*Journal of Experimental Botany, Vol. 58, No. 11, pp. 3047–3060, 2007*
doi:10.1093/jxb/erm162 Advance Access publication 30 August, 2007
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

**RESEARCH PAPER**

# 1-Aminocyclopropane-1-carboxylic acid and abscisic acid during the germination of sugar beet (*Beta vulgaris* L.): a comparative study of fruits and seeds

**Katrin Hermann[1], Juliane Meinhard[2], Peter Dobrev[3], Ada Linkies[1], Bedrich Pesek[3], Barbara Heß[1], Ivana Macháčková[3], Uwe Fischer[2] and Gerhard Leubner-Metzger[1,*]**

[1] *Institute of Biology II, Botany/Plant Physiology, Faculty of Biology, Albert-Ludwigs-University Freiburg, Schänzlestr. 1, D-79104 Freiburg i. Br., Germany*

[2] *KWS SAAT AG, Grimsehlstr. 31, D-37555 Einbeck, Germany*

[3] *Institute of Experimental Botany CAS, Rozvojová 263, CZ-16502 Prague 6, Czech Republic*

Received 30 March 2007; Revised 15 June 2007; Accepted 25 June 2007

## Abstract

**The control of sugar beet (*Beta vulgaris* L.) germination by plant hormones was studied by comparing fruits and seeds. Treatment of sugar beet fruits and seeds with gibberellins, brassinosteroids, auxins, cytokinins, and jasmonates or corresponding hormone biosynthesis inhibitors did not appreciably affect radicle emergence of fruits or seeds. By contrast, treatment with ethylene or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) promoted radicle emergence of fruits and seeds. Abscisic acid (ABA) acted as an antagonist of ethylene and inhibited radicle emergence of seeds, but not appreciably of fruits. High endogenous contents of ACC and of ABA were evident in seeds and pericarps of dry mature fruits, but declined early during imbibition. ABA-treatment of seeds and fruits induced seed ACC accumulation while ACC-treatment did not affect the seed ABA content. Transcripts of ACC oxidase (ACO, ethylene-forming enzyme) and ABA 8′-hydroxylase (CYP707A, ABA-degrading enzyme) accumulate in fruits and seeds upon imbibition. ABA and ACC and the pericarp did not affect the seed CYP707A transcript levels. By contrast, seed ACO transcript accumulation was promoted by ABA and by pericarp removal, but not by ACC. Quantification of the endogenous ABA and ACC contents, ABA and ACC leaching, and ethylene evolution, demonstrate that an embryo-mediated active ABA extrusion system is involved in keeping the endogenous seed ABA content low by 'active ABA leaching', while the pericarp restricts ACC leaching during imbibition. Sugar beet radicle emergence appears to be controlled by the pericarp, by ABA and ACC leaching, and by an ABA–ethylene antagonism that affects ACC biosynthesis and ACO gene expression.**

Key words: Abscisic acid (ABA), ABA 8′-hydroxylase (CYP707A), 1-aminocyclopropane-1-carboxylic acid (ACC), ACC oxidase (ACO), ACC and ABA leaching, ethylene, ethylene–ABA interaction, pericarp, radicle emergence, seed and fruit covering layers, sugar beet germination.

## Introduction

The seeds of higher plants contain an embryo surrounded by covering layers and function to ensure the establishment of a new plant generation (Bewley, 1997; Kucera *et al*., 2005). The tremendous structural biodiversity of the various covering layers is not only a hallmark of dispersal unit evolution (Finch-Savage and Leubner-Metzger, 2006), but is also of the utmost importance for germination responses to environmental cues and to plant hormones. Germination commences with the uptake of water by imbibition of the dry seed or fruit, followed by embryo expansion. Further increase in water uptake occurs as the embryo elongates and breaks through the covering layers. Once some portion of the embryo (usually the radicle) is through all the covering layers, germination is considered complete. The testa (seed coat) is a ubiquitous, and the endosperm is a widespread, covering layer of mature seeds.

* To whom correspondence should be addressed. E-mail: gerhard.leubner@biologie.uni-freiburg.de. Web: 'The Seed Biology Place'; http://www.seedbiology.de

Their role in the control of seed germination and their interplay with plant hormones has been studied in many species including core Eudicot model systems of the families Brassicaceae (Koornneef *et al*., 2002; Müller *et al*., 2006) and Solanaceae (Kucera *et al*., 2005; Manz *et al*., 2005). By contrast with the germination of seeds, far less is known about fruits as dispersal units, that is when upon dispersal the seeds remain enclosed by additional covering layers that originate from fruit tissues like the pericarp (fruit coat). Achenes are small, usually single-seeded, dry indehiscent fruits and are the dispersal units of many species including lettuce, sunflower, and sugar beet.

In the achenes of monogerm cultivars of *Beta vulgaris* L. (Amaranthaceae, a family of the Caryophyllid clade of the core Eudicots) the 'botanically true' seed is surrounded by a thick pericarp (Fig. 1; Artschwager, 1927; Bennet and Esau, 1936; Coumans *et al*., 1976). The sugar beet pericarp is known as a fruit tissue that can restrict water and oxygen uptake by the enclosed seed (Coumans *et al*., 1976; Richard *et al*., 1989; Santos and Pereira, 1989). Except for the basal pore, the pericarp is composed of a dense, impervious layer of sclerenchyma cells. The operculum, that is the ovary cap of the fruit is the upper part of the pericarp; and the basal pore, that is a pore-like pericarp structure filled with loose cells at the bottom part of the pericarp have both been proposed as major entry points for water and oxygen. Removal of the operculum and/or the use of 'isolated true seeds' removed these restrictions and promoted radicle emergence (Coumans *et al*., 1976; Richard *et al*., 1989; Santos and Pereira, 1989).

Abscisic acid (ABA) is a negative regulator of seed germination, while gibberellins (GA), brassinosteroids (BR), cytokinins, and ethylene are known to promote the germination of many species (reviewed by Kucera *et al*., 2005). The antagonisms of GA–ABA, BR–ABA, cytokinin–ABA, and ethylene–ABA control radicle emergence and, depending on the species, their modes of action have the embryo and/or the covering layers as their target. These antagonistic interactions have been thoroughly investigated for the testa rupture and endosperm rupture of Rosid and Asterid clade model species (Brassicaceae and Solanaceae, respectively; Leubner-Metzger *et al*., 1998; Beaudoin *et al*., 2000; Ghassemian *et al*., 2000; Leubner-Metzger, 2001; Steber and McCourt, 2001; Koornneef *et al*., 2002; Müller *et al*., 2006). By contrast, only few reports have been published for the perispermic seeds of the Caryophyllid clade (*Chenopodium* spp., dispersal units: true seeds, Karssen, 1976), and nothing is known about the hormonal interactions of fruits that harbour perispermic seeds (*Beta* spp., dispersal units: achenes with a thick pericarp). In the present work on sugar beet germination, comparative experiments are performed with fruits and seeds. First, how treatments with ABA, GA, BR, cytokinin, ethylene, auxin, jasmonates, and several 'hormone inhibitors' affect germination were investigated and, second, how endogenous levels of ABA, 1-aminocyclopropane-1-carboxylic acid (ACC, the ethylene precursor), transcripts of the ABA-degrading *ABA 8′-hydroxylase* (*CYP707A*) and ethylene-producing *ACC oxidase* (*ACO*) genes, ACC and ABA leaching, and ethylene evolution are regulated during germination. It was found that the pericarp has a decisive role and that an novel ABA–ACC/ethylene interaction is evident during sugar beet radicle emergence.

## Materials and methods

### *Plant material and permeation technique*

Fruits of a triploid monogerm sugar beet (*Beta vulgaris* L.) seed lot (302-688C) were produced in Italy in 2002 and processed (cleaned, polished, calibrated) after harvest according to the commercial standards (KWS SAAT AG, Einbeck, Germany). A representative sample of calibre fraction 3.35–3.60 mm was taken and dry fruits (moisture content $\leqslant 8\%$; dry weight) were stored in paper bags at room temperature until use.

For the permeation experiments, the technique with dichloromethane (DCM, Meyer and Mayer, 1971) was used. Dry sugar beet fruits were submerged in DCM and incubated for 20–24 h with gentle shaking. To introduce hormones or inhibitors into dry fruits these substances were freshly dissolved in DCM and the permeation was started immediately. Subsequently, the permeated fruits were washed briefly with DCM, dried for at least 30 min in an desiccator and stored in darkness in containers permeable to air. These fruits were used for the experiments with permeated fruits or seeds (after deoperculation).

### *Germination assays*

Throughout the paper the term 'fruit' refers to the achene, i.e. the sugar beet dispersal unit (Fig. 1, Richard *et al*., 1989). The operculum (ovary cap) is the upper part of the pericarp. The operculum opening is visible as the ovary cap lifts and exposes the radicle end of the seed. The term 'seed' refers to the botanically true seed and includes the embryo, the perisperm, the remnants of the endosperm, and the testa (seed coat). Radicle emergence is the visible protrusion of the radicle tip through all the (fruit and seed) covering layers, i.e. pericarp (operculum opening), testa (testa rupture), and endosperm (endosperm rupture). For the experiments with seeds, the operculum was removed from the fruit by prising it off with a mounted needle. Initially either 'deoperculated fruits' placed with the seed side onto the medium or 'isolated seeds' (the seed was carefully removed from the pericarp) were compared, but were found to provide similar temporal patterns of radicle emergence. If not otherwise stated, the 'seed experiments' presented in this paper are therefore performed with 'deoperculated fruits', which significantly reduced the number of artefacts due to dissection-generated seed wounding of the brittle testa. Seeds that were damaged during the deoperculation procedure were not used for the germination experiments.

For the germination experiments with sugar beet fruits, at least triplicates of 100 fruits were incubated in plastic boxes (120×160×60 mm) with pleated filter paper and 30 ml deionized water in the dark at 15 °C. Operculum opening and radicle protrusion through all the covering layers (testa and endosperm) were scored over time using an illuminated magnifier lens (type 277711, Eschenbach Optik GmbH, Nürnberg, Germany) and the percentages of the population responses were calculated. If indicated, *cis*-(±)-abscisic acid (ABA; Sigma, Taufkirchen,

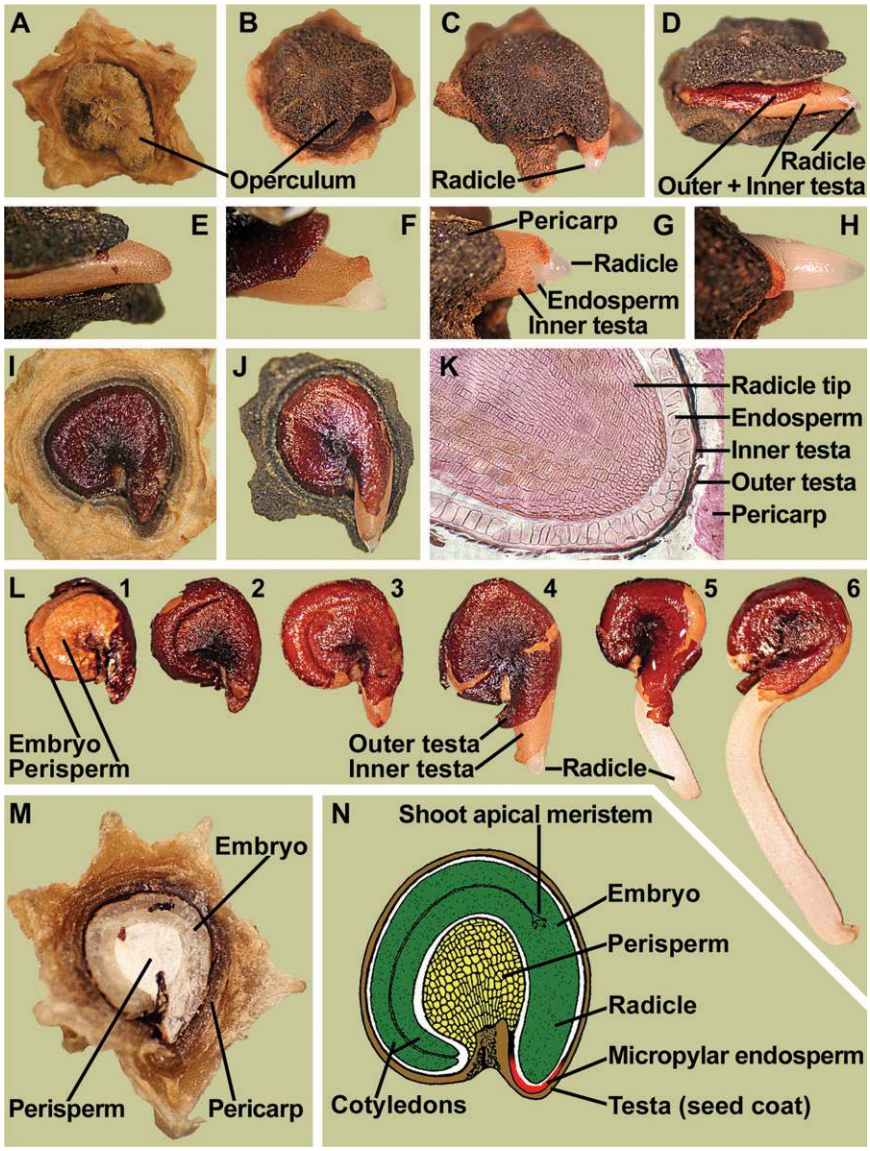



**Fig. 1.** Structure of mature fruits and seeds of *Beta vulgaris*. (A–H) Visible events during the incubation of sugar beet fruits in water: (A) Dry fruit. (B, E) Operculum opening; note that the radicle tip is still enclosed by the micropylar endosperm and the inner testa. (C, D, F–H) Radicle emergence through the seed covering layers (testa and endosperm) is the completion of germination. (I, J) Seed germination studied with deoperculated fruits. The sugar beet seed has a lentil-like structure (about 3 mm diameter and 1.5 mm thick) and occupies a horizontal position within the fruit. (J) Radicle emergence through the seed covering layers (testa and endosperm) is the completion of germination. (K) Microscopic section through a dry fruit showing the radicle tip enclosed by the covering layers. (L) Distinct stages of sugar beet seed germination: isolated dry seed (1, 2); note that the testa was removed in (1) to make the embryo and perisperm visible. Imbibed seed showing rupture of the outer testa (3) and radicle protrusion through all the seed covering layers (4–6). (M) Section through a mature sugar beet fruit. The curved embryo completely encloses the perisperm, which is dead starch storage tissue localized in the seed centre. (N) Drawing of a sugar beet seed; modified from Bennett and Esau (1936) and reproduced by the kind permission of the United States Department of Agriculture. Based on the peripheral location of the embryo, the sugar beet seed can be structurally classified as being perispermic and P-type (Finch-Savage and Leubner-Metzger, 2006).

Germany), gibberellin $A_{4+7}$ ($GA_{4+7}$; Duchefa), gibberellin $A_3$ ($GA_3$; Duchefa, Harlem, The Netherlands), the GA biosynthesis inhibitor flurprimidol (Duchefa), 24-epi-brassinolide (EBR; Duchefa), the BR biosynthesis inhibitor brassinazole (T Asami, RIKEN Institute, Japan, Asami *et al*., 2000; Kiran *et al*., 2006), indole-3-acetic acid (IAA; Sigma), the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma), the ethylene biosynthesis inhibitor aminoethoxy-vinylglycine (AVG; Sigma), kinetin (Duchefa), or methyl jasmonate (MeJA; Duchefa) were added to the medium. Treatment with ethylene itself and with 2,5-norbornadiene (NBD; Sigma) was via the gas phase by co-incubation of the open germination vessels with a small vessel with NBD or 2-chloroethylphosphonic acid (CEPA, ethephon; Sigma) in an air-tight 6.5 l container. The release of ethylene from CEPA was achieved by adding 500 µl 0.1 N NaOH to 500 µl 100 mM CEPA. As a positive control for the ethylene response the induction of β-1,3-glucanase activity of tobacco seeds was used (Leubner-Metzger *et al*., 1998).

For the germination experiments with sugar beet seeds, at least triplicates of 25 'deoperculated fruits' were placed with the seed side down onto two filter papers soaked with 5 ml medium in Petri

dishes. The Petri dishes were sealed with parafilm and incubated at 15 °C in the dark. Visible protrusion of the radicle tip through the covering layers (testa and endosperm) was used as a criterion for radicle emergence, which was scored using a binocular microscope. If indicated, hormones or inhibitors were added as described above.

For the hormone quantification experiments, triplicates of 100 fruits or 'isolated seeds' were incubated in plastic boxes with pleated filter paper and 30 ml deionized water (control) or 100 μM ABA or 1 mM ACC and incubated as described above. All samples were harvested at $T_{1\%}$, $T_{50\%}$, and $T_{max}$ which indicate the time the untreated control fruits required to reach 1%, 50%, and maximal radical emergence. $T_{1\%}$, $T_{50\%}$, and $T_{max}$ were determined prior to sample production in three independent germination assays with 4×100 fruits using the seed calculator 3.0 software (Plant Research International BV, Wageningen, The Netherlands). At harvest time, seeds were dissected out of fruits and frozen immediately in liquid nitrogen, freeze-dried, and used for endogenous hormone analyses.

### *Microscopy and image preparation*

Sugar beet fruits or seeds (Fig. 1) were incubated under standard conditions and appropriate developmental stages were selected for photographic documentation. Fixation of sugar beet fruits (Fig. 1K) was for 24 h at room temperature in a solution containing 5% formaldehyde, 5% acetic acid and 30% ethanol; followed by an ethanol dilution series (70% for 24 h; 80% for 5 h; 90% for 18 h; 100% for 6 h; 100% for 18 h) and embedding in Technovit 7100 (Heraeus-Kulzer, Haslab GmbH, Ostermundingen, Switzerland) according to the manufacturer's instruction and cutting into 5 μm sections using a microtome (RM 2145, Leica, Wetzlar, Germany). Section staining was carried out in 1% fuchsin basic in ethanol followed by thorough washing with water. For imaging, either a Canon Powershot G5 digital (Canon, Krefeld, Germany) or a Nikon F100 (Nikon, Düsseldorf, Germany) camera attached to a stereomicroscope (Stemi 2000-CS, Zeiss, Göttingen, Germany) or a microscope (Axioskop 40, Zeiss, Göttingen, Germany) were used. The Adobe Photoshop CS2 software was used for image processing.

### *Analysis of endogenous hormone contents*

Freeze-dried tissues, pericarp or 'isolated seeds', were used for hormone analyses. The detailed procedure for the extraction, purification, and quantification of IAA, ABA, ACC, and cytokinins has been described in Kiran *et al.* (2006). IAA, ABA, and cytokinins were extracted overnight at –20 °C using Bieleski solvent (Bieleski, 1964). [$^3$H]IAA (Sigma) and [$^3$H]ABA (Sigma) and 12 deuterium-labeled cytokinins ([$^2$H5]tZ, [$^2$H5]tZR, [$^2$H5]tZ7G, [$^2$H5]tZ9G, [$^2$H5]tZOG, [$^2$H5]tZROG, [$^2$H3]DHZ, [$^2$H3]DHZR, [$^2$H6]IP, [$^2$H6]IPR, [$^2$H6]IP7G, [$^2$H6]IP9G; Apex Organics, UK) were added as internal standards. The extracts were purified using Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA) and an Oasis MCX mixed mode, cation exchange, reverse-phase column (150 mg, Waters) (Dobrev and Kamínek, 2002). After washing with 1 M HCOOH, the hormones IAA and ABA were eluted with 100% MeOH and evaporated to dryness. Further, cytokinin phosphates and ACC were eluted with 0.34 M $NH_4OH$ in water and cytokinin bases, ribosides, and glucosides were eluted with 0.34 M $NH_4OH$ in 60% (v/v) MeOH. Phosphates were converted into ribosides with alkaline phosphatase. IAA and ABA were separated and quantified by 2D-HPLC according to Dobrev *et al.* (2005). ACC was determined as ethylene after oxidation with NaOCl according to Lizada and Yang (1979). Ethylene levels were determined on a 50 m capillary alumina 'S' 15 μm column, ID 0.53 mm on the apparatus of Fissons Instruments. The temperature of injection, column, and detector was 230 °C, 40 °C, and 200 °C, respectively (Fiserová and Hradilík, 1994). Carbon dioxide was determined on a Chrom 5 gas chromatograph (Laboratory Instruments, Czech Republic), with a catharometer on the 1.5 m long column filled with Porapak Q. Purified cytokinin samples were analysed by an LC-MS system consisting of HTS PAL auto sampler (CTC Analytics, Zwingen, Switzerland), Rheos 2000 quaternary pump (FLUX, Switzerland) with Csi 6200 Series HPLC Oven (Cambridge Scientific Instruments, England) and LCQ Ion Trap mass spectrometer (Finnigan, USA) equipped with an electro spray. 10 μl of sample was injected onto a C18 column (AQUA, 2 mm×250 mm×5 μm, Phenomenex, USA) and eluted with 0.0005% acetic acid (A) and acetonitrile (B). The HPLC gradient profile was as follows: 0–5 min, 10% B; 5–15 min, 10–17% B; 15–25 min, 17–46% B; at a flow rate of 0.2 ml $min^{-1}$. Column temperature was kept at 30 °C. The effluent was introduced into a mass spectrometer being operated in the positive ion, full-scan MS/MS mode. Quantification was performed using a multilevel calibration graph with deuterated cytokinins as internal standards.

### *Analyses of mRNAs by semi-quantitative RT-PCR*

Total RNA was extracted from sugar beet seeds at $T_{50\%}$ from either incubated fruits or from isolated seeds as described by Leubner-Metzger (2005). The RNA concentration was determined spectrometrically and by semi-quantitative RT-PCR amplification of rRNA. A thermocycler (Mastercycler, Eppendorf, Hamburg, Germany) was used for the 50 μl RT-PCR ('Qiagen OneStep RT-PCR Kit', Qiagen, Hilden, Germany) reactions with 250 ng total RNA as template. One-tube reverse transcription (30 min, 55 °C) was followed by inactivation of reverse transcriptase, activation of *Taq* polymerase, and template denaturation (15 min, 95 °C), by 30 cycles of denaturation (0.5 min, 95 °C), annealing (0.5 min, 58 °C), and extension (1 min, 72 °C), by a final extension cycle (7 min, 72 °C) and subsequent cooling. Specific primers were designed for the co-amplification of partial cDNAs of the known *Beta vulgaris ACO* (BI095869, NCBI database, http://www.ncbi.nlm.nih.gov) or *CYP707A* (BQ582685) transcripts together with 18S rRNA (AJ236016). Regions of the transcripts that are homologous to the corresponding sequences of other species were used and should result in the amplification of of 340 bp, 201 bp, and *c*. 0.44 kb PCR bands for ACO, CYP707A, and 18S rRNA, respectively. Primer sequences (5' to 3') were: ACO-forward CAGACTGGGAAAGCAGCTTCTT, ACO-reverse AATTCAAGGCCWGGVACTTGA, CYP707A-forward CTGGTTCMATGGGTT GGCCTTA, CYP707A-reverse AAGTTGG-TTTAAAGAGATGAGCTT, 18SrRNA-RRNA2 CGAGCTGATGA-CTCGCGCTTA, 18SrRNA-RRNA5 GAGTGGAGCC TGCGG-CTTA. 10 μl aliquots of these reactions were separated on 1.4% (w/v) agarose gels and the band sizes were determined in comparison to a 100 bp DNA ladder (Invitrogen, Karlsruhe, Germany).

## Results

### *Visible events during sugar beet germination: a comparative study of fruits and seeds*

In the initial time-course experiments on sugar beet germination, visible events were defined that can be quantified in populations of fruits and seeds (Figs 1, 2). In the mature sugar beet fruit the 'botanically true' seed is surrounded by a thick pericarp, which in the upper part forms a cap-like structure, the operculum (Fig. 1A–D). In the mature sugar beet seed the embryo is enclosed by the outer layer of the thin testa, which is very brittle and has a reddish brown colour (Fig. 1E–L). The endosperm is obliterated with the exception of a single cell layer

surrounding the radicle (Fig. 1K). Operculum opening was the first visible event potentially leading to radicle protrusion of fruits incubated in water (Fig. 1B, E). Operculum opening disclosed the radicle end of the seed, with the radicle still covered by the inner testa and the endosperm. Operculum opening revealing the testa layers (Fig. 1B–H), preceded radicle emergence (Fig. 2A). Operculum opening always occurred at the site above the radicle, which strongly suggests that operculum opening is achieved by radicle growth causing rupture of the pericarp at predetermined breaking points.

The experiments with (botanically true) seeds (Fig. 1I, J, L) showed that the onset of radicle emergence is earlier in populations of seeds compared to fruits. The $T_{10\%}$ for seeds and fruits were about 23 h and 44 h, respectively (Fig. 2A). Since the slope of the time-course was flatter for seeds compared with fruits, their $T_{50\%}$ values for radicle emergence were roughly equal (at about 55 h; Fig. 2A). Taken together, these results demonstrate that this system can be used in comparative experiments to test the effects of plant hormones on sugar beet seeds and fruits.

In several experiments, the permeation technique with dichloromethane (DCM; Meyer and Mayer, 1971) was used to introduce substances into dry sugar beet fruits. When DCM-permeated fruits were used in time-course experiments, the onset was advanced, but the final percentage of radicle emergence was decreased relative to unpermeated fruits (Fig. 2A, B). DCM permeation of fruits caused a temporal pattern of radicle emergence similar to that of seeds. CHX-permeation ($CHX^{perm}$), i.e. permeation of dry fruits with cycloheximide using the DCM technique, inhibited radicle emergence. These results demonstrate that the DCM-permeation technique can be used to 'permeate' substances into dry sugar beet fruits. DCM-permeated fruits ($Control^{perm}$) were used as controls in these experiments.

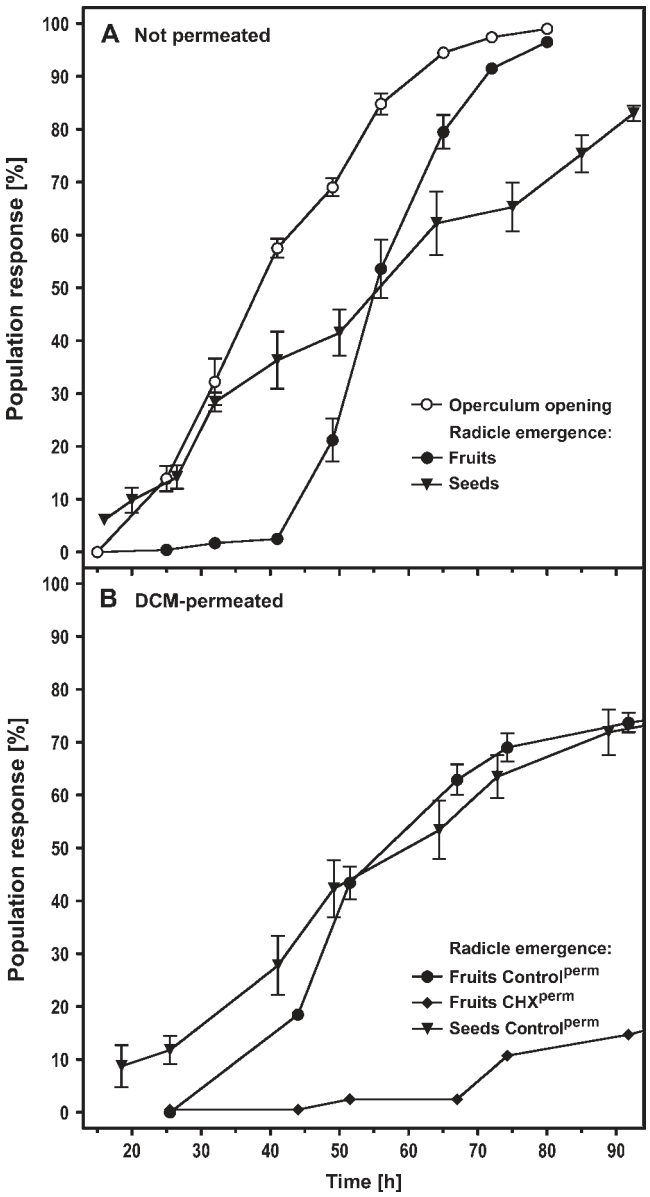



**Fig. 2.** Time-courses of visible events during the germination of *Beta vulgaris*. (A) Population responses of fruits and (botanically true) seeds. Fruits: triplicates of 100 fruits incubated at 15 °C in the dark were scored over time and the percentage of operculum opening (ovary cap lifting; Fig. 1B, E) and radicle protrusion (completion of seed germination; Fig. 1C, D, F, G, H) of the fruit populations were calculated. Seeds: Triplicates of 25 seeds were scored for radicle protrusion. Mean values ±SE of three (fruits) and six (seeds) independent experiments are presented. SE values <1.5% are not drawn. (B) Population responses of DCM-permeated fruits and seeds. Dry fruits were permeated with dichloromethane (DCM; $Control^{perm}$) or DCM plus cycloheximide ($CHX^{perm}$). Scoring, conditions, and statistics of one (fruits) and two (seeds) independent experiments as in (A).

### *Sugar beet fruits and seeds contain high endogenous ABA contents and differ in the ABA inhibition of radicle emergence*

Radicle emergence of sugar beet seeds is inhibited by treatments with ABA, but radicle emergence of fruits is only slightly delayed by this plant hormone (Fig. 3). The inhibitory effect of ABA added to the incubation medium of seeds appears to be dose-dependent and 100 μM ABA already caused a strong inhibition (Fig. 3B). In contrast to this strong effect, the addition of 100 μM ABA to the incubation medium of fruits just delayed radicle emergence by about 10 h, lower ABA concentrations were less effective, and higher ABA concentrations caused only a slightly longer delay (Fig. 3A; see Supplementary Fig. 1 at JXB online). Fluridone, an inhibitor of ABA biosynthesis, did not affect radicle emergence of seeds or fruits when added to the medium (Fig. 3A, B) or when fluridone-permeated fruits were used (Fig. 3C). ABA-permeation ($ABA^{perm}$) of dry fruits with the DCM-technique inhibited radicle emergence from fruits and seeds: A delay of about 30 h was evident for fruits (Fig. 3C), and a strong inhibition for seeds was observed (Fig. 3D). Thus, ABA

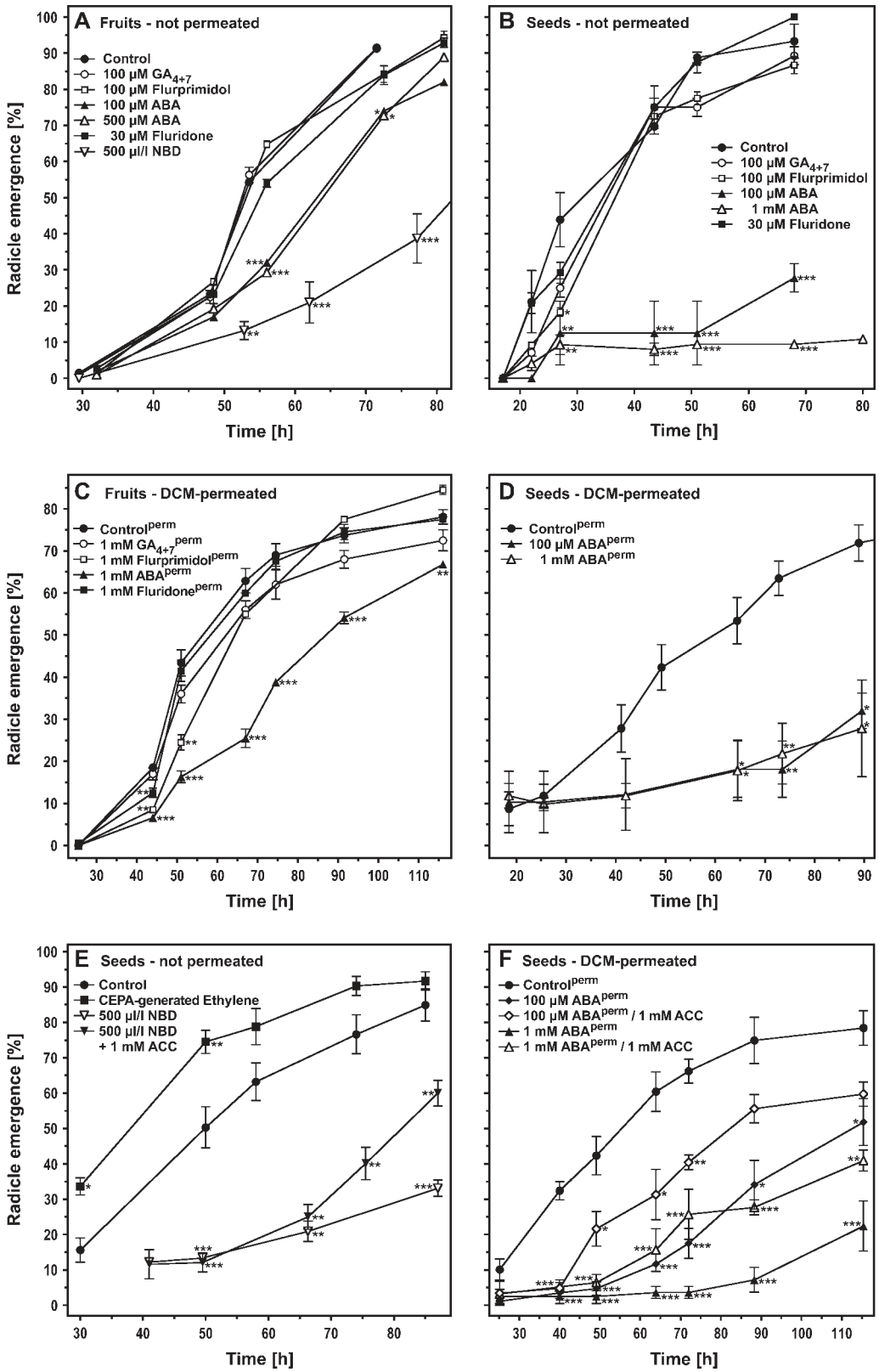



**Fig. 3.** The effects of abscisic acid (ABA), gibberellins (GA), and 1-aminocyclopropane-1-carboxylic acid (ACC, ethylene precursor)/ethylene on radicle emergence of fruits (A, C) and seeds (B, D–F) of *Beta vulgaris*. (A, B, E) Fruits or seeds were incubated with ABA, ACC, $GA_{4+7}$, fluridone (ABA biosynthesis inhibitor), or flurprimidol (GA biosynthesis inhibitor) added to the medium, or with 2,5-norbornadiene (NBD, ethylene action inhibitor, applied via the gas phase) or ethylene (applied via the gas phase as described in Materials and methods). (C, D, F) Dry fruits were permeated using the DCM-technique with ABA, $GA_{4+7}$, fluridone or flurprimidol as indicated ($ABA^{perm}$, $GA^{perm}_{4+7}$, $fluridone^{perm}$, $flurprimidol^{perm}$)

inhibits sugar beet radicle emergence from seeds and fruits, but this inhibitory effect is significantly lower for fruits.

The endogenous ABA contents of dry sugar beet fruits and seeds are 2–3-fold higher than those of tobacco seeds (Table 1). Similar contents were detected in seeds (embryo plus perisperm plus testa and endosperm) and pericarp. Within the pericarp, no difference in ABA content was detected between the operculum and the rest of the pericarp (data not shown). Thus, a considerably high endogenous ABA content was evident in seeds and pericarp of sugar beet fruits.

### *Treatments with GA, BR, auxins, cytokinins, and jasmonates do not appreciably affect radicle emergence*

Neither GA nor flurprimidol, an inhibitor of GA biosynthesis (Rademacher, 2000), affected radicle emergence of sugar beet seeds (Fig. 3). The addition of 100 μM $GA_{4+7}$ (Fig. 3A, B) or higher $GA_{4+7}$ or $GA_3$ concentrations (see Supplementary Fig. 1 at *JXB* online) to the medium of fruits or seeds had no appreciable effect. Permeation of dry fruits with $GA_{4+7}$ or the GA-biosynthesis inhibitor did not affect radicle emergence (Fig. 3C). Furthermore, the addition of 100 μM or 1 mM $GA_{4+7}$ to the medium of ABA-permeated (100 μM or 1 mM $ABA^{perm}$) fruits did not revert the inhibitory effect of ABA on radicle emergence (data not shown). Supplementary Fig. 1 shows, with pharmacological experiments, that no effect of GA, BR, auxin, cytokinins, or jasmonates on sugar beet radicle emergence was obtained.

### *ACC promotes sugar beet radicle emergence and counteracts the inhibitory effects of ABA*

High endogenous contents of 1-aminocyclopropane-1-carboxylic acid (ACC), the direct biosynthetic precursor of the plant hormone ethylene, were detected in dry sugar beet fruits (Table 1). ACC contents of 20.7 nmol $g^{-1}$ DW and 12.1 nmol $g^{-1}$ DW were evident in the seed and the pericarp, respectively. These values are at least 10-fold higher than the ABA contents (Table 1) and an ACC/ABA ratio of about 13 is evident in dry seeds. Figure 4 shows a rapid decline of the seed ACC and ABA contents during the early imbibition of sugar beet fruits; a 3-fold and a 6-fold decline was evident for ACC and ABA, respectively. Both compounds remained low in the seed at $T_{1\%}$ and $T_{50\%}$. However, although both declined, the ACC/ABA ratio within the seed increased to about 26. The completion of radicle emergence and the subsequent start of post-germination growth was associated with a rapid increase in the seed ACC content, while the ABA content continued to decline. Seedling growth directly after radicle emergence ($T_{max}$) was therefore associated with a further increase of the ACC/ABA ratio to about 127 (Fig. 4). By contrast with ACC and ABA, the endogenous contents of IAA remained roughly constant in dry seeds and during imbibition to $T_{1\%}$ and $T_{50\%}$. By contrast with ABA, but as for ACC, post-germination growth was associated with an increase in IAA content (Fig. 4). Thus, a high ACC/ABA ratio is evident in sugar beet seeds. Together with the temporal pattern of seed ACC contents, this suggests that ethylene production from ACC by the enzyme ACO may play an important role.

In agreement with this, the addition of 1 mM ACC to the medium promoted radicle emergence of sugar beet fruits (Fig. 5A) and seeds (Fig. 5B) by 12 h and 23 h, respectively. 2,5-Norbornadiene (NBD), a widely used ethylene action inhibitor (Sisler and Serek, 2003), inhibited radicle emergence of sugar beet fruits (Fig. 3A) and seeds (Fig. 3E). The addition of ACC to the medium partially reverted the inhibitory effects of NBD on seeds and seed radicle emergence was promoted by ethylene gas (Fig. 3E). To test further if the ACC/ABA ratio in the embryo is important, the DCM-technique was used to introduce two different ABA concentrations into dry fruits (100 μM

**Table 1.** *Endogenous hormone contents of dry sugar beet fruit tissues*

The contents of ACC, ABA, IAA, and various cytokinins in pmol $g^{-1}$ dry weight (DW). Note for comparison: an ABA content of 585±86 pmol $g^{-1}$ DW was measured for after-ripened seeds of the *Nicotiana tabacum* cultivar Havana 425.

| Hormones[a] | | Sugar beet fruit tissues[b] | |
|---|---|---|---|
| | | Seed | Pericarp |
| ACC | 1-Aminocyclopropane-1-carboxylic acid | 20700±200 | 12100±1100 |
| ABA | Abscisic acid | 1577±19 | 1241±116 |
| IAA | Indole-3-acetic acid | 520±43 | 324±21 |
| tZ | *trans*-Zeatin | 46.5±4.0 | 16.9±1.4 |
| tZR | *trans*-Zeatin riboside | 4.6±0.2 | 7.6±0.4 |
| tZ7G | *trans*-Zeatin glucoside | 49.7±0.8 | 1.1±0.3 |
| tZOG | *trans*-Zeatin O-glucoside | 7.3±0.0 | 0.5±0.0 |
| cZ | *cis*-Zeatin | 3.6±0.5 | 12.1±1.0 |
| cZR | *cis*-Zeatin riboside | 6.9±0.4 | 22.1±0.6 |
| cZROG | *cis*-Zeatin riboside *O*-glucoside | 105.6±2.6 | 2.5±0.2 |
| IP | Isopentenyladenin | 2.6±0.1 | 5.1±0.4 |

[a] Mean values ±SE of hormone contents (pmol $g^{-1}$ DW) of at least three independent samples prepared by dissection of 100 air-dry sugar beet fruits each.

[b] Dry sugar beet fruits were dissected into seed (embryo plus perisperm plus testa) and pericarp (includes in addition remnants of the perianth).

and fruits (C) or seeds dissected from these fruits (D, F) were incubated in medium without hormones or inhibitors. Note that DCM-permeated controls ($Control^{perm}$) were used for comparison. Radicle emergence of fruit or seed populations was scored over time; conditions as in Fig. 2. Mean values ±SE of a representative experiment with triplicates of 100 fruits or 25 seeds are presented; the results were confirmed in independent experiments; SE values <1.5% are not drawn. Statistical significance of results was analysed by one-way ANOVA with Tukey's multiple comparison test performed using GraphPad Prism software (version 4.0 for Macintosh, GraphPad Software, San Diego California USA, www.graphpad.com). Means of treatments labelled with asterisks differ significantly from the corresponding controls: (asterisk) $0.05 > P > 0.01$; (2 asterisks) $0.01 > P > 0.001$; (3 asterisks) $P < 0.001$.

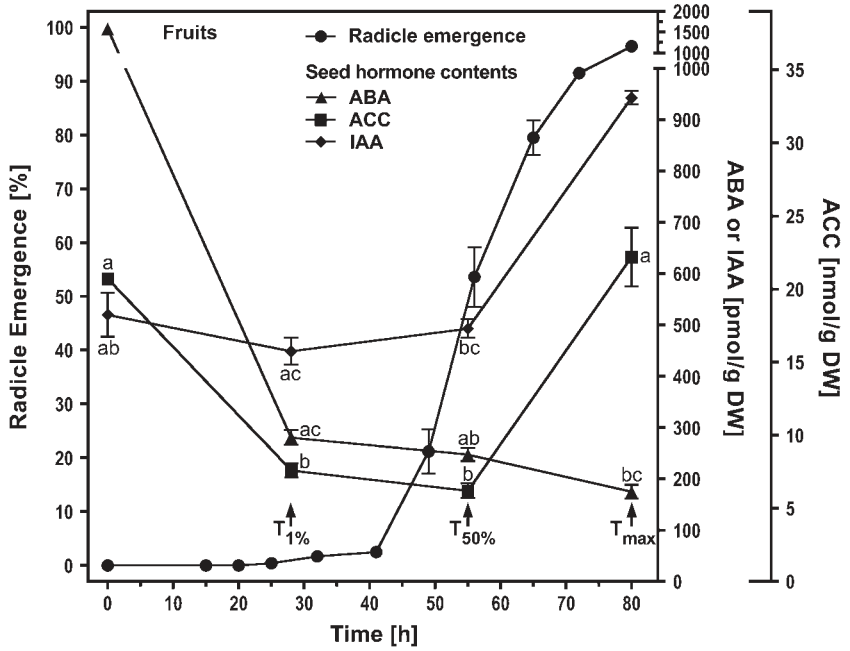



**Fig. 4.** The time-course of the endogenous contents of abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC, ethylene precursor), and indole-3-acetic acid (IAA, auxin) in the seed during the germination of *Beta vulgaris* fruits. The endogenous contents were determined in seeds (embryo plus perisperm plus testa) dissected out of dry fruits and fruits at the times indicated when radicle emergence of the population was 1% ($T_{1\%}$), 50% ($T_{50\%}$), and maximal ($T_{max}$). The seed samples were freeze-dried and each yielded *c*. 0.3 g in dry weight (DW). The results are presented in pmol (ABA, IAA) or nmol (ACC) $g^{-1}$ DW. Radicle emergence of fruit populations was scored over time; mean values ±SE from three independent experiments each with 4×100 fruits are presented for radicle emergence; mean values ±SE of triplicates of 100 seeds used for the hormone measurements are presented. Conditions and statistics as in Fig. 3; within each series (ABA, ACC, IAA), means denoted by the same letter do not differ significantly at $P$ <0.01 according to Tukey's multiple comparison test.

$ABA^{perm}$ and 1 mM $ABA^{perm}$). The timing of radicle emergence of the seeds from these ABA-permeated fruits was considerably delayed, but the addition of 1 mM ACC to the medium partially reverted the inhibitory effect of ABA-permeation (Fig. 3F). These results suggest that the seed ethylene/ABA ratio is important for radicle emergence and raises the question how it is regulated and if ACC action is associated with its conversion into ethylene.

### *ABA promotes ACC and ACO transcript accumulation during sugar beet germination*

Figures 5 and 6 show experiments in which ACC or ABA was added to the medium of fruits (Figs 5A, 6) and seeds (Figs 5B, 6). At 55 h, i.e. at the $T_{50\%}$ of the fruit control curve, the endogenous contents of ACC, ABA, and IAA were determined in seeds of the fruit (Fig. 5C) and the seed (Fig. 5D) series, and ACC and ABA leaching into the medium was determined (Fig. 6). Compared with the corresponding controls, these results show for fruits and seeds (Figs 5, 6): (i) that ACC is readily taken up from the medium into the seed by fruits and seeds (16–24-fold increase). By contrast, ABA uptake into the seed by fruits is not very efficient (1.7-fold increase), is more efficient by seeds (15-fold), but does not lead to endogenous contents as high as for ACC. (ii) The addition of ACC to the medium and uptake into the embryos of fruits or seeds did not affect the endogenous seed ABA contents. By contrast, the addition of ABA to the medium of fruits or seeds caused 53-fold or 70-fold increases, respectively, of the endogenous seed ACC contents. (iii) Addition of ACC to the medium induced ABA biosynthesis, but >90% of this ABA leached out into the medium of fruits and seeds. By contrast, the ACC accumulation caused by the addition of ABA to the medium was accompanied only by a minor ACC leaching into the medium from fruits. Interestingly, even at high endogenous ACC contents, only a fixed amount of *c*. 40 pmol $seed^{-1}$ ACC leaching was evident from fruits, while similar high contents were evident from seeds. (iv) Neither ACC nor ABA appreciably affect the seed IAA contents of fruits or seeds.

Figure 6 shows that ethylene evolution at $T_{50\%}$ was evident from seeds, but was below the detection limit for fruits and seeds at the start of imbibition (0 h). In agreement with this, a strong induction of the ACO transcript levels was evident in the seeds at $T_{50\%}$ compared with 0 h-seeds. Neither ACC nor ABA treatment appreciably affected this induction. ACO transcripts also accumulated in the embryos from fruits at $T_{50\%}$ (Fig. 6). Compared with seeds, the induction in fruits was much weaker; and, compared with control and ACC, ABA promoted the ACO transcript accumulation in seeds from fruits. By contrast with ACO, the transcript levels of CYP707A, the ABA-degrading enyzme ABA 8′-hydroxylase, accumulate in fruits and seeds (compared with the start of imbibition), but

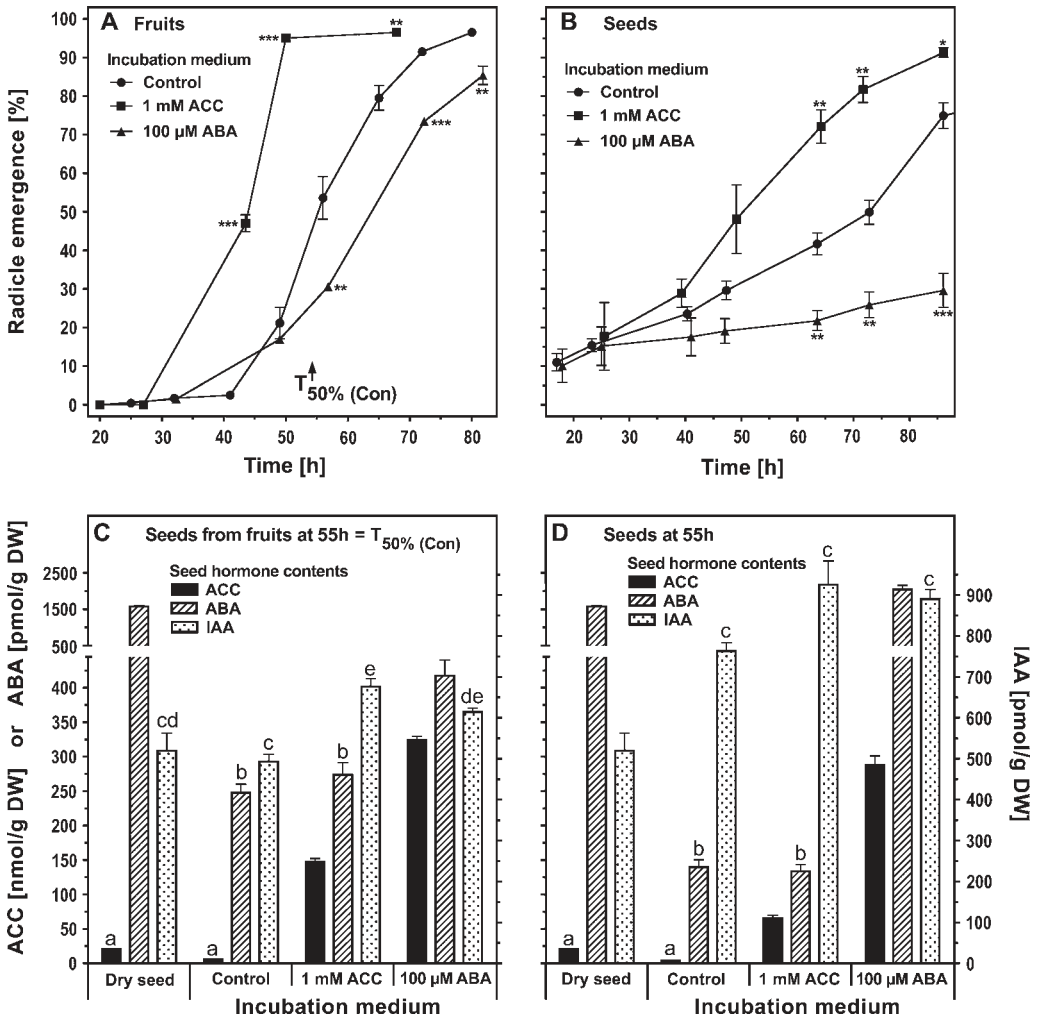


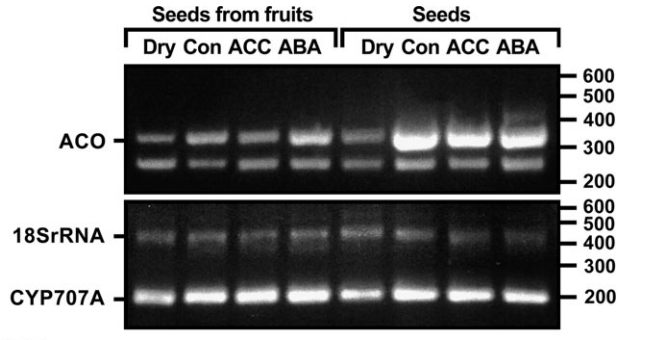

**Fig. 5.** The effect of 1-aminocyclopropane-1-carboxylic acid (ACC, ethylene precursor) and abscisic acid (ABA) added to the medium of *Beta vulgaris* fruits (A, C) and seeds (B, D) on the timing of radicle emergence (A, B) and the endogenous contents of ACC, ABA, and indole-3-acetic acid (IAA, auxin) in the seeds (embryo plus perisperm plus testa) (C, D). The endogenous contents were determined in seeds dissected out of fruits or in 'isolated seeds' at 55 h, i.e. the $T_{50\%}$ for the radicle emergence of fruit populations in control medium. The results are presented in pmol (ABA, IAA) or nmol (ACC) $g^{-1}$ DW. Mean values ±SE are presented; radicle emergence scoring, hormone measurements, conditions, and statistics with Tukey's multiple comparison test as in Figs 3 and 4. (A, B) Means of treatments labelled with asterisks differ significantly from the corresponding controls: (Asterisk) $0.05 > P > 0.01$; (2 asterisks) $0.01 > P > 0.001$; (3 asterisks) $P < 0.001$. (C, D) Means within each series, means denoted by the same letter do not differ significantly at $P < 0.01$ (ABA, ACC) or $P < 0.05$ (IAA); unequal or no letter denote differences.

were roughly similar in fruits and seeds at $T_{50\%}$ in any of the treatments. These results show that ABA is a positive key regulator of the ethylene biosynthetic pathway during sugar beet germination, and that ABA extrusion and the pericarp are involved in regulating a complex interaction between ethylene/ACC and ABA.

## Discussion

### *Plant hormones and the germination of sugar beet fruits and seeds*

The comparison of sugar beet radicle emergence of fruits and seeds lead to the important discovery that an ethylene–ABA antagonism and the pericarp affect germination and that ABA seems to be a positive regulator of the seed ACC and ACO contents. In the known model species for seed germination, the plant hormones GA, BR, cytokinins, and ethylene promote radicle emergence and act as ABA antagonists (reviewed by Kucera *et al*., 2005). The fact that, in our pharmacological experiments treatment with GA, BR, cytokinins, and corresponding hormone biosynthesis inhibitors did not affect sugar beet germination, does not provide evidence for or against a role of these hormones under natural conditions. For the classical GA–ABA antagonism it could simply be that the GA requirement for radicle emergence of non-dormant sugar beet fruits is very low or even absent, and that therefore it is not visible in pharmacological experiments. Similarily, endogenous BRs have been detected in dry




| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **ABA** endogen. | 5.7 | 0.9 | 1.0 | 1.5 | 5.7 | 0.5 | 0.5 | 7.4 | pmol/seed |
| **ABA** leaching | 0 | 10 | 67 | --- | 0 | 4 | 189 | --- | pmol/seed |
| **ACC** endogen. | 76 | 22 | 533 | 1175 | 76 | 15 | 239 | 1044 | pmol/seed |
| **ACC** leaching | 0 | 44 | --- | 36 | 0 | 9 | --- | 1654 | pmol/seed |
| **Ethylene** | nd | nd | nd | nd | nd | 0.04 | 0.33 | 0.01 | ppm/hour |
| **ACC** medium | --- | --- | 1000 | --- | --- | --- | 1000 | --- | pmol/µl |
| **ABA** medium | --- | --- | --- | 100 | --- | --- | --- | 100 | pmol/µl |

**Fig. 6.** The effects of abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylic acid (ACC, ethylene precursor) on the transcript expression of ACC oxidase (ACO, ethylene-forming enzyme) and ABA 8′-hydroxylase (CYP707A, ABA-degrading enzyme), ACC, and ABA leaching into the medium, ethylene evolution and endogenous ACC and ABA of either seeds dissected from incubated fruits (left) or incubated 'isolated seeds' (right). RT-PCR was performed from total RNA extracted from seeds at the incubation time $T_{50\%}$ (see Fig. 5). Specific primers for conserved regions of the known *Beta vulgaris ACO* (BI095869, NCBI database, http://www.ncbi.nlm.nih.gov) and *CYP707A* (BQ582685, NCBI database) mRNA sequences were used that resulted in the expected amplification of 340-bp and 201-bp bands, respectively; 18S rRNA primers amplifing 0.45 kb PCR bands served as internal controls. The endogenous ABA and ACC contents of seeds at $T_{50\%}$ were calculated from Fig. 5 (dry seed mass=3.6±0.4 mg; a similar volume in µl was determined). Ethylene evolution at $T_{50\%}$ was determined in the time window between 48 h and 62 h; nd=not detected, i.e. below the detection limit. ABA and ACC leaching into the medium was determined prior to radicle emergence (prior to $T_{1\%}$) in the time window between 0 h and 30 h. For comparison, the ABA and ACC concentrations of hormone additions to the medium are presented in the last rows. These experiments were performed with the experimental setup described in Figs 4 and 5.

sugar beet fruits (Schmidt *et al*., 1994). A BR-ABA antagonism however, was evident in pharmacological experiments with wild-type tobacco (Leubner-Metzger, 2001), but not with wild-type *Arabidopsis* seeds (Steber and McCourt, 2001). An antagonism was found between ACC/ethylene and ABA in our pharmacological experiments with sugar beet, and further investigation involving measurement of endogenous ACC and ABA contents, ACC and ABA leaching, as well as ethylene evolution and the transcript regulation of ACO (ethylene-forming enzyme) and CYP707A (ABA-degrading enzyme) supports the view that the control of radicle emergence of sugar beet is mediated, at least in part, by an ethylene–ABA antagonism in interaction with the pericarp.

### *ABA inhibits sugar beet germination and embryo-mediated ABA extrusion keeps endogenous seed ABA contents low*

In the present work regarding ABA it is demonstrated: (i) high and roughly equal endogenous ABA contents are localized in the pericarp and in the seed of dry sugar beet fruits. The seed ABA contents decline very rapidly upon imbibition and are already about 6-fold lower prior to the onset of radicle emergence. This decline seems to be due to ABA degradation (transcripts of *CYP707A*, the ABA-degrading enzyme ABA 8′-hydroxylase, accumulate in fruits and seeds), the absence of ABA biosynthesis (the ABA biosynthesis inhibitor fluridone has no effect), and ABA leaching (high ABA contents are detected in the medium prior to radicle emergence). (ii) ABA leaching appears to be the most important process for the decline of the endogenous seed ABA content. In the hypothetical model presented in Fig. 7 it is proposed that an 'embryo ABA level sensor', an embryo-mediated active ABA extrusion system, and the pericarp are involved in keeping the seed ABA content low at a fixed amount of *c*. 1 pmol seed$^{-1}$. While treatments like ACC do not cause an increase in the endogenous ABA content, they seem to cause a net ABA biosynthesis, but this ABA is extruded into the medium upon imbibition. This extrusion system works for fruits and seeds and appears to be 'active ABA leaching' even against a 50-fold ABA concentration gradient, for example ABA extrusion from seeds in 100 µM ABA-containing medium keeps the endogenous seed ABA content low at 7.4 pmol seed$^{-1}$ (a seed is *c*. 3.6 mg or µl; Fig. 6). This embryo-mediated active ABA extrusion system is supported in fruits by the pericarp, which keeps the seed ABA content even 5-fold lower (Fig. 6). (iii) ABA added to the medium inhibited radicle emergence of sugar beet seeds and fruits to a different degree. While 0.1–1 mM ABA almost blocked radicle emergence of seeds, it only delayed radicle emergence of fruits by 10 h. ABA concentrations between 0.1 µM and 10 µM are known to inhibit seed germination of model species like *Arabidopsis* and tobacco (Kucera *et al*., 2005; Müller *et al*., 2006) and correspond to similar endogenous ABA contents in dry seeds of these species: about 400 pmol g$^{-1}$ DW for *Arabidopsis* ecotype Col and Cvi (Ali-Rachedi *et al*., 2004; Okamoto *et al*., 2006) and 585±86 pmol g$^{-1}$ DW for tobacco (Table 1). Our results for sugar beet therefore demonstrate that radicle emergence of seeds is inhibited by ABA to a similar extent as shown for seeds of other species. Passive ABA uptake via the pericarp seems to be slow, but active extrusion from the seed via the pericarp seems to be a fast process. Thus, ABA leaching is mediated actively by the seed and the pericarp supports the ABA extrusion. This results in lower endogenous ABA contents of the fruits compared with the seeds and seems to be one reason for the low effectiveness of ABA treatment of fruits.

### *Ethylene promotes sugar beet germination, and the pericarp restricts ACC leaching and ethylene evolution*

The conclusion that ethylene promotes sugar beet germination is based on the following findings. (i) Treatment of fruits or seeds with the direct ethylene precursor ACC

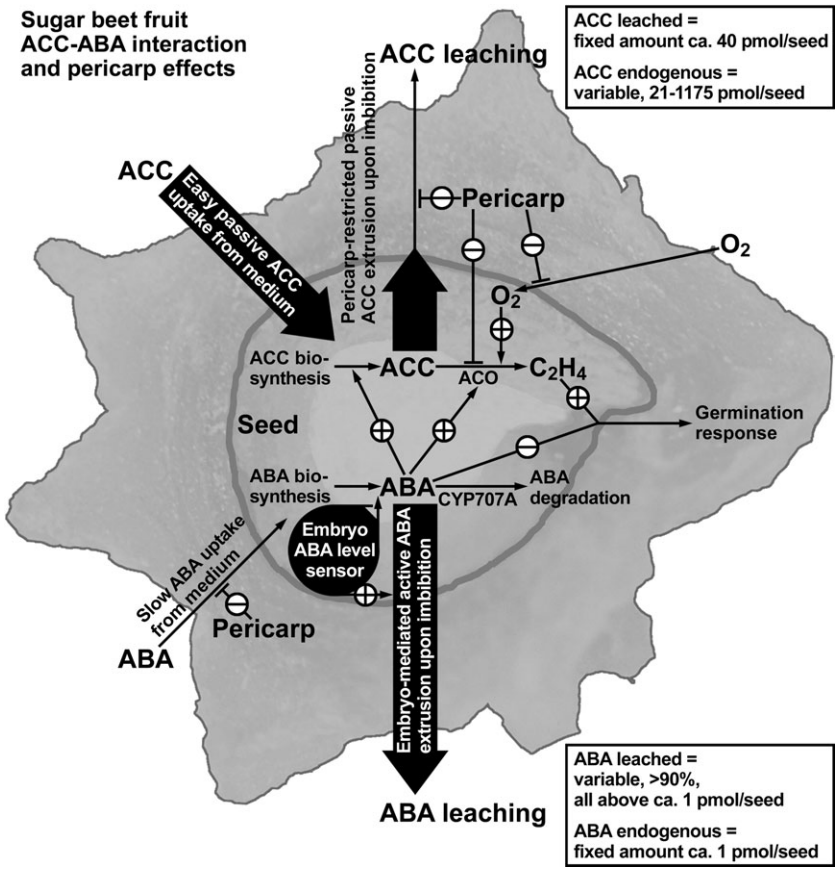



**Fig. 7.** Working model for the interaction between abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC, ethylene precursor), ethylene, and the pericarp on the germination response (radicle emergence) of sugar beet fruits. According to this model the ABA content of the seed is kept low at *c*. 1 pmol seed$^{-1}$ in accordance with the non-dormant state. This is achieved by the combined action of ABA degradation, an 'embryo ABA level sensor' and an embryo-mediated active ABA extrusion system. The pericarp supports this system and allows fast ABA extrusion leading to the low seed ABA contents. In addition, even minor elevations in the seed ABA contents cause increased ACC biosynthesis and ACO transcript expression. In contrast to this, ACC does not affect the endogenous seed ABA contents. ACC can accumulate in the seed to extremely high contents and the pericarp restricts ACC leaching. Only a fixed amount of *c*. 40 pmol seed$^{-1}$ leached out, irrespective of the endogenous ACC content. The pericarp restricts oxygen uptake and as the ACO enzyme requires oxygen, the conversion of ACC to ethylene is inhibited. In addition, the pericarp inhibits ACO gene expression, which might also be regulated by hypoxia. According to this model, sugar beet radicle emergence as the germination response depends on the antagonistic interaction between ACC/ethylene and ABA in the seed and on the physicochemical characteristics of the pericarp.

promotes radicle emergence. ACC is widely used in experiments because it is readily converted into ethylene by ACO (Petruzzelli *et al*., 2000; Kucera *et al*., 2005). That ACC acts *in vivo* via the ACO-mediated conversion into ethylene is further supported by our finding that gaseous ethylene promotes radicle emergence of sugar beet seeds. (ii) Our measurements of the endogenous ACC contents demonstrate that ACC uptake into the embryo occurs readily into imbibed fruits and seeds. The pericarp is therefore no barrier for ACC uptake and *in vivo* ethylene production from ACC could, therefore, be the cause for the promotion of radicle emergence. However, ACC leaching is restricted by the pericarp, it seems that even >50-fold differences in the seed ACC contents do not affect the amount of ACC leaching via the pericarp. This appears to be a fixed amount of *c*. 40 pmol seed$^{-1}$ for fruits, while no restriction is evident for seeds where it seems to diffuse passively. (iii) ACO transcripts accumulate in seeds and fruits during germination. This accumulation was much stronger in seeds compared with fruits, and ethylene evolution from seeds was detected, but was below the detection limit for fruits. ACC treatment of seeds caused an approximately 10-fold increase in seed ethylene evolution, but did not affect ACO transcript levels of seeds and fruits. This increase in seed ethylene evolution seems to be due to supplying a saturating ACC substrate concentration for ACO. The fact that this did not result in detectable ethylene evolution from fruits, suggest that a lack of oxygen required for ACO-mediated ethylene biosynthesis limits fruit ethylene evolution. This explanation is supported by the finding that the sugar beet pericarp is a physicochemical barrier for oxygen uptake (Coumans *et al*., 1976; Richard *et al*., 1989). Based on the enhanced ACO transcript accumulation in seeds, but not in fruits, it

is speculated if oxygen could also be a positive regulator of ACO gene induction. (iv) NBD is known to bind to the ethylene receptor and thereby prevents ethylene responses. It inhibits seed germination and this inhibition can be partially reversed by simultaneous treatment with ethylene or ACC in many species (Leubner-Metzger *et al*., 1998; Kucera *et al*., 2005) including sugar beet (this work). Ethylene biosynthesis and sensitivity are both important for the seed germination of *Arabidopsis* (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000; Kucera *et al*., 2005). In *Arabidopsis*, ethylene alone can possibly not act as a positive regulator of germination, but possibly acts by interfering with ABA signalling and synthesis. Based on these findings it is proposed that a complex interaction between the pericarp and the antagonists ACC/ethylene and ABA control sugar beet radicle emergence. However, this antagonistic ACC/ethylene–ABA interaction during sugar beet germination was found to be distinct compared with what is known in other species.

### *A novel type of antagonistic ACC/ethylene–ABA interaction during sugar beet germination*

ABA inhibits the seed germination of tobacco and *Arabidopsis* and this effect can be partially antagonized by ACC or ethylene treatment (Leubner-Metzger *et al*., 1998; Beaudoin *et al*., 2000; Ghassemian *et al*., 2000). Ethylene or ACC can have pronounced effects on ABA levels and ethylene evolution of seeds and young seedlings: Altered ethylene signaling in *Arabidopsis* seeds can increase their ABA contents (Chiwocha *et al*., 2005). Treatment of rice seedlings with ethylene or ACC induced ABA 8′-hydroxylase (Saika *et al*., 2007). Hypoxia inhibited ABA degradation and caused increased embryo ABA contents of cereal grains (Benech-Arnold *et al*., 2006). Increasing ethylene evolution accompanies germination of most Eudicot seeds and correlates positively with seed vigour (see review by Kucera *et al*., 2005). Based on the spatial induction of ACC synthase and ACO the site for ethylene production in germinating seeds is localized in the radicle (Petruzzelli *et al*., 2000, 2003; Gómez-Jiménez *et al*., 2001). Ethylene promotes ethylene biosynthesis during pea and chickpea seed germination by positive feedback regulation of ACO. ABA inhibits ACO expression, ACC accumulation, and ethylene production prior to and during chickpea radicle protrusion, but not after germination. In the present work, an antagonistic response of ACC/ethylene and ABA was also found on sugar beet germination. However, on the molecular level this ACC/ethylene–ABA interaction was distinct from the described ACC/ethylene–ABA interactions in seeds and seedlings of *Arabidopsis*, cereals, pea, and chickpea: ACC treatment of sugar beet fruits and seeds did not affect the endogenous seed ABA contents and the ACO and CYP707A transcript levels. ABA treatment induced ACC accumulation and caused increased ACO transcript levels without affecting the CYP707A transcript levels. Thus, there appears neither to be a positive autoregulatory feedback loop for ethylene, nor a negative impact of ABA on ACS and ACO in sugar beet seeds.

This novel type of ACC/ethylene–ABA interaction is also distinct from what is known in vegetative tissues of adult plants (Neill *et al*., 1986; Grossmann and Hansen, 2001; Fellner *et al*., 2005; Raghavan *et al*., 2006). There is no increase in endogenous IAA, ABA, or ACC prior to the onset of sugar beet radicle emergence; ACC/ethylene does not alter the endogenous seed ABA content and the CYP707A transcript levels; and there is an ABA-mediated increase in the seed ACC content and the ACO transcript levels.

These results with sugar beet support the finding from these other species that ACC/ethylene promotes and ABA inhibits germination, but they also show that the regulation of ACC content and ACO levels by ABA and ethylene/ACC are completely different. By contrast with pea and chickpea, ethylene/ACC do not affect ACO transcript levels. Thus, the positive autoregulatory feedback loop appears to be absent. By contrast with pea and chickpea, ABA induces ACC accumulation and does not inhibit ACO transcript accumulation in seeds and fruits. In seeds, ABA even promoted ACO transcript accumulation. Thus, there is no negative impact of ABA on ACS and ACO, but an ABA-mediated up-regulation. However, fruits and seeds differ in their quantitative responses and this is mediated by an embryo-mediated active ABA extrusion system and by the pericarp (Figs 6, 7).

It is a new finding that the pericarp-mediated control of substance exchange between the seed and the medium is highly selective: Together with the embryo-mediated active extrusion system, the pericarp restricts ABA uptake from the medium into the seed and promotes ‘active ABA leaching’ into the medium. By contrast, the pericarp restricts ACC leaching, but did not inhibit ACC uptake. The restriction of ACC leaching appears to be mediated by the pericarp itself and not by a retention system of the embryo; passive ACC leaching is evident for seeds. The thick pericarp is a barrier for oxygen and water uptake into the sugar beet seed and has been demonstrated to be a physicochemical barrier (Coumans *et al*., 1976; Richard *et al*., 1989; Santos and Pereira, 1989). Phenolic compounds are known to create seed hypoxia by oxygen fixation in the sugar beet pericarp (Coumans *et al*., 1976; Richard *et al*., 1989) and the barley glumellae (Benech-Arnold *et al*., 2006). Leaching of various endogenous germination inhibitors in sugar beet fruits has been shown (Chetram and Heydecker, 1967; Juntilla, 1976; Coumans *et al*., 1977; Morris *et al*., 1984), and it is demonstrated here that ABA is among them. Incubation of sugar beet fruit or seeds caused pH value increases of the medium from 5–6 (start of imbibition) to 6–7 (seeds) and 7–8 (fruits) before and at $T_{50\%}$ (data not shown). At pH

values between 6 and 8, ABA is almost completely present as an anion, but the embryo-mediated extrusion system explains the outward direction of the net ABA flux. By contrast with ABA, ACC is in the Zwitterion state at pH 5–8 (almost 100% at pH 5 to 7, *c*. 75% at pH 8). One possibility for the pericarp-mediated retention of ACC in the seed is that the pericarp restricts passage of molecules without net charge. The differences in ABA and ACC uptake into the seeds are not only important for germination-improving treatments ('seed technology'), but also suggests that the pericarp can be a selective barrier for other substances from the environment (in nature). Depending on the environmental conditions, the differences in ABA and ACC leaching can be important for germination and subsequent seedling growth. The dramatic seed ACC accumulation could, later, serve in the seedling for ethylene production and increased seedling vigour.

Taken together, the physicochemical properties of the pericarp can modify the complex interaction of ethylene and ABA in controlling sugar beet radicle emergence. Differences in the pericarp, in ABA and ACC leaching, in ethylene biosynthesis, ACO gene expression, and in ethylene–ABA signalling might therefore be key factors of sugar beet radicle emergence.

## Supplementary data

Figure S1 is available at *JXB* online as supplementary data of this manuscript. These data show that with pharmacological experiments (treatments with plant hormones and biosynthesis inhibitors) no effect of GA, BR, auxin, cytokinins, or jasmonates on sugar beet radicle emergence was obtained.

## Acknowledgements

We thank Tadao Asami (RIKEN Institute of Physical and Chemical Research, Japan) for kindly providing brassinazole, Wilhelm Rademacher (BASF AG, Agricultural Research Station Limburgerhof, Germany) for his valuable advice on plant growth retardants, Jiri Malbeck (Institute of Experimental Botany, Prague, Czech Republic) for performing the measurements of cytokinins, and Xiaofeng Wang (College of Life Sciences, South China Agricultural University, Guangzhou, China) for his critical reading. Expert technical assistance by Anke Jagonak and financial support for this project by the KWS SAAT AG (Einbeck, Germany) to Gerhard Leubner-Metzger and the grant AV0Z 50380511 to Ivana Macháčková are gratefully acknowledged.

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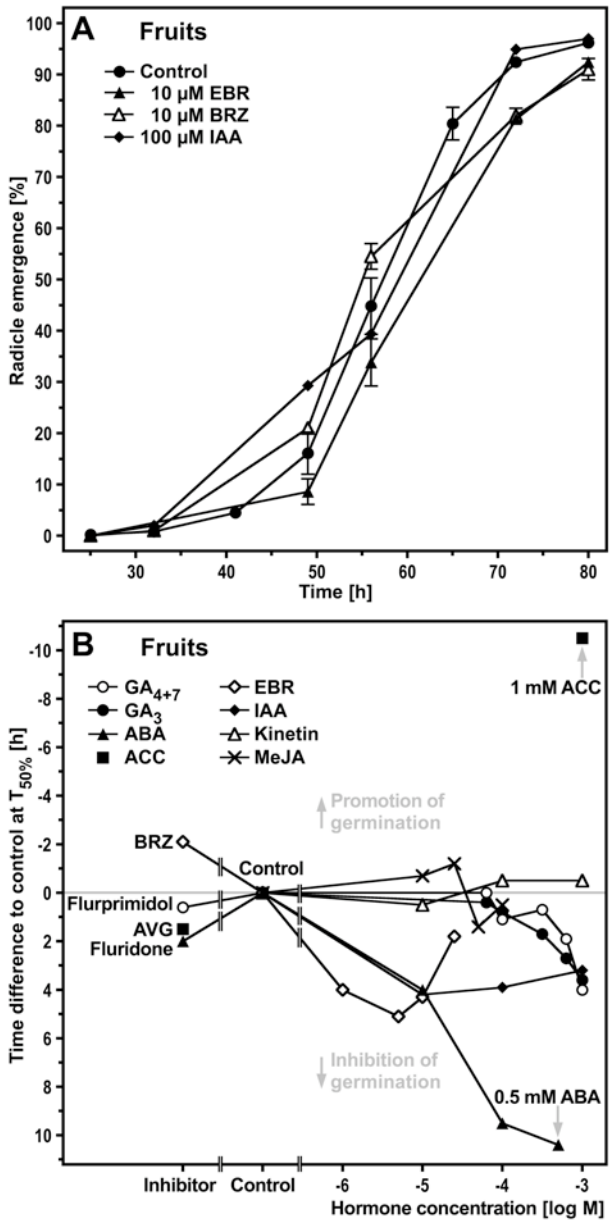


**Supplementary Figure 1.** The effects of various plant hormones and hormone biosynthesis inhibitors on radicle emergence of *Beta vulgaris* fruits. (A) Time course analysis of the effects of 24-epi-brassinolide (EBR), brassinazole (BRZ, BR biosynthesis inhibitor), and indole-3-acetic acid (IAA, auxin) on radicle emergence of fruit populations. The endogenous contents of the auxin indole-3-acetic acid (IAA)

are higher in the dry sugar beet seeds compared to the pericarp (Table 1). In addition, endogenous BRs have been detected in dry sugar beet fruits (Schmidt et al., 1994). Supplementary Figure 1 shows that Neither EBR, nor treatment with brassinazole (BRZ, Asami et al., 2000), the inhibitor of BR biosynthesis, nor IAA treatment affected radicle emergence. In addition, EBR-permeation had no effect on sugar beet germination (data not shown). (B) Dose-response curves for gibberellins ($GA_{4+7}$, $GA_3$), ABA, 1-aminocyclopropane-1-carboxylic acid (ACC, ethylene precursor), EBR, IAA, kinetin (cytokinin), methyljasmonate (MeJA), and the hormone biosynthesis inhibitors flurprimidol, fluridone, BRZ and aminoethoxyvinylglycine (AVG, ethylene biosynthesis inhibitor) on the radicle emergence of fruit populations. Based on these pharmacological experiments no effect of GA, BR, auxin, cytokinins or jasmonates on sugar beet radicle emergence was obtained. The times for 50 % radicle emergence ($T_{50\%}$) of the fruit populations were calculated for each treatment. The difference in hours of these $T_{50\%}$ values to the corresponding controls are presented; a negative value (upper part of the graph) is therefore a promotion and a positive value (lower part of the graph) an inhibition of radicle emergence. Note the logarithmic scale for the hormone concentrations. Radicle emergence of fruit populations was scored over time; mean values ± SE (A) or $T_{50\%}$ values (B) of at least one experiment with triplicates of 100 fruits are presented; conditions and statistics as in Fig. 2.