

Survival and Pathogenicity of *Ascochyta fabae* f. sp. *lentis* in Lentil Seeds After Storage for Four Years at 20 to -196 C

WALTER J. KAISER, Research Plant Pathologist, Western Regional Plant Introduction Station, USDA-ARS, Washington State University, Pullman 99164-6402; PHILLIP C. STANWOOD, Research Agronomist, USDA-ARS, National Seed Storage Laboratory (NSSL), Colorado State University, Ft. Collins 80523; and RICHARD M. HANNAN, Horticulturist, Western Regional Plant Introduction Station, USDA-ARS, Washington State University, Pullman 99164-6402

ABSTRACT

Kaiser, W. J., Stanwood, P. C., and Hannan, R. M. 1989. Survival and pathogenicity of *Ascochyta fabae* f. sp. *lentis* in lentil seeds after storage for four years at 20 to -196 C. *Plant Disease* 73: 762-764.

Survival of *Ascochyta fabae* f. sp. *lentis* in naturally infected lentil (*Lens culinaris*) seed was studied over 4 yr at temperatures of 20, 5, -18, and -160 to -196 C (liquid nitrogen). The pathogen was isolated from 52 to 78% of the infected seeds during the experiment. There was an 18-25% increase in the incidence of seedborne *A. f. f. sp. lentis* between the third and fourth years at all temperatures. Incubation of infected seeds for 4 yr at 20 to -196 C did not adversely affect the pathogenicity of the fungus to lentil. Germination of infected seeds was appreciably lower than that of healthy seeds at all temperatures on each sampling date (1 day, 1, 2, 3, and 4 yr). There was a significant reduction in germination of infected, but not of healthy, seeds at each temperature over the 4-yr period.

There has been an enormous increase in the international exchange of plant germ plasm in the last two decades (16,17). There are over 2.5 million accessions of cereals, pulses, root crops, vegetables, forages, and industrial crops in gene banks worldwide (17). Most of this germ plasm is propagated by true seeds (15), and many plant pathogens are associated with this germ plasm. Seed transmission is important in the spread and survival of different plant pathogens and in disease epidemiology (2). Most of the seeds in gene banks are stored at temperatures ranging from 4-6 to -18 C and at relative humidities of <50%. For many plant species, these storage conditions prolong the longevity of seeds and associated seedborne pathogens (8,12-14,18). Gene banks may serve as important reservoirs of many seed-transmitted pathogens (5,7,12,13,16,17).

The lentil (*Lens culinaris* Medik.) germ plasm collection in the United States, which currently numbers over 2,100 plant inventory (PI) accessions, is maintained at the USDA Western Regional Plant Introduction Station in

Pullman, WA. In 1986, Kaiser and Hannan (13) isolated *Ascochyta fabae* Speg. f. sp. *lentis* Gossen et al (= *A. lentis* Vassiljevsky) (6) from original seeds of 46 PI lentil lines introduced from both new- and old-world countries. The incidence of infection ranged from <1 to >60%. *A. f. f. sp. lentis* was the most prevalent and potentially important pathogen isolated from these seeds. The fungus was isolated from seeds of several lentil accessions that had been in cold storage since 1948.

Little information is available on survival of pathogenic fungi in plant tissues, including seeds, stored at sub-freezing temperatures. The objectives of this study were to examine the effect of storage temperatures (20, 5, -18, and -160 to -196 C) over a 4-yr period on 1) germination of healthy lentil seeds and those infected with *A. f. f. sp. lentis*, 2) survival of the fungus in infected lentil seed, and 3) pathogenicity of isolates of the pathogen to lentil.

MATERIALS AND METHODS

Seed source. Seeds of lentil PI 436518 from Turkey were harvested from healthy plants and from those naturally infected with *A. f. f. sp. lentis* at Central Ferry, WA. Seeds were sized by passing through metal screens with round holes graduated in 0.8-mm increments, and only those retained by the 4.0-mm screen were used. Seeds were sent by mail from Pullman, WA, to the National Seed Storage Laboratory (NSSL), Ft. Collins, CO, where they were stored at laboratory conditions (20-23 C, 10-30% relative humidity) for several days before preparing them for the experimental

storage conditions. Initial (day 0) germination was determined before sending the seeds to the NSSL for storage treatments.

Seed germination tests. Seeds were tested for viability by planting four replicates of 50 seeds each in rolled paper towels moistened with tap water. The towels were placed in a germination chamber operating on a 12-hr photoperiod at 20 C. The seeds were evaluated for germination using Association of Official Seed Analysts (AOSA) rules (1). Final germination counts were made after 10 days and the incidence of normal and abnormal seedlings was recorded. Data in Table 1 present only a percentage of normal seedlings. Normal seedlings possess essential structures indicative of the ability to produce plants under favorable conditions (1).

Seed moisture tests. Seed moisture content of healthy and infected seeds (wet weight basis) was determined at the beginning of the test (day 0) and on each sampling date. Triplicate samples of 10 seeds each were oven-dried for 24 hr at 103 C (20).

Seed storage. Seeds from healthy and infected lentils were stored in separate containers. Four hundred fifty seeds were placed in moisture-tight, heat-sealable aluminum foil packets. Enough packets were made to allow for subsequent testing at 1-yr intervals for approximately 4 yr. The packets were stored at 20, 5, -18, and -160 to -196 C (vapor phase to liquid phase of liquid nitrogen) (LN₂). Liquid nitrogen storage treatments cooled at approximately 20 C per min. Initially, healthy and infected seeds were placed in the liquid phase of LN₂, but after 1 yr they were transferred to the vapor phase. At specified intervals, one packet of healthy and one packet of infected seeds from each temperature were removed from storage and placed on a laboratory bench in their sealed container until they reached temperature equilibrium. The LN₂ storage treatments took approximately 30 min to reach laboratory temperature conditions. The packets were then opened and seed moisture and germination tests were conducted. The remaining seeds (approximately 200) were repackaged in moisture-tight, heat-sealable aluminum

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Table 1. Germination of healthy lentil seeds and seeds infected with *Ascochyta fabae* f. sp. *lentis* stored at 20 to -196 C for various time intervals

Temperature (C)	Germination (%) ^a									
	Healthy					Infected ^b				
	1 Day	1 Yr	2 Yr	3 Yr	4 Yr	1 Day	1 Yr	2 Yr	3 Yr	4 Yr
20	93.5	93.5	94.0	95.0	91.5	82.5	82.5	66.5	68.0	57.0 ^c
5	98.5	89.5	93.5	92.5	89.5	81.0	80.5	69.0	70.0	58.5*
-18	95.0	93.5	92.0	94.0	95.0	78.5	82.0	71.5	69.0	62.0*
-160 to -196	93.5	91.0	94.0	91.5	94.5	76.5	74.0	74.5	68.5	58.5*

^aData are the mean of four replicates of 50 seeds per replicate. Germination counts were taken after 10 days.

^bGermination of infected seeds was significantly lower than that of healthy seeds at all temperatures on each sampling date, according to the analysis of variance at $P = 0.05$.

^cThere was a significant reduction in germination of infected seeds at all temperatures between the third and fourth years according to the linear contrast at $P = 0.05$ (*), but there was no significant difference in germination at any given assay time across the temperatures using LSD at $P = 0.05$.

foil packets and mailed to Pullman for assay of seedborne *A. f. f. sp. lentis*.

Incidence of seedborne *A. f. f. sp. lentis* in lentil seeds. One hundred sixty seeds from each temperature were surface-disinfested in 0.25% NaOCl for 5 min, dried on paper towels, and placed on 2% water agar (WA). A completely random design of 20 seeds per 9-cm-diameter plate, with eight plates per temperature treatment, was used. Plates were incubated under fluorescent lights (12-hr photoperiod, 4,300 lx) at 20–24 C. Seeds were observed for the incidence of seedborne fungi after 6–7 and 10–12 days. An initial infection incidence level (day 0) was determined before sending the seeds to the NSSL.

Pathogenicity tests. Pathogenicity tests were conducted with *A. f. f. sp. lentis* isolated from seeds of the different treatment temperatures on each sampling date. Isolates of *A. f. f. sp. lentis* were cultured on potato-dextrose agar (PDA) plates under fluorescent lights (12-hr photoperiod, 4,300 lx) for 10–14 days. Conidia were collected by flooding plates with 10 ml of sterile distilled water and gently scraping the colony surface with a bent spatula. Conidial suspensions were counted with a hemacytometer and diluted to about 1×10^6 spores per milliliter with sterile distilled water. The foliage of four to six 20- to 30-day-old lentil plants (PI 477920, USA) (two or three plants per 15-cm-diameter plastic pot) was sprayed to runoff with the spore suspension of each isolate using a DeVilbiss atomizer. Control plants were sprayed to runoff with distilled water. Plants were incubated in a moist chamber for 72–96 hr at 18–22 C and were then moved to a greenhouse bench for symptoms to develop. Temperature in the greenhouse ranged from 18 to 25 C. Plants were rated for disease after 15–20 days. Isolations were made from lesions by surface-disinfesting the tissues in 0.25% NaOCl for 5 min and then placing pieces of tissue on WA. Pathogenicity of each isolate of *A. f. f. sp. lentis* to lentil PI 477920 was based on a disease index of 1–5, where 1 = healthy tissues, 2 = 1–10%, 3 = 11–25%, 4 = 26–50%,

Table 2. Incidence of *Ascochyta fabae* f. sp. *lentis* in naturally infected lentil seeds at temperatures of 20 to -196 C for different time intervals

Temperature (C)	Infection incidence (%) ^{a,b}				
	1 Day	1 Yr	2 Yr	3 Yr	4 Yr
20	59	56	63	54	65
5	60	60	59	66	78 ^c
-18	55	52	62	58	72*
-160 to -196	58	58	56	53	66*

^aTested 160 seeds at each temperature on all sampling dates. Seeds were surface-disinfested in 0.25% NaOCl for 5 min and placed on 2% water agar with 20 seeds per plate.

^bThere was no significant difference in infection incidence between temperatures at each assay time according to the analysis of variance with temperature as the source of variation at $P = 0.05$.

^cThere was a significant increase in incidence of infection between the third and fourth years as shown by a linear contrast over time at $P = 0.05$ (*).

and 5 = more than 50% necrosis of the foliage (13).

RESULTS

Seed germination tests. The initial germination (day 0) was 96% for healthy seeds and 76% for infected seeds. Germination of infected seeds was significantly lower than that of healthy seeds at all temperatures on each sample date (Table 1). The temperature treatments did not significantly reduce germination of the healthy seeds over the duration of the trial. Germination of healthy seeds during the 4-yr period varied from 89.5 to 98.5%, with a mean of 93.3%.

Germination of infected seeds declined significantly over the storage time, but there was no significant difference in germination within sampling dates at the different treatment temperatures (Table 1). There was a significant reduction in germination (10–16%) at all temperatures between the third and fourth year. Germination during the course of the study ranged from 57 to 82.5%, with a mean of 71%.

Seed moisture. The initial (day 0) seed moisture content of the healthy and infected seeds was 7.5 and 7.3%, respectively. There was no significant difference in the moisture content of healthy seeds at the different temperatures from the beginning to the end of the experiment. It varied from 7.3 to 8.3%. The moisture

content of infected seeds did not vary significantly over the 4-yr period at the different temperatures and ranged from 7.0 to 7.9%.

Incidence of seedborne *A. f. f. sp. lentis*. The initial incidence (day 0) of seedborne *A. f. f. sp. lentis* in infected seed was 54.5%. The pathogen was isolated from 52 to 78% of the seeds over the 4-yr period, with a mean infection incidence of 60.5% (Table 2). There were no significant differences in the incidence of seedborne *A. f. f. sp. lentis* between the different temperatures on any given sample date. There was, however, a significant increase in incidence (18–25%) between the third and fourth years at temperatures of 5, -18, and -160 to -196 C.

Ascochyta was isolated from most heavily infected seeds, which were often shriveled and discolored purplish-black, with whitish mycelium present in sunken lesions. In seeds with necrotic lesions, the fungus often produced pycnidia that oozed conidia. However, many seeds without visible symptoms also were infected. It was usually possible to isolate *A. f. f. sp. lentis* in pure culture from infected seed on WA. Other fungi pathogenic to lentil that were isolated infrequently from seeds of healthy and infected plants were *Botrytis cinerea* Pers. ex Pers., *Fusarium avenaceum* (Fr.) Sacc., *Macrophomina phaseolina* (Tassi) Goid., *Phoma medicaginis*

Malbr. & Roum. var. *pinodella* (L. K. Jones) Boerema, *Rhizoctonia solani* Kühn, and *Sclerotinia sclerotiorum* (Lib.) de Bary.

Pathogenicity tests. Isolates of *A. f. f. sp. lentis* from each sampling date at each temperature were tested for pathogenicity to lentil PI 477920. The pathogenicity of isolates of the fungus from lentil seeds stored for 4 yr at 20 to -196 C was not adversely affected. All isolates produced typical lesions on lentil stems, petioles, and leaflets. The mean disease index, 2.5-3.5, did not vary appreciably on lentil plants inoculated with different isolates of the fungus tested at the beginning or end of the experiment.

DISCUSSION

There was no significant decrease in germination of healthy lentil seeds over 4 yr of storage at any of the treatment temperatures. Storage for 4 yr was not long enough to affect germination at these temperatures. However, there was an indication that germination was declining at a slightly faster rate after 4 yr of storage for healthy seeds maintained at the higher temperatures (20 and 5 C) than at the lower temperatures (-18 and -160 to -196 C). Germination of seeds infected by *A. f. f. sp. lentis* was significantly reduced at all temperatures between the third and fourth years. It is evident that seeds infected by *A. f. f. sp. lentis* lose viability quicker than healthy seeds. The mean germination of seeds from infected plants declined from 79.6% after 1 day to 59.0% after 4 yr. However, the mean germination of healthy seeds declined by only 2.5% over the same time span. The adverse effect of infection by *Ascochyta* on germination of lentil seed also was noted by Cromey et al (3).

A. f. f. sp. lentis survived in infected lentil seeds stored for 4 yr at temperatures of 20, 5, -18, and -160 to -196 C. Although there was no significant change in the incidence of seedborne *A. f. f. sp. lentis* at the different temperatures over the 4 yr, there was a significant increase in the infection incidence (18-25%) between the third and fourth years. The reason for this increase is not clear. The increase could be due to a shift in survival of specific components of the microflora on or beneath the seed coat that earlier may have inhibited growth of the fungus from infected seeds. Another possibility is that more vigorous seeds suppress the pathogen occasionally, particularly

during the first 2-3 yr of storage. As the seed ages, the fungus is able to overcome the suppression that results in an increase in infection incidence.

Although it is unknown how long lentil seeds will survive at ultralow temperatures, it is not unrealistic to speculate that it will be in excess of 50-75 years. The *Ascochyta* blight fungus will probably survive as long or longer than the seeds remain viable, if past experience is a guide. For instance, the fungus was isolated from seeds of a number of lentil accessions that were stored for 33 yr at 35-40% relative humidity and 4-6 C (13). Cryogenic storage of lentil seeds infected with *A. f. f. sp. lentis* would be one method of preserving, almost indefinitely, isolates of the pathogen that vary in such traits as pathogenicity, geographic origin, or cultural characteristics. The risk of the isolates mutating in LN₂ is greatly reduced because the metabolic activity of living organisms at ultralow temperatures is retarded or stopped (19).

The spores and mycelia of many fungi have been preserved for varying periods up to 9 yr in LN₂ (4,9-11). Dahmen et al (4) reported little difference in survival of a wide range of pathogenic fungi when stored in the vapor or liquid phase of LN₂. Additionally, they observed that these pathogens could be preserved for several years without loss of viability and pathogenicity. There are only a few reports on cryogenic preservation of fungi in plant tissues (4). Our study is one of the few that has used naturally infected seeds to preserve a pathogen at ultralow temperatures.

Kaiser and Hannan (13) demonstrated that *A. f. f. sp. lentis* was present in lentil seeds (originally imported from 16 countries) that had been in cold storage for 2-33 yr. It was also isolated from seeds that had been subsequently increased under field conditions at Pullman. Lentil germ plasm collections in other countries undoubtedly harbor the pathogen. Seed transmission is important in spread and survival of the *Ascochyta* blight fungus (3,12,13). Unless special precautions are taken to prevent the introduction of the pathogen on imported lentil seeds, it is possible that the fungus will survive in infected seeds for extended periods of time when stored at ultralow temperatures. In this way, gene banks could become a repository of different isolates and/or new pathotypes of the pathogen that could be introduced into a country where *Ascochyta* blight does not occur or

virulent pathotypes of the pathogen are not present.

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