Does zinc concentration in the substrate influence the onset of flowering in *Arabidopsis arenosa* (Brassicaceae)?

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Abstract We investigated the impact of low zinc (Zn) concentrations in the substrate on the onset of flowering in *Arabidopsis arenosa* (Brassicaceae). Experiments were carried out in controlled conditions using plants from four different populations. The research was aimed to verify experimentally the following hypotheses: (1) Zn content in the growth medium promote the onset of flowering in *A. arenosa*, (2) Changes in the onset of flowering induced by Zn depend on Zn concentration employed; (3) Zn-induced early onset of flowering is an universal plant response present within the species and is not an effect of stress or physiological adaptation to high Zn content in the environment. Investigated plants were subjected to four different Zn concentrations: 0.4 (control), 155, 775 and 1,550 $\mu\text{M Zn}^{2+}$. To assess stress level in investigated plants we calculated biomass accumulation and employed fluorometric methods. Zn content was estimated in shoots using atomic absorption spectroscopy. Differences in the onset of flowering were assessed using Kaplan–Meier curves. Our results showed that Zn was transported from growth medium to roots and shoots of investigated plants and that the content of Zn increased with the increase of Zn concentration in the growth medium. We evidenced that apart from one (1,550 $\mu\text{M Zn}^{2+}$) applied Zn concentrations did not cause stress in investigated plants what was

confirmed by two independent experimental approaches: measurement of biomass accumulation and chlorophyll *a* fluorescence. Flowering curves obtained on the basis of calculation of Kaplan–Meier estimator showed that: (1) control plants originating from four different populations did not differ in terms of the onset of flowering, (2) plants from each population tested tend to enter flowering phase earlier in response to applied Zn concentrations than control plants, (3) plants treated with the lowest tested Zn concentration (155 $\mu\text{M Zn}^{2+}$) tend to flower earlier than plants treated with the higher concentration (775 $\mu\text{M Zn}^{2+}$), (4) the impact of Zn on the onset of flowering did not depend on the origin of the plant material used (Zn-rich or Zn-poor soils). Our results indicate that Zn ions present in the growth medium promote early flowering in *A. arenosa* and that this effect may depend on Zn concentration used. Zn-induced early flowering in *A. arenosa* seems to be an universal plant response present within the species and is not an effect of stress or physiological adaptation to high Zn content in the environment.

Keywords *Arabidopsis arenosa* · Flowering · Zinc · Zinc accumulation

Introduction

Zinc is an important metallic element for both plant and animal life. The metal is relatively abundant as its mean concentration in the earth crust is estimated to 70 mg/kg, ranging from 10 to 300 mg/kg (Malle 1992). There are, however, areas where low content of Zn in the soil, or its low bioavailability causes problems and impacts plant growth and human well-being (Cakmak et al. 1999; Ozturk et al. 2006). On the other hand in some areas Zn content in

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the substrate can significantly exceeds normal limits. Such areas are inhabited by Zn-tolerant plants, well adopted to elevated content of the metal in the environment (Ernst 2006). Mechanisms of Zn tolerance in plants as well as problems connected with its deficit have been widely discussed in the literature in recent years (Cakmak et al. 1999; Cakmak 2008; Ernst 2006). In plant cells zinc is required by large number of proteins and is involved in many major metabolic processes by the presence in active sites of different enzymes (Mengel and Kirkby 2001; Kabata-Pendias 2010). It has been shown that zinc-containing molecules are also involved in the metabolism of nitrates and phosphates, RNA, proteins and carbohydrates (Mengel and Kirkby 2001; Nahed Abd El-Aziz and Balbaa 2007; Kabata-Pendias 2010). Zn is also essential in the synthesis of tryptophan, a precursor to IAA synthesis (Mengel and Kirkby 2001; Nahed Abd El-Aziz and Balbaa 2007). Zinc has been also confirmed to increase plant resistance to drought and disease (Kabata-Pendias 2010) and to be present in the protein being the product of CONSTANS gene involved in the regulation of flowering time (Putterill et al. 1995; Robson et al. 2001).

Taking into account an important role that zinc plays in major life processes in plants we were interested to see if it has also an impact on the onset of flowering. This question has never been raised before and the metal has never been considered as a factor influencing flowering time in plants.

Flowering time is an important character that enable plants to adapt to their local environments (Nah and Chen 2010) and the onset of flowering is thought to be one of the key events in plant life cycle (Turnbull 2011) influencing most important plant characteristics as: seed set, interactions with pollinators and thus genetic variability (Neil and Wu 2006). It has been evidenced that plants adopt to changing environmental conditions such as seasonality of weather, day length (photoperiodism) and temperature (vernalization) as well as to environmental stress conditions by regulation of their flowering time (Brun et al. 2003; Nah and Chen 2010; Turnbull 2011). It has never been evidenced, however, that Zn content in the soil or in the growth medium can influence the onset of flowering. Taking into account that wild plants are able to grow on soils with dramatically different Zn content (Ernst 2006), it is also interesting to see whether the influence of Zn on the onset of flowering would be the same in both ecological groups: plants adapted to low and high Zn content in soil and thus showing two distinct physiological phenotypes. To accomplish this aim we needed a suitable model plant that is intraspecifically differentiated into two types of populations growing on Zn-rich and Zn-poor soils.

It seems that *Arabidopsis arenosa* (Brassicaceae) can serve as a good example of such species. It is common in central and northern part of Europe and closely related to

A. thaliana (estimated time of divergence between the two taxa is 5 Myr, Koch et al. 2000). *A. arenosa* can be considered as an ecologically unique within *Arabidopsis* due to the ability to inhabit wide range of different environments from natural habitats of grassy and sandy areas to disturbed habitats as roadsides, railroad tracks or waste heaps (Clauss and Koch 2006; Przedpelska and Wierzbicka 2007). One example of this ecological diversity is the ability of the species to grow in heavy-metal polluted sites as well as on soils free of these pollutants (Przedpelska and Wierzbicka 2007). Our previous research (Przedpelska and Wierzbicka 2007) showed that within *A. arenosa* exists two different types of populations: (1) growing on heavy metal polluted calamine soils rich in Zn (metallicolous populations, M) and (2) natural populations (non-metallicolous populations, NM) growing on soil with normal Zn content.

During our previous studies on *A. arenosa* focused on Zn tolerance (Przedpelska and Wierzbicka 2007) we observed that tested plants showed similar response to low zinc concentrations used during tolerance tests carried out in uniform conditions. We noticed that the metal present in low concentration in the experimental growth medium induced early flowering in these plants. This observation could not be easily confirmed without setting out an experiment focused on the impact of low zinc content in the substrate on the onset of flowering in *A. arenosa* in controlled conditions.

As *A. arenosa* is a monocarpic plant, its flowering strategy could be expected to be a key element in its adaptation to changing environmental conditions. If this is true, populations from contrasting environments (M vs. NM) should differ in the onset of flowering. It has been hypothesized that plants from Zn-enriched sites flower earlier and produce more seeds in order to increase survival in their harsh environment (Wierzbicka and Panufnik 1998; Zalecka and Wierzbicka 2002). This has never been tested experimentally.

We believe, that using *A. arenosa* as a model species creates unique opportunity to test possible Zn impact on the onset of flowering on two levels: (1) adaptive level—changes in the onset of flowering time between populations from Zn-rich soils and from natural soils (M vs. NM) that have developed during relatively long period of time as a result of adaptation to Zn-enriched soils, (2) non-adaptive level—the impact of low (physiologically non-toxic) concentrations of Zn on the onset of flowering in *A. arenosa* plants regardless their origin; an universal response to Zn treatment in low concentrations present within the species.

In our study we employed Zn concentrations ranging from 0.4 to 1,550 μM Zn^{2+} . The reader should bear in mind, however, that these concentrations cannot be simply compared with Zn content present in natural soils. The total content of Zn (usually measured and described in

geochemical literature) is not a good indicator of bioavailable Zn resources present in the substrate. Zn bioavailability depends on many different factors such as: pH, predominant chemical compound being a major Zn source, the content of organic matter in the soil etc. Cakmak et al. (1999) have shown that even in case of fairly high total Zn content in the soil (ranging from 40 to 80 mg kg⁻¹) the concentration of bioavailable Zn can be about 0.25 mg kg⁻¹, while widely accepted critical level of bioavailable Zn in soil for cultivated plants is 0.5 mg kg⁻¹ (Cakmak et al. 1999). In case of our study it can be assumed that the amount of total and bioavailable Zn was almost the same. In light of this it can be stated that concentrations employed by us were within the limits of natural soils (with no Zn deficit) or higher, reaching the concentration of 1,550 µM of bioavailable Zn²⁺ that has been found to be toxic for many wild and cultivated plant species (Kabata-Pendias 2010).

The present study was aimed to test the following hypotheses:

1. Zn content in the growth medium promote the onset of flowering in *A. arenosa*
2. Changes in the onset of flowering induced by Zn depend on Zn concentration employed
3. Zn-induced early onset of flowering is an universal plant response present within the species and is not an effect of stress or physiological adaptation to high Zn content in the environment

Materials and methods

Plant material

Seeds were sampled from four different populations inhabiting contrasting environments from calamine grassland and Zn-polluted sandy soils, through ruderal habitats of a railway line to relatively stable environment of forest edge. Seeds were sampled in bulk in July–August and subsequently used to grow plants used in experiments. Details on sampling locations were given in Table 1.

Plant cultivation

Seeds were germinated on artificial substrate (glass beads) in plastic trays using Knop's growth medium (pH = 6) containing: 205,45 g/l Ca(NO₃)₂·4H₂O; 71,5 g/l KNO₃; 35,5 g/l KCl; 71,5 g/l KH₂PO₄; 145,42 g/l MgSO₄ × 7H₂O, 28 g/l EDTA-Fe and micronutrients according to Hoagland recipe modified by Bassalik (Strebeyko 1967). Seeds were germinated in light using growth chamber (Liebherr FKU 1800) at 24 ± 1 °C.

After germination plants were transferred to plastic pots containing perlite as a substrate and grown in semi-hydroponic conditions in greenhouse at 24 ± 4 °C, relative humidity of 45 ± 5 %, light intensity of ~ 120 µmol/m²/s and photoperiod of 16/8 h (light/dark).

At this stage we also started the treatment with different concentrations of zinc

1. 10 mg/dm³ Zn²⁺ (155 µM) from Zn(NO₃)₂
2. 50 mg/dm³ Zn²⁺ (775 µM) from Zn(NO₃)₂
3. 100 mg/dm³ Zn²⁺ (1,550 µM) from Zn(NO₃)₂
4. control group (Knop's solution containing 0.4 µM Zn²⁺)

Zinc concentrations in the growth medium were established on the basis of pilot experiments in order to use both toxic and non-toxic concentrations. Concentrations between 0.4 and 50 µM Zn²⁺ were found to be optimal for development of tested plants, whereas concentrations equal to 100 µM Zn²⁺ and higher were toxic. There were 20 plants in each experimental unit, what gives a total of 320 tested plants (4 units and 4 populations tested).

In vivo chlorophyll fluorescence measurements

Measurements of chlorophyll *a* fluorescence were carried out in order to determine plant reaction to applied Zn concentrations. Measurements were performed in vivo on plants from each experimental unit (5 plants tested per one unit). In total 80 plants were tested (4 populations and 4 experimental units). FMS-1 chlorophyll fluorometer (Hansatech Instruments) was employed. Fluorescence measurements were carried out on dark adapted leaves (30 min). Due to dark adaptation all light dependent reactions were inhibited. This resulted in complete re-oxidation of PSII electron acceptor molecules, opening PSII reaction centers and maximized the probability that absorbed light can be used for in photochemical reactions. After adaptation to darkness leaves were illuminated with light of low intensity (0.05 µmol m⁻² s⁻¹). At this stage measurement of the fluorescence origin (*F*_o) was done. Subsequently, leaves were exposed to an intense saturating pulse of light from the chlorophyll fluorometer (4,500 µmol m⁻² s⁻¹) and the maximum fluorescence yield (*F*_m) was measured. On the basis of these measurements maximal photochemical efficiency ($\left(\frac{F_v}{F_m}\right)$ of the photosystem II (PSII) in dark adapted leaves was calculated as $\frac{F_v}{F_m} = \frac{F_m - F_o}{F_m}$.

Biomass accumulation

After the end of experiment plants from each experimental unit were harvested, washed with deionized water and

Table 1 *Arabidopsis arenosa* populations sampled in the present study

Population acronym	Locality	GPS coordinates		Elevation (m a.s.l.)	Edaphic type ^a
		N	E		
BI	Eastern Carpathians–Bieszczady Mts., Balnica, railway line in forest	49°11'08.4"	22°12'52.9"	696	NM
BO	Bolesław near Olkusz (S Poland), calamine grassland developing on Zn-Pb waste heap	50°17'31.89"	19°28'17.31"	319	M
KA	Forest edge in Kampinoski National Park (Central Poland), sandy soil	52°17'07.00"	20°50'28.00"	96	NM
MS	Miasteczko Śląskie (S Poland), surroundings of Zn smelter	50°30'09.33"	18°56'03.64"	300	M

^a Edaphic type of each sampled population was established on the basis of heavy metal content in the substrate: *NM* non-metallicolous population (substrate with natural, low content of Zn), *M* metallicolous populations (substrate with excessive content of Zn)

incubated in oven at 80 °C until constant weight and weighted using electronic scale. The biomass accumulation for control plants as well as for each treatment was expressed in grams as mean $\pm$ SD.

Observations of the onset of flowering

The onset of flowering was observed on 20 plants per each experimental unit. In total, the onset of flowering was recorded in 240 plants. Each day and at the same time flowering was assessed in experimental plants. The day in which first flower bud opened on a plant was treated as the onset of flowering.

Zn concentration in leaves

Zn content was assessed in leaves using plants treated with 10 and 50 mg/kg Zn²⁺ as well as for control plants. Measurements were done using 3 plants per experimental unit. In total Zn content was measured in leaves from 36 plants using atomic absorption spectroscopy (AAS). Leaves were washed with demineralized water, dried and grounded prior to chemical analyses. Subsequently samples of 0.1 g of grounded leaves were treated with the mixture containing HNO₃ (69 % solution) and H₂O₂ (30 % solution), 9:1 (v/v), in Teflon bombs. Certified plant reference material was used as a control sample. In order to test purity of chemicals used in the process pure mixture of HNO₃ and H₂O₂ (without any plant material) was used as a reference sample. Zinc content was then measured by flame AAS using Solar M6 spectrometer (Thermo Scientific) and expressed in mg/kg of dry weight.

Statistical analyses

Kaplan–Meier estimator

To explore differences between experimental groups regarding the onset of flowering we used Kaplan–Meier estimator (Kaplan and Meier 1958). The analytical technique is most widely used in medical sciences and is particularly useful when researcher has to cope with censored

observations (Kirkwood and Sterne 2003). Censored observations arise always when the variable of interest represent the time to a terminal event (in our case the onset of flowering), and when duration of the study have to be limited in time. We estimated survival function directly from continuous survival times (Kaplan and Meyer 1958):

$$\hat{S}_{(t)} = \prod j : t_j \leq t \left[1 - \frac{d_j}{n_j} \right]$$

where: $\hat{S}_{(t)}$ —represents estimated survival probability at time t : $P(T > t)$, $\prod j : t_j \leq t$ —multiply the probability of surviving event time t with the probabilities of surviving all the previous event times, d_j —is the number of deaths up to point t , n_j —is number of individuals at risk just prior to t , $1 - \frac{d_j}{n_j}$ —proportion surviving the event time t .

In case of our research survival time was defined as the number of days from the beginning of the experiment (seeds sowing) to the onset of flowering in each individual plant and corresponding Kaplan–Meier curve (here called “flowering curve”) depicts the probability of “no-flowering” at the time t . To assess statistical significance between experimental units log-rank, F-Cox and Cox-Mantel tests were used ($\alpha = 0.05$).

ANOVA and post hoc tests

Differences in Zn content in shoots were assessed using one-way ANOVA and post hoc Tukey’s HSD test ($\alpha = 0.05$). All the statistical analyses were carried out using Statistica 9.1 software package (Statsoft Inc., USA).

Results

Zn content in plants

Our results showed that increased content of Zn in the growth medium caused increased content of the metal in shoots (Fig. 1).

Mean Zn concentration in control plants was 31.88 mg/kg Zn in D.W. (Fig. 1). In experimental groups treated with increasing Zn concentration (155 and 775 $\mu\text{M Zn}^{2+}$) mean Zn content was 82.40 and 239.84 mg/kg Zn in D.W., for lower and higher Zn concentration respectively (Fig. 1).

Stress level in investigated plants

Table 2 shows mean values of $\frac{F_v}{F_m}$ ratio measured in investigated plants for all the experimental units. Values of $\frac{F_v}{F_m}$ ratio obtained for control plants varied narrowly between 0.83 and 0.84, what indicate that control plants were in good physiological state, showing no signs of stress. Similar results were obtained for two lower zinc concentrations (155 and 775 $\mu\text{M Zn}^{2+}$). We noticed that plants from BI population treated with 775 $\mu\text{M Zn}^{2+}$ showed slightly lower (0.78) mean value of the $\frac{F_v}{F_m}$ ratio. Table 2 shows that value of $\frac{F_v}{F_m}$ ratio decreased substantially in population KA. Plants from BI population subjected to the highest concentration of Zn (1,550 $\mu\text{M Zn}^{2+}$) showed signs of acute toxicity: substantially decreased biomass production, numerous chloroses and substantially smaller size, when compared with plants from BO and MS populations. Small size of leaves made impossible measurement of $\frac{F_v}{F_m}$ ratio for this experimental unit.

Table 3 presents mean biomass accumulation in investigated plants. Substantial differences in plant biomass (assessed as dry weight) between investigated populations was recorded. Significant decrease in mean dry weight of investigated plants was recorded for population BI and KA in case of the highest Zn concentration (1,550 $\mu\text{M Zn}^{2+}$). In case of remaining populations (BO and MS) accumulated biomass was the highest in plants subjected to high Zn concentrations.

Fig. 1 Zn content in *A. arenosa* plants treated with different Zn concentrations (0.4, 155 and 775 $\mu\text{M Zn}^{2+}$). Estimated using atomic absorption spectroscopy

The onset of flowering

Figure 2 shows flowering curves obtained for control plants originating from populations inhabiting contrasting environments: metalliferous (Zn-rich) and non-metalliferous soils (showing natural, low content of Zn). Significance tests evidenced that there is no differences in the onset of flowering between plants from M and NM populations in *A. arenosa* ($p \gg 0.05$).

Using Kaplan–Meier estimator we studied the effect of zinc concentration on the onset of flowering on population level. Figure 3a–d shows flowering curves obtained for each investigated population. We employed three tests (log-rank test, F-Cox test and Cox-Mantel test) in order to assess statistical significance of our observations. Our results evidenced that plants treated with the lowest concentration of Zn (155 $\mu\text{M Zn}^{2+}$) flowered earlier than control plants regardless the population studied (M vs. NM). Statistical significance of these differences was confirmed by three different tests (see above) at the significance level of $\alpha = 0.05$. Plants treated with 775 $\mu\text{M Zn}^{2+}$ also showed a tendency to flower earlier than control plants (Fig. 3a–d). These differences, however, were significant only in populations BO (Fig. 3d) and KA (Fig. 3a, $\alpha = 0.05$).

We investigated also the impact of zinc concentration on flowering using data from overall sample (all individuals and all populations). Results of this analysis were shown in Fig. 4. We evidenced that differences between control plants and both Zn-treated groups were statistically significant. Concordant results ($p < 0.05$) were obtained in case of all three tests. Analysis carried out on overall sample evidenced also that plants treated with lower Zn concentration flowered earlier than those treated with higher concentration of Zn ($p < 0.05$, concordant results for three tests employed).

We also compared flowering time in reaction to applied Zn concentration between populations growing on metalliferous (Zn-rich) and non-metalliferous soils (natural, low content of Zn). No significant ($\alpha = 0.05$) differences in flowering time between M and NM populations within the species were detectable in response to different Zn concentrations (Fig. 5).

Discussion

Zinc content in plants

In order to test whether Zn from the growth medium was effectively transported to plants and whether changes in the content of the metal in the growth medium influenced Zn

Table 2 Maximal photochemical efficiency ($\frac{F_v}{F_m}$) of the photosystem II (PSII) in dark adapted leaves of *A. arenosa* subjected to different concentrations of Zn

Zn concentration (μM)		0.4 (control)		155		775		1,550	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Population acronym	BI	0.84	0.007	0.83	0.006	0.78	0.032	–	–
	BO	0.84	0.008	0.83	0.015	0.85	0.004	0.82	0.023
	KA	0.83	0.012	0.82	0.016	0.83	0.010	0.49	0.141
	MS	0.84	0.006	0.83	0.016	0.85	0.005	0.80	0.024

Table 3 Mean biomass accumulation in investigated plants subjected to different concentrations of Zn

Zn concentration (μM)		0.4 (control)		155		775		1,550	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Population acronym	BI	1.09	0.19	0.90	0.12	0.86	0.17	0.03	0.01
	BO	0.24	0.07	0.31	0.12	0.63	0.18	0.51	0.10
	KA	0.67	0.15	0.60	0.29	0.53	0.20	0.25	0.13
	MS	0.94	0.31	1.08	0.33	1.55	0.67	1.71	0.49

Fig. 2 Kaplan-Meier curves showing predicted probability of “no flowering” in plants grown in control conditions ($0.4 \mu\text{M Zn}^{2+}$) and originating from *A. arenosa* populations belonging to two edaphic types: metallicolous—Zn-rich (M) versus non-metallicolous—Zn-poor (NM)

content in plants we carried out chemical analysis using AAS technique. This approach enabled us to verify experimentally Zn content in plants subjected to different Zn concentrations in the growth medium. We found that Zn concentrations measured by us in investigated plants were within physiological limits and were significantly lower than toxic level of the element reported for plants (Kabata-Pendias 2010).

Stress level in investigated plants

Many authors pointed out that stress can have substantial effect on early flowering (Roux et al. 2006; Wada and Takeno 2010; Yaish et al. 2011). In order to exclude Zn induced stress as a cause of the changes in the onset of flowering we examined stress level in *A. arenosa*.

The ratio of $\frac{F_v}{F_m}$ is widely used as a screening in vivo parameter for stress response in plants (Schreiber et al. 1994; Andrews et al. 1995; Waldhoff et al. 2002; Mallick and Mohn 2003). It has been show that in vivo fluorescence measurements are reliable and fast method used for assessing changes in photosynthetic activity in leaves induced by stress factors, both of natural and anthropogenic origin. Therefore we decided to use $\frac{F_v}{F_m}$ ratio as an indicator of stress caused by zinc ions.

It seems, that both Zn concentrations were well-tolerated and did not cause stress in investigated plants. It is widely accepted that the values of $\frac{F_v}{F_m}$ ratio ranging from 0.79 to 0.84 are optimal and were confirmed in different plant species (Maxwell and Johnson 2000; Kitajima and Butler 1975). Lower values, however, are indicator of stress (Maxwell and Johnson 2000; Kitajima and Butler 1975). We noticed that plants from BI population treated with $775 \mu\text{M Zn}^{2+}$ showed slightly lower (0.78) mean value of the $\frac{F_v}{F_m}$ ratio. This decrease was, however, minimal and negligible when taking into account the value of standard deviation. Situation was quite different in case of the highest Zn concentration.

Fig. 3 Kaplan-Meier curves showing predicted probability of “no flowering” in plants from different populations of *A. arenosa* grown in control conditions ($0.4 \mu\text{M Zn}^{2+}$) and treated with different Zn

concentrations (155 and $775 \mu\text{M Zn}^{2+}$). **a** population KA, **b** population BI, **c** population MS, **d** population BO

Fig. 4 Kaplan-Meier curves showing predicted probability of “no flowering” in overall sample of plants from different populations of *A. arenosa* grown in control conditions ($0.4 \mu\text{M Zn}^{2+}$) and treated with different Zn concentrations (155 and $775 \mu\text{M Zn}^{2+}$)

We investigated also biomass accumulation in investigated plants. Results of these analyses were in agreement with data from in vivo fluorescence measurements and confirmed that only the highest concentration of Zn caused significant decrease in biomass accumulation observed in two populations (BI and KA).

Data obtained from these experiments show clearly that control plants as well as plants treated with two lower zinc concentrations were in good physiological state, showing no signs of stress caused by applied Zn concentrations. It seems that these concentrations, although higher than found in most of natural environments (Kabata-Pendias 2010), are within tolerance limits for *A. arenosa*. Plants treated with the highest Zn concentration, showing clear signs of toxicity and stress, were excluded from further experiments in order to minimize the possibility that observed differences in flowering could be attributed to stress-related factors. It seems therefore, that in our

Fig. 5 Kaplan-Meier curves showing predicted probability of “no flowering” of *A. arenosa* plants originating from M and NM populations subjected to different Zn concentrations: **a.** 155 $\mu\text{M Zn}^{2+}$ and **b.** 775 $\mu\text{M Zn}^{2+}$

experiment stress can be excluded as a factor influencing observed phenomena.

Differences in the onset of flowering between populations in control plants

It is accepted that flowering is controlled by four main pathways promoting flowering phase: photoperiodic, vernalization, autonomous, and hormonal (Zeevaert 2006). Autonomous and hormonal pathways are thought to be independent from environmental factors, but connected with plant development and age (e.g. Mouradov et al. 2002; Wang et al. 2012). Photoperiodic and vernalization pathways are controlled by environmental factors such as day length or temperature (e.g. Mouradov et al. 2002; Wang et al. 2012) and thus are likely to be involved in local adaptation in plants.

Numerous studies focused on intraspecific variation and ecotypes pointed out that different onset of flowering is likely to be the result of natural selection. This phenomenon is usually associated with the notion that only those plants whose life history traits (including the onset of flowering) “fits” the environment of a particular area will survive in the long-term (Briggs and Walters 2000). In our case Zn content in the soil was the most interesting factor that could potentially influence flowering patterns in investigated populations. In our study we used four different M and NM populations of *A. arenosa*. If early flowering was an important factor contributing to plant survival in Zn-rich polluted environment we should expect that plants from metalicolous (M) populations should differ from plants from non-metalicolous (NM) populations in terms of flowering onset due to the selection of well adapted genotypes. To answer this

question we investigated differences in the onset of flowering between M and NM populations of *A. arenosa* in control plants.

Our results suggest that time to the onset of flowering is probably not a selected trait in M and NM populations of *A. arenosa* and that both types of populations do not differ in this aspect in controlled conditions. It also means that preexisting differences in the onset of flowering, present between populations and being an effect of local adaptation, had no impact on our observations.

Stimulation of the onset of flowering by low Zn concentrations

Many environmental factors can influence the onset of flowering (Bernier and Perilleux 2005) and plants are able to detect these environmental stimuli and react adjusting their development (Ausin et al. 2005; Yaish et al. 2011).

Different environmental factors such as light and temperature show predictable and repeatable pattern of variation during the year and have a major effect on flowering season in wild plants. There are also other factors, however, such as wind or nutrient content in the substrate that can influence the onset of flowering in different plant species. It seems that zinc, as a mineral nutrient, may play here a special role. Golcz and Seidler-Lozykowska (2009) investigated the content of mineral nutrients (including Zn) in plants at different stages of development. They showed that the maximal amounts of zinc were accumulated during the onset of flowering, full flowering stage and during seed setting by three different plant species: *Origanum majorana*, *Ocimum basilicum* and *Satureja hortensis*. These results suggest that zinc may play a role in regulation of flowering.

It seems that the impact of Zn on plant physiology can differ substantially between species or even between conspecific populations. It is widely known that Zn is an essential element which is indispensable for plants. On the other hand zinc deficiency (resulting from decreased Zn bioavailability in soil) is a widespread problem in many areas of the world. It is estimated that nearly 50 % of the cereal-cultivated soils have Zn deficiency problem, causing decreased crop yields (Cakmak 2008). It is also believed that Zn deficiency is the most widespread micronutrient deficiency in crop plants (Ozturk et al. 2006; Cakmak et al. 1999; Cakmak 2008). At the opposite pole from soils with Zn deficit there are places where substrate is unusually rich in this metal. Some of these soils are so rich in Zn that excess of this nutrient becomes a major factor limiting plant growth (Ernst 2006) and only some plants were able to adapt to such extreme environments (e.g. Przedpelska and Wierzbicka 2007; Abratowska et al. 2012). It is highly probable that Zn metabolism and the impact of this metal on physiological processes could be different in plants from Zn-poor and Zn-rich soils.

To investigate plant response to Zn and expressed as changes in the onset of flowering also in this context we choose *A. arenosa* as the most suitable model species having (1) well developed strategies facilitating adaptation to different environments and (2) occurring in places with both low and excessively high content of zinc in the soil. It seemed to us that investigating plants from two different physiological phenotypes (adopted to low and high concentration of Zn in the soil), we will be able to show whether plant response to Zn manifested as differences in the onset of flowering were an effect of the local adaptation to Zn-rich or Zn-poor soils, or constitutes an universal, “constitutional” plant response exhibited by both physiological phenotypes present within the species.

It has been shown that flowering time in *A. arenosa* is regulated by temperature and day length (Nah and Chen 2010). It has been also evidenced that the species need about 60 days from germination to the onset of flowering (Przedpelska and Wierzbicka 2007; Nah and Chen 2010). Hitherto, however, there was no observations showing the impact of zinc on early flowering both in *A. arenosa* as well as in other plant species. This is surprising, especially when taking into account that zinc has a special place among heavy metals. On the one hand the metal is able to cause phytotoxic effects, on the other hand, however, it plays a key role in many different metabolic processes in plants (Mengel and Kirkby 2001; Nahed Abd El-Aziz and Balbaa 2007; Kabata-Pendias 2010).

We found that in each investigated population at least one non-toxic concentration of zinc in the growth medium caused significant stimulation of the onset of flowering. The lowest concentration applied ($155 \mu\text{M Zn}^{2+}$) caused

significant acceleration of the onset of flowering in plants from all populations studied. The same phenomenon was observed when data from overall sample was taken into account in one analysis. Significant results of statistical test indicate that plants treated with Zn started to flower earlier than the control plants. It was also clear that differences existed not only between the control and treated plants, but also between treatments. The lowest applied Zn concentration ($155 \mu\text{M Zn}^{2+}$) stimulated the onset of flowering most efficiently. These results have shown that zinc present in the growth medium in low and non-toxic concentrations promote early flowering in *A. arenosa*. We have also shown that the effect of stimulation may depend on the Zn concentration applied. In our case the lowest Zn concentration had the strongest effect. Our previous experiments (see above) analyzing chlorophyll fluorescence and biomass accumulation have shown that the observed phenomenon cannot be interpreted as an effect of stress.

Our results suggest also that early flowering observed in Zn treated plants is an universal characteristic present within the species and is not influenced by the origin of plant material

Our previous studies (Przedpelska and Wierzbicka 2007) evidenced the presence of two physiological phenotypes present within *A. arenosa*: one inhabiting Zn-poor soils and the other one present in Zn-rich soils. These phenotypes were found to be different in terms of several morphological and physiological characteristics and were hypothesized to be a product of local adaptation to Zn-polluted soils (Przedpelska and Wierzbicka 2007). In light of these findings, it was reasonable to assume that plants from these two phenotypes can differ in terms of their response to Zn treatment in the present experiment. Surprisingly, however, we did not evidenced any differences in plant response to applied Zn concentrations manifested as changes in the onset of flowering. Analyses carried out on the data collected from plants originating from M and NM populations showed that regardless the concentration applied (155 or $775 \mu\text{M Zn}^{2+}$) no significant differences in the onset of flowering were observed.

It seems therefore that regardless the origin of the material studied, plant response to applied Zn concentrations was the same. This finding seem to confirm our previous observations that Zn-induced early flowering is not associated with particular phenotype well adapted to local environment (especially in terms of Zn excess in the substrate).

Explanation of the phenomena observed by us is difficult on the basis on contemporary knowledge on the regulation of flowering. Similar reaction—early flowering—was also observed by Wang et al. (2012) in *A. thaliana* in response to cadmium. They concluded that the phenomenon of early flowering was induced by up regulated

expression of CO and FT genes that play a key role in photoperiodic pathway of flowering regulation. We should bear in mind, however, that *A. thaliana* is highly sensitive to cadmium and that observed stimulation of the onset of flowering might have been induced by stress. It cannot be confirmed at present whether the mechanism of early flowering described in *A. thaliana* in response to cadmium can be the same or at least similar in *A. arenosa*. Both metals have similar chemical properties: they have similar atomic radius, similar oxidation state in chemical compounds and share similar geochemical properties (Emsley 1991; Kabata-Pendias 2010) and both have been evidenced to interact similarly with different macromolecules in plant cells (e.g. Przedpelska-Wasowicz et al. 2012; Przedpelska-Wasowicz and Wierzbicka 2011; Wierzbicka et al. 2007). Taking this into account, similar mechanism could be postulated for both phenomena. Highly similar genomes of both species (Clauss and Koch 2006) also seems to support this hypothesis. Surely, however, further research on gene expression in *A. arenosa* in response to Zn treatment is needed to elucidate the problem completely.

It seems that if response to low Zn concentrations is similar also in other plant species, particularly those of economic significance, some level of Zn surplus in fertilizers may induce early flowering if desired. This question requires also further research and confirmation.

Conclusions

Our research showed that increased Zn content in the growth medium caused increased Zn concentration in investigated plants and promoted the onset of flowering in *A. arenosa*. We evidenced also that applied Zn concentrations had no harmful effect on plants used in our experiments, and that stress can be excluded as a factor contributing to early onset of flowering in Zn treated plants. It seems that acceleration in the onset of flowering induced by Zn may depend on Zn concentration employed. Carrying out experiments on plants belonging to two different physiological phenotypes (adopted and non-adopted to high Zn content in the substrate) allowed us to confirm that Zn-induced early flowering in *A. arenosa* is an universal plant response present within the species and is not a part of physiological adaptation to high Zn content in the environment.

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