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Impact of Chemical Stimuli and Temperature on Water Transport and Mobility in Germinating Rape Seeds by Pulsed ^{1}H NMR Spectroscopy

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Pulsed ^{1}H NMR *in vivo* difference spectroscopy and germination were combined to elucidate the effect of exogenously applied estriol or ethanol on water retention in fully hydrated rape seeds. The presence of estriol or ethanol in the imbibition medium resulted in a reduction of the photoreversible phytochrome. An approach to the assessment of cell membrane permeability related to the intracellular water exchange rate, using the bi-exponential feature in the water proton spin–lattice relaxation rate R_1 and spin–spin relaxation rate R_2, is presented. The intracellular water exchange rate increases approximately two- and three-fold in seeds pre-treated with estriol or ethanol solution in comparison with water imbibed seeds. The activation energy for the germination rate was 61 ± 6, 42 ± 4 and 37 ± 4 kJ mol^{-1} and the transition temperature was 281 ± 2, 286 ± 2 and 284 ± 2 K for seeds imbibed with water, estriol and ethanol solution, respectively. The temperature dependence of the intracellular water exchange rate showed comparable values of activation energy and transition temperatures. All the results support the hypothesis that the two effects, thermal activation and increase in the seed cell membrane permeability, combine during water transport into the cell and result in the germination of rape seeds imbibed with estriol or ethanol solution. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Organic solvents provide a useful means of applying prophylactic treatments to stored seeds.[1] Particularly some insecticides or fungicides may be applied to dry seeds using ethanol. Although these treatments are widespread, relatively little is known of the consequences of exposing dry seeds to anaesthetics.

Generally, in terms of biophysics,[2] this problem concerns interactions between chemical or physical stimuli and water flux (water uptake rate)[3] in the early phases of the seed imbibition. Conditions during the initial phases of seed imbibition, when the hydrating membranes are becoming reorganized into a continuous bilayer configuration exhibiting efficient semipermeable properties, are crucial to survival[4] and successful germination. This is because membranes of dry seeds are in a disorganized or non-lamellar state and, as such, form inefficient barriers to the movement[5] of water and solutes. Consequently, excessive rates of water uptake before reorganization may lead to the displacement of membrane components,[6] deleterious mixing of cellular compounds and loss of cellular components through leakage into the aqueous medium.

Seeds are able to modulate the uptake of water by the light absorbed by the photoreceptor of germination,[7] phytochrome. Phytochrome is a red/far-red (R/FR) reversible pigment[8] which exists in two forms: a physiologically inactive red light-absorbing form P_r, which can be transformed by red light into the physiologically active far-red light-absorbing form P_{fr}, which in turn can be transformed by far-red light into P_r. Therefore, detection of phytochrome in plant tissue was made possible by means of dual-wavelength spectrophotometry,[9] following alternate irradiation of plant material with red and far-red light.

Rape seeds covered by a thin permeable coating are suitable for studying the water relationships influenced by physical and chemical stimuli. Preliminary studies showed[10] that the germination process of winter rape is delayed by imbibition in ethanol or estriol solution, depending on the alcohol concentration. Abnormal difference spectra for phytochrome phototransformation were observed in rape seedlings imbibed in the presence of estriol. The need for an adequate interpretation of these effects is emphasized by the current use of pulsed ^{1}H NMR spectroscopy as a putative probe of seed water mobility. In particular, attention has been

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directed towards the contrasting effects produced by a fairly narrow temperature range, on both the germination rate and seed water mobility in the presence or absence of estriol or ethanol.

THEORY

Recently, pulsed ^{1}H NMR methods have become common in studying water properties in cells[11,12] and whole plants.[13–15] It has been found that in spite of the complex composition of cells, the intracellular and the extracellular water fractions can be separated according to the spin–lattice and spin–spin relaxation rates. This provides an opportunity to apply to these systems theories developed by Zimmerman and Brittin.[16]

Let us consider two water fractions a and b existing in seeds. Their properties are characterized by the water proton spin–lattice relaxation rates R_{1a} and R_{1b} and the spin–spin relaxation rates R_{2a} and R_{2b}, respectively. We also assume that P_a and P_b represent the probability that spin can be in state a and b while $P_a + P_b = 1$. We introduce the average lifetime of spin in a and b states as τ_a and τ_b. The reciprocal lifetimes, $1/\tau_a$ and $1/\tau_b$ represent the exchange rate, i.e. probability of transferring a particular state in a given period of time, in compliance with the equilibrium condition $P_a/\tau_a = P_b/\tau_b$. Thermal mobility of spin carriers leads to an exchange between states a and b, which modifies the intrinsic parameters of particular states. As a result, the apparent relaxation rates (R'_{1a}, R'_{1b}, R'_{2a}, R'_{2b}) and the apparent populations of states a (P'_{1a}, P'_{2a}) and b (P'_{2a}, P'_{2b}) depend on the exchange rates represented by the equations

$$2P'_b = 1 - \frac{(P_b - P_a)(R_a - R_b) + 1/\tau_a + 1/\tau_b}{[(R_a - R_b + 1/\tau_a - 1/\tau_b)^2 + 4/\tau_a\tau_b]^{1/2}} \quad (1)$$

$$2R'_a = R_a + R_b + 1/\tau_a + 1/\tau_b - [(R_a - R_b + 1/\tau_a - 1/\tau_b)^2 + 4/\tau_a\tau_b]^{1/2} \quad (2)$$

$$2R'_b = R_a + R_b + 1/\tau_a + 1/\tau_b + [(R_a - R_b + 1/\tau_a - 1/\tau_b)^2 + 4/\tau_a\tau_b]^{1/2} \quad (3)$$

$$P_a' = 1 - P_b' \quad (4)$$

These expressions can be used to solve the reverse problem of finding the intrinsic parameters R_{1a}, R_{1b}, R_{2a}, R_{2b}, P_a and P_b and establishing the lifetimes τ_a and τ_b characterizing the exchange processes. However, the number of variables in Eqns (1)–(4) exceeds the number of equations, which complicates the subject. A similar subject has been solved[17,18] using some simplifying assumptions when $R_b \gg R_a$, $P_a \geqslant P_b$ and $1/\tau_b < R_b$. In order to fulfil the assumption $R_b \gg R_a$, paramagnetic ions have been used.[11,19,20] However, the parameters obtained in this way can be uncertain owing to the approximate nature of the applied equations. Furthermore, we cannot exclude[19] that paramagnetic ions may change the properties of cells and tissues. A simple situation occurs when Eqns (1)–(4) are applied to simultaneous measurements of both spin–lattice and spin–spin relaxation rates. In this case eight independent equations are formed for the eight parameters involved. Otherwise, the intrinsic spin–spin and spin–lattice relaxation rates of water protons and their fractional populations can be calculated in all cases when the ^{1}H relaxation measurements yield decay curves that appear to be optimally described by two exponentials.

In this work Eqns (1)–(4) were used for calculating the exchange rates $1/\tau_a$ of water protons in rape seeds influenced by the previous impact of estriol and ethanol. The effects of temperature on the rate of germination and water proton exchange rate determined by pulsed ^{1}H NMR spectroscopy were compared.

EXPERIMENTAL

Groups of 25 Yet Neuf rape seeds screened for size uniformity, layered on two 4.5 cm blotters in Petri dishes and hydrated with 3 cm^3 of distilled water, estriol solution (18 μM) or ethanol solution (2.5%, v/v) were incubated in a germination chamber at a constant temperature between 278 and 303 K. Estriol [$\Delta^{1,3,5(10)}$-estratiene-3,16α, 17β-triol] was purchased from Koch. Ethanol was of spectral grade. Incubation was performed in the dark except for a brief interval when the observations were made under a dim green light.

The difference spectra for phytochrome phototransformation $P_{fr} \rightarrow P_r$ in the samples were measured using a dual-wavelength spectrophotometer[9] operating with the reference beams set at 800 nm. Measurements were made at 276 K with whole seeds imbibed for 4 h in water, estriol solution (18 μM) or ethanol solution (2.5%, v/v) at 293 K, packed directly into the sample cells. The difference spectra were determined by irradiating seed samples with modified projector lamp assembly equiped with a 250 W quartz iodine lamp (Philips, Eindhoven, The Netherlands) and suitable interference filters (B40, Balzers, Liechtenstein). The total reversible phytochrome was indicated by the change in the optical density reading, ΔOD, following alternating irradiation of the sample with actinic sources of red (660 nm) and far-red light (720 nm). The average concentration of phytochrome in rape seeds can be estimated from the reversible optical density changes of OD values, referred to as Δ(ΔOD).

Prior to each ^{1}H NMR experiment the seeds were gently stirred (0.5 h) in darkness with deionized water, estriol solution (60 μM) or ethanol solution (10%, v/v) and dehydrated in darkness in dry air. Rape seeds screened for size uniformity layered on two 4.5 cm blotters in Petri dishes and hydrated with 3 cm^3 of distilled water were incubated in a germination chamber at 293 K up to the equilibrium water content (24 h). The water proton relaxation rates were measured between 277 and 308 K (±0.5 K) with a Bruker PC 20 pulsed NMR spectrometer operating at 20 MHz. The amplitude of the FID signal (M_0) was measured 60 μs after the first radiofrequency pulse. R'_1 values were determined on the basis of an inversion–recovery sequence (180°–τ–90°). The pulses were separated by a time τ ranging between 3 and 800 ms. The raw data were analysed according to the expression

$$M_0 - M(\tau) = M_0(P'_{2a}\,e^{-2\tau R'_{2a}}\,e^{-2\tau R'_{2b}}) \quad (5)$$

where $M(\tau)$ is the magnetization intensity at time τ and M_0 is the initial magnetization. R'_2 was determined on the basis of a Carr–Purcell–Meiboom–Gill sequence (90°–τ–180°); τ was 200 μs. The raw data were analysed according to the spin–echo amplitude $M(2\tau)$ expression

$$M(2\tau) = M_0(P'_{2a}\,e^{-2\tau R'_{2a}} + P'_{2b}\,e^{-2\tau R'_{2b}}) \qquad (6)$$

The longitudinal relaxation fractional populations (P_1') and longitudinal relaxation rates (R'_1) and transverse relaxation fractional populations (P'_2) and transverse relaxation rates (R'_2) are identified with subscripts *a* and *b*. The faster longitudinal and transverse relaxation rate component is arbitrarily designated *b*. The data were analysed using a non-linear least-squares curve-fitting procedure. Depending on the expected R'_1, the pulse repetition rate was set to 1–0.2 s^{-1}. Each point was the triplicate mean combined from four separate trials. The estimated standard deviation of the experimental points was not higher than 5%. The equilibrium sample water content (after 24 h of imbibition) in ^{1}H NMR experiments measured gravimetrically by drying in a vacuum oven at 353 K for 19 h was equalled 0.62 ± 0.03 g water g dry^{-1} mass basis.

In the case of germination measurements, groups of 25 rape seeds screened for size uniformity, layered on two 4.5 cm blotters in Petri dishes hydrated with 3 cm^3 of distilled water, estriol solution (18 μM) or ethanol solution (2.5%, v/v) were placed in plastic containers. These containers, wrapped with black paper to reduce evaporation and placed in light-tight wooden boxes, were incubated in a germination chamber at a defined constant temperature between 278 and 303 K (±0.5 K). The relative number of germinating seeds was determined using embryo axis growth (2 mm radicle) as a criterion. For 1–15 days seeds were scored for radicle emergence every 12 h and germinated seeds were removed from the containers. A complete combination of water, estriol or ethanol imbibed samples of seeds was employed with three replications.

A comparison of the effect of temperature on germination rate was made by calculating the germination rate k_G as the reciprocal time for half of the final germination value.[7] Incubation was performed in the dark except for a brief interval when the observations were made under a dim green light.

RESULTS

The difference spectrum between $P_{fr} \rightarrow P_r$ in rape seeds after imbibition with water (curve 1), estriol solution (curve 2) or ethanol solution (curve 3) is shown in Fig. 1. The presence of estriol or ethanol in the imbibition medium resulted in the reduction of photoreversible phytochrome [expressed in $\Delta(\Delta A)$ units].

Owing to the presence of P_{fr}, the Yet Neuf rape seeds germinate in darkness. Figure 2 shows examples of the germination of Yet Neuf rape seeds at (a) low and (b) high temperature. The results indicate that both estriol and ethanol stimulate rape seed germination at low temperatures but inhibit it at high temperatures.

The Arrhenius plot presented in Fig. 3 gives a

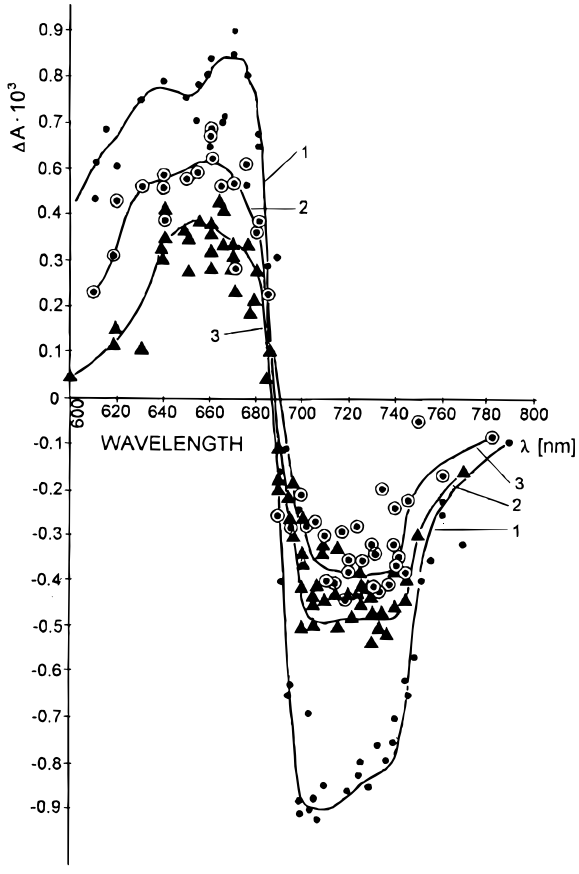

Figure 1. Phototransformation difference spectrum ($P_{fr} \rightarrow P_r$) of phytochrome in rape seeds after 4 h of imbibition at 293 K with (1) water, (2) estriol solution and (3) ethanol solution.

detailed description of the effect of temperature and exogenously applied chemical stimuli. This relationship consists of two lines with different slopes. The two lines intercept was at a temperature $T = 281 \pm 2$ K. As a result of the interaction with exogenously applied estriol or ethanol, the intercept of the two straight lines shifted to 286 ± 2 and 284 ± 2 K, respectively. The activation energy for the germination process in the temperature range below the transition points was 61 ± 6; 42 ± 4 and 37 ± 4 kJ mol^{-1} for water, estriol and ethanol pretreated rape seeds, respectively. At higher temperatures the activation energy decreased to 15 ± 2, 10 ± 2 and 17 ± 2 kJ mol^{-1}, respectively.

Two-phase behaviour for both longitudinal and transverse relaxation corresponding to two regions (*a* and *b*) of different water mobility were observed in the entire temperature range investigated (Figs 4–6).

The apparent populations P'_{1a} measured in the spin–lattice experiment are similar to the P'_{2a} values measured in the spin–spin experiment (Fig. 4). Therefore, apparent populations with lower R'_1 (namely R'_{1a}) and lower R'_2 (namely R'_{2a}) correspond to the same environment. Consequently, a group of nuclei *b* with a higher R'_1 has a higher R'_2 value. These population parameters, particularly R_2, also exhibit a readily discernible temperature dependence (Fig. 4), suggesting that they are predominantly determined by a thermally activated process such as nuclear transfer.

The most significant clue to the interpretation of the

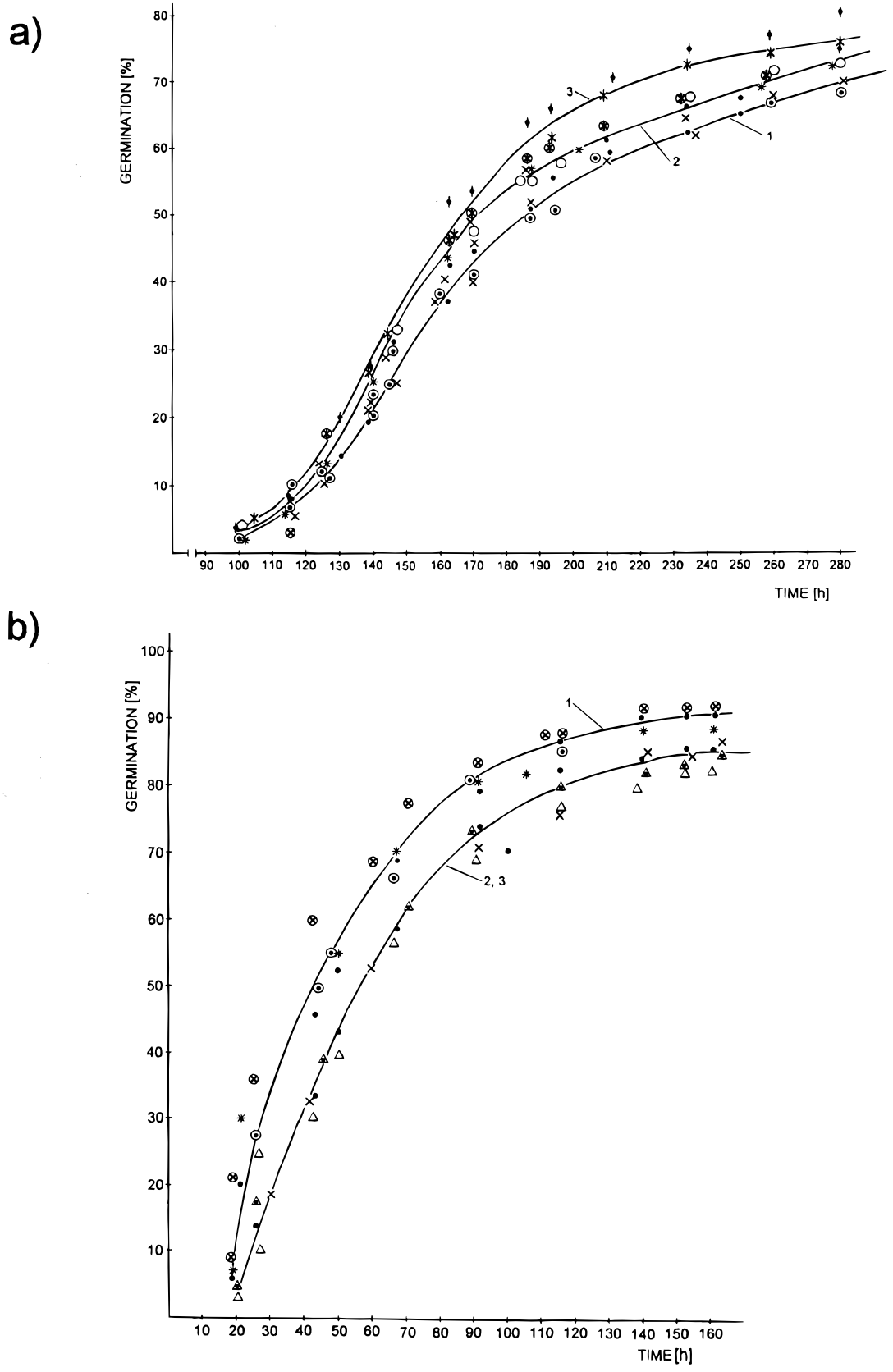

Figure 2. Time course of germination following imbibition in (1) water, (2) estriol solution and (3) ethanol solution in a germination chamber at (a) 278 and (b) 295 K.

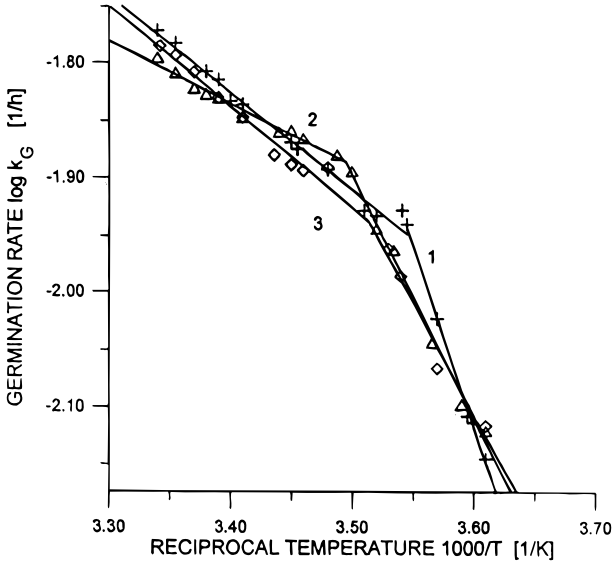

Figure 3. Arrhenius plot of the germination rate k_G in rape seeds imbibed with (1) water, (2) estriol solution and (3) ethanol solution.

data in Figs 4–6 is the observation that the lower longitudinal and transverse relaxation fractional populations P'_{1a} and P'_{2a} (Fig. 4) and relaxation rates R'_{1a} [Fig. 5(a)] and R'_{2a} [Fig. 6(a)] increase with increase in temperature. A similar temperature dependence has been observed in the case of hydrated silica gel.[17] According to the proposed mechanism,[16] these observations proved that a temperature increase results in an increase in the nuclear transfer rate between two different environmental states and causes nuclei of the lower R_1 and R_2 states to relax more rapidly by placing them in the higher R'_1 and R'_2 states. However, this analogy cannot be extended to the b water fraction because in the case of seeds the R'_{1b} [Fig. 5(b)] and R'_{2b} [Fig. 6(b)] values increase with increase in temperature, approach a maximum and subsequently decrease, whereas for silica gel these parameters appeared to be nearly independent of temperature. A possible explanation of this observation is that R'_{1b} and R'_{2b} are influenced by the changes in the physical properties of the different biopolymers forming a plasmalemma/cell wall barrier for water flow.

The intrinsic fractional population P_a determined on the basis of Eqns (1)–(4) appeared to be nearly independent of temperature but slightly influenced by pretreatment. The values were 0.23, 0.25 and 0.29% for water, estriol and ethanol pre-treated seeds, respectively.

Fig. 7 illustrates the main effect in the form of an Arrhenius plot for the exchange rate $1/\tau_a$. Exogenously applied estriol or ethanol influences the exchange rate of the seed water population a. The activation energy of exchange rate for the water population a was 33 ± 5, 19 ± 4 and 17 ± 3 kJ mol^{-1} in water (curve 1) estriol (curve 2) and ethanol (curve 3) pre-treated rape seeds, respectively, calculated on the basis of this plot. Figure 7 shows that the transition in exchange rate for water population a occurs at 283, 288 and 285 K in water (curve 1), estriol (curve 2) and ethanol (curve 3) pretreated rape seeds, respectively.

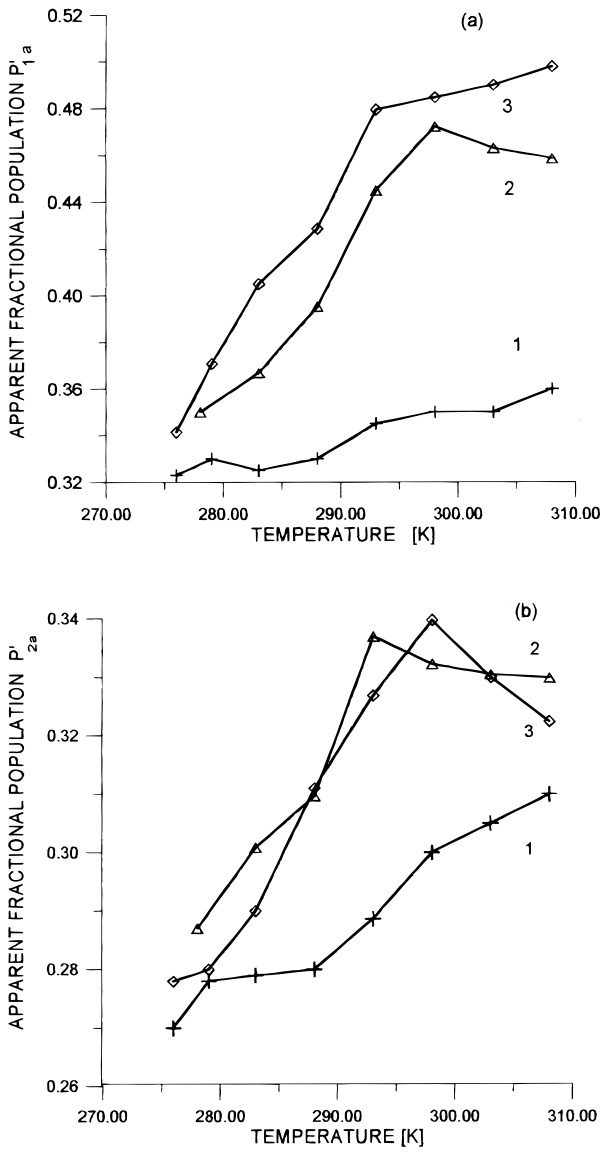

Figure 4. Temperature dependence of the apparent fractional populations of protons, P'_{1a} and P'_{2a}, in the low (a) R'_1 and (b) R'_2 state of water, respectively, absorbed by rape seeds following 24 h of imbibition at 293 K with (1) water, (2) estriol solution and (3) ethanol solution pre-treatment.

DISCUSSION

There are two important early events in germination—the formation of new membranes and transformation of existing membranes—which allow for changes in permeability to water and gases. The first phase of germination is imbibition. Imbibition of seeds which do not exhibit a dormancy mechanism initiates a sequence of events which result in the mobilization of food reserves to the embryo, elongation and often division of cells in the embryo and protrusion of the radicle through surrounding layers.

Alcohols may have at least two types of damaging effects on seed membranes:[6] (1) randomization of the configurations of proteins associated with membranes and (2) penetration into the lipid components of the

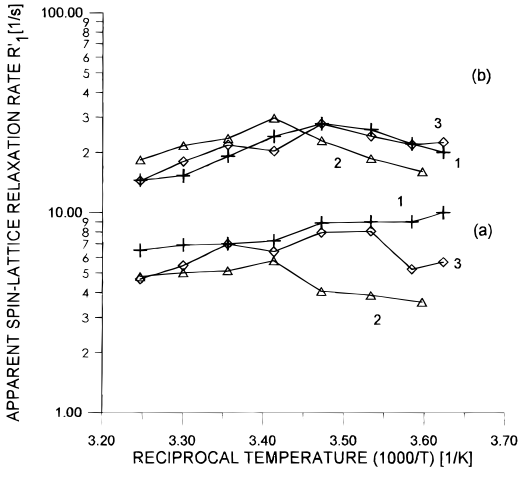

Figure 5. Temperature dependence of the apparent proton longitudinal relaxation rate for the (a) low R'_{1a} and (b) high R'_{1b} states of water absorbed on rape seeds following 24 h of imbibition at 293 K with (1) water, (2) estriol solution and (3) ethanol solution pre-treatment.

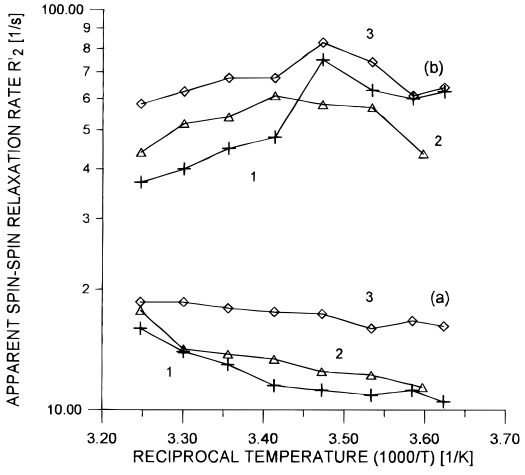

Figure 6. Temperature dependence of the apparent proton transverse relaxation rate for the (a) low R'_{2a} and (b) high R'_{2b} states of water absorbed by rape seeds following 24 h of imbibition at 293 K with (1) water, (2) estriol solution and (3) ethanol solution pre-treatment.

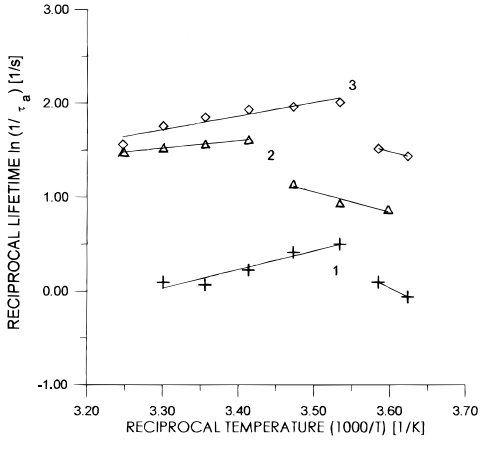

Figure 7. Temperature dependence of the exchange rate $1/\tau_a$ for the population of protons in the low R_1 and R_2 state, calculated from Eqns (1)–(4) using experimental spin–lattice relaxation data (Figs 4 and 5) and spin–spin relaxation data (Figs 4 and 6) of water absorbed by rape seeds following 24 h of imbibition at 293 K with (1) water, (2) estriol solution and (3) ethanol solution pre-treatment.

membranes, disrupting the lipid arrangement and even removing phospholipids from the cell. Although the mode of action of steroids in higher plants has not received much attention, one physiological study suggested[21] that these compounds influence the plant cell membrane.

The hydrated rape seeds were analysed with regard to the exogenously applied chemical stimuli (estriol and ethanol) in terms of physical parameters estimated from spectroscopy *in vivo* and pulsed ^{1}H NMR experimental data.

Pulsed ^{1}H NMR measurements on rape seeds revealed two populations of protons, each with a different magnetic environment that causes a different relaxation rate. The relaxation rates are too low to be accounted for by lipid and protein macromolecules,[22] and must reflect different water proton categories. These two populations may correspond to water molecules differing in mobility, such as free and bound water.[23] Several workers have already exploited this feature in attempts to determine tissue water partitioning.[22,24] A general conclusion is that high relaxation rates relate to extracellular water and low relaxation rates to intracellular water compartments. Intracellular and extracellular water mutually exchange[25] across the plasma membrane and the rate of exchange depends on the plasma membrane permeability. For example, low-temperature injury has been linked[20] with an increase in the exchange rate across the cell membrane of non-acclimated wheat crowns.

As the estriol or ethanol probably cause changes in the lipid composition paralleled by membrane injury (alcohol stress), it was reasonable to investigate the intracellular water exchange rate $1/\tau_a$ which is a marker of all the effects.

The present study offers an approach concerning the assessment of cell membrane permeability related to the intracellular water exchange rate using the bi-exponential feature in the water proton spin–lattice relaxation rate R_1 and spin-spin relaxation rate R_2. Generally, exogenously applied estriol and ethanol increase the intracellular water exchange rate. Figure 7 shows that the exchange rate of water through the seed cell membrane is between two and three times higher in estriol or ethanol pre-treated seeds than in water-imbibed seeds. It is possible that these cases correspond to changes in the lipid composition of the seed cell membrane; exogenously applied compounds are metabolized.

The temperature dependence of the water exchange rate through the seed cell membranes is characterized by values of the apparent activation energy similar to those of the rotational motion or the diffusion of water in mono- or submonolayers of adsorbed water.[17] The values lie between the activation energy of molecular rotation in ice[26] and that of liquid water[27] and are similar to the activation energy of water in the adsorbed state in other systems having statistical mono- or submonolayer coverage water.[28] The permeability of biological membranes has been explained in terms of aqueous pathways dispersed throughout a matrix of hydrophobic lipids. Even though the properties of the seed membranes are probably a function of the imbibition progress, it is reasonable to assume that water

crosses the lipid part of the membrane by diffusing through some dynamic aqueous pathways.[29] The activation energy results suggest that the mobility of seed water molecules during the passage across the membrane is similar to that of molecules in the adsorbed state.

It has been found that the exchange rate of water through seed cell membranes increased at 293 K approximately two- and three-fold in seeds with estriol or ethanol solution pre-treatment, respectively (Fig. 7). An inhibitory effect of both treatments on the phototransformation of phytochrome (Fig. 1) and on the rate of germination has been observed at 293 K [Fig 2(b); see Fig. 3 for comparison]. Although the seed cell permeability is still higher (Fig. 7), both chemical stimuli increase the germination rate at 278 K [Fig. 2(a); see Fig. 3 for comparison]. The temperature dependence of both the intracellular water exchange rate and germination manifested intercepts of two lines at 281, 286 and 285 K for water, estriol end ethanol, respectively. Such effects have been linked[30,31] to the phase transitions in the seeds cell membranes and/or to the changes in the cell membrane fluidity.[32] For example, the ability of ethanol to increase directly the fluidity of membranes *in vitro*[33] has been invoked to explain many of its pharmacological effects.

The possible molecular nature of the binding of the photoreceptor with cellular membranes was examined[34] and it was concluded that the physiological activity of the phytochrome may be associated with membrane integrity.[35] The effect of estriol and ethanol present in the incubation medium on rape seed germination [Fig. 2; see Fig. 3 for comparison] and phytochrome phototransformation in the early stages of seed imbibition (Fig. 1) implies that (1) both applied stimuli interact with the germination photoreceptor and (b) the above effects might have common origin.

Overall, the results of pulsed ^{1}H NMR and difference spectroscopy *in vivo* are consistent. The simplest explanation to the results is that estriol (similarly to ethanol) increases the cell membrane permeability of rape seeds. This in turn increases the water uptake rate. However, an increase in seed membrane permeability followed by an increase in water uptake rate induces different effects on the germination: the estriol or ethanol pre-treatment accelerates rape seed germination at low temperatures but retards it at high temperatures.

In other words, the two effects, thermal activation and increase in seed membrane permeability, combine during water transport into the cell. At low temperatures, when water uptake is a limiting factor in the case of germination, exogenously applied chemical stimuli can promote seed germination. At high temperatures, these two effects combine and consequently a very high flux of the water uptake into the plant cell can occur. This in turn disturbs the spatial and temporal organization of the active enzymes and results in delayed germination.

A simultaneous analysis of all the experiments performed determines the theoretical parameters and places a stringent criterion of self-consistency on the overall analysis of the effects of both chemical stimuli on the water transport and mobility in germinating rape seeds.

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