Onkar D. Dhingra
James B. Sinclair

BASIC PLANT PATHOLOGY METHODS

Second Edition

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Onkar D. Dhingra, Ph.D.
Professor of Plant Pathology
Departamento de Fitopatologia
Universidade Federal de Viçosa
Minas Gerais, Brazil

James B. Sinclair, Ph.D.
Professor of Plant Pathology
Department of Plant Pathology
College of Agriculture
University of Illinois at Urbana-Champaign
Urbana, Illinois

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PREFACE

With our international experience in plant pathology we are aware that with the expansion of world food production and intensive crop production comes increased losses due to plant diseases. There are many examples that could be given from the agriculture history of the world. In recent times, the expansion of food and crop production has taken place in the so-called "third-world" of agriculturally developing countries. Often when plant disease epidemics develop in these countries, agricultural scientists and plant pathologists are expected to seek an immediate solution to the problem. In many countries, library facilities are inadequate or inaccessible to provide the research material required to give leads to finding solutions to specific disease problems. This book was prepared with these colleagues in mind.

The book is designed to serve as a ready reference in the classroom or laboratory, and to bring to the attention of the novice in plant pathology the diversity of available basic plant pathology techniques that can be used to solve problems in the field.

Most of the methods are described in detail, eliminating the need to seek out original articles. Whenever possible, several methods are described so that the user can select one that best meets the need of the problem and can be used with facilities available. The assembling and construction of many instruments are described and illustrated to allow for the construction of apparatus from inexpensive materials that may be locally available.

Excellence in research depends upon the imagination and ingenuity of the worker, who can find new and better answers to problems even without the most sophisticated laboratory equipment. The advancement of any science depends upon the development and refinement of techniques used to study in the discipline. The results from an experiment are as good as the method or methods used to acquire the data. We hope that this book will serve as a starting point for the development of new and refined methods and techniques for plant pathology research.

There are a number of other books published dealing in whole or in part with methods used in plant pathology. Some of these books are out of print, limited in scope, or serve as a guide to the literature which may not be available to the user. This book is intended to serve as a resource for basic plant pathology methods dealing with diseases caused by bacteria and fungi. Books that have been published on research methods to study plant disease in 1980 or later, should also be consulted.

Blanchard, R.O. and Tater, T.A., *Field and Laboratory Guide to Tree Pathology*, Academic Press, New York, 1981.

Burgess, L.W. and Liddell, C.M., *Laboratory Manual for Fusarium Research*, University of Sydney Press, Sydney, 1983.

Commonwealth Mycological Institute, *Plant Pathologist's Handbook*, 2nd ed., Commonwealth Mycological Institute, Kew, Surrey, England, 1983.

Hampton, R., Ball, E., and De Boar, S., Eds., *Serological Methods for Detection of Viral and Bacterial Plant Pathogens: A Laboratory Manual,* APS Press, Inc., St. Paul, 1990.

Hickey, K.D., Ed., *Methods for Evaluating Pesticides for Control of Plant Pathogens*, APS Press, Inc., St. Paul, 1986.

Razin, S., and Tully, J., Eds., *Methods in Mycoplasmology*, Academic Press, New York, 1983.

Saettler, A.W., Schaad, N.W., and Roth, D.A., Eds., *Detection of Bacteria in Seed and Other Planting Material*, APS Press, Inc., St. Paul, 1989.

Schaad, N.W., Ed., *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, APS Press, Inc., St. Paul, 1988.

Schneck, N.C., Ed., *Methods and Principles of Mycorrhizal Research*, APS Press, Inc., St. Paul, 1982.

Singleton, L.L., Mihail, J.D., and Rush, C.M., Eds., *Methods for Research on Soilborne Phytopathogenic Fungi*, APS Press, Inc., St. Paul, 1992.

Sneh, B., Burpee, L. and Ogoshi, A., *Identification of Rhizoctonia Species*, APS Press, Inc., St. Paul, 1991.

Tuite, J., *Plant Pathology Methods — Laboratory Exercises*, Dept. Botany & Plant Pathol., Purdue University, West Lafayette, 1988.

The scientific names of many plant pathogens and some of their hosts have been changed since the first edition. The revised scientific names of selected fungal pathogens are provided (see Appendix J). For this second edition, the Latin names for hosts and pathogens listed in the following publications have been used:

Farr, F.F., Bills, G.F., Chamuris, G.P., and Rossman, A.Y., *Fungi on Plants and Plant Products in the United States*, APS Press, Inc., St. Paul, 1989.

Hansen, E.M., and Maxwell, D.P., Species of the *Phytophthora megasperma* complex, *Mycologia*, 83, 376, 1991.

Sneath, P.H.A., Mair, N.S., and Sharpe, N.E., Eds., *Bergey's Manual of Systemic Bacteriology,* Vol. 2, 9th ed., William & Wilkins, Baltimore, 1986.

As with many major publications, the results are due to the efforts of many people involved professionally and personally with the author or authors. It would be difficult to list the many people who have contributed in one way or another to this publication. The manuscript for the first edition was reviewed by two persons at the request of Thor Kommendahl, Publication Coordinator, American Phytopathological Society, St. Paul, Minnesota. The anonymous reviewers made many helpful suggestions for the improvement of this original effort. The authors are grateful to these two persons for their advice and suggestions, many of which were used in the final version. We thank the many authors who gave permission to use illustrative material in this second edition.

Our thanks are given to the many individuals who assisted in the preparation of this second edition. We thank Marcill J. Stadnik (Vicosa) and Nancy David and Susan Schmall-Ross (Urbana) who typed supplemental drafts of all or portions of the manuscript. We thank Pam Purcell Avenius (Urbana) who did portions of the art work. Particular thanks go to Richard D. McClary (Urbana) for his many trips to the libraries at the University of Illinois at Urbana-Champaign to search for articles.

Special thanks to students, colleagues, and friends for their patience during the preparation of the manuscript for the second edition.

O.D. Dhingra
J.B. Sinclair

THE AUTHORS

Onkar D. Dhingra, Ph.D., is a professor of plant pathology at the Federal University of Viçosa, Minas Gerais, Brazil. Since 1982 he has been on deputation to the National Center of Training in Storage (CENTREINAR) to develop research on the control of fungal deterioration of stored grains and grain products. Dr. Dhingra received his B.Sc. degree from Punjab Agricultural University, Ludhiana, India in 1967 and his M.Sc. in plant pathology from J.N. Agricultural University, Jabalpur, India in 1971, where he also worked as a research fellow. He joined the University of Illinois as a graduate research assistant in 1971, and completed his Ph.D. in 1974, under Dr. James B. Sinclair, with whom he continued to work with a postdoctoral appointment until 1975. In 1976 he joined the Federal University of Viçosa as a professor of plant pathology. Dr. Dhingra has taught three graduate courses in plant pathology and has published 75 refereed research papers and 26 research abstracts and co-authored three books in the area of seed- and soilborne plant pathogens. Currently he teaches seed pathology courses at the graduate level. He is a recipient of research grants from the National Research Council (CNPq) and FINEP (Brazil).

James B. Sinclair, Ph.D., is a professor of plant pathology in the Department of Plant Pathology, College of Agriculture, University of Illinois at Urbana-Champaign, Urbana (UIUC). Professor Sinclair received his B.Sc. degree from Lawrence University, Appleton, Wisconsin in 1951 and his Ph.D. in plant pathology from the University of Wisconsin, Madison in 1955 under J.C. Walker with whom he continued to work with a postdoctoral appointment until 1956, when he accepted a position in the Department of Plant Pathology, Louisiana State University, Baton Rouge (LSU). AT LSU he served as an Assistant Professor, Associate Professor, and then Professor until 1968. Also, he was an Administrative Assistant to the Chancellor from 1966 to 1968. He joined the Department of Plant Pathology, UIUC, in 1968 as a professor of international plant pathology. He was campus, then all-university coordinator for the Illinois-Tehran Research Unit, 1974–1978. He was named Interim-Director, National Soybean Research Laboratory, UIUC in 1992.

Professor Sinclair has taught five graduate courses in plant pathology; has planned, participated in, and given invitational lectures at numerous national and international conferences and workshops; has worked in over 40 countries professionally; and has directed the research of 71 graduate students, of whom 12 have completed a portion of their thesis research at an overseas institution. He is a member of many national and international professional organizations and served from 1979 to 1983 as the Chairman, Seed Pathology Committee, International Society of Plant Pathology.

Professor Sinclair's research has been primarily on seed- and soilborne pathogens of soybeans and other crops and their control, and on the uptake and translocation of systemic fungicides in various crop plants. He has published over 203 refereed research papers; 218 research abstracts; and authored, edited, or co-edited 18 books and monographs and 206 other articles. He has received the following awards: ICI/American Soybean Association Research Recognition Award, 1983; UIUC Paul A. Funk Award, 1984; U.S. Department of Agriculture Award for Distinguished Services, 1988; American Soybean Association Production Research Award, 1989; Honorary Member, Illinois Crop Improvement Association, 1990; North Central Division, American Phytopathological Society Distinguished Service Award, 1991; Land of Lincoln Soybean Association Research Award, 1991; the UIUC College of Agriculture Senior Faculty Award for Excellence in Research, 1992; and Fellow, American Phytopathological Society, 1993.

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CHAPTER 1

Sterilization of Apparatus and Culture Media

I. INTRODUCTION

Pure culture techniques are used for isolation, identification and multiplication of plant pathogens, for increasing inoculum, and for studying their biology and physiology. All require sterile conditions. Sterilization of apparatus and working areas involves the inactivation or physical elimination of all living cells and infective agents from the environment. It does not include the destruction or elimination of constitutive enzymes, metabolic by-products, or removal of dead cells.

Sterilization is achieved by exposing materials to lethal agents which may be chemical, physical, or ionic in nature or, in the case of liquids, physical elimination of cells or infective agents from the medium. Selection of a method depends on the desired efficiency, its applicability, toxicity, ease of use, availability and cost, and effect on the properties of the object to be sterilized. Several publications review the theoretical aspects of sterilization methods.[1-4] The methods commonly used for sterilization are gas, heat, and in the case of liquids, ultrafiltration.

II. HEAT STERILIZATION

Heat is the most reliable method for sterilization when the material to be sterilized is not modified by high temperature. High temperature can be attained by using either dry or moist heat. The mechanisms of cell destruction by heat were reviewed.[5] The chief mechanism of death is oxidation or coagulation of proteins.[6]

A. DRY HEAT

Dry heat is used for the sterilization of glassware, metal instruments, certain plastics, and heat-stable compounds. The action of dry heat is an oxidation process resulting from heat conduction from the contaminated object and not from the hot air surrounding it. Thus, the entire object must be heated to a temperature for a sufficient length of time to destroy contaminants. Dry heat requires higher temperatures for longer duration than moist heat for sterilization because heat conduction by the former is slower than the latter. Many bacteria in a desiccated vegetative state or as spores can survive dry heat at high temperatures.

Hot air ovens equipped with a thermostat and heated either by electricity or burning gas are used for dry heat sterilization. Heating of objects is by radiation from the oven walls, and unless equipped with a fan to circulate the air within the chamber, heating of objects is uneven,

especially if the heat source is in the oven base. To determine whether or not a uniform temperature occurs throughout the load, thermometers should be placed at different sites within the chamber.

The time required for sterilization is inversely proportional to temperature. Commonly used time per temperature regimes are 1 hr at 180°C, 2 hr at 170°C, 4 hr at 140°C, or 12 to 16 hr at 120°C. Exposure time is counted from when objects to be sterilized have reached the desired temperature inside the oven. The air in the oven heats faster than the objects to be sterilized; therefore, the duration of the heat treatment should be increased by 1.0 to 1.5 hr over suggested time allowing the objects to reach the sterilizing temperature.

Glassware should be completely dry before placing in a hot air oven since wet glassware may break. Objects, such as glass culture plates, should be placed in sealable metal or other heat-resistant containers to prevent recontamination during cooling, transport, or storage. The objects can be wrapped in heavy paper if metallic containers are not available. However, paper may leave organic residue and become brittle and charred. Dry heat sterilization using paper should be done at low temperatures and for longer times than if metal containers are used. Calibrated glassware should not be sterilized with dry heat since the expansion and contraction can cause changes in the graduations. Objects with tight-fitting joints or plugs should be separated during hot air sterilization; otherwise, they may break.

Sterilization chambers whether using dry or wet heat, should be loaded in such a way as to provide ample space between items allowing for air circulation and to avoid breakage. Containers plugged with cotton, plastic, or rubber stoppers should be sterilized at lower temperatures for longer times. Slip-on metal caps can be substituted for cotton for culture tubes. Rubber-stoppered or screw-cap bottles, flasks, or culture tubes should be sterilized with moist heat.

After sterilization, the oven and its contents should be allowed to reach ambient temperature before opening to prevent breakage and recontamination by cool air rushing into the chamber. Sterilized material may remain in the oven until used or stored in a dry area free of air currents, but should be used within a short time and not stored for long periods.

B. MOIST HEAT

Moist heat is usually provided by saturated steam under pressure in an autoclave or pressure cooker, and is the most reliable method of sterilization for most materials. It is not suitable for materials damaged by moisture or high temperature, or culture media containing compounds hydrolyzed or reactive with other ingredients at high temperature. Moist heat has advantages over dry heat in that conduction is rapid and the temperature required for sterilization is lower and the duration of exposure is shorter. Materials to be sterilized should be in contact with the saturated steam for the recommended time and temperature.

The process is usually carried out in an autoclave[7] or a kitchen-type pressure cooker equipped with pressure gauges, thermometer, automatic pressure control valves, and exhaust valves. Autoclaves may be nonjacketed (Figure 1-1) or jacketed (Figure 1-2). In jacketed types, the duration for heating is less than in the nonjacketed types, moisture does not condense on objects, and the steam is "dry", i.e., it does not contain particulate water.

Steam is supplied either from a central source or is generated within the autoclave (or pressure cooker) by electric or gas heating. Pressure cookers and autoclaves are available in a variety of sizes and models (follow operationing instructions provided by the manufacturer).

The temperature and length of time for sterilization with steam are different from that of dry heat. Thiel et al.[8] calculated the time required for sterilization at temperatures ranging from 100 to 130°C (Table 1). For most purposes 15 min at 121°C or 30 min at 115°C are suggested.

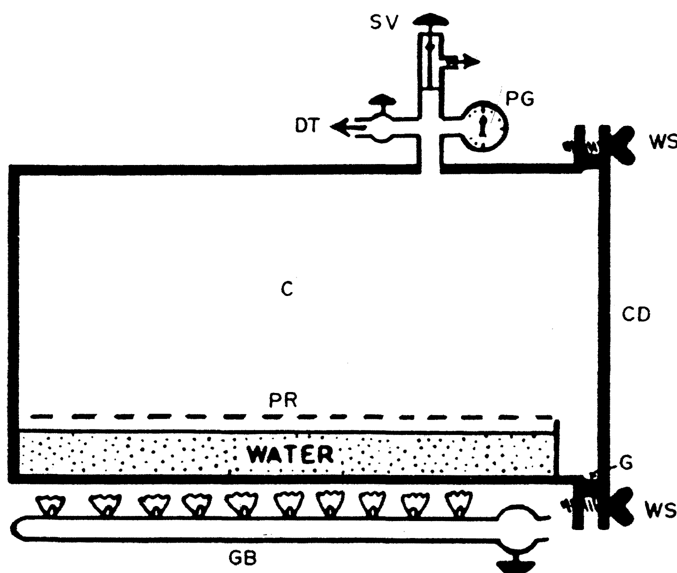

Figure 1-1. Schematic diagram of a simple horizontal nonjacketed autoclave: C, chamber; PR, perforated tray; GB, gas burner; G, gasket; WS, wing screw, CD, chamber door; PG, pressure gauge; SV, adjustable safety valve; DT, chamber discharge tap. (From Cruickshank, R., *Handbook of Bacteriology: A Guide to the Laboratory Diagnosis and Control of Infection*, E. & S. Livingston, Edinburgh, 1960. With permission.)

If the level of contamination is low, then 10 min at 121°C or 20 min at 115°C can be used. These temperatures are attained at 1.1 kg/cm^2 or 0.7 kg/cm^2, respectively.

All of the air must be removed from within the chamber before closing the exhaust valve. The effect of air removal on temperature is summarized (Table 2). Sterilization begins after the load has reached the desired temperature. The preheating times required for various liquid volumes are 2 min for loosely packed culture tubes containing 10 ml; 5 min if tightly packed; 5 min for flasks or bottles containing 100 ml plugged with cotton or loosely screwed caps loosely packed; 10 to 15 min for 500 ml, 15 to 20 min for 1 l; and 20 to 25 min for 2 l. If flasks or bottles are stacked or layered, the preheating time should be increased 5 to 10 min. It is not desirable to autoclave large and small volumes at the same time because of the different preheating and autoclaving times. Empty or dry containers should be loosely stoppered and placed horizontally to allow for the movement of air and steam; sterilization requires 35 min at 121°C.[2,3]

Culture media are altered by heat treatment. The effect may be harmful or beneficial, but no medium should be exposed to more heat than necessary. The medium pH usually is changed by 0.2 to 0.4 units; carbohydrates are partially hydrolyzed and the nature of proteins may be changed. Glucose and amino acids may react to form compounds inhibitory to microorganisms.[9–11] Excessive autoclaving partially hydrolyzes agar-agar, which can inhibit microbial growth.[12]

Acidified agar does not gel properly when autoclaved, thus, acidification is done after autoclaving. Additives, such as antibiotics, hormones, vitamins, and other compounds may be destroyed by heating and therefore should be sterilized by filtration or other means and added after autoclaving the medium. (Remember that when liquids are mixed a dilution factor must be considered.)

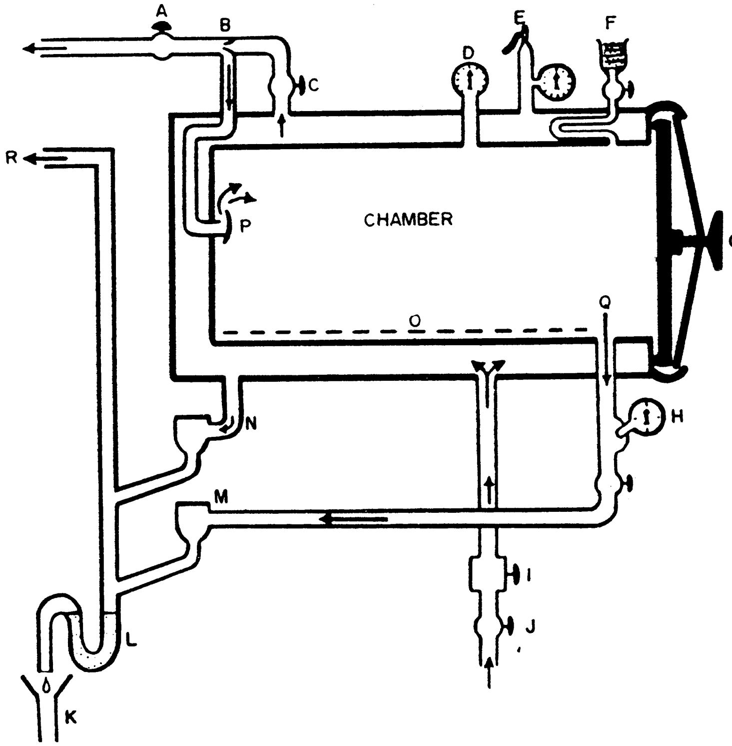

Figure 1-2. Schematic diagram of a steam jacketed autoclave with automatic gravity discharge of air and condensate, and system for drying by vacuum and intake of filtered air: A, chamber discharge and vacuum valve; B, venturi tube; C, steam to chamber valve; D, chamber pressure gauge; E, jacket safety valve and pressure gauge; F, air intake and filter; G, chamber door, H, thermometer; I, pressure regulator; J, steam supply valve; K, drain; L, vapor trap, M, chamber steam trap; N, jacket steam trap; O, perforated tray; P, baffle; Q, discharge channel; R, discharge to atmosphere. (From Cruickshank, R., *Handbook of Bacteriology: A Guide to the Laboratory Diagnosis and Control of Infection*, E. & S. Livingstone, Edinburgh, 1960. With permission.)

Table 1. Theoretically Calculated Time Required for Sterilization at Steam Temperatures Ranging from 100 to 130°C[8]

Temperature (°C)	100	110	115	121	125	130
Time	20 hr	2.5 hr	51 min	15 min	6.4 min	2.4 min

Table 2. Effect of Amount of Air Removed from the Autoclave at Various Pressure Gauge Readings on the Temperature Attained in the Autoclave[6]

	Internal autoclave temperature (°C)				
Pressure gauge readings ($lb/in.^2$)	**All air removed**	**2/3 air removed**	**1/2 air removed**	**1/3 air removed**	**No air removed**
5	109	100	94	90	72
10	115	109	105	100	90
15	121	115	112	109	100
25	130	126	124	121	115
30	135	130	128	126	121

Certain precautions must be taken when using an autoclave or pressure cooker. All vents, exhaust values, and safety valves as well as the chamber should be kept clean. Use nonabsorbent cotton for plugs, which should be loose enough to allow for access of steam and air exhaust during decompression. If the volume of liquids is critical, screw-cap containers may be used or a compensation of 3 to 5% water loss should be made. If screw-cap containers are used, treatment time may have to be increased 5 min over cotton-plugged containers. Always check the effect of heat on an object or material to be autoclaved before beginning the process. Containers should be no more than one- half to two-thirds full. All air should be replaced before closing the exhaust valve. Exhaust of steam after autoclaving should be slow to prevent blowing of stoppers and boiling of liquids.

C. FLAME STERILIZATION

Flame sterilization is used for metal objects, such as transfer needles and tips of forceps, and glass objects, such as the lips of flasks and culture tubes, microscope slides and cover slips, and the surface of certain plastics. The object to be sterilized is held at a 45° angle in the upper portion of a flame from a bunsen burner or alcohol lamp. Tempered metal can be heated to "red hot" and remains sterile as long as it is hot. Glass objects are passed through the flame several times and should not be placed immediately on a cool surface or they will crack.

III. GAS STERILIZATION

Gas sterilization is used on objects that cannot be sterilized by heat or liquid filtration. Advantages are that the process can be carried out at low temperatures and relative humidity; objects can be sterilized in their containers since most gases will diffuse out of most containers with time; the process can be carried out using simple equipment such as plastic or rubber bags, or metal or plastic drums. Major disadvantages are that a longer time is required for sterilization over that of heat, materials used are flammable and highly toxic, and the cost is higher than heat. If the gas is highly reactive, it may combine with organic matter. Some gases used are ethylene oxide, formaldehyde, propylene oxide, methyl bromide, ozone, and B-propiolactone. The first three are alkylating agents. The mechanism of gas sterilization was summarized.[13]

A. ETHYLENE OXIDE

Ethylene oxide is the most efficient and commonly used sterilizing gas. However, it is highly explosive when mixed with air, toxic at low concentrations, and a direct-contact skin irritant. Flammability is eliminated when mixed with inert gases. Ethylene oxide is available commercially in a mixture of 10% ethylene oxide plus 90% carbon dioxide. This mixture is available in metal cylinders containing 15 to 30 kg of gas as a liquid, in 100- to 350-g cans to be used with needle valves and a can holder, or in glass bottles. When in glass, the mixture must be stored refrigerated since the boiling point of ethylene oxide is 10.8°C.

The concentration of gas needed varies between 400 to 1000 mg/l of the chamber space, which is equivalent to 20 to 50% at atmospheric pressure. Sterilization requires a minimum of 3 hr; the time at a fixed temperature is inversely proportional to gas concentration. Exposure time is reduced by increased temperatures of 50 to 60°C, and/or pressure or prevacuuming of the chamber.[14] Relative humidity should be between 30 and 50%. Hygroscopic compounds cannot be sterilized with ethylene oxide. However, it can be used to sterilize aqueous solutions. The liquid is cooled to 3 to 5°C in an ice bath or refrigerator and chilled ethylene

oxide is added using a chilled pipette (with bulb) to bring the gas concentration to 1% of the liquid. Close, but do not seal, the container, and after 1 to 6 hr at 3 to 5°C, transfer the container to a warm place or into a water bath at 45°C under an exhaust hood to volatilize the gas.[4,15] Sterilization of culture media with ethylene oxide (see Section III.C) may change its composition by reaction with certain compounds.

B. FORMALDEHYDE

Formaldehyde is an excellent microbicide and viricide but its use is restricted because of its pungency, poor penetration and diffusion ability, and high toxicity. It is used generally as a surface sterilant although a thin film of organic matter can restrict its activity. Formaldehyde gas boils at –20°C, but in aqueous solution at 90°C. The usual concentration required for gas sterilization ranges from 3 to 10 mg/l of chamber space with a relative humidity of 75 to 90% at 55 to 60°C. A 5 to 10% formalin solution is a powerful and rapid disinfectant when applied directly to contaminated surfaces.

C. PROPYLENE OXIDE

Propylene oxide boils at 34°C and concentrations used for sterilization range between 800 and 2000 mg/l of chamber space. It is less effective than ethylene oxide and has less penetrating power. Expensive apparatus and valve systems for its use can be avoided if the chamber is not prevacuumed, the propylene oxide is chilled and removed with a chilled pipette (see Section III.A) into a chilled container such as a beaker, flask or culture plate, or soaked onto a chilled cotton wad, which than is placed in the chamber. Sterilization is carried out at a relative humidity of 30 to 50%. Any airtight chamber without modification or polyethylene bags can be used as sterilization chambers.

Sterilization of culture media in plates is accomplished with propylene oxide.[16–18] One ml of cooled propylene oxide is added to each plate of hardened medium (use a chilled pipette with bulb) and placed in an exhaust hood for 24 hr at room temperature. If the medium is to be stored, used in the field, or if small numbers of a variety of media are to be sterilized, stack 15 to 20 plates in a polyethylene bag, place a cotton wad with 10 ml of chilled propylene oxide inside the bag, and knot or seal with a rubber band; leave for at least 24 hr. Ethylene oxide at 3 ml per bag may also be used.

A variety of chambers can be used for gas sterilization if they are airtight, nonreactive with the gas, and nonpermeable. These range from various plastics, polyethylene or rubber bags, metal or plastic drums, to automatic commercial chambers. A laboratory autoclave can be modified for gas sterilization by placing a "T" joint at the exhaust pipe with a cut-off valve on each side.[19–21] One arm of the "T" joint is connected to the gas through a flow meter with the other arm acting as an exhaust. An adaptation of a jacketed autoclave for gas sterilization was described.[22]

Other simple chambers can be constructed, limited only by imagination and skill. Metal drums can be modified (Figure 1-3).[21] A rubber gasket is glued to the lid to make an airtight fit. A bolt or other device holds the lid in place during use. Two holes are drilled in the lid, one for a pressure gauge and the other for attachment to a vacuum pump. A gas inlet port with a needle valve and can holder is placed on one side near the bottom. If the gas source is a cylinder, a pressure reducing device and flow meter need to be installed.

Other containers can be used including 6- to 9-mil polyethylene bags with one heat-sealed seam; heavy-gauge rubber bags; terylene fabric treated with neopropylene; plastic, rubber or aluminum cans. Polyethylene bags are most suitable.[23] Materials are placed in the bag with the gas can equipped with a needle valve and sealed. The needle valve is opened and the gas can inverted holding the can from outside the bag.[21,22] Other improvised chambers are described.[21,22,24]

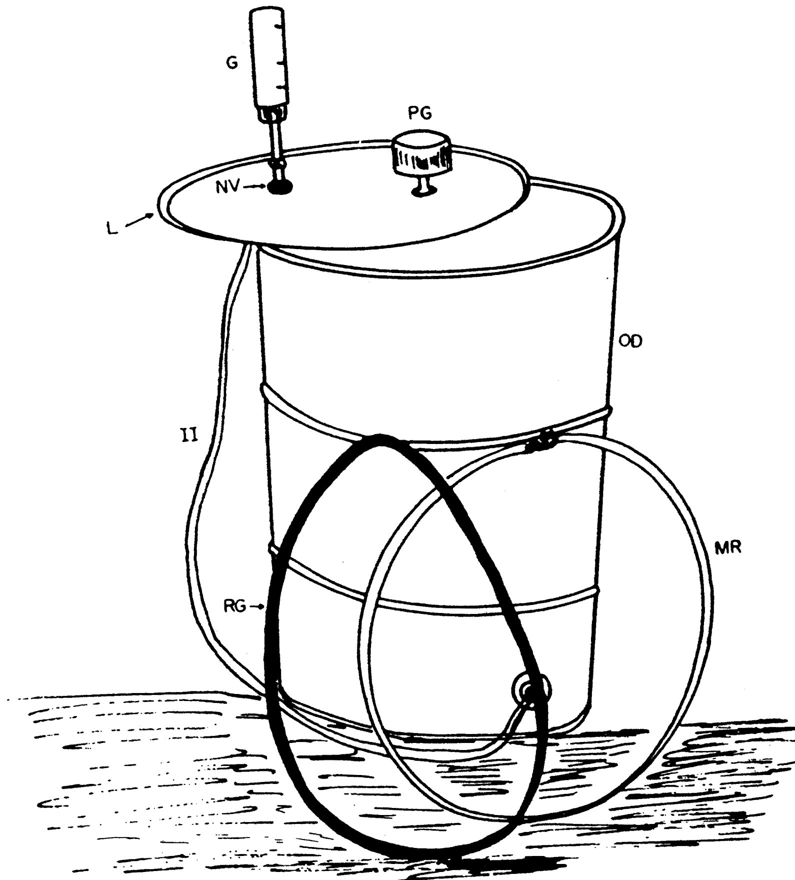

Figure 1-3. Schematic diagram of an oil drum modified for gas sterilization apparatus: G, gas can; NV, needle valve; L, lid; II, gas intake tube; RG, rubber gasket; PG, pressure gauge; OD, oil drum; MR, metal seal for lid. (From Schley, D. G., et. al., *Appl. Microbiol.*, 8, 15, 1960. With permission.)

For continuous or long-term use, an airtight box with perforated shelves (to allow for diffusion of gas) can be constructed from 1.5-cm thick plywood. The gas inlet is located at the bottom of the box on one side and the outlet on the opposite side near the top. Control valves and gauges also are required.[21]

IV. FILTRATION

Filtration physically separates microorganisms, cells, and debris from liquids, but not viruses or metabolic by-products. Except for these limitations, sterilization by filtration is superior to other methods since there is no change in the properties of the filtrate. Aqueous solutions, organic solutions, and oils can be sterilized by filtration. Filters used are sintered glass, asbestos pads, unglazed porcelain, diatomaceous earth disks or candles, and cellulose ester membranes. Microorganisms and other large particles are retained on the filter in part by the small size of the pores and dry adsorption onto pore walls.

Sintered glass filters — These are prepared by fusing fine glass fragments, can be reused but must be cleaned after each use. A new filter is washed before use by suctioning through

hot hydrochloric acid followed by several rinses of distilled water. Cleaning also is done in a vented hood suctioning through a mixture of concentrated sulfuric acid and nitric acid (see manufacturer's instructions), then flushing several times with distilled water followed by an acetone rinse. Caution must be taken when working with these materials. Do not collect the different solvents in the same container.

Asbestos pads or Seitz filter — These are made of washed asbestos and cellulose fibers in various combination. The amount of asbestos determines filter efficiency. Some grades are suitable for sterilization and others for clarification of liquids. Filters are used once. Asbestos pads are soft and easily damaged when wet, thus, need careful handling. These filters are strongly adsorbent and can remove active substances from solutions with the major loss occurring from the first small volume of solution passing through, then progressively declining as the adsorbing sites become saturated. The filters shed fibers, sometimes small amounts of alkalies and metals (especially iron) during filtration. Preliminary washing with distilled water or weak acid solution can overcome this problem. Because asbestos fibers can be carcinogenic, extreme caution is required and should be used in a vented hood.

Membrane filters — Made from cellulose ester, they are very thin and delicate, need careful handling and are used once. They are available in various diameters and pore sizes, but a pore size of 0.22 μm is suitable for most sterilization. Membrane filters can be autoclaved at up to 125°C in air and higher temperatures in the absence of air. They are solubilized in esters, ether alcohol and ketones, and attacked by strong alkalies. They do not absorb materials from solution nor release materials into the filtrates, and with clear liquids, allow for a rapid flow rate. The assurance of sterility is increased with two layers.

Asbestos or membrane filters and filter assembly must be autoclaved before use. Both types give the best results when used in their respective filter assemblies. The filter assembly with filter in place is mounted on the filtration flask and the side arm loosely plugged with cotton. If a small amount of liquid is to be filtered, place a culture tube over the delivery tube to collect the filtrate. Use rubber stoppers or other nonporous material to mount the filter apparatus in the flask. Wrap the entire assembly in aluminum foil or heavy paper and autoclave for 15 min at 121°C.

Since the gravity flow rate of liquids is slow, it is necessary to have negative pressure. Suction is most commonly used. When using suction, an autoclaved trap filled with cotton placed in the line between the filter assembly and suction source prevents accidental backflow of nonsterile air into the filtration flask. Use the least negative pressure that will produce a satisfactory flow rate. A high negative pressure draws small particles into the pores preventing further filtration. All liquid should be clarified by passing it through a coarse filter before filtering for sterilization.

REFERENCES

1. Perkins, J. J., *Principles and Methods of Sterilization*, C. C. Thomas, Springfield, IL, 1963.
2. Reddish, G. F., *Antiseptics, Disinfectants, Fungicides and Chemical and Physical Sterilization*, Lea & Febiger, Philadelphia, 1957.
3. Sykes, G., Methods and equipment for sterilization of laboratory apparatus and media, in *Methods in Microbiology*, Vol. 1, Norris, J. R. and Ribbons, D. W., Eds., Academic Press, New York, 1969, 77.
4. Sykes, G., *Disinfection and Sterilization*, D. Van Nostrand, New York, 1965.
5. Brown, M. R. W. and Melling, J., Inhibition and destruction of microorganisms by heat, in *Inhibition and Destruction of Microbial Cells*, Hugo, W. B., Ed., Academic Press, New York, 1971, 1.
6. Quensnel, L. B., Hayward, J. M., and Barnette, J. W., Hot air sterilization at 200°, *J. Appl. Bacteriol.*, 30, 578, 1967.

7. Cruickshank, R., *Handbook of Bacteriology: A Guide to the Laboratory Diagnosis and Control of Infection*, E. & S. Livingstone, Edinburgh, 1960.
8. Thiel, C. C., Burton, H., and McClemont, J., Some aspects of the design and operation of vertical laboratory autoclaves, *Proc. Soc. Appl. Bacteriol.*, 15, 53, 1952.
9. Hall, A. M., The culture of *Phytophthora infestans, Trans. Br. Mycol. Soc.*, 42, 15, 1959.
10. Hansen, H. N. and Snyder, W. C., Gaseous sterilization of biological material for use as culture media, *Phytopathology*, 37, 369, 1947.
11. Patton, A. R., Salances, R. C., and Piano, M., Lysine destruction in casein-glucose interaction measured by quantitative chromatography, *Food Res.*, 19, 444, 1954.
12. Robbins, W. J. and McVeigh, I., Observations on the inhibitory action of hydrolyzed agar, *Mycologia*, 43, 11, 1951.
13. Hoffman, R. K., Toxic gases, in *Inhibition and Destruction of Microbial Cells,* Hugo, W. B., Ed., Academic Press, New York, 1971, 226.
14. Phillips, C. R., The sterilization action of gaseous ethylene oxide. II. Sterilization of contaminated objects with ethylene oxide and related compounds: Time, concentration and temperature relationships, *Am. J. Hyg.*, 50, 280, 1949.
15. Judge, L. F., Jr. and Pelcazar, M., The sterilization of carbohydrates with liquid ethylene oxide for microbial fermentation tests, *Appl. Microbiol.*, 3, 242, 1955.
16. Klarman, W. L. and Craig, J., Sterilization of agar media with propylene oxide, *Phytopathology*, 50, 868, 1960.
17. Thompson, H. C. and Gerdemann, J. W., An improved method for sterilization of agar media with propylene oxide, *Phytopathology*, 52, 167, 1962.
18. Watson, R. D., Carley, H. E., and Hubber, D. M., Storage of culture media for laboratory and field use, *Phytopathology*, 56, 352, 1966.
19. Freeman, M. A. R. and Barwell, C. F., Ethylene oxide sterilization in hospital practice, *J. Hyg. Comb.*, 58, 337, 1960.
20. Newman, L. B., Colwell, C. A., and Jameson, E. L., Decontamination of articles made by tuberculous patients in physical medicine and rehabilitation, *Am. Rev. Tuberc. Pulm. Dis.*, 71, 272, 1955.
21. Schley, D. G., Hoffman, R. K., and Phillips, C. R., Simple improvised chambers for gas sterilization with ethylene oxide, *Appl. Microbiol.*, 8, 15, 1960.
22. Winge-Heden, K., Ethylene oxide sterilization without special equipment, *Acta Pathol. Microbiol. Scand.*, 58, 225, 1963.
23. Dick, M. and Feazel, C. E., Resistance of plastics to ethylene oxide, *Modern Plastics,* November 1960, p. 148 and 226, 1960.
24. Fulton, J. D. and Mitchell, R. B., Sterilization of footwear, *U.S. Armed Forces Med. J.*, 3, 425, 1952.

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CHAPTER 2

Culture of Pathogens

I. INTRODUCTION

Most pathological studies require culturing a pathogen either to increase infective propagules for inoculation or to study its taxonomy and genetics. Techniques for producing vegetative, asexual, or sexual propagules have been developed for many pathogens. The quality of inoculum is more important than the quantity because the nutritive status of infective propagules often is related to its infectivity; however, the importance of the quantity of inoculum should not be underestimated.[1–7] Sporulating cultures should be used whenever possible. Repeated transfers of a pathogen on artificial media usually result in loss of pathogenicity or sporulation or both. Transfers should be made using spores and cultured alternately on nutrient rich and poor culture media. (Reference to medium number refers to Appendix A.)

II. FACTORS AFFECTING THE CULTIVATION AND SPORULATION OF PATHOGENIC FUNGI

All microorganisms require a set of environmental conditions (aeration, light, moisture, temperature, etc.) under which they grow and sporulate best. The range of conditions permitting vegetative growth and sporulation is divided into minimum, below which, and maximum, above which, no growth will occur, and optimum conditions, under which it will grow best. The optimum range may be wide or narrow. Spores often are produced under conditions that are adverse to vegetative growth. Sometimes allowing a culture to desiccate slowly will induce sporulation. There is no universal set of conditions for culturing pathogenic fungi. In general saprophytic fungi are less exact in their requirements than are pathogenic fungi.

A. CULTURE MEDIUM

Culture medium is the major factor influencing fungal cultivation. The concentration of medium constituents determines the quality and quantity of growth and whether sporulation or vegetative growth will dominate. A good culture medium supports high sporulation and low mycelial growth. Generally, sporulation is favored by nutritional exhaustion. A weak medium with a low C and/or N source stimulates sporulation and suppresses vegetative growth. Natural media, in general, are more favorable to growth and sporulation than synthetic ones. The advantages of natural media prepared from plant parts has been discussed.[8,9] Such media are prepared as decoctions, extracts or juices from plant parts or powdered plant material added to agar. The plant parts used may not be the same as those on which the fungus is found in

nature, however, differences in growth rate and sporulation may occur among substrates.[10,11] Preparing a medium from parts of susceptible plants, may improve the chances of success.[12]

Gas-sterilized rather than heat-sterilized plant material, such as chopped leaves, stems, roots, fruits or straw of the host and sometimes of nonhosts are excellent substrates to induce fructification,[8,9,11] especially perithecia, pycnidia, or sporodochia. Such substrates after sterilization with propylene oxide, are placed on similarly sterilized 1.5% water agar, moist sand or in a moist chamber and seeded with inoculum at various points. For many fungi an agar surface is unsuitable for production of fruiting structures, therefore, placing substrates of different textures on the agar surface improves the chance of sporulation. Such methods have been useful for inducing sporulation in *Alternaria solani, Phoma pinodella* on pea straw, *Mycospherella (Ascochyta) pinodes* on wheat straw, and *Phytophthora cinnamomi* on avocado roots.[8,11,13] Sterilization can be achieved by placing them in a propylene oxide atmosphere for 24 to 48 hr. Large quantities of material can be collected when available, fumigated and stored in sterile jars for future use.

Green leaves dried in a plant press also can be stored for future use, or allowed to wilt overnight for immediate use.[10,14] A leaf is cut to fit loosely inside a culture plate, then floated on 30 ml of nonautoclaved, cool, but still molten 1.5% water agar. After solidification, the plates are placed in a propylene oxide atmosphere (1 to 1.5 ml propylene oxide/l of space) for 24 hr, then on a laboratory bench for 24 hr. The leaf is seeded with inoculum at various points and incubated under conditions favorable for the test fungus. Synthetic media can be costly and take more time to prepare than natural media. The choice depends on the purpose and requirements of the experiment. No universal rules or recommendations can be outlined for a synthetic medium suitable for sporulation of all fungi. Tuite[15] described certain principles and should be consulted. The formulae and preparation of multipurpose natural and synthetic media are presented in appendix A.

When a number of culture media are compared for fungal sporulation, the microplate assay technique[16] permits an easy analysis of a range of factors using a large number of replicates in a small space. Tissue culture plates with a number of wells or cavities are used. Each cavity is filled with a predetermined amount of culture medium. After solidification, each cavity is seeded with a droplet of a standardized spore suspension. The plates then are covered with a lid or placed in plastic bags to prevent desiccation and incubated. The technique also can be used with liquid media.

B. TEMPERATURE

The optimum temperature for vegetative growth may be different than that for sporulation; and the temperature range for sporulation generally is narrower than that for vegetative growth. Some pathogens grow and sporulate well at a constant temperature and others are favored by diurnal fluctuations. Although the optimum temperature for a specific fungus often is available from the literature, new isolates should be tested at a range of temperatures using 4 to 5°C intervals. Isolates from a cold region generally have a lower optimum than those from a warmer one. Some fungi and bacteria require longer incubation than others.

One useful device for measuring the temperature requirements of a pathogen is the temperature gradient plate, which provides a continuous range of temperatures between a preselected minimum and maximum[17] (Figures 2-1 and 2-2). The plate can be programmed for a gradient in a single direction for constant temperature, or both in a horizontal and vertical one for fluctuating temperatures. The advantage of using such an apparatus is that it requires less space than a series of incubators and may be more accurate. The apparatus consists of an aluminum plate (110 × 55 × 1.5 cm) heated at one end and cooled by circulating water at the other. Thermometers or thermocouples are placed at 10-cm intervals between the two ends.

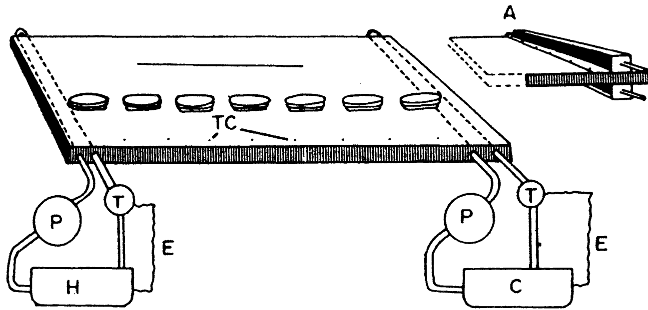

Figure 2-1. Single-direction temperature-gradient plate (110 × 55 × 1.5 cm). A, an alternate arrangement for circulating water through heating and cooling channels; C, refrigeration unit; P, water circulating pump; T, adjustable temperature switches connected electrically (E) to heating and cooling units; H, water heater; TC, thermocouples. (From Leach, C.M., *Seed Pathology — Problems and Progress,* Yorinori, J.T., et al., Eds., IAPAR, Londrina, Brazil, 1979, 89. With permission.)

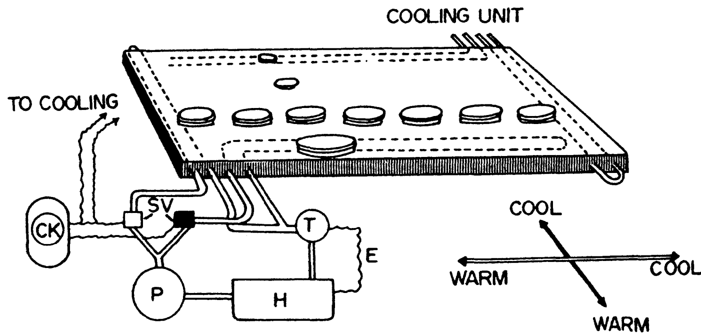

Figure 2-2. Two-directional temperature-gradient plate (110 × 55 × 1.5). CK, electrical time clock operating solenoid values (SV) for day and night temperatures cycle; P, water circulating pump; T, adjustable temperature switches connected electrically (E) to heating and cooling units; H, water heater. (From Leach, C.M., *Seed Pathology — Problems and Progress,* Yorinori, J. T., et al., Eds., IAPAR, Londrina, Brazil, 1979, 89. With permission.)

Culture plates are distributed on the plate where they attain the temperature of the plate. If light is used, radiation may increase media temperature by 1°C.

C. LIGHT

Light stimulates asexual and sexual reproduction in most fungi, and its effect is closely related to nutrition and temperature. The effect of light on the culture of fungi was discussed by Leach.[18] Fungi requiring light for sporulation were listed by Marsh et al.[19] and Tuite.[15] Ultraviolet (UV) and near ultraviolet (NUV) wavelengths of less than 340 nm usually induce sporulation, but excessive dosages may inhibit it.[17,18,20] Sunlight or UV and NUV lamps may be used. Red light rarely induces sporulation except for *Botrytis fabae*.[21] Most light-sensitive fungi sporulate when exposed to continuous light, but some, called diurnal sporulators, require a dark period followed by a light period.[20] Such fungi require light to initiate conidiophore formation and sporogenesis; however, sporulation is inhibited by light. *Alternaria, Choanephora, Helminthosporium, Peronospora,* and *Stemphylium* are examples of diurnal sporulators.

The artificial light source generally used is a NUV fluorescent lamp with a peak emission of 360 nm. The spectral quality of NUV lamps varies with manufacturer and will reduce with age. In the absence of NUV lamps, cool daylight-type white fluorescent lamps alone or with incandescent lamps, can be used in most cases.[22] General recommendations for inducing sporulation with light are (1) use black light fluorescent lamps emitting NUV radiation in a continuous spectrum from 320 to 420 nm, which are available in 20, 40, and 80 W; cool, white daylight-type fluorescent lamps also emit NUV and may be substituted; (2) place two lamps (40 W each) horizontally 20 cm apart at 40 cm above the culture plate; (3) use an alternating cycle of 12 hr of light and dark; diurnal sporulators as well as fungi sporulating under continuous light will respond; (4) begin irradiating 2 to 3 days after initial growth or after a small colony has formed and continue until the end of the growth period; old colonies do not respond to light; and (5) use plastic or glass (Corning® or Pyrex®) culture plates or tubes which allow NUV transmission.[23]

Optimum temperature requirements for light-induced sporulation vary among fungi and within the same species.[17,18] The interaction of light and temperature is important for most diurnal sporulators. *Alternaria* and *Stemphylium* form conidiophores under light at high temperatures and conidia in the dark at low temperatures. Low temperatures may nullify the effect of light in some fungi. *Alternaria* sporulates in continuous light if the night temperature is below 23°C. At temperatures above 26°C light inhibits sporulation.[24] A daytime temperature of 21 to 24°C and a night temperature of 18 to 24°C is satisfactory for most fungi. If room temperature does not exceed 25°C during the day, it may be used for incubating diurnal sporulators.

D. pH

The optimum pH range for vegetative fungal growth is different and broader than the optimum for sporulation.[25,26] For routine work media pH is adjusted using acetic acid, lactic acid, HCl, KOH or NaOH before autoclaving. However, pH generally changes after autoclaving. Adjusting pH either before or after autoclaving depends on the requirements of the experiment. Highly acidic agar media (pH <4) cannot be autoclaved because they do not gel on cooling. Thus, pH is adjusted after autoclaving. Most buffers used in fungal culture to maintain a constant pH during the growth period act as nutrients and their buffering capacity are reduced with age. To compensate for such changes the use of a high concentration of a buffer becomes necessary, which in turn may be toxic. A range of nontoxic and nonnutritive buffers are available commercially.[27]

To study the pH effect on mycelial growth, sporulation, or spore germination, a series of culture plates containing a medium with a range of pH are used. The method is laborious, time consuming and expensive. A range of pH values between two predetermined high and low limits can be obtained in a single plate using the gradient plate technique. The pH limits are adjusted using suitable buffers. Buffer solutions of preselected low and high pH limits at a suitable molarity are prepared and autoclaved. Two flasks containing equal amounts of the culture medium using 10% less water are autoclaved separately. At the time of dispensing the medium into the plates mix the buffer with the culture medium (1:9 v/v). Thus, one flask will have medium with the low and the other one with the high pH limit. Place a large culture plate, preferably 100 mm square plastic plate, in an inclined position, by resting one edge of the plate on a 3- to 4-mm glass rod. Dispense 15 to 20 ml of the high pH medium. After solidification place the plate in a leveled position and add the same quantity of the low pH medium. Keep the plates for 24 hr at room temperature, permitting for gradient stabilization. The pH at any point can be determined using a pH meter equipped with flat tipped, active ion surface electrode.[28,29]

Using the same principle, a two-way gradient plate was used for simultaneously studying the effect of pH and water potential on spore germination. The first two layers of the culture medium set the pH gradient in one direction, and the other two layers contained different concentrations of KCl and poured at a 90° angle to the pH gradient established the water potential in the other direction.[30]

E. AERATION

Excess ammonia or CO_2 may inhibit growth and sporulation of some microorganisms. Culture plates and cotton-plugged culture tubes usually allow sufficient gaseous exchange while autoclavable polypropylene bags do not and fungi in such containers generally do not sporulate.

III. SOME SPECIAL METHODS OF INDUCING SPORULATION IN FUNGI

A. SEEDING CULTURE MEDIA

The type of inoculum and seeding method has qualitative and quantitative effects on the sporulation of most fungi. Seeding the medium with mycelial inoculum at one point results in a small spore quantity, delayed sporulation, and spores of different ages. The medium should be seeded at various points using spores. A concentrated spore suspension spread over the medium yields the maximum spore number. The rapidity of sporulation and quantity of spores are directly related to spore concentration of the seeding suspension. If spores are not available, the medium should be seeded with a concentrated mycelial suspension in which the mycelium may or may not be macerated. The resulting spores are washed off with a small quantity of sterile water and the suspension used to seed fresh plates. This is repeated until the desired yield of spores is obtained.[31,32]

B. FILTER PAPER METHOD

A method developed to induce sporulation in *Alternaria solani* and *Drechslera poae* has been used for inducing the sporulation in *Drechslera* state (*Bipolaris*) of *Cochliobolus sativus* and *Pyrenophora dictyoides*.[33] The fungus is grown in a shake culture until sufficient mycelium is formed which then is harvested by centrifugation, blended in a blender for 2 min and centrifuged again. (Aseptic techniques may not be required after this process.) The pellet is resuspended in 0.02 *M* phosphate buffer (pH 6.4) and distributed in 2-ml portions onto dry filter papers in culture plates. No nutrients are added. Light may be needed for some fungi.

The following is used to induce sporulation in *Alternaria, Chaetomium, Cladosporium, Curvularia, Helminthosporium, Phoma, Plenodomus, Pleospora* and *Stemphylium*: seed moistened filter paper in culture plates with small agar disks of the test fungus and incubate at room temperature.[34] The hyphae grow from the disks onto the filter paper and produce abundant spores.

To induce sporulation in *Alternaria zinniae* place filter paper strips in culture tubes containing 5 ml of medium 147 without agar and autoclave.[35] The tubes are seeded with a mycelial or spore suspension and incubated for 14 days at room temperature. Spores may be difficult to remove by washing. If so, dip the filter paper disks in medium 147 without agar, place in a culture plate and autoclave. Just cover the disks with sterile water agar and spread the inoculum over the agar surface. The fungus will sporulate over the covered disks, which are lifted out and the spores washed off using a wash bottle.

Placing sterile filter paper, lens paper, or blotters over an agar surface may induce sporulation in many fungi.

C. GRASS-LEAF METHOD

This is used to induce sporulation in *Alternaria, Colletotrichum, Curvularia, Drechslera, Fusarium, Helminthosporium, Nigrospora, Pestalotia, Phaeotrichoconis, Plenodomus, Pyricularia* and *Trichoconis*. Small pieces of *Pennsetumum glaucum* leaves are autoclaved in water, the excess moisture removed by blotting on sterile blotter paper, and then transferred to culture plates with 1.5% water agar. The leaf strips or pieces are seeded with mycelial disks obtained from 48- to 72-hr-old colonies and incubated under optimum conditions for the test fungus.[36]

IV. CULTIVATION OF PATHOGENS FOR SOIL INFESTATION

Large quantities of inoculum of soilborne pathogens can be obtained by using one of the following substrates in large containers: (1) maize meal:sand mixture (2 to 5:98 to 95) moistened and autoclaved for 1 to 2 hr on 2 successive days; (2) vermiculite-mix (3:1) of the desired nutrient broth such as V-8® juice broth or potato-dextrose broth autoclaved for 1 to 2 hr; (3) grains of barley, maize, oats, peas, rice, or wheat, individually or mixed are excellent substrates for many fungi; the grains are soaked in water for 24 hr, drained and autoclaved; and (4) a grain mixture with sand, soil, or vermiculite (1:1) can be used. Large flasks, prescription bottles, metallic or wooden trays can be used for containers.

The system developed by Schroeder et al.[37] is useful and inexpensive. Wooden trays about 10 cm deep with a hardware cloth bottom are lined with butcher (heavy duty) paper, filled with the substrate to about 5 cm deep, covered with butcher paper and then autoclaved for 1 to 2 hr on 2 successive days. A mycelial/spore suspension of the test fungus is prepared by blending the cultures grown on an agar medium and pouring evenly over the substrate surface. After seeding, the paper is replaced and the boxes incubated for 10 to 14 days at room temperature. The inoculum then is air-dried, crumbled by hand, or ground mechanically and stored in a cold room. Drying is not necessary for immediate use.

The success of this method depends upon the quantity, concentration, and uniform distribution of the inoculum in the medium. Incubation should be done in an area free from air currents and the trays exposed or moved as little as possible. All equipment and the work area should be disinfested. If the substrate layer is too thick, some heating of inoculum occurs. This can be reduced by using a shallow layer of medium, adequate aeration, incubating the trays in a refrigerated room, or by stirring the inoculum at least once a day.

Large quantities of inoculum can be grown on agar media using culture plates, aluminum cooking or serving dishes, or trays covered with butcher paper.

V. OTHER SOURCES OF INOCULUM

In the absence of spores, mycelial inoculum may be used for spray inoculation. Blend the mycelial mat grown on agar or liquid medium in a blender to bits small enough to pass through the sprayer nozzle.[38]

Dry leaf inoculum, especially for bacterial pathogens, can be prepared from heavily infected leaves collected from greenhouse-grown, inoculated plants. After drying at 28 to 30°C, grind in a blender and sieve through a 500 μm sieve. Store in plastic bags at 4 to 20°C.

Quantification may be done by plating a dilution series on a suitable agar medium. At the time of use suspend the required amount of leaf powder in water.[39] Infected plant parts from the field that are washed and disinfested by soaking in 70% ethanol and placed in a moist chamber under conditions favorable for sporulation also can be used. A spore suspension is prepared by washing the spores from the plant parts.

VI. CULTIVATION AND SPORULATION OF SOME PATHOGENS

A. FUNGAL PATHOGENS

1. *Aphanomyces* spp.

A. euteiches grows well on natural or synthetic media containing reduced sulfur. (For the preparation of *Aphanomyces* oospores free of mycelium see *Phytophthora* and *Pythium* spp.). For growth and oospore production, media 55[40] and 63[41] are useful. Oospores can be produced on maize-decoction agar (45 g maize seeds per liter water). Autoclaved broth is seeded with a 2-day-old culture disk of the test fungus and incubated for 1 mon at room temperature.[42]

For zoospore production, use one of the following: (1) seed medium 153 in Erlenmeyer flasks with a 2-day-old culture disk of the test fungus grown on 2% water agar. After 72 hr pour off the medium and replace it with a solution containing 1.5×10^{-3} *M* $CaCl_2$, 0.2×10^{-3} *M* $MgSO_4$ and 1.5×10^{-3} *M* KCl. Change the replacement solution three times at 20-min intervals and incubate for 12 to 26 hr at 25°C. For oospore production incubate in the replacement solution for 8 days;[43] (2) grow the fungus in a solution containing 0.5% glucose and 0.2% peptone for 2 days at 24°C. Replace the medium with a solution containing 1.75×10^{-3} *M* $CaCl_2$ and 10^{-3} *M* each of KCl and $MgSO_4 \cdot 7H_2O$. Wash the culture with it twice, successively, and then at 1- and 4-hr intervals during a 16- to 20-hr period. To release zoospores, chill the cultures for 20 min at 9°C;[44] (3) grow the fungus on 100 ml of medium 87 in 500 ml Erlenmeyer flasks for 5 days at 24°C. Pour off the medium, rinse the mycelial mat with sterile tap water, pour off the rinse water, and add enough sterile distilled water to cover the mat. Mix the contents from four flasks and aerate by bubbling air for 7 hr. Transfer the entire culture from three or four flasks to a 2 l flask and incubate for 4 hr at 24°C. Zoospores are collected by filtering through cheesecloth;[45] or (4) grow the culture on maize or pea seed-decoction broth (41 g seeds per liter) for 5 days at 25 to 28°C. Pour off the medium and replace it with tap water; after 1 to 2 hr pour off the tap water and replace it with distilled water and aerate by bubbling air. Zoospores are released in 6 to 8 hr[46] (also see *A. cochlioides*).

A. cochlioides — For oospore production grow on 4% oat-decoction, 3% sugarbeet-decoction, 5% buckwheat groats, or 0.5% oatmeal in water, or a homogenate of 3% barley grain all at pH 6.5 to 6.6. Incubate in the dark for 30 days. Oospore inoculum for soil infestation is prepared by culturing on vermiculite-oatmeal broth (2.5:1 v/v) and incubated for 30 days at 25°C, dried on a paper-covered screen and stored in a refrigerator until used.[47,48] To produce zoospores, cultivate in 250 ml Erlenmeyer flasks containing a sterile decoction of five maize seeds per 50 ml of water for 7 days at 20°C. Pour off the decoction and add 125 ml sterile tap water. Zoospores are released after 12 to 36 hr at 20 to 25°C. Additional crops of zoospores are obtained by adding fresh sterile tap water to the cultures.[49] This method is useful for zoospore production of *A. euteiches*.[50]

A. raphani grows best on media 121 and 122, which are used for oospore production at 20 to 24°C.[51] For zoospore production cultivate on media 121 or 123 without agar. After 2 to 3 days, pour off the medium and replace it with distilled water, deionized water, or a NaCl solution (100 μg/ml) at pH 4 to 5.3 and incubate for a few hours at 20°C.[52]

2. *Alternaria* spp.

The following method induces sporulation in several *Alternaria*. Grow the fungus in culture plates containing 20 ml of either medium 81 or 111 in the dark at 25°C. Before aerial mycelium is apparent, generally after 48 to 72 hr, cut the colony into small blocks (ca. 4 mm^2). Transfer and evenly distribute the blocks on the sporulation medium (2% sucrose, 3% $CaCO_3$ and 2% agar) in a culture plate. Add 2 ml sterile water to each plate partially covering the agar blocks and incubate in the dark at 18°C. Older cultures also can be used to seed the sporulation medium. Remove all aerial mycelium by first scraping and then washing the culture with sterile distilled water. Several crops of conidia can be harvested by flooding the plates with an aqueous solution of 0.01% Triton X-100.[53]

A. brassicae — Place 10 ml autoclaved 20% V-8® juice in culture plates and seed with 0.2 ml of a mycelial or spore suspension prepared by homogenizing a PDA culture in a small amount of sterile water. Incubate under light (12-hr day) at 25°C. Sporulation begins when the culture medium begins to dehydrate.[54] The fungus also sporulates on alfalfa seed-decoction agar (100 g seeds per liter water). Culture washing and slow drying used for inducing sporulation of *A. solani* (see below) induces sporulation of this fungus.[55,56]

A. carthami — Cultivate the fungus on the medium 110 and incubate under NUV (12-hr day) at 25°C. After the colony has covered the plates, cut the cultures into small squares and reincubate.[57]

A. crassa — Nonsporulating cultures can be induced to sporulate by inoculating onto autoclaved *Datura* leaves placed on moist sterile filter paper in culture plates and incubated for 5 to 7 days at room temperature.[58]

A. cuscutacide grows and sporulates on liquid medium 37 with constant aeration.[59]

A. dauci — Culture on medium 18 (pH 6.3) and incubate at 15 to 30°C. The number of conidia per unit area is proportional to a temperature range of 15 to 28°C. To increase the spore quantity evenly seed the agar surface with a conidial suspension. V-8® juice (pH 7.0) can be used if covered with a sterile cellulose filter paper or pad before seeding. Incubate under light for a minimum of 4-hr day. Good sporulation also occurs on cellulose filter paper or pad moistened with medium 18 without agar and seeded with conidia.[60]

A. solani — Grow on medium 147 in culture plates for 2 wk at room temperature. Remove aerial mycelium by scraping the medium surface with the end of a microscope slide. Place the plates with the lids removed under gently running tapwater for 24 hr. The plates may be wrapped in cheesecloth to hold the medium in place. After washing stack the open plates in a tray in an inverted, slanted position so that each plate is partially covered by the bottom of the plate ahead of it. Incubate for 2 days at room temperature. Conidia are harvested using a jet of water from a wash bottle and the plates reincubated. Successive crops of conidia are obtained at 1- to 2-day intervals until the medium dries out.[61] The same method was used for inducing sporulation of *A. brassicae* except the washing time was 72 hr.[55]

Sporulation was induced in *A. solani* by growing it in culture plates of medium 113 for 10 to 14 days at room temperature under constant or diurnal light, then cutting the culture medium into 4-cm strips and placing them in 250 ml sterile water in flasks. Vigorously shake the flasks for 1 min, let stand for 10 min, then spread 1.5 ml of the fluid on fresh plates of medium 113 and incubate under constant fluorescent light at 20°C or in diurnal light if above 23°C.[62] Increased sporulation was reported in *A. solani* cultured on sterile cellophane disks on agar culture plates.[63] Transferring small block from a 2-day-old PDA colony to medium 36 and incubating under light for 4 hr, then in the dark at 18°C induces sporulation within 12 hr.[64]

3. *Ascochyta* spp.

A. chrysanthemi (Mycosphaerella ligulicola) — Field inoculum is prepared from infected flowers and stems dried at room temperature and stored in a dry place. To prepare a spore

suspension, the dried material is placed in deionized water for 15 min to allow pycnidia to eject their spores and then filtered through a double layer of cheesecloth.[65]

A. lethalis — Grow on PDA by covering the agar surface with a concentrated spore suspension or macerated mycelium fragments. Some isolates require light, while others sporulate under light or in the dark. *A. lethalis* produces spores on autoclaved *Melilotus* stems partially immersed in water agar, but incubation under light may not be necessary.[66] It will form perithecia by streaking conidia from different isolates on PDA and incubating for several weeks at 4°C and then returning to 24°C.[66]

4. *Aureobasidium zeae*

A. zeae sporulates on medium 147 seeded with 1 to 2 ml of a concentrated spore suspension, which is obtained by culturing in shake cultures using medium 30 or 31.[67,68] It grows well but does not sporulate on sterilized whole oat or sorghum kernels. It sporulates on oats in about 2 to 3 wk. To induce sporulation on whole kernels, partially dry the kernels and reincubate in a moist chamber.

5. *Bipolaris* spp. (also see *Drechslera* spp.)

For *B. sorokinianum* grow on 20% V-8® juice agar (4% agar) and incubate under light (12-hr day) at 22°C.[69] Maximum virulence occurs in 20-day-old cultures.

6. *Botrytis* spp.

Botrytis aclada — PDA is used for conidia production, but for large quantities, medium 96 is used, incubating cultures for 7 to 8 days at 22°C.[70]

B. cinerea* and *B. squamosa — For conidia production, cover the bottoms of culture plates with air-dried onion leaves from the field and cover with 20 to 25 ml of 2% water agar and autoclave for 20 min. After solidification, seed the plates with either a mycelial or conidial suspension taken from a vigorous colony. Incubate under fluorescent light (14-hr day) for 7 to 10 days at 18°C.[71] Medium 12 supports conidia production in both fungi if incubated for 7 to 8 days under light (12-hr day) at 21°C.[72]

To induce sclerotia cover the bottom of culture plates with onion leaf straw (approximately 2 g), add 20 ml water and autoclave for 30 min. After cooling, spread 2 ml of a conidial suspension over the straw surface and incubate in the dark for 3 wk at 18°C. To harvest, combine the leaf material from several plates in a large beaker and rub them together until the sclerotia separate from the leaf tissues. Add water and stir, allow sclerotia to settle to the bottom, then pour off water and floating debris. Repeat several times until the sclerotia are free of all foreign matter. Sclerotia can be produced on PDA but they are shortlived and not uniform in size.[71]

B. cinerea isolates from chickpea may not sporulate in agar cultures but on plant parts. Place about 7-cm long plant tips with four to five leaves taken from 3-wk-old plants, in culture tubes containing 2 ml water and autoclave. Seed with mycelial bits taken from a PDA culture and incubate in the dark for 3 days at 20°C and then under light (12-hr day) at the same temperature.[73] To induce apothecia formation cultivate on 2% malt extract agar for 30 days at 15°C to produce sclerotia. Then aseptically transfer sclerotia to culture tubes (200 × 24 mm) containing 9 ml water and spermatize by adding 3 ml of the suspension containing conidia and mycelial fragments from the same culture. Place the tubes in a slanted position so that the sclerotia become distributed in a thin layer and place in a growth chamber under light at 11°C. Apothecia formation begins after 4 to 5 wk.[74]

B. fabae sporulates well on medium 74, version X, amended with 10% sucrose, when incubated in the dark for 2 to 3 days at 18°C and then under NUV (12-hr day) at the same temperature.[75] Conidia also are produced on the version Y of the same medium.[76]

Medium 119 with amendments is useful for diagnostic work and sporulation of *B. aclada*, *B. byssoidea*, *B. cinerea* and *B. squamosa* isolated from onion and leeks. *B. aclada* may sporulate in the dark at 10 to 20°C, while other species required incubation under NUV. *B. cinerea* and *B. byssoidea* sporulate abundantly at 15°C. Sporulation also can be induced when cultured on onion leaf segments. Place cylindrical segments cut from green onion leaves on a moist cellulose pad in culture plates and seed at the cut ends. Incubate under fluorescent light (12-hr day) at 15°C. However, not all isolates of *B. byssoidea* may sporulate. Fleshy internal scales of onion bulbs can be used to induce sporulation in most isolates. The scales are wounded on the abaxial surface, inoculated with mycelial disks and incubated under NUV at 20°C.[77]

7. *Cercospora* spp.

Various decoction media are used. A decoction medium for one specie may not be suitable for another. *C. brachiata*, *C. canescens*, *C. capsici*, *C. festucae*, *C. kikuchii*, *C. penniseti*, *C. sesami*, *C. sorghi*, and *C. zebrina*; and *Pseudocercospora puerariicola* and *P. stizolobii* sporulate well on medium 19. Pour 25 ml of the medium in 9-cm culture plates and just before solidification, streak mycelium fragments, if spores are not available, into the medium and agitate briefly. Deep medium favors profuse sporulation.[78] Good sporulation of *C. apii* was reported on medium 19 with two to three harvests from the same plate[79] as well as on medium 23 where celery leaves are seeded with mycelial fragments and incubated at room temperature.[80] Sporulation of several *Cercospora* spp. can be induced on carrot disks on medium 140.[81] Media 102 and 103 support good sporulation of *C. arachidicola*. Seed plates with a conidial suspension and incubate under light at 28°C.[82,83]

C. beticola sporulates on medium 141 in culture plates seeded uniformly with 1 ml of a spore/mycelium suspension and incubated under alternating light and dark at 15 to 22°C;[84] or on autoclaved sugarbeet leaves placed on 1.5% water agar seeded with a mycelial suspension and incubated at high humidity.[85] Placing a PDA disk of *C. beticola* on sterile moist filter paper and incubating under high humidity also induced sporulation. High humidity can be obtained by lining the culture plate lids with moist sterile filter paper.[86] Several harvests of *C. beticola* can be obtained by growing it on PDA until the agar was covered with mycelium, then using the edge of a microscope slide, removing the aerial mycelium. Incubate under near 100% relative humidity for 48 hr at 25°C. After harvesting the conidia, reincubate and harvest a new crop. The process is continued for 10 to 14 days.[86]

To prepare *C. beticola* inoculum for field inoculation, inoculate susceptible sugarbeet cultivars in the field; at sporulation, harvest infected leaves, dry and store in a cool, well-ventilated place until needed. At use, wet the leaves, rub them together by hand in water and periodically squeeze the water from the leaves. Add more leaves and continue the process. Strain the suspension through a 60-mesh screen using the resulting suspension as a stock to be diluted as required.[87]

C. canescens sporulates on medium 19 under light at 28°C;[88] carrot leaves can be replaced by mung bean leaves.[89] *C. nicotianae* on medium 143 at 27°C (seed medium while still soft; isolates may vary considerably);[90] and *C. elaeidis* sporulates on medium 98 or PDA containing a palm leaf decoction of medium 98. Pour 15 ml of the medium into 9-cm culture plates and just before solidification spread 1 ml of a mycelial suspension on the surface and incubate in natural or artificial light for 7 to 10 days at 28 to 31°C.[91]

Three or four harvests of conidia were obtained from medium 147 for *C. sojina* and from V-8® juice plus dead-soybean-plant tissue agar for *C. kikuchii* incubated under 12-hr day for 4 to 5 days at 25°C.[92]

Spore production by poorly sporulating isolates of *C. davisii*, *C. medicaginis* and *C. zebrina* was increased by covering recently isolated cultures with water and dislodging the few conidia present, then pouring the suspension onto fresh culture plates of either PDA or

medium 147, and incubating for 4 to 7 days at 24 to 28°C and then repeating this procedure two to four times until adequate conidia are produced.[93,94] *C. zebrina* sporulates well on carrot, maize meal, *Trifolium pratense*-extract and V-8® juice agars, with maximum sporulation on agar 4 to 6 mm deep.[94]

C. zeae-maydis sporulates on either medium 147 (300 ml V-8® juice and 4g $CaCo_3$) or 35. Seed either medium in plates with 2 to 3 ml of spore/mycelial suspension prepared by homogenizing a few V-8® juice agar disks. After 15 min decant off the excess liquid and incubate under light (12-hr day) at 25 to 28°C.[95] Medium 25 was used to study biosynthesis and regulation of abscisic acid of *C. rosicola*.[96] *C. asparagi* is one of a few species that sporulates in continuous dark when cultured on medium 19 or 147. The media were seeded by flooding with a mycelium/spore suspension or the suspension is added to the cool, molten agar before dispensing into plates.[97]

8. *Claviceps purpurea*

Surface disinfest a sclerotium and place a slice of it on PDA for 7 days at 20°C, then transfer it to medium 81 without the agar and incubate on a rotary shaker for 10 days.[98]

9. *Colletotrichum* spp.

C. capsici — sporulates on medium 81 at 25 to 30°C.[99]

C. coccodes — For conidia production, cultivate the fungus on 30% V-8® juice agar, replacing $CaCO_3$ with celite, at pH 4.5 in continuous light at 23°C.[100] For sclerotia production, cover the surface of PDA culture plates with uncoated, washed, and autoclaved cellophane. Place inoculum on the cellophane and after 1 to 2 mon, scrape off the sclerotia, grind them in water containing fine mesh sand, and then wash through a 150- to 250-μm sieve.[101] Sclerotia also can be produced on medium 147 (30% V-8® juice, 3% agar, pH 4.6). Incubate in the dark for 2 mon at 22 to 25°C.[102] Sclerotia for soil infestation can be produced on medium 136.[103]

C. fusarioides — Grow on 20 ml of medium 154 in culture plates at 25°C until the fungus has covered the medium, then cover the cultures with alluvial soil (pH 8.0) to 1 cm depth. Moisten the soil to about 30% moisture. Place the covered plates on a laboratory bench in natural diffused light. After 3 to 4 days remove the plate cover. Basidiospore formation generally begins 3 to 4 days after removing the cover. Keep the soil moist by adding water when necessary.[104]

C. gloeosporioides — Sporulates well on media 95 and 96 under NUV (12-hr day),[105,106] but some isolates sporulate on PDA.[107] Conidial inoculum of *C. g. var. aeschynomene* can be obtained by culturing on pea juice agar (400 ml fluid from commercially canned, salt free peas; 20 g agar and 600 ml water). The medium should be seeded with conidia and incubated under light (12-hr day).[108,109] Media 95 and 96 also support sporulation of *C. graminicola* when incubated under light.[110]

C. lindemuthianum — Abundant conidia were produced on media 10a[111] and 91.[112] For large-scale production cultivate it on a mixture of autoclaved barley, oat, and wheat grains (1:1:1: w/w/w) in Erlenmeyer flasks seeded with a concentrated spore suspension cultivated on medium 10a and incubate for 7 days at 23°C. To harvest conidia, add sufficient water to each flask and shake; strain the suspension through cheesecloth.[111]

C. musae — Conidia were produced on medium 58 after 30 days at 27 to 30°C.[113]

C. orbiculare — Conidia were produced when grown on 2- to 4-cm long pieces of autoclaved green bean pods for 5 to 7 days at 27°C.[114] Culturing on medium 61 also yields a large number of conidia after 7 to 10 days at 24°C.[115]

C. trifolii — To produce conidia, trim bean leaves to fit the bottom of culture plates, press dry, and place one on filter paper in a culture plate and autoclave in a paper bag. After autoclaving, pour 30 ml of autoclaved medium 147 without agar, replacing $CaCO_3$ with 15 ml

of 0.1 *N* NaOH/l into each culture plate allowing the leaf to float. Seed the leaf with conidia or mycelium fragments from a stock culture and incubate under light for 2 wk at 25°C. Pour off the liquid and homogenize the filter paper and bean leaves in water.[116] To produce dry inoculum for field use, inoculate susceptible 14-day-old alfalfa seedlings, which die. The dead plants covered with acervuli, conidia, and sclerotium-like structures were dried to 12% moisture at 40°C and ground into a coarse powder which was used as inoculum.[117]

C. truncatum — Conidia were produced on autoclaved bean or soybean stems by placing the stems in wide culture tubes filled with water and autoclaved for 40 to 60 min. After cooling, most of the water was poured off and the stems seeded at various points with mycelial fragments and incubated under light for 4 to 6 days at room temperature. Good sporulation also occurs on PDA when incubated under light. Sclerotia producing isolates may be grown on PDA in the dark for 3 wk at 28°C.[118]

10. *Crinipellis perniciosa*

There is lack of consistency in inducing basidiocarp information; the following methods are useful. Add 150 ml of 2% water agar in a 150 ml flask. Place a 10-cm long section of witch's broom material from cocoa, half submerged in the agar and autoclave. After solidification seed each plate with 2-wk-old PDA culture disks. Incubate under fluorescent light (12-hr day) at fluctuating temperature (23 to 27°C). Basidiospore formation begins after 7 wk on the witch's broom material. Mature basidiocarps may release basidiospores for about 1 wk after expansion of the pileus.[119] Basidiospores can be produced without using witch's broom material. Seed 20 ml of the medium 79 in 50 ml flasks with a PDA culture disk. Incubate in the dark for 3 wk at 25°C and then transfer to natural light at ambient temperature. Sporulation may occur in 6 to 8 wk. Natural illumination and fluctuating temperature are important since no sporulation occurs in the dark at a constant temperature.[120]

11. *Cryphonectria parasitica*

Perithecia were produced by compatible strains on surface-disinfested stem segments of *Castanea dentata* placed on moist sand.[121] The method does not eliminate contamination from wild strains. To produce perithecia in axenic culture, cut fresh *C. dentata* stems 3 mm or less in diameter into 8-cm segments, split in half and place cut side down in a culture plate with one segment per plate, then autoclave for 30 min.[121] Pour medium 44 cooled to 40°C around the stem segments to 5 mm deep. Seed the medium on either side of the segment with a 7-day-old PDA culture disk from each mating type grown on PDA containing 1 µg/ml biotin and 100 µg/ml DL-metheonine. Incubate under cool white fluorescent light under a 16-hr day for 10 to 12 days at 28°C. When stroma with pycnidia exuding conidia are formed on the bark, flood with sterile water, repeatedly brush off the conidia from the surface of stroma and pour off the suspension. Reincubate under light with an 8-hr day at 25°C. Perithecia are formed in 10 to 15 days.

12. *Cylindrocladium* spp.

C. crotalariae — Microsclerotia are produced on medium 81 without agar after 6 to 8 wk at room temperature. Homogenize the culture mat in a blender and pass the suspension through a series of 246-, 149-, 104-, and 74-µm sieves at tandem. Sclerotia of different sizes are collected on the sieves, which are washed and collected in beakers and rinsed with distilled water to remove nutrients and mycelial fragments.[122]

C. quinqueseptatum — Cut 8- to 10-cm long pieces of 5- to 10-mm thick twigs of anona, cashew or clove. Slightly injure the bark. Place three to four pieces in a 250 ml flask containing

10 ml distilled water and autoclave. Inoculate the twigs by placing actively growing mycelium bits on injured areas. Incubate at room temperature. Perthecia may be formed in 7 to 10 days.[123]

13. *Cytospora* spp.

C. cincta — Pycnidia are produced by adding 5 ml of a dense conidial suspension in sterile water to 30 ml of the honey-peptone solution from medium 68. Mix this with pearl barley, shake vigorously, and incubate at room temperature. Break up the clumps each day by shaking and then on days 3 to 5 add sufficient honey-peptone solution to keep the barley seeds moist. Pycnidia are produced in 2 to 3 wk and the spores are released when cultures are flooded with water.[124]

C. leucostoma — It produces pycnidia on medium 75 in 2 to 3 wk.[125]

14. *Dothistroma septospora*

Seed culture plates of medium 81 at pH 6.2 with a concentrated spore suspension and incubate for 10 to 12 days at 20°C. Sporulation decreases with an increase in malt extract and a decrease in spore concentration in the seeding suspension; there should be 50 to 100 spores/mm^2 of agar surface.[126]

15. *Drechslera* spp.

Most *Drechslera* sporulate under 12-hr days on medium 139.[127,128] Conidia are obtained by leaching mature colonies on PDA under running tap water for 48 hr, then incubating for 2 wk at room temperature.[127]

The *Drechslera* state (*Bipolaris zeicola*) of *Cochliobolus carbonum* sporulates well on medium 73.[129] Large quantities of conidia can be produced on medium 10. Seed the medium in plates with a spore suspension and incubate under light at 25°C. After 2 wk, remove the plate cover and incubate in a drying chamber at 32 to 35°C.[130]

The *Dreschlera* state (*Ophiobolus heterostrophus*) of *Cochliobolus heterostrophus* can be produced on medium 10 following the technique used for *C. carbonus*.[130] If dry spores are required, using medium 34, remove the cover after sporulation, let dry and collect conidia with a cyclone separator. Conidia are produced on filter paper by first growing the fungus on medium 49 containing 20 μg/ml $FeSO_4 \cdot 7H_2O$, then macerating the mycelium in a blender, washing it and resuspending the mixture in 0.1 *M* phosphate buffer at pH 6.4. Spread portions of the suspension on dry filter paper in culture plates and after sporulation dry and harvest conidia with a cyclone separator.[131] Medium 32 is a defined medium for sporulation and vegetative growth of *C. heterostrophus*. On complete medium, mycelial growth is unaffected between pH 5.4 and 7.0, but sporulation is best at pH 6.0 and below 25°C. NUV stimulates more sporulation than fluorescent light.[132] Perithecia can be produced by seeding a naturally senescent autoclaved maize leaf (1 × 4 cm) on opposite ends with culture disks taken from the colony edge of sexually compatible isolates. The maize leaf is placed on the surface of medium 127 and incubated in the dark for 18 days at 25°C.[132]

The *Drechslera* (*Bipolaris oryzae*) state of *Cochliobolus miyubeanus* sporulates on media 126[133] or 120[134] incubated under NUV (12-hr day) at 27°C. To induce perithecia in the *Drechslera* state of *C. sativus* disinfest cereal seeds in a mixture of 0.5% NaOCl and 0.1% $HgCl_2$ for 5 min, then boil them in sterile distilled water for 1 min, rinse in two changes of sterile distilled water, and place six seeds, well spaced, in a culture plate and add medium 127 until the seeds are half immersed.[128] Inoculate each seed with a drop of a conidial suspension of each compatible strain and incubate at high humidity in the dark for 3 wk. Dry barley straw soaked in water for 2 hr and sterilized with propylene oxide can be used instead of maize

kernels. The culms from fresh barley straw are cut into 5-cm pieces, placed in culture plates and fumigated before inoculation.[135]

Drechslera graminea can be induced to sporulate on media 38 or 111 incubated for 7 days at 6°C followed by 5 days at 28°C.[136] When cultured on medium 148, incubate in the dark for 4 days at 25°C and then under NUV for 24 hr. Check for conidiophore formation, if only a few are present, irradiate for another 12 hr and incubate for 24 hr at 20°C. Besides conidia, spermogonia/pycnidia may be present. Unclarified V-8® can be used in the medium, however, clarification helps reduce aerial mycelium and increase conidiophore development.[137] Culturing on PDA amended with 2% hot water extract from rice straw and incubating under light also induces sporulation.[138]

The *Drechslera* state (*Exserohilum turcicum*) of *Setosphaeria turcica* sporulates well on medium 73.[129] Sporulation can be induced by culturing on oat hulls plus a small amount of maize meal in culture plates. When growth is visible, transfer oat hulls to PDA in culture plates. Sporulation occurs in 3 wk.[139]

Sporulation in *D. teres* is induced by growing on medium 144. When the colony reaches the edge of the plate, scrape off the aerial mycelium and cut the colony into 5 mm^2 blocks. Place the blocks, upside down, on a moist filter paper in culture plates. Incubate in the dark for about 5 days at 21°C.[140] The fungus also sporulates on PDA containing 80 µg/ml benomyl.[141] Abundant sporulation also can be achieved on barley seedling leaves. Harvest the first leaves of 14-day-old seedlings and discard the distal 5 mm portions. Place the leaves on sterile filter paper moistened with a 80 µg/ml benzimidazole solution. The leaves can be held in place with segments of 80 µm/ml benzimidazole water agar. Inoculate leaves at three or four points with droplets of a spore suspension and incubate under NUV (12-hr day) at 17°C.[140] *Bipolaris sacchari* is grown in 2 l screw cap jars or Erlenmeyer flasks containing 250 ml of medium 50.[142] Each container is seeded with 10 ml of a mycelial suspension and after 4 days, when the agar is covered with dense mycelial growth, shaken to break up the mycelium and reincubated for 10 days. Homogenize in a blender and use the suspension as inoculum.

16. *Diplocarpon earlianum*

Use medium 62.[143]

17. *Elsinoe veneta*

Conidia are produced in a three-stage system. Grow the fungus on medium 36 at 20°C until it produces mircosclerotia and conidia sufficient to seed other cultures. Cut 5-mm disks and agitate them in 5 ml sterile water. Spread 50 µl drops of the suspension on medium 39 amended with 5% v/v complete supplement of nutrients and vitamins from medium 133. After 14 to 21 days cut the culture disks and float them with mycelium side upwards in about 30 ml sterile distilled water in culture plates and incubate overnight in the dark at 25°C. Remove the conidia from the disks under water by brushing with a small brush.[144]

18. *Exserohilum* spp. (see *Drechslera* spp.)

19. *Fomes durissimus*

Grow on medium 81 in large flasks for 30 days, then aseptically place sterilized host sapwood blocks (5 × 2.5 × 0.8 cm) over the culture. Incubate in diffuse light at room temperature and at intervals, spray sterile water on the blocks. Fruiting structures mature in about 8 mon.[145] *F. igniarius* var. *populinus* can be cultured on medium 84 for 30 to 40 days at 27°C.[146]

20. *Fusarium* spp.[147–152]

Media used for general cultivation are 96, 111, and 117. Media 69 and 117 induced sporulation in most species.[152] Cultivation on medium 111 favors mycelial growth over sporulation. Conidia often are misshapen and atypical. The chances of mutation are enhanced. Therefore, for identification purposes cultivation on this medium is not recommended.[153] However, when necessary to use, it should be prepared from raw material instead of using commercial preparations, and avoid red skin potatoes. Best identification is carried out on medium 15 that favors sporulation rather than mycelial growth. Carnation leaves of this medium can be replaced by leaves of maize, wheat, and other grasses, keeping the sterilization and handling procedures as described.[153] Medium 109 is useful for identification of *Fusarium* in the 'Liseola' section. Sporulation and pigmentation are favored by fluctuating temperature (25°C day/20°C night) or when incubated in diffused day light from north window.[153] Perithecia formation is enhanced with the use of natural substrates,[154] particularly host tissue for pathogenic species, but substrates and environmental conditions that favor formation vary between species. Typical substrates include carnation leaf pieces, oat hulls, rice or wheat straw, and various cereal seeds using UV light and a temperature similar to that under which perithecia form in nature.[150]

Isolates of *F. roseum* 'graminearum' Group 2 form perithecia on medium 111 and on sterile nodal wheat straws standing in water in flasks; *F. roseum* 'heterosporum' on sterile wheat straw; *F. moniliforme* and *F. moniliforme* 'subglutinans' on moist wheat chaff and PDA; and some isolates of *F. solani* on PDA or 30% V-8® juice agar.

Although methods to induce chlamydospore formation generally are based on the conversion of macroconidia, the soil agar (250 g sieved soil, 7.5 g agar in 500 ml water, autoclave and agitate well before dispensing into culture plates) induces chlamydospore formation in most *Fusarium*. Seed the plates with 1-cm culture disks from medium 15 or other suitable medium. Growth is sparse and chlamydospores are formed adjacent to the original inoculum disk.[155] To produce chlamydospores of *F. oxysporum* f. sp. *batatas* macroconidia are harvested from a PDA culture, washed with sterile distilled water, concentrated by centrifugation or with a bacteriological filter, and then suspended in sterile distilled water for 20 to 30 days at 24 to 28°C.[156] A small disk of a PDA culture of *F. solani* placed in a culture tube with distilled water produces chlamydospores in 10 days.[157] High yields are obtained using a soil extract instead of distilled water, prepared by mixing 1 kg soil with 1 l of water, allowing it to stand for 24 to 48 hr, then filtering the supernatant through glass wool and then a bacteriological filter. The macroconidia are washed with distilled water, concentrated, and 1 ml of the suspension is added to 15 ml of the soil extract in culture plates and incubated for 4 to 6 days at room temperature. For *F. solani* f. sp. *pisi* macroconidia from a PDA culture are washed with sterile distilled water, mixed with 40 ml of potato-dextrose broth and incubated on a rotary shaker for 48 hr at room temperature. Germinating macroconidia are collected by centrifugation or filtration, washed with distilled water, suspended in 40 ml of a soil extract, and incubated for 7 days on a shaker. Chlamydospores are collected, washed in distilled water, and placed in a blender at low speed to break up aggregates.[158]

For *F. solani* f. sp. *pisi* and f. sp. *phaseoli* two methods were described.[159] Conidia are washed from 2- to 3-wk-old PDA cultures with water. A Czapek-Dox salt solution amended with 0.25 or 2% sucrose and 0.075 or 0.3% $NaNO_3$ and 0.5 or 1% nonsterile or radiation-sterilized soil is used as a conversion medium. Each 250-ml flask containing 100 ml of conversion medium is seeded with 10^5 per milliliter conidia and incubated on a rotary shaker at room temperature. At higher concentrations of *N* or sucrose chlamydospore formation begins after 64 hr at lower concentrations or with the use of nonsterile soil time is reduced, which also increases the number of chlamydospores. The second method allows conidia to germinate for 15 min in Czapek's salt solution amended with 1% sucrose and 0.3% $NaNO_3$

on a reciprocal shaker. Then the conidia are harvested using a bacteriological filter, washed three times with nonamended Czapek's solution, resuspended at 10^5 per milliliter in flasks containing 100 ml of nonamended Czapek's solution, and then incubated on a rotary shaker.

In another method, germinating conidia, harvested after 16 to 18 hr in potato-dextrose broth by filtration, are washed with distilled water and incubated in 0.03 *M* Na_2SO_4.[160] Maximum yields are obtained after 6 days with *F. oxysporum* f. sp. *lycopersici* and f. sp. *melonis, F. roseum* f. sp. *cerealis,* and *F. solani* f. sp. *phaseoli* and f. sp. *pisi.* The addition of 1 ml 20% celery stem extract amended with 0.03 *M* Na_2SO_4 to 9 ml of conidial suspension (10^3/ml) and incubated for 7 to 14 days at 24°C stimulated chlamydospore formation in *F. oxysporum* f. sp. *apii*, f. sp. *batatas*, f. sp. *lycopersici*, f. sp. *marmodicae*, f. sp. *niveum*, and f. sp. *spinaciae*. With the exception of f. sp. *spinaciae*, light inhibited chlamydospore formation in other *forma speciales*. To prepare celery stem extract, autoclave 20 g chopped stem in 100 ml water.[161]

For *F. sulphurum*, conidia are produced on medium 117 at 25°, harvested, and washed with distilled water, concentrated by centrifugation (300 G for 5 min) or filtration, then resuspended in medium 88 at 5×10^5 per milliliter and incubated for 3 to 6 days at 37°C.[162] For *F. oxysporum* f. sp. *elaeidis*, grow it on 9% malt extract and 1.2% agar under NUV light (18-hr day) for 14 days at 27°C, flood cultures with distilled water, scrape with a scalpel, cut into small pieces, and filter the entire suspension through lens paper on a Buchner funnel. The filtrate is mixed immediately with talc at 2 g/ml, the paste left in trays for 3 days, then ground in a mortar with a pestle to 500 μm. After 40 days most macroconidia will be converted to chlamydospores and microconidia will perish. Dilute the sample with water and plate on a selective medium to verify population.[163]

For *F. oxysporum* f. sp. *cannabis* use a basal medium containing 800 g barley straw and 2 l of distilled water amended with 160 g of either alfalfa straw, cottonseed oil meal, or soybean meal. The other basal medium contains 40 g of barley straw and 160 ml of a mixture of 0.1% each of Na succinate, glycine and $NaNO_3$. All media are autoclaved for 15 min and inoculated with an aqueous conidial-mycelial suspension taken from 2- to 3-wk-old PDA cultures (28°C) and incubated for 3 wk at room temperature. Filter the cultures, air dry for 2 wk, and store in plastic bags for up to 6 mon at room temperature. Maximum chlamydospore production occurs on barley straw plus cottonseed oil meal followed by alfalfa straw, succinate, glycine plus $NaNO_3$ and soybean meal.[164]

Increase of mixed inoculum — Mixed inoculum of *Fusarium* is increased in liquid shake culture or on media 38, 111, 117 or 147. Optimum sporulation on agar medium is obtained when incubated under NUV or cool white fluorescent lights (12- to 16-hr day) for 2 to 3 wk. Most *Fusarium* change morphological characteristics or lose pathogenicity or sporulation when transferred repeatedly. The source of *N* or the C:N ratio in media influences sporulation, *N* can quantitatively affect sporulation, spore size, morphology, viability, and infectivity potential.[6,165]

Conventional methods for increasing *Fusarium* for routine pathogenicity tests are adequate, but not for studies in soil.[166] Methods described for specific *Fusarium* spp. may be useful for other species.

For *F. graminearum (Gibbrella zeae)*, fill 1-l flasks fitted with stoppers, with glass tubes for air inlet and outlet, with 500 ml of medium 38 without agar and sterilize. Seed each flask with a disk from a PDA culture. Bubble sterile air through the cultures or incubate on a rotary shaker for 1 wk at room temperature followed by 2 wk at stationary growth. Then homogenize the cultures and dilute to the desired concentration. Conidia also are produced on medium 31 without special light after 10 days at room temperature.[167,168] Inoculum produced on PDA is more virulent than that on cooked rye grain.[169]

F. oxysporum f. sp. *batatas* sporulates poorly on PDA, but high yields of macroconidia are obtained using medium 60. Dispense 30 ml in 250-ml flasks, autoclave and seed with a cell

suspension, then incubate under continuous light at 25 to 29°C. Harvest conidia by washing cultures with water.[156]

For *F. oxysporum* f. sp. *lycopersici*, cultivate on PDA for 7 days, then blend the culture in water.[170] Most workers cultivate it in liquid media such as 21,[171] 27,[172] or 111[173] on a shaker at 28°C. When using medium 52 incubate for 3 days with occasional shaking.[174]

For *F. oxysporum* f. sp. *pisi*, grow on medium 38 without agar (50 ml/250 ml-flask) on a reciprocal shaker for 14 days at 20°C. For increasing inoculum for field infestation use medium 27 dispensing 3 l of medium into 6-l flasks, then seeding with a culture growing on the same medium. Incubate on a shaker for 3 days at room temperature.[175] Sterilized leaves of *Dianthus caryophyllus* also are used, where, after 4 wk growth, leaf cultures are air dried at room temperature and powdered in a micromill.[176]

For *F. oxysporum* sp. *tulipae*, add a dense conidial suspension from a PDA culture to cooked rice (50 g rice plus 50 ml water per 300-ml flask, and autoclaved), then incubate for 7 to 10 days at 25°C. Grind the contents in 300 ml water per flask.[177]

For *F. oxysporum* f. sp. *cepae* grow on PDA and incubate under 12-hr day for 3 wk at 26°C, homogenize in a blender using 50 ml water per plate, then filter through four layers of cheesecloth.[178]

For *F. roseum* 'avenaceum', produce conidia on medium 14.[179]

For *F. solani*, cultivate on PDA made from fresh potatoes under light at 22 to 30°C. Large quantities for field inoculation are prepared on medium 36 cultured on a shaker for 1 wk at 24°C, the suspension then strained through a double layer of cheesecloth, and stored in a cold room until needed.[180]

For producing perithecia of *F. solani* f. sp. *cucurbitae*, grow the fungus on PDA (pH 6.2) in large culture plates for 30 days or until perithecial primorida are visible. Spray with conidia of opposite mating type. If homothallic, spray with sterile water after 20 days growth. Incubate under NUV or cool fluorescent light,[181] or grow the fungus on 30% V-8® juice agar or medium 56 (either medium at pH 5.5 prior to autoclaving) in the dark for 15 days, then spray with conidia of opposite mating type or sterile water and incubate under continuous light at room temperature.[182] The type of *N* used in a synthetic medium is the major factor determining perithecia development. Seed the medium with agar culture disks taken from actively growing margins of a colony and incubate in the dark at 20°C. When the plate is completely covered by the fungus, add 5 ml sterile distilled water and wet the entire culture by gently shaking and tilting the plate. Incubate in natural diffused light at 20 to 22°C. Perithecia may be formed in 20 days and mature ascospore begin to extrude in another 10 days.[182]

21. *Gaeumannomyces graminis*[183,184]

Perithecia are produced on Lilly-Barnett medium containing 1% glucose and 0.2% asparagin incubated in the dark for a few days at 15 to 24°C (optimum 20°C), then under light for 3 to 4 wk at the same temperature,[185] or on Lilly-Barnett medium incubated until covered with mycelium, then immersing the plates in tap water or distilled water for 48 hr, decanting the water, and reincubating under diurnal light and dark.[186]

22. *Glomerella cingulata*

Conidia are produced on medium 17, but more conidia result when it is fortified with 50 g/l wet orange peel.[187,188] Pour 30 ml of the medium in 9-cm culture plates and after solidification seed each plate by spreading 1 ml of a concentrated suspension from a young sporulating culture over the agar surface. Incubate under light (12-hr day) at 25°C or alternating 15 and 25°C.[188]

23. *Helminthosporium* spp. (also see *Drechslera* spp.)

Most species sporulate on medium 139 when incubated under alternating light and dark. The techniques used for inducing sporulation in *Drechslera* are applicable to many *Helminthosporium*. The following is useful for having a continuous source of *H. solani* inoculum, that can be stored for over a year. Grow the fungus on medium 147 (177 ml V-8® juice). When the colony is 10 mm diameter, cut 3 mm disks, transfer them to rye grains in flasks and incubate at 20 to 25°C. During the first 6 wk, shake the flasks every 3 days to prevent clumping. To prepare the grains, soak them in equal amounts of water for about 16 hr. Decant the water, then distribute the grains in appropriate quantities into the flasks. Autoclave for 30 min on 2 consecutive days. To produce conidia, transfer a few colonized grains to medium 147 or water agar in culture plates and shake so that most of the agar surface comes in contact with the grains. Incubate at 20 to 25°C.[189]

24. *Hymenula cerealis*

For infesting soil in the field or greenhouse increase inoculum on 150 g of oat grain in an Erlenmeyer flask or glass jar moistened with 100 ml water.[190] If jars are used, punch a hole in the middle of the lid and cover with filter paper. After autoclaving, seed the substrate with 10 ml of a heavy spore/mycelium suspension produced in shake culture. Shake each container to distribute the inoculum and incubate for 8 wk at 22°C, occasionally shaking the culture to prevent caking. Then spread the kernels to dry and store in the cold. Inoculum will remain infective for up to 18 mon.[190]

25. *Leptosphaeria maculans*

For sexual reproduction, make a thick suspension of dried canola (rape) roots in water and after autoclaving, dispense into culture plates. Place small pieces of culture from two mating types 2 to 3 cm apart. Incubate under continuous fluorescent plus NUV light for 5 to 7 wk.[191]

26. *Leptosphaerulina trifolii*

Ascospores are obtained on medium 147 after 8 to 10 days at 20°C. Incubate plates in an inverted position under fluorescent light.[192]

27. *Macrophomina phaseolina*

Sclerotia are obtained on medium 135 dispensed in 1 l amounts into 5- to 6-l Erlenmeyer flasks and autoclaved before seeding the medium with four to five disks of a PDA culture of the fungus and incubated for 15 days at 30°C. The culture mat is homogenized and filtered through filter paper or a 44-µm mesh screen. Sclerotia are washed several times with distilled water and dried for 24 hr at 40°C. Break up the clumps by grinding in a mortar using a pestle and screen through a 125- or 150-µm mesh sieve to separate sclerotia.[193,194] Sclerotia can be produced on PDA or water agar in culture plates by covering the agar surface with cellophane, which first is boiled in water several minutes and then autoclaved. Seed the cellophane with a culture disk and after sclerotia appear, pick off the cellophane and wash with water to remove the sclerotia.[195]

Pycnidia are produced by various methods in strains that will respond. Cut hypocotyls from 20-day-old greenhouse-grown kidney bean seedlings into 2-cm pieces and dry to 8% moisture at 90°C, then sterilize with propylene oxide for 24 hr. Place two segments on 1.5% water agar in culture plates, inoculated with fresh inoculum and after at least 24 hr in the dark

at 30°C (mycelial growth seen), place under fluorescent lights or in natural shade at 30°C until pycnidia are formed.[196] Pycnidia are induced on propylene oxide sterilized leaves (fresh or dried) of cotton or bean.[14] When fresh leaves are used, allow to wilt overnight. Trim leaves to fit culture plates and then sterilize with propylene oxide for at least 24 hr in a sealed container. Then pour 30 ml of 1% water agar at 45°C until the leaf floats. When the agar is hardened, inoculate the leaf at various points from a fresh culture of the test fungus and incubate in the dark at 30°C until mycelium is seen and then under fluorescent lights for 7 days.[14]

Pycnidia were induced on medium 80 plus an ether extract of peanut meal or cooking oil (blend of peanut and sunflower oil, "COVO").[197,198] Whatman no. 1 filter paper disks (4.5 cm diameter) are soaked in a 20% extract solution in ether and allowed to evaporate, then autoclaved and the disk placed on the agar surface. Seed the plate with the fungus at the edge of the disks. After 24 hr in the dark, incubate under NUV for 5 days at 30°C. Pycnidia also are induced on autoclaved leaf bits (2 to 3 cm long) of *Agrostis semiverticillata*, barley, maize, oats, or wheat placed on 1.5% water agar in culture plates and inoculated with mycelial disks from the margin of a 48-hr-old culture and incubated under NUV light (12-hr day) for 7 to 10 days at 20°C.[199,200]

28. *Mycoleptodiscus terrestris*

Sporulation occurs on propylene oxide-sterilized natural media such as the leaves and petioles of alfalfa, blue grass, oats, red clover, or soybean.[201] Another method uses cotton, *Serica lespedeza*, snapbean, or soybean leaves dried in a plant press and trimmed to fit loosely into culture plates.[10] The largest amount of conidia were produced on cotton leaves. Add 30 ml of 1% molten water agar buffered with 0.1% $CaCO_3$ to 9-cm culture plates floating the leaf tissue. Sterilize using propylene oxide for 24 hr in a desiccator (1 ml propylene oxide per liter of desiccator volume) at room temperature. Seed the leaf at several points 24 hr after sterilization and incubate in the dark at 27°C until the mycelium covers the leaf and then incubate under fluorescent light for 6 days. Sclerotia are produced on media 81, 111, or 147, which inhibit conidia formation.

29. *Mycosphaerella populorum* (anamorph *Septoria musiva*)

For ascomata production maintain single ascospore or conidial cultures on clarified medium 147 with 0.2% $CaCO_3$. Grow isolates on the same medium for 2 wk. Cut the sporulating mat and streak across the surface of medium 108. After 2 days seal the cultures and incubate in the dark at 8°C. Spermagonia and spermatia may develop after 2 wk. Using a sterile needle remove the spermatia produced in milky drops, and transfer to sterile distilled water. Spread 1 to 2 ml of this suspension over the receiving culture. Incubate in the dark at 8°C. Ascomata develop in about 45 days. Crosses may not be always successful.[202]

30. *Nectria* spp.

N. coccinea grows well on medium 81. For perithecia production pour 15 ml sterilized 1% malt-extract agar in 9-cm culture plates, then place on the surface a piece of beech twig previously autoclaved for 1 hr. Seed opposite ends of the twig with inoculum plugs of compatible strains grown on medium 81 in the dark for 3 wk. Seal the plates with water-proof tape and incubate under NUV (12-hr day) for 7 wk at 25°C, then for 4 wk at 15°C. Perithecia appear on the twig in 15 wk.[203]

N. cosmariospora is cultured on medium 118. Some strains are biotin deficient. For perithecia production grow a mixture of two mating types on oatmeal agar (30 g oatmeal/l)

at pH 8 in the dark for 5 days at 25°C, then in the laboratory exposed to daylight for 3 to 5 wk. Perithecia also are induced when cultures of one thallus are sprayed with conidia from a compatible strain.[204]

N. galligena — Old cultures do not produce perithecia. Perithecia formation by single ascospore cultures was reported.[205] Perithecia are produced on an autoclaved mixture of 70 g of 1:1 mixture of oat and whole grain wheat in 1 l of 1% agar or 70 g powdered bark from yellow beech (*Betula alleghaninsis*) in 1 l of 1% water agar. After seeding, both media are incubated in a moist chamber exposed to daylight at room temperature. High moisture and diurnal light are needed. More perithecia are produced on grain than bark medium.[205]

31. *Neovossia indica*

It grows well on medium 38 amended with 10 g/l wheat germ, wheat embryo, or wheat seedlings. Good sporulation also occurs on medium 146 incubated for 15 days at 20°C.[206]

32. *Oncobasidium theobrome*

Basidiospores lack dormancy, germinate only in free water, are susceptible to desiccation, and lose viability after discharge if conditions are not favorable for germination. To collect basidiospores without losing viability, gather infected cacao stems bearing sporophores during the day, moisten and store under humid conditions in the laboratory, then in early evening lay them across an open culture plate containing 38% sucrose and 2% agar overlaid with disks of boiled cellophane so that the basidiospores are shed onto the cellophane. Place the plates on a tray with a damp cloth on the bottom, enclose the trays in polyethylene bags, and place out-of-doors overnight. Bring the trays indoors before sunlight reaches the trays and cover the plates with their lids and store in the dark for 12 hr, then wash the spores from the cellophane, centrifuge at 140 G for 5 min, discard a portion of the supernatant, determine spore concentration, and then dilute to the desired concentration.[207]

33. *Ophiostoma ulmi*

Hetero- and homothallic strains occur. Cultures should be grown on medium 59 or 86; for some strains yeast extract may be substituted for biotin. Cultures first are incubated for 6 days at 25°C and then spermatized with a conidial suspension of a compatible type. For higher yields of conidia use liquid medium 59.[208,209] For *O. ulmi* mycelial inoculum production, cultivate on medium 24 and incubate for 5 to 7 days at 25°C. A conidial suspension for spermatization is produced on the same medium without agar by incubating on rotary shaker. For perithecia production the receptive isolate should be grown on medium 42 or 43 for 2 wk at 25°C. Spermatize with 2 ml of the conidial suspension from a compatible isolate. Incubate in the dark for at least 3 wk.[210]

34. *Phoma* spp.

For *P. lingam*, steam pea seeds in equal volumes of water on 3 consecutive days for 30 min or autoclave for 30 min at 110°C, inoculate with culture disks from the margin of 10-day-old cultures, and incubate in the dark at 22°C or under fluctuating temperatures of 4 to 21°C in the laboratory; the latter results in greater sporulation in 10 to 12 days. Pycnidia begin to exude conidia within 30 to 60 min after flooding the culture with sterile water.[211]

For *P. medicaginis* autoclave seeds of barley, oat, soybean or wheat on 2 successive days in an equal volume of water, shaking to prevent caking. Inoculate by pouring in a portion of