Determinants of effective microspore embryogenesis in triticale (× *Triticosecale* Wittm.)

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INTRODUCTION

The discovery that microspores can start an alternative, sporophytic program of development created a very interesting model for manifold biological studies. Moreover, haploid (n)/doubled haploid (DH, 2n) plants produced in this process, called microspore embryogenesis (ME), can be utilised as objects of various biotechnologies. Besides, incorporation of DHs into breeding programs is highly beneficial, minimising time, cost and labour necessary for the development of a new variety. However, a fundamental requirement for commercial implementation of DH technology – high efficiency for a wide spectrum of genotypes – is not fulfilled in the case of many plant species, including triticale.

In order to gain a better understanding of the mechanisms controlling triticale ME, several experiments have been undertaken to explain the role of reactive oxygen species (ROS), antioxidative system activity, and plant growth regulators (PGRs) in the initiation and regulation of the process.

MATERIAL AND METHODS

Plant material: Five highly embryogenic (DH18, DH28, DH44, DH47, DH101) and five recalcitrant (DH2, DH19, DH72, DH119, DH144) lines of winter triticale derived from F1 cross between German inbred line Saka 3006 and Polish cv. Modus were used in the study (Fig. 1).

Anther culture: The responsiveness of DH lines was estimated by anther culture method according to Wędzony (2003). Collected spikes were kept under low temperature (4°C; LT) in the dark for 3 weeks. Aseptically excised anthers were placed on mod. C17 medium (Wang and Chen 1983) supplemented with 0.5 mg l^{-1} Kinetin, 1 mg l^{-1} Dicamba and 1 mg l^{-1} Picloram, 90 g l^{-1} maltose and 0.6 % agar; pH 5.8. The cultures were incubated in the dark at 28±1°C. The effectiveness of ME induction was based on the number of embryo-like structures produced per 100 anthers (ELS/100A). Each dish containing 100 anthers collected from one spike was considered as a replicate.

Cytological analyses: Microspore viability was determined by fluorochromatic reaction to 0.01% fluorescein diacetate (FDA) (Heslop-Harrison and Heslop-Harrison 1970). For light microscopy analysis chromatin was stained with 1% solution of orecin (Zeiling 1956). For fluorescent microscopy, chromatin was stained with 0.0001% 4'6-diamidino-2-phenylindole·2HCl (DAPI) (Custers et al. 1994).

Analysis of antioxidative system activity: The activity of antioxidative enzymes: superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) were measured spectrophotometrically (Droillard et al. 1987; Bergmeyer 1965; Aebi 1984). The total activity of low molecular weight antioxidants (LMW) was analysed by DPPH (2,2-Diphenyl-1-picrylhydrazyl) method (Brand-Williams et al. 1995).

Analysis of PGRs concentration: Auxins (IAA and IBA) and cytokinins (*trans* and *cis* isomers of zeatin and zeatin riboside) were analysed by HPLC using Agilent Technologies 1260 system (Dobrev and Kaminek 2002 mod. by Stefancic et al. 2007). ABA was measured by indirect enzyme-linked immunosorbent assay (ELISA, Walker-Simmons and Abrams 1991) using MAC 252 antibody (Babraham Technix, UK).

All studied parameters were measured in anthers isolated from (1) freshly cut (FC) tillers at the developmental stage optimal for ME initiation, and (2) in anthers isolated from LT-treated tillers in which ME had been initiated.

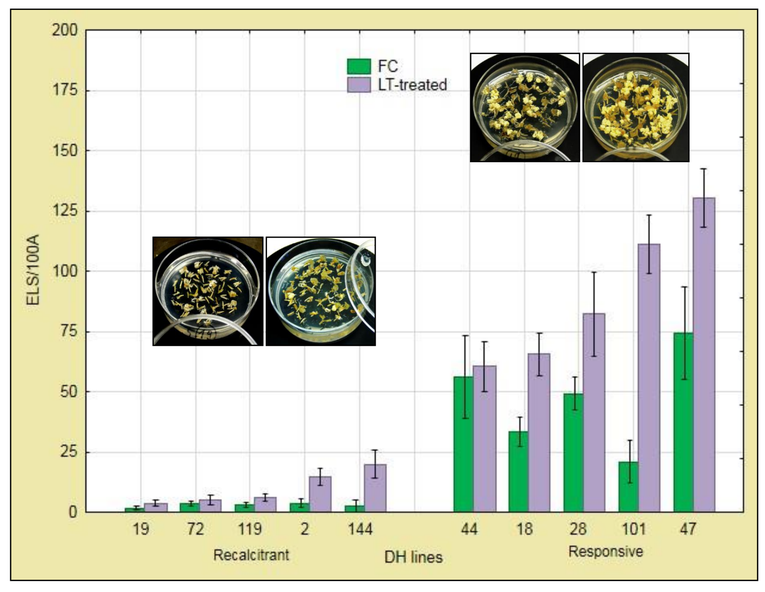

Fig. 1 The effect of LT tillers pre-treatment (3 weeks at 4°C) on ME effectiveness in anther culture of DH lines of winter triticale (× *Triticosecale* Wittm.). Data are the means of ten biological replications ± S_d. Enclosed photos show anthers six weeks after isolation from FC (a, c) and LT-treated spikes (b, d) of recalcitrant (a, b) and responsive (c, d) DH lines.

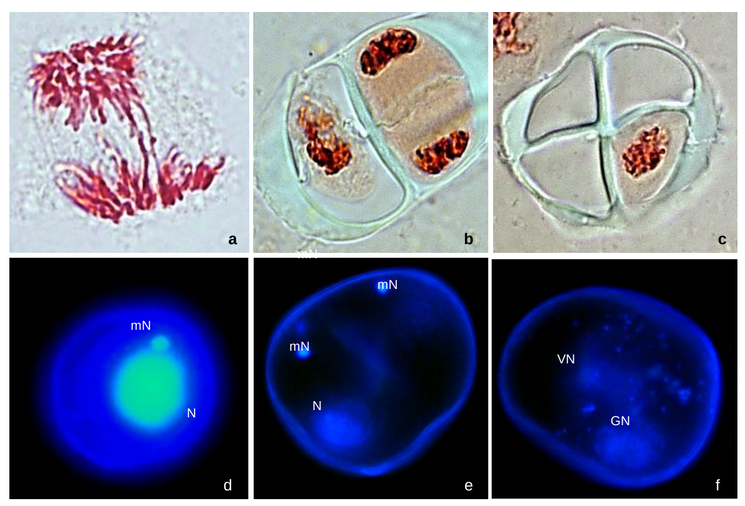

Fig. 2 Examplary disturbances in microspore- and gametogenesis observed in DH lines of triticale (× *Triticosecale* Wittm.).
a-c. *Microsporogenesis:* a). Anaphase I with chromosome bridges. b) Cytomixis. c) Degenerated tetrads. Differential interference contrast (DIC). d-f. *Gametogenesis:* d) Uni-nucleate microspore with nucleus (N) and micronucleus (mN) located centrally. e) Late uni-nucleate microspore with three micronuclei (mN). f) Differences in heterochromatin condensation in bi-cellular structure containing the vegetative (VN) and the generative nucleus (GN). Blue fluorescence (UV) caused by DAPI staining.

Table 1. Microspore viability, antoxidative activity and phytohormones levels in anthers of DH lines of triticale characterized by various embryogenic potential. All parameters were measured in anthers isolated from freshly cut tillers (FC) or low temperature treated tillers (3 weeks at 4°C; LT). Data are the means of five responsive and five recalcitrant DH lines and 3-5 biological replications for each DH line. Data marked with the same letter do not differ significantly according to Duncan test (p≤0,05)

Parameter		Recalcitrant DH lines		Responsive DH lines	
		FC	LT	FC	LT
ELS/100A		3.2	10.9	38.8	78.3
Microspore viability [%]		17.9	22.1	16.4	56.3
Enzymatic antioxidants [U g^{-1} p] [µM H_2O_2 $min^{-1}mg^{-1}$ p] [ΔAmin^{-1} mg^{-1}]	SOD	1.15 a	2.31 b	1.40 a	2.16 b
	CAT	2.0 b	1.0 a	1.4 a	1.9 b
	POX	0.039 b	0.032 a	0.039 ab	0.04 b
LMW antioxidants [µM Trolox g^{-1} DW]		31.3 b	33.8 c	28.6 a	31.5 b
Auxins [nM g^{-1} DW]	IAA	21.8 b	27.7 c	15.8 a	20.9 b
	IBA	0.2 a	0.3 b	0.2 a	0.4 c
Cytokinins [pM g^{-1} DW]	*t*Z	6.9 c	3.8 a	9.6 d	5.0 b
	*c*Z	203.6 b	328.2 c	164.7 a	357.4 c
ABA [nM g^{-1} DW]		0.9 a	1.9 b	0.8 a	3.2 c

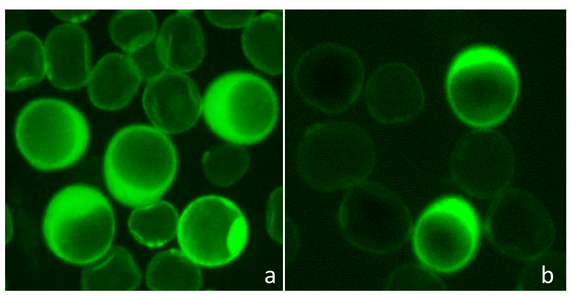

Fig. 3. Viability of isolated microspores of responsive (a) and recalcitrant (b) DH lines of triticale (× *Triticosecale* Wittm.). Green fluorescence (FITC) caused by FDA staining demonstrates vital microspores.

RESULTS AND CONCLUSIONS

1) The fundamental requirement for effective ME initialization was regular microsporogenesis leading to the formation of vital, properly developed microspores. In triticale, which possesses rather complex genetic organization, this condition is not always fulfilled. Many abnormalities, e.g. disturbances in the symmetry of nucleus division, dispersion of chromatin in the vegetative nucleus, and the presence of micronuclei, were revealed during mitosis (Fig. 2). Their frequency was distinctly higher for recalcitrant DH lines as compared to responsive ones (64% vs 14%).

2) Other important prerequisite for effective ME is tolerance to stress applied as a trigger of sporophytic development, and then connected with anthers isolation and transfer to *in vitro* culture conditions. In triticale (Żur et al. 2014), this feature was highly dependent on antioxidative system efficiency, especially on the activity of H_2O_2-decomposing enzymes: CAT and POX (Table 1).

3) Last but not least among the requirements is a specific endogenous level of PGRs. In triticale (Żur et al. 2015), anthers of responsive genotypes after induction of ME were characterized by significantly lower concentration of IAA together with significantly higher IBA, *trans* zeatin and ABA level as compared to recalcitrant genotypes (Table 2). LT-induced changes created a specific homeostasis between auxins, cytokinins and ABA, distinctly different for responsive (1/17/0.15) and recalcitrant (1/12/0.07) DH lines.

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