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ORIGINAL PAPER

Interaction of karrikinolide and ethylene in controlling germination of dormant *Avena fatua* L. caryopses

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Abstract Caryopses of *Avena fatua* L. are dormant after harvest and germinate poorly at 20 °C. Dormancy was released by after-ripening the dry caryopses in the dark at 25 °C for 3 months. Karrikinolide (butenolide, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, KAR_1), in contrast to exogenous ethylene and the precursor of ethylene biosynthesis 1-aminocyclopropane-1-carboxylic acid (ACC), completely overcame dormancy. The effect of KAR_1 was not affected by aminoethoxyvinylglycine (AVG), α-aminoisobutyric acid (AIB) and $CoCl_2$, inhibitors of ACC synthase and oxidase, respectively. 2,5-Norbornadiene (NBD), a reversible inhibitor of ethylene binding to its receptor, counteracted the stimulatory effect of KAR_1. Ethylene, ethephon and ACC counteracted and AVG reinforced inhibition caused by norbornadiene. Inhibition due to norbornadiene, applied during the first 3 days of imbibition in the presence of KAR_1, disappeared after transfer to air or ethylene. The obtained results confirm that KAR_1 breaks dormancy and indicate that ethylene alone plays no role in releasing dormancy of *Avena fatua* caryopses. KAR_1 probably did not relieve dormancy via the stimulation of ethylene biosynthesis. Some level of endogenous ethylene is probably required for ethylene action, which might be required for releasing dormancy by KAR_1 or for subsequent germination of caryopses after removing dormancy.

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Keywords *Avena fatua* L. · Butenolide · Caryopses · Ethylene · Karrikinolide · Primary dormancy · Germination

Introduction

It is well documented that smoke and smoke–water stimulate seed germination of many plant species from fire-prone and fire-free environments (Light et al. 2009). The role of smoke in releasing dormancy and accelerating germination has been extensively studied since the germination stimulant, a butenolide compound (karrikinolide, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, KAR_1), was isolated from plant-derived smoke (Van Staden et al. 2004) and burned cellulose (Flematti et al. 2004). Several studies showed that seeds sensitive to smoke also responded to KAR_1 (Light et al. 2009). There are also examples showing that KAR_1 and smoke are not equally effective in all species. Smoke contains many compounds besides the KAR_1, some of which may inhibit germination (Light et al. 2010). Both smoke and the KAR_1 stimulate germination of partially dormant caryopses (seeds) of *Avena fatua*, an important widespread, persistent weed, which infests agricultural soils for several years (Adkins and Peters 2001; Kępczyński et al. 2006b; Daws et al. 2007; Stevens et al. 2007). This compound completely removed dormancy in *A. fatua* caryopses at 15–25 °C when used continuously (Kępczyński et al. 2010). KAR_1 can also be effective if applied for shorter periods. It was also found that the stimulatory effect of KAR_1 on dormancy release of these caryopses is associated with cell cycle activation (Kępczyński et al. 2010). The physiological role of KAR_1 in the control of seed dormancy, especially in relation to plant growth regulators, is poorly understood. The only relationship that has received attention is between KAR_1 and gibberellic acid. Ethylene can stimulate the

germination of primary dormant seeds of several plant species (Kępczyński and Kępczyńska 1997; Matilla and Matilla-Vázquez 2008) and ethephon can stimulate germination of partially dormant *A. fatua* seeds (Adkins and Ross 1981).

In view of these considerations, *A. fatua* seeds were used as a model system to define the relationship between KAR_1 and ethylene. We determined: (1) the response of caryopses to KAR_1, ethephon, ethylene and the precursor of ethylene biosynthesis 1-aminocyclopropane-1-carboxylic acid (ACC); (2) the effect of inhibitors of ethylene biosynthesis aminoethoxyvinylglycine (AVG), α-aminoisobutyric acid (AIB) and $CoCl_2$ in the presence of KAR_1; and (3) the effect of 2,5-norbornadiene (NBD) (which inhibits ethylene binding to its receptor) when applied alone or in combination with ethylene, ethephon, ACC or AVG in the presence of KAR_1 on caryopses germination.

Materials and methods

Florets of *Avena fatua* L. were collected near Szczecin in Poland at the time of natural dispersal in July 2007. After collection they were dried in open air at room temperature for 7 days and then stored at −20 °C until required. Under these conditions dormancy was maintained. One batch of florets was stored dry at 25 °C for 3 months to break dormancy. In all experiments dehulled caryopses (lemma and palea removed) were used.

Germination

Three replicates of 25 caryopses each were incubated in Petri dishes (6 cm) on Whatman No. 1 filter paper moistened with 1.5 ml distilled water, KAR_1 (10^{-10}, 10^{-9}, 10^{-8} M), ethephon (10^{-4} M), ethylene (4.5×10^{-7} M), ACC (10^{-4} M), AIB (3×10^{-4}, 10^{-3} M), AVG (3×10^{-4}, 10^{-3} M) or $CoCl_2$ (3×10^{-4} M) applied alone or in combination with KAR_1 at 20 °C in the dark. Germination was recorded every day over 5 days or after 5 days depending on the experiment. All manipulations were performed under a green safe light at 0.4 μmol m^{-2} s^{-1}. Protrusion of the coleorhiza (2 mm) was used as the criterium of germination.

2,5-Norbornadiene (NBD) and ethylene treatment

Continuous treatment

For NBD ($5x10^{-5}$, 10^{-6} M) treatment, 3 uncovered Petri dishes with 25 caryopses on filter paper moistened with water, KAR_1, KAR_1 in combination with ethephon, ACC or AVG were placed in tightly sealed glass containers (2.6 L). Liquid NBD was applied by syringe onto pieces of filter paper placed under the cover of the container. The liquid evaporated completely. Caryopses were also incubated in water or KAR_1 in an atmosphere containing ethylene or ethylene in combination with NBD.

Preincubation in NBD

The caryopses were preincubated in water or in a solution of KAR_1 on Petri dishes in containers (2.6 L) with NBD for 3 days. Seeds were then transferred to fresh water or a solution of KAR_1 and then incubated for the following 4 days in glass containers (2.6 L) with air or an atmosphere containing ethylene. In one experiment caryopses were first preincubated in the presence of KAR_1 and NBD and were then transferred to air and KAR_1 in combination with AVG.

1-methylcyclopropene (1-MCP) treatment

Powdered 1-MCP in concentrations producing 25, 75 or 100 μl/l gas, were placed in open glass vials suspended under the cover in 500 ml glass containers with 3 Petri dishes each containing 25 seeds on filter paper moistened with KAR1. In order to liberate 1-MCP to the atmosphere, 2 ml of sterile water were injected through the stopper into a vial containing powdered 1-MCP.

Statistical analysis

The average ± standard deviation (SD) of three independent samples of 25 caryopses each are presented. Data were analyzed using one-way and two-way ANOVA (Statistica for Windows ver. 8.0, StatSoft Inc., Tulsa, Oklahoma, USA) to determine statistical differences between mean values ($p < 0.05$). Treatment means were then ranked by the Duncan's multiple-range test.

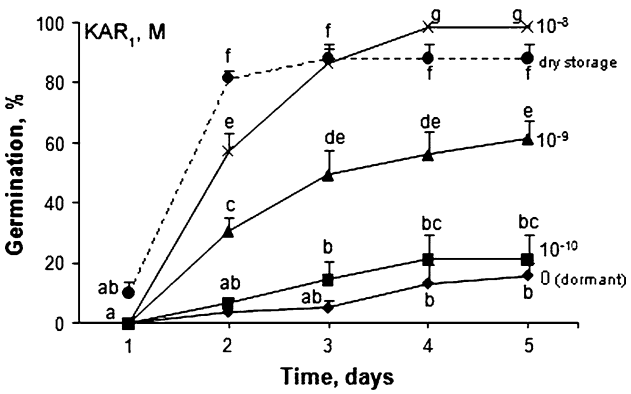

Fig. 1 The effect of KAR_1 and dry storage for 3 months at 25 °C on the germination of *A. fatua* caryopses

Results

Effect of dry storage and KAR_1 on germination of caryopses

Germination of caryopses stored at −20 °C (dormant) and dry-stored (25 °C for 3 months) was determined (Fig. 1). Caryopses of *A. fatua* germinated poorly at 20 °C after harvest; 17 % after 5 days. Dry storage for 3 months allowed 80 % of caryopses to germinate within 2 days and 90 % after 5 days.

KAR_1 at all concentrations used, stimulated germination of dormant caryopses. Even after 2 days a clear effect was observed; 30 and 60 % of caryopses were germinated at 10^{-9} and 10^{-8} M of KAR_1 respectively. After 3 days KAR_1 at 10^{-9} and 10^{-8} M caused 50 and 85 % germination, while at 10^{-8} M, the highest concentration used, 100 % germination occurred after 4 days.

Effect of KAR_1, ethephon, ethylene, ACC and inhibitors of ethylene biosynthesis on germination of dormant caryopses

Application of KAR_1 at 10^{-8} M resulted in all caryopses germinating after 5 days (Fig. 2). Ethephon, ethylene and the precursors of ethylene biosynthesis only had slight effects on germination.

To determine whether biosynthesis of endogenous ethylene is involved in the response of caryopses to KAR_1, inhibitors of ethylene biosynthesis in combination with KAR_1 were applied. Inhibitors of ACC synthase, α-aminoisobutyric acid (AIB) and aminoethoxyvinylglycine (AVG), an inhibitor of ACC oxidase activity, did not affect the stimulatory effect of KAR_1 (Table 1).

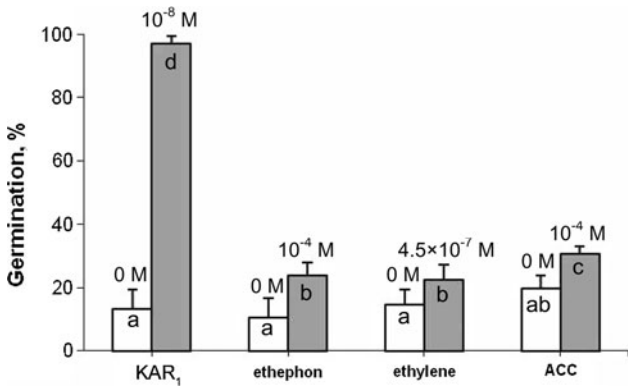

Fig. 2 The effect of KAR_1, ethephon, ethylene and ACC on the germination of *A. fatua* L. caryopses after 5 days

Table 1 The effect of KAR_1 applied alone and in combination with AIB, AVG and $CoCl_2$ on the germination of *A. fatua* caryopses after 5 days

Compounds, M	Germination (%)
KAR_1, 10^{-8}M	94.67 ± 2.3
KAR_1, 10^{-8}M + AIB, 3×10^{-4} M	97.33 ± 2.3
KAR_1, 10^{-8}M + AIB, 10^{-3} M	88.00 ± 4.0
KAR_1, 10^{-8}M + AVG, 3×10^{-4} M	84.00 ± 8.0
KAR_1, 10^{-8}M + AVG, 10^{-3} M	81.33 ± 10.0
KAR_1, 10^{-8}M + $CoCl_2$, 3×10^{-4} M	90.67 ± 2.3

Germination of caryopses in the presence of 2,5-norbornadiene and 1-MCP

The experiment with inhibitors of ethylene biosynthesis seem to suggest that ethylene is not part of the response of caryopses to KAR_1. Consequently it was interesting to determine whether some action of ethylene is required for germination of dormant caryopses in the presence of KAR_1. Therefore the competitive, reversible inhibitor of ethylene binding to its receptor, 5×10^{-5} M 2,5-norbornadiene (NBD), was applied. As in previous experiments germination of dormant caryopses incubated in water and air was

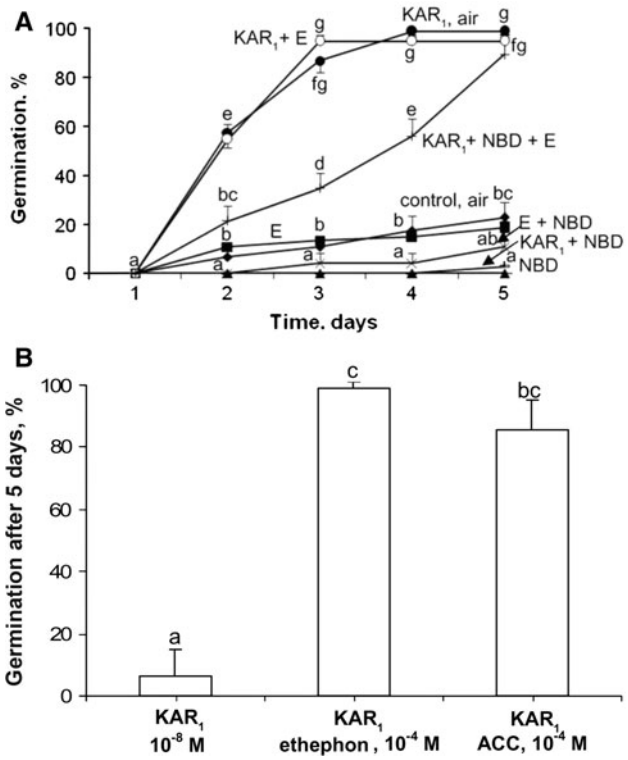

Fig. 3 The effect of KAR_1 on the germination of *A. fatua* caryopses incubated in air, an atmosphere containing ethylene, 2,5-norbornadiene, a combination of norbornadiene and ethylene (**a**), or in the presence of ethephon or ACC in atmosphere with norbornadiene (**b**). KAR_1 10^{-8} M; NBD 5×10^{-5} M; ethylene 4.5×10^{-7} M

very low and after 5 days reached about 20 %, while 10^{-8} M KAR_1 caused germination of almost all caryopses (Fig. 3a). Addition of ethylene at 4.5×10^{-7} M did not affect germination of dormant caryopses either in the presence or absence of KAR_1. Nobornadiene inhibited germination completely when caryopses were incubated in water and almost completely when incubated in a solution of KAR_1. When caryopses were incubated in KAR_1 and simultaneously in an atmosphere enriched with both NBD and ethylene, progressive germination during incubation was observed and after 5 days all caryopses had germinated. Likewise, incubation in the presence of KAR_1 in combination with ethephon or ACC and in an atmosphere containing NBD, allowed all, or almost all, caryopses to germinate (Fig. 3b). Application of NBD at a far lower concentration, 10^{-6} M, did not completely block the stimulatory effect of KAR_1 (Fig. 4). However, simultaneous incubation in NBD and KAR_1 in combination with AVG caused greater inhibition of germination. Instead of the reversible inhibitor of ethylene binding, NBD, the non-reversible inhibitor of ethylene binding, 1-MCP, was applied simultaneously with KAR_1 (Fig. 5). 1-MCP when applied with KAR_1 at the lowest concentration (25 μL/L) decreased the speed of germination without affecting overall germination after 5 days of incubation. When the inhibitor was applied at 75 μL/L it markedly antagonized the stimulatory effect of KAR_1 and only about 50 % of the caryopses germinated. Application of MCP at 100 μL/L almost totally inhibited the stimulatory effect of KAR_1 on germination.

Germination of caryopses preincubated in air enriched with 2,5-norbornadiene

To determine whether the NBD effect is toxic or non reversible, caryopses were preincubated in norbornadiene

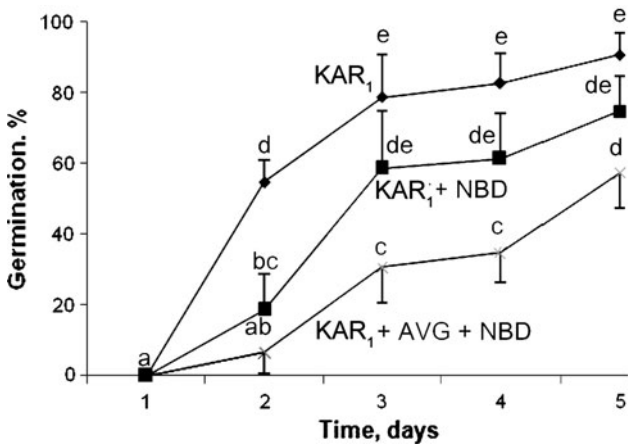

Fig. 4 The effect of KAR_1 on the germination of caryopses of *A. fatua* in the presence of AVG and norbornadiene after 5 days. KAR_1 10^{-8} M; AVG 3×10^{-5} M; NBD 10^{-6} M

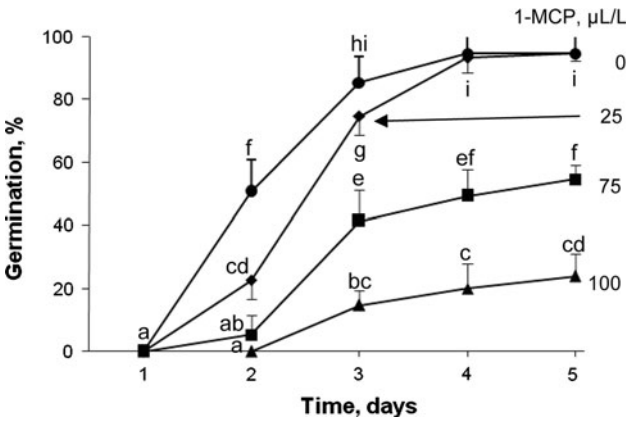

Fig. 5 The effect of KAR_1 on the germination of *A. fatua* caryopses in the presence of 1-MCP

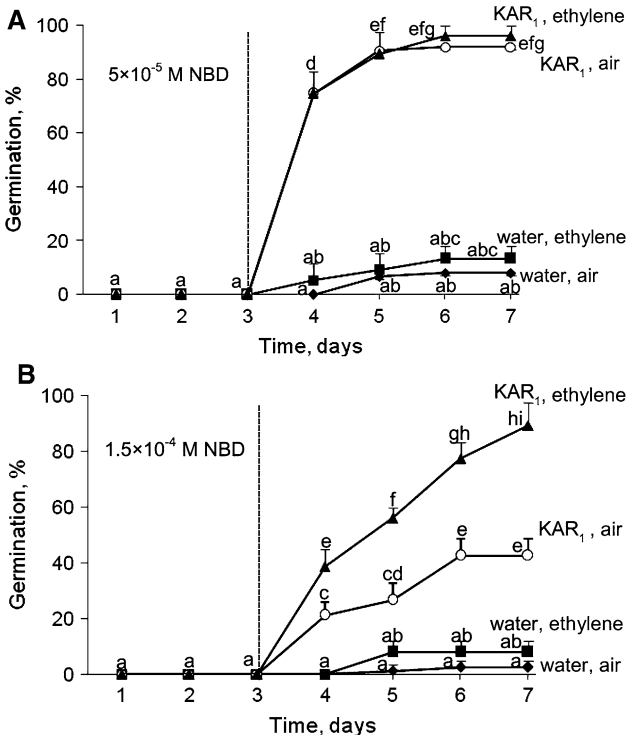

Fig. 6 The effect of transfer from norbornadiene to air or ethylene on the germination of *A. fatua* caryopses in the absence or presence of KAR_1. The caryopses were incubated for 3 days in 5×10^{-5} M (**a**) or 1.5×10^{-4} M (**b**) norbornadiene and from days 3 to 7 in air or 5×10^{-7} M ethylene. The caryopses were incubated constantly with water or in the presence of 10^{-8} M KAR_1

and in water or in a solution of KAR_1 for 3 days and then transferred to fresh water or fresh solution of KAR_1 and incubated in air or in an atmosphere enriched with ethylene (Fig. 6a). These caryopses did not germinate during preincubation in the presence of NBD. The inhibition of the stimulatory effect of KAR_1 caused by preincubation in 5×10^{-5} M NBD was relieved completely after transfer to air. The addition of ethylene to the atmosphere after transfer

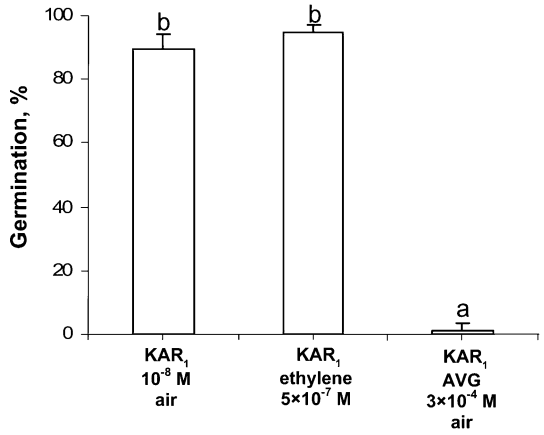

Fig. 7 The effect of transfer from an atmosphere enriched with norbornadiene to air or ethylene on the germination of *A. fatua* caryopses in the presence of KAR_1 or KAR_1 in combination with AVG. The caryopses were incubated for 3 days in 8×10^{-5} M NBD and for 4 days after transfer in air or 5×10^{-7} M ethylene. The caryopses were incubated constantly in the presence of 10^{-8} M KAR_1

did not accelerate the recovery of germination ability. When caryopses were preincubated in a solution of KAR_1 and simultaneously at an higher concentration of NBD (1.5×10^{-4} M) the inhibitory effect of norbornadiene was partially relieved after transfer to air and almost completely after transfer to an atmosphere containing ethylene (Fig. 6b). When caryopses were preincubated in the presence of KAR_1 in an atmosphere together with 8×10^{-5} M norbornadiene for 3 days and then incubated simultaneously in the presence of KAR_1 in combination with AVG, extremely low germination was observed (Fig. 7). Incubation in air or ethylene and simultaneously in the presence of KAR_1, after transfer from norbornadiene, similarly as in the previous experiment, resulted in almost total germination.

Discussion

Seed dormancy is a relative phenomenon the expression of which varies with the environment. More dormant seeds germinate within a narrower temperature range than less dormant seeds and fully dormant seeds are not able to germinate at any temperature (Hilhorst 2007). It was shown that the optimal temperature for dormant *Avena fatua* caryopses is 4–12 °C with little germination at 20–24 °C while non-dormant seeds germinated at 4–24 °C (Naylor and Fedec 1978). In this study caryopses of *Avena fatua* that were air-dried and then stored at −20 °C, germinated poorly at 20 °C, as in previous experiments (Kępczyński et al. 2010), 20 %. Dormancy of these seeds was removed by after-ripening during dry storage at 25 °C for 3 months (Fig. 1). In another experiment it was also demonstrated that dormancy in *A. fatua* caryopses can be released by dry storage at temperatures from 20 to 40 °C (Foley 1994). KAR_1, isolated from plant-derived smoke, has the ability to stimulate germination of several plant species from fire-prone and fire-free environments, including arable weeds and crop plants (Light et al. 2009). The observed stimulatory effect of KAR_1 on germination of dormant *A. fatua* caryopses (Fig. 1) is in agreement with results shown previously (Daws et al. 2007; Stevens et al. 2007; Kępczyński et al. 2010) and indicates that stimulation by this compound is a common response for this species. Dormancy of *A. fatua* caryopses was removed in our experiments by KAR_1 at very low concentration (10^{-8} M).

Ethylene, liberated from ethephon, applied as a gas or converted from the exogenous precursor of its biosynthesis, ACC, which was shown previously to increase ethylene biosynthesis in germinating dormant *A. fatua* caryopses (Kępczyński et al. 2006b) was not able to remove dormancy (Fig. 2). This is in contrast to the effect of KAR_1 and indicates that ethylene does not play a role in controlling dormancy in *A. fatua* caryopses. This is in good agreement with previous findings, where ethephon increased germination only in partially after-ripened, but not fully dormant seeds of *A. fatua* (Adkins and Ross 1981). After-ripening induced sensitivity to ethylene also in *Sisymbrium officinale* seeds (Iglesias-Fernández and Matilla 2009). There are seeds where dormancy can be broken by ethylene (Kępczyński and Kępczyńska 1997; Matilla 2000). Some hypotheses suggest that ethylene acts minimally during dormancy inception and that its major action is during imbibition to terminate dormancy and/or initiate germination (Ghassemian et al. 2000; Matilla and Matilla-Vázquez 2008). Inhibitors of ACC synthesis, AIB, AVG as well as ACC conversion to ethylene and $CoCl_2$ were not able to counteract the effect of KAR_1 (Table 1). This may suggest that ethylene biosynthesis is not involved in the response to KAR_1 during germination of dormant caryopses. Previously it was found that AVG did not affect germination of dormant *A. fatua* seeds in the presence of the dormancy breaking factor GA_3 (Lalonde and Saini 1992). It was reported that inhibitors of ethylene biosynthesis did not affect germination of dormant sunflower embryos in the presence of cyanide, which breaks dormancy, and it was suggested that cyanide seed dormancy alleviation does not involve ethylene production (Oracz et al. 2008). The antagonism of the stimulatory effect of KAR_1 by blocking ethylene binding to its receptor by norbornadiene (Fig. 3), might suggest that ethylene action is only involved in releasing dormancy by KAR_1 or for the germination process after prior release of dormancy by this compound. The reversal of the inhibitory effect of norbornadiene on the release from dormancy by KAR_1 suggests that ethylene binding to its receptor is required in caryopses response to karrikinolide. Likewise, results of experiments with the application of a non-reversible inhibitor of ethylene binding to its receptor,

1-MCP (Fig. 5), which counteracted the stimulatory effect of KAR_1 may confirm that ethylene action is necessary for germination of dormant caryopses. Previously it was reported that ethylene action is required in releasing primary and secondary dormancy of *A. retroflexus* and *A. caudatus* by gibberellin (Kępczyński et al. 2003; Kępczyński et al. 2006a). Antagonizing the stimulatory effect of KAR_1 was also possible by combining NBD, at a concentration not completely blocking germination, with AVG (Fig. 4). This might suggest that a certain concentration of endogenous ethylene is required for germination of dormant caryopses in the presence of KAR_1. Similarly as in other seeds (Kępczyński and Kępczyńska 1997) the effect of NBD was non-toxic and reversible), since inhibition of dormancy releasing by KAR_1 as a result of preincubation in an atmosphere enriched with this inhibitor was relieved after transfer to air (Fig. 6a). Relieving inhibition after transfer to air is probably associated with the diffusion of NBD from the receptor and its replacement by endogenous ethylene. In cases where higher concentrations of NBD were used during pre-incubation of caryopses in a solution of KAR_1, a slower recovery of germination ability after transfer to air (Fig. 6b) is probably related to a lack of sufficient amounts of endogenous ethylene required for expelling NBD molecules. Therefore exogenous ethylene effectively counteracted NBD-inhibition. The substitution of norbornadiene by endogenous ethylene, after transfer from a NBD atmosphere to air was impossible, because of the presence of AVG (Fig. 7), probably due to a lowering of ethylene biosynthesis.

The results presented here confirm that KAR_1 is a very active compound for releasing dormancy in caryopses of *A. fatua*. Ethylene alone plays no role in control of the dormancy in the caryopses. Butenolide releases dormancy but not via stimulation of ethylene biosynthesis. A certain level of endogenous ethylene is probably necessary for ethylene action, which might be required for dormancy removing by KAR_1 or for germination process after dormancy release.

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