

## Carnation Leaves as a Substrate and for Preserving Cultures of *Fusarium* species

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### ABSTRACT

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Carnation leaf pieces, sterilized with gamma irradiation, in a water agar medium (carnation leaf agar) promoted good growth, sporulation, and maintenance of the original cultural type of *Fusarium* spp. Isolates to be preserved were grown from single conidia on carnation leaf agar for 7-10 days. Several colonized carnation leaf pieces were then transferred from the plate to 5-ml vials and covered with sterile skim milk. The vials were loosely

stoppered, placed in a tray, quickly frozen with liquid nitrogen, lyophilized, then tightly stoppered, and stored at -30 C. Viability of lyophilized cultures has been 100% since 1978, and cultural variation minimal. The critical components of the procedure are the initial culturing, use of carnation leaf agar, and lyophilization of cultures of appropriate age.

*Additional key words:* *Dianthus caryophyllus*.

The identification of *Fusarium* species is often difficult, being based primarily on the morphology of asexual spores. Therefore, it is important that conidia be well shaped and uniform in appearance. The growth and sporulation of *Fusarium* spp. is highly dependent on culture conditions. One of the most critical factors is the medium used to grow the cultures. A medium rich in carbohydrates, such as potato-dextrose agar (PDA), may delay the formation of sporodochia, the typical fructifications. Growth on PDA also may result in atypical, nonuniform, misshapen conidia (Fig. 1). Natural media were developed to alleviate these problems (9).

Several methods have been used for long-term preservation of fungus cultures (2,7,13). The appropriate selection and success of a preservation method varies with the fungal species being preserved, the length of time the cultures of the fungi remain viable when preserved by that method, the size and characterization of the collection, and the size of the staff available for culture maintenance.

The Fusarium Research Center, Department of Plant Pathology, The Pennsylvania State University, maintains a collection of approximately 5,000 isolates of *Fusarium* spp. The majority of *Fusarium* spp. isolated from nature produce macroconidia on sporodochia. The original sporodochial culture type (wild-type) often varies or mutates in culture, a change frequently accompanied by a loss in plant pathogenicity (11). It is, therefore, important to maintain the original cultural type when the isolates are preserved.

Cultures stored at the Fusarium Research Center were originally maintained on PDA. The possibility of rapid cultural variation on this carbohydrate-rich medium necessitated frequent culture renewal.

Soil storage (11) has been used by many workers, but not all *Fusarium* spp. remain viable in soil. Isolates from tropical regions and *Fusarium nivale* were not viable in soil storage (5). Isolates from temperate regions varied in the length of time they remained

viable in soil. For instance, *Fusarium roseum* 'Avenaceum' remained viable in soil for 1-2 yr, while *Fusarium oxysporum* remained viable in soil for 8-10 yr. Cultural variation also occurred in soil storage, but not as frequently as on PDA. Owing to the number of isolates in the collection, the time involved in checking soil cultures for viability, and its unreliability for preserving all *Fusarium* spp., the soil storage method was not practical for preservation of the Fusarium Research Center culture collection.

Lyophilization is an effective method for preserving fungal cultures (1,8).

The purpose of this paper is to describe the procedure currently in use at the Fusarium Research Center for growing and preserving cultures of *Fusarium* spp., and to summarize the improvements made in the system and its components during the past 5 yr.

### MATERIALS AND METHODS

**Carnation leaves as a substrate.** Young carnation (*Dianthus caryophyllus* L.) leaves were harvested from actively growing, disbudbed plants free from pesticide residues. The leaves were cut into pieces ~5 × 5 mm and dried in an oven at 45-55 C for ~2 hr. When properly dried, the leaf pieces were green and crisp; loss of the green pigmentation indicated that the drying temperature had been too high. The leaf pieces were then placed in aluminum canisters (5 cm deep and 9 cm in diameter) and sterilized with 2.5 megarads of gamma irradiation from a Cobalt 60 source. Propylene oxide fumigation was used as an alternate method of sterilization (3,11), but sterilization of the green leaves was not as thorough as with gamma irradiation and required repeated fumigation.

Carnation leaf agar (CLA) was prepared by placing several sterile leaf pieces in a petri dish and floating them on 1.5-2.0% water agar cooled to 45 C. The dishes were left at room temperature for 3-4 days before use to allow the growth of possible contaminants from the leaf pieces.

**Preservation of cultures.** Isolates to be lyophilized were grown on CLA in petri dishes for 7-10 days, and then checked for adequate growth and lack of bacterial contamination. Bacterial contamination was assayed by observation of a slide mount of each culture under the microscope, or by growth in tryptic soy broth (Difco Laboratories, Detroit, MI 48201). Each lyophilization run

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was prepared under sterile conditions in a transfer chamber. Several colonized carnation leaf pieces were transferred to each of five replicate sterile 5-ml vials labeled with the isolate number. A 0.5-ml aliquot of sterile skim milk (Difco Laboratories, Detroit, MI 48201) was added to each vial. The vials were loosely stoppered with split rubber stoppers, which allowed for evacuation of air. The stoppered vials were placed in a tray and quick-frozen by pouring liquid nitrogen into the tray. A Lucite plate slightly larger than the tray was placed on top of the partially stoppered vials. A VirTis drying chamber (Model 10-MR-SA, The VirTis Co., Gardiner, NY 12525) on a refrigerated freeze-dryer was used for lyophilization. The tray was placed on the precooled (-35 C) shelf in the drying chamber. After 10 min, vacuum was pulled in the chamber and maintained at a reading of 10  $\mu$ m Hg on a McLeod gauge. Shelf refrigeration was then turned off and the shelf heat was turned on to 15 C for 16-20 hr, while the samples dried gradually.

After lyophilization, the vials were sealed under vacuum by inflation of a rubber diaphragm in the chamber over the tray, which pressed down on the Lucite plate, and forced the rubber stoppers to seal the vials.

## RESULTS AND DISCUSSION

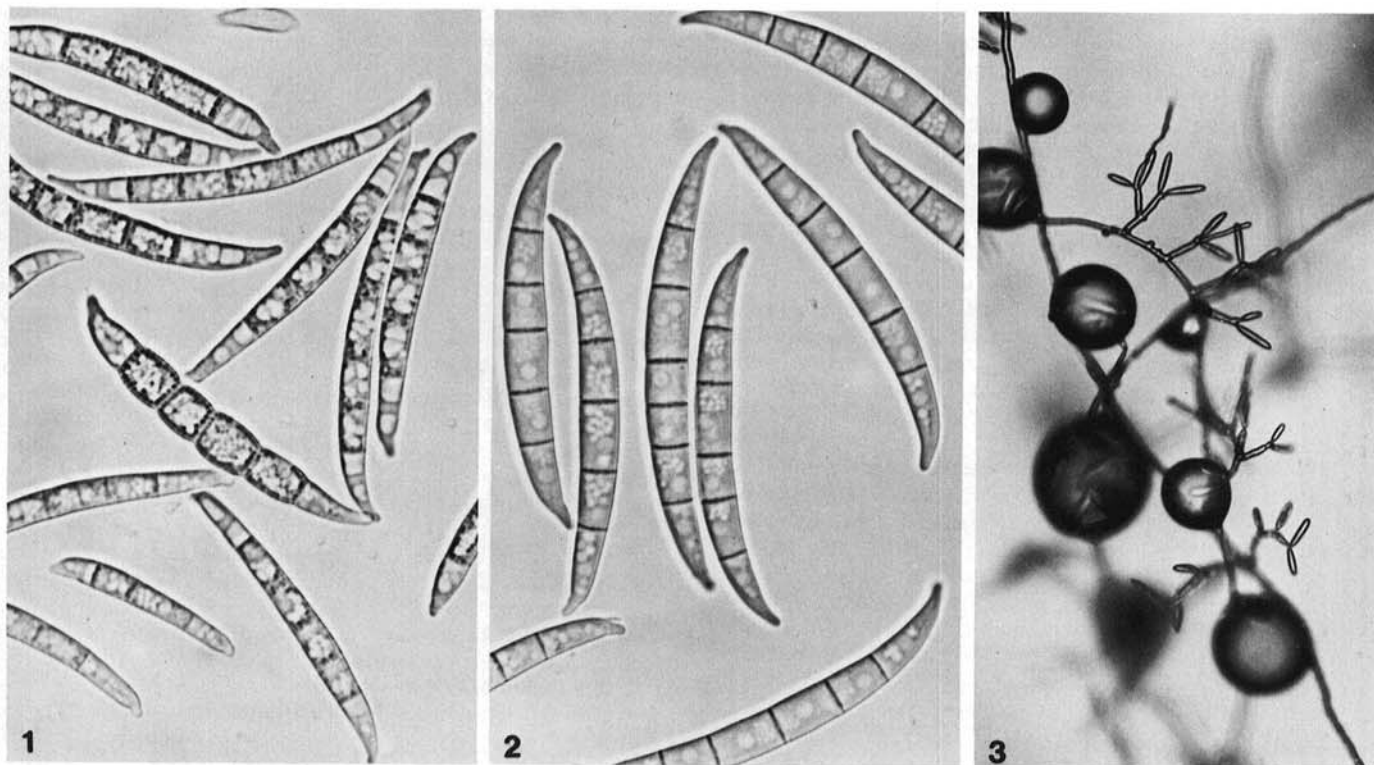
**Carnation leaves as a substrate.** Most *Fusaria* sporulate on CLA in 5-10 days (10,11). Sporulation and growth are also dependent on conditions of light and temperature, which should be standardized. The *Fusarium* Research Center uses a 12-hr alternating cycle of light and dark at a temperature of 20-22 C (11). Carnation leaf pieces promoted good growth, sporodochium formation, and the production of uniform conidia of typical morphology suitable for microscopic observation and identification of *Fusarium* spp. (Fig. 2). In addition, conidia produced on a medium rich in carbohydrates, such as PDA (Fig. 1), often gave rise to cultures of variant morphology such as pionnotal and mycelial types, whereas spores produced on CLA normally maintained the original sporodochial morphology. Therefore, although cultures were originally maintained on PDA slants, that medium is no longer used by us for successive transfers or storage of cultures.

Direct observation of a culture of *Fusarium* on CLA under a compound microscope also indicates the manner in which conidia are borne on conidiophores (Fig. 3). This characteristic is important in the identification of some *Fusarium* spp. Perithecia of the perfect states of homothallic *Fusarium* spp. also are formed readily on CLA under favorable conditions of light and temperature (Fig. 4) (12).

**Preservation of cultures.** The study of storage of *Fusarium* spp. as lyophilized cultures has been under way in the *Fusarium* Research Center since 1973. Initially, *Fusarium* spp. were lyophilized in a bovine-serum suspension of conidia prepared from PDA slants. The viability of 600 *Fusarium* isolates in bovine serum was highly variable after 1.5 yr. Viability was checked by placing lyophilized cultures on CLA or PDA and observing them for growth for 14 days. Results showed that only 20% of the isolates from tropical areas and isolates of *F. roseum* 'Avenaceum' remained viable while 80% of the isolates of *F. oxysporum* and *F. solani* were still viable after 1.5 yr. These cultures were not quick-frozen with liquid nitrogen. Rather, the tray was placed on a precooled shelf (-35 C) in the drying chamber and gradually frozen for 30-60 min before vacuum was applied and freeze-drying carried out.

Due to the failure of bovine serum as a cryoprotective substance, isolates were lyophilized using *Fusarium* spp. grown on carnation leaf tissue. In this case, the carnation leaf was considered to protect the isolates that had colonized the tissue. Colonized leaf pieces were scraped from the surface of CLA, put in vials, and gradually frozen on the shelf (quick-freezing with liquid nitrogen was not used) and lyophilized. A total of 3,000 cultures preserved in this manner showed initial viability when grown on PDA or CLA, but viability decreased under long-term storage at 5 C. Viability of cultures lyophilized in this manner was 80% after 3 yr and 70% after 4 yr when checked for growth on CLA or PDA. The loss of 20-30% of the isolates in our collection was not acceptable.

In 1977 a procedure was tested using colonized carnation leaf pieces with milk as an additional cryoprotective substance. Isolates were grown on PDA slants, and a conidial suspension in water was added to vials containing sterile leaf pieces and capped and placed under lights for 5 days. Following colonization of the leaf material,



Figs. 1-3. 1, Conidia of *Fusarium graminearum* produced on a culture grown on potato-dextrose agar ( $\times 950$ ). 2, Conidia of the same isolate as in Fig. 1, but from a culture grown on carnation leaf agar ( $\times 950$ ). 3, Conidia and conidiophores produced on carnation leaf agar ( $\times 210$ ).

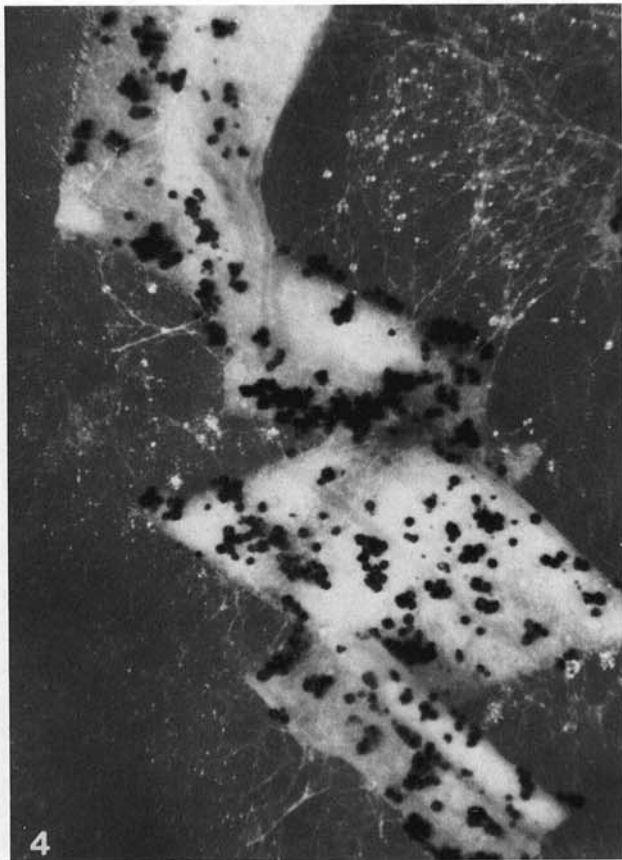


Fig. 4. Perithecia of *Gibberella zeae* produced on carnation leaf agar ( $\times 8$ ).

0.5 ml of sterile milk was added. Samples were quick-frozen with liquid nitrogen, put on the shelf at  $-35\text{ C}$ , and after several minutes, vacuum was pulled in the chamber. After the vacuum stabilized at a reading of  $10\ \mu\text{m Hg}$  on a McLeod gauge, refrigeration was discontinued and heat of  $27\text{ C}$  applied to the shelf to facilitate drying. This method resulted in excellent viability of approximately 2,000 isolates (close to 100% viability of isolates assayed over a 3-yr period). However, approximately 30% of the lyophilized isolates produced variant cultures such as pionnotal types. We believe this occurred because of the use of PDA as a growth medium to produce conidia for lyophilization. Therefore, the use of PDA for growth of isolates to be lyophilized was discontinued by us.

With the current procedure, utilizing CLA and sterile milk as a cryoprotective substance, the viability of 4,100 lyophilized cultures has been 100% since 1978 when checked for growth on CLA or PDA, and cultural variation has been less than 5%. The most important components of the procedure are the initial culturing, use of CLA, and lyophilization of cultures of appropriate age. The lyophilization of isolates grown for over 3 wk on CLA may result in the survival of variant conidia.

Isolates of *Fusarium* spp. to be maintained in the collection of the Fusarium Research Center are cultured using a standardized routine (11), stressing purity and maintenance of the original

cultural type. Single conidium cultures are grown on PDA to determine cultural morphology, pigmentation, and growth rate; and on CLA to promote production of conidia suitable for identification of the species, and for growth of isolates to be lyophilized. Carnation leaf agar slants are also used as a convenient method of short-term storage of an isolate, in the event of problems with viability or contamination during the lyophilization process.

After lyophilization, vials are capped and labeled showing the isolate number and date of lyophilization, and the vials are stored at  $-30\text{ C}$ . Recommended storage of lyophilized cultures is  $5\text{ C}$  or lower (4) and some fungus cultures are stored at temperatures as low as  $-52\text{ C}$  (6). The lyophilized pellet from one replicate vial is cultured immediately on water agar or CLA to check for viability and possible contamination.

Canisters containing sterilized carnation leaf pieces should be stored in a refrigerator until used and may be kept for 2–3 mo prior to use.

Since the fungus colonizes the leaf tissue by mycelial growth, preservation by this method may not depend solely on numbers of conidia produced. Mycelium as well as conidia may be lyophilized with the cryoprotective qualities provided by the leaf tissue and milk. We have successfully substituted natural substrates such as straw or wheat seed for carnation leaf tissue for isolates of *F. nivale* and isolates that produce few conidia. This method is being used for long term preservation of *Fusarium* in the Fusarium Research Center collection.

#### LITERATURE CITED

1. Bazzigher, G. 1962. Ein vereinfachtes Gefrier- und Trocknungsverfahren zur Konservierung von Pilzkulturen. *Phytopathol. Z.* 45:53-56.
2. Buell, C. G., and Weston, W. H. 1947. Application of the mineral oil conservation method to maintaining collections of fungous cultures. *Am. J. Bot.* 34:555-561.
3. Hansen, H. N., and Snyder, W. C. 1947. Gaseous sterilization of biological materials for use as culture media. *Phytopathology* 37:369-371.
4. Hatt, H. (editor) 1980. *Laboratory Manual on Preservation Freezing and Freeze-Drying*. American Type Culture Collection, Rockville, MD. 51 pp.
5. Jong, S. C., and Davis, E. E. 1978. Conservation of reference strains of *Fusarium* in pure culture. *Mycopathologia* 66:153-159.
6. Meyer, E. 1955. The preservation of dermatophytes at subfreezing temperatures. *Mycologia* 47:664-668.
7. Onions, A. H. S. 1971. Preservation of fungi. Pages 113-151 in: C. Booth, ed. *Methods in Microbiology*. Vol. 4. Academic Press, New York. 795 pp.
8. Raper, K. B., and Alexander, D. F. 1945. Preservation of molds by the lyophil process. *Mycologia* 37:499-525.
9. Snyder, W. C., and Hansen, H. N. 1947. Advantages of natural media and environments in the culture of fungi. *Phytopathology* 37:420-421.
10. Tio, M., Burgess, L. W., Nelson, P. E., and Toussoun, T. A. 1977. Techniques for the isolation, culture and preservation of the *Fusaria*. *Australian Plant Pathol. Soc. Newsl.* 6:11-13.
11. Toussoun, T. A., and Nelson, P. E. 1976. *A Pictorial Guide to the Identification of Fusarium species*, 2nd ed. Pa. State Univ. Press, Univ. Park. 43 pp.
12. Tschanz, A. T., Horst, R. K., and Nelson, P. E. 1975. A substrate for uniform production of perithecia in *Gibberella zeae*. *Mycologia* 67:1101-1108.
13. Weiss, F. A. 1957. Maintenance and preservation of cultures. Pages 99-119 in: H. J. Conn, ed. *Manual of Microbiological Methods*. Soc. Am. Bacteriol. 315 pp.