot be critically assessed. Thus, multiple isolates from the same plant should not be assumed to be independent events and probably are better handled in a qualitative, rather than a quantitative manner.

When collecting in a more-or-less uniform setting, *e.g.*, a monoculture agricultural field, the spatial pattern associated with the sample may be of interest as well. A rectangular grid often is used in settings where there are a series of parallel rows. The sample often is taken in a diagonal manner beginning at an arbitrary point in the field, and then moving over one row, and down the row the width of the row. The process can be continued for the width of the field, the length of the row, or until the sample size is large enough. If the samples at the sampling site are not arranged in rows, then setting up a grid based on rectangular co-ordinates can be time-consuming and at least somewhat frustrating. Under these conditions, using a polar grid for sample collection can be much more effective and time efficient.

To set up a polar grid, a central stake is needed, as is a protractor (or some other means of measuring an angle), a compass, and a string or rope in which knots have been tied at the distances from the centerpoint that are to be sampled. By sampling from the centerpoint, and from 12 points on each of five concentric circles around that centerpoint, a set of 61 isolates can be obtained. The central stake is driven into the ground, and a sample is taken immediately adjacent to it. Using the compass, the string is extended out sufficiently far to the north and samples are taken where the knots fall on the ground. The string is moved clockwise an angle of 30 degrees and another series of five points sampled. The process continues until the entire circle (with 12 samples per circle) is complete. We have used circles with a radius of 1, 4, 8, 10 and 12 m in our studies, but any five distances could be used.

3.2 Isolation Techniques – Plants

Isolation protocols have a significant impact on the recovery of the *Fusarium* species from diseased plants (1411). It is common to recover more than one species of *Fusarium* from individual pieces of diseased plant material, but some species may be pathogens, while others are saprophytes or endophytes that may have a role as secondary pathogens or which may have no role in the disease process whatsoever. In plant pathology settings, the isolation technique selected should maximize the recovery of the "true" pathogen while restricting the recovery of "weed" fungi. This problem can be particularly severe in

Table 3-1. Species of *Fusarium* regularly recovered from various parts of diseased plants as saprophytes.

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