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ORIGINAL ARTICLE

Interplay between nitric oxide, ethylene, and gibberellic acid regulating the release of *Amaranthus retroflexus* seed dormancy

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Abstract A very small percentage, below 10%, of *Amaranthus retroflexus* seeds were germinated at 25 °C in the light and thus the seeds were considered to be primary dormant. Nitric oxide (NO) applied for only 5 h stimulated germination of these non-deep physiologically dormant seeds. Likewise, ethephon, ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) and gibberellic acid (GA_3) induced dormancy release. The stimulatory effect of NO, ACC, and GA_3 on germination was associated with increased ethylene production prior to radicle protrusion. The nitric oxide scavenger, cPTIO, antagonized the stimulatory effect of GA_3, ethephon, and ACC on seed germination, indicating that the presence of endogenous NO is required for dormancy release by these compounds. This scavenger inhibited both germination and ethylene production stimulated by NO and GA_3. An inhibitor of ethylene binding to its receptor, 2,5-norbornadiene (NBD), antagonized the beneficial effect of ethylene and NO, indicating that ethylene action is necessary for the appearance of seed response to these gases. The ACC synthase inhibitor, aminoethoxyvinylglycine (AVG), and the ACC oxidase inhibitor, α-amino-isobutyric acid (AIB), strengthened the effect of NBD on the germination of NO-pretreated seeds. Induction of germination of dormant seeds by NO, ethephon or GA_3 was associated with initiation of the cell cycle prior to radicle protrusion. The data presented indicate NO crosstalk with ethylene and GA_3 in regulating dormancy release in *A. retroflexus* seeds.

Keywords *Amaranthus retroflexus* · Ethylene · Germination · GA_3 · Nitric oxide · Primary dormancy

Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ABA	Abscisic acid
AIB	α-Amino-isobutyric acid
AVG	Aminoethoxyvinylglycine
cPTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
ETH	Ethephon
GA_3	Gibberellic acid
GA_s	Gibberellins
NBD	2,5-Norbornadiene
NO	Nitric oxide

Introduction

Primary seed dormancy, which is common in wild plants, is induced during seed development and maturation. These seeds are not able to germinate in conditions favorable to the germination of non-dormant seeds (Bewley et al. 2013). Thus, dormancy prevents germination of intact viable seeds during temporary suitable conditions in an unfavorable season for seedling establishment. It enables the formation of weed seed banks in the soil, which can remain viable for many years. Seed dormancy, depending on the plant species, can be released during burial, stratification or dry after-ripening and/or by various chemicals (Bewley et al. 2013). Dormancy may be absolute, when seeds are unable

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to fully germinate under any environmental conditions (Rodríguez et al. 2015). However, relative dormancy also occurs in seeds; these seeds can germinate only in a narrow range of conditions compared to non-dormant seeds. Therefore, dormancy release in these seeds broadens the range of conditions suitable for germination (Bewley et al. 2013). It is commonly accepted that the balance between ABA and GAs and/or sensitivity to these hormones is responsible for control of the dormancy state and seed germination (Finkelstein et al. 2008; Arc et al. 2013a, b; Corbineau et al. 2014). ABA has been found to play a crucial role in the induction and maintenance of dormancy in seeds and GA_s have been considered as factors involved in dormancy release and/or germination (Finkelstein et al. 2008; Rodríguez-Gacio et al. 2009). Moreover, ethylene plays a key role among hormones participating in the regulation of germination and seed dormancy (Kępczyński and Kępczyńska 1997; Matilla 2000; Matilla and Matilla-Vazquez 2008; Arc et al. 2013a; Corbineau et al. 2014). Ethylene stimulatory effect may occur via the involvement of C_2H_2–GA_s–ABA crosstalk (Corbineau et al. 2014). Interaction between ethylene and reactive oxygen species (ROS) in the regulation of seed germination and dormancy is also considered.

In addition to hormones, such as gibberellins and ethylene, nitrogen-containing compounds, such as nitrates, nitrites, hydroxylamines, and azides, can also break dormancy in seeds, and it was suggested that the response of seeds to nitrogenous compounds was caused by NO (Hendriks and Taylorson 1974). NO from various donors, such as nitroprusside (SNP), *S*-nitroso-*N*-acetylpenicillamine (SNAP), *S*-nitrosoglutathione (GSNO), and acidified KNO_2, removes dormancy in apple, *Arabidopsis*, barley, lettuce, and redroot pigweed seeds (Beligni and Lamattina 2000; Bethke et al. 2004; Gniazdowska et al. 2010; Liu et al. 2011; Kępczyński and Sznigir 2014). NO produced by seeds is now recognized as a signaling molecule involved in regulating germination of dormant and non-dormant seeds in cooperation with plant hormones (Arc et al. 2013b; Krasuska et al. 2015). NO-dependent protein posttranslational modifications are proposed as a key mechanism underlying NO signaling during early seed germination (Arc et al. 2013b). Participation of plant hormones in seed dormancy release and germination involves several metabolic changes. Once dormancy has been released seeds can germinate when suitable conditions specific for the species are ensured. The germination process includes three phases distinguished on the basis of water uptake. The first two phases are regarded as germination "sensu stricto", ending in radicle protrusion. Phase I is associated with rapid water uptake, activation of respiratory metabolism, transcription, and translation. During phase II, known as the "lag phase", water content remains constant or increases slowly, and metabolic processes required for embryo growth and the completion of germination are activated. Dormant seeds are able to pass through two phases, but cannot enter phase III, which is associated with further uptake of water, major reserve mobilization, and seedling establishment. Metabolic activity in phase II of dormant seeds is lower than in non-dormant seeds (Bewley et al. 2013). Data obtained for maize, *Arabidopsis*, and barley have shown that radicle protrusion occurring by cell elongation or cell cycle induction precedes radicle growth (Baíza et al. 1989; Masubelele et al. 2005; Gendreau et al. 2008, 2012).

The seeds of *Amaranthus retroflexus*, a common annual weed of 60 crops in many parts of the world (Holm et al. 1997), including Poland, are dormant after harvest and can remain viable in the soil for at least 6–10 years (Costea et al. 2004). *A. retroflexus* seeds express relative dormancy and therefore germinate completely after harvest at relatively high temperatures, usually 35–40 $^{\circ}$C (Kępczyński et al. 1996; Liu et al. 2011), but do not germinate at lower temperatures until their dormancy is interrupted. There are *A. retroflexus* seeds which are unable to germinate after harvest or germinated only partially at 35 $^{\circ}$C (Schönbeck and Egley 1980; Kępczyński and Sznigir 2014), indicating that the level of dormancy depends on the harvest. *A. retroflexus* seed dormancy can be removed by dry storage, the effect which depends on the duration and temperature of storage (Schönbeck and Egley 1980; Kępczyński and Sznigir 2014). Dormancy in these seeds also disappears as a result of burial in late autumn–winter (Egley 1989; Kępczyński and Sznigir 2013). Likewise, cold stratification has been found to remove dormancy in *A. retroflexus* seeds (Kępczyński and Sznigir 2013). As in the case of other seeds, *A. retroflexus* seed dormancy can be controlled by exogenous hormones. Ethephon, compound releasing ethylene in plant tissue, ethylene, and the ethylene biosynthesis precursor, ACC, remove dormancy, and the seeds were able to germinate at a wider range of temperatures than dormant ones (Kępczyński et al. 1996, 2003a). Likewise, GA_3 was effective in removing *A. retroflexus* seed dormancy. The response of these seeds to GA_3 and ethylene increased progressively with the duration of dry storage and stratification. Both GA_3 and ethylene can partially substitute for autumn–winter burial (Kępczyński and Sznigir 2013). In contrast to GA_3 and ethylene, the response to ABA decreased as the duration of stratification and burial increased by up to 3–6 months depending on the harvest (Kępczyński and Sznigir 2013). The effect of GA_3 on germination of dormant *A. retroflexus* seeds involves ethylene biosynthesis and action (Kępczyński et al. 2003a). Seed dormancy in this species has also been partially removed by gases released from SNP, NO + HCN, and from Fe(II)CN, HCN (Liu et al. 2011;

Kępczyński and Sznigir 2014). The importance of ABA crosstalk with ethylene and nitric oxide in seed dormancy and germination was discussed in detail by Arc et al. (2013a).

There are no available data on the effect of NO on germination of physiologically non-deep dormant seeds in relation to endogenous ethylene. Information on NO interaction with gibberellin in the regulation of seed dormancy release is still inadequate. There is scant information on the role of cell cycle in relation to germination of dormant seeds. However, it was found only that dormancy release in the seeds of tomato (de Castro et al. 2001) and caryopses of *Avena fatua* (Cembrowska-Lech and Kępczyński 2016) was associated with the induction of DNA replication before radicle protrusion. Expression of barley grain dormancy at 30 °C is associated with a blocking of the nuclei in the phase S (Gendreau et al. 2012). It is unknown whether the cell cycle is activated before radicle protrusion through the *A. retroflexus* seed coat. The effect of NO on cell cycle activity has yet to be examined in any dormant seeds.

Therefore, the aim of this work was to study the relationship between NO, ethylene, and gibberellins in regulating dormancy release in *A. retroflexus* seeds. To achieve this purpose, the effect of NO, ethephon, ACC, and GA_3 in the absence or presence of the NO scavenger cPTIO on the germination of dormant *A. retroflexus* seeds was determined. Likewise, the ACC synthase inhibitor, AVG, and the ACC oxidase inhibitor, AIB, were used to determine whether ethylene biosynthesis is necessary in the response of seeds to NO or GA_3. To explain whether the action of ethylene is required for NO and GA_3 to exert an effect, we applied 2,5-norbornadiene (NBD), a competitive inhibitor of ethylene binding to its receptor. In addition, we estimated ethylene production by seeds treated with NO, ACC or GA_3 before radicle protrusion through the seed coat. To clarify whether the cell cycle is activated before germination of dormant seeds, we determined the effect of NO, ethephon, and GA_3 on nuclear DNA content in radicle tips prior to protrusion through the seed coat.

Materials and methods

Plant material

Amaranthus retroflexus L. (redroot pigweed) plants were collected in September 2006 near Lubniewice in Poland. The inflorescences were dried at room temperature and then gently shaken to remove the seeds. The air-dried seeds were stored at − 20 °C until use. Some experiments were done in 2013 and others in 2016.

Seed treatments

Treatment with NO, ETH, ACC, GA_3, NO + ACC, GA_3 + ACC and cPTIO alone or in combination with NO, ETH, ACC or GA_3 (Scheme 1)

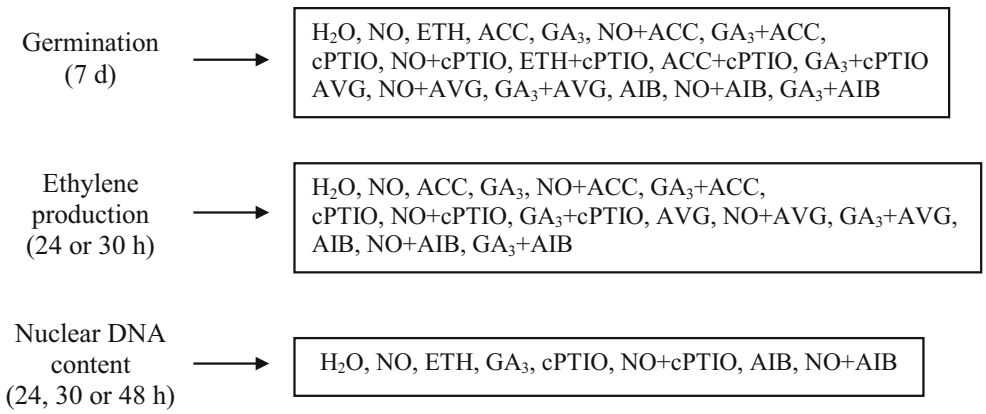
Three uncovered Petri dishes (ø 6 cm) with seeds on filter paper moistened with distilled water or solution of various compounds and one open Petri dish (ø 6 cm) containing water or 10^{-2} M KNO_2 (NO donor) (5 ml 2×10^{-2} M KNO_2 acidified with 5 ml 2×10^{-1} M HCl) were enclosed in a 19-cm (0.5 l) Petri dish, which was sealed with Parafilm. After treatment for 5 h, the Petri dish with the NO donor solution was replaced by a Petri dish with water. The concentration used compounds: ETH (10^{-4} M), ACC (10^{-3} M), GA_3 (10^{-3} M), GA_3 (10^{-3} M) + ACC (10^{-3} M), cPTIO (10^{-4} M), ETH (10^{-4} M) + cPTIO (10^{-4} M) or GA_3 (10^{-3} M) + cPTIO (10^{-4} M) (results in Fig. 1).

Treatment with NO or GA_3 applied alone or in combination with AVG or AIB (Scheme 1)

Seeds were incubated in water, in the presence of an inhibitor of ACC synthase activity (aminoethoxyvinylglycine, AVG) (10^{-3} M) or an inhibitor of ACC oxidase activity (α-amino-isobutyric acid, AIB) (10^{-3} M) in air enriched with NO. After incubation for 5 h, the Petri dish with the NO donor solution was replaced by a Petri dish with water. Seeds were also incubated in air in water, GA_3 (10^{-3} M), AVG (10^{-3} M), AIB (10^{-3} M), GA_3 + AVG or GA_3 + AIB solutions (results in Fig. 2).

Treatment with NO, applied alone or in combination with ethylene, 2,5-norbornadiene, AVG or AIB (Scheme 2)

Experiment 1 Seeds were incubated for 5 h in Petri dishes with water and air or air enriched with NO. Then, three uncovered Petri dishes with untreated or NO-pretreated seeds were placed in glass containers (0.5 l) with air, air enriched with NBD (10^{-6}, 3×10^{-5}, 10^{-4} M), air enriched with ethylene (5×10^{-7} M) or air enriched with NBD (10^{-4} M) + ethylene (5×10^{-7} M). Liquid NBD (0.05–5.0 µl) was applied by syringe via the stopper onto filter paper placed under the lid of the container. The liquid evaporated completely and the final gas concentration ranged from 10^{-6} to 10^{-4} M. Each day during the 7-day incubation period the containers were opened and the seeds were transferred to Petri dishes with filter paper moistened with fresh water. The Petri dishes were placed in glass containers and NBD was again applied. In the treatment involving ethylene, a standard of ethylene was injected into the containers via stoppers to obtain the required



Scheme 1 The schemes of experiments with NO, ETH, ACC, GA_3, cPTIO, AVG, and AIB

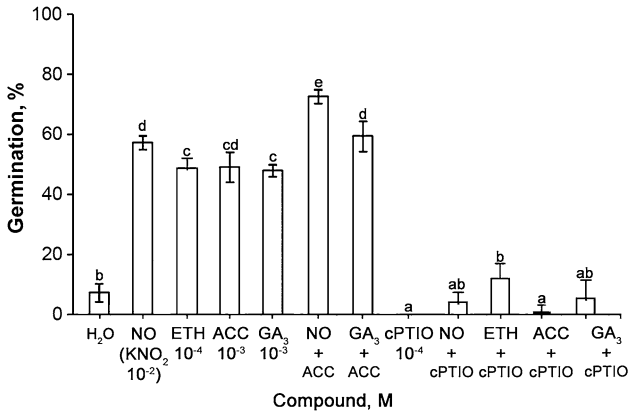


Fig. 1 The effect of NO, ETH, ACC, GA_3, NO + ACC, GA_3 + -ACC, cPTIO, and cPTIO in combination with NO, ETH, ACC or GA_3 on germination of *Amaranthus retroflexus* L. seeds. The seeds were incubated in water or solution of appropriate compound(s) in air or for first 5 h in air enriched with NO before transfer to air. Vertical bars indicate ± SD. One-way ANOVA with the Duncan's post hoc test on arcsin (x/10) transformed data was used to determine the significance of differences. Mean values with different letters (a–e) are significantly different ($P < 0.05$)

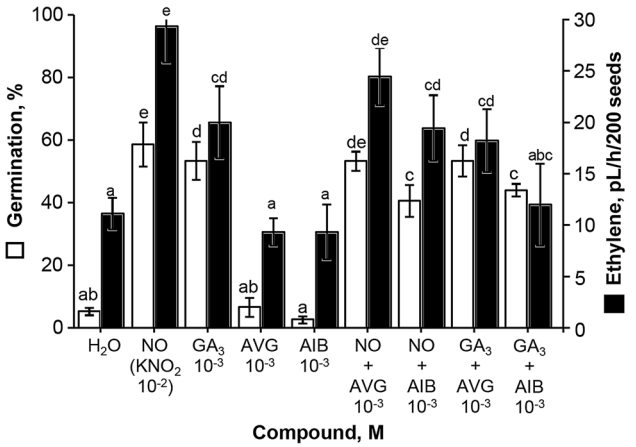


Fig. 2 The effect of NO or GA_3 in the absence or presence of AVG or AIB on germination and ethylene production by *A. retroflexus* L. seeds after incubation for 30 h. After incubation of seeds for 27 h in water, GA_3, AVG, AIB, GA_3 + AVG or GA_3 + AIB solution in air or air enriched with NO for first 5 h, seeds were transferred to glass vials with fresh water or solutions and incubated for 3 h before ethylene measurement. Vertical bars indicate ± SD. One-way ANOVA with the Duncan post hoc test was used to determine significant differences. Mean values with different letters (a–e) are significantly different ($P < 0.05$)

concentration. The ethylene concentration in the containers was tested with a gas chromatograph (results in Figs. 3, 4).

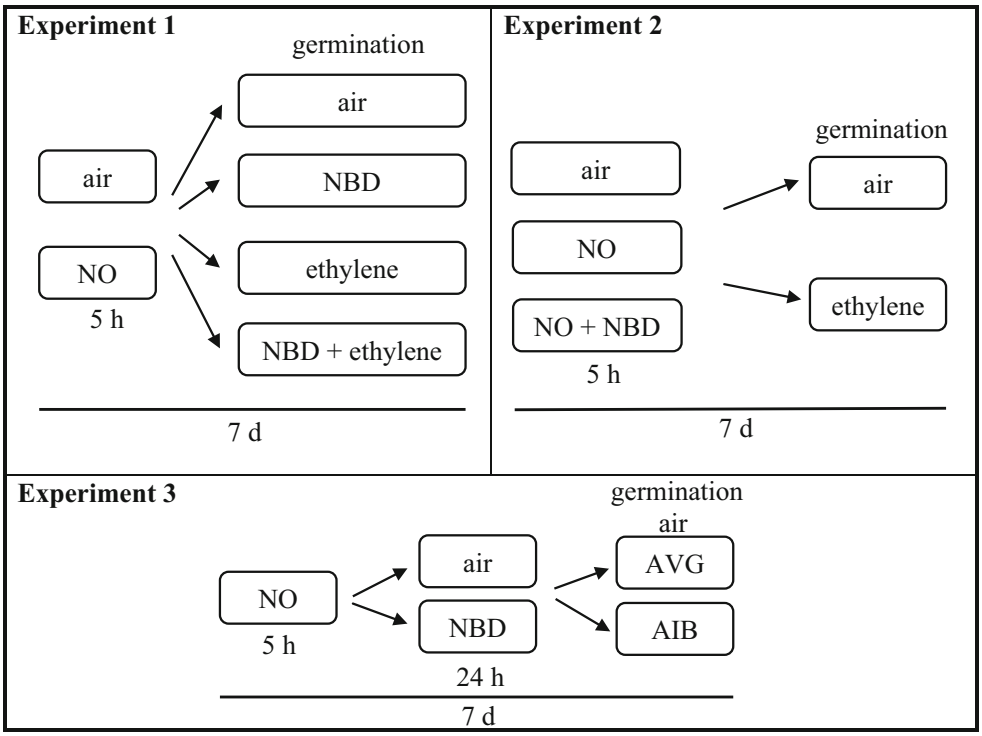
Experiment 2 Seeds were incubated for 5 h in Petri dishes with water and air or in water and air enriched with NO or NO + NBD (10^{-4} M). After 5 h, the seeds were transferred to Petri dishes containing filter paper with fresh water. Then Petri dishes with untreated seeds or seed pretreated with NO or NO + NBD were placed in glass containers (0.5 l) with air or air enriched with ethylene (5×10^{-7} M) (results in Table 2).

Experiment 3 Seeds were incubated for 5 h in Petri dishes with water and air enriched with NO. Then Petri dishes with NO-pretreated seeds were placed for 24 h in glass containers (0.5 l) with air or air enriched with NBD (10^{-4} M). Seeds pretreated with NO or NO + NBD were transferred to Petri dishes with filter paper moistened with fresh water, AVG (10^{-3} M) or AIB (10^{-3} M) solution and were incubated in air (results in Table 3).

Measurement of seed germination

In all experiments, primary dormant untreated or treated seeds (50 in three replicates) were incubated at 25 °C in the light (light intensity 120 µmol m^{-2} s^{-1}; 16/8 h photoperiod), in Petri dishes (ø 6 cm) with one layer of filter paper (Whatman no. 1) moistened with 1.5 ml of distilled water or a solution. Seed germination was determined after

Scheme 2 The scheme of experiments with NO, ethylene, NBD, AVG, and AIB



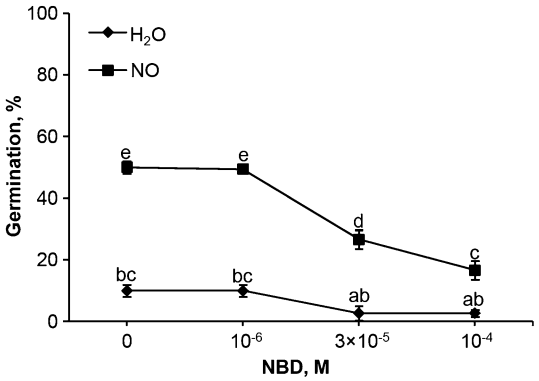


Fig. 3 The effect of NBD on germination of untreated and NO-pretreated *A. retroflexus* L. seeds. The seeds were incubated for the first 5 h in water and air or in air enriched with NO before transfer to air or air enriched with NBD. Vertical bars indicate ± SD. Two-way ANOVA with the Duncan post hoc test was used to determine significant differences. Mean values with different letters (a–e) are significantly different ($P < 0.05$)

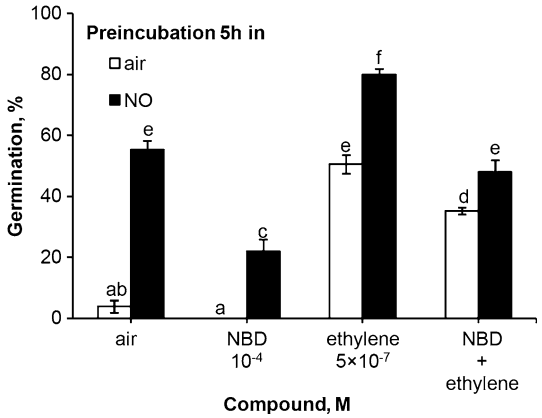


Fig. 4 The effect of NBD and ethylene on germination of untreated or NO-pretreated *A. retroflexus* L. seeds. The seeds were incubated for 5 h in water and air or air enriched with NO before transfer to air, NBD, ethylene or NBD + ethylene. Vertical bars indicate ± SD. Two-way ANOVA with the Duncan post hoc test was used to determine significant differences. Mean values with different letters (a–f) are significantly different ($P < 0.05$)

7 days of incubation. Seeds were regarded as germinated when the radicle protruded through the coat was about 2 mm in length.

Measurement of ethylene production (Scheme 1)

Seeds (200 in three replicates) were incubated in Petri dishes (ø 6 cm) for 21 or 27 h in the light at 25 °C in air and water or a solution of GA_3 (10^{-3} M), ACC (10^{-3} M), AVG (10^{-3} M), AIB (10^{-3} M), cPTIO (10^{-4} M), GA_3 + AVG, GA_3 + AIB, GA_3 + cPTIO, or GA_3 + ACC. Seeds were also incubated in water or solutions of ACC, cPTIO, AVG or AIB for the first 5 h in air enriched with NO before transfer to air up to 24 or 27 h. Following these treatments, the seeds were transferred to 4-ml glass vials containing filter paper moistened with 0.2 ml of fresh water or solution. The vials were sealed with rubber stoppers. After incubation for 3 h in the same conditions as before, gas samples of 1 ml were taken with a syringe and

injected into a Hewlett-Packard 5980 gas chromatograph equipped with an FID and a stainless steel column with Poropack Q 80/100 mesh. No seeds with seed coat pierced by radicle were observed in the glass vials after ethylene determination (results in Fig. 2, Table 1).

Determination of nuclear DNA (Scheme 1)

Nuclear DNA content in the radicle was determined using flow cytometry. To determine cell cycle activity, 50 seeds in three replicates were incubated in Petri dishes (ø 6 cm) at 25 °C for 0, 24, 30 or 48 h in the light in distilled water, cPTIO (10^{-4} M) or AIB (10^{-3} M) and air. Seeds pretreated with NO during 5 h of incubation in water, solutions of cPTIO (10^{-4} M) or AIB (10^{-3} M) were transferred to Petri dishes containing one layer of filter paper moistened with fresh water or solutions and incubated in air for 24 or 30 h. In the case of treatment with ETH (10^{-4} M), seeds were incubated for 24 and 48 h, and with GA_3 (10^{-3} M) for 30 h. Fifty radicles were isolated from the imbibed seeds. Tips of radicles (approx 1 mm) were chopped using a razor blade and placed in 2 ml of a nuclei isolation buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% Triton X-100, and 2 μg ml^{-1} DAPI) (Galbraith et al. 1983) for 2 min, after which they were incubated for 10 min at 25 °C. Subsequently, the suspension was passed through 20-μm nylon mesh. The DAPI-stained nuclei were analyzed using a Partec PAII flow cytometer (Partec). Populations of 2C and 4C nuclei were measured on 10,000 nuclei (results in Table 4).

Table 1 The effect of NO, ACC, GA_3, NO + ACC, GA_3 + ACC, cPTIO and cPTIO in combination with NO or GA_3 on ethylene production by *A. retroflexus* L. seeds after incubation for 24 or 30 h

Compound, M	Ethylene, pL/h/200 seeds Time, h	
	24	30
H_2O	5.8 ± 3.1b	7.1 ± 2.8b
NO (KNO_2 10^{-2} M)	12.4 ± 1.5bc	32.0 ± 4.8e
ACC 10^{-3} M	15.4 ± 1.9bc	26.5 ± 1.4de
GA_3 10^{-3} M	14.7 ± 1.3bc	18.6 ± 4.2cd
NO + ACC	23.6 ± 4.3d	56.9 ± 5.7g
GA_3 + ACC	30.7 ± 4.8de	44.0 ± 3.7f
cPTIO 10^{-4} M	nd	0 ± 0a
NO + cPTIO	nd	9.3 ± 2.6bc
GA_3 + cPTIO	nd	6.7 ± 5.8b

The seeds were incubated in water or in solution of appropriate compound(s) in air on for first 5 h in air enriched with NO before transfer to air. After incubation for 21 or 27 h, seeds were transferred from Petri dishes to glass vials with fresh water or solutions and incubated for 3 h before ethylene measurement. Ethylene production was measured after 24 or 30 h of incubation, 7 or 1 h before radicle protrusion, respectively. Vertical bars indicate ± SD. Two-way ANOVA with the Duncan post hoc test was used to determine significant differences. Mean values with different letters (a–g) are significantly different ($P < 0.05$)

nd not determined

Statistical analysis

The mean ± standard deviation (SD) of three replicates is shown. The means were also analyzed for significance using one-way or two-way analysis of variance (Statistica for Windows v. 10.0, Stat-Soft Inc., Tulsa, OK, USA). Duncan's multiple range test was used to determine the significance of differences ($P \leq 0.05$) between germination percentage and biochemical assay results for *A. retroflexus* L. seeds.

Results

Response of seeds to NO, ETH, ACC or GA_3 applied alone or with cPTIO

Only a very small percentage (below 10%) of *A. retroflexus* seeds is germinated at 25 °C in the light (Fig. 1). NO, released from the KNO_2 applied at 10^{-2} M, markedly enhanced the percentage of seed germination resulting in ca. 50% of seed germination. Ethephon and GA_3 enhanced seed germination to a similar degree as in the case of NO. The ethylene biosynthesis precursor ACC also stimulated seed germination, resulting in ca 45% of seed germination. ACC slightly increased stimulatory effect of NO. Seeds were unable to germinate in the presence of the NO scavenger cPTIO. cPTIO markedly decreased the stimulatory effect of NO, ethephon, ACC, and GA_3.

Ethylene production by dormant seeds incubated for 24 or 30 h was detected (Table 1). ACC and GA_3 caused a similar level in ethylene production after 24 h as in the case of NO. ACC increased ethylene production induced by NO or GA_3. After 30 h, seeds pretreated with NO produced ethylene 4.5 times more effectively than untreated seeds. ACC, and GA_3 also increased ethylene production after 30 h of incubation. Ethylene was not produced when seeds were incubated in the presence of cPTIO. cPTIO reduced ethylene production in seeds pretreated with NO. Likewise, application of cPTIO in combination with GA_3 decreased ethylene production to a similar degree.

Seed response to NO or GA_3 in the absence or presence of AVG or AIB

Inhibitors of ethylene biosynthesis were used to determine whether ethylene biosynthesis is required to obtain the stimulatory effect of NO and GA_3. AVG, an inhibitor of ACC synthase, and AIB, an inhibitor of ACC oxidase, did not affect seed germination (Fig. 2). AVG had no effect on the germination of NO-pretreated seeds, while AIB only slightly decreased the germination percentage. The presence of AVG during incubation with GA_3 did not affect germination, but AIB again slightly decreased the percentage of germination induced by GA_3. AVG and AIB did not affect ethylene production after 30 h. AVG had no influence on ethylene production when seeds were pretreated with NO. Stimulation of ethylene production by GA_3 also did not decrease under the influence of AVG. AIB slightly decreased ethylene production by seeds pretreated with NO. Simultaneous application of AIB and GA_3 reduced ethylene production as well.

Seed response to NO in combination with NBD, ethylene, AVG or AIB

To examine whether ethylene action is necessary for NO to influence seed germination, NBD, a competitive inhibitor of ethylene binding to its receptor, was applied. NBD inhibited germination of dormant seeds (Fig. 3). As reported in the previous experiment, NO enhanced the percentage of seed germination. Germination of NO-pretreated seeds was markedly, 2.5 times, reduced by NBD applied continuously to these seeds. NO was also applied alone or simultaneously with NBD for 5 h, after which the seeds were incubated in air or in air enriched with ethylene. Both NO and ethylene increased the germination percentage (Table 2). The stimulatory effect of NO was not registered when NO was applied together with NBD. Ethylene reinforced the effect of NO and markedly counteracted the inhibition of the stimulatory effect of NO by NBD. In the following experiment, untreated or NO-pretreated seeds were then incubated in air or in air enriched with NBD, ethylene or NBD with ethylene. As in the previous experiment, NO and ethylene-stimulated germination and NBD inhibited it (Fig. 4). Ethylene increased the germination percentage in comparison with the untreated seeds and intensified the effect of NO. NBD reduced the beneficial effect of NO. Stimulatory effect of ethylene and also NO was less manifested when ethylene in combination with NBD was applied.

In the next experiment, NO-pretreated seeds were incubated for 24 h in air or air enriched with NBD and then incubated in air and in the presence of AVG or AIB. Application of NBD for 24 h antagonized the effect of the previously applied NO (Table 3). AIB decreased the effect of NO, and both inhibitors strengthened the inhibitory effect of NBD on the germination of NO-pretreated seeds; the percentage of inhibition caused by AVG and AIB in the presence of NBD reached 35–46%, respectively.

Table 2 The effect of ethylene on germination of untreated and pretreated with NO and NBD *A. retroflexus* L. seeds

Treatment	Germination, %	
	Ethylene, M	
	0	5×10^{-7}
Air	6.0 ± 3.4b	52.7 ± 5.0c
NO	66.0 ± 4.0de	86.0 ± 3.5f
NO + NBD	0 ± 0a	72.7 ± 1.2e

The seeds were incubated for 5 h in water and air, in water and air enriched with NO, or in air enriched with NO + NBD (10^{-4} M). After incubation, seeds were transferred to air or air enriched with ethylene. Vertical bars indicate ± SD. Two-way ANOVA with the Duncan post hoc test was used to determine significant differences. Mean values with different letters (a–f) are significantly different ($P < 0.05$)

Nuclear DNA content in seeds treated with NO, ethephon, GA_3, cPTIO, and AIB

Nuclear DNA content was determined by flow cytometry in the radicle tips of seeds incubated in the presence of NO, ethephon or GA_3. Analysis of the dry seeds indicated that while the cells contained nuclei in 2C, S, and 4C, but most were in 2C (Table 4a). Imbibition in water for 30 or 48 h decreased the percentage of nuclei in 2C. NO and GA_3 markedly decreased the percentage of nuclei in 2C and increased the percentage in phase S and in 4C after 30 h of incubation. The percentage of nuclei was 1.6 lower in 2C and about 1.5 and 2.5 times higher in S and 4C, respectively. Ethephon reduced the percentage of nuclei in 2C after 24 h by about 50% and increased the percentage of nuclei 1.9 and 4 times in phase S and in 4C, respectively. Prolongation of incubation in the presence of ethephon decreased the percentage of nuclei in phase S and increased the percentage in 4C. cPTIO and AIB did not affect the percentage of nuclei in all phases after 24 and 30 h of incubation and after 24 h when applied to seeds pretreated with NO (Table 4b). After 30 h of imbibition in the presence of cPTIO or AIB seeds pretreated with NO, the number of nuclei in 2C was by 51 or 42%, respectively, higher compared to the pretreated only with NO. However, cPTIO and AIB decreased the number of nuclei in 4C phases in NO-pretreated seeds by 3.4 and 2.1, respectively.

Table 3 The effect of AVG or AIB on germination of *A. retroflexus* L. seeds pretreated with NO or NO + NBD

Inhibitor, M	NO → air		NO → NBD → air	
	Germination, %	Inhibition, %	Germination, %	Inhibition, %
0 (H_2O)	50.0 ± 2.0c	–	32.0 ± 3.5b	–
AVG 10^{-3}	46.7 ± 1.2c	6.7 ± 2.3a	20.7 ± 1.2a	35.4 ± 3.6cd
AIB 10^{-3}	36.7 ± 3.0b	26.7 ± 6.1bc	17.3 ± 3.0a	45.8 ± 9.5d

Seeds were incubated in water and for the first 5 h in air enriched with NO (10^{-2} M), then for 24 h in air or air enriched with NBD (10^{-4} M). Then they were incubated in air and water or in air and AVG or AIB solution. Vertical bars indicate ± SD. Two-way ANOVA with the Duncan post hoc test was used to determine significant differences. Mean values with different letters (a–e) are significantly different ($P < 0.05$)

Discussion

The seeds of *A. retroflexus*, a common and important summer annual weed all over the world, are physiologically non-deep dormant after harvest (Baskin and Baskin 2014). This dormancy, which can be considered as relative dormancy, is expressed as the inhibition of germination only at relatively low temperatures. The application of various physical or chemical factors enables them to germinate at lower temperatures thereby extending the temperature range suitable for germination. The *A. retroflexus* seeds used in the present experiments were dormant, and therefore germination at 25 °C in the light was very poor, not exceeding 10% (Figs. 1, 2, 3, 4). Dormancy in these seeds was removed by NO released from acidified KNO_2. Likewise, the application of SNP, NO, and HCN donors, for 7 days (Liu et al. 2011) or for 1 day, have stimulated germination of these seeds (Kępczyński and Sznigir 2014). The results presented here showed that NO released from the KNO_2 applied for only 5 h was sufficient to remove dormancy (Figs. 1, 2, 3, 4). Similarly, short-term treatment of dormant apple embryos with NO has been shown to stimulate their germination (Gniazdowska et al. 2007). Reducing the stimulatory effect of NO with the widely used scavenger cPTIO, which is highly specific to NO (D'Alessandro et al. 2013), confirmed that NO is responsible for dormancy release in *A. retroflexus* seeds. The beneficial effect of NO from various donors, usually applied for at least 1 day, on the germination of dormant and non-dormant seeds of several plant species has been described previously (Bethke et al. 2007; Arc et al. 2013a, b; Krasuska et al. 2015).

Seed dormancy of *A. retroflexus* can be removed by ethylene liberated from ethephon taken up by seeds (Kępczyński et al. 1996; Fig. 1). The response of *A. retroflexus* seeds to ethylene applied as a gas (Kępczyński et al. 2003a; Fig. 4) is evidence of the significance of this hormone in releasing dormancy. The important role of ethylene in releasing dormancy of *Amaranthus* seeds is supported by the very high sensitivity of both primary dormant *A. retroflexus* (Kępczyński et al. 1996) and secondary dormant *A. caudatus* seeds (Kępczyński et al. 2003b), as it can substantially or completely remove dormancy at very low concentrations. Stimulation of germination of *A. retroflexus* seeds by exogenously applied ACC, a precursor of ethylene biosynthesis which is converted to ethylene (Fig. 1, Table 1), also supports the role of ethylene in releasing dormancy. Moreover, it indicates that ACC oxidase is partially active in these dormant seeds. Induction of germination of these seeds by exogenous ACC, which is metabolized to ethylene (Table 1), as well as the lower content of endogenous ACC in dormant seeds than in non-dormant seeds (Kępczyński et al. 2003a), suggests that dormancy is associated with insufficient production of ethylene caused by too low content of endogenous ACC. The significant role of ethylene in the germination of primary and secondary dormant seeds as well as non-dormant seeds has been discussed in previously published reviews (Kępczyński and Kępczyńska 1997; Matilla 2000; Corbineau et al. 2014).

However, additional knowledge is still required, especially regarding the interaction of ethylene with other molecules. The significant role of ethylene in removing *A. retroflexus* seed dormancy also stems from the increase in its production as soon as 1 h, before radicle protrusion through the coat of seeds treated with dormancy-releasing factors (Table 1).

The opposite effects of NO scavengers derivatives of PTIO to those of NO donors are usually considered to be reliable evidence of NO implication in physiological processes (Arc et al. 2013b). Removal of NO by application of its scavenger, cPTIO, simultaneously with ethephon or ACC prevents the stimulatory effect of these compounds (Fig. 1), indicating that NO is required for the seed response to ethylene. The fact that ethylene or ACC increases the beneficial effect of NO (Figs. 1, 4) on germination indicates a positive interaction between these compounds in regulating the removal of dormancy. In addition to ethylene, GA_3 was also able to induce the germination of dormant *A. retroflexus* seeds (Figs. 1, 2),

Table 4 Effects of NO (KNO_2 10^{-2} M), GA_3 (10^{-3} M), ETH (10^{-4} M), cPTIO (10^{-4} M), AIB (10^{-3} M), NO + cPTIO or NO + AIB on the nuclear DNA content in radicles of *A. retroflexus* L. seeds after incubation for different periods

Imbibition, h	Percentage of nuclei					
	2C		S		4C	
	Untreated	NO	Untreated	NO	Untreated	NO
(a)						
0	81.6 ± 1.0d	–	12.2 ± 0.6ab	–	6.3 ± 0.6a	–
30	72.3 ± 1.4c	46.9 ± 1.1b	15.6 ± 0.6b	21.4 ± 2.3c	12.1 ± 0.9b	31.7 ± 1.2c
		GA_3		GA_3		GA_3
30	–	46.1 ± 0.6b	–	23.2 ± 0.7c	–	30.7 ± 0.3c
	Untreated	ETH	Untreated	ETH	Untreated	ETH
24	75.4 ± 1.6c	39.1 ± 0.8a	17.4 ± 1.5b	32.3 ± 1.2d	7.1 ± 0.6a	28.7 ± 1.0c
48	75.1 ± 0.8c	40.8 ± 1.0a	17.3 ± 1.2b	24.7 ± 0.8c	7.6 ± 1.0a	34.5 ± 0.4d

Treatment	Percentage of nuclei		
	2C	S	4C
(b)			
Dry	82.1 ± 1.4 cd	12.0 ± 0.7a	5.9 ± 1.1a
24 h			
Control	76.9 ± 0.9 cd	16.7 ± 0.7ab	7.0 ± 0.5a
NO (KNO_2 10^{-2} M)	71.9 ± 0.4bc	20.5 ± 1.7c	7.6 ± 0.2a
cPTIO 10^{-4} M	76.9 ± 0.4 cd	16.0 ± 1.2ab	7.1 ± 1.0a
NO + cPTIO	72.1 ± 2.1bc	19.3 ± 0.9bc	8.6 ± 1.4ab
AIB 10^{-3} M	73.3 ± 1.8bc	17.3 ± 1.9ab	9.4 ± 1.1ab
NO + AIB	72.6 ± 2.6bc	18.7 ± 0.4bc	8.7 ± 2.3ab
30 h			
Control	73.3 ± 1.5bc	15.3 ± 1.0a	11.4 ± 0.7b
NO (KNO_2 10^{-2} M)	46.9 ± 1.2a	22.4 ± 0.9c	31.3 ± 1.9c
cPTIO 10^{-4} M	76.3 ± 1.6bc	13.5 ± 2.5a	10.2 ± 1.7b
NO + cPTIO	70.9 ± 3.2b	19.8 ± 1.2bc	9.1 ± 2.5ab
AIB 10^{-3} M	68.8 ± 1.5b	18.5 ± 1.3ab	12.7 ± 0.6b
NO + AIB	66.7 ± 1.8b	18.5 ± 0.6ab	14.8 ± 1.2b

The first seeds with seed coat pierced by the radicle were observed ca 1 h after the determination of nuclear DNA content in seeds incubated for 24 h with ETH or 30 h with NO or GA_3. After 48 h of incubation in ETH solution, about 60% of seeds were germinated. Vertical bars indicate ± SD. Two-way ANOVA with the Duncan post hoc test was used to determine significant differences. Mean values with different letters (a–d) are significantly different ($P < 0.05$)

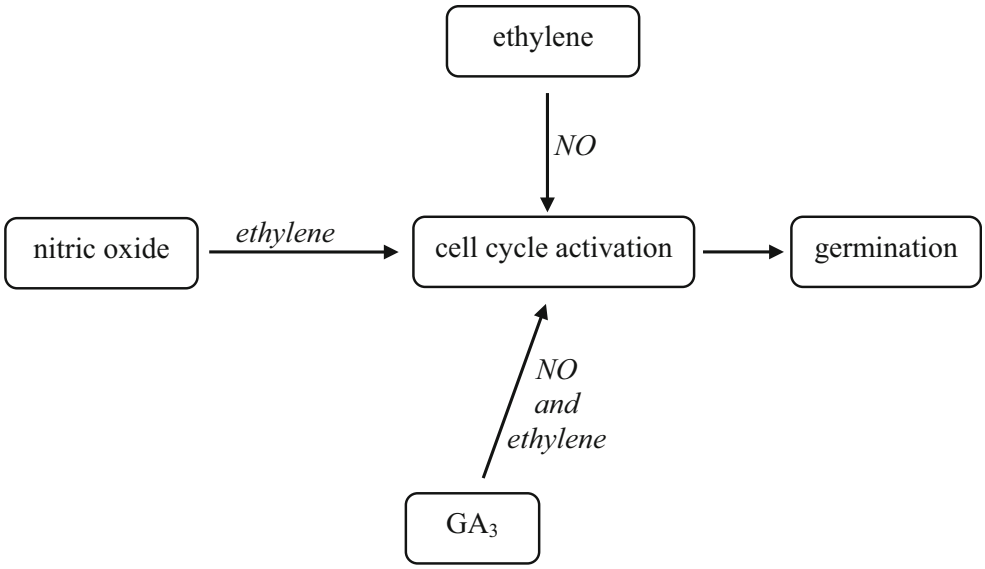
which is in agreement with previous findings (Kępczyński et al. 1996). The impact of GA_3 can also be blocked through the removal of endogenous NO by cPTIO (Fig. 1), showing that not only ethylene but also gibberellin provides evidence of the need for nitric oxide. Dormancy removal in *Arabidopsis* seeds by NO was associated with enhanced transcription of *GA3ox1* and *GA3ox2* (Bethke et al. 2007). Likewise, an interaction between gibberellin and ethylene in regulating seed germination has been shown in several experiments. Inhibition of germination of non-dormant *A. caudatus* seeds by tetcyclacis, an inhibitor of gibberellin biosynthesis, was reversed by GA_{4+7} and also by ethephon or ACC (Kępczyński 1986a). In experiments with *Arabidopsis* seeds, GA_3 was found to induce germination of the *etr1* mutant (Bleecker et al. 1988) and ethylene-stimulated germination of the GA-deficient *ga1* mutant (Karssen et al. 1989).

Moreover, induction by GA_3 and NO ethylene production before radicle protrusion of *A. retroflexus* dormant seeds (Table 1) suggest that release dormancy in these seeds by above compounds involves control of ethylene biosynthesis. This result also indicates that dormancy release was ethylene-dependent. Moreover, cPTIO, in addition to reducing germination, also decreased ethylene production enhanced by NO and GA_3 (Table 1), confirming that the presence of NO is necessary for the stimulatory effect of both compounds.

In light of these data, we can postulate NO crosstalk with ethylene and GA_3 in regulating dormancy release of *A. retroflexus* seeds. Crosstalk between NO and ethylene in the regulation of dormancy release of apple embryos (Gniazdowska et al. 2010) and germination under salinity of non-dormant *Arabidopsis* seeds (Lin et al. 2013) has previously been demonstrated. The stimulatory effect of NO and GA_3 on *A. retroflexus* seed germination was not affected by the ACC synthase inhibitor AVG, which also had no effect on ethylene production (Fig. 2). However, AIB, an inhibitor of ACC oxidase, slightly influenced both ethylene production and germination of seeds treated with NO or GA_3. This may to a small degree suggest action of NO and GA_3 via the stimulation of ethylene biosynthesis. Previous studies in which inhibitors of ethylene biosynthesis were applied to seeds have provided varied data. Significant reduction in the stimulatory effect of GA_3 on *A. retroflexus* seed germination was obtained by applying $CoCl_2$, a nonspecific inhibitor of ACC conversion to ethylene (Kępczyński et al. 2003a). An earlier study showed that AVG simultaneously inhibited the release from secondary dormancy of *Amaranthus caudatus* seeds by GA_3 as well as ethylene production stimulated by this compound (Kępczyński et al. 2006). An inhibitor of ACS activity, α-aminoisobutyric acid, inhibited the germination of partially dormant and non-dormant apple embryos (Kępczyński et al. 1977), and AVG inhibited germination of dormant apple embryos pretreated with NO (Gniazdowska et al. 2010). It has also been demonstrated that both AVG and $CoCl_2$ suppress the stimulatory effect of glutamate on the germination of cucumber seeds under salt stress (Chang et al. 2010). In the case of non-dormant *Amaranthus caudatus* seeds, even strong reduction of ethylene production by AVG did not inhibit germination, but the seeds still produced a certain amount of ethylene, which probably was sufficient for their germination (Kępczyński and Karssen 1985). However, while AVG did not affect the germination of non-dormant *A. caudatus* seeds, it did reduce the acceleration of seed germination by GA_{4+7}, indicating the ethylene-dependent effect of this gibberellin (Kępczyński 1986a). Moreover, AVG increased germination inhibition caused by tetcyclacis or ABA (Kępczyński 1986a, b).

As an alternative to using inhibitors of ethylene biosynthesis, manipulation of the availability of ethylene receptors provides very important information on the functions of ethylene alone or together with other compounds, since ethylene action is crucial for the germination of dormant and non-dormant seeds. NBD, an ethylene inhibitor binding to its receptor, counteracts the effect of ethylene on germination of non-dormant and secondary dormant *A. caudatus* seeds (Kępczyński and Karssen 1985; Kępczyński et al. 2006) and primary dormant *A. retroflexus* seeds (Kępczyński et al. 2003a; Fig. 4), indicating the necessity of ethylene action for germination of these seeds. NBD applied simultaneously with NO for 5 h (1), for 24 h to NO-pretreated seeds (2) or continuously after pretreatment with NO (3) reduced the beneficial effect of NO on the release of *A. retroflexus* seed dormancy (Table 2, 3; Figs. 3, 4), which indicates that ethylene action is required to reveal the effect of NO, and also demonstrates the importance of ethylene in response to this compound. Blocking of ethylene receptors by NBD in NO-pretreated apple embryos also prevented the stimulating influence of nitric oxide (Gniazdowska et al. 2010). The release of dormancy in *A. retroflexus* seeds by GA_3 (Kępczyński et al. 2003a; Fig. 3) was also strongly antagonized by NBD (Kępczyński et al. 2003a), which suggests that ethylene action is required for dormancy release by this compound. Application of AVG and AIB in combination with NBD intensified the inhibitory effect of NBD on germination of NO-pretreated seeds (Table 3), demonstrating the significance of ethylene and the need for its synthesis in response to NO.

To determine whether dormancy breaking by NO, ethylene, and GA_3 involves cell cycle activation, flow cytometry was used to analyze the content of nuclear DNA in the tips of radicles isolated from seeds. As in previous experiments with tomato, barley, and *A. fatua* (Bino et al. 1993; Gendreau et al. 2012; Cembrowska-Lech and Kępczyński 2016), most of the cells in the radicles from dry seeds were arrested in phase G_1 of the cell cycle (Table 4). The percentage of nuclei in dry *A. retroflexus* seeds in phase S and G_2 was much lower than the percentage in phase G_1. Imbibition of dormant seeds in water for 24 or 30 h slightly decreased the percentage of nuclei in G_1 and doubled the percentage of G_2 nuclei after 30 h. NO and GA_3, stimulating germination of *A. retroflexus* dormant seeds, decreased the percentage of nuclei in G_1 and increased in the percentage in S, and more so in G_2, 1 h before radicle protrusion (Table 4a). Ethylene also decreased the percentage of nuclei in G_1 and increased the percentage in S and G_2 1 h before radicle protrusion (after 24 h of incubation). The inhibition of seed germination of NO-pretreated seeds by cPTIO and AIB, was associated with lowering number of nuclei in phase G2 (Table 4b).



Scheme 3 The model of interaction between NO, ethylene, and GA_3 in germination induction of dormant *A. retroflexus* L. seeds

Above data may indicate that induction of dormancy release and germination of seeds by these compounds involves initiation of the cell cycle prior to radical protrusion. Previously, *A. fatua* seed dormancy release by smoke water, KAR_1, and GA_3 was found to initiate the cell cycle before germination (Cembrowska-Lech and Kępczyński 2016, 2017).

The results presented here show the essential role of ethylene in releasing dormancy in *A. retroflexus* seeds (Scheme 3). Nitric oxide applied for only 5 h was able to release dormancy to a similar degree as did ethylene or GA_3. The germination of dormant seeds due to NO or GA_3 treatment was preceded by an increase in the production of ethylene, indicating that the dormancy-releasing effect of both compounds is ethylene-dependent. The response of seeds to NO, as shown earlier for GA_3 (Kępczyński et al. 2003a), required ethylene action. On the other hand, induction of germination of dormant seeds by NO, ethylene or GA_3 required the presence of NO. Dormancy release and germination caused by NO, ethylene, and GA_3 was associated with activation of the cell cycle prior to radicle protrusion.

Author contribution statement JK initiated and designed the research, interpreted the results, wrote the manuscript and provided the funding. DC-L conducted most experiments and statistical analysis. PS conducted some experiments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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