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In situ FTIR assessment of desiccation-tolerant tissues

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Abstract. This essay shows how Fourier transform infrared (FTIR) microspectroscopy can be applied to study thermodynamic parameters and conformation of endogenous biomolecules in desiccation-tolerant biological tissues. Desiccation tolerance is the remarkable ability of some organisms to survive complete dehydration. Seed and pollen of higher plants are well known examples of desiccation-tolerant tissues. FTIR studies on the overall protein secondary structure indicate that during the acquisition of desiccation tolerance, plant embryos exhibit proportional increases in α-helical structures and that β-sheet structures dominate upon drying of desiccation sensitive-embryos. During ageing of pollen and seeds, the overall protein secondary structure remains stable, whereas drastic changes in the thermotropic response of membranes occur, which coincide with a complete loss of viability. Properties of the cytoplasmic glassy matrix in desiccation-tolerant plant organs can be studied by monitoring the position of the OH-stretching vibration band of endogenous carbohydrates and proteins as a function of temperature. By applying these FTIR techniques to maturation-defective mutant seeds of *Arabidopsis thaliana* we were able to establish a correlation between macromolecular stability and desiccation tolerance. Taken together, *in situ* FTIR studies can give unique information on conformation and stability of endogenous biomolecules in desiccation-tolerant tissues.

Keywords: Desiccation-tolerance, macromolecular stability, glasses, protein stability, FTIR

1. Background

1.1. Anhydrobiosis

Drying of cells generally causes massive damage to cellular structure and organization, which eventually may lead to cell death. At the molecular level, the dissipation of water from membrane lipids, proteins and nucleic acids changes the hydrophobic and hydrophilic interactions determining structure and function [22]. The loss of water from the polar headgroups alters the physical properties of membranes, which can be measured as an increase in the gel-to-liquid crystalline phase transition temperature [27]. The consequences of such phase transitions are thought to include increased membrane permeability and lateral phase separation of membrane components [24]. Intracellular proteins may undergo irreversible structural alterations with dehydration [48]. In addition, proteins and lipids are exposed to transient, but highly reactive oxygen free radicals. Because enzymatic scavenging systems are nonfunctional at low moisture contents, free radical-induced injury ensues, including mainly lipid peroxidation and phospholipid de-esterification [42,53], but also DNA breakage, and accumulation of carbonyl derivatives in proteins [47]. Furthermore, proteins may be involved in Amadori and Maillard reactions with reducing sugars, particularly at low water contents [45,60]. These reactions refer to a series of complex reactions

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that occur following an initial carbonyl-amine reaction. Proteins may also be degraded by proteases originating from lysosomes that lost membrane integrity during dehydration. Additional injury may occur upon rehydration. The changed physical properties and chemical composition of the plasma membrane may lead to an extensive leakage of cytoplasmic solutes during rehydration of the tissue [37,52,54,65].

However, some organisms have the remarkable ability to cope with the deleterious effects of drying and survive in a state of almost complete dehydration, known as anhydrobiosis [25]. This phenomenon occurs in all major taxa. Although widely spread in pollens and seeds of higher plants and in mosses, anhydrobiosis of whole vascular plants is relatively rare, including approximately 300 species [1]. The leaves and roots of the resurrection plant *Craterostigma plantagineum* (Scrophulariaceae), for example, can be dehydrated to water contents of less than 10% on a dry weight basis, and yet the plant resumes vital metabolism after rehydration [5,7]. Under cold and dry conditions, dried organisms may survive anhydrobiosis for decades or even centuries. One of the most spectacular examples of long-term survival in nature is the ancient seed of Sacred Lotus from China [56]. These seeds that remained in anhydrobiosis for more than 1000 years have proved to be still capable of germination.

1.2. Accumulation of sugars and stress proteins in anhydrobiotes

The ability to withstand damage from desiccation requires several biochemical adaptations. One such adaptation, which anhydrobiotes appear to have in common, is the accumulation of large amounts of carbohydrates [25]. In desiccation-tolerant seeds, usually sucrose is present as the major disaccharide [2]. Pollens may contain as much as 25% of their dry weight in the form of this carbohydrate [36]. In seeds, not only sucrose, but also oligosaccharides and cyclitols are found in large quantities [2,39,41]. Fresh leaves of the resurrection plant *Craterostigma plantagineum* contain large quantities of the metabolically inactive monosaccharide, D-glycero-D-ido-2-octulose. On drying, the octulose is rapidly converted into sucrose [7]. The sucrose contents in the leaves can be as high as 50% on a dry weight basis. In bacteria and yeasts the analog of sucrose is trehalose [3].

Together with the sugars, stress proteins are produced. An example of such proteins related to dehydration tolerance is the family of proteins known as dehydrins, found in both higher [20] and lower [43] plants. The synergy of sugars and stress proteins appears to confer tolerance to multiple stresses, including dehydration, chilling, freezing, and osmotic stress [20,21,62,77]. Dehydrin production is promoted by the plant hormone abscisic acid (ABA) in maturing seeds and in vegetative organs exposed to water deficiency [8].

The carbohydrates that are abundantly present in desiccation-tolerant organisms are thought to play a role in the protection of cellular proteins and membranes in the dried state. Crowe and co-workers [22,25] have proposed that sugars play a role in the protection of cellular and macromolecular structure in anhydrobiotes by replacing the water that is normally hydrogen bonded to polar residues. In addition, differential scanning calorimetry (DSC) studies indicate that sugars are involved in the formation of a glassy matrix in the cytoplasm of anhydrobiotes [17,68]. The glassy state is a liquid state with solid-like properties. Glassy materials have no defined molecular structure, and the molecular mobility is low. Formation of a glassy matrix in the cytoplasm of anhydrobiotes results in immobilisation of cytoplasmic molecules and organelles, which gives protection to the dried organism [12–14]. The molecular mobility in the dried cytoplasm appears to be negatively correlated with the lifespan of the anhydrobiote.

Also, stress proteins may be involved in the stabilization of membranes and proteins in the dried state. Dehydrins (also referred to as LEA proteins) in plants are located in several different cellular compartments. They are present in nuclei, mitochondria and the cytoplasm [11,21]. The major dehydrin

in maize embryos has been found to be associated with a cytoplasmic endomembrane [30], and a wheat dehydrin has been found to be located in the vicinity of the plasma membrane [28], suggesting that these proteins are involved in the protection of plasma and internal membranes. LEA-like proteins have been proposed to protect seed tissues against free radical damage by reactive oxygen species during the later stages of seed development and early imbibition [58]. Together with sugars, dehydrins may determine the physical properties of the dried cytoplasm of anhydrobiotes. An FTIR spectroscopy study has shown that, in comparison with a pure sucrose glass, the presence of LEA proteins increases both the glass transition temperature and the average strength of hydrogen bonding of the amorphous sugar matrix [73]. The protein appears to act synergistically with sugars in the formation of a glassy matrix.

1.3. Application of FTIR in the study of biological tissues

The dried state of desiccation-tolerant tissues limits the number of techniques that can be applied to study conformation and stability of intracellular biomolecules. One of the few suitable techniques for dried tissue analysis is FTIR spectroscopy. The advantage of FTIR is that it can be used, irrespective of the hydration state of the tissue. On account of characteristic molecular vibrations that absorb in the infrared region, information can be derived on the molecular conformation and the inter-molecular interactions of biomolecules in their native environment. With an FTIR microscope, a specific sample area as small as 100 μm^2 can be selected for FTIR analysis.

For a macromolecule, there are many vibrational transitions absorbing in the IR region, which can be assigned to particular bonds or groupings. This forms the basis of characteristic group frequencies. The main experimental parameter is the position of the maximum of the absorption band, often presented as wavenumber (cm^{-1}). The band position of a molecular group depends on the intrinsic molecular vibration and on the microenvironment of the oscillating atoms. Information can be obtained about the molecular structure and interaction with other molecules. Characteristic group frequencies also form the basis for the analysis of biological tissues. It should be realized that the observed group frequencies in IR spectra of biological tissues may contain contributions from various types of biomolecules. Nevertheless, some of the *in situ* IR bands are dominated by one type of biomolecule. The characteristic CH_2 stretching vibrations of lipids have been used to detect lipid phase transitions in isolated biological membranes and in whole cells [18,23,26]. Lipids contain a relatively high proportion of CH_2 groups compared to other biomolecules, which show up as two characteristic absorption bands at around 2924 and 2854 cm^{-1}, denoting the asymmetric and symmetric stretching mode of the lipid CH_2 groups, respectively. Proteins in biological tissues can be detected on account of two characteristic absorption bands at around 1650 cm^{-1} (amide-I band) and 1550 cm^{-1} (amide-II band), arising from the peptide backbone. The amide-I band, which is most often used for protein analysis, is usually a complex band, because the different types of protein secondary structure have different IR transitions in the amide-I region. The amide-I band has been extensively studied to determine the relative proportion of the different types of protein secondary structure [4,33,61]. The observed protein bands in biological tissues are the average of all the proteins in the cell, but are often dominated by one type of protein. For example in *Lathyrus sativus* seeds, globulins and albumins comprise 60% and 30% of the total protein fraction, respectively [50]. The OH stretching band between 3600 and 3000 cm^{-1} is dominated by water in hydrated biological tissues, but in dried, desiccation-tolerant tissues, this band arises from carbohydrates and proteins [69,74].

The temperature dependence of a molecular vibration can be used to verify further the assignment of the type of biomolecule that is observed. IR spectra of biological tissues as a function of temperature show shifts of bands, associated with the melting of lipids in membranes (CH-stretching vibrations), with protein denaturation (C=O stretching vibration) and with the melting of cytoplasmic glasses

(OH-stretching vibration), which can be measured simultaneously. FTIR has an advantage over other methods such as DSC that, besides thermodynamic parameters, information can be derived on molecular conformation and intra- and inter-molecular interactions. In the next section, an overview is given of how we apply FTIR microspectroscopy to study thermodynamic properties of desiccation-tolerant tissues and the conformation of endogenous biomolecules.

2. *In situ* FTIR microspectroscopy measurements on desiccation-tolerant tissues

We focus on the following subjects: (a) characterization of seed tissue according to chemical composition; (b) changes in protein secondary structure during the acquisition of desiccation tolerance; (c) stability of protein secondary structure and of membranes during ageing; and (d) macromolecular stability of cytoplasmic glasses. The latter two subjects involve heating scans, from which melting of lipids and glasses, and unfolding of proteins can be derived. We show that the stability of proteins and glasses are linked with the life span of the anhydrobiotic organism.

FTIR spectra were recorded using an IR-spectrometer equipped with a liquid N_2-cooled MCT detector and a microscope, as described previously (see [69–76] for details). Samples were assayed in transmission mode, which requires them to be sufficiently translucent for IR to avoid distortion of the shape of absorption bands. For that purpose, the material was cross-sectioned and, in the case of dried material, pressed gently between two diamond windows. In the case of hydrated material, the sample was loaded between two circular CaF_2 windows. Samples were hermetically sealed by a rubber ring in a temperature-controlled brass cell to avoid exchange of water between sample and environment. Samples were sectioned and loaded in the brass cell under defined conditions of relative humidity.

As the experimental materials, pollen (cattail [*Typha latifolia*] and the resurrection plant [*Craterostigma plantagineum*]), various seeds (*Arabidopsis thaliana*, maize [*Zea mays*], wheat [*Triticum aestivum*], and some commercial crops), somatic embryos (carrot [*Daucus carota*] and alfalfa [*Medicago sativa*]), and leaves (*Craterostigma plantagineum*) were used.

2.1. Tissue analysis of a wheat kernel

Figure 1 depicts the IR absorbance spectra of different tissues in a wheat kernel. The dead covers form a protective layer around the kernel. The non-viable endosperm functions as a food reserve for the embryo and is mainly composed of starch. The aleurone layer is a live, single cell layer surrounding the endosperm, which secretes hydrolytic enzymes that play a role in the degradation of the endosperm during germination [49]. The embryo grows into a plantlet after germination of the kernel. The differences in the IR spectra of these tissues reflect differences in relative contribution of endogenous carbohydrates, lipids and proteins. Overall, the IR spectrum of the endosperm is characteristic of a tissue enriched in carbohydrates (starch), whereas the IR spectrum of the embryo is characteristic of a tissue enriched in proteins and lipids. The band at 3330 cm^{-1}, which is visible in all the tissue sections corresponds to OH-stretch vibrations, mainly arising from carbohydrates, cell wall material, and proteins. Striking differences in the CH stretching region between 3000 and 2800 cm^{-1} are evident between embryo and endosperm. In the endosperm, the CH stretch region is a broad and featureless band, reflecting a wide range of CH stretching oscillations in different micro-environments. In the embryo and, to a lesser extent, in the aleurone layer more pronounced bands at 2929 and 2856 cm^{-1} are visible in the CH stretching region, which denote the asymmetric and symmetric lipid CH_2 stretching vibrations, respectively. In the 1800–1500 cm^{-1} region, at least 3 bands can be observed in the embryo tissue. The distinctive amide-I

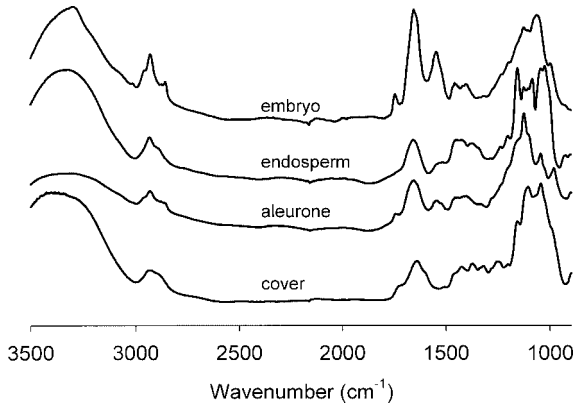

Fig. 1. IR absorption spectra of an embryo, endosperm, aleurone layer and covers of a dried wheat (*Triticum aestivum*) kernel.

band at 1655 cm^{-1} and amide-II band at 1545 cm^{-1} arise from the endogenous proteins in the embryo. The IR spectra of the endosperm and the kernel covers show reduced protein absorption bands. In the region below 1500 cm^{-1}, a variety of characteristic IR group frequencies can be observed, but the bands in this region are difficult to assign. The shape of the spectra below 1500 cm^{-1} is characteristic and can be used as a "finger print" of the tissue.

2.2. Adaptations in overall protein secondary structure during the acquisition of desiccation tolerance

Generally, seeds acquire desiccation tolerance during their development, but before physiological maturity (maximum dry matter) [51,59]. The acquisition of desiccation tolerance in seeds is associated with biochemical adaptations that allow the seed to survive desiccation. In maize, excised immature embryos acquire the ability to germinate at 14 days after pollination, but they are not yet desiccation-tolerant [9]. The rate of drying further determines how early in development isolated embryos acquire desiccation tolerance [10]. Slow drying over a 6 d period renders them desiccation-tolerant from 18 days after pollination onwards, whereas rapid dehydration over a 2 d period is tolerated only from 22 days after pollination onwards. Figure 2A depicts the amide-I band profile of rapidly and slowly dried maize embryos that were excised at 20 days after pollination. The amide-I band is composed of various bands, which denote different types of protein secondary structure. The band at 1655 cm^{-1} visible in both rapidly and slowly dried embryos is indicative of α-helical structures. The bands at 1638 and 1680 cm^{-1} reflect β-sheet and turn structures, respectively. The relative proportion of α-helical structures is higher in the slowly dried than in the rapidly dried embryos. The differences in amide-I band profile coincide with differences in viability of the embryos: 90% of the slowly dried embryos were still able to germinate, whereas all of the rapidly dried embryos lost the ability to germinate. The amide-I band profile in dried, mature embryos resembled that of immature embryos after slow drying (see [70] for details).

Studies on the effect of drying rate on the amide-I band profile of carrot and alfalfa somatic embryos yielded similar results. Somatic embryogenesis is an in vitro regeneration system, whereby an embryo is formed out of one somatic cell, morphologically resembling a zygotic embryo. Just like zygotic embryos in seeds, somatic embryos may grow into plantlets. Because it is practically difficult to store hydrated somatic embryos, methods have been developed to render somatic embryos desiccation-tolerant, which allows for extended storage in the dry state. Tetteroo et al. [63,64] have shown that carrot somatic embryos acquire complete desiccation tolerance when they are treated with the plant hormone abscisic acid

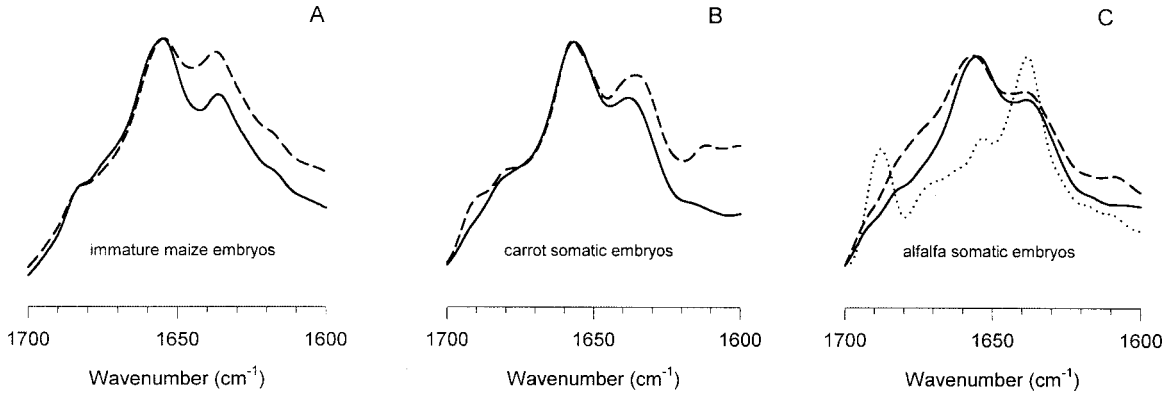

Fig. 2. Deconvolved IR absorption spectra of zygotic and somatic embryos subjected to rapid (dashed line) or slow drying (solid line). (A) Maize embryo axes excised at 20 days after pollination; data adapted from [70]. (B) Carrot somatic embryos precultured in ABA; data adapted from [75]. (C) Alfalfa somatic embryos precultured in ABA; the dotted line represent slow-dried embryos that were cultured in the absence of ABA; data adapted from [57].

(ABA) during culture and the subsequent exposure to slow drying. During slow drying, changes in sugar and protein composition take place allowing the embryo to cope with desiccation-stress [38]. Somatic embryos are a good system to study the factors involved in the acquisition of desiccation tolerance, because the culture medium, in which the embryos are grown, can be easily manipulated.

When carrot somatic embryos are slowly dried over a period of 6 days, approximately 90% of the embryos germinate after rehydration, whereas rapidly dried embryos are not able to resume growth [64]. Figure 2B shows that slowly dried embryos contain a higher relative content of α-helical structures compared to rapidly dried embryos, which coincides with an increased survival of the slowly dried embryos.

Also, in ABA-pretreated alfalfa somatic embryos, a higher proportion of α-helical structures was observed after slow drying than after rapid drying (Fig. 2C), and this coincided with increased survival of the slowly dried embryos. In the absence of ABA in the growth medium, these embryos showed very low survival of desiccation, irrespective of drying rate (see [57] for details). Slow-dried embryos cultured in the absence of ABA exhibited clear signs of protein denaturation (Fig. 2C). This can be deduced from the pronounced bands at 1638 and 1688 cm^{-1}, characteristic of β-sheet structures. Apparently, massive protein denaturation coincides with a complete loss of viability.

Taken together, the available data indicate that slow drying is pivotal for optimal embryonic survival of carrot and alfalfa somatic embryos and immaturely dissected maize zygotic embryos. Slow drying coincides with an increased proportion of α-helical structures in these tissues, which could be due to protection of endogenous proteins from degradation. Alternatively, the increased proportion of α-helical structures in desiccation-tolerant tissues might be due to the stress proteins that are synthesized during slow drying.

Dehydrins (or LEA proteins) are an example of stress proteins that are synthesized during dehydration. Dehydrin-like transcripts are expressed during slow drying of carrot somatic embryos, but not during rapid drying [75]. During normal development in the kernel, the expression of LEA proteins in maize embryos is initiated at 22 days after pollination [44], which coincides with the time, at which the embryos improve their survival of drying [10]. Specific, predominantly α-helical secondary structures for some of the LEA proteins have been predicted [29]. However, the structure of a LEA-like protein isolated from pollen has been shown to be highly dependent on the environment [73]. In solution, the protein adopts random coil conformation, but when dried in the presence of sucrose, the protein adopts predominantly

α-helical conformation. It can be expected that the conformation of the protein in a dried sucrose matrix resembles that in the dried tissue. We conclude that the slow drying-induced synthesis of stress proteins that adopt α-helical structures in the dried state could explain the observed differences in amide-I band profile between slowly (desiccation-tolerant) and rapidly (desiccation-sensitive) dried tissues.

2.3. Changes in overall protein secondary structure during ageing

Dried seeds have certain life spans, generally ranging from months to decades. Survival times of dried pollens are at least ten times less than those of dried seeds at similar storage conditions [34]. Apart from this intrinsic factor, life spans are a function of relative humidity (RH) and temperature [16]. Tissues will accumulate small cellular injuries during storage until a critical point is reached at which the damage becomes irreparable upon imbibition [32].

Cattail pollen, for example, has a maximum lifespan of approximately 120 days at 24°C at 40% RH. At 75% RH and 24°C, ageing is considerably accelerated, and viability is lost in a few weeks. Viability can be inferred from the pollen's capacity to form pollen tubes *in vitro*. Figure 3 depicts the effects of accelerated ageing (storage at 75% RH and 24°C) on the overall protein secondary structure *in situ* and the thermotropic response of membranes isolated from the pollen. Viability under these conditions drops to 0% after 12 days of storage and is approximately 40% after 5 days [67]. Despite the loss of viability during accelerated ageing, protein secondary structure is mostly conserved, as can be seen from the lack of changes in the amide-I band profile (Fig. 3A). The amide-I band profile shows that α-helical structures are the main type of protein secondary structure in the pollen. In contrast to the considerable stability of the protein secondary structure, drastic changes occur in the thermotropic response of membranes that were isolated from the pollen during viability loss (Fig. 3B). Membranes isolated from fresh pollen exhibit a major phase transition at approximately -10°C, which increases to 35°C after 12 days of storage, indicating that drastic changes have occurred in the lipid composition. These changes include increases of free fatty acids and lysophospholipids [66].

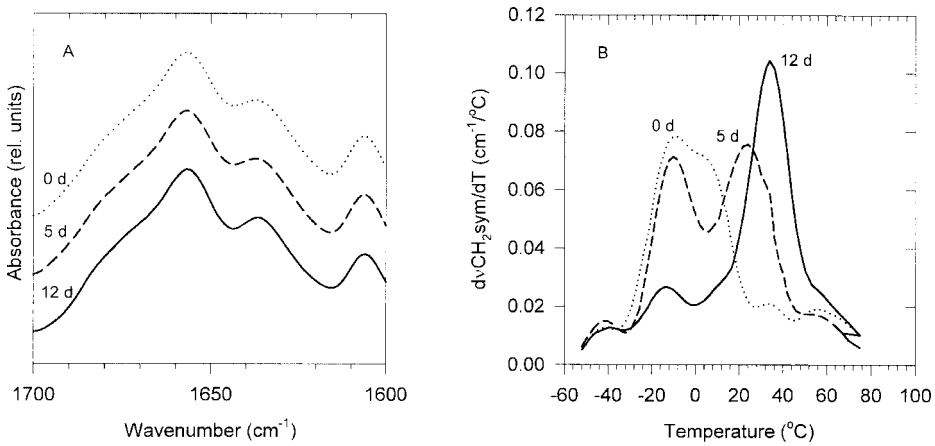

Fig. 3. (A) Deconvolved absorbance IR spectra of 0-days-aged (dotted line), 5-days-aged (dashed line) and 12-days-aged (solid line) redried cattail pollen in the amide-I region; ageing conditions: 75% RH at 24°C. (B) Thermotropic response of microsomal membranes isolated from the aged cattail pollen in (A). The lines represent the first derivative of a wavenumber versus temperature plot of the symmetric CH_2 stretching mode around 2850 cm^{-1}($d\nu CH_2sym/dT$ in $cm^{-1}/°C$). Data adapted from [71].

A study conducted on the overall protein secondary structure in freshly harvested and long-term (25 years) stored seeds yielded similar results as observed with the pollen [32]. The overall protein secondary structure of the seeds did not appreciably change during long-term dry storage (25 years), whereas viability was lost at least after 10 years of storage, which coincides with the loss of plasma membrane integrity [31].

2.4. Macromolecular stability in desiccation-tolerant tissues

2.4.1. Different thermodynamic parameters of desiccation-tolerant tissues can be measured simultaneously

Thermodynamic parameters of desiccation-tolerant tissues can be deduced from temperature dependent shifts in characteristic IR group frequencies. IR spectra from *Craterostigma plantagineum* pollen as a function of temperature show shifts of bands, associated with melting of lipids (Fig. 4A), denaturation of endogenous proteins (Fig. 4B) and melting of cytoplasmic glasses (Fig. 4C).

The thermotropic response of the symmetric CH_2 stretching vibration shows that melting of lipids (neutral and polar lipids) in this pollen mainly occurs between -20 and 20°C. The increase in wavenumber from 2851.0 to 2854.5 cm^{-1} with a temperature increase from -20 to 20°C denotes a transition from an ordered to a disordered phase of the lipids [26]. The observed melting could originate from either neutral lipids (storage oil droplets) or from polar lipids in the membrane [35]. At 60°C a small transition is visible in the νCH_2 versus temperature plot, which might be attributed to the melting of a hydrocarbon layer that surrounds the pollen.

Heat-induced denaturation of proteins in the dried pollen commences above 80°C as can be seen from changes in the amide-I band profile. Protein denaturation can be monitored as an abrupt shift in position of the band around 1635 cm^{-1} to lower wavenumbers, coincident with an increase in relative proportion of the band. These changes denote the formation of extended β-sheet structures characteristic of protein denaturation in biological tissues [69,72,75]. Also, protein denaturation of dried cattail pollen occurs at an onset temperature of approximately 80°C [72]. The protein denaturation temperature is a function of the water content. In addition, the protein denaturation temperature is affected by the sucrose in the pollen. Sugars protect protein structure and function during drying [19], but decrease the heat-induced protein denaturation temperature of dried proteins [6].

The OH stretching band shifts to higher wavenumber with increasing temperature, which indicates a decrease in hydrogen bonding with increasing temperature. The OH stretching mode exhibits two linear regions in an νOH against temperature plot. At temperatures above 35°C the slope of νOH versus temperature increases sharply, denoting a transition in the hydrogen bonding network of the pollen. Such an inflection can be assigned to the melting of the cytoplasmic glass in the pollen from the glassy to the rubbery state. The glass transition is a second order transition associated with an increase in heat capacity of the system [55]. With FTIR the glass transition can be measured as an abrupt decrease in the average strength of hydrogen bonding upon melting of the glass [74].

2.4.2. Dried leaves of Craterostigma plantagineum are in a glassy state

Glasses can also be detected in the vegetative tissues of *Craterostigma plantagineum*. Figure 5 depicts the temperature dependence of the OH stretching vibration in dried leaves and that of pure amorphous sucrose. The latter one reveals an inflection point around 57°C, which denotes the glass transition (Tg). The dried leaves exhibit an νOH *vs* temperature plot, which is comparable with that of amorphous sucrose with an inflection point (Tg) at 63°C. This high Tg of ca. 63°C, indicates that the dried leaves are in a protective glassy state at ambient temperatures. The similarity of the νOH versus temperature plots in

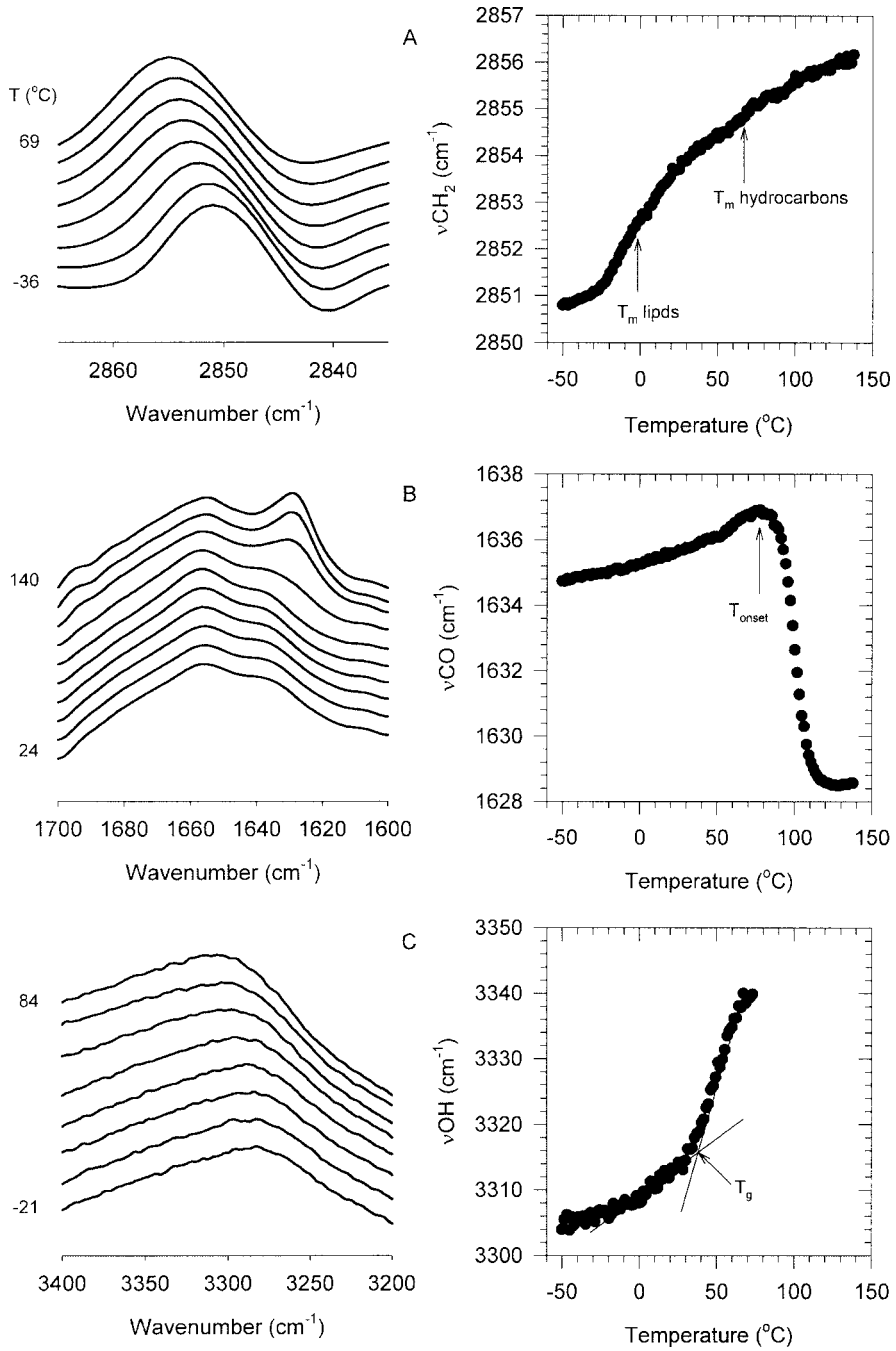

Fig. 4. Melting of lipids (A), denaturation of proteins (B), and melting of cytoplasmic glass (C) in *Craterostigma plantagineum* pollen. Panel A: inverted second derivative IR spectra of the 2865–2835-cm^{-1} region (symmetric CH_2 stretching band, left panel) and νCH_2 versus temperature plots (right panel). Panel B: deconvolved IR spectra of the 1700–1600-cm^{-1} region (amide-I band, left panel) and wavenumber versus temperature plot of the band around 1635 cm^{-1} (right panel). Panel C: absorbance IR spectra of the 3400–3200-cm^{-1} region (OH stretching band, left panel) and νOH versus temperature plots (right panel). The onset temperature of protein denaturation (T_{onset}), the T_m of lipids, and the Tg of the cytoplasm are indicated in the figures. Pollen was equilibrated at 30% RH at 24°C for one day prior to FTIR analysis.

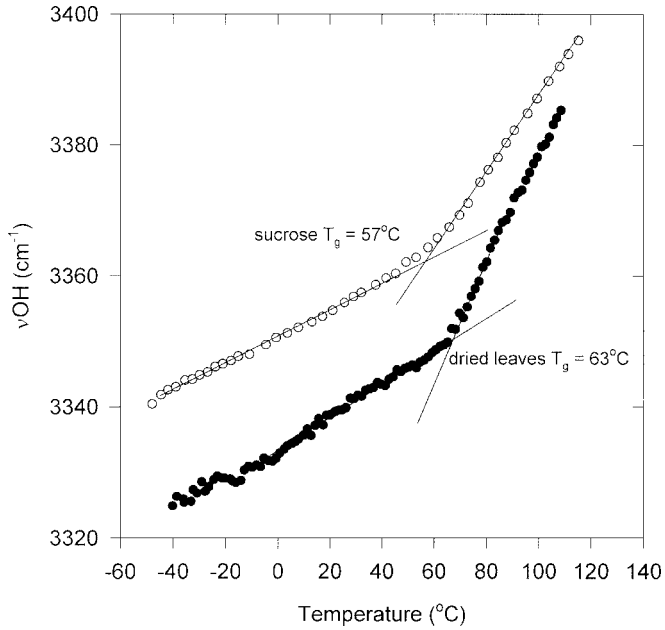

Fig. 5. Wavenumber versus temperature plot (FTIR) of dried *C. plantagineum* leaves and amorphous sucrose. The data points represent the OH-stretching vibration of dried leaves (filled circles) and sucrose (open circles). The Tg values are also indicated. Data adapted from [74].

Fig. 5 suggests that the sucrose in the cytoplasm of the dried leaves is a primary factor contributing to the glassy state.

In dehydrating leaves, octulose is rapidly converted into sucrose (up to 50% on a dry weight basis). The conversion of octulose into sucrose is pivotal for the survival of the plant in the desiccated state. When cytoplasm isolated from fresh leaves (with octulose abundantly present) is dried, Tg is at approximately 19°C (Fig. 6), which is close to the Tg of pure octulose [74]. However, when cytoplasm is isolated from dried leaves (with sucrose as the main carbohydrate) and dried, Tg is at approximately 73°C, close to the Tg of dried leaves. This implies that the conversion of octulose into sucrose in the cytoplasm of the leaves results in a marked increase in Tg when the plant is dried.

2.4.3. Macromolecular stability in maturation-defective mutant seeds of Arabidopsis thaliana

Desiccation-tolerant cells are not only programmed to survive drying, but also to retain viability for prolonged periods of time. The plant hormone ABA is thought to play an important role in the biochemical changes associated with these adaptive programs [40,46]. Therefore, mutations affecting ABA responsiveness are particularly suitable to study these biochemical adaptations in more detail. In an attempt to link the structure of cytoplasmic glasses and the heat stability of proteins to desiccation tolerance, various maturation-defective (ABA-insensitive) mutant seeds of *A. thaliana* were studied (see [69] for details).

Three different ABA-insensitive mutant seeds of *A. thaliana* with mutations in the *ABI3* locus were studied with respect to protein stability and hydrogen bonding (Figs 7 and 8). The severity of the mutation increases in the order *abi3-1 – abi3-7 – abi3-5*. Mutation in the *ABI3* locus does not affect desiccation tolerance per se, but survival in the dry state. *abi3-5* Seeds are desiccation-tolerant, but loose viability within a couple of weeks during dry storage. Figure 7 shows that the severity of mutation in the *ABI3* locus is manifested in protein stability of the seeds. In *abi3-5* seeds, the most desiccation-sensitive mutant

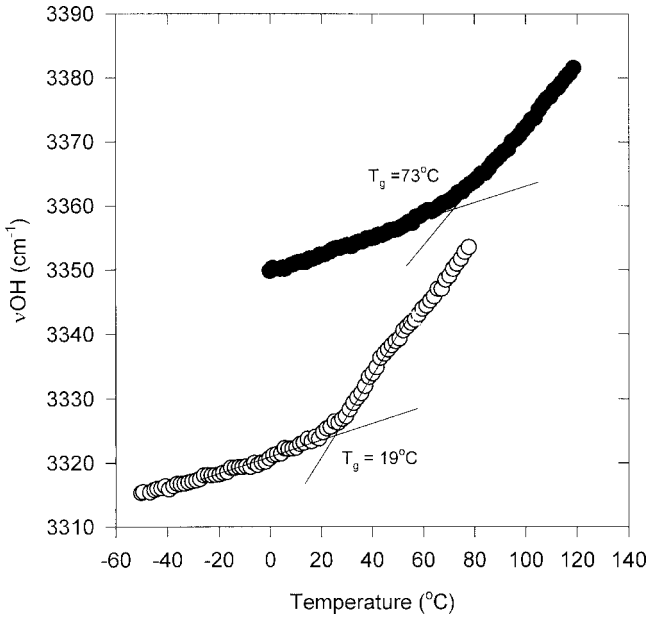

Fig. 6. Wavenumber versus temperature plot (FTIR) of dried extracted cytoplasm of fresh and dried *C. plantagineum* leaves. The data points represent the OH-stretching vibration of dried extracted cytoplasm of fresh (open circles) and dried (filled circles) leaves. Tg values are also indicated. Data adapted from [74].

of the series, denaturation commences at temperatures just above 70°C. Seeds from the *abi3-1* mutant exhibit signs of protein denaturation only at temperatures above 130°C. The intermediate *abi3-7* mutant shows signs of reduced protein heat stability: part of the protein fraction starts to denature above 80°C and another fraction above 130°C. Endogenous proteins in wild type seeds do not denature up to 150°C. The apparent relation between protein stability and desiccation tolerance has also been found in seeds of the leafy cotyledon mutants of *A. thaliana* [69]. Leafy cotyledon (*lec1*) mutants exhibit even more defects in seed maturation than the *abi3* mutants. The mutant seeds *lec1-1* and *lec1-3* are extremely desiccation-sensitive (no germination after drying). *lec2-1* Mutant seeds are different from the lec1 seeds in having a desiccation-tolerant axis, but sensitive cotyledons. Heat-induced protein denaturation of *lec1-1* and *lec1-3* seeds was found to occur at an onset temperature of 50 and 65°C, respectively, whereas proteins in desiccation-tolerant *lec2-1* seeds were considerably more heat stable.

A plot of νOH versus temperature can be used to determine the Tg of anhydrobiotes. In addition, the shift of the OH band with temperature, the wavenumber temperature coefficient (WTC, $cm^{-1}/°C$), can be used as a measure of the average strength of hydrogen bonding, i.e. low WTC indicates high average strength [74]. Figure 8 depicts the νOH *vs* temperature plots of seeds of wild-type and the ABA-insensitive mutants of *A. thaliana*. In dried *abi3-5* seeds an inflection point in the νOH *vs* temperature plot is observed at 37°C, indicative of a glass transition. No clear inflection points were observed in the other mutants and in wild type seeds. Figure 8B shows that in wild type and *abi3-1* seeds WTC remains below 0.22 $cm^{-1}/°C$ throughout the measured temperature range, whereas *abi3-5* seeds exhibit a drastic increase in WTC above 20°C to a maximum of 0.51 $cm^{-1}/°C$ at 55°C. Seeds from the *abi3-7* mutant show a maximum WTC of 0.27 $cm^{-1}/°C$. The WTC reflects the expansion of hydrogen bonding with increasing temperature. Apparently WTC can reach higher values in the more ABA-insensitive mutant

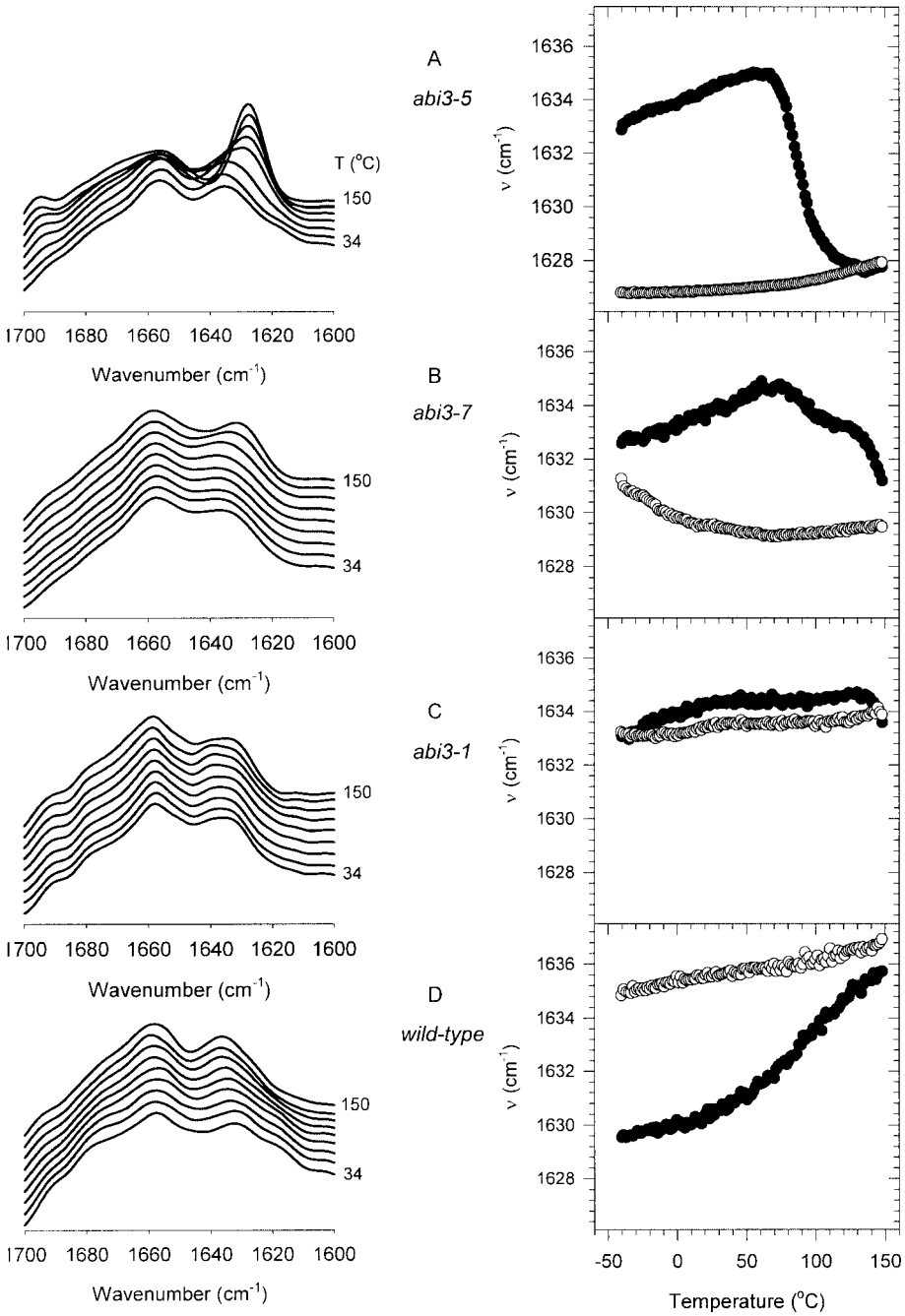

Fig. 7. Deconvolved IR spectra of the 1700–1600-cm^{-1} region (left panels) and wavenumber versus temperature plots (right panels) of the amide-I band denoting turn and β-sheet protein structures in *A. thaliana abi3-5* (panel A), *abi3-7* (panel B), *abi3-1* (panel C) mutant seeds and wild-type (panel D) seeds. The data points in the wavenumber versus temperature plots denote the first (filled circles) and second (open circles) heating scan. The severity of the mutation in the *ABI3* locus (insensitivity to the plant hormone abscisic acid (ABA), which is associated with a reduced level of desiccation tolerance) increases in the order *abi3-1 – abi3-7 – abi3-5*. Data adapted from [69].

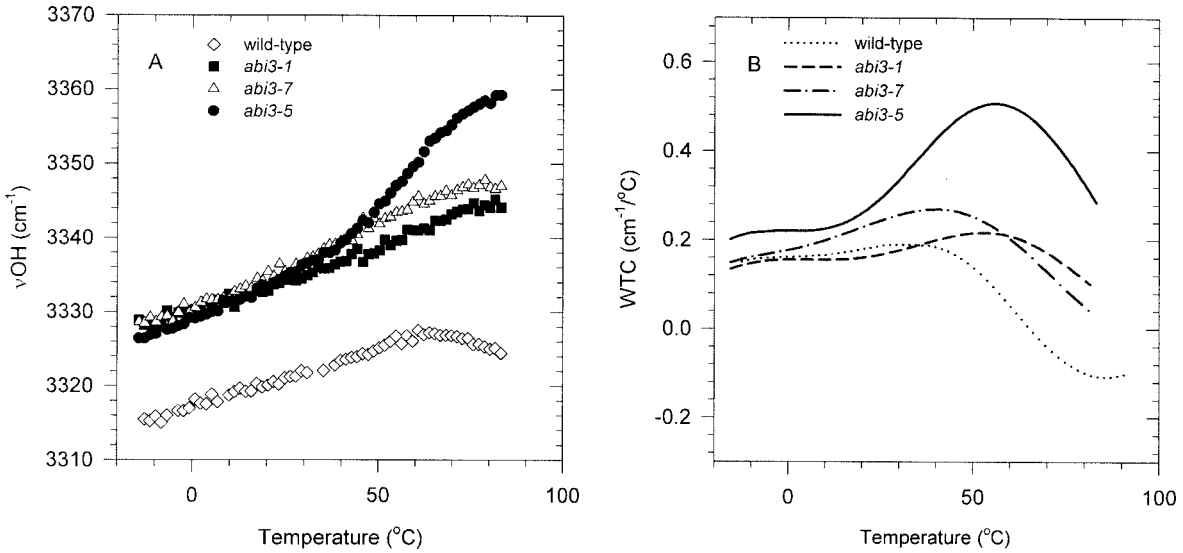

Fig. 8. (A) Wavenumber versus temperature plots of the OH-stretching vibration band in dried *A. thaliana abi3* mutant seeds. The data points represent wild-type, *abi3-1*, *abi3-7* and *abi3-5* seeds. (B) First derivative of the νOH versus temperature plots in (A). Data adapted from [69].

seeds. We conclude that the average strength of hydrogen bonding in the glassy matrix decreases in the order wild type - *abi3-1* - *abi3-7* - *abi3-5*, coincident with a decreased survival in the dry state.

ABA induces changes in the molecular composition and organization of the cytoplasm, which are involved in long-term stability, in particular. The most important of these changes are found in carbohydrate and protein composition. Raffinose and stachyose are synthesized, monosaccharides disappear completely, and maturation-specific proteins, such as LEA proteins are synthesized. The ABA-insensitive mutant seeds have reduced contents of LEA and other maturation-specific proteins, and different sugar composition. *abi3-5* Seeds, for example, have reduced oligosaccharide contents, and lack many of the maturation-specific proteins. We suggest that these maturation-specific proteins increase Tg (we could not determine a clear Tg in the wild-type and the more desiccation-tolerant mutant seeds) and average strength of hydrogen bonding. High WTC values appear to correspond with a generally low heat stability of endogenous proteins in the more extreme *abi3* mutant seeds.

3. Concluding remarks

FTIR analysis has proven to be a very useful method to study conformation and stability of macromolecules in the dry cellular environment of anhydrobiotes. Inspection of the overall protein secondary structure has shown that proteins are generally extremely stable in dried desiccation-tolerant seeds. It is interesting to note that maturation-defective (ABA insensitive) mutant seeds, which lack many of the adaptations that allow for long-term survival in the dry state, also exhibit reduced heat stability of their endogenous proteins. This reduced heat stability in these mutant seeds coincides with reduced strength of hydrogen bonding in the glassy matrix, thus allowing these properties to be used as indicators of life span. Apparently, ABA is involved in the macromolecular stabilization of dried seeds.

The glassy matrix that is formed in dehydrating tissues depends on hydrogen bonding, likely involving sugars and proteins. In pollen and leaves of *Craterostigma plantagineum*, the dried glassy matrix

predominantly consists of sugars. Apart from sugars, maturation-specific proteins such as LEA proteins play an important role in the glassy matrix of seeds, where they may be involved in the molecular immobilisation of the cytoplasm. Proteins increase the average strength of hydrogen bonding and increase Tg. In addition, Buitink et al. [15] have reported that proteins have pronounced effects on the physical properties of intracellular glasses by maintaining a slow molecular motion in the cytoplasm even at temperatures far above Tg. Thus, both sugars and proteins may play a role in the molecular organization of the dried cytoplasm of anhydrobiotes.

Seeds were found to have extremely high protein denaturation temperatures, whereas the denaturation temperature of pollen is considerably lower. An explanation might be that seeds are adapted to survive for considerable longer periods of time in the dry state than pollen. While longevity of seed is of crucial importance for bridging unfavorable growing seasons, pollen may only require short-term desiccation tolerance during its transport from the anther to the receptive stigma.

In summary, *in situ* FTIR studies can give additional information on molecular adaptations associated with the development of desiccation tolerance and longevity, when compared with other biochemical and physiological studies. The added value of this approach is that molecules can be studied in the intact biological system.

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