The influence of the oxidative stress on androgenesis induction in rye (Secale cereale L.).

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INTRODUCTION

Androgenesis is applicable in protocols for haploids/doubled haploids (DHs) for over 250 crop species (Wędzony et al. 2009; Ferrie and Mollers 2010). However, for many economically valuable plant species, among them rye (*Secale cereale* L.), the efficiency of the process is still not satisfactory, limiting its incorporation on a wider scale. The complex and composed nature of androgenesis make it a very difficult object of the study and despite the fact that the process has been known for almost 50 years, still the mechanisms of molecular control and regulation are not precisely described. Recently published results (Jacquard et al. 2009; Żur et al. 2014) suggest, that recalcitrancy to androgenesis could be caused by low ability to counter the oxidative stress induced by the procedures of androgenesis induction and transfer to *in vitro* culture conditions. As antioxidative defence ability might be elevated by changes in endogenous content of reduced (GSH) and oxidised (GSSG) glutathione forms, their concentrations have been analyzed in stress-induced to androgenesis anthers of several rye genotypes and related to the effectiveness of androgenesis initiation.

MATERIAL AND METHODS

Donor plant growth conditions

Germinated seeds of 15 rye (*Secale cereale* L.) genotypes, provided by Polish breeding companies, were vernalized for 8 weeks (2°C, 8h/16h; day/night) photoperiod. Vernalized seedlings were planted into pots containing a mixture of soil, deacidified substrate peat and sand (3/2/1; v/v/v) and grown at glasshouse conditions (20°C; 16h/8h; day/night photoperiod). Tillers were collected when the majority of microspores were at mid- to late uni-nucleated stage of development, optimal for androgenesis induction.

Induction of androgenesis by stress pre-treatment

Collected tillers were placed in Hoagland's salt solution or 0.7 M mannitol (Mn) or Hoagland's salt solution with GSH (0.3 mM) and stored at 4°C in the dark for 3 weeks.

Anther cultures

Anther culture procedure described by Immonen and Tenhola-Roininen (2003) was used after several modifications. The spikes were sterilized in 'Domestos' (20%, 15 min.) and rinsed with sterile water. Anthers were aseptically excised and cultured in modified 190-2 (Zhuang and Xu 1983) or W-14 (Ouyang et al. 1988) induction media contained 0.5 mg dm^{-3} kinetin, 1.0 mg dm^{-3} Dicamba and 1.0 mg dm^{-3} Picloram. Cultures were incubated in the dark at 28± 1°C. Starting from the sixth week of culture, androgenic structures (AS) of 1 mm size were transferred onto 0.6 % agar solidified regeneration medium 190-2 supplemented with 0.5 mg dm^{-3} kinetin, 0.5 mg dm^{-3} NAA and 30 g dm^{-3} sucrose (pH 6.0). The regeneration phase took place at 26°C, with 16 h/8 h (day/night) photoperiod, at about 80 µmol m^{-2} s^{-1} light intensity (Fig. 1).

Parameters of androgenesis effectiveness

The androgenic efficiency was evaluated for each genotype and was expressed by the following parameters: AS/100A –the number of androgenic structures per 100 anthers and R/100AS - the number of regenerants per 100 androgenic structures. GR/100AS - the number of green regenerants per 100 androgenic structures. The parameters were calculated as the mean from at least seven replications, with a 60×15 mm Petri dishes containing 100 anthers from one spike considered as one replication.

Measurement of oxidized (GSSG) and reduced (GSH) glutathione

GSSG and GSH concentrations were measured spectrophotometricly in 6 rye genotypes (in anthers isolated from freshly cut and pre-treated tillers; Fig. 2), significantly different in their response to androgenesis induction (two-highly responsive (8, 14), one-moderate (1) and three-recalcitrant (2, 3, 13). GSH and GSSG were determined by the method of Knörzer et al. (1996). To measure GSH, sample (100 µl supernatant) was neutralized with 150 µl 0.1 M potassium phosphate buffer (pH 7.8). To measure GSSG sample (100 µl supernatant) was neutralized with 200 µl 0.1 M potassium phosphate buffer (pH 7.8), blocked with 8µl 2- Vinylpyridine (97%), mixed and incubated for 1 hour at RT. Next, reaction mixture, in case of GSH and GSSG, was prepared according to Tab 1. The absorbance of GSH and GSSG was measured at 412 nm for 3 min. The activity of glutathione reductase was proportional with glutathione concentrations. The glutathione concentration was calculated from a calibration curve prepared with glutathione solutions of known concentrations.

Table 1. Reaction mixture for GSH and GSSG analyses.

Ingredients	Volume [µl]	Concentration
KP buffer	2600	0.1 M
DTNB	100	18 µM
NADPH	100	6.9 µM
GR	100	1.5 U
Sample	100	-

Statistical analysis

All statistical analyses were performed using STATISTICA package version 10.0 (Stat Soft Inc., USA, 2011). The effect of tested variables, describing androgenesis effectiveness, was examined by multi-factor analysis of variance (ANOVA). Post-hoc comparison was conducted with the use of Duncan's multiple range test ($p \leq 0.05$).

Fig. 1. Rye anther cultures.

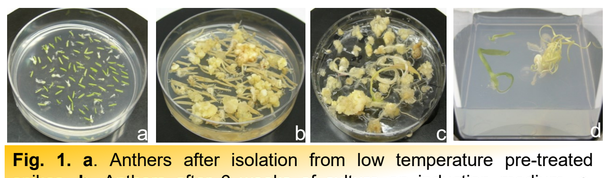


Fig. 1. a. Anthers after isolation from low temperature pre-treated spikes. **b**. Anthers after 6 weeks of culture on induction medium. **c**. Embryo-like structures on regeneration medium. **d**. Green and albino plants in rooting medium.

Fig. 2. The scheme of the experiment.

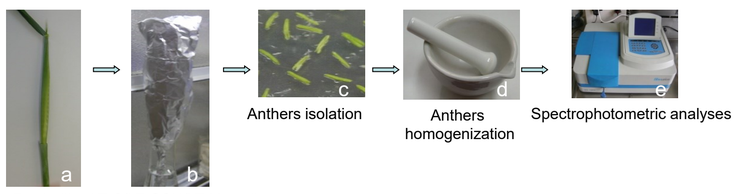


Fig. 2. a. Freshly cut tiller. **b**. Pre-treated spikes. LT-C control, low temperature spikes treatment in Hoagland medium; LT-GSH low temperature spikes treatment in Hoagland medium contained GSH, LT-Mn low temperature spikes treatment in Mannitol. **c**. Anthers isolated from the fresh/pre-treated spikes. **d**. Anthers homogenisation. **e**. Spectrophotometric analyses of GSH and GSSG.

Fig. 3. Efficiency of androgenesis in rye genotypes.

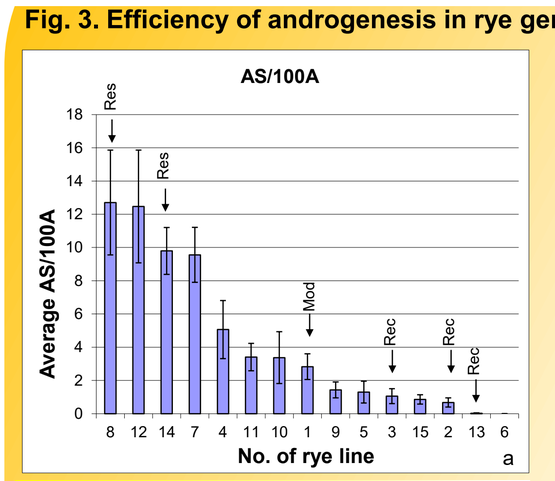


Fig. 3 a. Androgenesis induction ability of rye anthers. Presented data for each genotype are the mean of seven replicates ± SD. AS/100A the number of androgenic structures (AS) produced per 100 anthers (A) of the parent plant, Res – rye lines chosen for biochemical analyses as the most responsive to androgenesis induction. Mod - rye lines chosen for biochemical analyses as moderate to androgenesis induction. Rec - rye lines chosen for biochemical analyses as recalcitrant to androgenesis induction.

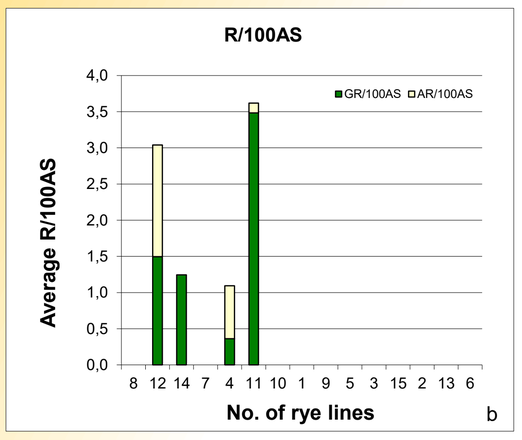


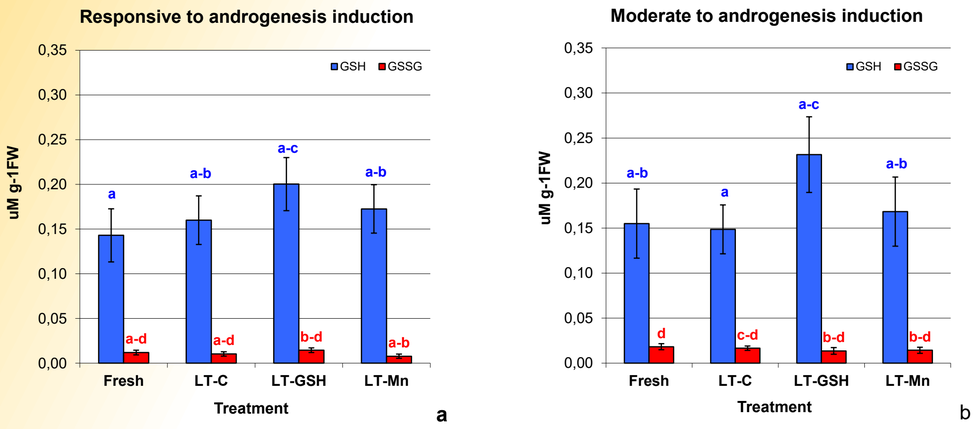
Fig. 3 b. Average regeneration ability of rye androgenic structures. Presented data for each genotype are the mean of seven replicates ± SD. R/100AS - the total number of regenerants (R) per 100 androgenic structures (AS) transferred to the regeneration medium. GR - the total number of green plants. AR - the total number of albino plants.

Table 2. The influence of spikes pre-treatment on androgenesis induction effectiveness (AS/100A).

Type of genotypes	Spikes treatment		
	C	G	Mn
Responsive	12.2	12.7	8.1
Moderate	3.6	10.1	3.7
Recalcitrant	2.7	0.9	1.2

C- Control, low temperature spikes treatment in Hoagland medium,G- low temperature spikes treatment in Hoagland medium contained GSH, Mn- low temperature spikes treatment in Mannitol.

Fig. 4. Endogenous level of reduced and oxidized glutathione isolated anthers of rye genotypes differed in response to androgenesis induction.



Figs. 4. a. Rye lines described as he most responsive to androgenesis induction. **b.** Rye lines described as moderate to androgenesis induction. **c.** Rye lines described as recalcitrant to androgenesis induction.
Mean values ± standard errors are presented in the graph. Mean values marked with the same letter do not differ significantly according to Duncan's multiple range test ($P<0.05$). FW-fresh weight. GSH - Reduced glutathione. GSSG – oxidized glutathione. Fresh-anthers isolated from the plants growing at glasshouse conditions. LT-C– control, anthers isolated from the low temperature pre-treated tillers. LT-GSH- anthers isolated from the low temperature pre-treated tillers immersed in Hoagland medium supplemented with GSH. LT-Mn-anthers isolated from the low temperature pre-treated tillers incubated in Mannitol.

RESULTS AND CONCLUSIONS

Examined rye genotypes differed in both, response to androgenesis induction (Fig. 3) and glutathione content (Fig. 4).

Androgenesis efficiency. The analysis of androgenic responsiveness among 15 studied rye genotypes showed variation in tested components of this feature: androgenic structure induction and plant regeneration ability. The average androgenesis induction efficiency for the most responsive (7, 8, 12, 14) lines was almost 3-fold higher in comparison to the moderately responsive (1, 4, 10, 11) lines and 14-fold higher in comparison to the recalcitrant ones (2, 3, 5, 6, 9, 13, 15). The parameter AS/100A was 11, 3 and 0.7 for those groups (Fig. 3a). Spikes pre-treatment had influence on androgenesis induction (Tab. 2). Average regeneration ability was 2.2 R/100AS for 4, 11, 12, 14 lines. Average green plants regeneration ability was 1.6 GR/100AS for 4, 11, 12, 14 lines (0.4, 3.5, 1.5 and 1.2 GR/100AS, respectively, Fig. 3 b). The number of regenerated green plants in line 11 was almost 9-fold higher than in line 4 (3.5 vs. 0.4 GR/100AS).

Glutathione content. GSH was the most abundant glutathione form in anthers under control and experimental conditions, present in µM-concentrations (Fig. 4). Endogenous content of GSH in anthers excised from the fresh cut tillers was different in lines described as responsive, moderate and recalcitrant to androgenesis (0.143 µM g^{-1} FW, 0.155 µM g^{-1} FW, 0.252 µM g^{-1} FW, respectively; Figs. 4 a-c). Temperature, osmotic stress and exogenous GSH treatments affected the contents of endogenous GSH and GSSG. These changes in GSH and GSSG content varied in lines described as responsive and recalcitrant to androgenesis (Figs. 4 a-c). Interestingly, in recalcitrant lines, Mn treatment at low temperature, significantly increased GSH content 0.281 µM g^{-1} FW (1.6-fold) in comparison to moderate (0.168 µM g^{-1} FW) and highly responsive (0.173 µM g^{-1} FW) to androgenesis lines. In anthers isolated from Mn-treated tillers, negative correlation (r=-0.56, -0.52 $p<0.05$) was detected between glutathione (GSH, GSSG) and androgenesis initiation (AS/100A).

The GSH/GSSG ratio increased in anthers subjected to androgenesis-inducible treatments. The GSH/GSSG ratio ranged 15.5-22.2, 8.9-17.0 and 13.1-65.5 under low temperature spikes treatment for lines described as highly responsive, moderate or recalcitrant to androgenesis induction, respectively.

- **Endogenous content of GSH in anthers excised from the fresh cut tillers was associated with the level of androgenic responsiveness**.
- **Tested modifications of spike pre-treatment influenced endogenous GSH and GSSG content.**
- **High content of GSH in anthers seems to be a defence reaction, which reflect the stress level, and do not have a positive effect on androgenesis induction effectiveness.**

Literature
Ferrie AMR, Mollers C (2010) Plant Cell Tiss Organ Cult. DOI: 10.1007/s11240-010-9831-4; **Immonen S, Tenhola-Roininen T** (2003) Kluwer Academic Publishers, Dordrecht, The Netherlands; pp. 141-150; **Jacquard C et al.** (2009) Plant Cell Rep 28: pp. 1329-1339; **Knörzer OC, Durner J, Boger P** (1996) Physiol Plant 97: pp. 388–396; **Ouyang JW et al.** (1988) Ann Rep Inst Genet Aca Sci, Beijing, pp 91-92; **Wędzony M et al.** (2009) Advances in Haploid Production in Higher Plants, pp. 1-33; **Zhuang JJ, Xu J** (1983) Cell and Tissue Culture Techniques for Cereal Crop Improvement, Science Press. Beijing: pp. 431; **Żur I et al.** (2014) Plant Cell Tissue and Organ Culture 119:pp. 79-94.

The research was supported by the IPP PAS statute project T1Zb1/2015 and individual national research project financed by the Ministry of Agriculture and Rural Development in 2015 No 84.