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Ageing-induced changes of reduced and oxidised glutathione in fragments of maize seedlings

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Abstract: A trial with four maize inbred lines with the ability to have different durations of seed germination in the course of the accelerated ageing (AA) treatment was set up. Changes of the content of total, reduced and oxidized glutathione (expressed as monomers) were observed in the seeds and seedlings before and after the treatment. For the first time, changes of glutathione in whole seedlings, as well as in the rest of the seed, were analysed. It was noticed that maize inbreds with a smaller decrease of the total glutathione but with an increase of the oxidized form had the ability of prolonged germination. In the control seedlings, the amount of total glutathione was lower than in the treated ones. Maize seeds which lost germination faster had greater losses of total glutathione with an increased content of the oxidized form in seedlings. The ability of prolonged germination together with the possibility of glutathione synthesis in seedlings are genotypic traits.

Keywords: ageing, maize seedling, reduced and oxidised gluthatione.

INTRODUCTION

The processes of ageing have primarily been investigated in anthropo-animal organisms, as they were initially observed in them. The development of new trends in agriculture required ageing processes in plant organisms to be investigated, firstly in seed material for biological (germplasm conservation) and economic reasons (production of commercial seeds). The reason for this phenomenon lies in complex biochemical changes of the reserve substances of seeds, the production of toxic reactants and inhibitory substances (free radicals, methyl jasmonates, *etc.*).[1,2] Glutathione is synthesized in living systems as a highly important antioxidant for the maintenance of equilibrium.

The role of glutathione has been much more investigated within anthropo-animal systems,[3,4] but in recent times the increase in the knowledge of its functions in plants has be-

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come more essential. The glutathione system is one of the most important non-enzymic protective factors.[4] From a biochemical point of view, it is a small, mobile molecule consisting of three amino acids: glutamine, cysteine and glycine, and it is important in oxidation-reduction reactions in tissues. In physiologically active tissues, reduced glutathione (GSH) presents a reducing substrate, whereby oxidized glutathione (GSSG) is generated. Then glutathione reductase catalyses the NADPH-dependent reduction of GSSG to glutathione GSH by a reversible reaction. Water elimination out from a system over a series of reactions is known as the ascorbate-glutathione cycle.[4] Since there is no free ascorbate in seeds, GSH directly participates in the oxidation, by the proposed mechanism of substitution:[5,6]

$$\text{Propagation: } {}^{\bullet}OH + GSH \rightarrow GS^{\bullet} + H_2O \quad (1)$$

$$\text{Termination: } 2\,GS^{\bullet} \rightarrow GSSG \quad (2)$$

The $GS^{\bullet}$ radical is relatively stable, while the formed GSSG is non-toxic to living systems.

Pastori and Trippi[7] and Kocsy *et al.*[8] indicated in their studies that plants with a higher resistance to stress factors are characterized by an increased level of glutathione. Furthermore, changes in the content of the total, as well as the reduced and oxidized glutathione were observed in aged seed.[9–11] Therefore, it is important to determine the influence of seed ageing on changes of the content of total, as well as of reduced and oxidised glutathione in maize genotypes of different germination ability. Additionaly, changes of the glutathione levels in seedlings retarded in growth, originating from aged seeds, should be determined and, therefore, these are also the objectives of this investigation.

EXPERIMENTAL

The following four maize inbreds were used in the experiment: two dent inhreds (ZP PL 175-L_1 and ZP PL. 188-L_2) and two sweet maize inbreds (ZP PL51-L_3 anD ZP PL67-L_4). Maize seeds were subjected to the AA treatment[12] at a temperature of 42 ºC and a relative air humidity of 100 %, for a duration of three, six or nine days up to the moment when the slope of the germination curve becomes steep and the seedlings are significantly retarded in growth. This occurred with L_1 and L_2 after 9 and 6 days (germination decreased from the initial 91.5 to 46 %, *i.e.*, 89 to 15.2 % respectively), while with the inbreds L_3 and L_4 it was registered after 3 days (germination decreased from the initial 28.7 to 13.7 %, *i.e.*, 88.5 to 77 %, respectively). Further exposure of the seeds to the treatment led to germination hold-up.

A further step was to determine the germination capacity according to the ISTA Rules[13] in four replicates of 100 uniform seeds. The germination capacity was evaluated seven days later. All seedlings were grouped into four replicates of 25 plants, and then the radicle, shoots and the rest of seed were fractioned. Also, four replicates of 25 uniform seeds were formed of the treated and untreated seeds, and average weight of a seed was determined.

The plant material was dried in a ventilation drier at 60 ºC to constant weight from which the average weight of each fraction individually was determined and expressed per unit, *i.e.*, seedling. Then, the plant material was pulverised (pulveriser Fritsch IZP-119-UP11).

The content of reduced (GHS) and oxidized glutathione (GSSG) was determined according to the method of Kok *et al.*[14] After shaking 1 g of sample with 10 mL 0.15 % Na ascorbate solution in a shaker, the sample was centrifuged at 20,000 g for 20 minutes, and then the supernatant was deproteinised in a water bath

at 95 ºC for 3 min. After repeated centrifugation at 15,000 g for 15 min, the content of total glutathione in the supernatant was analysed in the following manner: 1.5 mL 0.2 M potassium phosphate buffer (pH 8.0) and 0.2 mL 10 mM DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] were added to 1.5 mL of the extract, as well as to 1.5 mL 0.02 M potassium phosphate buffer (pH 7.0). The absorbance was read at 415 nm. In the other 1.5 mL of supernatant, 0.5 mL 0.25 M potassium phosphate buffer (pH 6.8), 0.3 mL albumin, 0.02 mL glyoxalase I (Sigma grade IV) and 0.08 mL 0.1 M methylglyoxal are added. After incubation at 30 ºC for 15 min. the content of reduced glutathione (GHS) was determined in the above described manner. GSH (Sigma Ultra 98–100 %) in the concentration range 0–0.1 μmol GSH mL^{-1} was used as the standard. The content of oxidized glutathione (GSSG), calculated as the difference between the total and the reduced glutathione, was expressed as monomer.

Statistical evaluation was performed using the Student's t-test. The control without AA treatment was used as the reference. The minimum level of statistical significance accepted was $p < 0.05$.

RESULTS

Changes of the GSH and GSSG contents were observed in seeds, as well as in parts of the seedlings originating from the seeds, before and after AA treatment.

The AA treatment lowered the level of total glutathione in the seeds (Table I). Thus, the total glutathione in L_1, L_2, L_3 and L_4 decreased by 12 %, 20 %, 30 % and even 55 %, compared to the control. Furthermore, the treatment resulted in an increase of the GSSG content in seeds of L_1 and L_2 from 90 to 395 and from 716 to 726 nmol g^{-1}. In contrast, the levels of GSH and GSSG decreased significantly from 814 to 619 and from 161 to 62 nmol g^{-1}, respectively, in L_3, and from 894 to 548 and from 822 to 222 nmol g^{-1}, respectively, in L_4.

TABLE I. The impact of accelerated ageing on the distribution of GSH and GSSG among seedling parts, [nmol g^{-1}].

Genotype	Ageing treatment	Seed			Radicle			Shoot			Rest of seed		
		GSH	GSSG	Σ	GSH	GSSG	Σ	GSH	GSSG	Σ	GSH	GSSG	Σ
L_1	control	963	90	1053	1090	672	1762	435	1453	1888	624	401	1025
	treatment	528	395	923	1312	1132	2444	664	993	1657	913	536	1449
L_2	control	793	716	1509	553	2407	2960	404	1949	2353	689	191	880
	treatment	474	726	1200	1196	2067	3263	378	1688	2066	805	394	1262
L_3	control	814	161	975	5778	4312	10090	3384	1613	4997	2241	2994	5235
	treatment	619	62	681	5019	2126	7145	3709	2273	5982	2173	3866	6039
L_4	control	894	822	1716	4900	2245	7145	356	1837	2193	1589	3328	4917
	treatment	548	222	770	4509	1032	5541	141	2400	2541	1730	3910	5640
$p < 0.05$*		18	21	37	44	86	130	43	66	108	28	72	101

*Significant differences from controls (*t*-tests)

It is interesting that the employed treatment provided a classification of the seedlings into two groups. Namely, the content of total glutathione increased from 4,675 to 5,550 nmol g^{-1} and from 6,193 to 6,528 nmol g^{-1} in the whole seedlings of L_1 and L_2 respectively (Table I). On the other hand, this content decreased in L_3 and L_4 from 20,322 to 19,166 nmol g^{-1} and from 14,255 to 13,722 nmol g^{-1} in the whole seedlings of L_3 and L_4,

respectively. The increase in the level of total glutathione in L_1 and L_2 was pronounced in the radicle (39 and 10 %) and the rest of the seed (41 and 43 %), while in L_3 and L_4 it was in the shoots (20 and 16 %) and the rest of the seed (15 %).

In order to express the changes in the glutathione levels in maize seeds and seedlings after AA treatment more clearly, the GSH:GSSG ratio was calculated (Fig. 1).

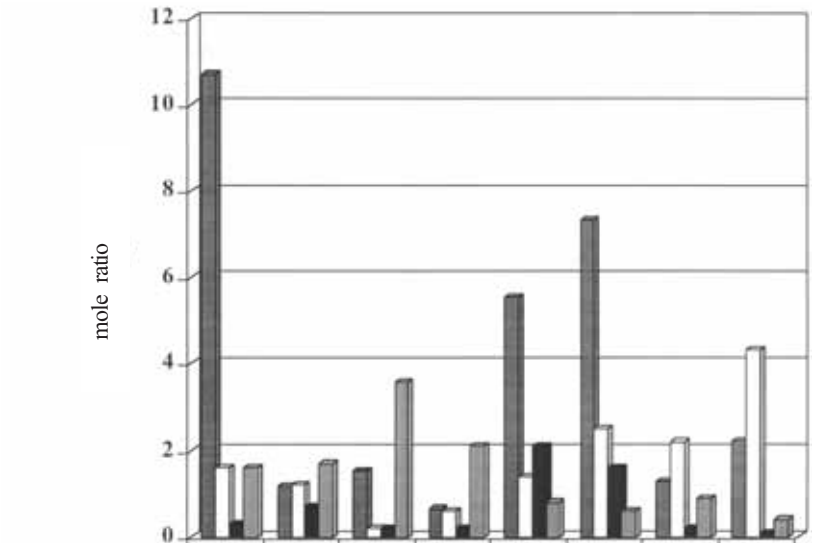
The GSH:GSSG ratio in the seeds of L_1 (the genotype most resistant to the treatment in whose seeds the ability to germinate is preserved for up to 9 days) decreased from 10.7:1 to 1.3:1 (Fig.1). There were no significant changes of this ratio in seeds of L_2 (the ability to germinate is preserved for up to 6 days in the course of accelerated ageing), *i.e.* the share of GSH in relation to GSSG decreases only from 1.1:1 to 0.7:1. However, this ratio increases in L_3 and L_4 (inbreds with the shortest ability to germinate, up to 3 days) from 5.1:1 to 10.3:1, and from 1.1:1 to 2.4:1, respectively.

The GSH:GSSG ratio distinguishes seedlings of L_1 from seedlings of the other inbreds (Fig. 1) - the GSH share in relation to GSSG decreases in the radicle from 1.6:1 to 1.2:1, while it increases in the shoots from 0.3:1 to 0.7:1, and shows no changes in the rest of the seed. Significantly higher variations of this ratio were recorded for all other inbreds. Thus, the treatment increased the GSH level in the radicle by 94 % on average, and increased the GSSG level in the shoots and the rest of the seed on average by 32 and 72 %, respectively.

DISCUSSION

The inbreds investigated under the effects of AA treatment were classified into the following groups: more sensitive (L_3 and L_4), in whose seeds the level of total glutathione decreased (Table I), in accordance with the results observed in tomatoes[10] and less sensitive (L_1 and L_2), in which the decrease of the level of total glutathione in the seeds was not significant, but with increased share of GSSG, similar to sunflower seeds.[11] The increase in the content of GSSG dimers in the seeds of the maize inbreds more resistant to the treatment can indicate an interruption of further propagation of radicals by the mechanism of substitution.[5,6] The antioxidative capacity of glutathione should be based on this mechanism.[4,7,8]

Considering numerous studies of other authors, such as Narayan *et al.*[1], McDonald,[5] Torres *et al.*,[11] Walters,[15] seed ageing is foremost oxidative stress that leads to a decline of germination and to the loss of viability. In constrast to seeds, where metabolic processes are mild and slow, seedlings are an active metabolic system in which catabolic and anabolic processes are simultaneously present. Therefore, changes of the glutathione content in seedlings are more complex. Oxidation of the greatest part of the GSH into GSSG in the fractions of seedlings originating from treated L_1 seeds (Fig. 1) did not affect changes of the GSH:GSSG ratio. It can be assumed that a lower content of total glutathione (Table I) and a smaller range of the GSH:GSSG ratio in seeds (Fig. 1) of other genotypes result in a lowering of the total glutathione content in the whole seedlings, with an increased accumulation of GSSG in the radicle and its decrease in the



	C L1	T L1	C L2	T L2	C L3	T L3	C L4	T L4
Seed	10.7	1.17	1.54	0.65	5.54	7.33	1.28	2.23
Radicle	1.6	1.2	0.2	0.6	1.4	2.5	2.2	4.3
Shoot	0.3	0.7	0.2	0.2	2.1	1.6	0.2	0.1
Rest of seed	1.6	1.7	3.6	2.1	0.8	0.6	0.9	0.4

Fig. 1. The GSH:GSSG mole ratio before and after accelerated ageing of seeds and seedling parts. C- control. T- treatment.

shoots and the rest of the seed in comparison with control seedlings, which is in accordance with the results of De Vos *et al.*,[9] De Paula *et al.*[10] and Torres *et al.*,[11] obtained with sunflowers and tomatoes. These results were also confirmed by investigations of Pastori and Trippi[7] and Kocsy *et al.*,[8] according to which the accumulation of total glutathione in the radicle and shoots of maize seedlings increased after they had been subjected to a stress factor. In the presented studies, changes of glutathione in the rest of the seed were analysed for the first time. Based on the significant share of glutathione in this part of seedlings, especially in inbreds more sensitive to the treatment, it seems that the seed rest plays a significant role in the synthesis and distribution of glutathione in 7-day old maize seedlings. This was confirmed by studies of Ruegsegger and Brunold[16] who determined that synthesis of GSH *de novo* in the 7-day period is mainly done in the scutellum (as a maize seed fraction).

According to our results, maize inbreds with a prolongated ability to germinate (9 and 6 days of treatment of L_1 and L_2) are characterized by a slower decrease of the total glutathione with an increased GSSG share, in contrast to seeds of L_3 and L_4, which lose their ability to germinate after a 3-day treatment, with the greatest decrease of the total glutathione. On the other hand, seedlings originating from treated seeds have a higher or a

lower content of total glutathione depending on their sensitivity to the treatment. The total glutathione in seedlings of L_3 and L_4, as sensitive inbreds, was lower, while the equilibrium was shifted towards GSSG accumulation, in contrast to seedlings of L_1 and L_2 where total glutathione was higher, with the equilibrium shifted towards GSH accumulation. Therefore, the obtained results indicate that seeds of dent maize inbreds (L_1 and L_2) can preserve their ability to germinate for a longer period of time due to both the smaller loss of total glutathione and a greater GSH synthesis *de novo* and/or GSSG reduction during germination and emergence, which could be bined to the rest of the seed. Seeds of sweet maize inbreds (L_3 and L_4) are less capable of preserving germination, which is probably a result of the greater loss of total glutathione during the ageing treatment. The significantly higher share of GSSG in these seedlings indirectly indicates oxidative stress which makes germination more difficult.

CONCLUSION

From the obtained results, it could be assumed that changes in content and form of the glutathione redox system have a significant influence on the retention of germination ability of seeds. Firstly, the degree of sensitivity of an individual genotype (maize inbreds) to the treatment can be caused by the decrease of total glutathione in the seeds, and, on the other hand, by its uneven distribution and *de novo* synthesis in certain parts of formed seedlings (greater glutathione content in the rest of the seed), as well as by the increased share of GSSG within the total glutathione. These processes result in the classification of maize genotypes into more sensitive, such as sweet maize (L_3 and L_4) and less sensitive, such as dent maize (L_1 and L_2); furthermore, within each group, more sensitive (L_2 and L_4) and less sensitive genotypes (L_1 and L_3) are observed. Based on the presented results, it could be purported that the retention of germination ability, as well as the potential of GSH synthesis *de novo* are genotypic traits, and as such, could be used in practice, *i.e.*, in selection of less sensitive genotypes, as carriers of these traits.

ИЗВОД

УТИЦАЈ УБРЗАНОГ СТАРЕЊА НА ПРОМЕНЕ РЕДУКОВАНОГ И ОКСИДОВАНОГ ГЛУТАТИОНА У КЛИЈАНЦИМА КУКУРУЗА

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Постављен је оглед са четири линије кукуруза различите дужине очувања клијавости семена током третмана убрзног старења. Испитиване су промене укупног, као и редукованог и оксидованог глутатиона (изражени као мономери) у семену и клијанцима пре и након убрзаног старења. У истраживањима су први пут анализиране промене глутатиона у целим клијанцима, као и остатку семена. У семену линија кукуруза које имају способност дужег очувања клијавости био је мањи губитак укупног глутатиона, уз повећање удела оксидованог

облика. Код њихових клијанаца дошло је до повећања садржаја укупног глутатиона у односу на контролне клијанце. Семе кукуруза које брже губи клијавост имало је веће губитке укупног глутатиона, уз већи садржај оксидованог облика код формираних клијанаца. Дужина очувања клијавости, као и синтеза глутатиона код клијанаца је генотипска особина.

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