Plant Physiol. 76, 155–160
0032-0889/84/76/0155/06/$01.00/0

Control of Seed Germination by Abscisic Acid[1]

II. EFFECT ON EMBRYO WATER UPTAKE IN *BRASSICA NAPUS* L.

Received for publication November 22, 1983 and in revised form March 20, 1984

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ABSTRACT

Germination of rape (*Brassica napus* L.) seeds proceeds in two phases, an initial imbibition phase and a subsequent growth phase. The time courses of water uptake, O_2 uptake, and ATP accumulation demonstrate that exogenous abscisic acid (ABA, 0.1 millimoles per liter) specifically prevents the embryo from entering the growth phase. The inhibition of water uptake by ABA is a rapid (lag-phase about 1 hour) and fully reversible process which appears to be the cause rather than the result of changes of the energy metabolism. In untreated seeds, an osmotic pressure (polyethylene glycol 6000) of 11 bars is required for a simulation of the ABA effect on water uptake. However, in ABA-treated seeds an osmotic pressure of only 3 bars is sufficient to suppress water uptake. Thus, ABA lowers the ability of the embryo to absorb water under osmotic stress. In a two-factor analysis of the simultaneous action of ABA and osmoticum on germination, a complete synergistic interaction between these factors was found while ABA and cycloheximide exhibit independent (multiplicative) coaction. These results are interpreted in terms of a common controlling point of ABA and osmotic stress in the water relations of germinating seeds.

The action mechanism of ABA, a potent physiological inhibitor of seed germination, is not yet understood. In a previous paper (9) we suggested that the hormone induces seed dormancy by restricting water uptake of the imbibed seed during a critical period of germination, where an active push of embryo expansion is needed to support embryo growth. This hypothesis implies that the biochemical changes previously related to the primary action of ABA such as the modification of nucleic acid or protein synthesis (*e.g.* 1, 2, 4, 10) are not direct causal links in the action mechanism of ABA but merely later consequences of the growth inhibition through restricted water uptake.

In the present communication we attempt to clarify the role of ABA in water uptake of germinating rape seeds. This type of seed is similar to the previously used mustard seed but lacks the mucilaginous seed coat which makes the study of embryo water relations in mustard rather cumbersome. In contrast to mustard, the very thin and brittle testa of rape absorbs only insignificant amounts of water and is rapidly ruptured by the swelling embryo soon after imbibition. Rape produces nondormant seeds which require only imbibition at a suitable temperature (*e.g.* 25°C) for rapid germination. There is no indication of physiologically significant levels of endogenous ABA in the mature rape seed. Exogenous ABA inhibits the completion of germination of these seeds in much the same way as described for mustard (9). As outlined in detail in the previous report (9), we define germination as the period from the increase of metabolic activity after imbibition of the dry seed up to the 'point of no return' which marks the loss of desiccation tolerance of the embryo. ABA prevents completion of germination by arresting embryo growth specifically before the point of no return is reached. This ABA-induced dormancy can be broken (*i.e.* germination can be induced) by washing the seeds briefly with water.

[1] Supported by the Deutsche Forschungsgemeinschaft (SFB 206).

MATERIALS AND METHODS

Preparation of Plant Material. Seeds of *Brassica napus* L. cv Diamant (winter rape, purchased in 1979 from Hambrecht, Freiburg, Germany) were selected for uniformity and sown using standard conditions. Batches of 570 mg dry mass (about 110 seeds) were placed on five layers of Schleicher and Schüll chromatography paper (No. 2043b Mgl, 8 × 8 cm) soaked with distilled H_2O (or a solution) in closed plastic boxes (10 × 10 × 6 cm). After adding further 5 ml of liquid, a small meniscus formed around the seeds allowing uniform wetting of the seeds without impairing gas exchange. Germination took place in darkness at 25.0 ± 0.3°C. Since germination was insensitive to light, all further manipulations were performed in normal laboratory light. Before transferring to a different medium, the seeds were thoroughly washed by incubation for 10 min under vigorous agitation in 100 ml of new medium. Except in Figure 8, ±ABA (Fluka AG, Buchs, Switzerland) was used at a concentration of 25 mg l^{-1} (=0.1 mmol l^{-1}), which was sufficient to inhibit germination completely for at least 5 d (see concentration response curve for 0 bar in Fig. 8). CHI[2] was obtained from Serva Entwicklungslabor (Heidelberg, Germany). The osmotic pressure (at 25°C) of PEG solutions (PEG 6000; Roth, Karlsruhe, Germany) was adjusted using the formula of Michel and Kaufmann (7). A calibration curve relating refractive index to osmotic pressure was used to check for any changes of osmotic pressure during experiments. Seeds incubated in PEG solution of 20 bars for 7 d rapidly germinated and produced perfectly normal seedlings, indicating that PEG had no side effects besides controlling the water status of the seeds. Germination media containing PEG were routinely replaced every 24 h. Due to water uptake of the seeds and to evaporation, the osmotic pressure of the media increased slightly (less than 10%) during this time.

Analytical Methods. The percentage of seeds with broken testa and of seeds with growing embryo axis (radicle protruding for more than 2 mm) was determined at suitable intervals. Oxygen uptake of intact seeds was determined with an oxygen electrode (Rank Bros, Bottisham, UK) using samples of 10 seeds in 3 ml of distilled H_2O (or ABA solution) equilibrated with air at 25°C. ATP content was determined by the luciferin-luciferase assay (Firefly lantern extract; Sigma, München, Germany) after grinding 20 seeds in liquid nitrogen and extracting the powder with

[2] Abbreviations: CHI, cycloheximide; π_o, osmotic pressure of the germination medium.

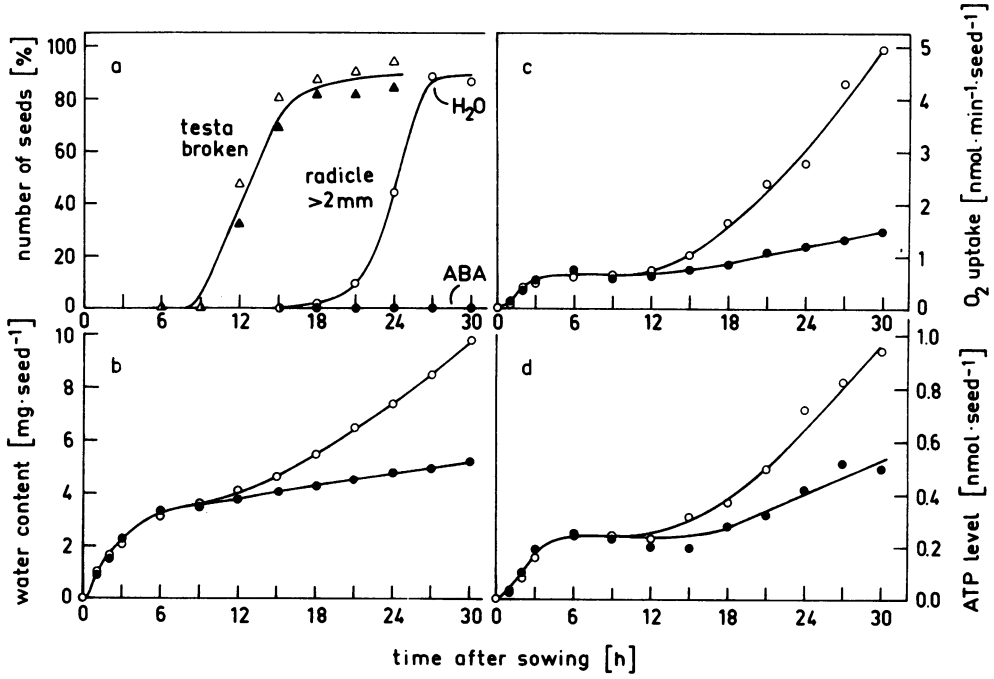

FIG. 1. Time course of germination as measured by (a) rupturing of the testa and onset of seedling axis growth, (b) water uptake, (c) respiration increase, and (d) ATP accumulation, in rape seeds incubated on water (○, △) and ABA solution (0.1 mmol l^{-1}; ●, ▲) from sowing onwards. (Four repetitions, SE ± 4 to 10%.)

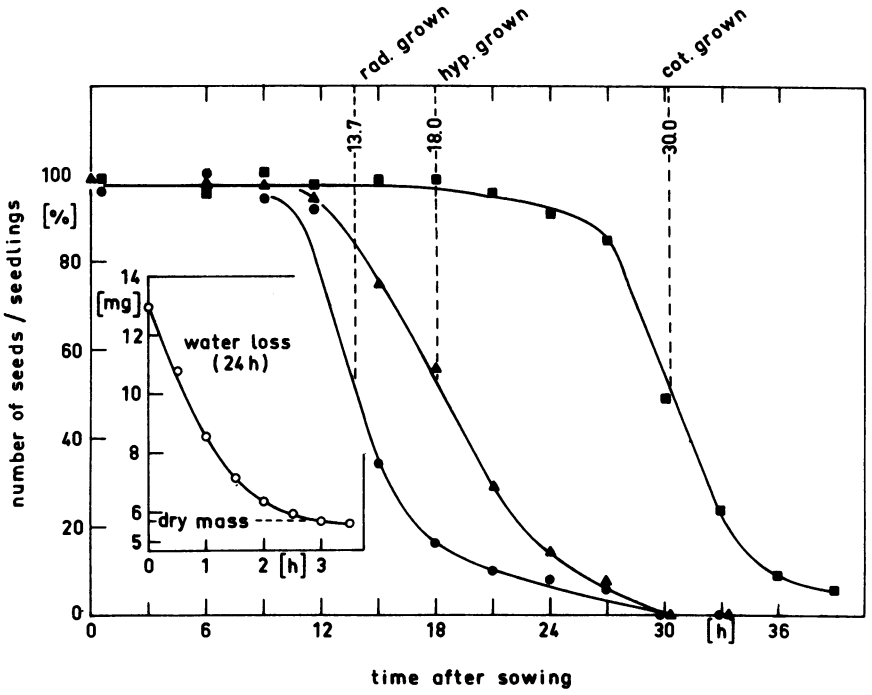

FIG. 2. Disappearance of desiccation tolerance ('point of no return') in the three organs of germinating rape embryos. Seeds were sown on water. At the times indicated on the abscissa, the germination process was interrupted by rapidly redrying the seeds at 25°C on silica drying beads (half-time about 40 min; the inset shows the drying kinetics after 24 h of germination). The ordinate indicates the percentage of embryos still able to increase visibly in radicle length (●), hypocotyl length (▲), or cotyledon area (■) upon resowing, determined after 96 h of incubation. The broken lines indicate the time points where half-final values are reached in the three organs. (Five repetitions, SE ± 8 to 12%.)

10 ml of boiling Pradet buffer (pH 7.4) according to Refs. 3 and 8. For the determination of fresh mass, seed batches (570 mg dry mass) were briefly blotted between two sheets of filter paper and weighed within 10 s using an electronic balance (±1 mg). The water content was calculated by subtracting the original dry mass of 570 mg (which did not change significantly during the first 24 h of germination) from the fresh mass. Desiccation tolerance was determined as described previously (9).

Each group of experiments shown on one figure or table was performed with seed batches (110 seeds) treated in parallel and repeated *in toto* as indicated in the legends.

RESULTS AND DISCUSSION

Inhibition of Germination Process by ABA. Based on water uptake, the time course of germination of many dicotyledonous seeds upon transfer to water can be divided into two phases, an initial imbibition phase and a later growth phase. In rape seeds the imbibition phase (period of physical swelling, until about 6–9 h after sowing) is accompanied by the establishment of a basic metabolic activity which starts very soon after sowing (Fig. 1). About 12 h after sowing, the seed enters the growth phase during which the embryo organs reach the 'point of no return', *i.e.* the irreversible loss of desiccation tolerance (9). This germination period proper is characterized by a second rise of water uptake and a corresponding respiration increase indicating active embryo expansion (=growth). Shortly thereafter, radicle elongation provides macroscopic evidence for successful germination (Fig. 1a). As previously shown for mustard (9), the disappearance of the ability to survive redrying (*i.e.* the point of no return) occurs at different times in the three seedling organs, starting with the

Table I. *Conservation by ABA of the Ability to Survive Desiccation*

Seeds were sown on ABA (0.1 mmol l^{-1}), dried back to their dry mass within 3 h on silica drying beads, and resown after thorough rinsing with water. Germination (radicle length >2 mm) was determined 3 d later. All germinated embryos developed into normal seedlings. The experiment was repeated four times.

Treatment after Sowing	Germinated
	%
3 d ABA, drying, 3 d water	75 ± 4
3 d water (control)	84 ± 2

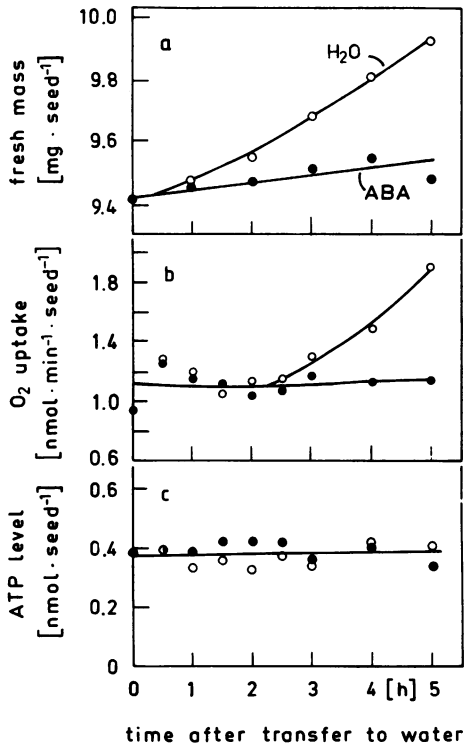

FIG. 3. Time course of the reversion of the ABA effect on (a) water uptake, (b) respiration, and (c) ATP level. Seeds were sown on ABA solution (0.1 mmol l^{-1}) and transferred to water (or ABA) 3 d after sowing. (Five repetitions, SE ± 3 to 7%.)

radicle and ending with the cotyledons (Fig. 2).

As in mustard, ABA inhibits specifically the initiation of the growth phase of germination, *i.e.* the passing of the point of no return in the radicle (Table I). However, Figure 1 strongly suggests that neither water uptake nor energy metabolism as such are direct targets of ABA inhibition. It rather appears that the effects of ABA on these parameters are merely indicator responses for a more central change which prevents the fully imbibed embryo from entering into the growth phase.

The effect of ABA on water uptake can be separated from its effects on energy metabolism. Short-term kinetics after removal of the hormone from inhibited seeds indicate that the resumption of water uptake starts after a lag of less than 2 h, preceding a measurable change in respiration by about 2 h. Furthermore, there is no measurable change of the ATP level within 5 h after removal of ABA (Fig. 3). It appears, therefore, that the effect of ABA on energy metabolism is a relatively late event, compared with the effect on water uptake.

Effect of ABA on Water Uptake Kinetics. Detailed measurements of the water influx kinetics during phase 2 of germination in the presence and absence of ABA are shown in Figure 4. The hormone adjusts the rate of water uptake to a much reduced

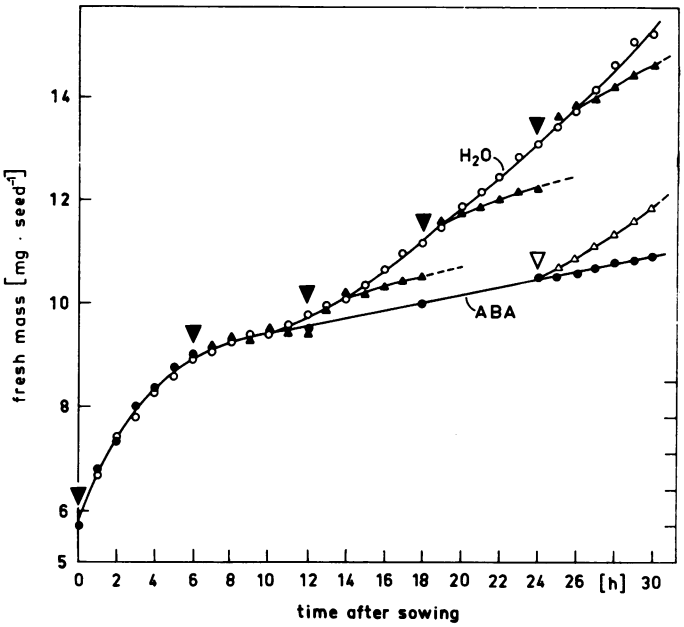

FIG. 4. Control of water uptake of germinating seeds by ABA. Seeds were sown on water (O) or ABA (●; 0.1 mmol l^{-1}) and transferred to ABA (after 6, 12, 18, 24 h; ▲) or to water (after 24 h; △). (Four repetitions, SE smaller ± 2%.)

value after less than 2 h. Likewise, removal of ABA by simply washing the ABA-treated seeds for 10 min with distilled H_2O readjusts water uptake to the rate of the water control after less than 2 h.

Even after prolonged treatment with ABA (*e.g.* for 3 d; Fig. 3a), removal of the hormone results in a rapid resumption of water uptake. Since the ABA-treated seeds are fully saturated with water (with respect to physical swelling) and all seed coats are ruptured under these conditions (Fig. 1a), such seeds are particularly suited for the elaboration of precise short-term water exchange kinetics of the embryo. Figure 5 shows a set of ABA/water transfer kinetics demonstrating the rapidity (lag-phase of about 1 h in both directions) and reversibility of ABA action on water uptake. These data, confirming our previous results with mustard (9), indicate that the regulatory processes involved in ABA control operate symmetrically in switching water uptake on and off and exhibit a low degree of hysteresis. This makes it rather unlikely that time-consuming metabolic processes, producing relatively stable products (such as the synthesis of enzymes) are directly involved in the action mechanism of ABA.

Germination of rape seeds can be inhibited, within a period of 3 d after sowing, by an external osmotic stress of ≧10 bars (Table II). Additional experiments (data not shown) demonstrated that seeds kept on water for 12 or 24 h after sowing

Table II. *Effect of Osmotic Stress (π_o = 0–12 bars) on Germination*

Seeds were sown on PEG solutions. Germinated embryos (radicle length >2 mm) were counted after incubation for 3 d. The experiment was repeated four times.

π_o	Germinated
bars	%
0	95 ± 2
4	94 ± 3
6	96 ± 2
8	80 ± 3
10	38 ± 4
12	6 ± 1
14	1 ± 2

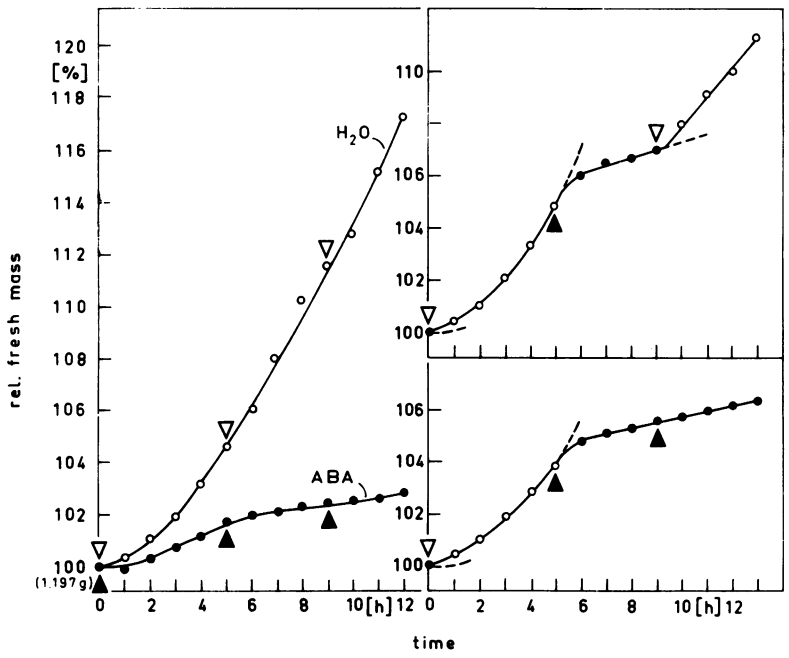

FIG. 5. Effect of ABA on water uptake kinetics. Seeds were sown on ABA solution (0.1 mmol l^{-1}) and transferred at 72 h after sowing (= time zero in the figure) to new germination paper soaked with water (O) or ABA (●). After 5 and 9 h, the germination medium was again changed (▽, transfer to water; ▲, transfer to ABA). Before each transfer, the seeds were submersed in 100 ml of fresh medium for 10 min under vigorous agitation. (Three repetitions, SE smaller ± 0.5%.)

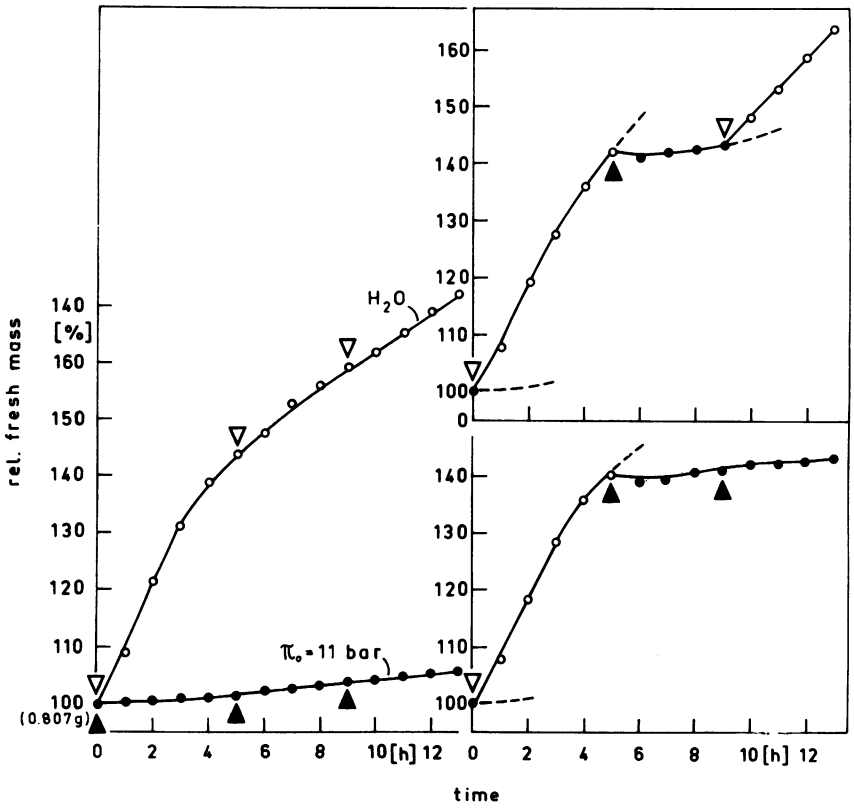

FIG. 6. Effect of π_o on water uptake kinetics. Seeds were sown on PEG solution (11 bars) and transferred at 72 h after sowing (= time zero in the figure) to new germination paper soaked with water (O) or PEG solution (●). Transfers between water and PEG as in Figure 5. (Three repetitions, SE smaller ± 0.5%.)

require likewise a π_o of about 10 to 11 bars for a complete inhibition of water uptake, indicating that the whole seed is representative for the visibly germinating embryo radicle with respect to the water uptake under osmotic stress. The effect of ABA on water uptake can be closely mimicked by a PEG solution of an osmotic pressure of 11 bars (Fig. 6). However, in contrast to Figure 5, there is no measurable lag-phase of water exchange after such an osmotically induced transition, indicating that the lag observed in the experiments of Figure 5 is not due to experimental handling of the seeds and/or any physical impediment of water flux by permeation barriers. It may be concluded, therefore, that the transport of ABA to its site of action in the embryo and the signal transduction chain connecting the site of primary hormone action (ABA receptor) to water uptake require together a time period of about 1 h.

Although the kinetics of Figures 5 and 6 appear to be similar, there is a striking difference between these two experiments. Whereas the osmotically inhibited seeds require a π_o of 11 bars for essentially complete inhibition of water uptake (Fig. 6), a π_o of only 3 bars is required to establish a similar situation in the ABA-pretreated seeds (Fig. 7). This result shows that ABA dramatically lowers the ability of the seeds to cope with osmotic stress.

Interaction of π_o and ABA in Inhibiting Germination. The

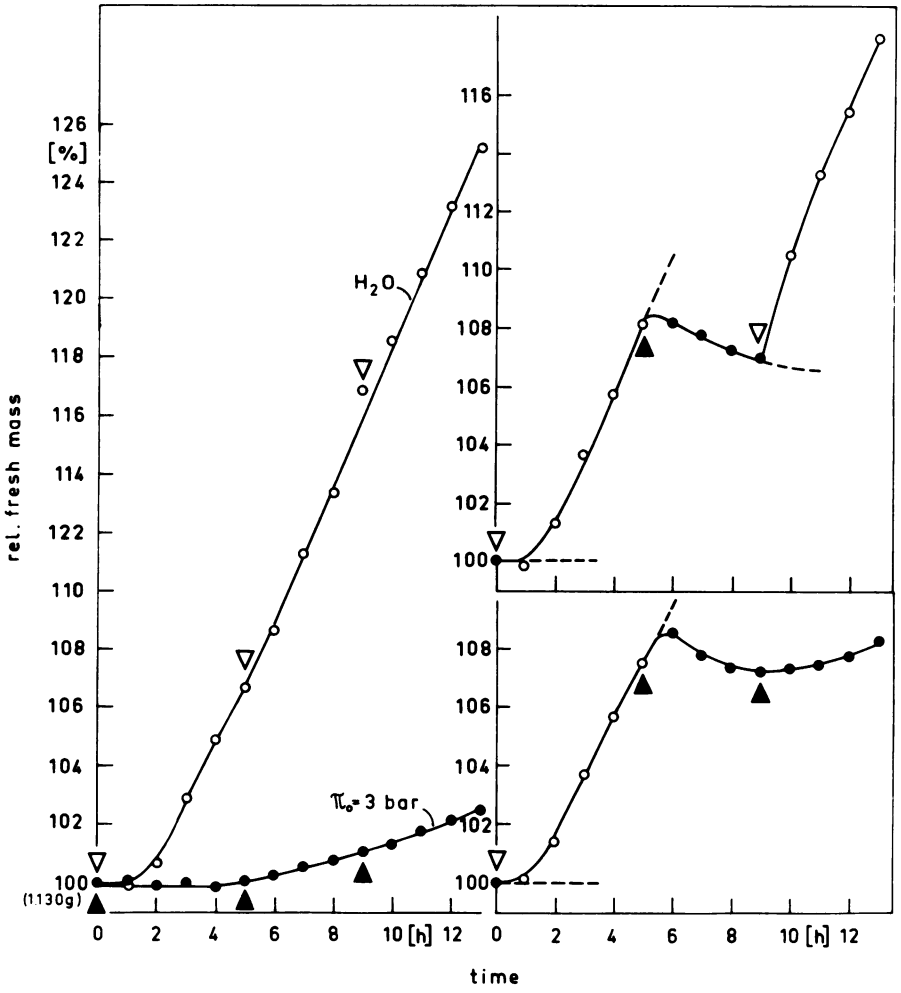

FIG. 7. Effect of π_o on water uptake kinetics in ABA-pretreated seeds. Seeds were sown on ABA solution (0.1 mmol l^{-1}) and transferred at 72 h after sowing (= time zero in the figure) to new germination paper soaked with water (O) or PEG solution (3 bars; ●). Transfers between water and PEG as in Figure 5. (Three repetitions, SE smaller ± 0.5%.)

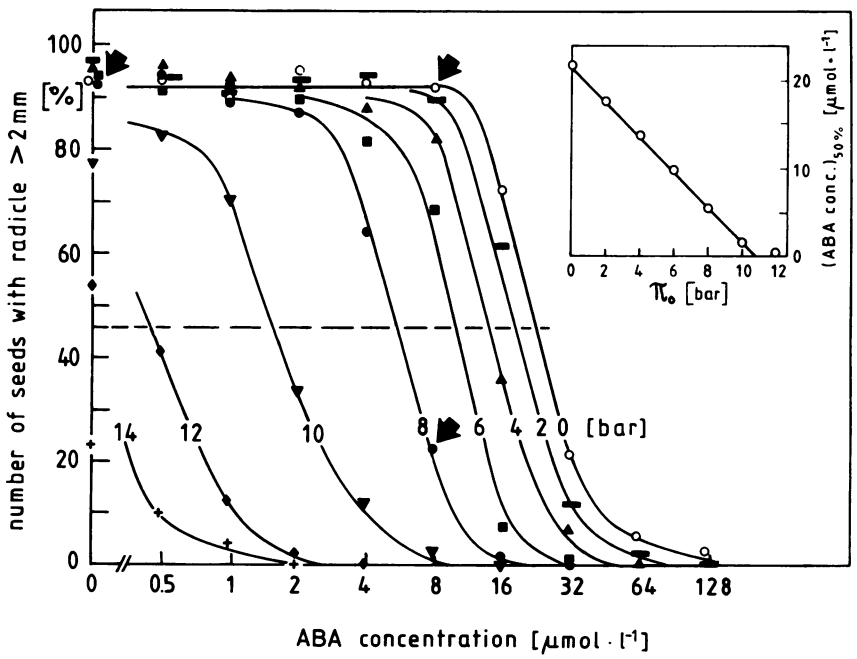

FIG. 8. Inhibition of germination by ABA and osmoticum (two-factor analysis). Seeds were sown in various combinations of ABA concentrations (indicated on the abscissa) and PEG solutions adjusted to different osmotic strengths (π_o [bar], indicated at the curves). Germination percentages were counted after 3 d, where germination was essentially complete in all treatments. The dashed line indicates 50% inhibition based on the germinable seed population (about 92% of the total population). The arrows at π_o = 8 bars / 8 µmol ABA l^{-1} illustrate the effect of additive subthreshold factor strengths: each factor strength is ineffective if applied alone but simultaneous application leads to 80% inhibition. The inset illustrates the linear decrease of sensitivity toward ABA (with respect to 50% inhibition) with increasing osmotic strength. (Five repetitions, SE ± 3 to 5%.)

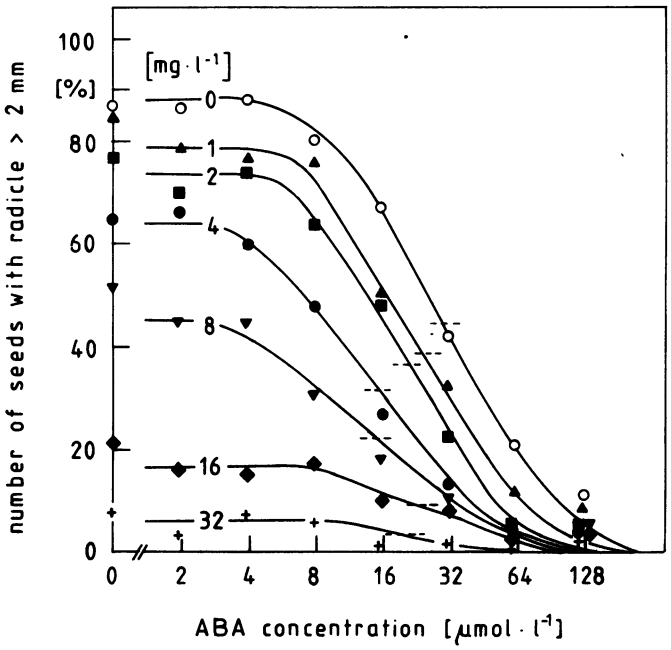

FIG. 9. Inhibition of germination by ABA and CHI. This two-factor analysis was performed in the same way as in Figure 8 with the exception that osmoticum was replaced by the protein synthesis inhibitor CHI (0–32 mg l^{-1}, indicated at the curves). The dashed lines indicate 50% inhibition based on the seed populations germinable in the absence of ABA. (Four repetitions, SE ± 5 to 11%.)

experiments described so far indicate that ABA (0.1 mmol l^{-1}) changes the water relations of germinating embryos inasmuch as it lowers the capacity for water uptake in a manner similar to that of an external osmoticum. Hence, one would expect that ABA and osmoticum (π_o) exhibit a synergistic (competitive) interaction (6) in a two-factor analysis. Figure 8 shows that this is indeed the case. Osmoticum of increasing strength (0–14 bars) shifts the concentration response curve for ABA inhibition of germination toward lower ABA concentrations. This shift is linearly related to π_o (see inset in Fig. 8). Since the interaction between ABA and osmoticum is symmetric, the situation can be equally described as an ABA-dependent shift of the π_o response curve toward lower π_o values. Thus, ABA can fully replace osmoticum in a quantitatively predictable manner. The inset of Figure 8 shows that an ABA concentration change of 1 μmol l^{-1} is equivalent, in terms of germination inhibition, to a $\Delta\pi_o$ of 0.5 bars. The response size of 50% inhibition is obtained whenever the sum of both factor strengths is equivalent to π_o = 11 bars. Therefore, *e.g.* 22 μmol ABA l^{-1} + 0 bars, 10 μmol ABA l^{-1} + 6 bars, or 0 μmol ABA l^{-1} + 11 bars represent equally effective treatments. A characteristic feature of this type of two-factor coaction is that sub-threshold factor strengths are also additive, *e.g.* in Figure 8 an ABA concentration of 8 μmol l^{-1} (equivalent to 4 bars) as well as π_o = 8 bars are without effect if applied separately whereas the combined application of these factor strengths (equivalent to 12 bars) yield 80% inhibition of germination.

Figure 9 shows that also independent coaction of germination factors can be demonstrated in the germination response of rape. In a two-factor analysis investigating the mode of coaction of ABA and CHI, an inhibitor of protein synthesis, in suppressing germination, clear evidence for a multiplicative action (6) of this pair of factors has been found. In this case each one of the two factors produces the same relative response, regardless of the action of the respective other factor (*e.g.* the ABA concentration for 50% inhibition of germination is always in the interval of between 16 and 32 μmol l^{-1}, independent of the simultaneous effect of CHI). This type of coaction is indicative for independent action mechanisms of the two germination factors at different 'sites of recognition.'

It would be premature to draw the opposite conclusion, namely that the two factors act on a common site of recognition, in the case of the interaction of ABA and osmotic stress (Fig. 8). In principle, a two-factor analysis can produce only formal results which can safely be used to exclude certain types of mechanisms but do not permit an unequivocal localization of recognition sites. However, Figure 8 does indicate that the system controlling water uptake and germination of the rape embryo cannot discriminate between the primary effects of ABA and osmotic stress, *i.e.* both signal transduction chains must meet somewhere at a common point controlling the water status of the embryo. A similar conclusion has been reached from an investigation of the effect of ABA, osmotic stress, and other factors on the germination of tomato seeds (5). The next paper of this series is devoted to the identification of the controlling point of water uptake in the germinating rape seed.

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