DANÚBIA GONÇALVES CARDOSO

PHYTOTOXIC ACTIVITY OF AUXIN ON GERMINATION AND SEEDLING GROWTH OF *Stylosanthes humilis*

Thesis presented to the Universidade Federal de Viçosa as part of the requirement of the Pos-Graduate Program in Plant Physiology for obtention of the degree of Magister Scientiae

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ABSTRACT

CARDOSO, Danúbia Gonçalves, M.Sc., Universidade Federal de Viçosa, July, 2015. **Phytotoxic activity of auxin on germination and seedling growth of Stylosanthes humilis.** Advisor: Dimas Mendes Ribeiro. Co-advisor: Wagner Luiz Araújo.

Although auxins, at high concentrations, are well known for their effects on plant growth control, little is known about their effects during seed-to-seedling transition. This study was carried out to evaluate the modulation of ethylene biosynthesis and metabolic adjustment in response to auxin during seed germination and postgermination growth of Stylosanthes humilis. In freshly harvested seeds (dormant seeds) of S. humilis, auxin, at high concentration, repressed the 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase activity. On the other hand, ACC biosynthesis and ethylene production by seedlings were increased by auxin treatments, which was associated with seedling growth inhibition. Furthermore, auxin inhibited ACC biosynthesis in aged seeds (non-dormant seeds). Together, these data indicate that the effect of high levels of auxin in the control of ethylene metabolism is developmentally separated. In addition, 2,4-dichlorophenoxyacetic acid (2,4-D) induced secondary dormancy in aged seeds of S. humilis, which was associated with the operation of energy-saving metabolism. Moreover, secondary dormancy of 2,4-D treated seeds was broken by ACC solution. Thus, this study has revealed a process in which storage reserves mobilization and ethylene crosstalk in regulating the developmental transition from metabolic quiescent seed to actively growing seedling.

RESUMO

CARDOSO, Danúbia Gonçalves, M.Sc., Universidade Federal de Viçosa, Julho, 2015. **Atividade fitotóxica das auxinas na germinação e no crescimento de plântulas de Stylosanthes humilis.** Orientador: Dimas Mendes Ribeiro. Co-orientador: Wagner Luiz Araújo.

Apesar dos efeitos das auxinas, em altas concentrações, no crescimento das plantas ser bem conhecido, pouco se conhece sobre os seus efeitos durante a transição de sementes para plântulas. Este estudo foi realizado para avaliar o efeito da biossíntese de etileno e do ajuste metabólico em resposta a auxina, durante a germinação das sementes e no crescimento de plântulas de Stylosanthes humilis. Em sementes dormentes de S. humilis, auxina, em elevada concentração, inibiu a atividade da oxidase do ácido 1-aminociclopropano-1-carboxílico (ACO). Por outro lado, a biossíntese do ácido 1-aminociclopropano-1-carboxílico (ACC) e a produção de etileno em plântulas aumentaram quando tratadas com auxinas, o que foi associado com a inibição do crescimento das plântulas. Além disso, auxina inibiu a biossíntese do ACC em sementes não-dormentes de S. humilis. Em conjunto, estes dados indicam que o efeito da auxina, em alta concentração, no controle e no metabolismo do etileno é de fato temporalmente separado. Além disso, o ácido 2,4-diclorofenoxiacético (2,4-D) induziu dormência secundária em sementes de S. humilis, o que foi associado com um mecanismo de reserva de energia. Além disso, a dormência secundária de sementes tratadas com 2,4-D foi quebrada por solução de ACC. Assim, este estudo revelou a existe de um mecanismo em que a mobilização de reservas e a biossíntese de etileno se conectam para regular a transição do desenvolvimento das sementes dormentes para o crescimento plântulas.

1. INTRODUCTION

Auxin is a key morphogenetic hormonal class involved in numerous developmental programs in plants (De Semet et al 2011). At low concentrations indole-3-acetic acid (IAA), the main auxin in higher plants, notably contributes to the regulation of cell division and elongation (Teale et al 2006, Grossmann 2010). However, at high concentrations IAA induces a variety of growth abnormalities, including epinasty and inhibition of shoot and root growth (Grossmann 2010, Pazmiño et al 2012). These symptoms provided the basis for the use of synthetic mimics of auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 1-naphthalene acetic acid (1-NAA), as herbicides in agriculture (Grossmann 2007, Song 2014). In this context, stimulation of ethylene biosynthesis is a common response when auxins, at high concentration, are applied to sensitive plant species (Grossmann and Scheltrup 1997, Grossmann 2010) or overproduced in transgenic plants (Klee and Lanahan 1995). The current model of auxin action emphasizes that elevated levels of auxin increase the activity of 1-aminocyclopropane-1-carboxylic acid synthase (ACS, EC 4.4.1.14) few hours after treatment, accompanied by increased 1-aminocyclopropane-1-carboxylic acid (ACC) concentration and ethylene formation in the tissue (Grossmann 2010). The stimulation of ethylene biosynthesis, which in turn triggers the biosynthesis of abscisic acid accompanied by overproduction of reactive oxygen species, is thought to be responsible for tissue damage and cellular death (Hansen and Grossmann 2000, Pazmiño et al 2011).

In the model dicot Arabidopsis, auxin has been shown to stimulated ethylene production by increased transcription of eight of the nine ACS genes (Tsuchisaka and Theologis 2004). In addition, the post-germination accumulation of ACC oxidase (ACO) and ethylene production of Cicer arietinum (chick-pea) seedlings was promoted by IAA

(Gómez-Jiménez et al 2001). However, IAA has no appreciable effect on ACO expression prior to or during germination of chick-pea. Together, these observations indicate that auxin and ethylene cascades share some common features in terms of mediation of seedling growth. In general, seedling growth is inhibited by high concentration of auxin through ethylene biosynthesis (Grossmann 2010). In contrast with the situation in seedlings, ethylene is known to promote seed germination of many species, including the germination of several agricultural weeds (Matilla and Matilla-Vázquez 2008, Corbineau et al 2014). Auxinic herbicides are widely used in agriculture to selectively control broadleaf weeds in cereal crops (Praczyk et al 2012). Despite auxinic herbicides do not have prolonged soil residual activity (with the exception of the pyridine carboxylic acids), previous studies provided information about the evolution of resistance to auxinic herbicides in plants (reviewed by Mithila et al 2011). In studies of mechanisms of auxinic herbicides resistance, the sensitive biotypes of Echinochloa crusgalli var. zelayensis and Sinapis arvensis produced more ethylene than resistant biotypes (Peniuk et al 1993, Xu et al 2013). This response was attributed with differential activity of ACS and ACO enzymes, concomitant with auxinic herbicide treatment. Although these studies have advanced our understanding of how ethylene is involved in auxin toxicity stress, relatively little is known concerning the role of the functional relationship between auxin and ethylene biosynthesis during seed-to-seedling transition, particularly in the ethylene-requiring seeds.

Townsville stylo (Stylosanthes humilis), an annual tropical forage legume, is distributed widely in tropical and subtropical regions of the Americas, Africa and Southeast Asia (Williams et al 1984, Costa 2006). The species has been considered as a potential contributor for pasture improvement in tropical zones due to its high-quality forage for livestock, high seed production, and wide adaptability to low fertility soils (Gardner 1984, Edye 1987). Seeds of Townsville stylo exhibit a relatively hard tegument

requiring a physical or chemical scarification to enable germination. S. humilis seeds also present a physiological dormancy, when freshly-harvested, which is gradually overcome with seed ageing (Vieira and Barros, 1994). Furthermore, free and total ACC accumulate to levels much higher in aged seed than in freshly harvested seeds (Pinheiro et al 2008). As with ACC, aged seeds produce as much as 10- to 12-fold more ethylene than freshly harvested seeds (Pelacani et al 2005). In this regard, inhibitors of ethylene biosynthesis such as 2-aminoethoxyvinylglicine (AVG), which blocks ACS activity, and Co^{2+}, which interferes with ACO activity, inhibit germination of aged seeds (Ribeiro and Barros 2006). This means that a greater capacity to synthesize ethylene is associated with germination of Townsville stylo seeds (Silva et al 2014). Together, these biochemical and physiological findings in Townsville stylo seeds are in agreement with what is known for the ethylene-mediated regulation of gene during seed germination of several species. Thus, Townsville stylo seeds were used as model system to determine the effects of auxin toxicity on seed germination and seedling development of dicotyledonous plants. These analyses indicate that the ethylene metabolism is flexibly regulated by auxin treatments during seed germination and postgermination growth. Furthermore, the functional characterization of ethylene biosynthesis during seed-to-seedling transition reveals that the relationships linking ethylene and seed germination are modified by auxin treatment, thus leading to the induction of the secondary dormancy in the seeds.

2. MATERIAL AND METHODS

2.1 Plant material and germination conditions

Plants of S. humilis were grown in 3.0 L plastic pots in a greenhouse in Viçosa (20º45’S, 42º15’W), Minas Gerais, Brazil. Matures pods were harvested and stored in the

laboratory (25-30 ºC). Dormant (about 10 post-harvest days old) and non-dormant (about 420 post-harvest days old) seeds were dehusked, scarified with fine sand paper (nº 150) for a few seconds, sterilized with 0.5 % NaOCl for 10 min and thoroughly washed with deionised water. Seeds were placed in glass Petri-dishes 150 mm diameter (for quantification of ACC and ACO activity) or in 50 ml Erlenmeyer flasks (for ethylene determinations) containing two layers of Whatman nº 1 filter paper moistened with 15 or 5 ml test-solution, respectively. Petri-dishes and sealed flasks containing seeds were placed into the darkness in a day/night growth chamber (Forma Scientific Inc., Ohio, USA), at 30 ºC. A seed was considered as germinated upon protrusion of its radicle.

2.2 Early growth assays

Non-dormant seeds were imbibed in deionised water for 24 h. Afterwards, germinated seeds with a protruded radicle about 5 mm long were transferred to Erlenmeyer flasks with two layers of filter paper, and incubated with 5 ml of deionised water (control) or solution of IAA and 2,4-D (both at 1 mM), alone or to each one combined with 0.1 mM AVG. The Erlenmeyer flasks containing the seedlings were placed in the growth chamber under a 16/8 h day/night cycle (30/25 ºC) with 150 μmol m^{-2} s^{-1} light intensity. Following 48 h exposure period, root and hypocotyl lengths as well as ethylene production by the seedlings were determined.

2.3 Ethylene measurement

Erlenmeyer flasks containing seeds or seedlings were stoppered with a rubber serum cap, and kept in growth chamber, under the conditions previously described. An air sample (1 ml) was taken from the flask headspace and injected in a gas chromatograph (Hewlett Packard 5890, Series II), equipped with a stainless-steel column (1.0 m x 6.0 mm)

packed with Porapak-N 80-100 mesh. Analyses were conducted under the following conditions: nitrogen carrier gas and hydrogen fluxes 30 ml min^{-1}; and air flux 320 ml min^{-1}. Column, injector, and detector temperatures were 60, 110, and 150°C, respectively. Ethylene peaks were registered by a peak simple software (Peak Simple, Version 3.92) coupled to the chromatograph, and quantified by comparison with authentic ethylene standards.

2.4 ACC measurement

In order to evaluate the relationship between auxin and ACC biosynthesis in seed germination and early seedling development, the contents of ACC were measured in dormant and non-dormant seeds as well as seedlings of S. humilis treated with IAA and 2,4-D solutions for 24 h. Afterwards, seeds and seedlings were thoroughly washed with deionised water, paper-dried and frozen in liquid nitrogen and stored at −80 ºC until analysis. ACC level was quantified as described by Bulens et al (2011) with slight modifications. Seeds were ground in a pestle and mortar with 80 % ethanol plus 5 % (w/v) polyvinyl-polypyrrolidone (PVPP). The extract was centrifuged (12,000 g, 4º C, 20 min); the supernatant was evaporated at 45 ºC until dryness and the residue was dissolved in 2.0 ml deionised water. Half the extract was taken for free ACC analysis by its chemical conversion to ethylene (Lizada and Yang, 1979). The other half was used to determine total (free plus conjugated) ACC. This portion of the extract was hydrolyzed with HCl (2.0 N) at 100 ºC for 4 h. Following hydrolysis, the extract was neutralized with NaOH (2.0 N), evaporated until dry and dissolved in 2 ml deionised water for ACC determination as before.

2.5 ACO activity measurement

Dormant seeds of S. humilis were incubated with deionised water or solution of 1 mM ACC alone or ACC containing IAA, 2,4-D or $Co(NO_3)_2$ at 10 µM, for 24 h. Afterwards, seeds were thoroughly washed with deionised water, paper-dried and frozen in liquid nitrogen and stored at −80 ^oC until analysis. ACO activity was quantified as described by Bulens et al (2011) which slight modifications. Seeds were ground with mortar and pestle with 4 ml of extraction buffer containing 0.1 M Tris-HCl (pH 7.0), 10 % (w/v) glycerol, 30 mM sodium ascorbate, 1 mM dithiothreitol, 0.1 % (v/v) Triton X-100 and 5 % (w/v) PVPP. The slurry was centrifuged at 12,000 g for 20 min and the supernatant was desalted by passing through a Sephadex G-25 column (Pharmacia PD-10) eluted with reaction buffer (without ACC). Desalt extract was used for the assay of ACC oxidase activity immediately. Afterwards an aliquot (0.2 ml) of the extract was incubated in a reaction buffer containing 1.8 ml extract buffer (without PVP), 50 µM $FeSO_4$, 30 mM $NaHCO_3$ and 1 mM ACC. Tubes were stoppered with serum caps and incubated in a water-bath at 32 ^oC for 1 h. Ethylene produced was determined as described above. No ACC oxidase activity was observed with boiled extract or when extract was omitted from the incubation medium.

2.6 GC-MS-based metabolite analysis

Metabolite analysis by gas chromatography-mass spectrometry (GC-MS) was carried out by a method described by Cross et al. (2006). Townsville stylo seed tissue (100 mg) was extracted in 1.4 mL of methanol, and 60 µL of internal standard (0.2 mg ribitol mL^{-1} water) was subsequently added as a quantification standard. The mixture was extracted for 15 min at 70°C and mixed vigorously with 1.4 mL of water. To separate polar and nonpolar metabolites, 750 µL of chloroform was then added to the mixtures. After centrifugation at 2,200 g, the upper methanol/water phase was taken and reduced to

dryness in vacuum. Residues were redissolved in and derivatized in 40 µL of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine at 37°C for 120 min. Afterwards the extract was treated with 60 µL of N-methyl-N-[trimethylsilyl]trifluoroacetamide at 37°C for 30 min. Eight microliters of a retention time standard mixture (0.029% [v/v] n-dodecane, n-pentadecane, n-nonadecane, n-docosane, n-octacosane, n-dotracontane, and n-hexatriacontane dissolved in pyridin) was added before trimethylsilylation. Sample volumes of 1 µL were then injected in the GC-MS system comprised an AOC-20i autosampler, and a QP2010 SE gas chromatograph-quadrupole mass spectrometer (Shimadzu, Tokyo, Japan), equipped with a column Rtx-5MS (Restek, Bellefonte, CA, USA). GC-MS data processing into a standardized numerical data matrix and compound identification were performed using the TagFinder software (Luedemann et al 2008). Compounds were identified according to standardized guidelines (Dethloff et al 2014) by mass spectral and retention time index matching to the mass spectral collection of the NIST11 database. Laboratory and reagent contaminations were identified by non-sample control experiments and removed for further analysis. Numerical analyses were based on the peak height values of the recoded mass feature, i.e., the response values. These values were corrected for the dry weight of each sample and by the response of the internal standard,13C6-sorbitol, from each respective GC-MS chromatogram to obtain normalized responses.

2.7 Respiration measurement

S. humilis seeds were incubated with either deionized water (control) or 2,4-D solution for a period of 12 h. Following exposure period, seeds were rinsed three times with deionized water and transferred to the Erlenmeyer flasks containing deionized water, 2,4-D or ACC solution. Erlenmeyer flasks containing seeds were stoppered with a rubber

serum cap, and kept in growth chamber, under the conditions previously described, for additional 12 h. An air sample (1 ml) was taken from the flask headspace and injected in a gas chromatograph (ShimadzuCrop, CG-14B), equipped with a stainless-steel column packed with Porapak-Q. Carbon dioxide peaks were registered by a peak simple software (Peak Simple, Version 3.92) coupled to the chromatograph, and quantified by comparison with authentic carbon dioxide standards.

2.8 Metabolites and protein content measurements

Sucrose and glucose were measured in ethanolic solutions prepared from seeds samples as described by Cross et al (2006). Starch and protein contents were determined on the pellet suspended in NaOH 100 mM. Protein amounts were assessed with the Bio-Rad Bradford reagent (Bio-Rad Laboratories) according to the manufacturer's instructions. Assays were prepared in 96-well microplates and absorbance was read in VersaMax ELISA microplate readers (Molecular Devices, Sunny Valle, EUA).

2.9 Statistical analysis

The statistical design of the assays was based on a completely randomized distribution with six replicates with 50 seeds or seedlings each for ethylene and carbon dioxide determinations in Erlenmeyer flasks. Germination percentage was transformed to arcsin $(\% \ G/100)^{1/2}$ prior to analysis and all data were checked for normality. For quantification of ACC levels, ACO activity and metabolite levels six replicates with 150 seeds or seedlings each were assayed. Analysis of variance (ANOVA; $P < 0.05$) was used to compare treatment effects (Sokal and Rohlf 1995). If ANOVA showed significant effects, a Tukey or t-test ($P < 0.05$) was used to determine differences among treatments.

All mean comparisons were performed with SPSS (Statistical Package for the Social Sciences) 11.0 for Windows Statistical Software Package.

3. RESULTS

3.1 Comparison of the response of the ethylene biosynthesis to auxin toxicity during seed germination and seedling development

IAA and 2,4-D at the concentration used promoted a large decrease on seed germination, with a correspondingly low ethylene production by the seeds (Fig 1A). IAA and 2,4-D also inhibited seedling growth of Townsville stylo, but ethylene production by seedlings was substantially increased by auxin treatments (Fig 1B). To further characterize the effect of auxin on ethylene biosynthesis during seed-to-seedling transition, we next monitored the levels of ethylene in non-dormant seeds and seedlings treated with the ethylene biosynthetic precursor ACC plus 2,4-D and AVG, which blocks ACS activity, plus 2,4-D, respectively. Ethylene biosynthesis of seeds increased in response to ACC treatment (Fig. 2A). Moreover, germination of auxin-inhibited seeds increased markedly with increasing exogenous ACC concentration. The maximum effect induced by applied ACC occurred at 1000 µM, whereby germination of 2,4-D-inhibited seeds was rescued, reaching 90 %, and ethylene production was increased by 48-fold, as compared with the control (without ACC). On the other hand, AVG increased growth of 2,4-D-inhibited seedling in a dose-dependent manner, with a correspondingly dose-response inhibition in ethylene production by the seedlings (Fig. 2B). Similar results were obtained both in the IAA- and 2,4-D-inhibited seed and seedling (see Supplementary Fig. S1), suggesting that type of auxin does not influence the response to ethylene biosynthesis.

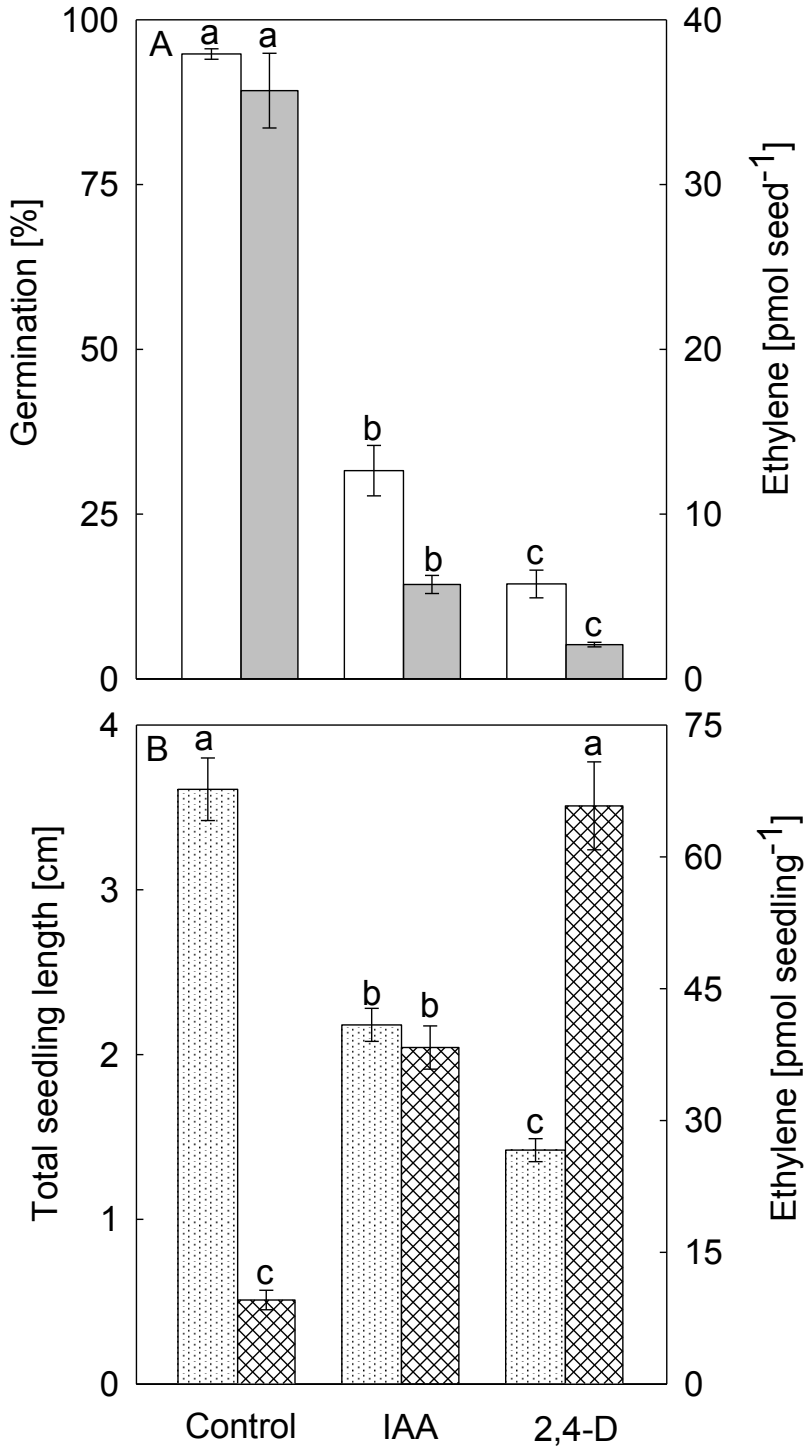

Fig. 1. Ethylene biosynthesis during seed germination and postgermination growth of S. humilis treated with auxins. Effects of IAA and 2,4-D solutions on the germination (open bars) and ethylene production (grey bars) by seeds (A). Effects of IAA and 2,4-D solutions on the early growth (dotted bars) and ethylene production (checkered bars) by seedlings (B). Data points are means of six replicates ± standard error

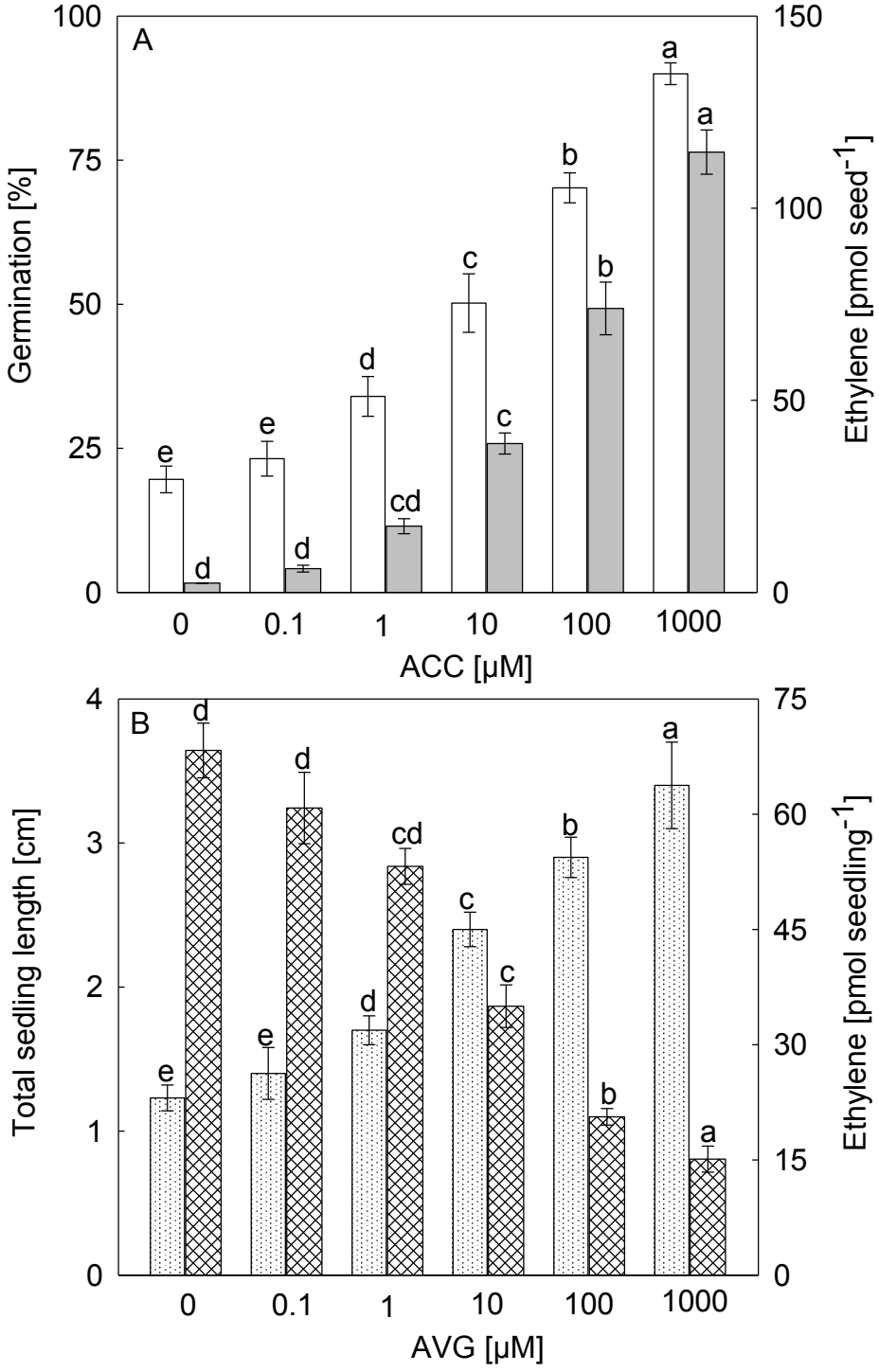

Fig. 2. Effects of ACC supplied in 2,4-D solution on the germination (open bars) and ethylene production (grey bars) by seeds (A). Actions of AVG supplied in 2,4-D solution on the early growth (dotted bars) and ethylene production (checkered bars) by seedlings (B). Seed germination, seedling length and ethylene production were determined following a 24 h exposure period. Seed germination and ethylene production in deionised water were 98.2±1.5% and 41.4±3.2 pmol seed^{-1}, respectively. Seedling growth and ethylene production in deionised water were 3.8±0.4 cm and 9.3±1.0 pmol seedling^{-1}, respectively. Bars followed by the same letters do not differ statistically at the 5 % level by Tukey test. Data points are means of six replicates ± standard error

Non-dormant seeds and seedlings treated with IAA and 2,4-D also showed a distinct response in levels of free and total ACC. For instance, free ACC content decreased by 82 % and 90 % in seeds treated with IAA and 2,4-D, respectively, whereas the same treatments resulted in seedlings of S. humilis having an increasing in free ACC by 11- and 14-fold, respectively (Fig 3A, B). The amount of total ACC was decreased by 91 % and 94 % in non-dormant seeds treated with IAA and 2,4-D, respectively (Fig 3A). However, exposure of S. humilis seedling to IAA and 2,4-D led to a marked increase in tissue total ACC concentration (Fig 3B).

3.2 Regulation of ethylene biosynthesis on dormant seed in response to auxin

We also investigated the effects of auxin, at high concentration, on physiological dormancy breakage of S. humilis. The content of free and total ACC of dormant seeds were no affected by IAA and 2,4-D treatment, seeds contained about 3.5 pmol free ACC $seed^{-1}$ and 12 pmol total ACC $seed^{-1}$. In addition, treatment of dormant seeds with IAA and 2,4-D had no effect on the germination response or on ethylene emanation along the time course of the experiment (Fig 4A-F). As expected, germination kinetics of imbibed seeds over 24 h showed that dormancy was broken quite rapidly by ACC (Fig 4A). At 1 mM ACC the time taken for 50 % germination (t_{50}) to occur was 10.4 h, with a maximum germination (G_{max}) of 90 %. On the other hand, IAA and 2,4-D largely depressed germination of ACC-stimulated seeds, as showed by G_{max} values of 39 % and 25 %, respectively (Fig 4B, C). Furthermore, ethylene production of dormant seeds treated with ACC was greatly increased along the time course of the experiment (Fig 4D). However, ethylene production was decreased by 71 % and 79 % in dormant seeds treated with ACC plus IAA or ACC plus 2,4-D, respectively, as compared with seeds imbibed just with ACC solution for 24 h (Fig 4E, F). In keeping with these responses, the ACC-induced increase in activity of ACO

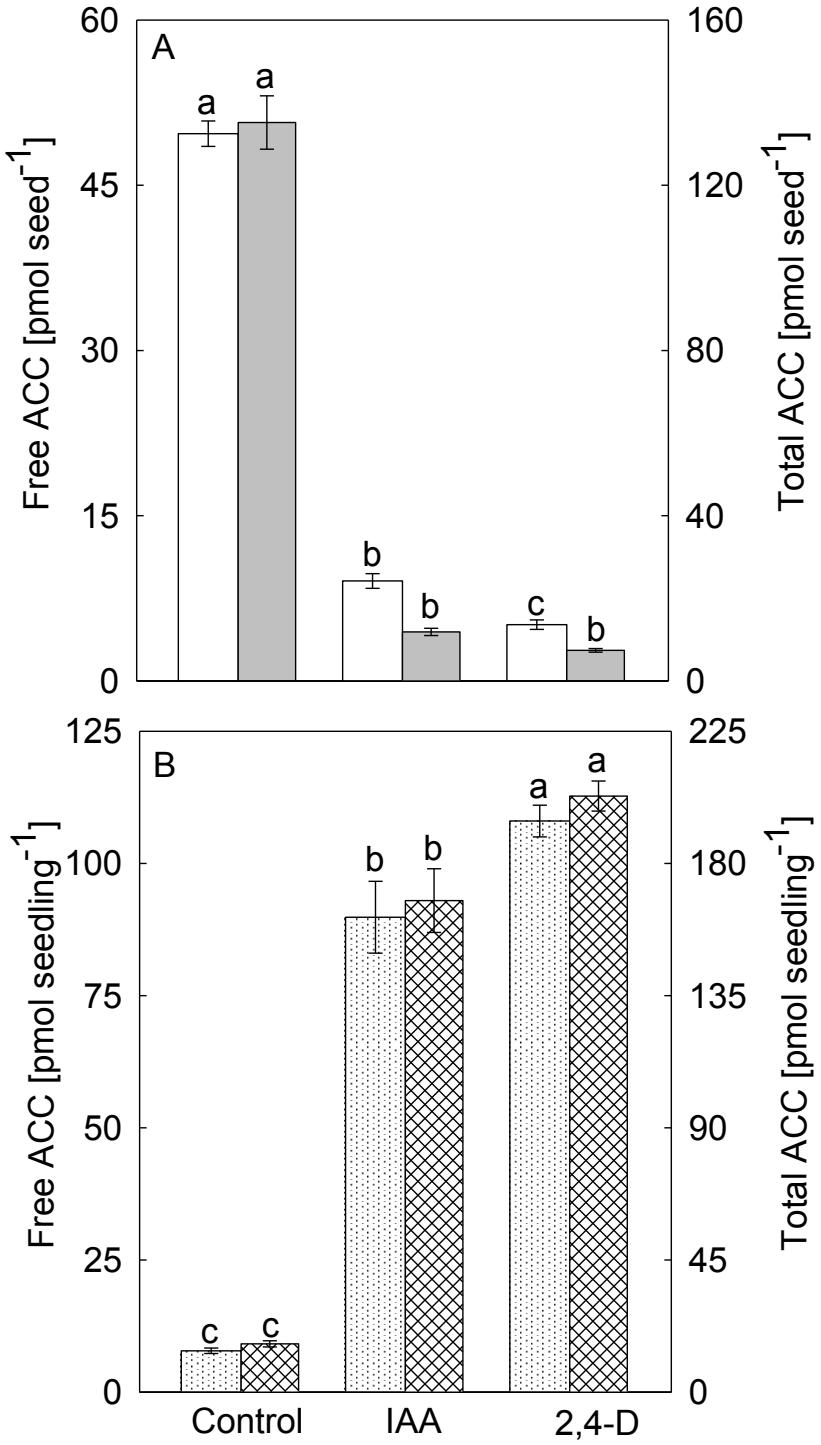

Fig. 3. Changes in contents of ACC in seed and seedlings treated with auxins. Effects of IAA and 2,4-D solutions on the free (open bars) and total (grey bars) ACC contents by seeds (A). Effects of IAA and 2,4-D solutions on the free (dotted bars) and total (checkered bars) ACC contents by seedlings (B). ACC levels were determined following a 24 h exposure period. Bars followed by the same letters do not differ statistically at the 5 % level by Tukey test. Data points are means of six replicates ± standard error

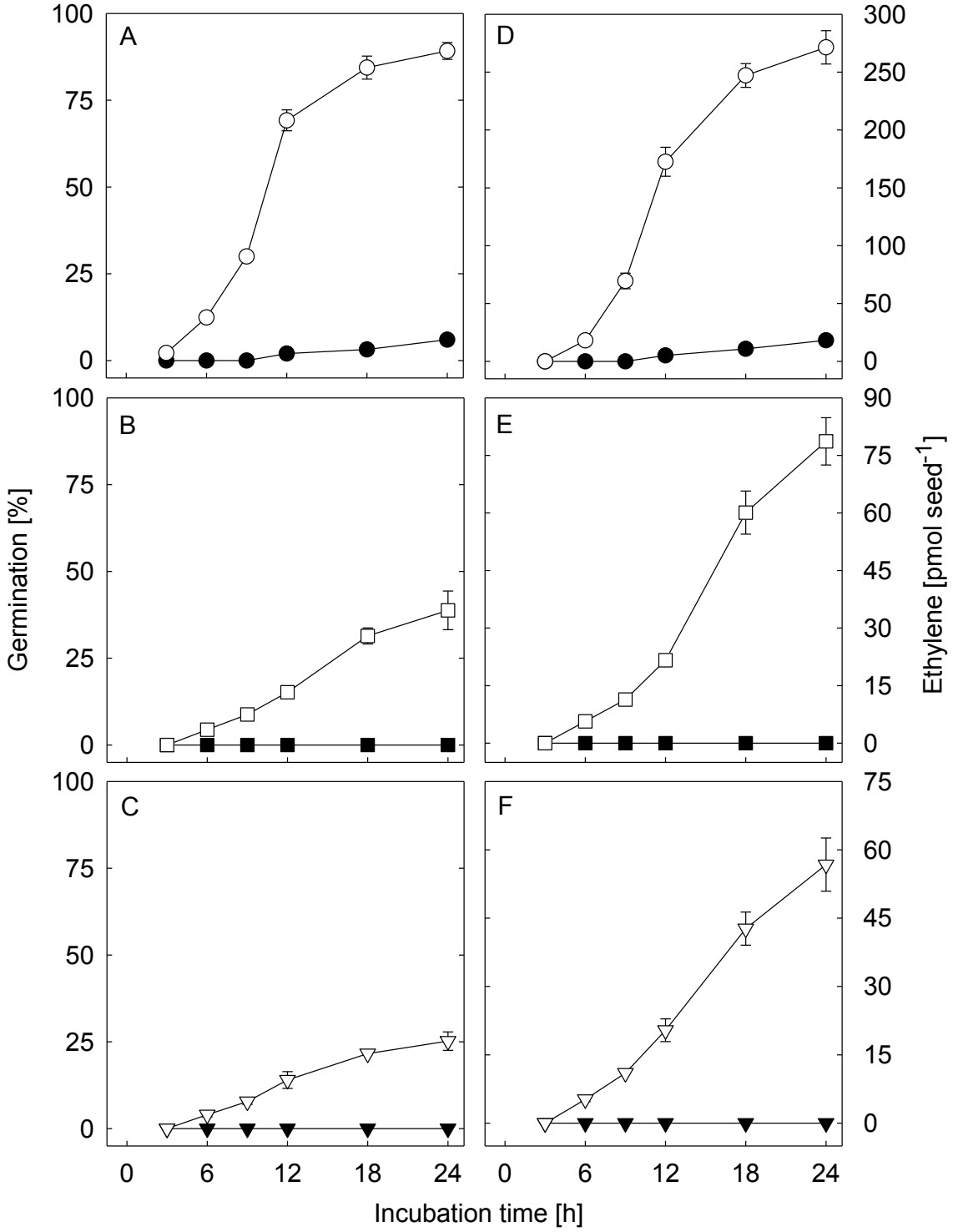

Fig. 4. Time course changes of germination and ethylene production by dormant seeds (A-F). (1) Filled circle, deionised water (control); (2) open circle, 1mM ACC; (3) filled square, 1mM IAA; (4) open square, IAA plus ACC; (5) filled triangle, 1mM 2,4-D; (6) open triangle 2,4-D plus ACC. Total germination recorded as the sum of the percentage of seed germinated under test solutions and germination recovery recorded after an extra 24 h upon seed transfer to 0.1 mM ethephon was 94 ± 1.8 % (control), 92 ± 2.5 % (IAA), 96 ± 1.2 % (IAA plus ACC), 90 ± 3.7 % (2,4-D) and 92 ± 2.2 % (2,4-D plus ACC). Data points are means of six replicates ± standard error

was substantially reduced by IAA and 2,4-D (Table 1). Moreover, $Co(NO_3)_2$ promoted a decrease in activity of ACC oxidase of ACC-treated seeds, which kept a close relationship with the germination process.

Table 1 Effects of auxins and $Co(NO_3)_2$ on germination and activity of ACC oxidase of ACC-stimulated dormant seeds. Both parameters were assayed 24 h from start of seed incubation in water, 1mM ACC, ACC plus 1mM IAA and ACC plus 1mM 2,4-D. Seed germination and activity of ACC oxidase in IAA and 2,4-D solution were nil. In each column means do not differ significantly at the 5 % level by Tukey test, when followed by the same letter. Data points are means of six replicates ± standard error

Treatment	Germination [%]	Ethylene [pmol $(mg^{-1}$ protein$)^{-1}$ h^{-1}]
Water (control)	5.6 ± 0.4 d	48.6 ± 2.8 c
ACC	92.2 ± 1.7 a	287.2 ± 21.3 a
ACC + IAA	37.0 ± 2.3 b	95.7 ± 3.8 b
ACC + 2,4-D	25.6 ± 2.0 c	70.3 ± 7.2 bc
ACC + $Co(NO_3)_2$	38.6 ± 2.9 b	69.4 ± 2.7 bc

3.3 Induction of secondary dormancy by auxin

In order to investigate whether auxin toxicity might be involved in the induction of secondary dormancy, non-dormant seeds were incubated with 2,4-D and, then transferred to water or ACC. Seeds treated just with 2,4-D showed a reduction of 75 % on germination and 84 % on ethylene production after 72 h of incubation in auxin solution (Fig. 5A, B). On the other hand, seed germination was recovered when seeds were incubated for 12 h in 2,4-D and then transferred to ACC. In addition, ethylene production of seeds treated with 2,4-D was greatly increased upon their transfer to ACC. In contrast, germination and ethylene production by the seeds treated with 2,4-D for 12 h and afterwards transferred to water were not significantly altered from those of treated solely with 2,4-D. Importantly,

germination of seeds treated with 2,4-D→2,4-D and 2,4-D→water was rescued by application of ethephon (2-chroethylphosphonic acid), an ethylene-releasing compound, indicating that the seeds were alive. Together, these data suggest that secondary dormancy was induced by auxin toxicity.

To assess the overall metabolic response to the auxin-induced secondary dormancy of Townsville stylo seeds, an established GC-MS protocol was used for metabolite profiling. Glucose and fructose showed a clearly reduced level in seeds treated with 2,4-D alone or following their transfer from 2,4-D solution to deionised water, while ACC only induced a slight increase in the fructose level (Fig 6A, see Supplementary Table S1). ACC increased the glucose-6-P and fructose-6-P content of 2,4-D treated seed, while there were no significant differences in hexose-phosphates in seeds treated just with 2,4-D or after 12 h of incubation in 2,4-D and then transferred to deionised water. Trehalose content was unaltered in seeds treated with 2,4-D for 12 h and then transferred to ACC, but 2,4-D alone or 2,4-D following deionised water led to an increase at trehalose content. Moreover, there was a significant increase in glyceric acid-3-phosphate level across all treatment. Interestingly, the level of galactinol was increased >16-fold and >11-fold in seeds treated just with 2,4-D or after 12 h of incubation in 2,4-D and then transferred to deionised water, respectively (Fig 6A). ACC decreased the galactinol content of 2,4-D-treated seeds. Furthermore, the effects of the 2,4-D on lactic acid, glyceric acid, GABA and pyruvic acid levels were reversed upon transferring the inhibited seeds to ACC. The analysis also revealed that the 2,4-D promoted a large decrease in the levels of a wide range of amino acids and TCA cycle intermediates (Fig 6A). A significant decreased in TCA cycle intermediates and amino acids levels was also observed in seeds incubated for 12 h in 2,4-D and then transferred to deionised water. On the other hand, the effects of 2,4-D on inhibition of TCA cycle intermediates and amino acids (except for arginine and serine)

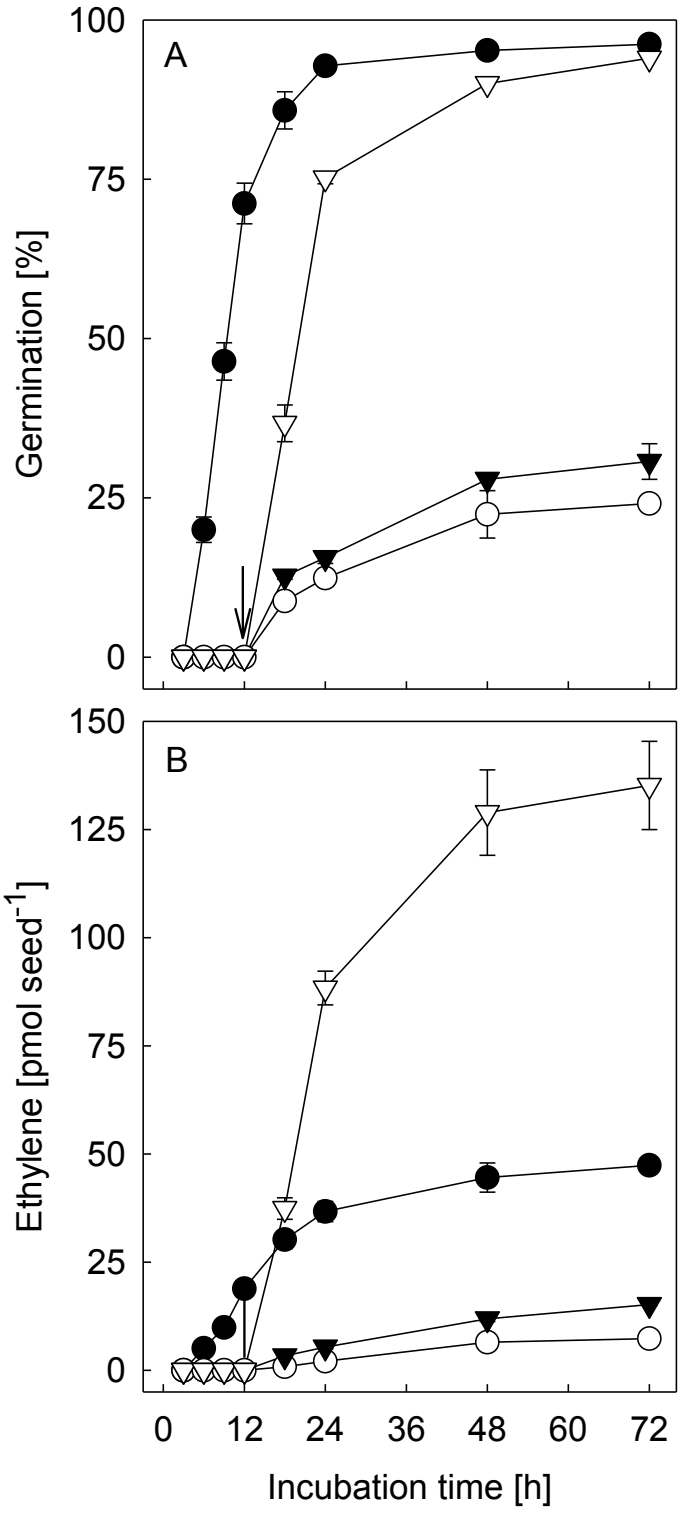

Fig. 5. Induction of secondary dormancy in Townsville stylo seeds by 2,4-D. Time-course changes of germination (A) and ethylene production (B) by seeds. (1) Filled circle, seeds were incubated in deionised water (control); (2) open circle, seeds were treated just with 1 mM 2,4-D; (3) open triangle, seeds were treated with 1 mM 2,4-D for 12 h and, then transferred to 1 mM ACC solution (as indicated by arrow); (4) filled triangle, seeds were treated with 1 mM 2,4-D for 12 h and, then transferred to deionised water (as indicated by arrow). Data points are means of six replicates ± standard error.

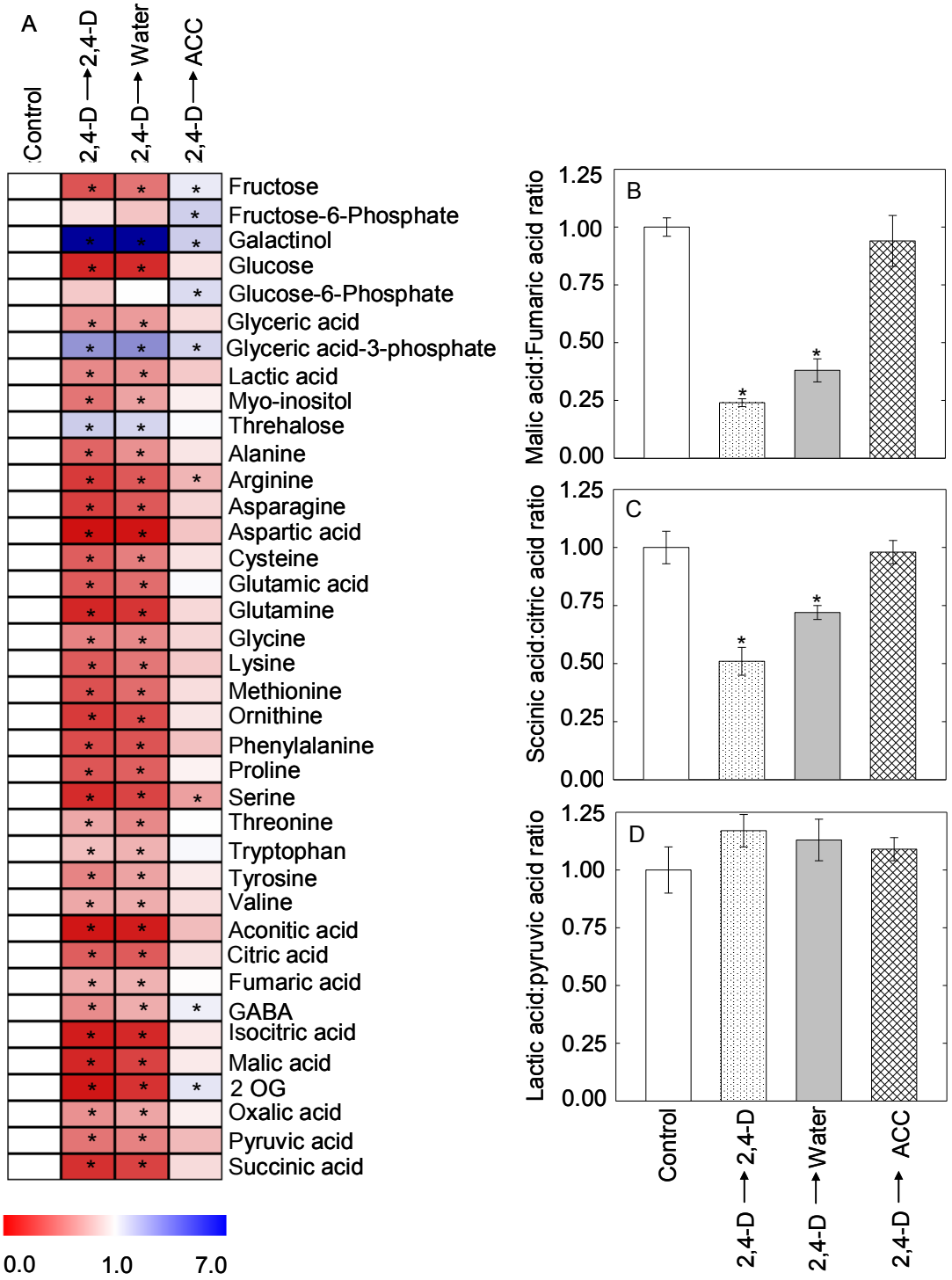

Fig. 6. Metabolic changes in seeds treated just with 2,4-D (2,4-D→2,4-D) or after 12 h of incubation in 2,4-D and then transferred to deionised water (2,4-D→water) or ACC (2,4-D→ACC) in comparison with control (deionised water). Heat map (A). Different shades of red and blue express the extent of the change according to the colour bar provided; white indicates no change. Heat map was generated with the MultiExperiment Viewer (MeV) software (www.tm4.org/mev). For absolute values, see Supplementary Table S1. Metabolic ratios relative to that in the control selected from the heat map. The malic acid-to-fumaric acid ratio (B), succinic acid-to-citric acid ratio (C) and lactic-to-pyruvic acid ratio (D). Asterisks indicate values determined by the student's t-test to be significantly different from the control ($P < 0.05$). Data represent means ± standard error of six independent replicates (each replicate is a pool of 150 seeds).

levels were reversible following transfer of their seeds to ACC. Overall, according to the principle component analysis (PCA) of metabolite data, the global composition changes induced by 2,4-D→2,4-D and 2,4-D→water treatments in seeds were clearly distinguishable from that of the control and 2,4-D→ACC condition (see Supplementary Fig. S2).

In the present experiments, the malic acid-to-fumaric acid ratio was >4-fold and >2-fold lower in seeds treated just with 2,4-D or after 12 h of incubation in 2,4-D and then transferred to deionised water, respectively (Fig 6B). The malic acid-to-fumaric acid ratio remained at same level in seeds treated with 2,4-D following transfer to ACC as in the control. The pattern of change in the citric acid-to-succinic acid ratio was largely consistent as that observed in malic acid-to-fumaric acid ratio (Fig 6C). Furthermore, the lactic acid-to-pyruvic acid ratio remained at the same level in all treatments (Fig 6D). In agreement with metabolite profiles results, 2,4-D promoted a large decrease in seed respiration rate as compared with control (Fig 7A), which was associated with germination percentage (Fig 7B). A low respiration rate and germination was also observed for seeds treated with 2,4-D for 12 h and then transferred to deionized water. In contrast, seed respiration rate and germination showed a distinct increase upon transferring the inhibited seeds to ACC. Starch and glucose levels were higher in seeds imbibed in water (control) than in other treatments (Fig 7C, D). Sucrose was slightly decreased in seeds treated just with 2,4-D, while 2,4-D→water and 2,4-D→ACC increased the sucrose level as compared with control (Fig 7E). The results also showed an increase in total protein content of 47 % and 37 % as compared with control, in seed treated with 2,4-D→2,4-D or 2,4-D→water, respectively. (Fig 7F). In seeds treated with 2,4-D→ACC, protein levels were slightly higher than control. Interestingly, protein content was slightly increased in non-imbibed seeds (481 µg seed^{-1}) compared to seeds imbibed in water (464 µg seed^{-1}). Furthermore,

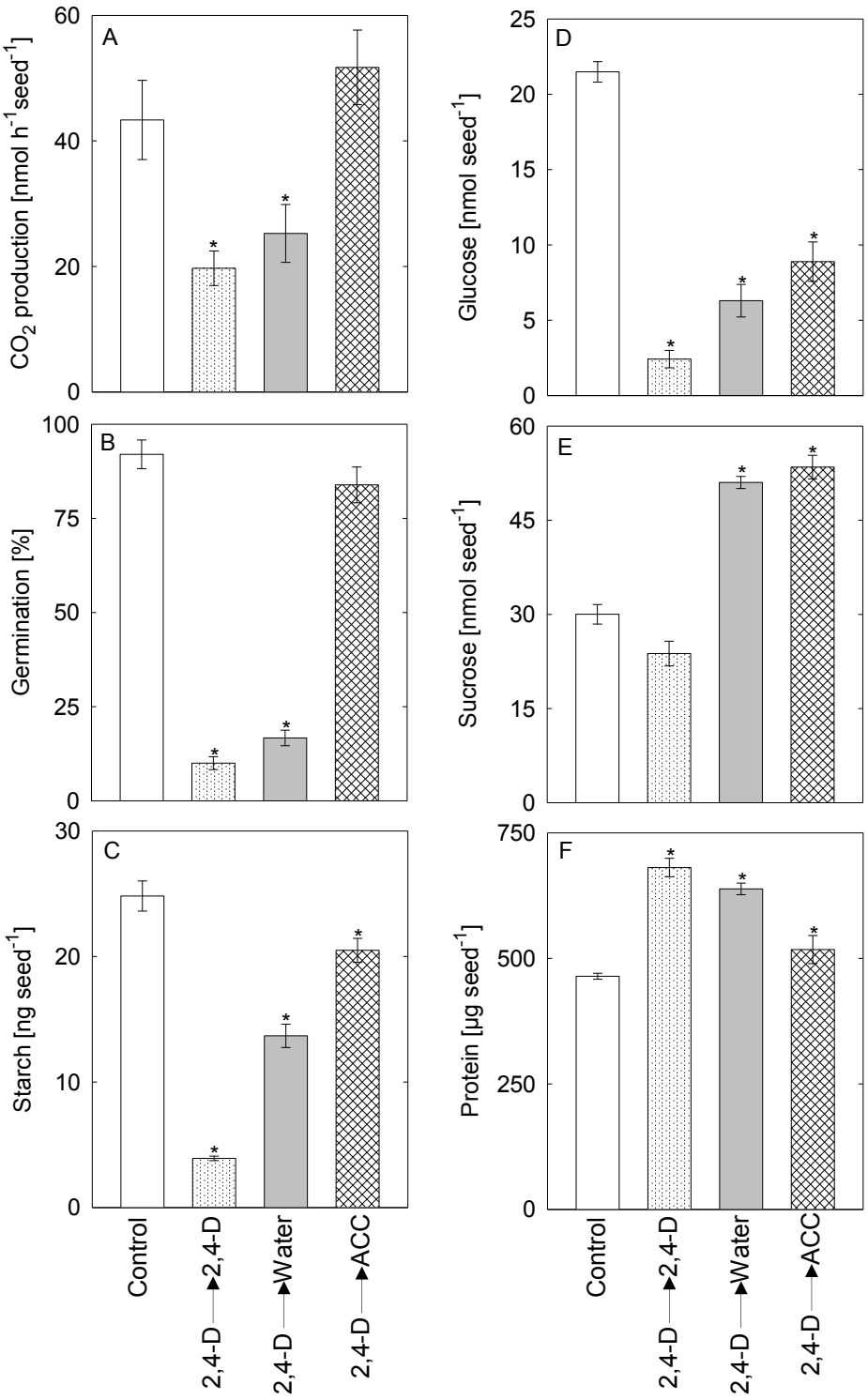

Fig. 7. Physiological analysis of seeds treated just with 2,4-D (2,4-D→2,4-D) or after 12 h of incubation in 2,4-D and then transferred to deionised water (2,4-D→water) or ACC (2,4-D→ACC) in comparison with control (deionised water). Respiration rate (A), germination (B), starch (C), glucose (D), sucrose (E) and total protein (F). Asterisks indicate values determined by the student's t-test to be significantly different from the control ($P < 0.05$). Data represent means ± standard error of six independent replicates (each replicate is a pool of 150 seeds).

starch level declined from an initial value of 33 ng seed^{-1} in non-imbibed seeds to about 25 ng seed^{-1} in seeds imbibed in water (control).

4. DISCUSSION

4.1 Responses of high concentrations of auxin in the control of ethylene biosynthesis depends on the developmental stage of plant material

The TIR1/AFB, a small family of F-box proteins including the transport inhibitor response 1 (TIR1) and homologue auxin-binding F-box (AFB) proteins, link binding of auxin and auxins herbicides at supraoptimal concentrations directly to overexpression of auxin-responsive genes of ACC synthase in ethylene biosynthesis (Dharmasiri et al 2005, Grossmann 2010). While several reports illustrate the biological relevance of auxin-mediated ethylene production, exactly how auxin is involved in this process (and to what extent they are intertwined) is not completely understood, particularly during seed germination and postgermination growth. To approach this problem, seeds and seedlings of S. humilis were treated with IAA and 2,4-D. Auxins largely decreased ethylene biosynthesis in non-dormant seeds, indicating that the inhibitory effect of the auxin on seed germination resulted from a reduced ethylene biosynthesis (Fig 1). On the other hand, ethylene production by seedlings was increased by auxin treatments, which was associated with seedling growth inhibition as compared with control. These data indicate that auxin, at high concentration, affects seed germination by uncoupling it from ethylene biosynthesis, whereas auxin is required for connecting ethylene biosynthesis and inhibition of seedling growth. This is supported by our finding that ACC increased ethylene biosynthesis and germination of auxin-inhibited seeds (Fig 2). Furthermore, growth of auxin-inhibited seedlings was fully recovered, with corresponding decrease in ethylene production by

AVG treatment. Several studies, performed mainly on leaves or complete plant, propose that high level of auxin stimulates ACS activity increasing ethylene levels in vegetative tissues (Grossman 2003, Raghavan et al 2006, Gleason et al 2011). Also in our experiments, seedlings of S. humilis showed the classical response of auxin-triggered induction of ethylene biosynthesis. However, auxin was shown to negatively affect the ethylene biosynthesis in germinating seeds. Taking these results into account, this work argues for the importance of spatial and developmental resolution to fully understand different aspects of the relationship between auxin and ethylene of multicelular organisms.

Free and conjugated ACC accumulated to levels much lower in auxin-treated non-dormant seeds than in control seeds, which was also reflected in germination levels (Fig 3). In contrast, the amounts of free and conjugated ACC produced by seedlings imbibed in deionised water were greater than those by S. humilis seedlings treated with auxin solutions, which correlates well with the lowest seedling growth. These data indicate the existence of distinct auxin controls of ACC biosynthesis, leading in turn to regulation of seed germination and seedling growth. The evidence that auxin has opposite effect on ACC biosynthesis during seed-to-seedling transition is interesting in light of the fact that ACS genes in numerous plant species have both unique and overlapping expression patterns during plant development and in response to internal and external stimuli (Tsuchika and Theologis 2004, El-Sharkawy et al 2008, Muday et al 2012). The picture is further complicated by discovery that dormant seeds treated with auxin did not exhibit significant changes in the ACC levels. However, germination and ethylene production of dormant seeds treated with ACC was inhibited by auxin (Fig 4). In this context, auxin seems to exert an inhibitory effect on ACO activity. This finding is somewhat surprising given the wealth of literature indicating an important role of auxin, at high concentration, in the control of ACS (Frankowski et al 2009, Grossmann 2010, Gleason et al 2011). The fact

that cobalt ion and auxin decreased the ACO activity of ACC-stimulated dormant seeds implies that the mechanisms underlying the ethylene inhibition of both cobalt- and auxin-treated seeds are likely the same (Table 1). Together, these data indicate that the effect of high levels of auxin in the control of ethylene metabolism is in fact developmentally separated.

4.2 Reduction of ethylene biosynthesis by the seeds is an important adaptive response to auxin herbicide

Stimulation of ethylene biosynthesis is a common response when auxins, at high concentration, are applied to sensitive plants (Grossmann 2010, Song 2014). However, it is still not clear whether this effect of auxin plays similar roles on the physiological properties associated with germination. Townsville stylo seeds imbibed in water were able to germinate faster and exhibit much higher germination percentages than seeds treated just with 2,4-D or after 12 h of incubation in 2,4-D and then transferred to deionised water (Fig. 5). As ethylene is required for the germination of S. humilis seeds (Silva et al 2014), ethylene production by seeds was then measured. Ethylene production by seeds was largely depressed by 2,4-D treatment. On the other hand, germination of seeds was rapidly fully recovered, with a corresponding increase in ethylene production following their transfer from 2,4-D to ACC solution (Fig. 5). The intensive crop production systems are likely to impose greater selection pressures for seed dormancy and selective herbicide resistance (Délye et al 2013, Owen et al 2015). In this context, the results presented here showed that secondary dormancy can be induced by 2,4-D in Townsville stylo seeds. Interestingly, secondary dormancy of 2,4-D-treated seeds is lost very slowly in soil as compared with the non-treated seeds of S. humilis (see Supplementary Fig. S3). Therefore, the secondary dormancy induced by 2,4-D can form a long-lived persistent soil seed bank.

In order to discern metabolic networks during seed dormancy and germination, we next performed metabolic profiling analysis (Fig. 6). Galactinol has been suggested as important in energy storage and membrane protection during seed desiccation (Zhao et al 2004, Verdier et al 2013). Furthermore, it was previously shown that high galactinol levels correlate with seed dormancy (Downie et al 2003), which is accordance with the observation that its levels are highly induced upon 2,4-D and 2,4-D → water treatments and reduced upon ACC treatment. Similarly, a significant increase in trehalose levels in seeds treated with 2,4-D alone or following their transfer from 2,4-D solution to deionised water suggests an important functional role for this metabolite during secondary dormancy (Fig. 6). Since trehalose is known to stabilize proteins (Paul et al 2008, Jain and Roy 2009) and galactinol performs protective functions by scavenging the free radicals (Nishizawa et al 2008, Keunen et al 2013), a possible function of these metabolites during period of secondary dormancy may be in the protection of the conformation of storage and housekeeping proteins. In the present studies, it was found that 2,4-D and 2,4-D → water treatments trigger an enhanced glyceric acid-3-phosphate level, and a decreased seed germination, suggesting a modified relationship between energy metabolism and germination. It is well known that glyceric acid-3-phosphate play a major role in membrane/phospholipid biosynthesis (Sulpice et al 2010). Thus, the reduced level of glyceric acid-3-phosphate in seeds treated with 2,4-D and, then transferred to ACC indicate that glyceric acid-3-phosphate is being utilized more rapidly when seed germination is stimulated by the ethylene. In keeping with this observation, seeds imbibed in 2,4-D and then transferred to ACC showed a fast increase in the germination, with a corresponding increase in ethylene production.

Glycolysis and the subsequent TCA cycle play a key role in metabolic activities during seed germination (Fait et al 2006). Indeed, the heterotrophic nature of the seed

makes it dependent on TCA cycle activities to supply reducing equivalents for mitochondrial respiration, which is essential for completion of the earliest stages of germination (Angelovici et al 2010). Seeds treated with 2,4-D and 2,4-D→water exhibited a general reduction in sugars levels, implying a reduced incorporation into glycolysis. Furthermore, The major reduction in the levels of the TCA-cycle intermediates in seeds treated with 2,4-D alone or following their transfer from 2,4-D solution to deionised water indicates a reduced flux through this pathways. In agreement with this model, the citric acid-to-succinic acid ratio was lower in seeds imbibed in 2,4-D or 2,4-D→water, suggesting a lower commitment of citric acid oxidation to succinic acid by the TCA cycle (Fig. 6). In the same treatments, the malic acid-to-fumaric acid ratio was also lower, showing a weak displacement of the oxireductive equilibrium toward malate. Moreover, there was no evidence of fermentative metabolism since lactic-to-pyruvic acid ratio remained steady across all treatments. A completely different picture emerged when 2,4-D-treated seeds were transferred to ACC solution. ACC increased the hexose phosphates levels of 2,4-D treated seeds, which apparently signifies the entrance of sugars into the glycolytic pathway. During ACC treatment, the observed enhancement of TCA-cycle intermediates and amino acids levels reflects the boost of biosynthetic processes during seed germination. In keeping with these observations, ACC supplementation rescues seed germination and increases ethylene biosynthesis in 2,4-D treated seeds. Together, these data indicate that ethylene is required for connecting energy metabolism and secondary dormancy breakage of Townsville stylo seeds. Ethylene has also been implicated in seed germination of several weed species such as Amaranthus caudatus (Kepczynski et al 2003), Striga hermonthica (Babiker et al 2000), Chenopodium album (Machabée and Saini 1991) and red rice (Gianinetti et al 2007). Furthermore, there is evidence that intensive cropping selects higher weed seed dormancy because only the early-germinating cohort of

population is removed by weed control measures (Délye et al 2013). Thus, it seems reasonable to presume that the induction of secondary dormancy by auxin herbicide in the ethylene-requiring seeds confers a selective advantage in the extreme selection pressures of arable environment, and determines plant persistence and coexistence.

Seed storage reserves such as starch, storage proteins, and lipids are predominantly mobilized during and after radicle protrusion (Bewley 1997). Indeed, legume seeds store large quantities of storage proteins to support germination and seedling emergence (Gallardo et al 2008). Our study shows that the changes in starch are accompanied by coordinated changes in the protein content and the induction of secondary dormancy in Townsville stylo seeds (Fig. 7). The decreased in amino acids levels in seeds treated with 2,4-D→2,4-D and 2,4-D→water was accompanied by an increase in total protein. In the same treatments, the starch and glucose levels were lower. The fact that protein synthesis represents a major sink for energy (Ishihara et al 2015) strengthens the idea that a tight link between carbon metabolism and protein synthesis is necessary to prevent acute carbon starvation. In this context, sucrose showed a slightly reduced level in seed treated with 2,4-D alone, while there were a clear increases in sucrose in seeds treated with 2,4-D for 12 h and the transferred to water (Fig. 7). Storage proteins in seeds are not only an important source of amino acids during germination, but are also important for energy production (Angelovici at al 2011). Aspartate and glutamate are among the most important abundant amino acids in seed storage protein, and serve as substrate for aspartate and alanine aminotransferases (Miyashita et al 2007, Weitbrecht et al 2011). The increase in aminotransferases during seed imbibition is accompanied by ATP accumulation, respiration and other metabolic processes related to energy production (Isola et al 2000, Pawlowski 2009). At the metabolite level, our results imply that the 2,4-D negatively regulates the amino acids levels and the activity of the TCA cycle and, as consequence,

might lead a limitation of energy need for proper seed germination. The progression of germinating ACC treated seeds was associated with increasing levels of the TCA cycle metabolites, implying a stimulation of energy-associated metabolism during early germination. Thus, the induction of secondary dormancy in 2,4-D-treated seeds may be associated with the operation of energy-saving metabolism. Since ethylene is a key hormone promoting germination of Townsville stylo seeds (Silva et al 2014), this study has revealed a process in which storage reserves mobilization and ethylene crosstalk in regulating the developmental transition from metabolic quiescent seed to actively growing seedling.

5. CONCLUSIONS

In summary, the data described herein demonstrate that application of auxin, at high concentration, elicits differential responses on ethylene biosynthesis during seed-to-seedling transition: (1) in freshly harvested seeds (dormant seeds) of Townsville stylo, auxin represses the ACO activity; (2) in aged seeds (non-dormant seeds), auxin inhibits ACC biosynthesis and induces secondary dormancy; (3) in young seedlings, auxin increases ACC biosynthesis. Thus, our results demonstrate both the stunning complexity and diversity of the co-action mechanism between auxin and ethylene during germination and seedling establishment. Moreover, the metabolite profiles indicate an intriguing link between the induction of secondary dormancy and the metabolic networks in seeds. The above lines of evidence open new directions for research on the molecular mechanisms involved and their concerted regulation in seed dormancy, which will contribute to our current understanding of seed biology.

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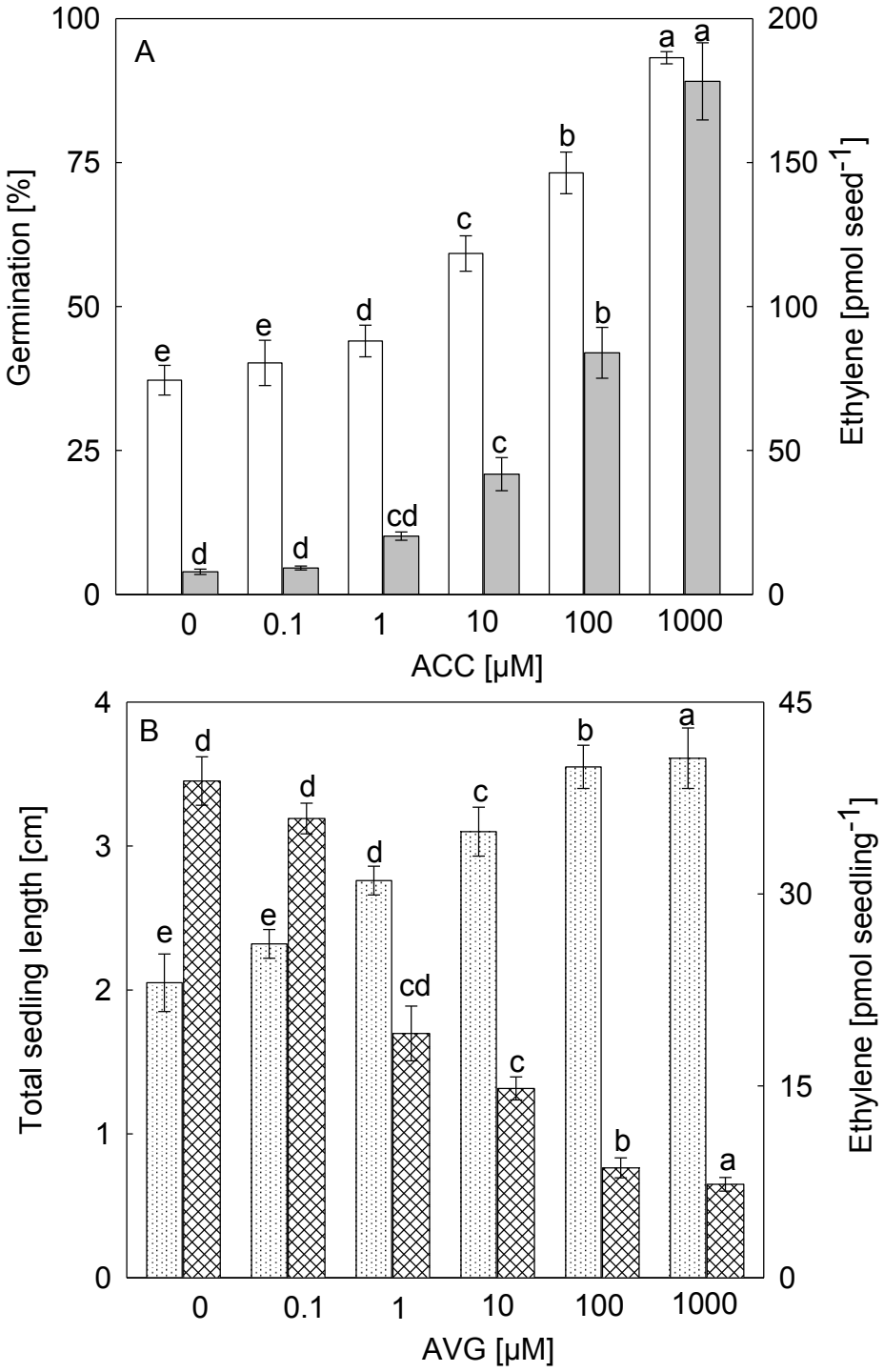
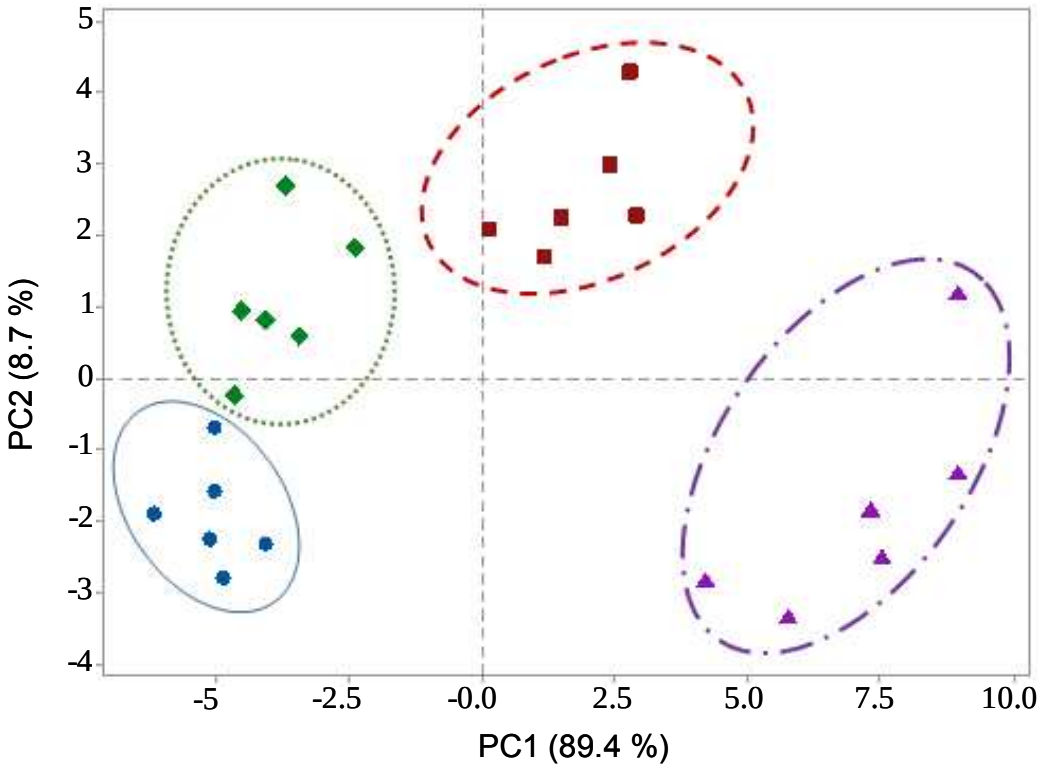
7. SUPPLEMENTARY DATA

Supplementary Table S1. Metabolic changes in seeds treated just with 2,4-D or after 12 h of incubation in 2,4-D and then transferred to deionised water or ACC in comparison with control. Values in boldface were determined by the student's t-test to be significantly different from the control ($P < 0.05$). Data represent means ± standard error of six independent replicates

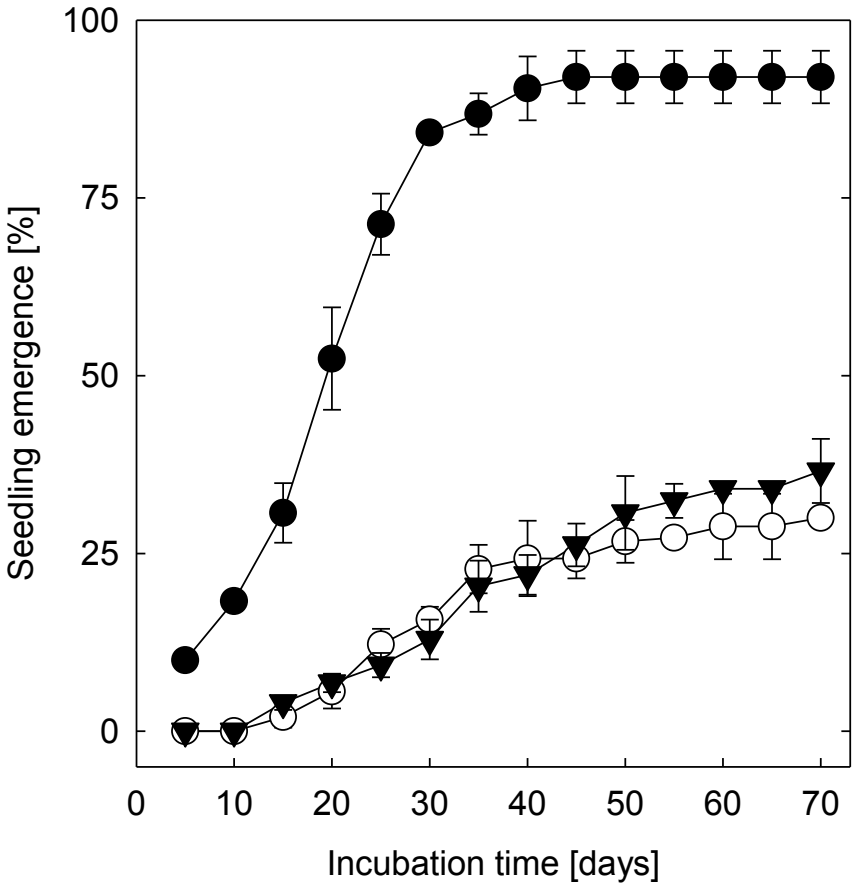
Metabolite	Treatment			
	Control	2,4-D→2,4-D	2,4-D→Water	2,4-D→ACC
Fructose	1.00±0.03	**0.33±0.05**	**0.46±0.03**	**1.50±0.12**
Fructose-6-Phosphate	1.00±0.16	0.89±0.11	0.77±0.08	**2.14±0.18**
Galactinol	1.00±0.09	**16.89±1.77**	**11.75±1.08**	**2.22±0.19**
Glucose	1.00±0.03	**0.15±0.02**	**0.17±0.01**	0.89±0.05
Glucose-6-P	1.00±0.10	0.79±0.10	1.04±0.11	**1.87±0.20**
Glyceric acid	1.00±0.14	**0.57±0.08**	**0.61±0.10**	0.86±0.13
Glyceric acid-3-P	1.00±0.10	**3.54±0.22**	**3.72±0.20**	**2.02±0.13**
Lactic acid	1.00±0.08	**0.54±0.10**	**0.58±0.10**	0.79±0.12
Myo-inositol	1.00±0.03	**0.46±0.02**	**0.64±0.04**	0.94±0.05
Threhalose	1.00±0.10	**2.20±0.06**	**2.01±0.22**	1.11±0.10
Alanine	1.00±0.07	**0.40±0.04**	**0.57±0.02**	0.90±0.08
Arginine	1.00±0.10	**0.23±0.03**	**0.35±0.05**	**0.71±0.06**
Asparagine	1.00±0.08	**0.25±0.02**	**0.35±0.04**	0.84±0.09
Aspartic acid	1.00±0.05	**0.07±0.03**	**0.08±0.01**	0.77±0.12
Cysteine	1.00±0.11	**0.37±0.06**	**0.50±0.05**	0.89±0.12
Glutamic acid	1.00±0.08	**0.36±0.04**	**0.43±0.03**	1.13±0.07
Glutamine	1.00±0.13	**0.15±0.03**	**0.21±0.05**	0.85±0.09
Glycine	1.00±0.11	**0.51±0.05**	**0.54±0.08**	0.84±0.10
Lysine	1.00±0.04	**0.36±0.04**	**0.47±0.03**	0.79±0.11
Methionine	1.00±0.06	**0.32±0.03**	**0.43±0.07**	0.87±0.09
Ornithine	1.00±0.08	**0.23±0.02**	**0.30±0.04**	0.90±0.07
Phenylalanine	1.00±0.12	**0.30±0.03**	**0.33±0.02**	0.76±0.10
Proline	1.00±0.05	**0.34±0.03**	**0.38±0.05**	0.95±0.06
Serine	1.00±0.06	**0.17±0.06**	**0.27±0.10**	**0.63±0.05**
Threonine	1.00±0.10	**0.66±0.06**	**0.54±0.03**	1.04±0.11
Tryptophan	1.00±0.07	**0.75±0.06**	**0.70±0.10**	1.18±0.14
Tyrosine	1.00±0.03	**0.52±0.02**	**0.64±0.03**	0.92±0.06
Valine	1.00±0.10	**0.66±0.04**	**0.68±0.05**	0.87±0.05
Aconitic acid	1.00±0.12	**0.09±0.03**	**0.11±0.06**	0.74±0.04
Citric acid	1.00±0.08	**0.37±0.05**	**0.36±0.03**	0.88±0.08
Fumaric acid	1.00±0.07	**0.67±0.07**	**0.70±0.05**	0.99±0.10
GABA	1.00±0.10	**0.55±0.08**	**0.68±0.09**	**1.38±0.12**
Isocitric acid	1.00±0.11	**0.11±0.04**	**0.16±0.03**	0.91±0.08
Malic acid	1.00±0.03	**0.15±0.01**	**0.26±0.07**	0.92±0.02
2 OG	1.00±0.06	**0.09±0.06**	**0.20±0.04**	**1.63±0.13**
Oxalic acid	1.00±0.09	**0.57±0.05**	**0.65±0.08**	0.94±0.09
Pyruvic acid	1.00±0.06	**0.46±0.07**	**0.51±0.04**	0.73±0.12
Succinic acid	1.00±0.05	**0.19±0.01**	**0.26±0.03**	0.86±0.08



Supplementary Fig. S1. Effects of ACC supplied in IAA solution on the germination (open bars) and ethylene production (grey bars) by seeds (A). Actions of AVG supplied in IAA solution on the early growth (dotted bars) and ethylene production (checkered bars) by seedlings (B). Seed germination, seedling length and ethylene production were determined following a 24 h exposure period. Seed germination and ethylene production in deionised water were 94.0±2.2% and 39.6±2.9 pmol seed^{-1}, respectively. Seedling growth and ethylene production in deionised water were 3.6±0.5 cm and 8.7±1.4 pmol seedling^{-1}, respectively. Bars followed by the same letters do not differ statistically at the 5 % level by Tukey test. Data points are means of six replicates ± standard error



Supplementary Fig. S2. PCA of metabolite data. Cluster of metabolites from control (triangles), 2,4-D→2,4-D (circles), 2,4-D→water (diamonds) and 2,4-D→ACC (squares). PCA was performed using the informant program MeV 4.0



Supplementary Fig. S3. Time course of germination by S. humilis seeds. Seeds were imbibed in 2,4-D for 24 h (Open circle) or after incubation in 2,4-D for 12 h, then transferred to deionised water (Filled triangle). Afterwards, non-germinated seeds were thoroughly washed with deionised water and then buried at a depth of 0.5 cm in 3.0 L plastic pots filled with soil and maintained in the greenhouse. For control, non-dormant seeds (Filled circle) were first scarified and then transferred to soil. Emergence of seedlings was registered at 5 days intervals from 0 to 70 days after sowing. Data are means ± standard error of three separate experiments, and in each experiment five plastic pots with 100 seeds each was evaluated for each treatment