PLANT GERMPLASM VIABILITY: BIOCHEMICAL INSIGHTS AND NONINVASIVE ASSESSMENTS[1]

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ABSTRACT

Biochemical research has been conducted at the National Seed Storage Laboratory to examine parameters important to plant germplasm viability. Emphasis was placed on the key respiratory enzyme cytochrome *c* oxidase. Studies using effector molecules to probe *Phaseolus* respiration on a variety of physiological levels (whole seed to isolated enzyme) showed a direct correlation between rate of respiration and vigor, and implicated oxidase in loss of vigor/viability. Recalcitrant seed storage in anesthetic atmospheres was shown to increase longevity. Infrared (FTIR) spectroscopy, with a variety of sampling techniques to accommodate (intact) biological samples, is a powerful analytical tool to examine biochemical structure/function relationships to plant germplasm viability. FTIR experiments conducted on suspension cultured cells showed measurement of CO_2 production to be a noninvasive viability indicator. In pollen, structural changes in membrane lipids were correlated with imbibitional chilling injury, and distinct changes in structure and function were observed *in vivo* during germination. FTIR-photoacoustic spectroscopy, which can detect CO_2 production during minimal hydration of intact seed, holds promise as a new noninvasive viability assessment method.

Additional index words: **Respiration, cytochrome *c* oxidase, vigor, storage, infrared spectroscopy, FTIR, seeds, pollen, suspension cultured cells**

INTRODUCTION AND OBJECTIVES

The analysis of viability is a complex biochemical problem. Although several hypotheses have been formulated about the loss of viability with germplasm storage, such as genetic damage, depletion of food reserves, enzyme denaturation, membrane damage, alteration of chemical composition (Priestley, 1986), a clear biochemical explanation of viability loss from aging remains to be elucidated.

The NSSL biochemistry research project objectives are: 1) To examine the biochemical properties of cytochrome *c* oxidase as related to seed viability/vigor; 2) to examine biochemical factors essential for plant germplasm viability in orthodox and recalcitrant seeds and clonal propagules; 3) to understand biochemical changes during storage/deterioration; and 4) to measure these changes noninvasively.

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Table 1. Summary of the effects of external molecules on bean seed respiratory activity and root growth during germination. For the gases, the remaining 20% of the atmosphere was composed of O_2; the concentration of D_2O was 99.8%.

	Seed Germination	Seed Respiration	Root Length	Mitochondrial Respiration	CcO Activity
Control	100%	100%[a]	100%[b]	100%[c]	100%[d]
80% CO	99%	88%	46%	42%	42%
80% N_2O	98%	81%	93%	87%	67%
D_2O	100%	85%	68%	43%	52%

[a] 34.6 + 1.4 mmol $min^{-1}kg^{-1}$, fresh mass basis
[b] 22.2 ± 0.4 mm, measured after 72h
[c] 440.8 ± 46.1 mmol $min^{-1}kg^{-1}$, protein basis
[d] 924 ± 12 mmol $min^{-1}kg^{-1}$, protein basis
Values are taken from reference 19.

RESPIRATION AND CYTOCHROME *c* OXIDASE

The enzyme cytochrome *c* oxidase (CcO) plays a key role in efficient cellular energy production, the aerobic respiratory pathway to ATP, by completely reducing oxygen to water. CcO is an inner mitochondrial membrane protein containing two heme prosthetic groups and other metal ion and amino acid components necessary for the four-electron reduction process, which is accompanied by proton pumping (Einarsdóttir, et al., 1988). CcO can also be implicated in the loss of germplasm viability; release of partially reduced oxygen species by a denatured enzyme, i.e., one-, two-, or three-electron compounds such as superoxide, peroxide, and hydroxyl radical, are associated with oxyradical damage, for example, as described in the "lipid peroxidation model of seed aging" (Wilson and McDonald, 1986). In terms of overall biochemical metabolism, more than 90% of the oxygen used on Earth passes through cytochrome *c* oxidase (Slater, et al., 1965).

One of the first research goals was to examine the properties of this enzyme as it occurs in plants, specifically in snap bean seed, *Phaseolus vulgaris* L., and to determine its role in seed germination/vigor (Sowa, 1988). Individual seed respiration, measured polarographically during the first 48h of germination, showed a direct correlation between increase in seed moisture and oxygen uptake; upon root emergence, rates increased at least 33% (Sowa and Roos, 1989). Enzyme preparations were isolated from imbibed bean seed, and studies using known CcO effector molecules were conducted to probe enzyme activity/respiration on a variety of physiological levels ranging from whole seed to isolated enzyme (Sowa, et al., 1993). Results (Table 1) indicate a close relationship between CcO and vigor, especially using carbon monoxide (CO), the most direct effector molecule, which binds at the enzyme ligand site. It could be hypothesized that loss of CcO activity correlates to loss of vigor. Studies with onion seeds demonstrated that a loss in respiratory activity accompanied loss of vigor (evaluated as root length) after storage at different temperatures

(Stanwood and Sowa, 1989). Vigor loss is the first sign of deterioration during storage (Roos, 1986); it therefore is reasonable to assume that loss in respiratory enzyme activity, especially CcO, would eventually lead to loss of viability.

Results of the effector molecule experiments suggested that the gaseous anesthetic nitrous oxide (N_2O) could be used to enhance storage of desiccation-sensitive tissues with active metabolism. Mitochondrial (Sowa, et al., 1987) and cell (Sowa and Towill, 1991a) respiration could be reversibly inhibited in a dose-dependent manner. The anesthetic was used to (reversibly) inhibit aerobic respiration during recalcitrant seed storage. After 12 weeks in a moist atmosphere containing N_2O and O_2, lychee (*Litchi chinensis* Sonn.) maintained 92% germination compared to 44% under air, and longan (*Dimocarpus longan* Lour.) germinated 70% when viability under air was completely lost after 7 weeks (Fig. 1) (Sowa, et al., 1991, reference 20). Contaminating microorganism growth was also suppressed under anesthetic storage. Although the "Sleeping Seeds" concept (Discover, 1990) has not been tested for long-term applicability, it does provide a novel approach to extending the longevity of recalcitrant seeds.

SPECTROSCOPIC TECHNIQUES AS VIABILITY DETERMINANTS

To examine biochemical factors essential for plant germplasm viability, we explored the use of spectroscopic techniques. Three major techniques were employed: nuclear magnetic resonance (NMR), uv-visible, and infrared. NMR experiments were initiated in collaboration with Colorado State University to examine phosphorous metabolism during bean seed germination. Uv-visible spectroscopy proved to be very helpful in identifying and quantifying mitochondrial cytochromes, measuring enzyme activities, and, when used in conjunction with HPLC, as a detection method for analyzing products of chromatographic separations, e.g. enzyme aggregation state and subunit composition. Infrared spectroscopy proved to be the most valuable technique for pinpointing viability markers by providing quantitative and qualitative information about chemical functional groups in cells.

Infrared spectroscopy measures vibrational transitions of molecular dipoles, and the energy of the transitions is determined by the strength of the bond holding the atoms together and their reduced mass (Griffiths and de Haseth, 1986). Functional groups that are informative in biological samples include peptide bonds (amide I & II vibrations), membrane lipid side chains (C–H vibrations) and ester linkages (C=O vibrations), carbohydrates (C–O–H vibrations), phosphates (P–O vibrations), and CO_2 production (measurement of respiratory metabolism). Infrared spectroscopy of heme-bound carbonyls can also provide information about the ligand binding site of CcO (Einarsdóttir, et al., 1988).

Fourier transform infrared (FTIR) instrumentation collects all spectral information simultaneously, and many accurate scans can be averaged in a short time (Griffiths and de Haseth, 1986). Sampling techniques such as reflectance and photoacoustic detection have provided innovative approaches to *in vivo* spectroscopy, including the analysis of plant germplasm.

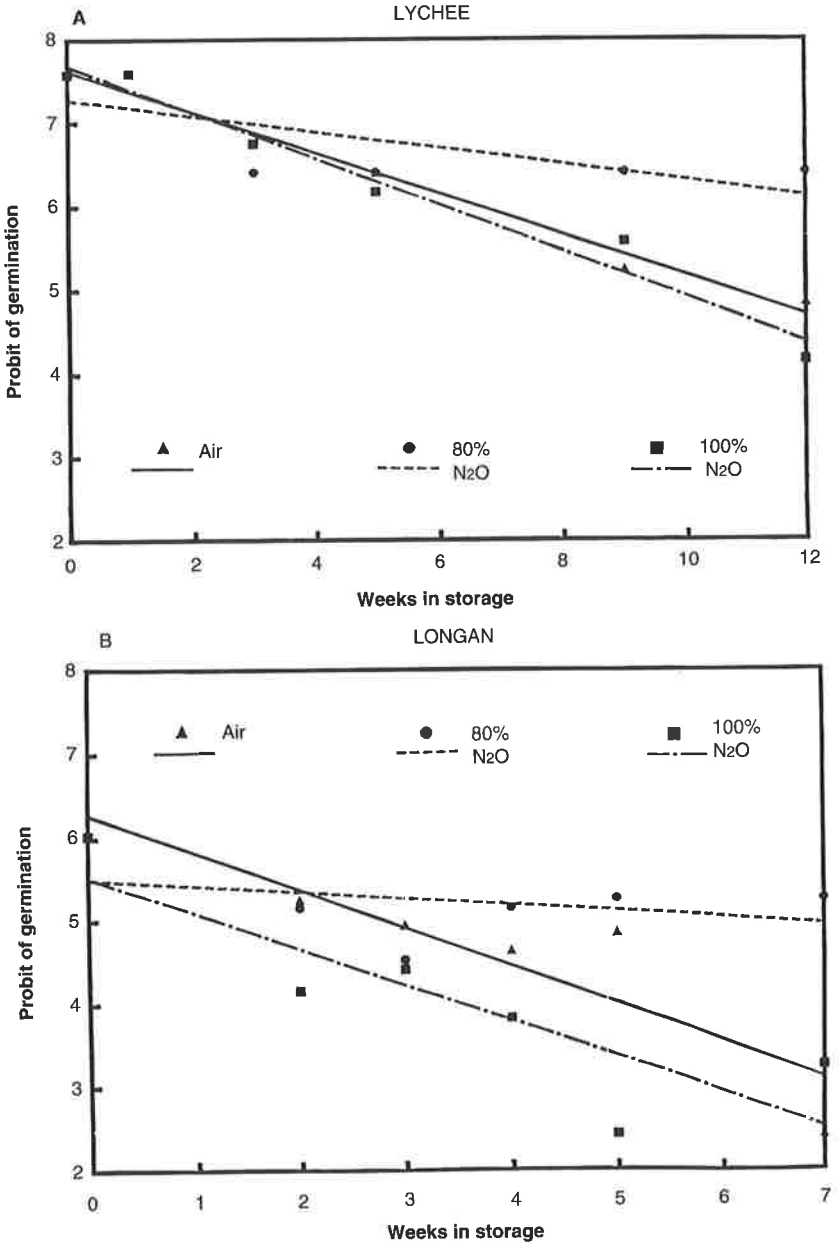

Figure 1. Probits of germination of lychee (A) and longan (B) seed after storage under various atmospheres. To obtain a probit value for 100% germination, a value of 99.5% was arbitrarily used. (From reference 20.)

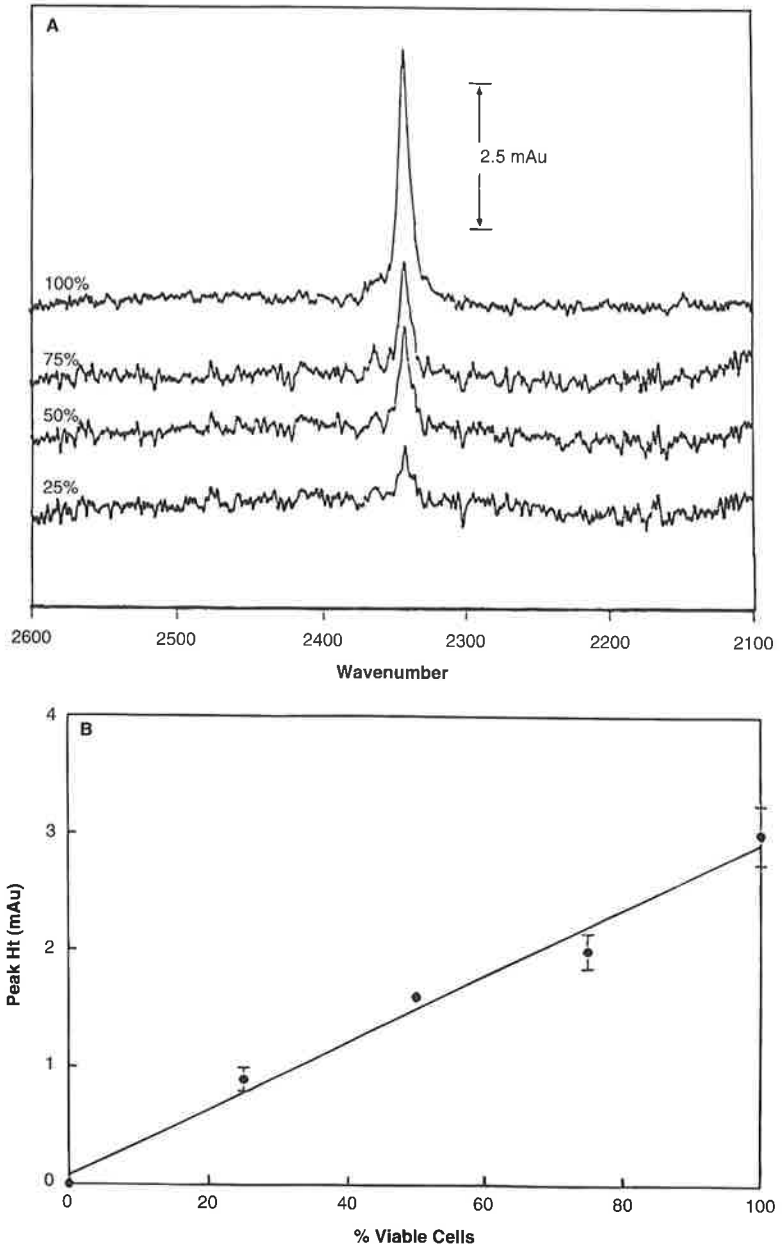

Figure 2. (A) Stack plots of IR absorbance in milliabsorbance units (mAu) of artificial mixtures of *S. pectinata* cells ranging in viability from 0 to 100%, and (B) regression of peak height at 2343 cm^{-1} to percentage of viable cells in the mixtures; each point represents the mean ± standard deviation of the measurements. (From reference 22.)

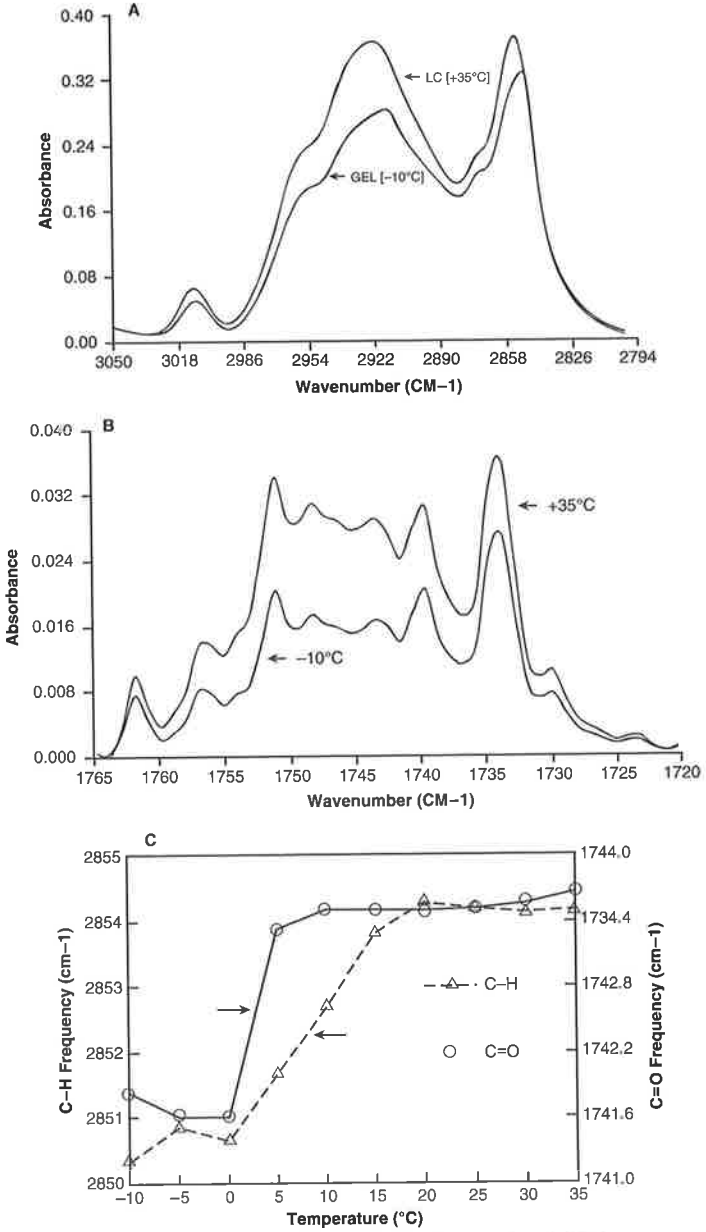

Figure 3. (A) FTIR spectra of hydrated pecan pollen in the lipid frequency range at −10°C (gel phase) and +35°C (liquid crystalline phase). (B) FTIR spectra of hydrated spruce pollen in the ester carbonyl region at −10°C and +35°C. (C) Phase transition behavior of membrane lipids in hydrated pecan pollen. Frequencies correspond to the symmetric CH_2 (triangles) and ester carbonyl (circles) vibrations; arrows indicate Tm values. (From reference 14.)

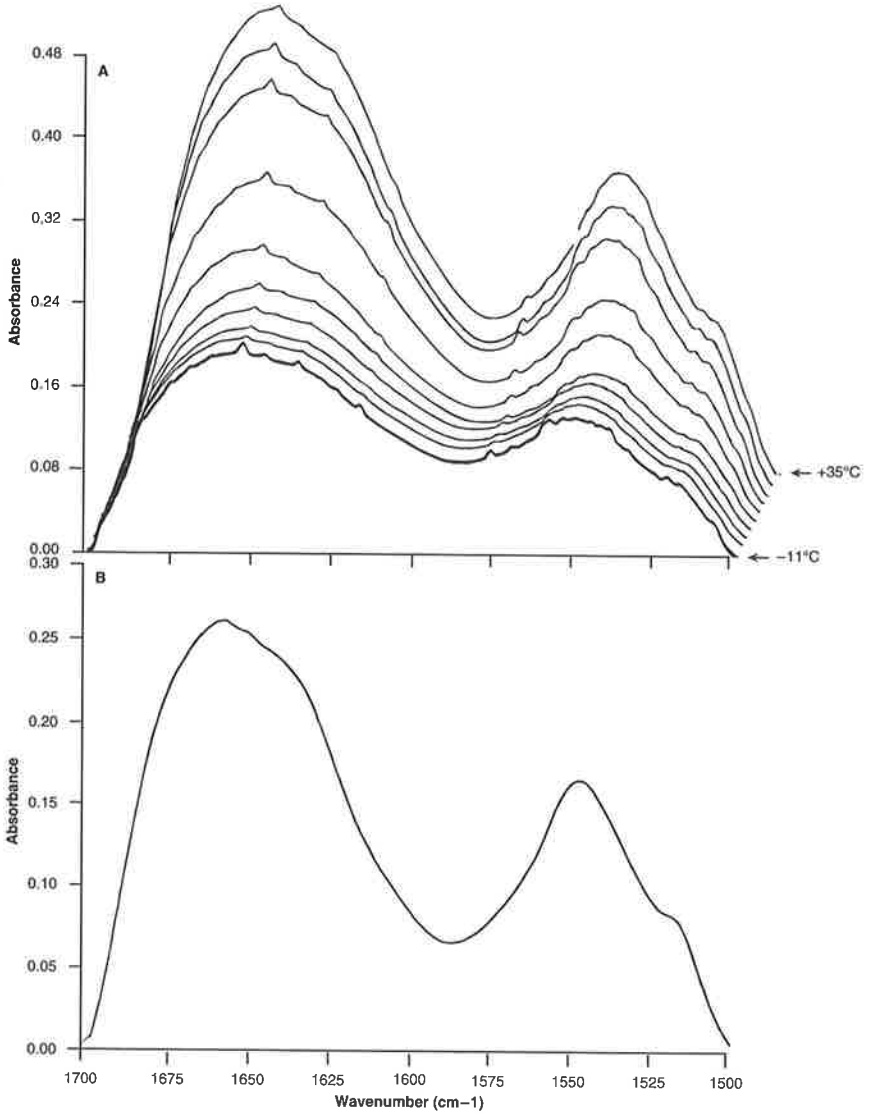

Figure 4. FTIR spectra of dry pecan pollen in the protein frequency range at increasing temperature (A) and difference spectrum (B) illustrating overall secondary structural changes with temperature. (From reference 14.)

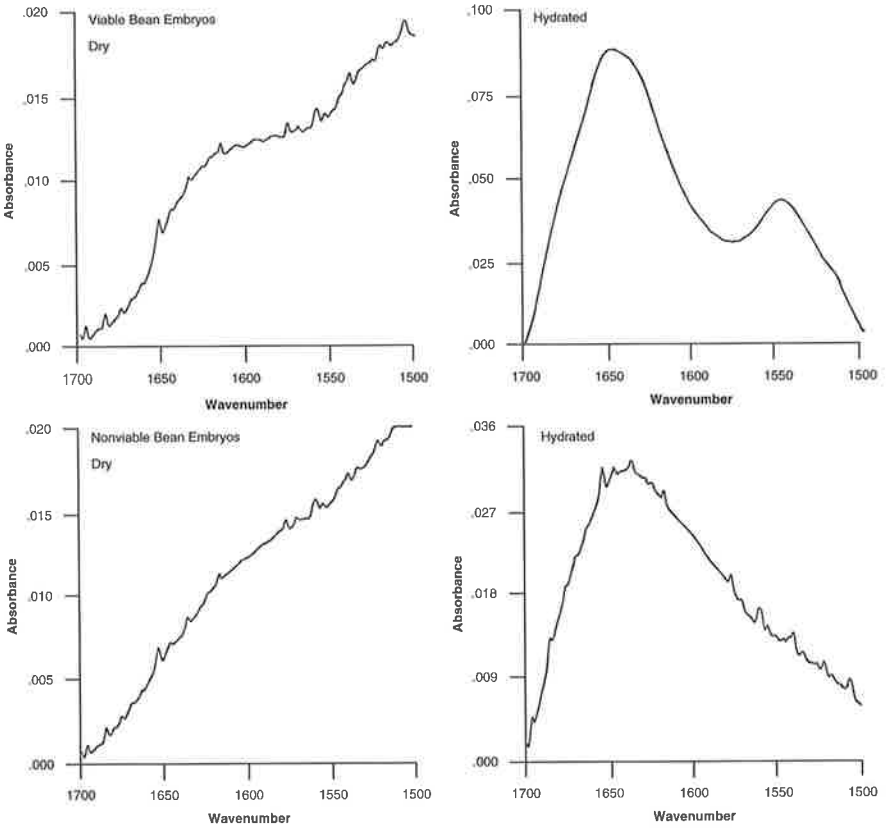

Figure 5. FTIR transmission spectra of excised viable and nonviable bean embryos in dry and hydrated states. The "spikes" in the spectra are due to water vapor absorbances which were not subtracted so that data manipulation was minimized.

Our first experiments using FTIR to examine biochemical properties of viability were conducted using suspension cultured cells (Sowa and Towill, 1991b). Cylindrical internal reflectance (CIRCLE) sampling was used to collect spectra noninvasively; suspensions were simply poured into the "open boat" of the accessory, and cells settled around the internal reflectance element crystal. Full-frequency spectra showed an obvious difference between live and dead cells, the appearance of a peak at 2343 cm^{-1}. A more detailed analysis of mixtures of live and dead cells (0–100%) showed that peak height (absorbance) was directly proportional to cell viability (Fig. 2). FTIR viability results also matched tetrazolium staining results. We verified the absorbance as dissolved CO_2, and could therefore directly correlate cell viability to the noninvasive measurement of cellular respiration.

Intact pollen grains were also analyzed using FTIR spectroscopy. Previous experiments by Crowe et al. (1989a,b) showed that membrane

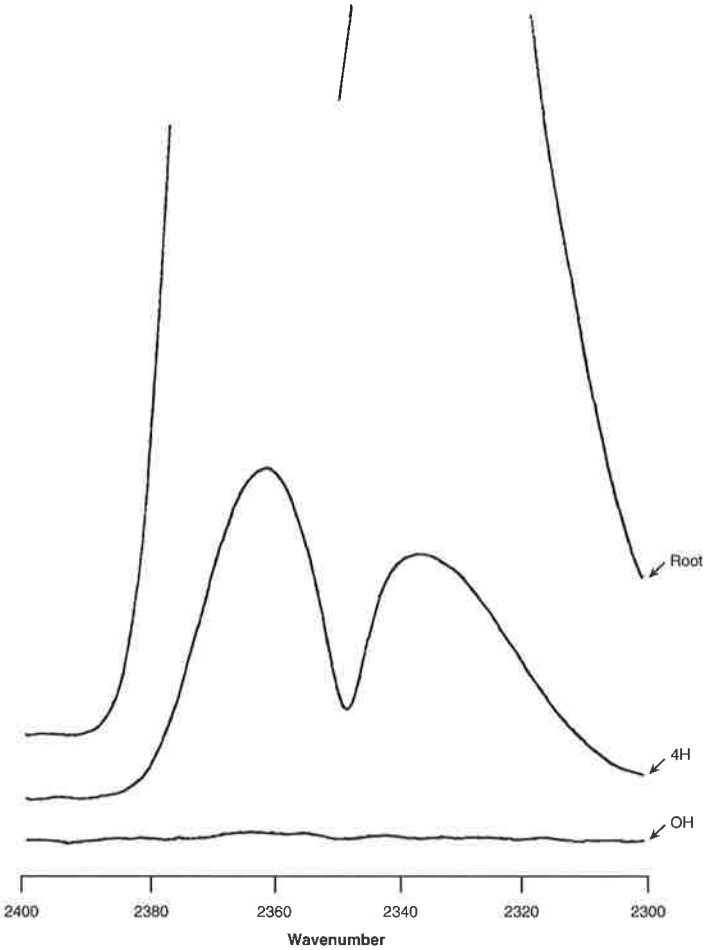

Figure 6. CO_2 production measured by FTIR-PAS of (intact) germinating bean seed at 0 h, 4 h, and root emergence (40 h). Spectra are plotted on the same scale, and peak height is directly proportional to the amount of gaseous CO_2 produced by the seed.

phase transitions could be measured in intact cells using FTIR, and that the changes caused imbibitional damage in pollen. We conducted temperature studies to examine the potential of FTIR to evaluate cryopreservation protocols. Our experiments showed that we could gain information about pollen membrane lipid and cellular protein structure as a function of temperature and hydration (Sowa, et al., 1991, reference 14). Using transmission sampling, we found that membrane phase transition temperature could be determined from the frequencies of the symmetric CH_2 and ester carbonyl vibrations (Fig 3), and that protein secondary structure (Fig. 4) was not a factor in imbibitional chilling injury. Using CIRCLE sampling, we

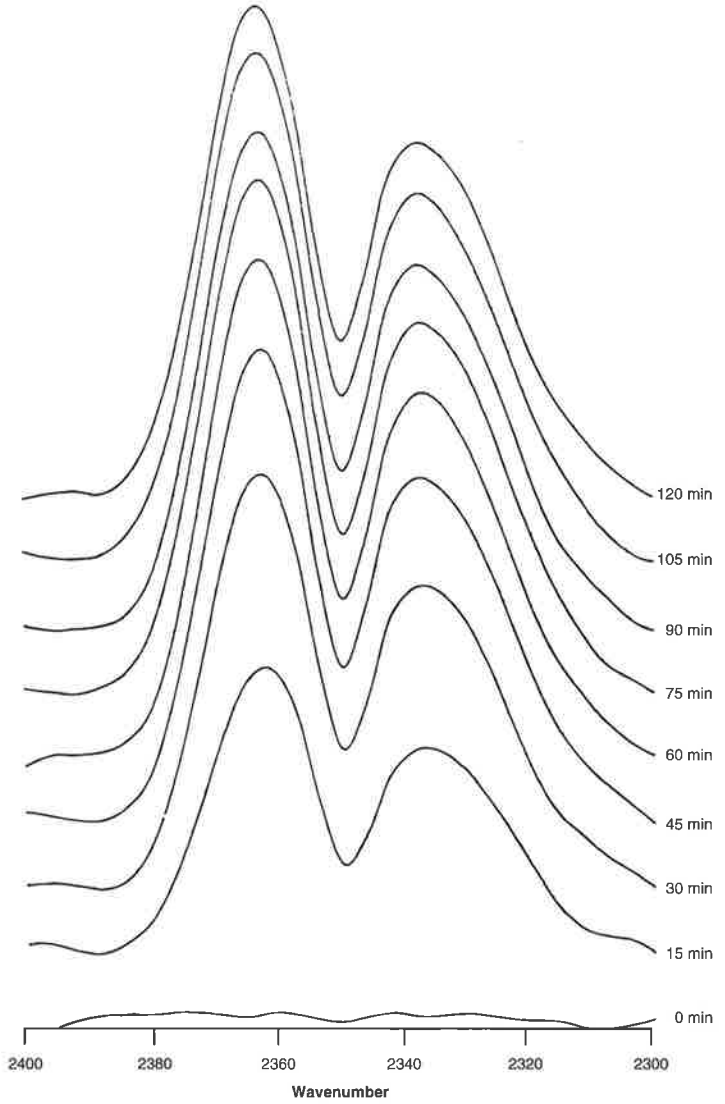

Figure 7. Difference spectra (live minus dead) of CO_2 evolution during the first 2 h of bean seed imbibition measured by FTIR-PAS.

found that we could measure biochemical changes during pollen germination *in vivo*, including structural differences and the onset of respiratory metabolism (Sowa and Connor, 1993).

Two FTIR sampling techniques were used to measure spectral characteristics of seed viability. Transmission spectroscopy of excised bean seed embryos, although invasive, was used to examine structural changes that occur during hydration of viable and nonviable samples. Membrane

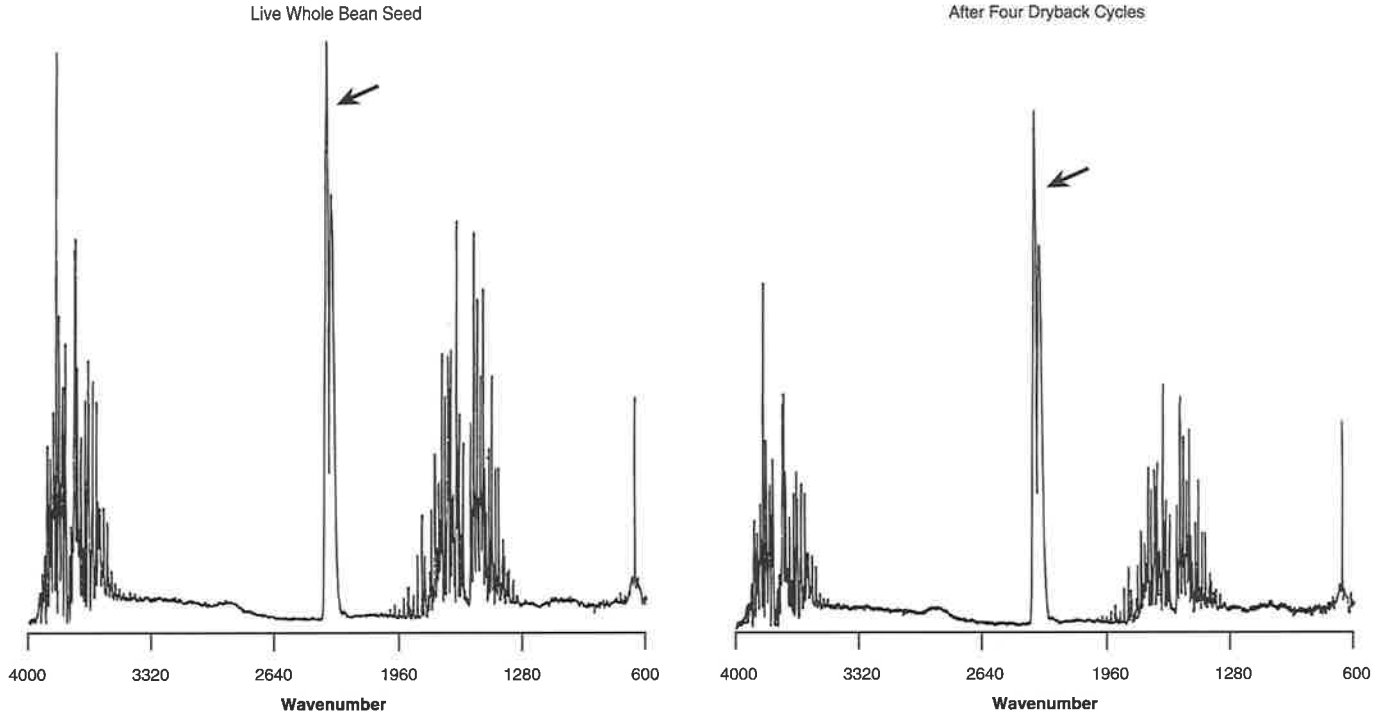

Figure 8. FTIR-PAS detection of CO_2 production by a live whole bean seed after 2 h of hydration (A), and after four cycles of testing (B). Spectra are plotted on the same scale; CO_2 peaks are marked by arrows.

phase change and the development of protein secondary structure were observed in embryos as in pollen (data not shown). Change in protein secondary structure was the most sensitive indicator of viability loss (Fig. 5). Structural changes during slow drying and rehydration of viable orthodox embryos were completely reversible, which was not the case in recalcitrant seed embryos (Sowa, et al., 1991, reference 23).

FTIR-photoacoustic spectroscopy (FTIR-PAS) is designed for the analysis of solid samples, and was used to collect data from intact seed (Sowa, 1991). This technique measures sample deexcitation as heat, transferred to the atmosphere above the sample which causes pressure/volume changes detected by a sensitive microphone; the resulting signal can be acoustically detected because the incoming absorbed beam is modulated (Rosencwaig, 1980).

FTIR-PAS analysis of *Phaseolus* seed imbibition showed that changes in structure and metabolism could be detected noninvasively (Sowa and Roos, 1991). The strongest signal represented CO_2 production upon seed hydration, which increased significantly with root emergence (Fig. 6), and correlated with our polarographic measurements of O_2 uptake (Sowa and Roos, 1989). Our experiments showed that onset of CO_2 production could be detected with minimal seed hydration, within 30 min of imbibition (Sowa and Roos, 1992), and, that viable seeds produced significantly more CO_2 than nonviable seeds (Fig. 7). Further experiments designed to predict viability of maize seed lots resulted in 80–90% success, when CO_2 production during early imbibition was compared to germination (data not shown). Prediction errors occurred in seeds contaminated with microorganisms when extraneous CO_2 production was mistakenly attributed to seed respiration. These preliminary results indicate a potential use of FTIR-PAS as a noninvasive seed viability indicator if non-seed CO_2 production can be controlled by surface sterilization or otherwise detected by characteristic absorbance frequencies in the spectrum (Greene, et al., 1992). There was little effect of multiple testing (imbibition/dryback) on seed sample viability; CO_2 production after four tests is comparable to that during initial hydration (Fig. 8).

SUMMARY

Experiments conducted in my laboratory have shown that cytochrome *c* oxidase plays an important role during seed germination, and that enzyme activity is directly correlated to root growth, an estimation of vigor. Studies with respiratory effector molecules have provided the basis for a new approach to recalcitrant germplasm preservation, the storage of these seeds under a mixture of the anesthetic gas nitrous oxide and oxygen. Investigation of the role of cytochrome *c* oxidase in seed storage longevity, and the potential for oxyradical release by deteriorated enzyme can be approached using infrared spectroscopy to examine the integrity of the ligand site. Modern spectroscopic techniques can provide biochemical insight into plant germplasm viability. FTIR analyses show that respiration and structural changes, especially in protein conformation, are correlated with viability, and new sampling/instrumentation can provide ways to measure viability on a noninvasive basis.

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