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Induction of microspore embryogenesis in bread wheat by mannitol pre-treatment is associated with the disruption of endogenous hormone balance and substantial accumulation of auxins

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Abstract

Background Hormonal homeostasis plays a critical role in the regulation of microspore embryogenesis (ME). The balance between endogenous phytohormones must be altered to induce microspore reprogramming from the classical pollen-formation pathway to embryogenic development, but too extensive changes may be detrimental. In the present study, the levels of auxins, cytokinins and abscisic acid were monitored in the anthers of two Polish winter wheat F1 lines and the spring cultivar Pavon highly differentiated in terms of ME effectiveness. Analyses were carried out at subsequent steps of the ME induction procedure that combined low temperature, sodium selenate and mannitol tiller pre-treatment.

Results Of all the factors tested, mannitol induced the most profound effect on phytohormones and their homeostasis in wheat anthers. It significantly increased the accumulation of all auxins and decreased the levels of most cytokinins, while the change in ABA content was limited to cv. Pavon. In an attempt to alleviate this hormonal shock, we tested several modifications of the induction medium hormonal composition and found thidiazuron to be the most promising in stimulating the embryogenic development of wheat microspores.

Conclusions The lack of ABA-driven stress defence responses may be one of the reasons for the low effectiveness of ME induction in winter wheat microspore cultures. Low cytokinin level and a disturbed auxin/cytokinin balance may then be responsible for the morphological abnormalities observed during the next phases of embryogenic microspore development. One possible solution is to modify the hormonal composition of the induction medium with thidiazuron identified as the most promising component.

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Key message
Mannitol, used to induce microspore embryogenesis in wheat, stimulates intense auxin accumulation, which disrupts hormonal homeostasis and can lead to disturbances in the next stages of embryogenic development.

Keywords Abscisic acid, Auxins, Cytokinins, Hormonal homeostasis, Microspore embryogenesis, *Triticum aestivum*

Background

Microspores are the cells of the male gametophyte. They are evolutionarily programmed to develop into pollen grains which initiate a new sporophyte generation upon double fertilisation [1]. However, stress-treated microspores transferred to in vitro culture are able to change their genetically programmed development by initiating a process called microspore embryogenesis (ME). This alternative developmental programme begins with a symmetric cell division which is followed by a sequence of randomly oriented divisions resulting in the formation of the so-called embryo-like structure (ELS) [2–4]. The process runs through successive stages that correspond to the developmental stages of the zygotic embryo; however, various abnormalities and the development of callus tissue, are quite common [5]. Properly developed ELS has the ability to regenerate into plant with a single paternal set of chromosomes (haploid) which could be transformed into fully homozygous doubled haploid (DH) after spontaneous or chemically induced genome doubling. DHs are used in plant breeding to accelerate biological progress and reduce the costs of developing new varieties. They are also highly beneficial in genetic research, bioengineering and basic studies [6, 7].

Among the world's most important crops, wheat occupies one of the top positions [8]. As a result, there are high expectations to increase the efficiency of breeding programmes e.g. by incorporating DH technology. However, in order to effectively alter the developmental fate of microspores for commercial application, it is necessary to recognize the factors that induce the desired reprogramming and to apply the appropriate in vitro culture conditions [9]. Unfortunately, the effectiveness of ME is strongly determined by the genotype of the donor plant and influenced by environmental growth conditions. As a result, the optimal procedure must be developed experimentally through a laborious trial-and-error process. Despite extensive efforts, efficient protocols for ME induction have not yet been developed for many genotypes. These include many wheat genotypes of high breeding value, particularly of winter growth habit.

The most commonly used inducers of ME in wheat are low temperature and heat shock, applied on their own or in combination with starvation and osmotic stress induced by the application of 0.3–1.5 mol/L mannitol [10–13]. Recent studies have shown that the highest effectiveness of ME for Polish highly recalcitrant wheat lines of F1 generation, was obtained as a result of low temperature (4°C) tiller pre-treatment, followed by application of 50 µmol/L sodium selenate (Na_2SeO_4; SSe) and 0.7 mol/L mannitol [14]. However, many of the reprogrammed microspores showed disturbances in the next stages of embryogenic development and died at a phase when multicellular structures were still surrounded by the sporoderm wall or shortly after its rupture.

Any irregularities in microspore embryogenic development are result from some perturbations in molecular, physiological or cytological mechanisms. Disturbances in hormonal homeostasis have particularly severe consequences as phytohormones regulate all physiological and developmental processes in plants [15, 16]. Among different groups of phytohormones, auxins and cytokinins are known to be extremely important in the context of ME induction, while the role of ABA is still questionable [17, 18]. All these compounds not only dynamically interact to control developmental processes, but also play an important role as signalling molecules that initiate defence mechanisms in response to various stress stimuli [19–21].

In order to recognize the causes of abnormalities observed in the embryogenic development of wheat microspores [14], changes in auxins, cytokinins and ABA contents and their homeostasis were monitored in wheat anthers subjected to the ME induction procedure. Based on our previous studies [14, 22], we examined two recalcitrant winter wheat F1 breeding lines (K393 and PO19) and responsive spring wheat cultivar Pavon. A step-by-step analysis was combined with the evaluation of ELS development effectiveness and their ability to regenerate green plants in anther and isolated microspore cultures. Finally, the effectiveness of ME was analysed using the induction media modified in terms of their hormonal composition.

Due to the highly complex and interwoven system of hormonal interactions, it seems impossible at the moment to determine the exact hormonal relationships that determine high ME effectiveness. Gradually uncovering more pieces of this complex puzzle, however, brings us closer to success. The results obtained will have practical application and help to develop a strategy to increase the effectiveness of the ME process in recalcitrant wheat genotypes.

Results

Modifications of auxin profiles and their accumulation in wheat anthers induced by the subsequent steps of the ME induction procedure

Active IAA, two amide conjugates (IAA-*L*-aspartate; IAA-Asp and IAA-*L*-glutamate; IAA-Glu) and the oxidised form of IAA (2-oxoindole-3-acetic acid; oxIAA) were identified in wheat anthers collected from freshly cut tillers (control; Fig. 1).

Total auxin levels ranged from 980 pmol/g DW in PO19 to about 1200 pmol/g DW in K393 and over 4800 pmol/g DW in cv. Pavon (Additional file: Figure S1). Active IAA represent between 33% and 42% of the total auxin pool.

Low temperature had almost no effect on the auxin content in the anthers of all wheat lines/cultivar analysed (Fig. 1). The only exception was a significantly increased level of IAA-Asp in the anthers of K393. At the same time, low temperature caused visible changes in the auxin profiles, reducing the proportion of active IAA in the total auxin pool (Additional file: Figure S1). In K393 anthers, IAA decreased to about 5% of the total auxin content, which was associated with a 30% higher participation of oxIAA and a 7% higher participation of IAA-Asp. A small amount of IAA-Glu was also detected (Fig. 1). A similar decrease in IAA participation was observed in the anthers of cv. Pavon, but it was associated with an almost 30% increase in IAA-Asp. Minor changes were observed in PO19 anthers where the decreased proportion of IAA was accompanied by a slightly increased proportion of both IAA-Asp and oxIAA in the total auxin pool (Fig. 1, Additional file: Figure S1).

Low temperature tiller pre-treatment combined with sodium selenate application also had no effect on the levels of IAA, IAA-Glu and oxIAA in the anthers of all wheat lines/cultivar compared to the control and low temperature treated anthers (Fig. 1). The only significant effect was observed in K393 anthers where the content of IAA-Asp decreased to the level characteristic of the control. The change in auxin profile was only visible in PO19 anthers where the decrease in IAA and IAA-Asp

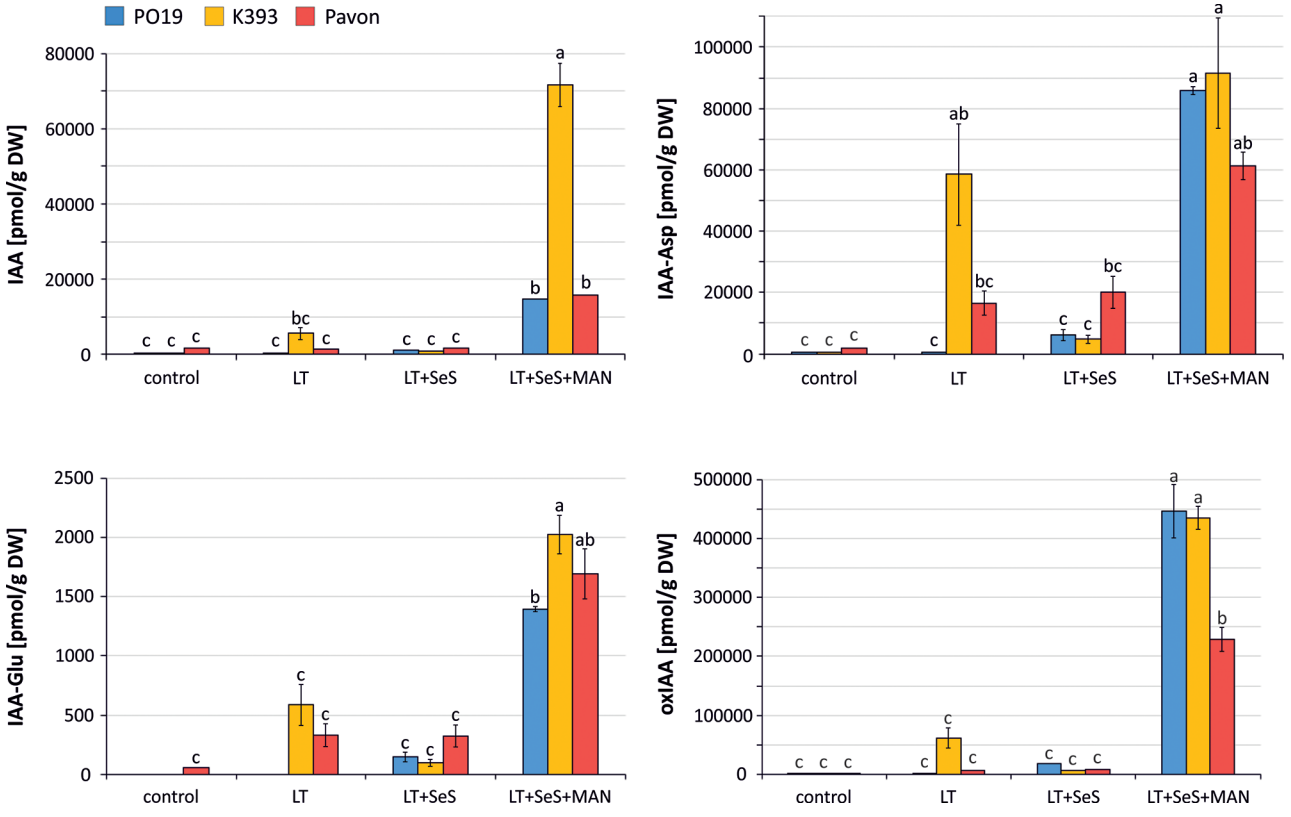


Fig. 1 The content of auxins detected in wheat anthers at the subsequent stages of the procedure used for microspore embryogenesis induction. Data represent mean values ($n = 3$) ± standard error (SE). Data marked with different letters are significantly different at $p \leq 0.05$ according to Duncan's multiple range test. K393 – highly recalcitrant wheat F1 line; PO19 – semi-recalcitrant wheat F1 line; Pavon – highly responsive wheat cultivar; Control – anthers collected from freshly cut tillers; LT – anthers collected from low-temperature pre-treated tillers (21–28 days at 4ºC); LT + SSe – anthers collected from tillers after pre-treatment with low temperature and sodium selenate (last 3 days in 50 µmol/L SSe); LT + SSe + MAN anthers collected from tillers after pre-treatment with low temperature, sodium selenate and mannitol (4 days in 0.7 mol/L mannitol at 20ºC); IAA – indolilo-3-acetic acid; IAA-Asp – IAA-*L*-aspartate; IAA-Glu – IAA-*L*-glutamate; oxIAA – 2-oxoindole-3-acetic acid

proportions was accompanied by a high (more than 3-fold) increase in the oxIAA share (Additional file: Figure S1).

A significant increase in the content of all auxin forms was detected in the anthers of all wheat lines/cultivar studied after the tiller pre-treatment that combined low temperature with sodium selenate and mannitol. As a result of this pre-treatment, oxIAA became dominant, accounting for between 72% and 81% of the total auxin pool (Additional file: Figure S1). Its content was almost twice higher in the anthers of the two winter wheat lines K393 and PO19 compared to cv. Pavon (Fig. 1). Mannitol also induced a significant increase in IAA content, particularly high in the anthers of K393. The percentage of IAA in the total auxin pool ranged from about 3 to 12% (Additional file: Fig. 1S). The levels of IAA-Asp and IAA-Glu also increased significantly in the anthers of all wheat lines/cultivar (Fig. 1). Among them, IAA-Asp was much more abundant, accounting for 15–20% of the total auxin pool, while the level of IAA-Glu was in the range of 0.3–0.6%.

Modifications of cytokinins profiles and its accumulation in wheat anthers induced by the subsequent steps of ME induction procedure

Free base cytokinins: *trans*-zeatin (*t*Z), *cis*-zeatin (*c*Z), isopentenyloadenine (iP), and a number of their conjugates (ribosides, O-glucosides 5'-nucleotides and N-glucosides) were identified in the anthers of the wheat lines/cultivar (Fig. 2).

In the anthers collected from freshly cut tillers (control), the total content of active, free base cytokinins ranged from 115 pmol/g DW in PO19 to 199 pmol/g DW in K393 and 708 pmol/g DW in cv. Pavon. Their contribution to the total cytokinins pool ranged from 7–15% (Additional file: Figure S2). Among them, iP and *c*Z were more abundant, while *t*Z was present in trace amounts (Fig. 2). Similarly, low levels were found for two conjugates – *trans*-zeatin riboside (*t*ZR) and dihydrozeatin riboside (DHZR). The levels of iPR and 5'-nucleotides (*c*ZR5'MP and iPR5'MP) accounted for up to 11% and 9% of the total cytokinins pool, respectively. Three conjugates (*cis*-zeatin riboside (*c*ZR), *cis*-zeatin-O-glucoside (cZOG) and *cis*-zeatin riboside-O-glucoside (cZROG)) dominated among detected cytokinins (Fig. 2). The highest level of *c*ZR, reaching 43% of the total cytokinins pool, was detected in the anthers of cv. Pavon. In the anthers of PO19, the highest percentage (34%) was represented by *c*ZROG. The accumulation of these two conjugates was almost equal (at 21–22%) in the anthers of K393. The proportion of *c*ZOG was also high in all the analysed wheat lines/cultivar, ranging from 10 to 25% of the total cytokinins pool. The total content of irreversibly deactivated cytokinins forms (*trans*-zeatin-N9-glucoside (*t*Z9G), *cis*-zeatin-N9-glucoside (*c*Z9G) and N6-isopentenyloadenine-N9-glucoside (iP9G) represented 3–10% of total cytokinins pool (Additional file: Figure S2).

A comparison of hormonal profiles between the studied wheats revealed that anthers of cv. Pavon contained relatively high level of free, biologically active cytokinins (particularly *c*Z), that accounted for 15% of the total cytokinins pool (Fig. 2, Additional file: Figure S2). Similarly, much higher were the levels of *t*ZR, *c*ZR, *c*ZR5'MP, DHZR and iPR detected in cv. Pavon anthers compared to the PO19 and K393 anthers (Fig. 2).

The total content of active cytokinins (free bases) after low temperature tiller pre-treatment increased to over 500 pmol/g DW in PO19 anthers, remained unchanged in K393 anthers and decreased to 150 pmol/g DW in anthers of cv. Pavon. Their share in the total pool of cytokinins ranged from 4 to 10% (Additional file: Figure S2). The content of the three dominant cytokinins conjugates (*c*ZR, *c*ZOG and *c*ZROG) was very similar in the anthers of all lines/cultivar studied (Fig. 2).

Low temperature increased the levels of most active and reversibly conjugated cytokinins in PO19 and K393 anthers (Fig. 2). Significant decrease was observed only in the case of *t*Z in K393 anthers. The opposite trend was observed in cv. Pavon anthers where low temperature caused a decrease in the levels of *t*Z, *t*ZR, DHZR, *c*Z, *c*ZR, *c*ZR5'MP and iP. It simultaneously increased the levels of *c*ZOG, *c*ZROG and iPR5'MP. The levels of irreversibly conjugated cytokinins (*t*Z9G, *c*Z9G, iP9G) remained unchanged, even though their share in the total cytokinins pool decreased slightly to 3–4% in anthers of K393 and PO19 (Additional file: Figure S2).

Application of sodium selenate only slightly altered the profile of cytokinins in PO19 anthers (Additional file: Figure S2). A decrease in the accumulation of *c*ZR was most pronounced as its share in the total cytokinin pool dropped from 30 to 17%. The levels of some other forms of zeatin (*t*Z, *t*ZR, DHZR, *c*ZR, *c*ZR5'MP) also decreased, as opposed to the levels of iP and iPR5'MP (Fig. 2). The same tiller pre-treatment had almost no effect on cytokinin content and composition in the anthers of K393. The increased level of iP (Fig. 2) was the only one significant change. A significant increase in the level of *t*ZR, DHZR, *c*Z, *c*ZR and iPR was observed in Pavon anthers (Fig. 2). With only one exception (iP9G in PO19 anthers), the content of N-glucosides remained unchanged and represented 3–5% of the total cytokinins pool (Fig. 2, Additional file: Figure S2).

In the anthers of all lines/cultivar studied, low temperature treatment, combined with sodium selenate and mannitol, significantly elevated the accumulation of *c*ZOG (Fig. 2), which was associated with its increased share in the total cytokinin pool to 33–34%. The share of biologically active forms in the total cytokinins pool reached

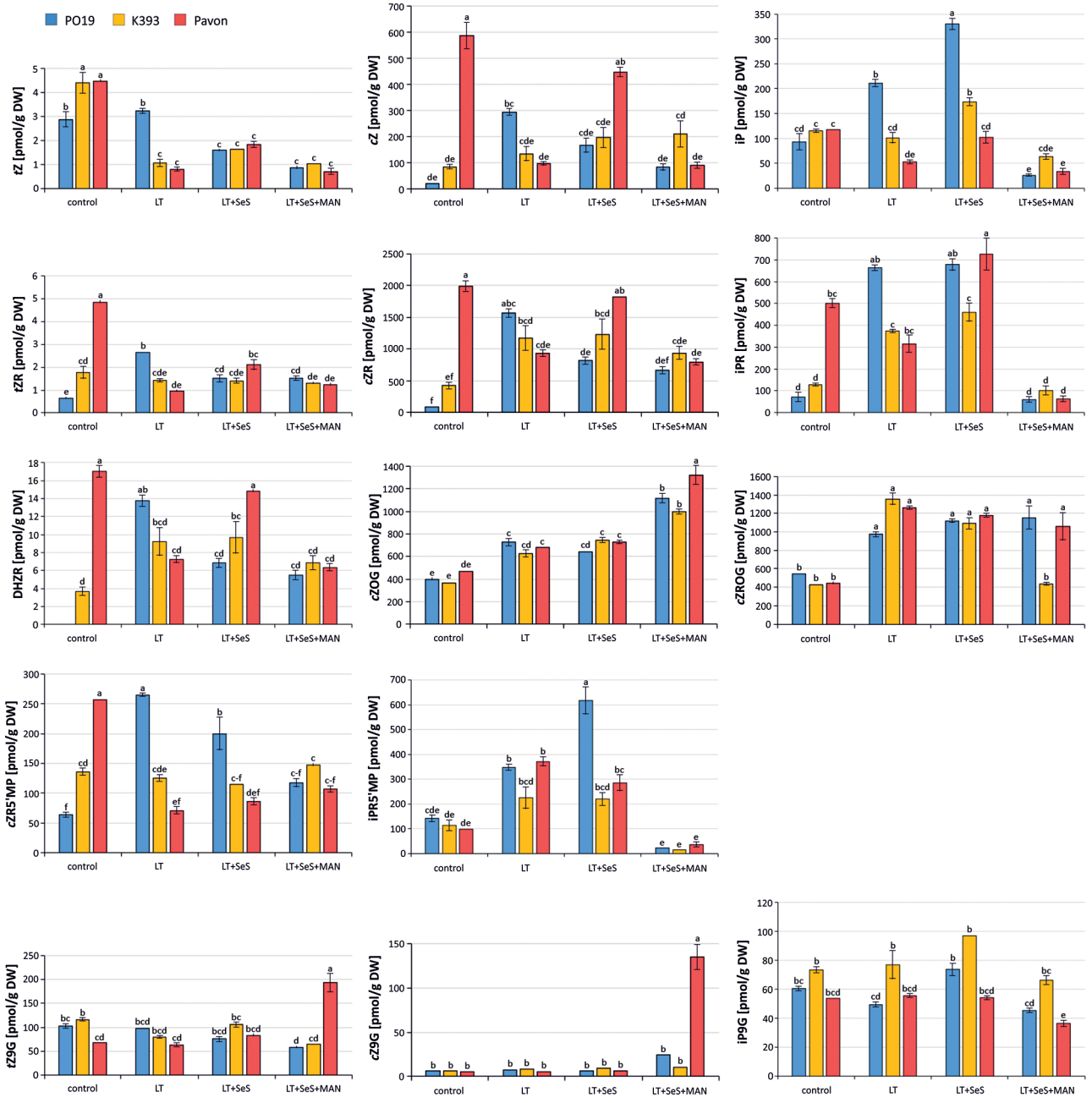


Fig. 2 The content of cytokinins [pmol/g DW] detected in wheat anthers at the subsequent stages of the procedure used for microspore embryogenesis induction. Data represent mean values ($n = 3$) ± SE. Data marked with different letters are significantly different at $p \leq 0.05$ according to Duncan's multiple range test. K393 – highly recalcitrant wheat F1 line; PO19 – semi-recalcitrant wheat F1 line; Pavon – highly responsive wheat cultivar; Control – anthers collected from freshly cut tillers; LT – anthers collected from low-temperature pre-treated tillers (21–28 days at 4°C); LT + SSe – anthers collected from tillers after pre-treatment with low temperature and sodium selenate (last 3 days in 50 µmol/L SSe); LT + SSe + MAN anthers collected from tillers after pre-treatment with low temperature, sodium selenate and mannitol (4 days in 0.7 mol/dm^3 mannitol at 20°C); *t*Z – *trans*-zeatin; *t*ZR – *trans*-zeatin riboside; *t*Z9G – *trans*-zeatin-N9-glucoside; DHZR – dihydrozeatin riboside; *c*Z – *cis*-zeatin; *c*ZR – *cis*-zeatin riboside; *c*ZOG – *cis*-zeatin-O-glucoside; *c*ZROG – *cis*-zeatin riboside-O-glucoside; *c*Z9G – *cis*-zeatin-N9-glucoside; *c*ZR5'MP – *cis*-zeatin riboside-5'-monophosphate; iP – N6-isopentenyladenine; iPR – N6-isopentenyladenosine riboside; iP9G – N6-isopentenyladenine-9-glucoside; iPR5'MP – N6-isopentenyladenosine riboside-5'-monophosphate

3% for PO19 and cv. Pavon and 9% for K393. Uniform for all wheat lines/cultivar was a strong reduction in the levels of iP and its conjugates, iPR and iPR5'MP (Fig. 2). The decrease of *c*ZR5'MP level in PO19 anthers and of *c*ZROG level in K393 anthers was genotype specific. This combined tiller pre-treatment was associated with reduced levels of *c*Z, *c*ZR, *t*ZR and DHZR and increased accumulation of *t*Z9G and *c*Z9G only in the anthers of cv.

Pavon. The content of iP9G decreased in the anthers of PO19 and cv. Pavon. The share of non-active N9-glucosides in the total cytokinin pool remained unchanged at 4–5% in the anthers of K393 and PO19 and increased to 9% in the anthers of cv. Pavon (Additional file: Figure S2).

Modifications of ABA accumulation in wheat anthers induced by the subsequent steps of ME induction procedure

The level of ABA in anthers collected from freshly cut tillers of winter wheat lines PO19 and K393 ranged from approximately 350 to 400 pmol/g DW (Fig. 3). It remained relatively stable throughout the whole ME induction procedure. ABA content in anthers collected from freshly cut tillers of cv. Pavon was circa 1000 pmol/g DW. It changed profoundly in response to the subsequent steps of the procedure. Pre-treatment with low temperature increased ABA content more than four times. After the application of sodium selenate it decreased to the level characteristic of the control. Additional pre-treatment with mannitol again rapidly boosted its accumulation (Fig. 3).

Changes in hormonal homeostasis of wheat anthers during ME-induction procedure

Detailed analysis of the entire spectrum of auxins and cytokinins in wheat anthers of the studied lines/cultivar made it possible to show changes in hormonal homeostasis associated with the subsequent stages of the ME induction procedure (Fig. 4a–b).

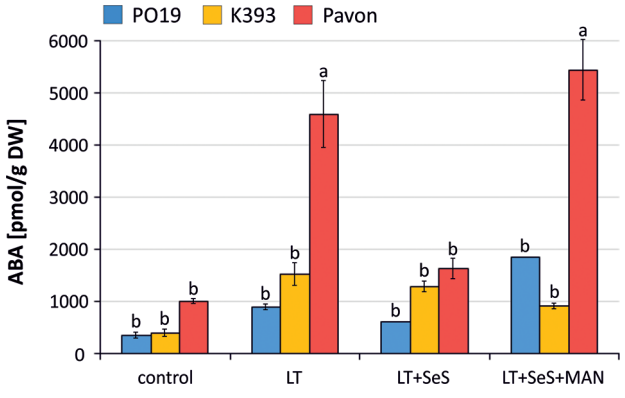


Fig. 3 ABA content [pmol/g DW] detected in wheat anthers at the subsequent stages of the procedure used for microspore embryogenesis induction. Data represent mean values ($n = 3$) ± SE. Data marked with different letters are significantly different at $p \leq 0.05$ according to Duncan's multiple range test. K393 – highly recalcitrant wheat F1 line; PO19 – semi-recalcitrant wheat F1 line; Pavon – highly responsive wheat cultivar; Control – anthers collected from freshly cut tillers; LT – anthers collected from low-temperature pre-treated tillers (21–28 days at 4ºC); LT + SSe – anthers collected from tillers after pre-treatment with low temperature and sodium selenate (last 3 days in 50 µmol/L SSe); LT + SSe + MAN anthers collected from tillers after pre-treatment with low temperature, sodium selenate and mannitol (4 days in 0.7 mol/L mannitol at 20ºC)

Low temperature tiller pre-treatment induced some genotype-specific changes in the hormonal homeostasis of wheat anthers (Fig. 4a). It visibly increased the ratio of active auxin/active cytokinins (IAA/CK_A) in K393 and cv. Pavon anthers, and only slightly decreased this ratio in the anthers of PO19 (Fig. 4b). For all wheat lines/cultivar studied, the application of sodium selenate restored the hormonal balance characteristic of the anthers collected from non-pretreated tillers (control). The whole procedure, combining low temperature, sodium selenate and mannitol induced a significant accumulation of IAA associated with a 1.4- to 4.5-fold decrease in active cytokinins content. It resulted in a rapid increase in the IAA/CK_A ratio. This effect was particularly strong in K393 anthers, causing an approximately 100-fold increase in IAA/CK_A compared to the control. The direction and amplitude of changes observed in ABA levels were also genotype specific (Fig. 4a). However, the effect of IAA accumulation prevailed, visibly increasing IAA/ABA ratio in all wheat lines/cultivar which was most pronounced in K393 anthers (Fig. 4b). The CK_A/ABA ratio remained stable at a relatively low level in all the wheats studied, irrespective of the tiller pre-treatment.

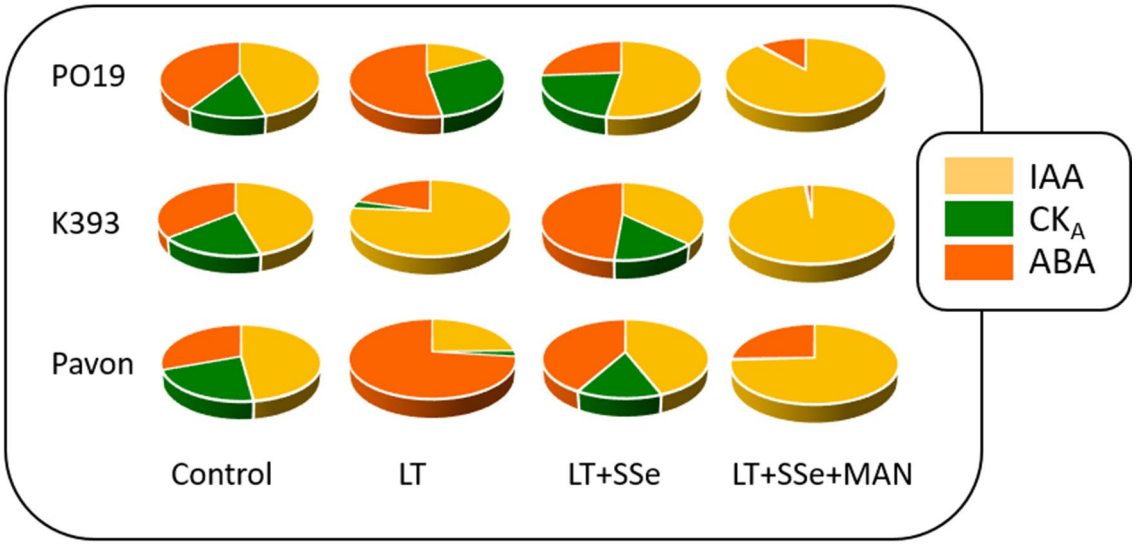
Effectiveness of microspore embryogenesis in anther cultures and isolated microspore cultures of wheat lines PO19 and K393, and cv. Pavon

The effectiveness of ME induction in anther cultures on a standard induction medium varied significantly between two F1 winter hybrid lines (PO19, K393) and the spring cv. Pavon. Tiller pre-treatment with low temperature, sodium selenate and mannitol resulted in an average number of 3 ELS/spike produced in the anther cultures of PO19 and K393 (Fig. 5). Green regenerants with an effectiveness of 0.5 GR/spike were obtained for PO19 only. No albino regenerants (AR) were produced for either hybrid line. The same pre-treatment of cv. Pavon tillers resulted in the formation of 39 ELS/spike, whereas plant regeneration reached 8 GR/spike and 9 AR/spike (Fig. 5).

Mean yield of isolated microspores varied visibly among the studied wheat lines/cultivar. Approximately 22,000 microspores were isolated per spike of K393 after tiller pre-treatment with low temperature, sodium selenate and mannitol. The microspore yield for PO19 (48,000 microspores per spike) and cv. Pavon (54,500 microspores per spike) was more than two times higher. Mean viability of microspore suspensions varied from 40% for K393 to 52% for cv. Pavon and 75% for PO19.

Microscopic observations revealed that many potentially embryogenic, enlarged microspores showed various aberrations from the first day of in vitro culture (Fig. 6). These included structures resulting from a supposedly asymmetric division of the microspore with two nuclei that exhibited different patterns of organization (Fig. 6a)

a.



b.

Line/ Cultivar	Pre-treatment	IAA/CK$_A$	IAA/ABA	CK$_A$/ABA
PO19	Control	3.4	1.1	0.3
	LT	0.6	0.3	0.6
	LT+SeS	2.5	2.0	0.8
	LT+SeS+MAN	136	7.9	0.1
K393	Control	2.5	1.3	0.5
	LT	24.3	3.7	0.2
	LT+SeS	2.7	0.8	0.3
	LT+SeS+MAN	263	78.4	0.3
Pavon	Control	2.2	1.6	0.7
	LT	10.3	0.3	0.03
	LT+SeS	3.1	1.1	0.3
	LT+SeS+MAN	130	2.9	0.02

Fig. 4 Hormonal composition of wheat anthers at the subsequent stages of the procedure used for microspore embryogenesis induction. **a**. The percentage of biologically active auxin (IAA), cytokinins (CK$_A$) and abscisic acid (ABA). **b**. The ratio of IAA/CK$_A$, IAA/ABA and CK$_A$/ABA. CK$_A$ (content of biologically active cytokinins) = *tZ* + *cZ* + iP. K393 – highly recalcitrant wheat F1 line; PO19 – semi-recalcitrant wheat F1 line; Pavon – highly responsive wheat cultivar; Control – anthers collected from freshly cut tillers; LT – anthers collected from low-temperature pre-treated tillers (21–28 days at 4°C); LT + SSe – anthers collected from tillers after pre-treatment with low temperature and sodium selenate (last 3 days in 50 µmol/L SSe); LT + SSe + MAN anthers collected from tillers after pre-treatment with low temperature, sodium selenate and mannitol (4 days in 0.7 mol/L mannitol at 20°C)

or different sizes (Fig. 6b). Many microspores accumulated starch grains after division, typically in close proximity to the symmetric nuclei (Fig. 6c). There were also structures with a fragmented vacuole, but with vegetative and generative nuclei (Fig. 6d). During the next three weeks of in vitro culture, many multicellular structures ruptured and the cytoplast burst (Fig. 6e-f). For the first time, we observed multicellular structures producing single elongated suspensor-like cells (Fig. 6g-h). The development of some aberrant phenotypes stopped at a few cells or at a more advanced stage after they were released from the exine (Fig. 6i-n). Some of the proembryos resembled more or less compact callus-like structures composed of a few to several cells (Fig. 6o-u).

Abnormal phenotypes were arrested in their development and did not reach the size of > 1 mm required for transfer to the regeneration medium. Consequently, no properly developed ELS were produced in the isolated

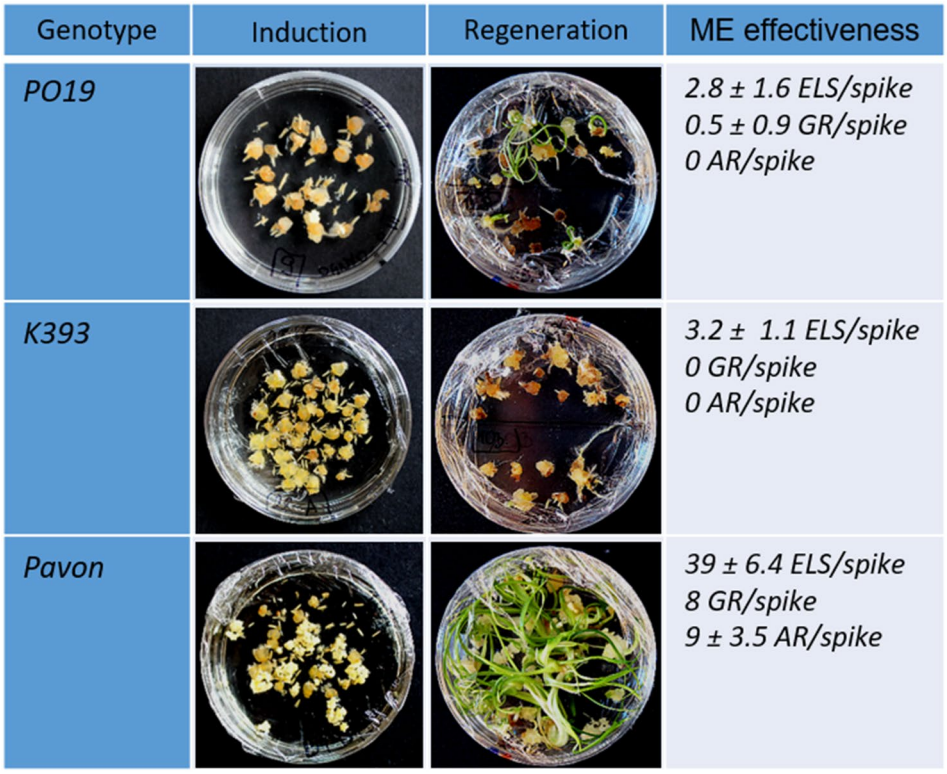


Fig. 5 Effectiveness of microspore embryogenesis (ME) in anther cultures of wheat lines PO19 and K393 and cv. Pavon after tiller pre-treatment that combined low temperature, sodium selenate and mannitol. ELS/spike – the average number of embryo-like structures per spike ± SE; GR/spike – the average number of green regenerants per spike ± SE; AR/spike – the average number of albino regenerants per spike ± SE

microspore cultures of PO19 and K393 (Fig. 7). The same pre-treatment resulted in the formation of 9 ELS per 100,000 microspores in the isolated microspore cultures of cv. Pavon. ELS regenerated plants with the effectiveness of 0.7 GR per 100,000 microspores, while albino regenerants were not observed.

The effect of the induction medium's hormonal composition on wheat ME effectiveness in anther and isolated microspore cultures

Several modifications of the induction medium's hormonal composition were tested following an analysis of the endogenous hormonal profile using isolated microspore cultures of cv. Pavon and K393 (Fig. 8). Compared with the standardly used KBP medium that contained 0.9 mg/L BAP, all the modifications improved ME induction effectiveness. For cv. Pavon, the number of ELS varied from 22 to 103 per 100,000 microspores with the highest effectiveness obtained on KBP medium supplemented with 1 mg/L Picloram. A slightly lower average number of ELS was observed on KBP medium supplemented with 2 mg/L TDZ, but this medium was the most effective in stimulating plant regeneration (Fig. 8). ELS production was observed on two modified KBP media in isolated microspore cultures of line K393. Both of them contained 1 mg/L TDZ. Plant regeneration was observed only on the medium additionally supplemented with 0.5 mg/L *t*Z (Fig. 8).

Discussion

Plant hormones, in particular auxins and cytokinins, are key players in the regulation of ME, starting from microspore reprogramming, through ELS formation and plant regeneration, to acclimation to *ex-vitro* conditions [18]. Modern analytical tools make it possible to precisely identify these signalling molecules even at very low levels, but drawing clear conclusions is not easy because both auxins and particularly cytokinins exist in various free and conjugated forms with different biological activities. Their biosynthesis is genetically and epigenetically controlled, but then their activity is altered by tissue specificity, developmental stage and plant physiological condition that regulate their metabolism, transportation and perception/signal transduction [23–26]. In addition, hormonal homeostasis changes dynamically in response to different environmental stimuli as phytohormone crosstalk coordinates plant stress response and determines the level of stress tolerance and adaptation [27].

In our previous studies on ME in rapeseed and triticale, phytohormone contents and hormonal homeostasis was monitored on a much smaller scale [17, 28, 29]. The results obtained in this study are comparable as

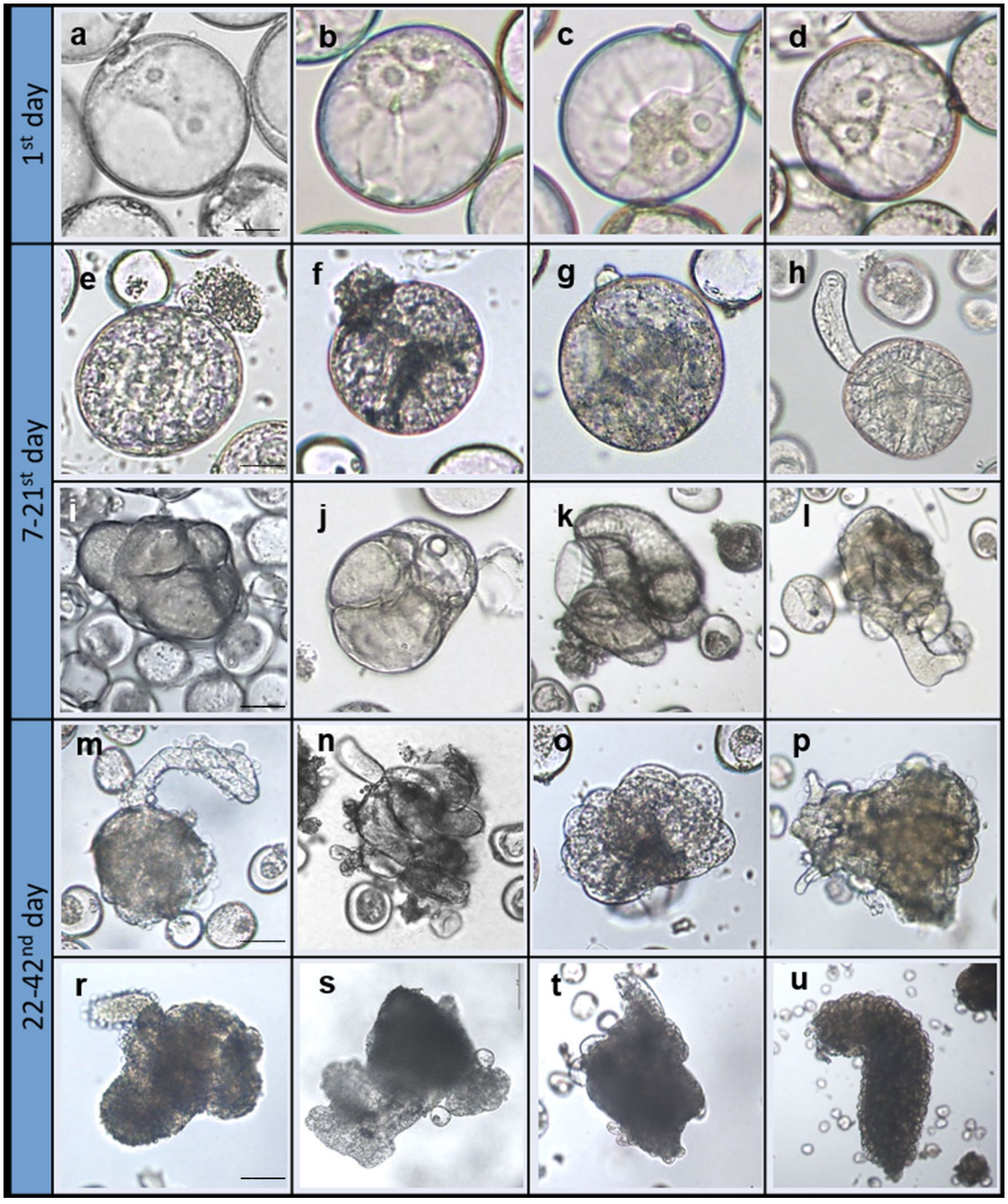


Fig. 6 Disturbances in the embryogenic development of microspores after tiller pre-treatment that combined low temperature, sodium selenate and mannitol. Randomly selected aberrant phenotypes of embryogenic structures at different developmental stages from day 1 to 42 of in vitro culture. Scale: a–d 20 µm; e–h 30 µm; i–l 40 µm; m–p 50 µm; r–u 100 µm

auxins identified in wheat anthers have previously been detected in isolated triticale microspores [28]. According to some authors, the relatively high level of biologically inactive IAA-Asp found in wheat anthers isolated from freshly cut tillers may serve as an IAA reserve [15, 30, 31]. In contrast, a recent study in *Arabidopsis thaliana* showed that two IAA-amino acid conjugates (IAA-Asp and IAA-Glu) can enter the catabolic pathway. Both molecules are first converted to oxIAA-Asp and oxIAA-Glu by dioxygenase for auxin oxidation 1 (DAO1) and then hydrolysed by ILR1/ILL aminohydrolase to oxIAA [32, 33]. There are also some studies that suggests a direct signalling function of IAA-Asp in some developmental processes, including embryo development [34, 35]. A

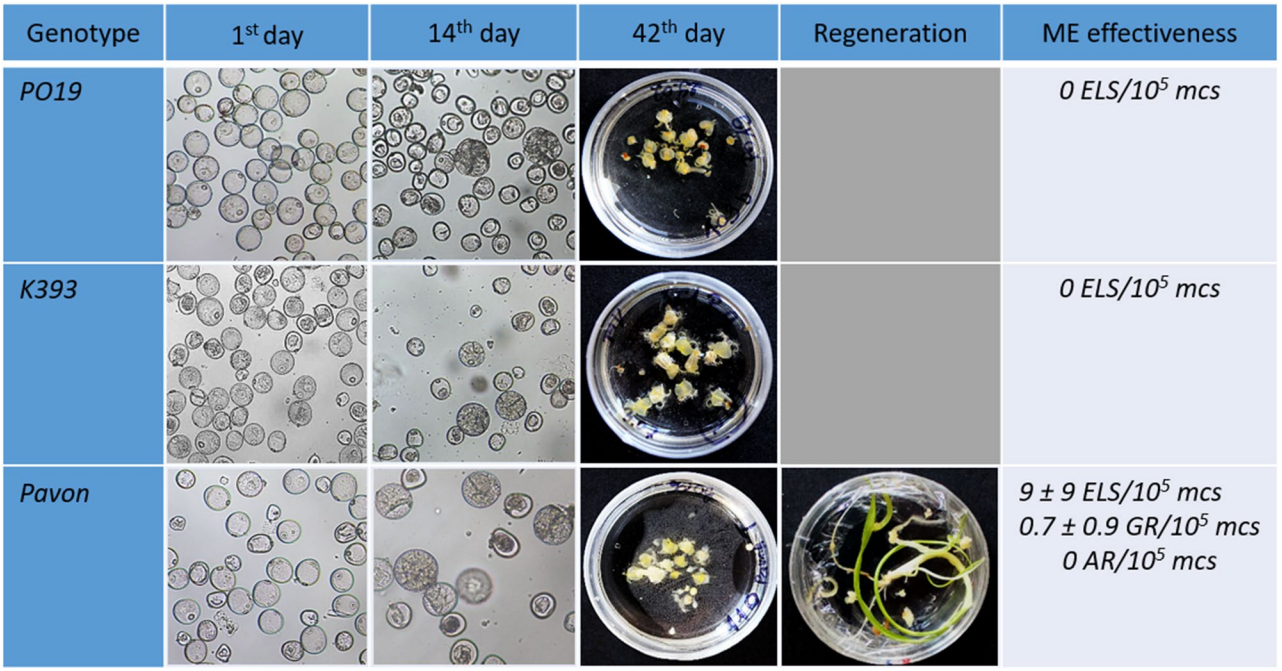


Fig. 7 Effectiveness of microspore embryogenesis (ME) in isolated microspore cultures of wheat lines (PO19, K393) and cv. Pavon after tiller pre-treatment that combined low temperature, sodium selenate and mannitol. Microspore suspensions on the 1st, 14th and 42th day of in vitro culture on the standard induction medium and after about 21 days of in vitro culture on the regeneration medium. ELS/10^5 mcs – the average number of embryo-like structures per 100,000 microspores; GR/10^5 mcs – the average number of green regenerants per 100,000 microspores; AR – the average number of albino regenerants per 100,000 microspores

very low level of the most active cytokinin (*t*Z) detected in wheat anthers is also consistent with data reported for triticale [28, 29]. Similarly, the content of two other free bases *c*Z and iP and their derivatives was much higher in both in triticale and wheat. The levels of reversible conjugates, ribosides (the main forms used for transportation) and O-glucosides (chemically stable storage forms resistant to enzymatic degradation), were particularly high in wheat anthers [36]. Wheat and triticale anthers collected from non-pretreated tillers were also characterised by quite similar levels of ABA accumulation [29].

In general, the hormonal homeostasis of anthers isolated from non-pretreated tillers of the wheat lines/cultivars studied was very similar. We found only a few differences in the levels of *c*Z and some riboside derivatives which were significantly higher in the anthers of cv. Pavon compared to two F1 winter breeding lines. This seems to confirm the hypothesis that indicates an active role of *c*Z in plant development and stress defence [37]. It has been suggested that *t*Z, which promotes intensive cell division, is replaced by *c*Z at the stage of suppressed growth, reorganization of energy expenditure and metabolic adjustment [38]. The fact that all these processes accompany microspore reprogramming and ME induction is supported by data from transcriptome and metabolome analyses [39, 40].

The response to low temperature tiller pre-treatment (21–28 days at 4°C) was genotype-specific, which indicates the induction of different stress defence mechanisms. The effects of low temperature observed in the anthers of two F1 wheat lines were much less pronounced (limited to increased accumulation of IAA-Asp and some cytokinins), probably due to higher cold tolerance typical of winter-type forms. Similarly, the increased level of ABA detected only in the anthers of cv. Pavon suggests its higher sensitivity to low temperature. Both auxins and cytokinins are known to interact with typical stress hormones, such as ABA, in an interconnected network that coordinates plant stress defence [41]. ABA accumulation was also observed in our study in responsive cv. Pavon after mannitol application and in other studies as a response to the stresses used for ME induction [42–47]. Even though some of these reports suggest its positive function, the level of ABA accumulation was not associated with the responsiveness to ME induction in our previous studies on winter triticale and oilseed rape [17, 48]. It was also demonstrated that pre-treatment with exogenous ABA had either no or negative impact on the effectiveness of ME induction, while the application of inhibitors of ABA biosynthesis reduced the frequency of plant regeneration [29, 49]. Considering all this data, it seems that a certain genotype-specific endogenous level of ABA is required for effective microspore

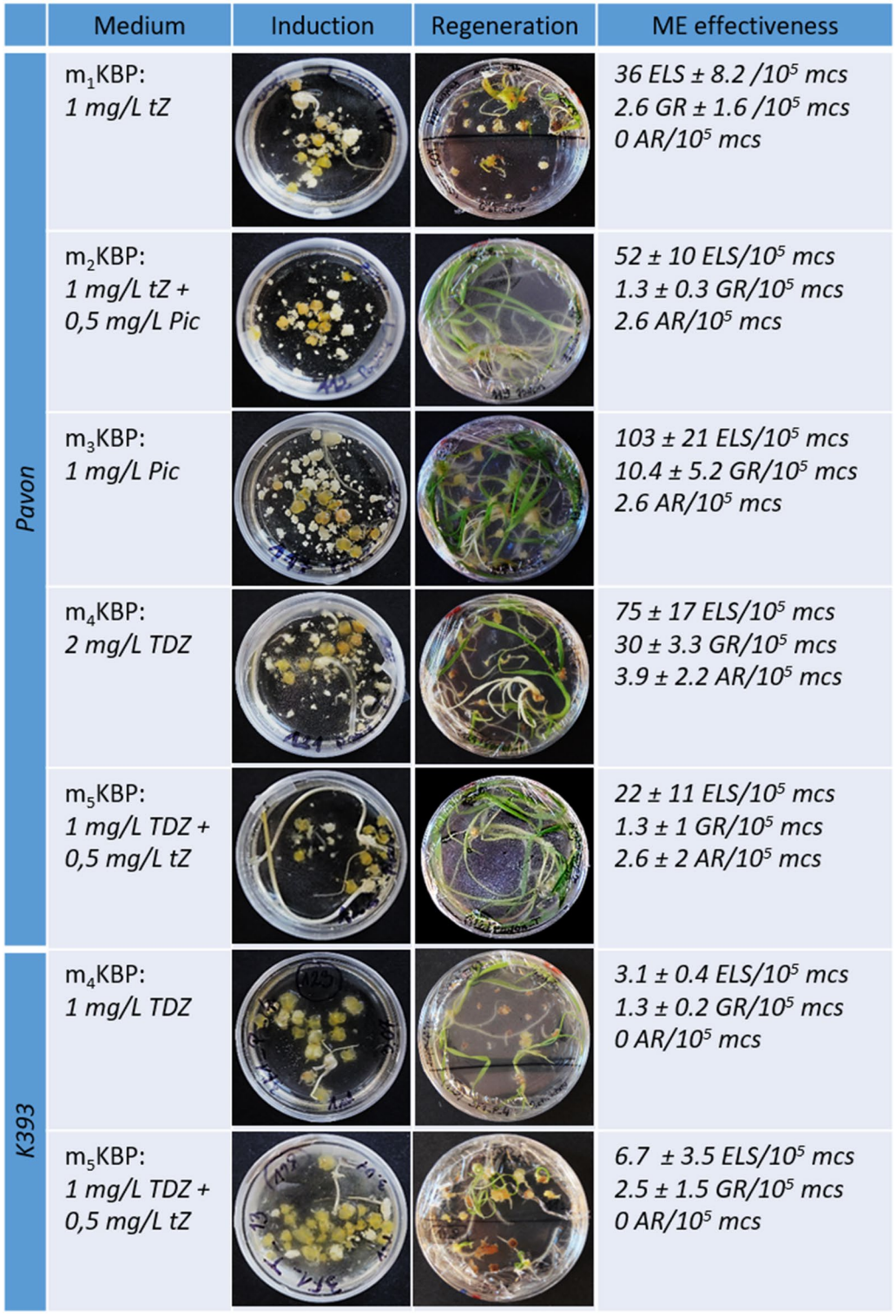


Fig. 8 Effectiveness of microspore embryogenesis (ME) in isolated microspore cultures of cv. Pavon and line K393 on modified induction media after tiller pre-treatment that combined low temperature, sodium selenate and mannitol. Isolated microspore cultures after 28 days of in vitro culture on modified induction media and after 21 days of in vitro culture on the regeneration medium. *tZ* – *trans*-Zeatin; Pic – Picloram; TDZ – thidiazuron; ELS/10^5 mcs – the average number of embryo-like structures per 100,000 microspores; GR/10^5 mcs – the average number of green regenerants per 100,000 microspores; AR – the average number of albino regenerants per 100,000 microspores

reprogramming. The beneficial effect of ABA could result from the induction of the cell stress defence system or upregulation of some ABA-responsive genes. It was reported that the accumulation of ABA in barley microspores inhibited apoptosis and increased microspore viability [50]. Similar conclusions were drawn from our studies on triticale where stress-induced increase in ABA level was associated with higher microspore viability [51]. ABA-responsive genes that encode LATE EMBRYOGENESIS ABUNDANT (LEA) proteins may also play a role. Their accumulation is usually associated with the late stage of seed maturation, but their protective functions could also be significant for the in vitro ELS development [52, 53]. A recently published transcriptome analysis of barley microspores subjected to mannitol pre-treatment revealed that five genes of the LEA family were among the candidates for ME markers [54], which supports the hypothesis on this specific pathway of ABA involvement in microspore reprogramming.

Most of the effects described were alleviated when low temperature tiller pre-treatment was combined with the application of selenium (Se). At low doses, Se was shown to have positive effects on plant growth, development and stress tolerance [55]. It stimulated photosynthesis, increased accumulation of carbohydrates and secondary metabolites. It additionaly activated antioxidant enzymes, such as superoxide dismutase, catalase, glutathione or ascorbate peroxidases, reducing the generation of reactive oxygen species (ROS) and protecting cells from the effects of various stress factors such as low temperature, dehydration or heavy metals [56]. The beneficial effect of sodium selenate on wheat microspore viability and reprogramming frequency has been reported previously [14]. Fluctuations in auxin and ABA levels observed in our study seem to confirm the stress-relieving effect of Se application. However, the changes in auxins and cytokinins induced by low temperature were not reversed and even exacerbated in some cases. The precise mechanisms that underlie these effects remain to be revealed.

The most profound changes in the hormonal balance of wheat anthers were induced by pre-treatment with mannitol. This six-carbon sugar alcohol is naturally synthesised by over a hundred species of higher plants for which it acts as a reservoir carbohydrate and osmoprotectant [57]. It stabilises the aqueous layers around various molecules, hence protecting them from desiccation. It also increases cell tolerance to oxidative stress by protecting some thiol-regulated enzymes, such as phosphoribulokinase, thioredoxin, ferredoxin and glutathione, from inactivation by ROS [58, 59]. Finally, it has been proposed to act as a scavenger of hydroxyl radicals, but such direct involvement in antioxidant defence requires further confirmation [60]. Although mannitol can cross plant cell membranes, many plant species, including those of the Poaceae family, are unable to incorporate it into metabolic pathways [61–63]. For this reason it is often used in experiments to simulate osmotic stress and to regulate the osmotic potential of various media and solutions. Mannitol as an ME-inducing factor was first used by Roberts-Oehlschlager and Dunwell [64] and has since been used in many procedures dedicated to various plant species. Compared to low temperature (28 days at 4 °C), short pre-treatments of anthers with 0.3–1.5 M mannitol enhanced ELS production and plant regeneration in isolated microspore cultures of barley [65–67], durum wheat [68, 69], wheat and triticale [70–72]. Stimulation of embryogenic potential is probably the effect of combined osmotic stress induced by reduced water potential and starvation associated with mannitol pre-treatment [4, 66, 73–75]. Both types of stress induce extensive changes in cell growth, metabolism and function, and initiate a complex defence network [76, 77]. Hu and Kasha [78] reported that pre-treatment of wheat anthers with 0.4 M mannitol at a low temperature (7 days at 4°C) delayed nuclear divisions and arrested microspores at the uninucleated stage. Applied at 28°C, mannitol induced a high number of first symmetric nuclear divisions frequently followed by nuclear fusion and spontaneous chromosome doubling. Microscopic analysis of wheat microspores isolated after 5 days of 0.7 M mannitol pre-treatment at 25°C revealed severe fragmentation of cortical and endoplasmic microtubules [22]. Although this effect proved to be reversible, even a transient disorganisation of the cell cytoskeleton could have major implications on microspore fate as it plays an important role in cell division, wall formation and stress defence. Another phenomenon following mannitol pre-treatment is autophagy, i.e. self-recycling induced in sugar-starved plant cells [77]. Morphologically, it is associated with an enlarged vacuole, degradation of organelles and the absence of starch granules also observed in re-programmed microspores [79]. Corral-Martínez et al. [80] reported that ME induction in *Brassica napus* microspores was associated with autophagy entirely restricted to embryogenic microspores. Further studies showed that autophagy and the activity of proteolytic enzymes (cathepsin-like, caspase-3-like) regulated microspore reprogramming and the first stages of ME in *B. napus* and *Hordeum vulgare*, regardless of the stress factor (cold, heat) used for ME induction [81–83]. Recently published data [84] confirmed the importance of autophagy for microspore reprogramming and ME induction also in *Nicotiana tabacum*.

To the best of our knowledge, this study is the first one to demonstrate a significant effect of mannitol (used as a trigger for ME induction) on the hormonal homeostasis of wheat anthers. Intense accumulation of auxins detected (particularly high in the case of oxIAA) suggests that the stress induced by 0.7 M mannitol tiller

pre-treatment stimulated IAA biosynthesis, its conjugation with aspartate and glutamate and, finally, its oxidation [33, 85]. Based on the findings discussed, it can be suggested that the visibly lower levels of oxIAA in the anthers of cv. Pavon may be indicative of a protective mechanism induced by the low-temperature tiller pre-treatment. Potentially using ABA as a signalling molecule, microspores induce a number of defensive responses that mitigate the effects of stress, prevent the overaccumulation of auxins and protect the cells from hormonal shock [33, 85].

At the same time, the total amount of active cytokinins in wheat anthers decreases, which leads to a profoundly different hormonal homeostasis. This is clearly visible when compared to the effect of low temperature on it own which is the most common and effective pre-treatment used for ME induction in cv. Pavon [86, 87]. Compared to the effect of low temperature, mannitol pre-treatment resulted in more than 12-fold higher IAA/CK_A and almost 10-fold higher IAA/ABA ratios. This imbalance could lead to disturbances in cell divisions and differentiation, i.e. processes typically promoted by specific cytokinins/auxins ratio. Mannitol pre-treatment effectively induced microspore reprogramming, but the development of many embryogenic microspores was characterised by some abnormalities which typically led to their death at the stage of multicellular structures. In addition, it almost inhibited ELS formation and completely blocked plant regeneration in microspore suspensions. These data also confirmed the vital role of cytokinins in controlling cell proliferation in shoot meristems [88] and in establishing the shoot-root axis of the embryo [89]. The results obtained in anther cultures revealed that the low level of cytokinins detected at the start of the in vitro culture can be somehow compensated. It could be hypothesised that these phytohormones are produced by tRNA degradation detected in senescing somatic anther tissues [37]. Wang et al. [50] reported DNA fragmentation in the cells of the loculus wall, tapetum and epidermis following 0.4 M mannitol pre-treatment of barley anthers. During a 4-day pre-treatment, the amount of both genomic DNA and total RNA decreased to 7% of the initial value. This also explains the high level of *c*Z and its conjugates as this form is supposed to be produced as a result of tRNA turnover [90].

When the three wheat lines/cultivar were compared, it was found that the potentially responsive line PO19 shared more similarities with cv. Pavon than with the second, winter-hardy line K393 highly recalcitrant to ME. This confirmed that genotype-dependent changes in hormonal homeostasis induced by stress pre-treatment play a key role in determining the ultimate effectiveness of ME.

The results of the next experiment showed that properly selected, exogenously applied phytohormones can at least partly overcome the problem of disturbed hormonal homeostasis and aberrant ELS development. Among the phytohormones and their analogues tested in isolated microspore cultures, the best effect was induced by 1–2 mg/L TDZ. This synthetic diphenylurea shows both cytokinin-like and auxin-like activity, but is structurally different from these two groups of phytohormones [91]. It has been used for in vitro culture since 1982 and has been found to be particularly effective in micropropagation of recalcitrant woody plant species [92]. Currently, it is widely used for in vitro culture of a large number of different plant species [93]. Due to its high biological activity and stability, it can be used at very low concentrations (2.5-5 µmol/L) to promote cell division, proliferation and shoot organogenesis. At higher concentrations (5–10 µmol/L), TDZ is used for callus induction, proliferation and initiation of somatic embryogenesis. However, prolonged exposure or overdose of TDZ results in tissue necrosis, dedifferentiation and various morphological abnormalities of the regenerated shoots. Due to the diversity of physiological effects, TDZ's mode of action remains unclear. It is unknown whether the effects of TDZ result from direct interactions with cytokinin/auxin receptors, reduced catabolism, increased synthesis or the conversion of inactive forms into their active counterparts. All these mechanisms are quoted based on the data obtained [91]. TDZ has also been used as a component of media for anther and isolated microspore cultures, as it improves the effectiveness of ME in some plant species [94–96]. For example, replacing BAP with TDZ and DIC in the induction medium significantly increased the ability to regenerate green plants in isolated microspore cultures of barley [94]. A similar effect was observed in our study for cv. Pavon on the induction medium supplemented with TDZ and PIC. A more efficient plant regeneration for the winter wheat line K393 required a higher supplementation of cytokinins (combination of TDZ and *t*Z), which was probably caused by a decisively higher accumulation of IAA and a higher increase in the IAA/CKA ratio observed in response to mannitol pre-treatment.

Conclusions

Based on the data obtained, it could be concluded that the lack of ABA-driven stress defence responses is one of the reasons for the low effectiveness of ME induction in winter wheat microspore cultures. The isolated microspores are then subjected to the high-intensity stress associated with mannitol-induced hormonal shock followed by mechanical isolation and transfer to in vitro culture. Low cytokinin levels and a disturbed auxin/cytokinin balance are likely to be responsible for the

morphological abnormalities observed later during in vitro development of isolated microspores which lead to the abortion of developing embryogenic structures. A potential solution to this problem i.e. the modification of hormonal composition of the induction medium has been suggested; however, the development of optimised medium composition requires further study and extensive documentation to fully understand and mitigate the problem.

Materials and methods

Donor plants

The two breeding lines of winter wheat (*Triticum aestivum* L.) used in this study (K393 and PO19) were F1 hybrids obtained from Strzelce Hodowla Roślin Sp. z o.o. IHAR Group and Danko Hodowla Roślin Sp. z o.o., respectively. Based on our previous studies, K393 was identified as highly recalcitrant, while PO19 was identified as semi-recalcitrant and potentially responsive to ME induction treatment [14]. Seeds of the ME-responsive spring wheat cultivar Pavon [22] were provided by the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain. Germinating seeds of winter lines K393 and PO19 were transferred to perlite with Hoagland's salt solution [97] and kept at 4°C for 7 weeks with an 8/16 h (day/night) photoperiod. Vernalized young plants were then transplanted into pots with a mixture of soil and sand (3:1; v/v). Pavon seeds were planted directly into pots after two days of germination on agar on a Petri dish. Plants were grown under semi-controlled conditions in an open foil tunnel located in an experimental field in Kraków, Poland.

Tiller pre-treatments

Tillers were collected when the majority of microspores had reached the mid-uninucleate stage of development as determined by 4′,6-diamidine-2′-phenylindole (DAPI) staining. Collected tillers were stripped of leaves, except for the flag leaf, placed in plastic bags, wrapped in aluminium foil and kept in a flask with tap water for 21 days at 4°C. For the last 3 days of pre-treatment, the tillers were transferred to 50 µmol/L sodium selenate (Na_2SeO_4) solution. They were then removed from plastic bags and aluminium foil and placed in 0.7 mol/L mannitol at 20°C in the dark for the next 4 days.

Anther culture protocol

The pre-treated spikes were sterilised with 96% ethanol for 1 min and then with 4.85% sodium hypochlorite (NaClO) solution for 4 min. Anthers were manually isolated with forceps and transferred to modified C17 medium [97, 98]. The medium contained 2 mg/L 2,4-D and 0.5 mg/L Kinetin and was solidified with 2.7 g/L Phytagel. Cultures were incubated at 26°C in the dark. The developed ELS larger than 1 mm were transferred to KBP4P for the first two weeks and subsequently to K4NB [99]. Cultures were maintained at 26°C with a photoperiod of 16/8 h (day/night) in dim light [80–100 µmol (hν) m^{-2} s^{-1} (PAR)]. The regenerated plants were then transferred to MS medium without phytohormones and replanted into pots with a mixture of soil and sand (3:1; v/v) after root formation. The effectiveness of ME was expressed as the number of ELS per spike (ELS/spike) and the number of green regenerated plants per spike (GR/spike).

Isolated microspore culture protocol

Pre-treated spikes were sterilised as described above in the anther culture protocol. Spikes were then rinsed three times with sterile distilled water. Microspores were isolated mechanically using a Waring blender (Fisher Scientific Inc.) according to the standard procedure detailed by Dubas et al. [14]. To summarise, spikes were cut into 2–3 cm sections with sterile scissors and homogenised in cold 0.3 mol/L mannitol. The obtained homogenate was filtered through a metal sieve (74 µm, Tissue Grinder Homogenizer Kit, Sigma-Aldrich) and centrifuged at 4°C (100 × *g*, 4 min). Microspores were then collected by density gradient centrifugation (0.3 mol/L mannitol/21% maltose, 80 × *g*, 4 min), resuspended in 0.3 mol/L mannitol and centrifuged again (100 × *g*, 4 min). The pelleted microspores were resuspended in 1 ml KBP medium with 0.9 mg/L 6-benzylaminopurine (BAP) according to Kumlehn et al. [99]. The number of isolated microspores was counted using a Neubauer counting chamber. The final culture density was set at 80,000 microspores per mL of induction medium. Microspore suspensions were transferred to 15 × 60 mm Petri dishes, co-cultured with immature ovaries (10 per 1.5 mL of medium) and incubated at 26 °C in the dark.

ELS larger than 1 mm were transferred to KBP4P for the first two weeks of regeneration and then retransferred to K4NB for the following weeks [99]. Cultures were maintained at 26°C and 16/8 h (light/dark) photoperiod. During the first week, light intensity was 30 µmol/m^{-2}/s^{-1} and it then increased to 80–100 µmol/m^{-2}/s^{-1}. Green regenerates were transferred to ½ MS medium without phytohormones and planted into a pot with a mixture of soil and sand (3:1; v/v) after root formation. The effectiveness of ME was expressed as the number of ELS and green regenerants per 100,000 microspores (ELS/10^5 mcs; GR/10^5 mcs).

Hormonal composition of the induction medium

In the next experiment, several modifications of the induction medium's hormonal composition were tested using the isolated microspore culture technique. Based

on the results of hormonal profiling, 0.9 mg/L BAP was replaced in the tested media by:

i) 1 mg/L *trans* Zeatin; (ii) 1 mg/L *trans* Zeatin and 0.5 mg/L Picloram; (iii) 1 mg/L Picloram; (iv) 2 mg/L Thidiazuron; (v) 1 mg/L Thidiazuron + 0.5 mg/L *trans* Zeatin.

Cytological analysis

The developmental stage of microspores was determined by reaction with 4′,6-diamidine-2′-phenylindole (DAPI). Observations were made under UV light (λ_{Ex} = 340 nm, λ_{Em} = 435 nm, blue fluorescence) using a Nikon Eclipse E600.

Microspore viability was estimated by fluorochrome reaction using fluorescein diacetate (FDA; 0.01%). Samples were observed under UV light (λ_{Ex} = 465 nm, λ_{Em} = 515 nm, green fluorescence) using a Nikon Eclipse E600 epifluorescence microscope fitted with a Zyla 4.2 (Andor) camera.

Microspore cultures were observed using a Nikon Eclipse TS100 inverted microscope at subsequent points in time, i.e. on the day of isolation (0 d), after two (14 d) and four (28 d) weeks of in vitro culture.

Collection of samples

Anthers were isolated from (i) freshly cut tillers, (ii) tillers after low temperature pre-treatment (2 weeks at 4°C), (iii) tillers after low temperature and sodium selenate pre-treatment (2 weeks at 4°C with the last 3 days in a solution of 50 µmol/L SSe) and (iv) tillers after low temperature, sodium selenate and mannitol pre-treatment (2 weeks at 4°C with the last 3 days in a solution of 50 µmol/L SSe followed by a 4-day pre-treatment with 0.7 mol/L mannitol at 20°C). Each sample contained anthers randomly collected from approximately 30 spikes. Samples were immediately frozen in liquid N_2 and stored at -60°C.

Analysis of the content of plant growth regulators

For the determination and quantification of endogenous levels of cytokinins and auxins, samples of approximately 0.8 mg DW were used.

Cytokinin samples were extracted with 1 mL of modified Bieleski buffer (-20°C) with the addition of a mixture of isotope-labelled internal standards (0.4 pmol per sample of B, R, 7G, 9G and 1 pmol per sample of OG, NT). Each sample was divided into three technical replicates after extraction and purified by the StageTips method using three layers of C18, SDB-RP and cation exchange stationary phases and conditions according to Svačinová et al. [100]. Samples were evaporated to dryness and dissolved in 30 µL of 10% MeOH and analysed by means of ultraperformance liquid chromatography (Acquity UPLC® I-class system; Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer fitted with an electrospray interface (Xevo TQ-S, Waters, Manchester, UK) according to the method described by Svačinová et al. [100]. Quantification was performed by multiple reaction monitoring of [M + H] + and the corresponding product ion. Optimal conditions, residence time, cone voltage and collision energy in the collision cell were optimised for each cytokinin metabolite [101, 102]. Quantification was performed with Masslynx software using a standard isotope dilution method.

Samples for auxin determination were extracted with 0.8 mL of 50 mM Na-phosphate buffer (pH 7.0; 4°C) containing a mixture of isotope-labelled internal standards of auxins (5 pmol per sample). Each sample was split into technical triplicates and purified by in-tip micro solid-phase extraction using a combination of three layers of C18 and SDB-XC. The extraction and purification protocol was performed according to Pěnčik et al. [83]. The eluates were evaporated to dryness, dissolved in 30 µL of 10% MeOH and injected onto a reversed-phase column (Kinetex 1.7 µm C18 100 A, 50 × 2.1 mm; Phenomenex) and analysed using an Acquity UPLC® I-class system (Waters, Milford, MA, USA) coupled to Xevo™ TQ-S (Waters, Manchester, UK) fitted with an electrospray interface (ESI) according to the method described by Pěnčik et al. [103]. Quantification was performed with Masslynx software using a standard isotope dilution method.

Statistical analysis

Statistical data analysis started with descriptive statistics (mean and standard deviation). Normally distributed variables were examined using one-way analysis of variance (ANOVA) followed by post-hoc comparison using Duncan's multiple range test ($p \leq 0.05$). All statistical analyses were performed using STATISTICA version 13.0 (Stat Soft Inc., USA).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06389-x.

Supplementary Material 1

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Author contributions

AS, MK, ED and PK prepared plant material and performed in vitro culture experiments. LP and AS performed hormonal analyses. KD validated the data. IŻ and DW were responsible for obtaining the funding. IŻ developed the concept, experimental design and managed the project. AS and IŻ prepared the first draft of the manuscript. All authors commented and approved the manuscript's final version.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethical approval
Not applicable.

Consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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