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Field priming with cytokinins enhances seed viability of wheat after low temperature storage

*Radoslav Chipilski[1], Irina Moskova[2], Albena Pencheva[1], Konstantina Kocheva[2]**

[1]Institute of Plant Genetic Resources, Sadovo, Bulgaria
[2]Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria
**Corresponding author: konstvk@abv.bg*

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Abstract: Field experiments were conducted with two winter wheat cultivars that were primed with 6-benzylaminopurine (6-BA) or kinetin at the concentration 10 mg/L twice during the grain filling stage. After priming, wheat physiological parameters were measured in the field, and the analysis of yield was performed after harvest. Harvested seeds were subjected to low temperature storage for 12 months at –18 °C simulating conservation conditions in genebanks. In field experiments, treated plants exhibited up to 14% higher productivity, higher fresh and dry weight, and chlorophyll content index of flag leaves. Priming significantly improved germination, seedling vigour and growth parameters. In 5-days-old seedlings developed from low temperature stored seeds of field primed plants, the average accumulation of malondialdehyde and H_2O_2 was estimated 25% lower, which contributed to higher cell membrane stability. These results correlated positively with growth characteristics of 15-days-old seedlings. The stimulating action of cytokinin priming was more pronounced in the modern cv. Geya-1 compared to the older cv. Sadovo 772 and could be attributed to improved anti-aging mechanism connected with better protection against oxidative damage.

Keywords: phytohormone; cold storage; oxidative stress; seed conservation; *Triticum aestivum* L.

Efficient conservation of plant genetic resources is critical to sustainable crop production and safeguards food and nutrition security (FAO 2014). A means for accomplishing this goal is the formation and support of genebanks in which short- and long-term low temperature conservation of embryonic germplasm is realised under controlled standard conditions (Li and Pritchard 2009). An essential task for managing stored germplasm is to maximise seed longevity and keep operational costs and logistics manageable through monitoring seed deterioration (Engels and Visser 2003). Thus, one of the main purposes in long-term conservation in genebanks is to preserve seed viability and usability after low temperature storage and subsequent use as sowing material (Desheva 2016, van Treuren et al. 2018). Generally, loss of germination and seedling vigour results from the aging effect of low temperature stress. As a common consequence of most abiotic stresses, an imbalance arises between the production of reactive oxygen species (ROS) and the scavenging capacity of the antioxidant defense system (Foyer and Noctor 2000, Jajic et al. 2015). Excessive ROS accumulation causes lipid peroxidation, and eventually, oxidative damage leads to accelerated leaf senescence (Sade et al. 2018). Cytokinins (CKs) are phytohormones that regulate many aspects of plant aerial and subterranean organ development and growth (Werner and Schmulling 2009) but also participate in the responses to biotic and abiotic stress (Hai et al. 2020). CKs reduce ROS accumulation either through increasing the activity of antioxidant enzymes or by inhibiting ROS formation (Hönig et al. 2018). Cytokinin action is also connected with affecting grain number and size (Jameson and Song 2016) and causing delayed senescence (Hönig et al. 2018). Endogenous CK levels could be elevated *via* enhancing their biosynthesis by isopentenyl transferase (IPT) or *via* decreasing their

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degradation by cytokinin oxidase/dehydrogenase (CKX). Newest transgenic approaches have shown that down regulation of CKX genes offered promising results towards yield improvement in cereals (Chen et al. 2020). On the other hand, the application of exogenous CKs retained chlorophyll levels of flag leaf in wheat and thus delayed leaf senescence, which could also contribute to improving grain filling and yield (Yang et al. 2016).

This study attempted to elucidate the effect of exogenous cytokinin application on seedling performance following seed conservation. The objective of the study was to investigate whether *in situ* field priming with CK-type growth regulators could overcome the negative consequences of low temperature storage on seed germination and seedling viability. In this regard, the effect of one natural (kinetin) and one synthetic (6-benzylaminopurine) adenine-type CK were applied in the field by spraying wheat plants at the grain filling stage. After cold storage, seeds were germinated, and physiological and biochemical parameters of seedlings were assessed.

The study could be useful for improving the efficiency of seed conservation in genebanks by displaying the possibility for increasing seed longevity after low temperature storage through the preliminary application of CKs in the field.

MATERIAL AND METHODS

Field experiment. Field experiment was carried out during 2018–2019 vegetation period with two winter wheat (*Triticum aestivum* L.) cultivars Sadovo 772 and Geya-1. The cultivars were produced through the conventional selection method of inter-varietal hybridisation at the Institute of Plant Genetic Resources and established in 1998 (cv. Sadovo 772) and in 2004 (cv. Geya-1). Seeds were sown at a planting rate of 550 seeds per m^2. The experimental area for each cultivar was 3 m^2 divided in three replicates per variant as follows: (1) untreated control plants in natural conditions throughout the vegetation; (2) *in situ* priming with 6-benzylaminopurine (6-BA) at the concentration 10 mg/L twice during the grain filling stage (first spraying 15 days after flowering, Zadoks scale No. 83, and second spraying 25 days after flowering, Zadoks scale No. 91) (Zadoks et al. 1974); (3) *in situ* priming with kinetin (6-furfurylaminopurine) at the concentration 10 mg/L twice during the grain filling stage as in variant (2). Chemical reagents were purchased from the Merck KGaA (Darmstadt, Germany). After priming, physiological parameters of the plants from different variants were measured in the field, and the analysis of yield was performed on harvested plants at the end of June.

Sample preparation for long-term seed conservation. Two samples were prepared from 250 g seeds for each variant of the field experiment. Seeds were dried to 6% moisture content and transferred for storage for 12 months at –18 °C.

Analysis of seed viability after exposition to low temperature stress. Laboratory experiments were conducted with mean sample of 60 seeds from the two cultivars. Seeds from control and CK-primed plants were superficially sterilised and germinated on moist filter paper in Petri dishes with 20 mL distilled water at 22 °C in thermostat for 120 h. After 5 days of germination, the degree of coleoptile and root growth was assessed.

Soil pot experiment was conducted with seedlings descended from the three variants of the field experiment. Seeds were sown in pots containing 1 kg dry soil and grown for 15 days in the greenhouse under ambient conditions of light and temperature and 40–60% relative humidity. Second, fully developed leaves were collected and used for the analyses.

Physiological measurements. Physiological measurements were performed on 5-days-old seedlings and leaves from pot and field experiments. Relative water content (RWC) was measured according to Turner (1981) and calculated from the formula:

$$RWC\ (\%) = (FW - DW)/(TW - DW) \times 100$$

where: FW – fresh weight; DW – dry weight, obtained after drying the leaves for 24 h at 80 °C; TW – turgid weight after soaking the leaves or coleoptiles in water for 24 h. Mean values were calculated from 10 flag leaves of mature field-grown plants or second fully developed leaves of 15-days-old pot grown seedlings (n = 10).

Cell membrane stability (CMS) was determined following the method of Premachandra et al. (1990). The 100 mg samples of plant parts were cut and kept in test tubes containing 10 mL of distilled water in two sets. One set was kept at 40 °C for 30 min, and another set at 100 °C in a boiling water bath for 15 min and respective electrical conductivities C1 and C2 were recorded. CMS was estimated from the formula:

$$CMS\ (\%) = [1 - (C1/C2)] \times 100.$$

Chlorophyll content index (CCI) was measured with portable Chlorophyll Content Meter-CCM 200 Plus (Opti-Sciences, Inc., Hudson, USA). From each

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variant, 20 leaves were analysed by readings from the central part of the leaf (n = 20).

Oxidative stress markers of 5-days-old seedlings. Malondialdehyde (MDA) was determined by the method of Heath and Packer (1968). Specific absorption was read spectrophotometrically at 532 nm, and a molar extension coefficient of 155 mmol/cm was used in calculations. Content of H_2O_2 was assayed by the method of Alexieva et al. (2001). Plant material was homogenised (at 4 °C) with 0.1% TCA (trichloroacetic acid) and subsequently centrifuged for 15 min at 15 000 × g. Aliquots of the supernatant reacted with 1 mol/L KJ. Extinction was measured at 390 nm, and H_2O_2 quantity was estimated from a previously prepared standard curve.

Statistical analysis. Results are presented as means ± standard error. Differences in mean values were subjected to a one-way ANOVA dispersion analysis between variants of each cultivar, followed by the Fischer's *LSD* (least significant difference) test.

RESULTS

CK priming in the field during the grain filling stage slightly affected growth parameters in both cultivars (Table 1). The modern cv. Geya-1 exhibited an increase in more parameters of productivity after CK priming than the older cv. Sadovo 772. Statistically significant differences between control and CK-primed plants from cv. Geya-1 were found regarding grain number and grain weight per spike as well as grain weight per plant and thousand kernels weight (TKW). In cv. Sadovo 772 only grain number per spike and TKW were slightly increased. Plants from cv. Geya-1 primed with 6-BA had the highest average grain yield (Figure 1A). Statistically significant differences were proved between yields of the three variants of cv. Sadovo 772 with the highest values obtained for kinetin (Figure 1B). Enhanced viability of field primed plants was expressed by higher fresh and dry weight, as well as higher CCI of flag leaves (Figure 2A, B, D). The strongest effect of CK was found on dry weight in cv. Geya-1 and on CCI in cv. Sadovo 772 (Figure 2B, D).

In 5-days-old seedlings from cold stored seeds of primed plants, greater fresh and dry weight was measured in modern cv. Geya-1 compared to older cv. Sadovo 722 (Figure 3). Seedlings of kinetin-primed cv. Geya-1 plants had higher fresh weight than controls and in cv. Sadovo 772 6-BA caused a more prominent increase (Figure 3A). Regarding dry weight, only in cv. Sadovo 772 both CK caused a significant increase (Figure 3B). RWC of 5-days-old seedlings did not show significant variations between variants (Figure 3C). In cv. Geya-1, the highest germination percentage was detected in seeds of 6-BA followed by kinetin-primed plants, while in cv. Sadovo 772, the two CK caused a similar increase (Figure 3D). In cv. Sadovo 772 6-BA priming increased coleoptile length and in cv. Geya-1, a stronger positive effect of kinetin on root growth was found (Figure 3E, F).

Table 1. Growth parameters of field grown wheat plants primed with CK during the grain filling stage

Parameter	Geya-1			Sadovo 772		
	untreated control	priming with 6-BA	priming with kinetin	untreated control	priming with 6-BA	priming with kinetin
Number of grains from spike	38.0 ± 1.61[a]	41.5 ± 1.72b	41.3 ± 1.77[b]	51.1 ± 2.17[a]	56.6 ± 2.65[b]	56.5 ± 2.81[b]
Number of grains from tiller	25.83 ± 2.13[a]	26.33 ± 2.27[a]	26.06 ± 1.84[a]	30.64 ± 1.92[a]	31.61 ± 2.15[a]	33.34 ± 2.45[a]
Number of grains per plant	202 ± 13.38[a]	208.7 ± 16.97[a]	206.8 ± 10.60[a]	265.6 ± 14.01[a]	274 ± 15.52[a]	293.2 ± 20.43[a]
Grain weight per spike (g)	1.68 ± 0.13[a]	1.93 ± 0.12[b]	1.79 ± 0.12[a]	1.85 ± 0.15[a]	2.02 ± 0.14[a]	2.08 ± 0.14[a]
Grain weight per tiller (g)	1.12 ± 0.10[a]	1.19 ± 0.11[a]	1.14 ± 0.09[a]	1.11 ± 0.56[a]	1.15 ± 0.47[a]	1.22 ± 0.91[a]
Grain weight per plant (g)	8.81 ± 0.30[a]	9.54 ± 0.40[b]	9.11 ± 0.39[b]	9.65 ± 0.68[a]	10.16 ± 0.50[a]	10.76 ± 1.01[a]
1 000 kernel weight (TKW, g)	43.6 ± 1.30[a]	47.1 ± 1.20[b]	44.2 ± 1.20[a]	34.6 ± 0.81[a]	36.6 ± 0.68[b]	36.8 ± 0.8[b]

Data are presented as means ± standard error (n = 20). Letters represent significant differences at P < 0.05

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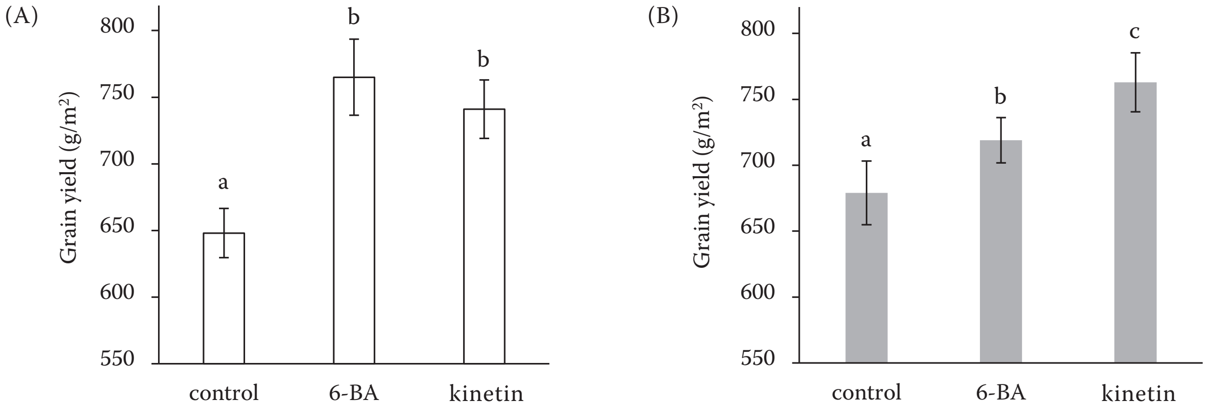

Figure 1. Grain yield of field grown wheat cultivars (A) Geya-1 and (B) Sadovo 772 after priming with 6-benzylaminopurine (6-BA) or kinetin at the grain filling stage. Data are means ± standard error (n = 20). Letters represent significant differences at P < 0.05

Pot grown 15-old-days seedlings of CK-primed plants exhibited a better start and development, which correlated with data from 5-days-old seedlings (Figure 4). A stronger positive effect of kinetin was assessed on fresh weight in cv. Geya-1 exceeding controls by 52%. In both cultivars, germination of 15-days-old seedlings (Figure 4D) was slightly affected, similarly as in 5-days-old seedlings (Figure 3D). Modern cv. Geya-1 was more affected by both CK than the older cv. Sadovo 772, where only kinetin priming significantly increased germination (Figure 4D). CMS values of 5-days-old seedlings were increased only in kinetin-primed cv. Geya-1 (Figure 5A). In 15-days-old seedlings, CMS was lower in controls and increased in 6-BA and kinetin-primed plants of both cultivars (Figure 5B). Accumulation of H_2O_2 in 5-days-old

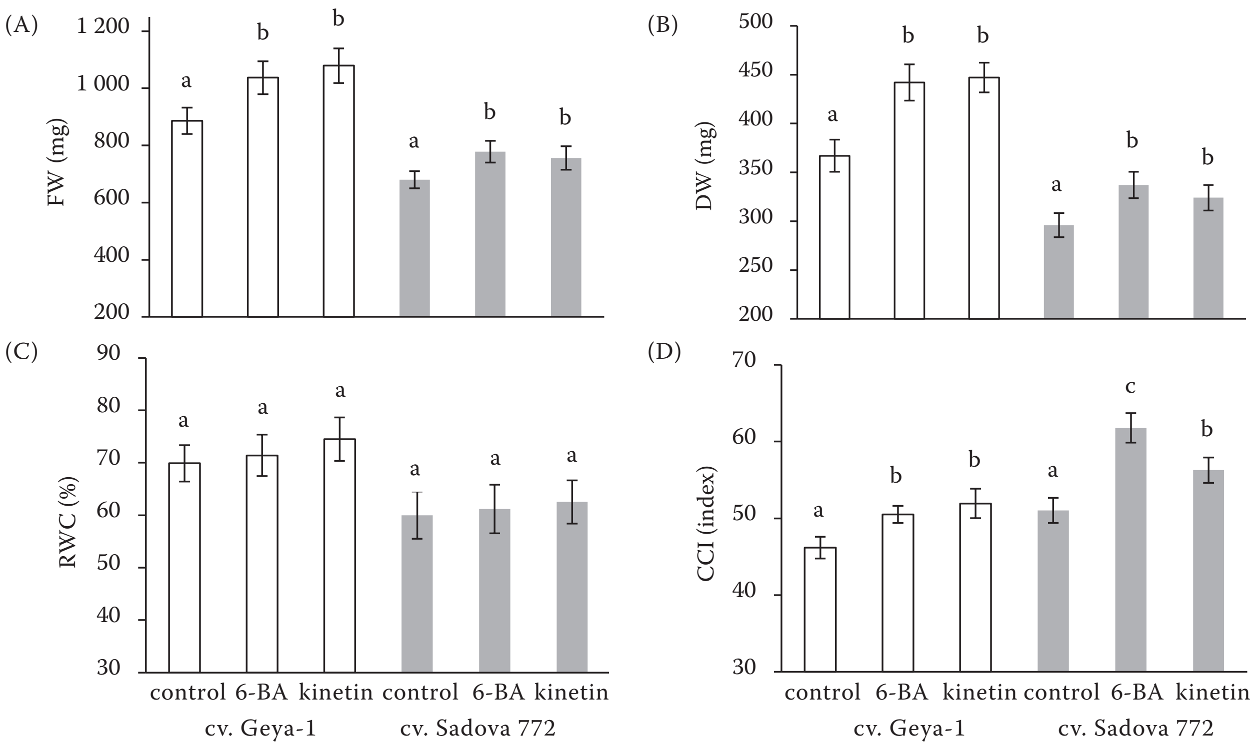

Figure 2. Growth parameters of flag leaves from two wheat cultivars field primed with 6-benzylaminopurine (6-BA) and kinetin during the grain filling stage: (A) fresh weight (FW); (B) dry weight (DW); (C) relative water content (RWC), and (D) chlorophyll content index (CCI). Data are means ± standard error (n = 20). Letters represent significant differences at P < 0.05

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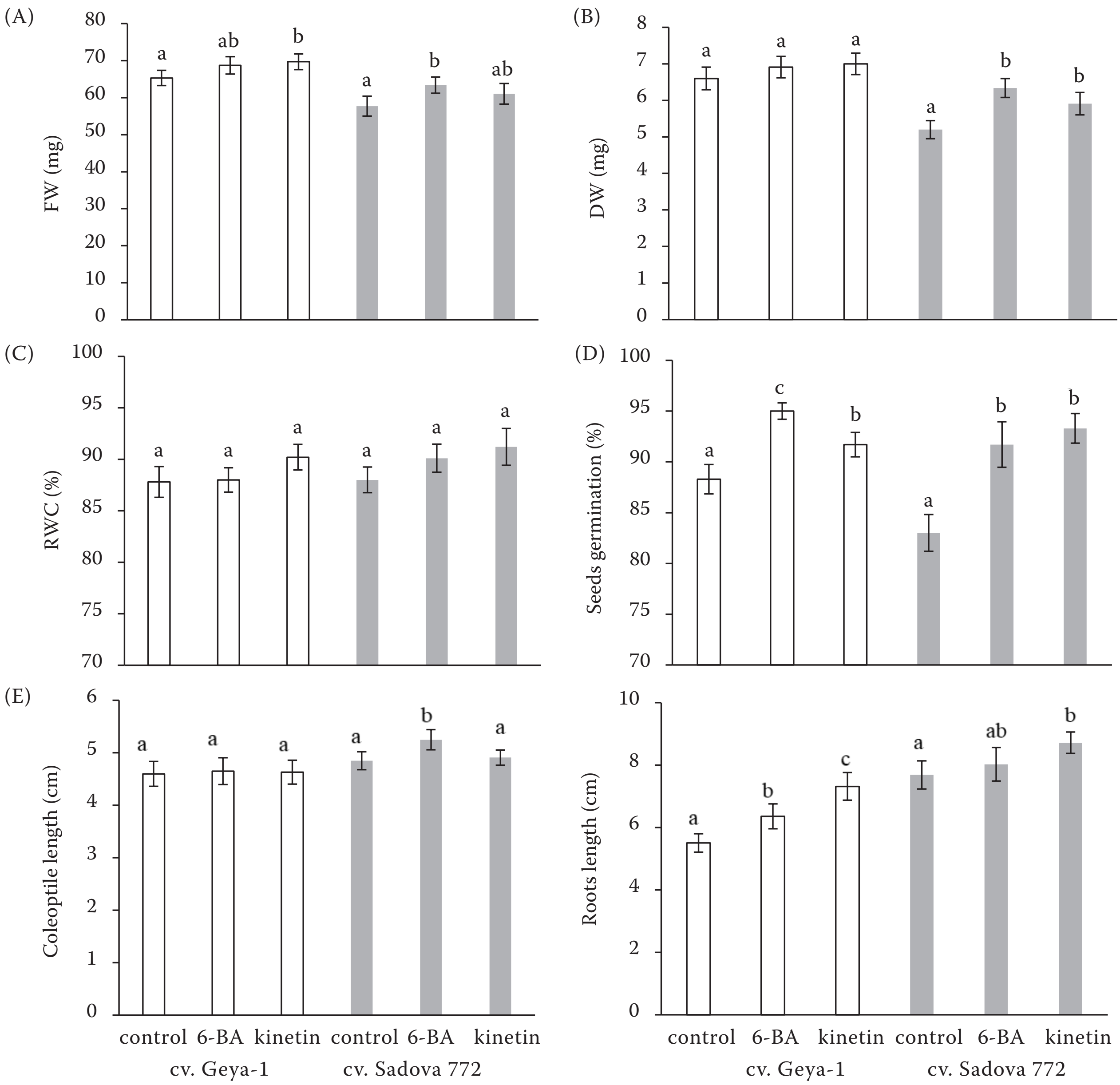

Figure 3. Viability after cold storage of 5-days-old seedlings from wheat plants field primed with cytokinin (CK) during the grain filling stage. (A) fresh weight (FW); (B) dry weight (DW); (C) relative water content (RWC); (D) percentage of germination; (E) coleoptile length, and (F) root length. Data are means ± standard error (n = 10). Letters represent significant differences at $P < 0.05$

seedlings was higher in untreated controls of both cultivars (Figure 6A). In cv. Geya-1, 6-BA caused a greater decrease in hydrogen peroxide (46.6%) than kinetin (10.8%). In cv. Sadovo 772, the lowest H_2O_2 concentration was measured in seedlings of kinetin-primed plants. Lower MDA values were measured in seedlings from CK-primed plants and subsequently cold stored seeds (Figure 6B). A stronger reduction in MDA was observed in 6-BA over kinetin-primed cv. Geya-1 plants while in cv. Sadovo 772, the effect of the two cytokines was similar.

DISCUSSION

Plant hormones, and CKs in particular, play an important role in substances accumulation in seeds and their weight formation while simultaneously defining the response towards specific types of stress (Hai et al. 2020). It was documented that CKs could greatly increase leaf chlorophyll content, chloroplast stability, and net photosynthetic rate (Chang et al. 2015). In this regard, higher values of yield and yield components in plants exogenously primed with

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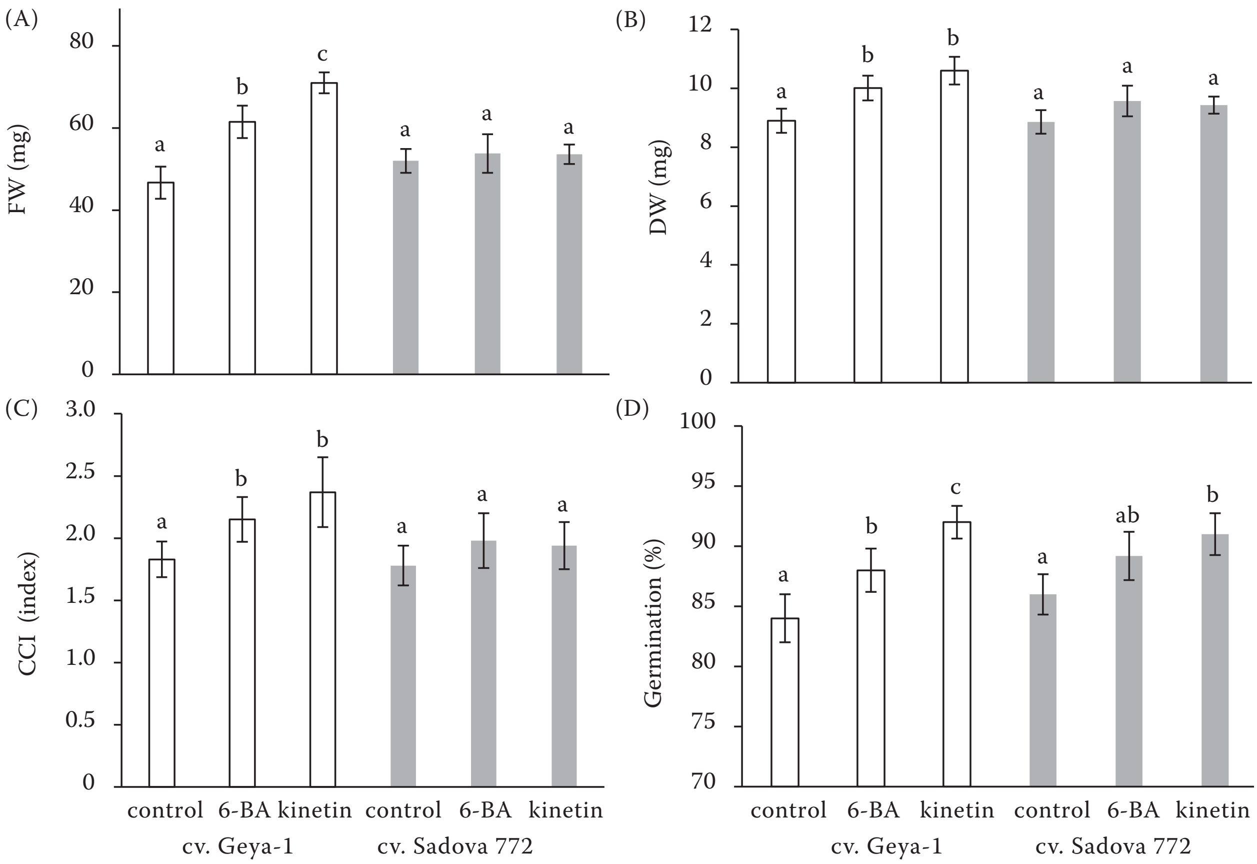

Figure 4. Growth parameters of pot grown 15-days-old seedlings planted from cold stored seeds of 6-benzylaminopurine (6-BA) or kinetin field primed wheat plants and untreated controls: (A) fresh weight (FW); (B) dry weight (DW); (C) chlorophyll content index (CCI), and (D) germination. Data are means ± standard error (n = 20). Letters represent significant differences at $P < 0.05$

6-BA or kinetin at the grain filling stage compared to untreated controls are quite natural and sought effect aimed at increasing seed viability. In our field experiments, increased plant viability after CK priming was conveyed by higher biomass and CCI in both cultivars. Yang et al. (2016) reported similar effects of exogenous 6-BA treatment of wheat cultivars under various meteorological conditions and accounted

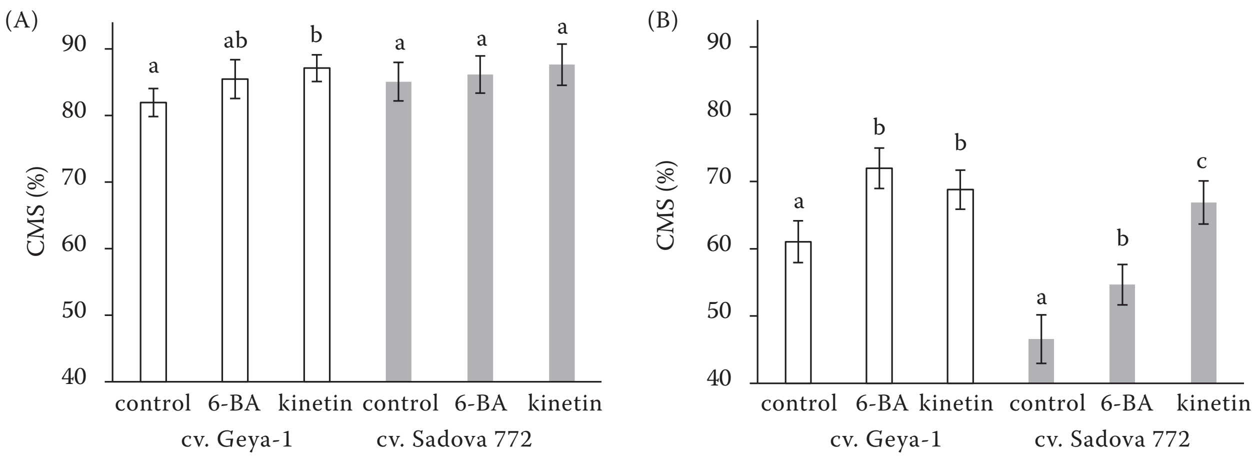

Figure 5. Cell membrane stability (CMS) in leaves of (A) 5-days-old seedlings and (B) 15-days-old seedlings from cold stored seeds 6-benzylaminopurine (6-BA) and kinetin field primed of at the grain filling stage wheat plants. Data are means ± standard error (n = 20). Letters represent significant differences at $P < 0.05$

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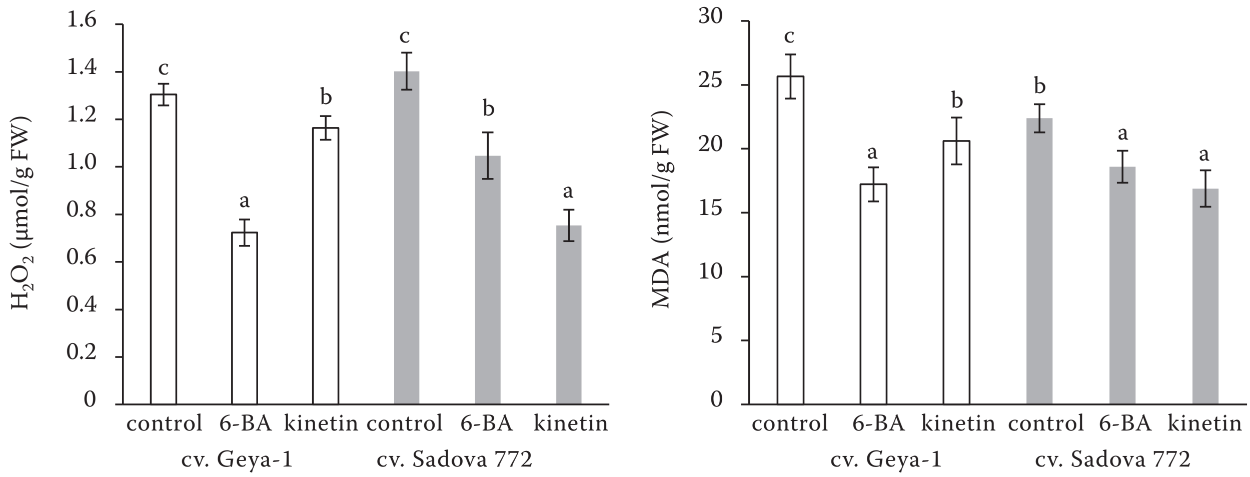

Figure 6. Oxidative stress markers of (A) hydrogen peroxide (H_2O_2) and (B) malondialdehyde (MDA) in 5-days-old seedlings from cold stored seeds of wheat plants field primed with 6-benzylaminopurine (6-BA) or kinetin at the grain filling stage and untreated controls. Data are means ± standard error (n = 10). Letters represent significant differences at $P < 0.05$. FW – fresh weight

higher yield and TKW in stay-green cultivars, such as cv. Geya-1. Exogenous BAP (benzylaminopurine) increased the duration of the grain filling stage in wheat, thus supporting longer active photosynthesis and improved transfer of assimilates toward reproductive organs (Chen et al. 2010, Yang et al. 2016). Our results demonstrate the positive action of exogenously applied CKs, which also contributed to higher seedling vigour and the significant increase in coleoptile length and dry weight of 5-days-old seedlings compared to untreated control plants. A lower accumulation of stress markers (MDA and H_2O_2) and higher CMS in coleoptiles of CK treated plants correlated with higher morphometric parameters and better viability and proved the positive effect of *in situ* added 6-BA and kinetin. These results proved that the induction of oxidative stress and ROS accumulation in seeds could be attributed to the low temperature effect and remaining seed moisture, which was also reported by many other authors (Bailly 2004, Wiebach et al. 2020). The antioxidant effect of CK through inactivation of ROS additionally increased treated seeds viability immediately before germination (Wang et al. 2015, Shu et al. 2016). CK had a more significantly stimulating effect on all morpho-physiological parameters in the modern wheat cultivar than in the older one. Exogenous CK application probably disturbed the balance between CK and auxins in developing seeds during grain formation. Improved seed viability of primed plants could be explained by CK influencing metabolism as well as gene expression. Tissues with higher CK levels attract more nutrients and are metabolically more active. On the other hand, transgenic wheat plants overexpressing the isopentenyl transferase (IPT) gene under developmentally activated modified AtMYB32xs promoter displayed delayed leaf senescence and reduced chlorophyll degradation, which had a beneficial effect on yield (Joshi et al. 2019). Reduced expression of a CKX gene correlated with significantly increased grain number in wheat (Li et al. 2018). CKs are synthesised in roots and are subsequently transported to leaves, but during the grain filling stage, their flow is redirected towards developing seeds. This process of CKs translocation towards seeds at a certain point induces leaf senescence activation (Davies and Gan 2012). CKs sustain longer active photosynthesis during grain filling, enhance the transfer of assimilates to grains, improve yield, and promote higher germination activity under heat stress (Yang et al. 2016). In our study, exogenous application of CKs during the grain filling stage enhanced metabolic activity and assimilate accumulation in seeds, thus increasing growth, yield, germination and seedling vigour following low temperature conservation in genebanks.

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