# Respiration of Pea Seeds (Pisum sativum) Infected with Aspergillus ruber

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ABSTRACT

Respiration of testae, and probably of cotyledons, of unimbibed peas (*Pisum sativum*) infected with *Aspergillus ruber*, was primarily fungal. Respiration of embryonic root-shoot axes from infected peas was correlated with activity of pea mitochondria and decreased as early as sporulation was detected. Respiration of axes from infected peas increased much less than respiration of axes from noninfected peas after imbibition. Mitochondria isolated from axes of infected peas were much less active than mitochondria isolated from axes of comparable noninfected peas. This evidence for mitochondrial dysfunction suggests that damage to host mitochondria induced by *A. ruber* may play a role in deterioration of infected pea seeds.

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*Additional key word:* mitochondria.

Loss of viability of stored seed results from aging or, more rapidly, from infection by storage fungi such as those of the *Aspergillus glaucus* group (2). Harman (6) and Harman & Nash (8) reported that *Aspergillus ruber* (Konig, Spieckermann, & Bremer) Thom & Church, a member of the *A. glaucus* group, is highly pathogenic to stored pea seeds. They suggested that a toxin produced by *A. ruber* killed embryonic root-shoot axes when the mycelium of *A. ruber* was still primarily confined to the testae. Electron microscopy (7) revealed that mitochondria from tissue of peas infected with *A. ruber* were damaged. Mitochondria from similarly aged, but noninfected, peas were not as severely damaged (7). The purpose of this study was to determine the respiratory pattern of pea seeds infected with *A. ruber* and to determine whether the mitochondria in the embryonic root-shoot axes were damaged from infection by *A. ruber*.

MATERIALS AND METHODS.—Seeds of *Pisum sativum* L. cultivar 'Alaska' were chosen for this study because they could be obtained free of internal infection (5,8). Samples from a single lot of seeds were placed in a small bottle, surface-sterilized in 1.75% NaOCl, rinsed in sterile distilled water, dried, and inoculated or not inoculated with *A. ruber* (U.S.D.A., Northern Regional Research Laboratory Culture 52) as described previously (6). They were then placed in a desiccator over a saturated $NH_4H_2PO_4$ solution at 30 C which maintained the relative humidity at 92% (13). When in equilibrium with the atmosphere in the desiccators, the moisture content of the peas was ca. 21% (5,6). Nonaged seeds from the same lot were stored at 10 C and 20% relative humidity. Under these conditions, deterioration of pea seeds is slight over the time period considered in this work (ca. 5 months). Thus, the peas used were (i) unaged, (ii) comparably aged but uninfected, and (iii) aged and infected. Throughout, they are called nonaged, aged noninfected, and aged infected, respectively.

In all experiments, the samples of peas used were tested for infection by *A. ruber* and contamination by other microorganisms. Sub-samples (30 seeds) were removed at the beginning of each experiment, surface-sterilized 1 min in 0.85% NaOCl, rinsed in sterile water, and plated on Czapek-Dox agar containing 30% sucrose as the carbon source (11). Data from any experiments where contamination occurred were discarded. Nonaged peas were also surface-sterilized, dried, and adjusted to 18 to 21% moisture content, as described elsewhere (7).

A Gilson respirometer was used to measure gas evolution, and oxygen consumption, by whole peas, pea testae, and peas from which the testae had been removed. Sample groups of inoculated and comparable noninoculated peas were taken after various lengths of storage, and the percentage of infected peas determined by plating subsamples as described above. Each 100 ml respirometer flask contained 100 peas or parts from 100 peas, and 1 ml of water in the sidearm to maintain high relative humidity. High relative humidity in the respirometer flasks was required to prevent evaporation from the peas which had been equilibrated with a relative humidity of 92%. Samples of aged infected and aged noninfected peas were run concurrently. After equilibration in the water bath at 30 C overnight, gas evolution was measured for 6 hr at intervals of 1 hr. After addition of 0.2 ml 6N KOH and a paper wick to the center well of each flask, and again allowing equilibration overnight, oxygen consumption was measured for 6 hr at 1-hr intervals.

In all respirometer experiments, rates of evolution and consumption were calculated as the linear regression coefficients.

For other Gilson respirometer experiments or for isolation of mitochondria, embryonic root-shoot axes were removed from nonaged, aged noninfected, and aged infected peas, all imbibed for 16 hr at 25 C and, unless otherwise noted, under reduced pressure (ca. 430 mm Hg). The axes were rinsed in cold distilled water immediately after removal. Regardless of the length of imbibition, no axes were ever found to be infected when plated on Bacto Nutrient Agar after a 20-sec rinse in sterile distilled water. The Gilson respirometer experiments measured the rate of oxygen consumption of 20 axes for 60 min, following a 15-min equilibration in a water bath at 25 C. Each flask contained 2 ml distilled water to moisten the axes, 1 ml of water in the side arm, and 0.2 ml of 6N KOH and a paper wick in the center well.

Mitochondria were isolated from 200 axes as described elsewhere (3) except that the media contained 3 rather than 1.5 mg/ml bovine serum albumin fraction V, 35 ml of grinding medium was used, and the axes were homogenized with a mortar and pestle. A similar procedure (12) was recently reported for the isolation of mitochondria from pea cotyledons. Oxygen consumption by 100 to 150 $\mu$liters of mitochondrial suspension was measured in a 2-ml flask using a Clark-type $O_2$ electrode (Yellow Springs Instruments, Yellow Springs, Ohio) in the presence and absence of adenosine 5′-diphosphate (ADP) with potassium succinate as the substrate (3). Mitochondrial protein was determined colorimetrically (10). Respiratory control ratios were calculated by the method of Chance & Williams (1). Less than 1,200 bacterial colonies per 150 $\mu$l were found when aliquots of mitochondrial suspensions were mixed with Bacto Nutrient Agar at 55 C and incubated at room temperature in petri dishes. There was no detectable $O_2$ consumption with the $O_2$ electrode when as many as 16,000 freshly harvested cells of *Bacillus subtilis* from cultures in the logarithmic phase of growth were added to the medium used to assay mitochondrial activity.

Embryonic root-shoot axes were used as the experimental material, because they became infected later than other portions of the pea seeds; at least some of the axes died before invasion occurred; and microscopic examination revealed no hyphae in these tissues until after most of the axes were dead (8). Thus, we could use axes to study respiration of host tissue from infected peas before gross infection by *A. ruber*.

In some experiments, it was necessary to obtain

axes from infected peas soon after infection occurred. The only symptom of infection was sporulation from the hila of infected peas; this could first be seen 7 to 8 weeks after inoculation. At that time, *A. ruber* sporulated on 20 to 80% of the peas. When whole peas or pea parts were plated on Czapek-Dox agar with 30% sucrose, some peas (up to 50%) were found to be infected 6 weeks after inoculation, although sporulation was usually not evident, and only 3% of the axes were infected. However, 8 weeks after inoculation, nearly 40% of the axes from peas exhibiting *A. ruber* sporulation were found to be infected. This indicated that infection of the axes did not frequently occur before 7 to 8 weeks after inoculation; even at 7 to 8 weeks, less than 50% of the axes in infected peas were themselves infected.

All experiments were replicated at least twice and, in the data reported here, the number following the ± symbol is the standard error.

RESULTS.—After 8 to 14 weeks of storage at 30 C and 92% relative humidity, whole, aged, noninfected peas and similar peas with their testae removed consumed 19.2 ± 3.5 and 19.3 ± 6.2 nl $0_2$/hr/seed, respectively. Oxygen consumption of samples of whole seeds increased rapidly as the proportion of seeds infected by *A. ruber* increased (Fig. 1). Respiration of samples of seeds without their seed coats also increased as the proportion of infected seeds increased, but at a slower rate than that for whole seeds (Fig. 1). Direct manometric measurement of the respiration of testae was not possible due to evolution of an unidentified gas. This evolution appeared to be an artifact due to removal of the testae since the respiration rates of uninfected whole peas and uninfected peas with their testae removed was similar. The respiratory activity of testae of intact peas can, however, be inferred as the difference between the two lines in Fig. 1. The respiratory quotient for infected peas was 0.91 ± 0.07. Some of the respiration in these experiments could have been from contaminating microorganisms introduced during the lengthy equilibration and experimental periods required. However, the contribution of contaminants to the observed respiratory rates should be insignificant. Respiration from contaminants would not be expected to exceed the respiration rate of comparably aged peas noninfected with *A. ruber* (0% infection in Fig. 1), since experiments with peas and pea parts infected and noninfected with *A. ruber* were run concurrently.

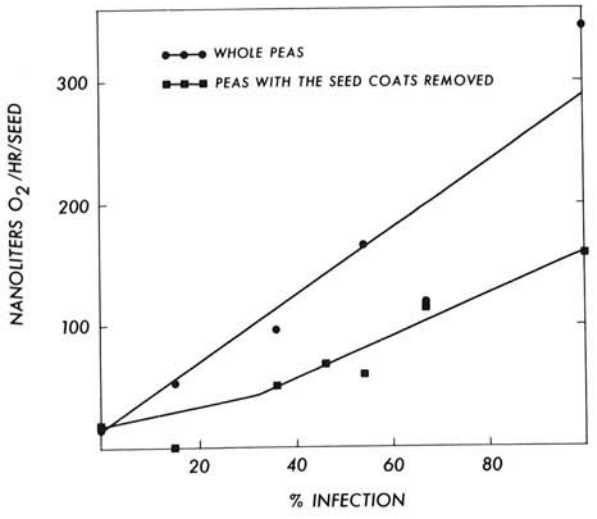


**Fig. 1.** Respiratory rates of whole peas and of peas with testae removed in samples of peas infected with *Aspergillus ruber*. Percentage of infection indicates the proportion of whole peas in each sample infected with *A. ruber*. Data presented are regression lines obtained from five separate experiments.

TABLE 1. Respiratory rates of mitochondria obtained from axes isolated from nonaged, aged noninfected, or aged infected peas infected with *Aspergillus ruber*

| Treatment | Respiratory rate[a] (nl $0_2$/min/mg protein) | |
|---|---|---|
| | Prior to ADP[b] addition | After ADP[b] addition |
| Nonaged | 426 ± 34 | 695 ± 47 |
| Aged noninfected | 319 ± 45 | 472 ± 47 |
| Aged infected | 175 ± 7 | 208 ± 23 |

[a]Values are the means of four experiments ± the standard error. Of the single degree of freedom comparisons implied, all involved significant differences except within infected, without ADP vs. with ADP.

[b]ADP stands for adenosine 5′-diphosphate. The substrate was 20 mM potassium succinate at pH 7.5. Each sample contained 1.2 ± 0.6 mg mitochondrial protein.

Mitochondria from axes obtained from aged noninfected peas respired more rapidly than those from infected peas aged 12 to 14 weeks but respired less rapidly than mitochondria from axes of nonaged peas (Table 1). The respiratory control ratio for mitochondria from nonaged axes was 2.07 ± 0.14 after the second addition of adenosine 5′-diphosphate, a figure similar to that obtained for mitochondria from pea cotyledons shortly after imbibition began (12). The respiratory rates of mitochondria from axes isolated from aged noninfected and aged infected peas were too low to accurately measure the respiratory control ratio. However, the first addition of ADP to mitochondria isolated from axes from nonaged and aged noninfected peas resulted in a significant increase in respiration ($\alpha$ = 0.005 and 0.025, respectively). There was no significant increase in respiration when ADP was added to mitochondria from axes from aged infected peas (Table 1).

It seemed desirable to compare mitochondrial activity of seeds as early as infection could be detected (7 to 8 weeks after inoculation as determined by sporulation from the hila) with the activity of comparable aged noninfected peas. It was not possible to obtain a sufficient number of axes from infected peas at 7 to 8 weeks for direct mitochondrial determinations, but a sufficient

number could be obtained for manometric measurements of whole axes. Therefore, we determined whether respiration rates of whole axes were correlated with mitochondrial activity. The partial correlation coefficients were 0.90, 0.97, 0.92, for nonaged, aged noninfected, and aged infected samples, respectively. The over-all correlation coefficient between pea axis respiration and mitochondrial respiration was 0.96. Thus, it appeared reasonable to make deductions of mitochondrial activity based on respiratory measurements of pea axes.

The respiratory rate of axes from infected peas aged 7 to 8 weeks (2.0 ± 0.23 μl/min/g fresh weight) was significantly lower ($\alpha = 0.025$) than that of axes from similar aged uninfected peas (3.2 ± 0.21 μl $O_2$/min/ g fresh weight) which, in turn, was significantly lower ($\alpha = 0.01$) than that of axes from unaged peas (5.4 ± 0.23 μl $O_2$/min/g fresh weight). None of the aged noninfected or nonaged peas was infected, while 90% of the aged infected peas were infected with *A. ruber*. Most (98%) of the nonaged and aged noninfected seeds germinated normally, whereas only 15% of the aged infected seeds germinated normally; 50% germinated abnormally; and 35% failed to germinate.

Other experiments were conducted to determine how rapidly the respiratory activity of axes from the differently treated peas increased during imbibition. Aged infected peas selected for sporulation 7 to 8 weeks after inoculation, comparable aged noninfected peas, and nonaged peas were soaked for 3 hr in distilled water at 25 C. The axes were removed from a portion of these peas. The remaining peas were placed between moist sterile paper towels for another 24 hr before removal of their axes. The respiratory rates of all axes increased during the period from 3 to 27 hr of imbibition (Table 2). After 3 hr, the respiratory rate from axes from nonaged peas was significantly higher than that of axes from aged infected or aged noninfected peas ($\alpha = 0.1$ and 0.25, respectively), but the rates of the latter two were not significantly different from one another (Table 2). However, after 27 hr of imbibition, the rates from axes from aged infected peas were significantly different ($\alpha = 0.005$) from those for axes from aged noninfected peas. The increase in the respiratory rates of aged infected peas was only 60% that of aged noninfected and 50% that of nonaged peas (Table 2). The fresh weight per axis increased from 6 mg after 3 hr imbibition to 7.1, 12.5, and 15.8 mg after 27-hr imbibition for aged infected, aged noninfected, and nonaged peas, respectively.

DISCUSSION.—Other workers (4,9) have shown that infection of wheat seeds by storage fungi increased the respiratory rate of infected seeds. They did not demonstrate whether this increase was due to fungal respiration or to stimulation of respiratory systems of the seeds themselves. Since the testae of peas are composed of dead tissue, any respiration that occurred in them was probably due to microorganisms. The respiratory quotient of 0.91 for whole peas suggests that nonmitochondrial terminal oxidases did not contribute significantly to the observed respiratory rates. At least 50% of the respiration in infected seeds occurred in the testae (Fig. 1) and is probably attributable to *A. ruber* growing there. The increase in the respiratory rate in *A. ruber*-infected peas with the testae removed may also be fungal, since the cotyledons became infected when sizable percentages of whole seeds were infected (8).

TABLE 2. Respiration of pea axes from nonaged, aged noninfected, or aged infected peas during imbibition

| Treatment | Respiration (μl $O_2$/min/g fresh weight) after imbibition for[a] | | Ratio of respiration |
|---|---|---|---|
| | 3 hr | 27 hr | $\frac{27}{3}$ hr |
| Nonaged | 5.9 ± 0.3 | 17.1 ± 1.1 | 2.9 ± 0.3 |
| Aged noninfected | 4.2 ± 0.7 | 10.0 ± 1.7 | 2.4 ± 0.0 |
| Aged infected | 3.4 ± 0.5 | 5.4 ± 1.2 | 1.5 ± 0.2 |

[a]Values are means of four replicates ± standard error. Of the single degree of freedom comparisons implied, all except those within 3 hr involved significant differences. Within 3 hr, nonaged vs aged and nonaged vs infected were significantly different.

A previous study (7), indicated that severe structural damage to mitochondria occurred in axes from peas infected with *A. ruber*. In the present study mitochondria from axes of aged infected peas had a lower respiration rate than mitochondria from axes from noninfected peas, and the respiration of mitochondria from infected peas was not significantly stimulated by ADP (Table 1). If the observed mitochondrial dysfunction was an important factor in pea seed deterioration induced by *A. ruber*, it would have to occur shortly after infection. The reduced respiration rates of axes obtained from aged infected peas 7 to 8 weeks after inoculation indicated that mitochondrial dysfunction occurred as early as sporulation could be seen on intact peas. This was at a time when less than 50% of the axes were infected.

This lower respiratory rate of axes from infected peas as compared with noninfected peas would affect germination only if the respiratory mechanisms of axes failed to recover during imbibition. The respiratory rate of axes from infected peas increased only 50 to 60% as fast as did the respiratory rate of axes from noninfected peas (Table 2). This, coupled with the observed ultrastructural mitochondrial damage (7), the mitochondrial dysfunction found in this work, and the decrease in the respiratory rate of axes from infected peas, suggests that mitochondrial damage may play a role in the deterioration of stored peas infected with *A. ruber*.

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