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ORIGINAL PAPER

Necessity of gibberellin for stimulatory effect of KAR_1 on germination of dormant *Avena fatua* L. caryopses

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Abstract *Avena fatua* L. florets (caryopses enclosed by lemma and palea) were partially dormant at 10–20 °C and did not germinate at temperatures outside this range. After-ripening florets at 25 °C for 12 weeks completely removed dormancy. Caryopses (florets without lemma and palea) were able to germinate totally at 20 °C. Karrikinolide (KAR_1) and gibberellic acid (GA_3) applied at 10–25 °C partially or markedly induced germination of dormant florets and caryopses, respectively. Both florets and caryopses were more sensitive to KAR_1 than to GA_3. To obtain similar effects, 1,000 to 10,000 times lower concentrations of KAR_1 than GA_3 were required. After-ripening with time gradually increased sensitivity of caryopses to these regulators. Likewise, after-ripened, non-dormant caryopses were sensitive to KAR_1 and GA_3. Inhibitors of gibberellin biosynthesis, ancymidol, paclobutrazol and flurprimidol inhibited the effect of KAR_1. This inhibition was reversed by GA_3. Caryopses pre-incubated in water with ancymidol or paclobutrazol in the presence or absence of KAR_1 germinated completely but with different rates after transfer to GA_3. KAR_1 probably requires gibberellin biosynthesis to stimulate germination of dormant *Avena fatua* L. caryopses. Both KAR_1 and GA_3 increased α-amylase, β-amylase and dehydrogenases activities during imbibition before visible germination occurred.

Keywords *Avena fatua* · Caryopses · Florets · Gibberellin · Karrikinolide · Primary dormancy

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Introduction

Smoke derived from burning plant material stimulates germination of dormant and non-dormant caryopses of a number plant species from fire-prone and fire-free ecosystems (Thomas and Van Staden 1995; Van Staden et al. 2000). Smoke can also increase seed vigor of several crop plants (Light et al. 2009). For many years, scientists looked for chemicals responsible for the stimulatory effect of smoke. It was difficult to isolate the active component(s) from smoke as it contains several thousand compounds (Maga 1988). Over two hundred compounds extracted from smoke did not affect seed germination. Only in 2003 was the active compound, a butenolide (3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, KAR_1) responsible for the stimulatory action, isolated. It was isolated simultaneously from plant-derived smoke (Van Staden et al. 2004) and from burned cellulose (Flematti et al. 2004). KAR_1 stimulate seed germination of many, but not all, plant species. Usually seeds which are sensitive to smoke responded positively to KAR_1 (Light et al. 2009) with more than 60 species reported as responsive to both smoke and to KAR_1 (Chiwocha et al. 2009). Later KAR_2, KAR_3 and KAR_4 from smoke were identified (Nelson et al. 2009) and different sensitivity to these compounds, depending on species, was noted (Chiwocha et al. 2009).

Avena fatua L. is a persistent weed in cereal growing regions of the world. Caryopses of this plant, after harvest,

can be primarily dormant or non-dormant (Li and Foley 1995). Dormancy corresponds to an inability to germinate at relatively high temperatures (above 12 or 15 °C) and is associated with the testa, pericarp and/or embryo (Adkins and Peters 2001). Germination of dormant caryopses can be stimulated by various factors e.g. dry storage (Foley 1994), coumarin (Hsiao and Quick 1985), strigol (Bradow et al. 1990) and gibberellins (Adkins et al. 1986; Kępczyński et al. 2006, 2010). Germination of dormant or partially dormant *A. fatua* caryopses from various regions of the world can be stimulated by smoke and KAR_1 (Adkins and Peters 2001; Kępczyński et al. 2006, 2010; Daws et al. 2007; Stevens et al. 2007). The response of dormant *A. fatua* L. caryopses to KAR_1 requires ethylene action (Kępczyński and Van Staden 2012). KAR_1 has similar effects to GA_3 on seed germination of Australian *Asteraceae* (Merritt et al. 2006) and arable weeds (Daws et al. 2007; Stevens et al. 2007).

The aim of this study was to determine the effect of KAR_1 and GA_3 on germination of *Avena fatua* L. dormant florets and caryopses. To determine if response to KAR_1 involves gibberellin biosynthesis, inhibitors of its biosynthesis in combination with KAR_1 and GA_3 on germination of dormant caryopses were examined. To compare the mechanism of KAR_1 and GA_3 action activities of α-amylase, β-amylase and dehydrogenases during imbibition of dormant caryopses in the presence of these regulators were measured.

Materials and methods

Plant material

Avena fatua L. spikelets were collected in Poland near Szczecin in July 2009. Spikelets contains 2–3 florets covered with glumes. The floret is a single caryopsis (fruit) covered by the lemma and palea (Simpson 2007). After collection, florets were dried at room temperature for 7 days to a constant moisture content (ca 11 %). In order to maintain primary dormancy florets were stored at −20 °C until required.

Isolation of karrikinolide (KAR_1)

Highly active KAR_1 compound (butenolide; 3-methyl-2*H*-furo[2,3-c]pyran-2-one) was isolated, purified and identified from plant-derived smoke–water as described by Van Staden et al. (2004).

Incubation of dormant florets and caryopses in the presence of KAR_1 and GA_3

Primary dormant florets and caryopses (25 in each of three replicates), were incubated in Petri dishes (60 mm) on one layer of filter paper (Whatman No 1) moistened with 1.5 ml distilled water, KAR_1 or GA_3 (Sigma Aldrich) solutions. Florets and caryopses were incubated in darkness at 5, 10, 15, 20, 25, 30 and 35 °C in the presence of KAR_1 (10^{-10}, 3×10^{-10}, 10^{-9}, 10^{-8} M) or GA_3 (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M) solutions. Germination was determined after 5 days of incubation in darkness. Florets or caryopses were regarded as germinated when the radical protruded through the coleorhiza. All manipulations were performed under a green safe light at 0.5 μM^{-2} s^{-1}, which did not stimulate germination.

Incubation of dormant caryopses in the presence of KAR_1 or GA_3 after various periods dry storage of florets

Dormant florets were stored dry under ambient relative humidity for up to 4 months in darkness at 25 °C to break dormancy. After 2, 4, 8 and 12 weeks of dry storage, florets were sorted and husks (lemma and palea) were removed. Caryopses were incubated in darkness at 20 °C in the presence of KAR_1 (10^{-10}, 3×10^{-10}, 10^{-9}, 10^{-8} M) or GA_3 (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) solutions for 5 days. Germination was determined every day under green safe light. Timson's index was used to determine rate of caryopses germination and to quantify differences in germination. The index was calculated by summing the progressive total of daily cumulative germination percentage over 5 days (Timson 1965).

Pre-incubation of dormant caryopses in the presence of KAR_1 or GA_3 before transfer to water

Caryopses were pre-incubated at 4 °C in distilled water, KAR_1 (3×10^{-9} M) or GA_3 (10^{-5} M) for 6, 12 or 24 h. Caryopses were then rinsed once with 100 ml of distilled water and transferred to Petri dishes with distilled water. They were then incubated at 20 °C in darkness. Germination was determined 5 days after transfer.

Treatment of dormant caryopses with KAR_1, GA_3, ancymidol, paclobutrazol and flurprimidol

In one experiment, caryopses were incubated in the presence of KAR_1 (10^{-8} M) or GA_3 (10^{-5} M) alone or in combination with flurprimidol (10^{-5}, 10^{-4} M) (Sigma Aldrich) in darkness at 20 °C. In a second experiment, caryopses were incubated in the presence of KAR_1, ancymidol (10^{-4} M) (Sigma Aldrich) or paclobutrazol (10^{-4} M) (Sigma Aldrich) in combination with GA_3 (10^{-5} M). Germination was determined after 2 and 5 days (experiment 1) or after 5 days (experiment 2).

In a follow-up experiment, dormant caryopses were pre-incubated at 20 °C in darkness for 7 days in the presence of distilled water, ancymidol (10^{-4} M) or paclobutrazol (10^{-4} M) alone or in combination with KAR_1 (10^{-8} M). Thereafter caryopses were rinsed once with 100 ml of water and transferred to new Petri dishes containing GA_3 (10^{-5} M) solution. Seeds were incubated in 20 °C in darkness for 7 days. Germination of caryopses was determined daily.

α-Amylase (EC 3.2.1.1) activity

After incubation in the presence of KAR_1 (10^{-8} M) or GA_3 (10^{-5} M) for 4, 8, 12, 16, 20, 24, 26 and 29 h caryopses (0.1 g fresh weight), were ground in a pre-chilled mortar and pestle in 1.0 ml ice-cold extraction buffer. All extraction steps were performed at 4 °C. The extraction buffer, pH 6.2, contained 20 mM Tris–maleate with 1.0 mM $CaCl_2$. Analysis of α-amylase activity was conducted as previously described by Kępczyński et al. (2006). The results are expressed as U mg^{-1} protein. One unit (U) is equivalent to the amount of enzyme liberating 1 mg maltose from starch at 37 °C at pH 6.2. The results presented correspond to the mean ± SD of the values obtained with five different extracts (one measurement per extract).

β-Amylase (EC 3.2.1.2) activity

Caryopses (0.1 g fresh weight), incubated in the presence of KAR_1 (10^{-8} M) or GA_3 (10^{-5} M) for different times (4, 8, 12, 16, 20, 24, 26 and 29 h) were homogenized in 1.0 ml of ice-cold 16 mM sodium acetate buffer, pH 4.8. The homogenate was centrifuged at 12,000×*g* for 15 min at 4 °C, and the supernatant then used for enzyme activity and protein content assay. β-Amylase activity was measured according to Bernfeld (1955). The results are expressed as U mg^{-1} protein. One unit (U) is defined as the amount of enzyme liberating 1 mg maltose from starch in 5 min at 37 °C and pH 4.8. The results presented correspond to the mean ± SD of the values obtained with five different extracts (one measurement per extract).

Total dehydrogenase activity

Samples of caryopses (0.1 g fresh weight), incubated in the presence of KAR_1 (10^{-8} M) or GA_3 (10^{-5} M) for different times (22, 26 and 29 h) were homogenized in a pre-chilled mortar and pestle with ice-cold 0.1 M potassium phosphate buffer, pH 7.2, containing 1.5 % TTC (2,3,5-triphenyltetrazolium chloride). Measurement of total dehydrogenase activity was performed as previously described by Kępczyńska et al. (2003). The results are expressed as μg formazan mg^{-1} protein. The results presented correspond to the mean ± SD of the values obtained with five different extracts (one measurement per extract).

Protein determination

The protein content in the enzymatic extracts was assayed by the method of Bradford (1976), using bovine serum albumin (BSA) as standard.

Statistical analysis

The average ± standard deviation (SD) of three independent determinations from 25 caryopses each are presented. Data were analyzed for significance using one-way or two-way analysis of variance, ANOVA (Statistica for Windows ver. 9.0, StatSoft Inc., Tulsa, OK, USA). Duncan's multiple range test was used for determination of significant differences between values of germination and enzymatic activities in wild oat florets or caryopses ($p \leq 0.05$). Similar results were obtained in two independent experiments.

Results

Germination of dormant florets and caryopses in the presence of KAR_1 or GA_3 at various temperatures

Freshly harvested *A. fatua* L. florets were not able to germinate at temperatures between 5 and 35 °C (Fig. 1a). KAR_1 did not affect germination at 5 and 35 °C. However, at temperatures between 10 and 30 °C, in the presence of KAR_1 at 10^{-8} and 10^{-7} M between 30 and 50 % germination was observed. Similarly GA_3 also stimulated germination at 10–30 °C. The highest stimulatory effect was found after application of GA_3 at 10^{-4} and 10^{-3} M (Fig. 1b). In another experiment, the effect of KAR_1 and GA_3, used at the same concentrations as above, on germination of dormant caryopses were compared. Caryopses did not germinate at 5 and above 20 °C (Fig. 1c). The highest level of germination was observed at 10 and 15 °C. More than half of the caryopses germinated. KAR_1 at the highest concentration (10^{-7} M) slightly increased caryopses germination at 5 °C, markedly at 15–25 °C and had no affect at 35 °C (Fig. 1c). Caryopses used in this experiment were a little less sensitive to KAR_1 at 25 °C than in previous experiments (Kępczyński et al. 2010) probably due to different conditions during development. At temperatures between 10 and 25 °C, 60–100 % of the caryopses germinated. GA_3 at 10^{-4}–10^{-3} M had a slight stimulatory effect at 5 °C (Fig. 1d). However, all or almost all caryopses germinated due to this regulator at 10–30 °C.

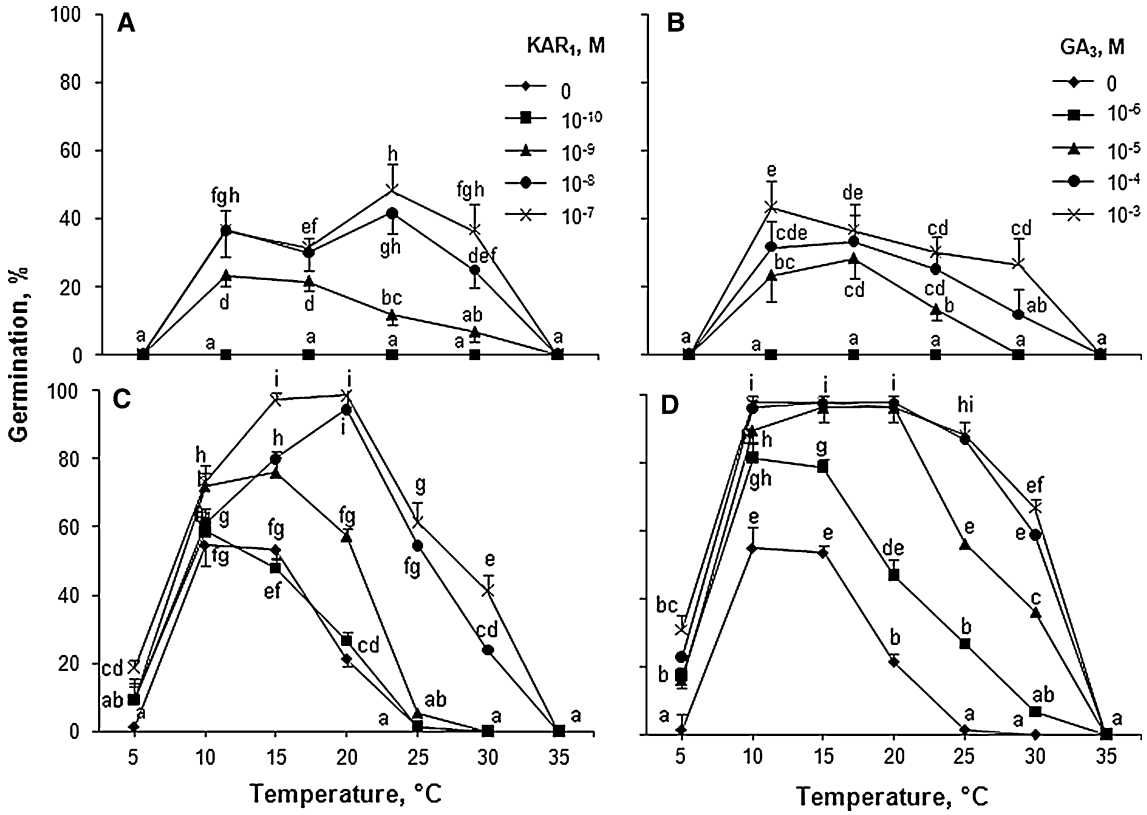

Fig. 1 The effect of KAR_1 (**a**, **c**) or GA_3 (**b**, **d**) on the germination of florets (**a**, **b**) and caryopses (**c**, **d**) of *Avena fatua* after 5 days of incubation at different temperatures. *Vertical bars* indicate ± SD. Two-way ANOVA with the Duncan post hoc test was used to determine significant differences. Mean values with different letters (**a**–**i**) are significantly different ($P < 0.05$)

Germination of caryopses in the presence of KAR_1 or GA_3 after dry storage of florets

A. fatua L. dormant caryopses germinated poorly at 20 °C (Fig. 2a). Dry storage of florets at 20 °C for 2 weeks did not affect this germination, however, increasing the period of dry storage progressively increased germination. All caryopses were able to germinate after 12 weeks of storage. KAR_1 used at lower than 10^{-8} M stimulated partial germination of fully dormant caryopses (Fig. 2a). Its application at low concentrations 3×10^{-10} or 10^{-9} M, caused 37 or 61 % germination of these caryopses in comparison to 17 % in the control. Dry storage for 2 weeks subsequently increased germination to 47 or 72 % in the presence of KAR_1 at the above concentrations. Caryopses after dry storage of florets for 4 weeks reached 80 or 90 % germination due to 3×10^{-10} or 10^{-9} M of KAR_1, respectively, in comparison to around 40 % in the control. All caryopses, unstored or stored for various periods, germinated almost completely at 10^{-8} M. A similar effect was observed when GA_3 at 10^{-5} M was used (Fig. 2b). GA_3 at low concentration (10^{-6} M) improved germination at dry storage for 2 and 4 weeks when compared to unstored caryopses. Both KAR_1 and GA_3 increased not only percent of caryopses germination, but also the Timson's index, which expresses the rate of germination (Table 1). This index was increased sixfold in case of the dormant caryopses. The rate of germination increased with increasing time of dry storage. The effect of KAR_1 and GA_3 in comparison to control was decreased with prolonged storage. These compounds increased the rate of germination after 12 weeks of storage but did not influence final percent germination.

Germination of dormant caryopses after transfer from KAR_1 or GA_3 solution to water

Pre-incubation of caryopses at 4 °C for 6 h in 3×10^{-9} M KAR_1 or 10^{-5} M GA_3 induced partial germination after transfer to water (Table 2). Germination reached 25 or 40 % due to KAR_1 or GA_3 application, respectively. Pre-incubation for 12 or 24 h in the presence of these regulators caused almost complete germination after transfer to water.

Germination of dormant caryopses with KAR_1, GA_3, ancymidol, paclobutrazol and flurprimidol treatments

To check if the stimulatory effect of KAR_1 on seed germination is associated with synthesis of endogenous

gibberellins, ancymidol, paclobutrazol and flurprimidol, inhibitors of its biosynthesis alone and combination with KAR_1 were used. Flurprimidol applied at 10^{-4} M inhibited germination of dormant caryopses (Table 3). GA_3 at 10^{-5} M, as in previous experiments, caused almost complete caryopses germination after 5 days. Likewise in the presence of flurprimidol at 10^{-5} or 10^{-4} M in combination with GA_3 almost all caryopses were able to germinate (Table 3). KAR_1 at 10^{-8} M again allowed almost all caryopses to germinate. Application of 10^{-5} M flurprimidol decreased the effect of KAR_1 and only about 40 % of the caryopses germinated (Table 3). Inhibitors of gibberellins biosynthesis at 10^{-4} M markedly antagonized the stimulatory effect of KAR_1 and only 17–25 % germination was recorded (Table 3; Fig. 3). The inhibition of the stimulatory effect of KAR_1, caused by ancymidol and paclobutrazol, was completely reversed by GA_3, applied at 10^{-5} M (90 %) (Table 3). Caryopses pre-incubated in water at 20 °C for 7 days still had a high response to GA_3 (90 %) (Fig. 4a). GA_3 also stimulated germination of caryopses pre-incubated with ancymidol. These caryopses germinated more slowly but almost completely. Likewise caryopses pre-incubated in a combination of KAR_1 and ancymidol, before transfer to GA_3, germinated more slowly than when pre-incubated in water and achieved a similar level of germination as when pre-incubated in water. A similar relationship was observed when instead of ancymidol, paclobutrazol was applied. Likewise gibberellin caused slower but complete germination of caryopses, pre-incubated in the presence of paclobutrazol or paclobutrazol in combination with KAR_1, than when pre-incubated only in water (Fig. 4b).

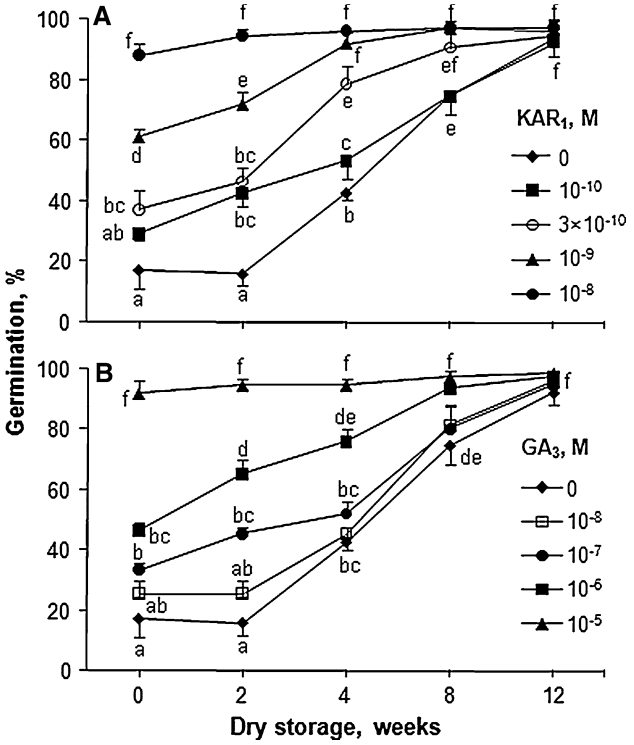

Fig. 2 The effect of KAR_1 (**a**) or GA_3 (**b**) on the germination after 5 days of *Avena fatua* caryopses at 20 °C after previous dry storage of florets at 25 °C for different times. *Vertical bars* indicate ± SD. One-way and two-way ANOVA with the Duncan post hoc test was used to determine significant differences. Mean values with *different letters* (**a**–**f**) are significantly different ($P < 0.05$)

α-Amylase, β-amylase and dehydrogenases activities in caryopses imbibed in KAR_1 or GA_3 solutions

Activity of α-amylase did not change during imbibition of dormant caryopses up to 29 h (Fig. 5a). Both KAR_1 and GA_3 increased activities of α-amylase. KAR_1 started to

Table 1 The effect of KAR_1 or GA_3 on the Timson's index (%) in *Avena fatua* caryopses at 20 °C after previous dry storage of florets at 25 °C for different times

Compound (M)		Dry storage (weeks)				
		0	2	4	8	12
KAR_1	0	53.3 ± 19.7a	53.3 ± 14.1a	152.0 ± 14.4a	309.3 ± 26.0a	393.3 ± 6.1a
	10^{-10}	82.7 ± 12.9ab	129.3 ± 16.2b	214.7 ± 26.6b	321.3 ± 25.7a	412.0 ± 6.9ab
	3×10^{-10}	114.7 ± 15.1b	160.0 ± 14.4b	297.3 ± 26.6cd	388.0 ± 21.2bc	426.7 ± 16.2ab
	10^{-9}	204.0 ± 12.0c	253.3 ± 14.1c	357.3 ± 20.5d	414.7 ± 20.5c	462.7 ± 18.9b
	10^{-8}	290.7 ± 12.9d	350.7 ± 16.2d	400.0 ± 10.6e	437.3 ± 12.2c	480.0 ± 12.0b
GA_3	10^{-8}	82.7 ± 12.9ab	74.7 ± 10.1a	158.7 ± 20.1a	334.7 ± 20.1a	433.3 ± 4.6b
	10^{-7}	112.0 ± 4.0b	160.0 ± 14.4b	204.0 ± 18.3b	332.0 ± 22.3a	429.3 ± 12.2b
	10^{-6}	157.3 ± 10.1c	214.7 ± 19.7c	309.3 ± 11.6c	400.0 ± 10.6b	456.0 ± 8.0d
	10^{-5}	326.7 ± 14.Id	370.7 ± 9.2d	428.0 ± 6.9d	456.0 ± 14.4c	481.3 ± 10.1d

Vertical bars indicate ± SD. One-way and 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 ($P < 0.05$)

Table 2 The germination after 5 days of *Avena fatua* caryopses at 20 °C, preincubated at 4 °C for different periods in the presence of KAR1 (3×10^{-9} M) or GA3 (10^{-5} M)

Compound (M)	Preincubation (h)		
	6	12	24
Germination (%)			
Water	8.0 ± 4.0a	8.0 ± 4.0a	9.3 ± 2.3a
KAR_1, 3×10^{-9}	25.3 ± 4.6b	81.3 ± 4.6d	85.3 ± 4.6d
GA_3, 10^{-5}	42.7 ± 6.1c	85.3 ± 4.6d	82.7 ± 2.3d

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 ($P < 0.05$)

Table 3 The effect of KAR_1 (10^{-8} M) or GA_3 (10^{-5} M) on the germination of *Avena fatua* caryopses in the absence or presence of flurprimidol (10^{-5}, 10^{-4} M) at 20 °C

Compound (M)	Days	Flurprimidol (M)		
		0	10^{-5}	10^{-4}
Water	2	6.7 ± 6.1a	0.0 ± 0.0a	0.0 ± 0.0a
	3	12.0 ± 6.9ab	5.3 ± 2.3a	0.0 ± 0.0a
	5	16.0 ± 4.0bc	13.3 ± 2.3bc	2.7 ± 2.3a
KAR_1, 10^{-8}	2	52.0 ± 6.9fg	22.7 ± 6.1cd	6.7 ± 2.3a
	3	77.3 ± 6.1hij	41.3 ± 9.2ef	17.3 ± 4.6bc
	5	85.3 ± 6.1kl	41.3 ± 9.2ef	17.3 ± 4.6bc
GA_3, 10^{-5}	2	74.7 ± 2.3hi	70.7 ± 2.3hi	49.3 ± 2.3fg
	3	81.3 ± 2.3jk	77.3 ± 2.3hij	77.3 ± 6.1hij
	5	90.7 ± 6.1kl	88.0 ± 0.0kl	86.7 ± 4.6jkl

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–l) are significantly different ($P < 0.05$)

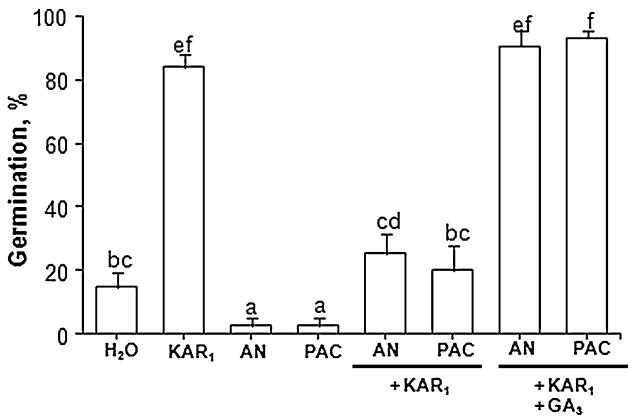

Fig. 3 The effect of KAR_1 (10^{-8} M) in the presence of ancymidol (10^{-4} M) or paclobutrazol (10^{-4} M) with or without GA_3 (10^{-5} M) on the germination of *Avena fatua* caryopses at 20 °C after 5 days. *Vertical bars* indicate ± SD. Two-way ANOVA with the Duncan post hoc test was used to determine significant differences. Mean values with *different letters* (**a**–**f**) are significantly different ($P < 0.05$)

increase activity of this enzyme after 20 h of imbibition and highest activity was measured after 29 h (approximately 2 h before germination); activity of this enzyme was increased about threefold. In the presence of GA_3 activity of α-amylase increased from 16 h of imbibition reaching the highest level in non-germinated caryopses after 26 h (approximately 2 h before start of germination); four times higher than in the controls. Ancymidol decreased the activity of α-amylase when used in the presence of KAR_1. GA_3 reversed this inhibition (Table 4). Activity of β-amylase was increased to a similar level by both compounds, but only 20 % in comparison to control (Fig. 5b). Activities of total dehydrogenase were on a par during imbibition of dormant caryopses (Table 5). As in the case of α-amylase activity, both KAR_1 and GA_3 increased activities of dehydrogenases. The highest effect on activity was found before coleorhiza protrusion.

Discussion

Dormant *Avena fatua* florets were not able to germinate at 5–35 °C (Fig. 1). Germination of dormant *A. fatua* L.

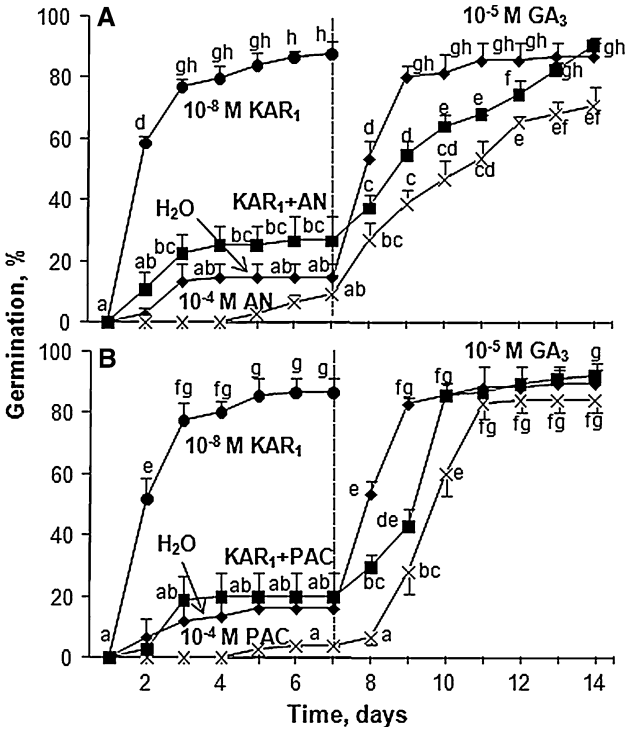

Fig. 4 The effect of GA_3 (10^{-5} M) on the germination at 20 °C *Avena fatua* caryopses transferred after 7 days of imbibition in water, ancymidol (10^{-4} M) (**a**) or paclobutrazol (10^{-4} M) (**b**) alone or combined with KAR_1 (10^{-8} M). *Vertical bars* indicate ± SD. Two-way ANOVA with the Duncan post hoc test was used to determine significant differences. Mean values with *different letters* (**a**–**h**) are significantly different ($P < 0.05$)

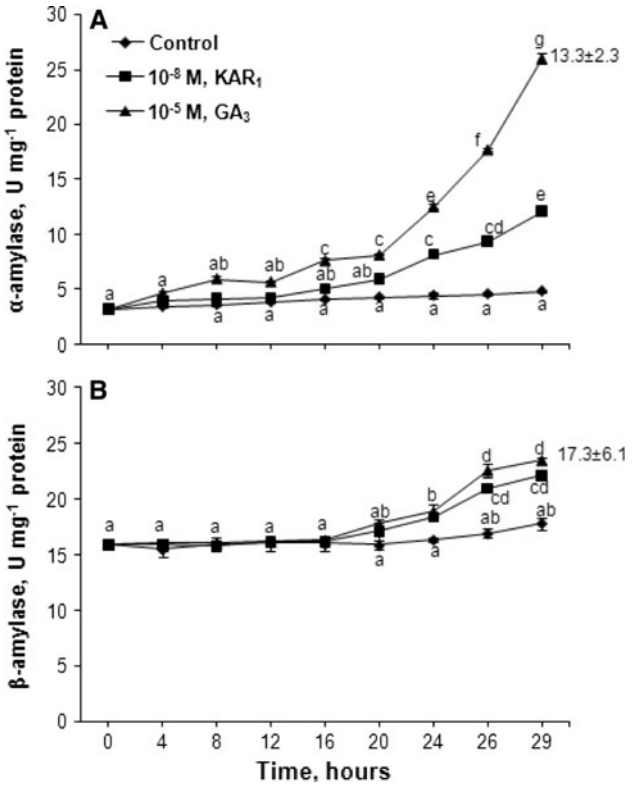

Fig. 5 The effect of KAR_1 (10^{-8} M) or GA_3 (10^{-5} M) on the α-amylase (**a**) and β-amylase (**b**) activities in *Avena fatua* caryopses after different periods of incubation at 20 °C. After 29 h of incubation in the presence of GA_3 caryopses began to germinate (percentage of germination shown in figure). 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 ($P < 0.05$)

Table 4 The effect of KAR_1 (10^{-8} M) or GA_3 (10^{-5} M) on the α-amylase activity in *Avena fatua* caryopses after 26 h incubation in the absence or presence of ancymidol (10^{-4} M) at 20 °C

Treatment	α-Amylase (U mg^{-1} protein)
Control	4.6 ± 0.1a
KAR_1, 10^{-8} M	9.4 ± 0.1c
AN, 10^{-4} M	3.9 ± 0.0a
KAR_1 + AN	6.3 ± 0.1b
GA_3, 10^{-5} M	17.7 ± 0.1e
GA_3 + AN	14.7 ± 0.2d
GA_3 + KAR_1	21.7 ± 0.2f
KAR_1 + AN + GA_3	18.0 ± 0.5e

One-way ANOVA with the Duncan post hoc test was used to determine significant differences. Mean values with different letters (a–f) are significantly different ($P < 0.05$)

Table 5 The effect of KAR_1 (10^{-8} M) or GA_3 (10^{-5} M) on the total dehydrogenases activity in *Avena fatua* L. caryopses after different periods of incubation at 20 °C. Dehydrogenases activity was expressed as μg formazan mg^{-1} protein

Compound (M)	Time (h)		
	22	26	29
Water	15.5 ± 0.1a	15.9 ± 0.5a	15.7 ± 0.2a
KAR_1, 10^{-8}	16.2 ± 0.4a	20.0 ± 0.4b	33.9 ± 1.1d
GA_3, 10^{-5}	19.3 ± 0.6b	28.9 ± 0.4c	–

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 ($P < 0.05$)

caryopses reached 50 % only at 10 and 15 °C. Lower and higher temperatures were unsuitable for germination. The inability of *A. fatua* L. florets and caryopses to germinate at temperatures corresponds to its dormancy state. Florets were completely dormant at all tested temperatures but caryopses were partially dormant at 10 and 15 °C and dormant at other temperatures. Previously it was reported that the optimum temperature for germination of *A. fatua* L. dormant caryopses was 4–12 °C (Naylor and Fedec 1978). An inverse relationship between caryopses germination and temperature, in studies using crosses between parents with high and low levels of dormancy in *A. fatua* was shown previously (Fennimore et al. 1998). Difference in germination of florets and caryopses at 10 and 15 °C may indicate different mechanisms of dormancy probably associated with the presence or absence of the lemma and palea. In *A. fatua*, several dormancy mechanisms may be exhibited with at least one expressed via the glumes, at least one by the testa and pericarp and in some biotypes, a physiological dormancy in the embryo (Adkins et al. 1988). Both KAR_1 and GA_3 partially (up to 50 %) stimulated germination of dormant florets in the 10–30 °C range (Fig. 1a, b). KAR_1 was found to be more active since a low concentration (10^{-8} M) was sufficient in comparison to high (10^{-4} M) GA_3 to obtain a comparable response. Thus, florets were about ten thousand times more sensitive to KAR_1 than GA_3. There are other examples that seeds of several plant species which respond to GA_3 are also sensitive to KAR_1 (Merritt et al. 2006; Daws et al. 2007; Stevens et al. 2007). As in our experiments, these seeds required a higher concentration of GA_3 than KAR_1 for germination. Dormant caryopses were more sensitive to KAR_1 and GA_3 (Fig. 1c, d) than dormant florets probably due to different level of dormancy. Continuous application of KAR_1 or GA_3 was not necessary since 12 h imbibition at 4 °C in the presence of these regulators was sufficient to complete germination (Table 2). The preincubation experiments also indicated higher sensitivity of caryopses to KAR_1 than to GA_3. Previously, it was also demonstrated that KAR_1 (Daws et al. 2007; Kępczyński et al. 2010; Kępczyński and Van Staden 2012; Long et al. 2011; Stevens et al. 2007) and GA_3 (Adkins et al. 1986; Daws et al.

2007; Fennimore and Foley 1998; Kępczyński et al. 2006; Stevens et al. 2007) stimulated germination of dormant and partially dormant *A. fatua* caryopses.

Dry storage (after-ripening), is commonly used to remove seed dormancy in many plant species, including *A. fatua* caryopses. After-ripening of *A. fatua* florets at 25 °C gradually removed dormancy and after 12 weeks all caryopses were able to germinate at 20 °C (Fig. 2). Dormancy in caryopses of this species was also released by dry storage at 20–40 °C (Foley 1994). Application of KAR_1 and GA_3, at suboptimal concentrations to caryopses, after different periods of dry storage (Fig. 2a, b), indicated that the requirement for these regulators decreased with after-ripening. Thus, after-ripening of *A. fatua* caryopses, as in the case of *Arabidopsis thaliana* (Karssen et al. 1989) and seeds of many other species (Kucera et al. 2005), increased sensitivity to GA_3 and also to KAR_1. Similarly as in previous experiments caryopses were more sensitive to KAR_1 than to GA_3. Both KAR_1 and GA_3 (Fig. 2) increased the rate of germination of caryopses from florets stored dry for various periods (Table 1). Non-dormant (after-ripened) caryopses germinated faster in the presence of KAR_1 or GA_3 indicating that non-dormant caryopses are also sensitive to these regulators. Thus KAR_1 cannot only break dormancy, but also stimulates the germination process after releasing dormancy by dry storage.

In order to determine if the action of KAR_1 involves endogenous gibberellins, the inhibitors of GA_3 biosynthesis, ancymidol, paclobutrazol or flurprimidol, were applied. These inhibitors were not toxic since no change in the stimulatory effect of GA_3 on germination of dormant caryopses was observed (Table 3). Moreover caryopses pre-incubated for 7 days in their presence also did not lose sensitivity to GA_3 (Fig. 4). The stimulatory effect of KAR_1 was not apparent in the presence of the inhibitors of gibberellin biosynthesis, suggesting involvement of gibberellin biosynthesis in response to this compound (Table 3; Fig. 3). GA_3 antagonized the inhibitory effect of ancymidol and paclobutrazol on caryopses germination in the presence of KAR_1. The reversal of inhibition due to above inhibitors by GA_3 may confirm that gibberellin biosynthesis is required for the stimulatory effect of KAR_1 (Fig. 3). Involvement of gibberellin biosynthetic genes in the response of *Arabidopsis* seeds to KAR_1 was demonstrated (Nelson et al. 2009). GA_4, but not KAR_1, stimulated germination of *Arabidopsis* GA-deficient mutants. KAR_1 did not enhance sensitivity to GA_4 seeds of an *Arabidopsis* GA-deficient mutant. This compound reduced the amounts of exogenous GA_3 and GA_4 required to promote germination of *Stylidium maritimum* seeds (Chiwocha et al. 2009). Prolonged pre-incubation of caryopses in the presence of inhibitors of gibberellin biosynthesis alone or in combination with KAR_1 did not change the response to gibberellin measured as final percent germination. There was, however, a decreased rate of germination (Fig. 4).

Germination of cereals and *A. fatua* caryopses is associated with reserve mobilization by hydrolytic enzymes, mainly α-amylase, synthesized in aleurone cells, participating in starch breakdown and other polymers required for embryo and seedling growth (Lovegrove and Hooley 2000). GA_3 stimulates synthesis of α-amylase in aleurone protoplasts of *A. fatua* (Smith and Hooley 2002). In the present experiments GA_3 and KAR_1 increased α-amylase and β-amylase activity during imbibition (Fig. 5). Total dehydrogenase activity, as α-amylase activity, did not change during imbibition of dormant caryopses in water (Table 5). However, both KAR_1 and GA_3 increased activity of dehydrogenases and this was lower than on α-amylase activity and appeared later. Previously, it was suggested that the pentose phosphate pathway might play a role in the metabolic regulation of germination and dormancy (Roberts and Smith 1977). Activity of glucose-6-phosphate dehydrogenase, a rate limiting enzyme of the pentose phosphate pathway, was increased in dormant *A. fatua* embryos after treatment with GA_3 (Gahan et al. 1986). Taken together with the obtained data, KAR_1 and gibberellin could at first stimulates degradation of starch and later utilization of glucose via the pentose phosphate pathway. The stimulatory effect of KAR_1 on α-amylase activity was decreased by ancymidol and was reversed by GA_3 showing a similar relationship when germination is considered (Fig. 3; Table 4).

To summarize, KAR_1 was found to be a more efficient stimulator of germination of dormant florets and caryopses than GA_3. Dormant *A. fatua* florets required higher concentrations of KAR_1 or GA_3 for germination than dormant caryopses possibly due to the presence of lemma and palea. Releasing dormancy by dry storage increased sensitivity to both regulators. KAR_1 and GA_3 also increased the rate of germination of after-ripened non-dormant caryopses. Gibberellin biosynthesis is probably indispensable for the stimulatory effect of KAR_1 on germination of dormant caryopses. KAR_1 and GA_3 increased α-amylase and dehydrogenase activities during imbibition of caryopses. Thus, probably preparation of dormant caryopses for germination and seedling growth by KAR_1 and GA_3 involves increasing metabolic activity via stimulation of starch breakdown amylases and respiratory enzymes.

Author contribution JK designed research, analyzed data and wrote the paper. DC conducted experiments and statistical analysis, JvS supplied KAR_1, corrected english.

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References

Adkins SW, Peters NCB (2001) Smoke derived from burnt vegetation stimulates germination of arable weeds. Seed Sci Res 11:213–222

Adkins SW, Loewen M, Symons SJ (1986) Variations within pure lines of wild oat (*Avena fatua* L.) in relation to degree of primary dormancy. Weed Sci 34:859–864

Adkins SW, Symons SJ, Simpson GM (1988) The physiological basis of seed dormancy in *Avena fatua*. VIII Action of malonic acid. Physiol Plant 72:477–482

Bernfeld P (1955) Amylases, α and β. Methods Enzymol 1:149–158

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Bradow JM, Connick WJ, Pepperman AB, Wartelle LH (1990) Germination stimulation in wild oats (*Avena fatua* L.) by synthetic strigol analogs and gibberellic acid. J Plant Growth Regul 9:35–41

Chiwocha SDS, Dixon KW, Flematti GR, Ghisalberti EL, Merritt DJ, Nelson DC, Riseborough JAM, Smith SM, Stevens J (2009) Karrikins: a new family of plant growth regulators in smoke. Plant Sci 177:252–256

Daws MI, Davies J, Pritchard HW, Brown NAC, Van Staden J (2007) Butenolide from plant-derived smoke enhances germination and seedling growth of arable weed species. Plant Growth Regul 51:73–82

Fennimore SA, Foley ME (1998) Genetic and physiological evidence for the role of gibberellic acid in the germination of dormant *Avena fatua* caryopses. J Exp Bot 49:89–94

Fennimore SA, Nyquist WE, Shaner GE, Myers SP, Foley M (1998) Temperature response in wild oat (*Avena fatua* L.) generations segregating for seed dormancy. Heredity 81:674–682

Flematti GR, Ghisalberti EL, Dixon KW, Trengove RD (2004) A compound from smoke that promotes seed germination. Science 305:977

Foley ME (1994) Temperature and water status of seed affect after-ripening in wild oat (*Avena fatua*). Weed Sci 42:200–204

Gahan PA, Dawson AL, Black M, Chapman JM (1986) Localization of glucose-6-phosphate dehydrogenase activity in caryopses and its possible involvement in dormancy breakage. Ann Bot 57:791–799

Hsiao AI, Quick WA (1985) Wild oats (*Avenu fatua* L.) seed dormancy as influenced by sodium hypochlorite, moist storage and gibberellin A_3. Weed Res 25:281–288

Karssen CM, Zagórski S, Kępczyński J, Groot SPC (1989) Key role for endogenous gibberellins in the control of seed germination. Ann Bot 63:71–80

Kępczyńska E, Piękna-Grochala J, Kępczyński J (2003) Effects of matriconditioning on onion seed germination, seedling emergence and associated physical and metabolic events. Plant Growth Regul 41:269–278

Kępczyński J, Van Staden J (2012) Interaction of karrikinolide and ethylene in controlling germination of dormant *Avena fatua* L. caryopses. Plant Growth Regul 67:185–190

Kępczyński J, Białecka B, Light ME, Van Staden J (2006) Regulation of *Avena fatua* seed germination by smoke solution, gibberellin A_3 and ethylene. Plant Growth Regul 49:9–16

Kępczyński J, Cembrowska D, Van Staden J (2010) Releasing primary dormancy in *Avena fatua* L. caryopses by smoke-derived butenolide. Plant Growth Regul 62:85–91

Kucera B, Cohn MA, Leubner-Metzger G (2005) Plant hormone interactions during seed dormancy release and germination. Seed Sci Res 15:281–307

Li B, Foley ME (1995) Cloning and characterization of differentially expressed genes in imbibed dormant and after-ripened *Avena fatua* embryos. Plant Mol Biol 29:823–831

Light ME, Daws MI, Van Staden J (2009) Smoke-derived butenolide: towards understanding its biological effects. S Afr J Bot 75:1–7

Long RL, Stevens JC, Griffiths EM, Adamek M, Powles SB, Merritt DJ (2011) Detecting karrikinolide responses in seeds of the *Poaceae*. Aust J Bot 59:610–620

Lovegrove A, Hooley R (2000) Gibberellin and abscisic acid signalling in aleurone. Trends Plant Sci 5:102–110

Maga JA (1988) Smoke in food processing. CRC Press, Boca Raton, pp 1–160. ISBN: 0-8493-5155-3

Merritt DJ, Kristiansen M, Flematti GR, Turner SR, Ghisalberti EL, Trengove RD, Dixon KW (2006) Effects of a butenolide present in smoke on light-mediated germination of Australian *Asteraceae*. Seed Sci Res 16:29–35

Naylor JM, Fedec P (1978) Dormancy studies in seed of *Avena fatua*. 8. Genetic diversity affecting responses to temperature. Can J Bot 66:2224–2229

Nelson DC, Riseborough JA, Flematti GR, Stevens J, Ghisalberti EL, Dixon KW, Smith SM (2009) Karrikins discovered in smoke trigger *Arabidopsis* seed germination by a mechanism requiring gibberellic acid synthesis and light. Plant Physiol 149:863–873

Roberts EH, Smith RD (1977) Dormancy and the pentose phosphate pathway. In: Khan AA (ed) The physiology and biochemistry of seed dormancy and germination. Elsevier/North Holland Biomedical Press, Amsterdam, pp 385–411

Simpson GM (2007) Seed dormancy in grasses. Cambridge University Press, Cambridge

Smith SJ, Hooley R (2002) An increase in the speed of response, and sensitivity, of *Avena fatua* aleurone layers and protoplasts to gibberellins. J Plant Physiol 159:355–360

Stevens JC, Merritt DJ, Flematti GR, Ghisalberti EL, Dixon KW (2007) Seed germination of agricultural weeds is promoted by the butenolide 3-methyl-2*H*-furo[2, 3-c]pyran-2-one under laboratory and field conditions. Plant Soil 298:113–124

Thomas TH, Van Staden J (1995) Dormancy break of celery (*Apium graveolens* L.) caryopses by plant derived smoke extract. Plant Growth Regul 17:195–198

Timson J (1965) New method of recording germination data. Nature 207:216–217

Van Staden J, Brown NAC, Jäger AK, Johnson TA (2000) Smoke as a germination cue. Plant Species Biol 15:167–178

Van Staden J, Jäger AK, Light ME, Burger BV (2004) Isolation of the major germination cue from plant-derived smoke. S Afr J Bot 70:654–659