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ORIGINAL ARTICLE

Plant-derived smoke induced activity of amylases, DNA replication and β-tubulin accumulation before radicle protrusion of dormant *Avena fatua* L. caryopses

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Abstract Florets of *Avena fatua* (wild oat) did not germinate at temperatures from 5 to 35 °C, nor did caryopses at 5 and above 15 °C. Smoke–water (SW) was found to partly or substantially induce dormant florets and caryopses to germinate at temperatures from 10 to 25 °C, whereby almost all caryopses germinated at 15 and 20 °C. When florets were dry-stored at 25 °C for 4 months, their dormancy, and that of florets was markedly or completely, respectively, removed at 20 °C. Floret and caryopsis SW demand decreased with time of dry storage. Paclobutrazol, a gibberellin biosynthesis inhibitor, completely antagonized the stimulatory effect of SW on germination of caryopses. Gibberellic acid (GA_3) reversed inhibition caused by paclobutrazol applied alone or in combination with SW. SW enhanced α- and β-amylase activities in caryopses before radicle protrusion. SW increased α-amylase activity and reduced starch content more effectively in intact caryopses than in embryoless ones. SW enhanced β-tubulin accumulation and the transition from G_1 to S and also from S to G_2 phases before radicle protrusion. The results presented indicate that florets are more dormant than caryopses and less sensitive to SW at incubation temperatures from 20 °C. Germination induction of dormant *Avena fatua* caryopses by SW required gibberellin biosynthesis. SW induction of dormant caryopsis germination involves mobilization of starch and cell cycle activation.

Keywords *Avena fatua* · Caryopses · Florets · Germination · Plant-derived smoke · Primary dormancy

Introduction

Fire plays an important role in germination and growth of plants in many parts of the world. In 1990 De Lange and Boucher (1990), two South African researchers, identified smoke as the key agent in stimulating germination of a threatened fynbos species *Audouinia capitata*. The smoke derived from burning plants and an aqueous smoke extract are known to stimulate the release of seed dormancy and germination of 1335 species, such as arable weeds and crop plants, from 120 families in fire- and non-fire-prone ecosystems (Jefferson et al. 2014). Little is known about the influence of smoke–water (SW) on the physiology of weed seeds. We used caryopses and florets of *Avena fatua*, an important weed distributed worldwide, as a model on which to study dormancy to better understand the state of dormancy and to develop new strategies for controlling the weed. Dormant caryopses can be induced to germinate by a variety of factors, e.g. dry storage (Foley 1994; Kępczyński et al. 2013), gibberellins (Adkins et al. 1986; Kępczyński et al. 2006, 2013), plant-derived smoke (Adkins and Peters 2001; Kępczyński et al. 2006) and karrikinolide (3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, KAR_1) (Daws et al. 2007; Stevens et al. 2007; Kępczyński et al. 2010, 2013), a compound identified in smoke (Flematti et al. 2004; Van Staden et al. 2004).

KAR_1 was demonstrated to require ethylene action (Kępczyński and Van Staden 2012) and gibberellin biosynthesis to stimulate germination of *A. fatua* dormant caryopses (Kępczyński et al. 2013). KAR_1-mediated *Avena fatua* caryopsis dormancy release is associated with control of the

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abscisic acid content, cell cycle, metabolic activity and homeostasis between reactive oxygen species and antioxidants in the embryo (Kępczyński et al. 2013; Cembrowska-Lech et al. 2015; Cembrowska-Lech and Kępczyński 2016). Germination of seeds of some plant species may be stimulated by SW and not by KAR_1; however KAR_1 but not SW stimulates germination in others (Stevens et al. 2007; Downes et al. 2010). There are also species seed germination of which was enhanced by KAR_1 and inhibited by smoke. The stimulatory effect of smoke, although mainly attributed to KAR_1, can be also related to other karrikins, e.g. KAR_2 and KAR_3 and/or glyceronitrile, found in smoke, which act as germination stimulators (Flematti et al. 2009, 2011). Inhibitory effect of smoke or an effect lower than that produced by KAR_1 can be associated with the presence of some inhibitors, e.g. 3,4,5-trimethylfuran-2(5*H*)-one in smoke (Pošta et al. 2013).

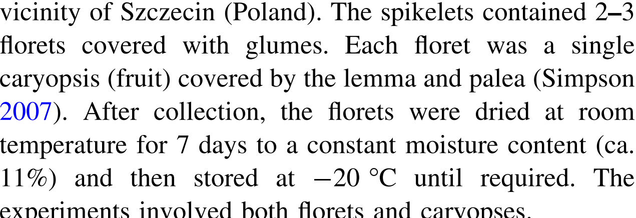
In contrast to the knowledge on KAR_1 involvement in dormancy release, that of smoke is much less known. There are no data with which to compare the response of dry-stored *A. fatua* florets, and seeds from florets which were dry-stored for different periods of time, to SW. The literature contains scant information only on the interaction between smoke and endogenous gibberellins; seeds of dicot such as *Lactuca sativa* (Van Staden et al. 1995) and *Nicotiana attenuata* (Schwachtje and Baldwin 2004) have been studied only. Moreover, effects of SW on α-amylase activity and starch degradation in intact and embryoless caryopses were not compared. Likewise, effects of SW on DNA synthesis and β-tubulin content before radicle protrusion have not been checked.

Consequently, this study was aimed to examine the role of smokein germination of dormant *Avena fatua* caryopses. To address this aim, effects of SW on germination of dormant florets and caryopses were followed (1) at various temperatures, and (2) after various periods of floret dry storage. It was also of interest to find out whether the effect of SW on germination of dormant caryopses is associated with biosynthesis of gibberellins, and whether effects on α-amylase and β-amylase activity, DNA replication and β-tubulin content appear prior to radicle protrusion through the coleorhiza. Effect of SW on α-amylase activity and starch content in caryopses and embryoless caryopses was also examined. Also, isoenzymes of α-amylase in untreated and SW-treated caryopses were analyzed.

Materials and methods

Plant material

Avena fatua L. (wild oat) spikelets were collected on July 21, 2010, during the time of their natural dispersal, in the vicinity of Szczecin (Poland). The spikelets contained 2–3 florets covered with glumes. Each floret was a single caryopsis (fruit) covered by the lemma and palea (Simpson 2007). After collection, the florets were dried at room temperature for 7 days to a constant moisture content (ca. 11%) and then stored at −20 °C until required. The experiments involved both florets and caryopses.



Preparation of smoke–water (SW)

SW was generated by burning 100 g of dry grass leaves in a 3-L metal drum. The smoke was bubbled through distilled water (100 mL) in a glass jar for 45 min (Baxter et al. 1994). This smoke extract was filtered through filter paper (Whatman No. 1) and was used as the stock solution. Different concentrations of SW were prepared by diluting the stock solution with distilled water.

Moisture content and dry weight determination

Dry weight (DW) of caryopses, 25 in three replicates, was determined gravimetrically on fully dried (105 °C for 24 h) specimens. The water content (WC) is expressed relative to the fresh weight (FW) to represent the percentage of water in the total mass, and is calculated as $WC = [(FW - DW)/FW] \times 100$.

Germination tests

Primary dormant florets or caryopses (25 in each of three replicates) were incubated in darkness in Petri dishes (ø 60 mm) on one layer of filter paper (Whatman No. 1) moistened with 1.5 mL of distilled water or various SW dilutions.

In experiment 1, florets or caryopses were incubated in the presence of distilled water or SW (1:10,000, 1:1000, 1:500, 1:100 v/v) in darkness at 5, 10, 15, 20, 25, 30 and 35 °C for 5 days.

In experiment 2, dormant florets were stored dry in the open air under ambient relative humidity for up to 4 months in darkness at 25 °C to break their dormancy. After 2, 4, 8 and 12 weeks of dry storage, both florets and caryopses (from dry-stored florets) were incubated in darkness at 20 °C in the presence of SW (1:50,000, 1:30,000, 1:10,000, 1:1000 v/v) for 5 days.

In experiment 3, dormant caryopses were preincubated in the presence of SW (1:1000 v/v) alone or in combination with paclobutrazol (PAC) (10^{-4} M) at 20 °C in darkness for 5 days. Thereafter, the caryopses were rinsed once with 100 mL of distilled water and transferred to new Petri dishes containing GA_3 (10^{-5} M) solution. The caryopses were incubated in the same condition for 5 days.

In all the experiments, counting was performed every day up to day 5 of germination. All manipulations were

performed under a green safe light at 0.5 μmol m^{-2} s^{-1} which did not affect germination. Florets and caryopses were regarded as germinated when the radicle protruded through the coleorhiza. In experiments 1 and 2, Timson's index was calculated by summing the progressive total of daily cumulative germination percentage over 5 days (Timson 1965).

α-Amylase (EC 3.2.1.1) activity

The activity of α-amylase was analyzed as described by Black et al. (1996). After incubation of intact caryopses for 24, 26 or 28 h or embryoless caryopses, 25 in five replicates, for 28 h in darkness at 20 °C in the presence of distilled water or SW (1:1000 v/v), the caryopses were ground using a pre-chilled mortar and pestle in ice-cold extraction buffer (fresh weight: extraction buffer, 1:10, w/v). All the extraction steps were performed at 4 °C. The extraction buffer contained 20 mM Tris–maleate, pH 6.2, with 1.0 mM $CaCl_2$. Barley malt α-amylase was used for constructing the calibration curve. Results were expressed as U mg^{-1} protein. One unit (U) was equivalent to the amount of enzyme liberating 1 mg of maltose from starch at 37 °C and pH 6.2.

For the analysis of α-amylase isoenzymes activity, total proteins (50 μg) extracted from untreated and treated caryopses, were separated on 7% native-PAGE gel containing 0.3% (w/v) starch in the Laemmli (1970) buffer system without sodium dodecyl sulfate (SDS). The gels were incubated overnight at 35 °C in 50 mM sodium acetate (pH 5.6) containing 1 M $CaCl_2$. α-Amylase isoenzymes activity was examined by staining the gel with I_2/KI solution.

β-Amylase (EC 3.2.1.2) activity

β-Amylase activity was measured according to Bernfeld (1955). Caryopses, 25 in five replicates, incubated in the presence of distilled water or SW (1:000 v/v) for 24, 26 or 28 h in darkness at 20 °C were homogenized in 1.0 ml of ice-cold 16 mM sodium acetate buffer, pH 4.8 (fresh weight: extraction buffer, 1:40, w/v). All the extraction steps were performed at 4 °C. The results were expressed as U mg^{-1} protein. One unit (U) is defined as the amount of enzyme liberating 1 mg of maltose from starch in 5 min at 37 °C and pH 4.8.

Determination of starch content

Total starch content in intact or embryoless caryopses, 25 in five replicates, incubated in the presence of distilled water or SW (1:000 v/v) for 28 h in darkness at 20 °C was estimated using the Total Starch Assay Kit (Megazyme International Ireland Ltd.) according to manufacturer's protocol.

Protein assay

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

Determination of nuclear DNA and β-tubulin contents

The nuclear DNA contents in radicle with coleorhiza (RC) were determined using flow cytometry. For cell cycle activity determination, caryopses, 25 in 5 replicates, were incubated at 20 °C for 0, 24, 26 and 28 h in the dark in distilled water or SW (1:1000 v/v). Twenty-five RCs were isolated from the imbibed caryopses and, using a razor blade, were chopped and placed in 2 ml of a nucleus isolation buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% Triton X-100 and 2 μg/mL DAPI) (Galbraith et al. 1983) for 2 min, following which they were incubated for 10 min at 25 °C. Subsequently, the suspension was passed through a 20-μm nylon mesh. The DAPI-stained nuclei were analyzed using a Partec PAII flow cytometer (Partec). The populations of 2C and 4C nuclei were measured on 10,000 nuclei.

The extraction and detection of β-tubulin by Western blotting was conducted following de Castro et al. (1995). Proteins were extracted from radicles with coleorhiza cell tips (50) isolated from caryopses after imbibition for various periods of time in distilled water or SW (1:1000 v/v) for 24, 26 or 28 h in darkness at 20 °C. Samples containing 50 μg of protein were separated on 15% SDS-PAGE gel following Laemmli (1970). Pure bovine brain tubulin (Cytoskeleton, Inc.) was loaded as a control in amounts of 10 and 30 ng (molecular mass ~50 kDa). After electrophoresis, the gels were electroblotted onto PVDF membranes (Millipore). The blotting membranes were probed with the mouse monoclonal anti-β-tubulin antibody (clone KMX-1) (Millipore). The data were expressed as immunoblot band visualization and densitometry analysis of immunoblot β-tubulin (ng). Band intensities were determined using the Fiji ImageJ software.

Statistical analysis

The mean ± standard deviation (SD) values of three or five replicates are shown. The means were also analyzed for significance using one-way or two-way analysis of variance, ANOVA (Statistica for Windows v. 10.0, StatSoft Inc., Tulsa, OK, USA). Duncan's multiple range test was used to test for significance of differences ($P \leq 0.05$)

for germination percentage and biochemical assay results for *A. fatua* caryopses.

Results

Effects of SW on the uptake of water and germination of caryopses

Uptake of water by dormant caryopses kept in water or exposed to SW was increasing for 12 h of incubation (Fig. 1). This period is regarded as the first phase of imbibition. At the second phase, which started after 12 h, the water content was observed to increase somewhat in the caryopses both kept in water and exposed to SW. Beginning from hour 20, the percentage of ruptures increased with incubation time in SW-treated caryopses. As of hour 28, SW resulted in increased protrusion of coleorhiza through the covers. After the subsequent 2 h, the beginning of the third phase was marked by the appearance of caryopses with coleorhiza ruptured by the radicle. During this phase, the water uptake was observed to increase, which was associated with a progressively growing protrusion rate of coleorhiza and radicles; after 48 h, about 80 and 40% caryopses were with coleorhiza and radicle, respectively. The third phase was observed only in the treated caryopses. After 48 h, the untreated caryopses somewhat increased their water uptake; as little as 10 and 5% of the caryopses featured coat break-down and coleorhiza protrusion, respectively. Extended incubation increased the proportion of untreated caryopses with protruding radicle only up to about 20%. After 96 h of incubation in SW, almost all the caryopses showed radicles protruding through the coleorhiza. Such caryopses were regarded as germinated.

Effects of SW on germination of florets and caryopses at different temperatures

Freshly harvested florets were dormant and did not germinate in the dark at temperatures from 5 to 35 °C (Fig. 2a). SW at dilution of 1:10,000 (v/v) did not affect germination at any of the temperatures tested. SW at dilutions of 1:1000, 1:500 or 1:100 (v/v) markedly

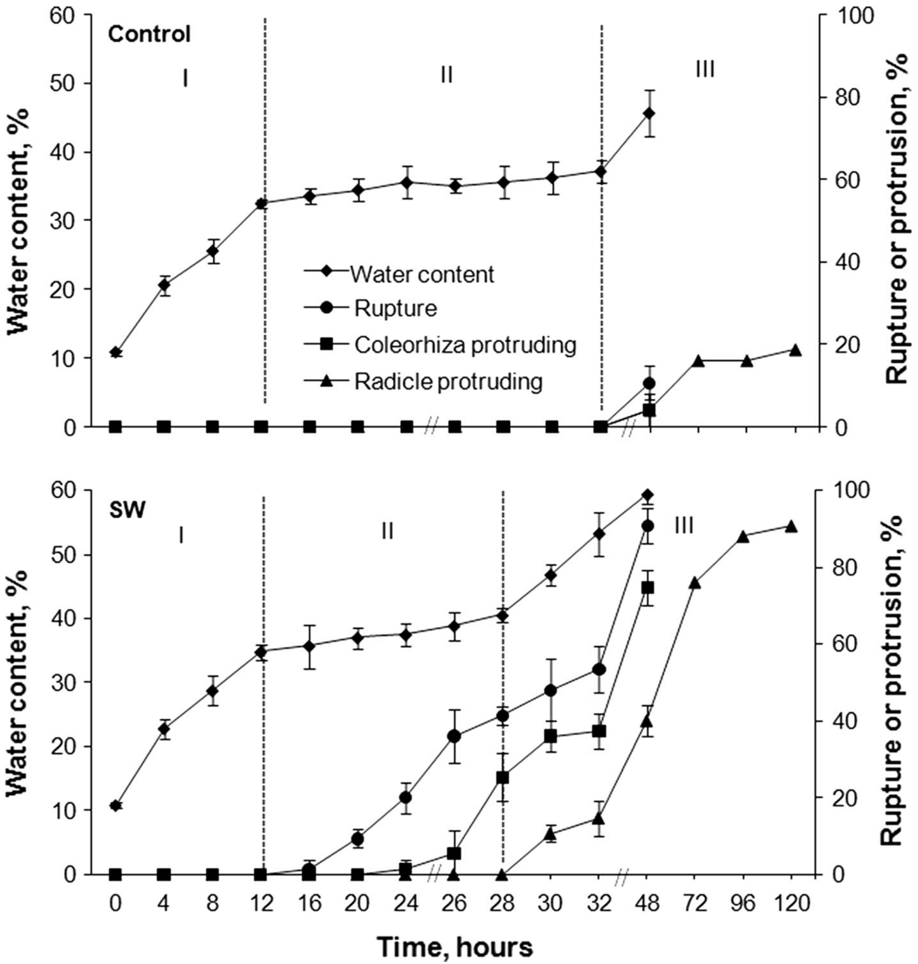


Fig. 1 Effects of smoke–water (1:1000 v/v) on the water content, coat rupture, coleorhiza protrusion and radicle protrusion in *Avena fatua* caryopses during imbibition at 20 °C for various times. *Vertical bars* indicate ±SD

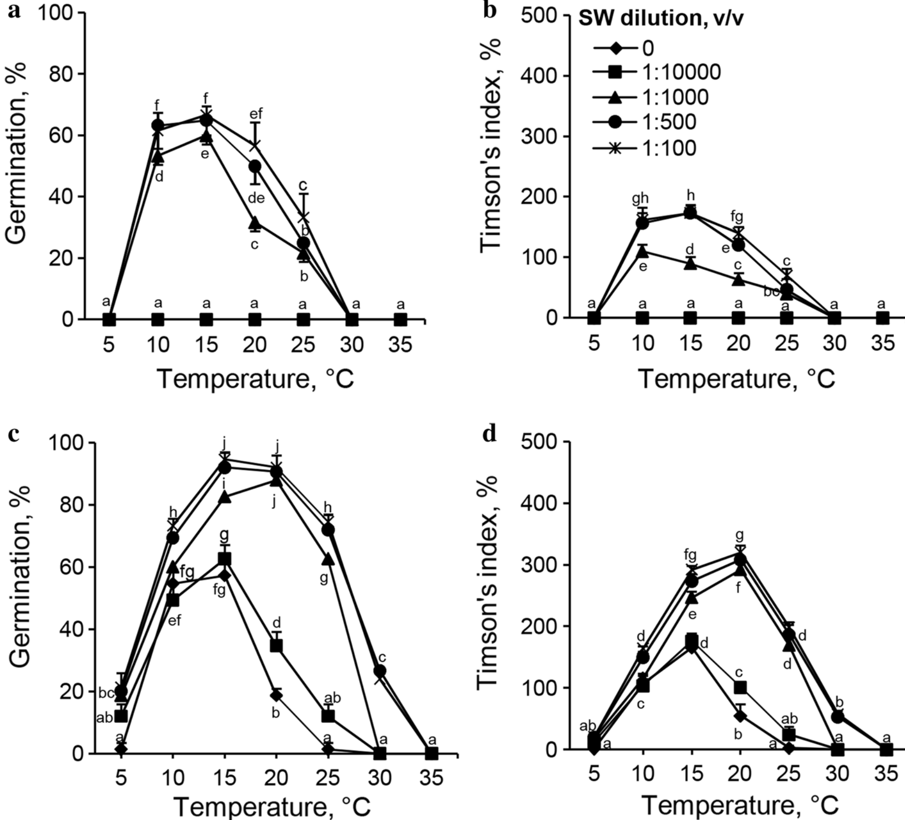


Fig. 2 Effects of SW on the germination (**a**, **c**) and Timson's index (%) (**b**, **d**) in *A. fatua* florets (**a**, **b**) or caryopses (**c**, **d**) after 5 days of incubation at different temperatures. *Vertical bars* indicate ± SD. Two-way ANOVA with the Duncan's post hoc test on arcsine (×/10)-transformed data was used to determine the significance of differences. Mean values with *different letters* (*a–j*) are significantly different ($P < 0.05$)

stimulated germination and 53–67% of florets were germinated at 10 and 15 °C after 5 days (Fig. 2a). At 20 °C, SW was most effective at dilutions of 1:500 and 1:100 (v/v); 50 and 57% of florets, respectively, being germinated. The SW-induced germination extent at 25 °C varied between 20 and 33%. SW applied at all the dilutions did not affect germination at temperatures above 25 °C. Not only the percentage of germinated florets, but also Timson's index, a measure of germination rate, was affected by the SW treatment. Florets treated with 1:500 and 1:100 (v/v) SW germinated faster at 10–20 °C, compared to florets imbibed in the presence of 1:1000 (v/v) SW (Fig. 2b).

Untreated caryopses did not germinate at the lowest temperature, 5 °C, but at 10 and 15 °C, about 55% of the seeds germinated (Fig. 2c). However, at 20 °C, only ca. 20% of caryopses germinated, no germination being observed at a temperature higher than 20 °C. At the highest dilution (1:10,000), SW increased germination percentage at 5, 20 and 25 °C only slightly; however, dilutions of 1:1000, 1:500 or 1:100 (v/v) induced almost complete caryopsis germination at 15 and 20 °C. The germination extent due to the presence of SW at 25 °C ranged between 63 and 72%. At 30 °C, only SW at dilutions of 1:500 and 1:100 (v/v) induced germination which amounted to 24%. SW also increased Timson's index. Caryopses treated with SW at dilutions of 1:1000, 1:500 and 1:100 (v/v) germinated faster at 15–25 °C, compared to caryopses imbibed in presence of water and 1:10,000 (v/v) SW (Fig. 2d).

Effects of SW on germination of florets and caryopses following floret dry storage

Dormant *A. fatua* florets are not able to germinate at 20 °C (Fig. 3a). Dry storage of florets at 25 °C for 2 weeks resulted in germination of 10% of florets. However, extension of the dry storage period progressively increased the germination, with 67% of the florets germinating after 12 weeks of storage. SW at the highest dilution did not affect germination of florets stored for up to 12 weeks. SW, only at its lowest dilution of 1:1000 (v/v), induced germination in only 26% of the completely dormant florets (Fig. 3a). After 2 weeks of storage, germination increased up to 23, 36 and 76% in the presence of SW at dilutions of 1:30,000, 1:10,000 and 1:1000 (v/v), respectively, compared to 10% in the control. All the florets, stored for 12 weeks, germinated in the presence of SW at the above dilutions, while about 70% of florets germinated in water. Likewise, the rate of caryopsis germination was observed

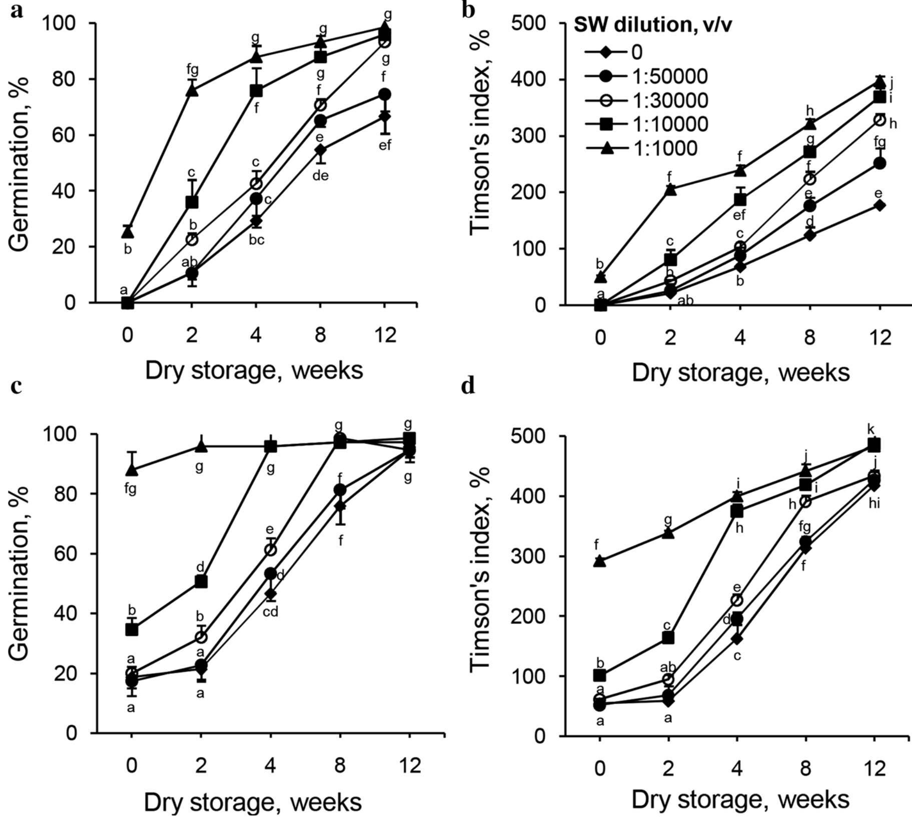


Fig. 3 Effects of SW on the germination (**a**, **c**) and Timson's index (%) (**b**, **d**) after 5 days of incubation of *A. fatua* florets (**a**, **b**) or caryopses (**c**, **d**) at 20 °C after previous dry storage of florets at 25 °C for various times. *Vertical bars* indicate ± SD. Two-way ANOVA with the Duncan's post hoc test on arcsine (×/10)-transformed data was used to determine the significance of differences. Mean values with *different letters* (*a*–*k*) are significantly different ($P < 0.05$)

to increase with the time of dry storage (Fig. 3b). The highest effect of SW on the germination rate, regardless of storage time, was found at the lowest dilution. SW at all dilutions increased Timson's index of florets stored for 8 or 12 weeks.

Dormant *A. fatua* seeds germinated poorly at 20 °C (Fig. 3c), as only ca. 20% of caryopses germinated after 5 days. Seed germination was not changed after the florets were stored dry at 25 °C for 2 weeks. However, extension of dry storage time tended to increase the germination success. After 12 weeks of storage, all the seeds could germinate. SW used at dilutions of 1:10,000 and 1:1000 (v/v) increased the germination of dormant caryopses from non-stored florets to 35 and 88%, respectively, compared to ca. 20% in the control. SW dilutions of 1:30,000 and 1:10,000 (v/v) increased the germination success after various dry storage times. After 4 and 8 weeks, all the dry-stored caryopses germinated completely in the presence of SW at dilutions of 1:30,000 and 1:10,000 (v/v), respectively. All the caryopses from florets stored for 12 weeks germinated in the presence of SW, regardless of the dilution. Like the caryopsis germination success, Timson's index was dependent on SW dilution (Fig. 3d). The highest effect of SW occurred with the lowest dilution. SW at 1::10 000 slightly enhanced the germination rate of seeds from florets kept dry for 12 weeks.

Effects of SW and gibberellin biosynthesis inhibitor on germination of caryopses

To find out if SW stimulated caryopsis germination because of the associated synthesis of endogenous gibberellins, PAC, an inhibitor of gibberellin biosynthesis was used alone and in combination with SW. As reported in the previous experiment, dormant seeds germinated poorly at 20 °C (ca. 15%), and the treatment involving 1:1000 (v/v) SW led, after 5 days, to a germination of 97% (Table 1). PAC administered at the concentration of 10^{-4} M hampered the germination of dormant seeds. In the presence of SW, PAC distinctly interfered with the enhancing effect of SW, resulting in germination of as few as 20% of caryopses. After 5 days of preincubation in water, caryopses showed a strong response to GA_3 applied at 10^{-5} M; 5 days after the transfer from water to GA_3, the germination success was 95%. PAC-induced inhibition of the stimulatory effect of SW was completely reversed after an additional 5-day incubation in the presence of GA_3.

Table 1 Effects of GA_3 (10^{-5} M) on the germination at 20 °C of *A. fatua* caryopses transferred after 5 days of imbibition in water, SW (1:1000 v/v), PAC (10^{-4} M) or SW combined with PAC

Compound	GA_3 (M)	
	0	10^{-5}
Water	13.3 ± 2.3bc	94.7 ± 2.3d
SW 1:1000 (v/v)	93.3 ± 2.3d	97.3 ± 2.3d
PAC 10^{-4} M	5.3 ± 2.3a	92.0 ± 4.0d
SW + PAC	20.0 ± 8.0c	93.3 ± 2.3d

Vertical bars indicate ± SD. Two-way ANOVA with Duncan's post hoc test was used to identify significant differences. Means with different letters (a–d) are significantly different ($P < 0.05$)

Effects of SW on α- and β-amylase activities in caryopses

After 24-h imbibition of caryopses in water, the α-amylase activity was similar to that in dry caryopses (Table 2). The α-amylase activity was not changed when dormant seeds were kept in water for as long as 28 h. SW increased the α-amylase activity from 24 to 28 h of imbibition. The highest activity of the enzyme was observed after 28 h, 2 h before the start of radicle protrusion through the coleorhiza; the activity was about 2.4 times higher than that recorded in caryopses kept in water for the same period. The beta-amylase activity was similar throughout the time the seeds were kept in water. SW enhanced the activity to a similar level after 26 and 28 h of imbibition. After 28-h incubation in SW, the enzyme's activity was almost 1.4 times higher than that in caryopses incubated in water.

Gel staining after separation of proteins from the caryopses revealed the presence of two α-amylase isoforms, AMY-1 and AMY-2 (Table 3). The isoforms were detected in dry seeds and in the seeds kept in water or SW for 24, 26 and 28 h. AMY-1 representing dry seeds and those kept in water for 24, 26 and 28 h remained slightly active; more intense bands were related to AMY-2. Intensity of bands was independent of the incubation time in water. SW increased the intensity of the bands representing AMY-1 and AMY-2, compared to the control (Table 3). Intensity of these bands was the highest after 28h incubation.

Table 3 Effects of SW (1:1000 v/v) on α-amylase isoenzyme activities in *A. fatua* caryopses after incubation at 20 °C for various times

Treatment	Time of imbibition, h			
	0	24	26	28
Control (AMY-1, AMY-2)				
SW	-			

Effects of SW on the α-amylase activity and starch content in intact caryopses and embryoless ones incubated for 28 h were also determined (Table 4). Incubation of intact caryopses in water increased the α-amylase activity and decreased the starch content. The α-amylase activity in intact caryopses treated with SW was 3.3 times higher than that in the untreated intact caryopses. SW, too, decreased the starch content after 28 h by a factor of 2.9. Imbibition of embryoless caryopses in water tended to increase the α-amylase activity and reduced the starch content. In embryoless caryopses, SW increased the α-amylase activity and reduced the starch content by factors of 1.6 and 1.3, respectively.

Effects of SW on nuclear DNA and β-tubulin contents in radicle tip with coleorhiza

In dry dormant caryopses, 93% of cells in the radicle with coleorhiza (RC) contained 2C DNA (Table 5). Imbibition of dormant caryopses in water for 24–28 h did not affect the distribution of nuclei with different DNA contents. SW,

Table 2 Effects of SW (1:1000 v/v) on the α- and β-amylase activity in *A. fatua* caryopses incubated at 20 °C for various times

Activities	Treatment	Time of imbibition (h)			
		0	24	26	**28**
α-Amylase (U mg^{-1} protein)	Control	3.2 ± 0.1a	3.9 ± 0.9ab	4.2 ± 0.2b	4.7 ± 0.1b
	SW	–	6.0 ± 0.6c	8.0 ± 0.9d	11.1 ± 0.4e
β-Amylase (U mg^{-1} protein)	Control	15.9 ± 0.4ab	14.5 ± 1.6ab	14.8 ± 1.3ab	15.0 ± 1.0ab
	SW	–	17.1 ± 1.0bc	19.4 ± 1.5cd	20.6 ± 0.4d

One-way ANOVA with Duncan's post hoc test was used to identify significant differences. Means with different letters (a–e) are significantly different ($P < 0.05$)

Table 4 Effects of SW (1:1000 v/v) on α-amylase activity and starch content in caryopses and embryoless caryopses of *A. fatua* after incubation at 20 °C for 28 h

	Treatment	α-amylase (U mg^{-1} protein)	Starch (mg g^{-1} FW)
Intact caryopses	Dry	2.6 ± 0.2a	40.5 ± 0.5c
	Water	5.8 ± 0.2b	32.5 ± 0.4b
	SW	19.2 ± 0.4c	11.3 ± 0.5a
Embryoless caryopses	Dry	3.1 ± 0.5a	38.9 ± 0.6c
	Water	4.1 ± 1.1a	33.7 ± 0.5b
	SW	6.6 ± 0.4b	26.5 ± 0.5a

Two one-way ANOVAs with Duncan's post hoc test were performed to identify significant differences: one for α-amylase activity, and one for starch content. Means in columns with different letters (a–c) are significantly different ($P < 0.05$)

Table 5 Effects of SW (1:1000 v/v) on the nuclear DNA content in *A. fatua* radicle with coleorhiza after caryopses incubation at 20 °C for various times

Imbibition (h)	Percentage of nuclei					
	2C		S		4C	
	Control	SW	Control	SW	Control	SW
0	92.8 ± 1.0d	–	3.7 ± 0.8a	–	3.5 ± 0.4bc	–
24	92.0 ± 3.9d	82.2 ± 2.9b	5.2 ± 1.7ab	12.3 ± 3.3c	2.7 ± 0.2a	5.5 ± 1.6c
28	88.5 ± 1.5 cd	55.2 ± 2.6a	7.6 ± 1.3b	26.0 ± 1.5d	3.8 ± 1.0bc	18.8 ± 1.4d

One-way ANOVA with Duncan's post hoc test on arcsine (×/10)-transformed data was used to identify significant differences in the percentage of nuclei separately for each cell cycle phase. Means for each parameter with different letters (a–d) are significantly different ($P < 0.05$)

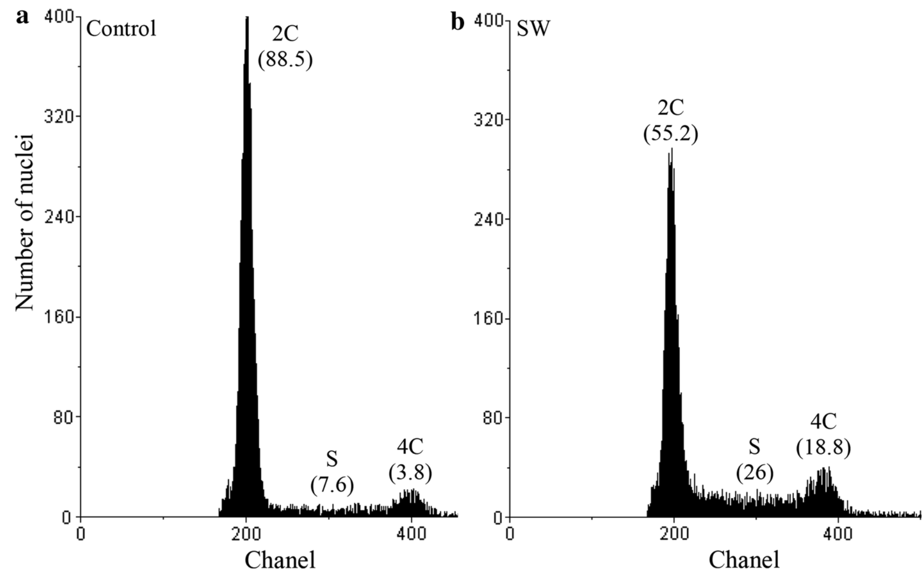


Fig. 4 Flow cytometric analysis of nuclei from radicle with coleorhiza isolated from *A. fatua* caryopses after incubation in water (**a**) or SW (1:1000 v/v) (**b**) at 20 °C for 28 h. *Number* in *parentheses* indicate the percentage of nuclei in 2C, S and 4C cell cycle phases

applied for 24 or 28 h, decreased the percentage of nuclei with 2C, but increased in phase S, and 4C DNA.

SW treatment for 28 h resulted in about 1.6-fold decrease in the percentage of nuclei with 2C DNA in RC of the caryopses, compared to RC from untreated caryopses, with the percentage of nuclei in phase S increasing by about 3.5 times (Fig. 4). The percentage of nuclei with 4C DNA in RC from SW-treated caryopses increased by a factor of 5.

Beta-tubulin, which is contained in the microtubular cytoskeleton, was not detectable in RC from dry seeds and those kept in water for 24 h (Table 6). Accumulation of β-tubulin was detected only just 28 h after imbibition in water. Beta-tubulin was detected in RC just after 24 h as a result of the exposure to SW. The signal was intensified when incubation in the presence of SW was extended to up to 28 h. The signal intensity was 7.5 times higher than that for RC of untreated caryopses.

Table 6 Effects of SW (1:1000 v/v) on the β-tubulin content in *A. fatua* radicle with coleorhiza after caryopses incubation at 20 °C for various times

β- tubulin, ng

10 30 — 55 kDa

Treatment	β-tubulin		
	Time of imbibition, h		
	0	24	28
Control	nd	nd	
	-	-	1.5 ± 0.2a
SW	-		
	-	2.7 ± 0.1b	11.3 ± 0.2c

nd-non-detected

Data are presented as immunoblot band visualization and densitometry analysis of immunoblot β-tubulin (ng). Positions of tubulin control in amounts of 10 and 30 ng (molecular mass ~55 kDa) are shown above the table. One-way ANOVA with Duncan's post hoc test was used to identify significant differences. Means with different letters (a–c) are significantly different ($P < 0.05$)

nd non-detected

Discussion

Most annual weed species in agricultural systems in temperate climate are dormant when dispersed in late spring and early summer. Dormancy can be defined simply as a seed trait that blocks the germination of viable seeds under favorable conditions (Bewley 1997). Dormancy in freshly harvested cereal grain is seldom absolute at all under any environmental condition (Rodríguez et al. 2015). In temperate cereals, dormancy is expressed strongly at relatively high temperatures, usually above 15–20 °C. Florets (caryopses covered with the lemma and palea) of *Avena fatua* after harvest did not germinate at temperatures from 5 up to 35 °C (Fig. 2a; Kępczyński et al. 2013). Like in earlier experiments (Kępczyński et al. 2013), the removal of the hulls from *A. fatua* florets resulted in germination at 10 and 15 °C (up to ca. 60%) (Fig. 2c). The results presented indicate that florets were totally dormant at all the temperatures tested, but seeds were partly dormant at 10 and 15 °C and fully dormant above 15 °C. Thus, dormancy expression in *A. fatua* caryopses, like in cereal grains (Rodríguez et al. 2015), depends on the incubation temperature. Difference in germination between florets and caryopses at 10 and 15 °C indicates difference in the depth of dormancy as well as various mechanisms of dormancy associated with the presence or absence of the hulls. The inhibitory effect of the lemma and palea on germination of *A. fatua* florets could be related to the presence of phenolic compounds which restrict the access of oxygen to the embryo. Limitation of oxygen supply to the embryo by oxygen fixation as a result of polyphenol oxidase-mediated oxidation of phenolic compounds in the lemma and palea has been suggested to be responsible for the dormancy of *Hordeum vulgare* (Lenoir et al. 1986).

SW produced from burnt dry grass leaves proved to be an effective germination stimulant in dormant florets and caryopses at temperatures from 10 to 25 °C, respectively (Fig. 2a, c). This finding is in agreement with results of Adkins and Peters (2001) who demonstrated that a commercially available SW solution (Seed Starter®) stimulated germination of both dormant florets and caryopses of *A. fatua*. Moreover, not only did SW enhance the final germination success, but it also accelerated germination of both *A. fatua* florets and caryopses (Fig. 2b, d). The data presented are also in agreement with earlier results showing that aqueous smoke extract produced from burnt *Themeda triandra* leaves also removes dormancy in *A. fatua* florets and caryopses (Kępczyński et al. 2006). The reaction of *A. fatua* florets or seeds to SW, like in the case of *T. triandra* seeds (Baxter et al. 1995), was independent of plant material used for smoke production. Seed dormancy of several monocot plant species such as *Bromus tectorum* (Allen et al. 1995), *Oryza sativa* (Doherty and Cohn 2000) and *Hordeum vulgare* (Barrero et al. 2009) can be released after dry storage. Likewise, dormancy of *A. fatua* caryopses and florets was released as a result of floret dry storage (Fig. 3). Dormancy release in *A. fatua* caryopses, as a result of dry storage of florets, has already been reported (Foley 1994). The release of dormancy in *A. fatua* caryopses due to dry storage could be associated with changes in the ABA level; the release of dormancy in *H. vulgare* caryopses by dry storage was related to a decrease of the ABA content in embryo during imbibition (Jacobsen et al. 2002). Earlier studies found dormancy breaking factors such as KAR_1 or GA_3 to reduce the ABA content in *A. fatua* embryos (Cembrowska-Lech et al. 2015). At suboptimal dilutions higher than 1:1000 v/v, responses to SW in florets (Fig. 3a) or caryopses (Fig. 3c), stored dry for different periods of time, indicated that the demand for this compound dwindled as the dry storage proceeded. Partly dormant seeds were more responsive to both SW (Fig. 3c) and KAR_1 (Kępczyński et al. 2013) than the completely dormant caryopses. Both florets (Fig. 3b), and the seeds from the florets (Fig. 3d), kept dry for different times, germinated in the presence of SW faster than in water. A similar response was observed when caryopses from stored

florets were incubated in the presence of KAR_1 (Kępczyński et al. 2013).

To find out whether the SW mode of action might be associated with endogenous gibberellin, dormant *A. fatua* seeds were imbibed in the presence of PAC, a gibberellin biosynthesis inhibitor (Table 1). The enhancing effect of SW was not visible with PAC, suggesting the gibberellin biosynthesis involvement in the response to SW. Reversal of the inhibitory effect of PAC on caryopsis germination in the presence of SW was possible by exogenous GA_3. Thus, it confirms the involvement of gibberellin biosynthesis in caryopsis response to SW, which was the case with KAR_1 (Kępczyński et al. 2013). Earlier experiments performed on *Lactuca sativa* (Van Staden et al. 1995) and *Nicotiana attenuata* (Schwachtje and Baldwin 2004) seeds showed, too, that gibberellin synthesis could be considered as a possible component of the mechanism involved in smoke-stimulated germination.

Uptake of water is essential for germination. Like in the case of non-dormant seeds (Bewley et al. 2013), uptake of water by *A. fatua* caryopses in the presence of SW was triphasic (Fig. 1). Both non-treated and SW-treated dormant caryopses can take up water during two phases. Phase II (germination sensu stricto) has usually been considered as the most important stage because all of the germination-required metabolic reactions are reactivated during this period (He and Yang 2013). Caryopses incubated in the presence of SW, in contrast to those incubated in water, were able to complete germination (radicles protruded through the coleorhiza; the end of germination sensu stricto). Thus, only these caryopses could enter phase III (post-germination).

At the late stage of phase II of water uptake, SW enhanced the activity of α-amylase, two isoforms, and β-amylase (Tables 2, 3), i.e., before the radicle protrusion and even before the coleorhiza protrusion, like in the case of KAR_1 or GA_3 application (Kępczyński et al. 2013). Thus, before dormant seeds are prepared for germination through SW treatment, their metabolic activity is enhanced by stimulation of starch-breaking amylases. Similarly, a large increase in the α-amylase activity was found at phase II of rice seed germination (He and Yang 2013). Earlier publications proposed that GA_3-induced hydrolysis of starch reserves is a post-germinative event not associated with GA_3-induced breaking of dormancy (Simpson and Naylor 1962; Foley et al. 1993). According to the present views, starch is a major carbon source for generating the energy required for seed germination and early seedling growth (Hong et al. 2012; Yan et al. 2014; Shaik et al. 2014). Moreover, α-amylase was considered as a candidate biomarker for rice seed germination (He and Yang 2013). The aleurone layer of caryopsis is a secretory tissue surrounding the starchy endosperm wherein the synthesis and secretion of hydrolytic enzymes, including amylases, respond to the release of gibberellins from the embryo (Lovegrove and Hooley 2000; Kaneko et al. 2002). To clarify the influence of SW on the α-amylase activity, assays to determine the enzyme's activity in intact and embryoless caryopses were conducted. The embryoless caryopsis responded to SW by increasing α-amylase activity. However, the α-amylase activity and starch degradation in embryoless caryopses were lower than in the intact caryopses (Table 4). These results may indicate that SW can only partially replace the influence of endogenous gibberellins from embryo on the aleurone layer. On the other hand, it cannot be ruled out that the effect observed can be due to the cooperation between SW and endogenous gibberellins which could accumulate at some amount during caryopsis maturation.

The role of cell cycle in the germination of non-dormant and dormant seeds is still not completely clear. There are data showing that radicle protrusion does not require the cell cycle activation (Baíza et al. 1989; Jing et al. 1999), whereas others indicate that activation of the cell cycle takes place prior to radicle protrusion (de Castro et al. 2000; Barrôco et al. 2005; Masubelele et al. 2005; Gendreau et al. 2008). In dry and dormant *A. fatua* caryopses imbibed in water at 20 °C, 93% of cells in the radicle with coleorhiza (RC) were arrested at the G_1 phase (Table 5; Cembrowska-Lech and Kępczyński 2016). Cells arrested at G_1 were also observed in dry dormant *Lycopersicon esculentum* seeds (de Castro et al. 2001) and in *H. vulgare* caryopses (Gendreau et al. 2012). Analysis of nuclear DNA content during imbibition of dormant caryopses in water may suggest that the dormancy in *A. fatua* caryopses is associated with inhibition of the cell cycle. SW decreased the percentage of nuclei at the G_1 cell cycle phase and increased the percentage at S and G_2, after 24 and 28 h of imbibition. SW-induced germination of dormant caryopses, like in the case of KAR_1 application (Cembrowska-Lech and Kępczyński 2016), was associated with cell cycle activation found 4 h before the coleorhiza protrusion and 6 h prior to protrusion of radicles (Table 5). Thus, it can be concluded that induction of germination of dormant seeds by both SW and KAR_1 involves onset of the cell cycle at phase II, prior to the radicle protrusion. Likewise, dormancy release in seeds of *L. esculentum* (de Castro et al. 2001) was associated with induction of DNA replication before the radicles started protruding through the covering structures. An experiment involving the detection and densitometry analysis indicates that β-tubulin, necessary in the cell cycle, appeared due to SW treatment after 24 h, i.e. when the cell cycle was initiated. Earlier studies showed that releasing dormancy in *L. esculentum* seeds was associated with accumulation of β-tubulin (de Castro et al. 2001).

To summarize, plant-derived smoke is an effective stimulant of germination not only in *A. fatua* caryopses, but also in florets. Florets were completely dormant at all the incubation temperatures, the dormancy expression of caryopses being dependent on the incubation temperature. Florets were less sensitive to SW at temperatures above 20 °C than caryopses. A progressive release of dormancy by dry storage increased the smoke sensitivity in both florets and caryopses. SW-induced germination in dormant caryopses involves the initiation of stored starch mobilization and the cell cycle activation at the late stage of phase II germination sensu stricto. The release of dormancy by SW requires gibberellin biosynthesis.

Author contribution statement JK initiated and designed the research, interpreted the results and wrote the manuscript. DC-L conducted the experiments and statistical analyses, interpreted the results and wrote the manuscript. All authors read, reviewed and approved the manuscript.

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Compliance with ethical standards

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

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