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LIPID PEROXIDATION LEVELS IN SOYBEAN (*Glycine max* (L.) Merr.) SEED PARTS AS A CONSEQUENCE OF IMBIBITION STRESS

M. Lisjak⁽¹⁾, *I. D. Wilson*⁽²⁾, *L. Civale*, *J.T. Hancock*⁽²⁾, *Tihana Teklić*⁽¹⁾

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SUMMARY

High rainfall and rapid water uptake by dry seed after sowing in the field can result in so-called seed imbibitional damage. Here, lipid peroxidation levels were evaluated in seed testa, embryos and cotyledons of three soybean cultivars (Podravka 95, Tisa and Vita), after 3, 6, 12 and 24 h of seed imbibition in water at 20°C. In general, lipid peroxidation was enhanced in soybean embryos and the lowest values were observed in seed testa. With respect to imbibition duration, the highest lipid peroxidation was observed after 3 h of imbibition and decreased thereafter in seed of Podravka 95 and Vita, with similar trend regarding seed of the same age.

Key-words: *Glycine max* (L.) Merr., imbibitional damage, lipid peroxidation, seed vigour, soybean, cultivar, TBARS

INTRODUCTION

Lipid peroxidation processes are considered as the primary cause of soybean (*Glycine max* (L.) Merr.) seed deterioration during storage (Hailstones and Smith, 1988; Ferguson et al, 1990; Sungand Chiu, 1995), as the high lipid and protein content in soybean seeds make them prone to oxidative stress and is related to fast seed vigour decline with seed age. After Shulaev and Oliver (2006), measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage and has been extensively used in plants.

Reactive oxygen species (ROS) play a dual role in seed physiology. On one hand they act as signalling pathways in cell, but on the other hand, they are toxic products accumulating under stress conditions (Bailly et al., 2008). In orthodox seeds, ROS are produced from embryogenesis to germination, i.e., in metabolically active cells. However, ROS are also generated in quiescent dry tissues during ripening and storage, with various mechanisms implicated depending on the seed moisture content (El-Maarouf-Bouteau and Bailly, 2008). Seed imbibition is a critical stage in successful soybean [*Glycine max* (L.) Merr.] crop establishment (McDonald et al., 1988). However, immediately after sowing in the field, dry seeds can be exposed to hypoxia due to high rainfall and rapid water uptake, especially in heavy-textured soils with low water percolation to deeper soil levels. This can result in so-called imbibitional damage that happens if seed parts absorb irregularly large amounts of water. The lack of oxygen in water-saturated soil causes a shift towards anaerobic seed metabolism, ROS production and lipid peroxidation-mediated cell membrane destruction. These events are exacerbated by adverse environmental conditions during seed maturation, harvest and storage and by either mechanical or imbibitional damage as well as during seed natural ageing (Andrić et al., 2008). Kühn and Borchert (2002) suggested that due to the oxidation of the lipid bilayer, biomembranes can lose their role as a barrier. This jeopardises the integrity of cells and their compartments. It is followed by the leaching of ions and diverse seed low-molecular components. Such seed decay much faster in adverse seed-bed conditions due to microbial attack and inadequate nutrient supply to the developing seedlings. This will result in unsatisfactory crop stand and final crop yield.

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This study investigates the time-dependent changes in lipid peroxidation in seeds of three soybean cultivars. The specificity of seed structural parts with regard to lipid peroxidation levels, seed age and the influence of imbibition duration were a particular focus.

MATERIAL AND METHODS

This study investigates the intensity of lipid peroxidation (TBARS – thiobarbituric acid reactive substances content) in seed testa, embryos and cotyledons of three Croatian soybean cultivars (Podravka 95, Tisa and Vita), after 3, 6, 12 and 24 hours of seed soaking (imbibition) in deionized water at 20°C. Selected cultivars differ according to their maturity group, so Vita belongs to maturity group 0, Podravka 95 to the group 0-I and Tisa is in the group I (Vratarić and Sudarić, 2008).

Soybean seeds were produced at the Agricultural Institute in Osijek, Croatia. The seeds were stored for 6 or 18 months prior to experimentation in closed paper bags in an open type warehouse, characterized by daily and seasonal fluctuation of air relative humidity and temperature. The experiment was carried out with 50 grains per replicate, 4 replicates per cultivar and seed lot using 250 mL of deionized water per replicate. Glass beakers with seeds and water were covered with plastic film to prevent water evaporation during a 24-h trial at 20°C. After the particular imbibition period, seed was dried gently by means of paper tissues and seed testa, cotyledons and embryos were carefully detached by hand ensuring minimal tissue damage.

TBARS levels (thiobarbituric acid-reactive substances) were evaluated using the method of Heath and Packer (1968) with some modifications. Firstly seed part samples were ground in liquid nitrogen with mortar and pestle. Subsequently, 0.2 ± 0.001 g of tissue powder was extracted in 1 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 3,000 g for 5 min at 4°C. One mL of 20% TCA containing 0.5% thiobarbituric acid was added to 0.5 mL of the supernatant and vortexed. The mixture was heated at 95°C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10,000g for 15 min at 4°C and the absorbance was measured at 532 nm. The absorbance value for non-specific absorption was subtracted at 600 nm. The concentration of TBARS (nmol g^{-1} tissue) was calculated using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

Three-factorial ANOVA with F test was applied for testing the influences of seed age (factor A), seed part (factor B) and imbibition duration (factor C) on TBARS content.

RESULTS AND DISCUSSION

The high lipid and protein content in soybean [*Glycine max* (L.) Merr.] seeds make them prone to oxidative stress and is related to their rapid age-associated decline in vigour. Here, seed age had a significant effect on lipid peroxidation levels in all the cultivars examined, but was not consistent between those tested. The mean TBARS level was higher in older seed of Podravka 95 (11.07 vs. 8.16 nmol g^{-1} tissue; $P \leq 0.05$) and Vita (12.66 vs. 11.75 nmol g^{-1} tissue; $P \leq 0.01$), but lower in older seed of the cultivar Tisa (7.84 vs. 9.58 nmol g^{-1} tissue, $P \leq 0.05$). The effect of seed age was statistically more significant in cultivar Vita ($P \leq 0.01$), in comparison with the other two cultivars ($P \leq 0.05$).

As stated by Shao et al. (2007) seeds of different soybean cultivars have strikingly different rates of water imbibition. The research of McDonald et al. (1988) showed that the soybean embryonic axis hydrates more than the cotyledons, so that after 72 h of imbibition the embryonic axis was the most hydrated portion of the seed. Imbibitional damage is a common occurrence comprising both the physical and metabolic consequences of rapid water uptake by dry seed during conditions of high water availability. Intense changes of water pressure within the seed structures provoke the formation of ROS and consequently oxidative stress in under what are essentially hypoxic conditions. With regard to the observed differences between the particular seed structures examined here, it was clear that the highest level of lipid peroxidation intensity occurred in the embryo and the lowest in the seed testa, regardless the cultivar examined and the age of the seeds (Table 1). The significance of the difference between the types of seed tissue studied for lipid peroxidation was confirmed by F test ($P \leq 0.01$).

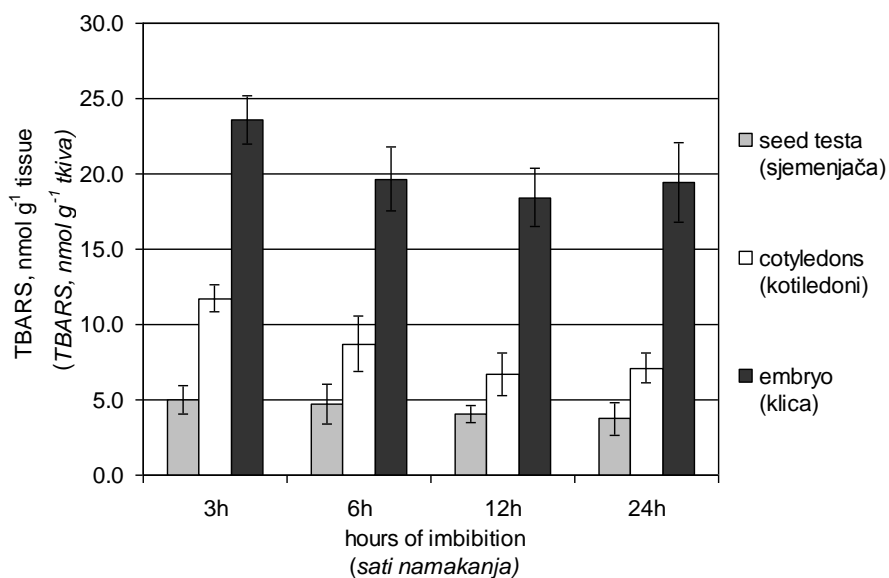
Table 1. Mean lipid peroxidation levels (TBARS) in seeds of three soybean cultivars, affected by seed age (factor A), seed part (factor B) and imbibition duration (factor C) in controlled conditions. (F test values: * significant at $P \leq 0.05$, ** significant at $P \leq 0.01$)

*Tablica 1. Prosječna razina lipidne peroksidacije (TBARS) u sjemenu tri kultivara soje, pod utjecajem starosti sjemena (faktor A), dijela sjemena (faktor B) i trajanja namakanja (faktor C) u kontroliranim uvjetima. (F test: * značajno pri $P \leq 0.05$, ** značajno pri $P \leq 0.01$)*

Treatment (tretman)		Cultivar (sorta)	Podravka	Tisa	Vita
		TBARS (nmol g ⁻¹ tissue) TBARS (nmol g ⁻¹ tkiva)			
Seed age (A) (starost sjemena, A)	6 months (6 mjeseci)		8.16	9.58	11.75
	18 months (18 mjeseci)		11.07	7.84	12.66
Seed part (B) (dio sjemena, B)	Seed testa (sjemenjača)		3.91	4.66	6.05
	Cotyledons (kotiledoni)		7.16	7.76	10.63
	Embryo (klica)		17.78	13.71	19.07
Imbibition duration (C) (trajanje namakanja, C)	3 h		11.92	9.08	13.74
	6 h		8.86	8.50	11.29
	12 h		9.12	9.57	11.10
	24 h		8.56	7.67	11.53
F test			A*, B**, C**	A*, B**, AxB*, BxC*	A**, B**, C**, BxC**

Regardless seed age and seed part, soybean cultivars tested here showed very different lipid peroxidation dynamics during 24 h of imbibition (factor C, Table 1). Podravka 95 and Vita had the highest intensity of lipid peroxidation after only 3 h of imbibition (11.92 and 13.74 nmol g⁻¹ tissue, respectively), while cultivar Tisa had the highest TBARS level after 12 h (9.57 nmol g⁻¹ tissue). The effect of imbibition duration was very significant ($P \leq 0.01$) in Podravka 95 and Vita, the latter also showed a significant effect of the interaction between seed part and imbibition duration (BxC, Table 1). This interaction was not significant in Podravka 95, but significant at $P \leq 0.05$ in Tisa. The level of lipid peroxidation was also significantly influenced by the interaction between seed age and seed part (AxB) in this cultivar. The observed variability of lipid peroxidation levels over the 24 hours of imbibition, regarding particular cultivars and seed age is shown in Graphs 1-6. Seed of the cultivars Podravka and Vita of the same age responded similarly to imbibition and the patterns of their lipid peroxidation levels showed analogous trends, particularly in the embryo tissues (Graphs 1-4). Interestingly, in comparison with other cultivars, Tisa showed the lowest lipid peroxidation levels in embryo tissues, especially in old seed (Table 1, Graphs 5 and 6). Thus, the process of lipid peroxidation in soybean seed during imbibition appears to involve complex genotype-specific traits, such as seed testa structure and chemical composition, seed vigour, as well as cellular signalling and ROS scavenging potential.

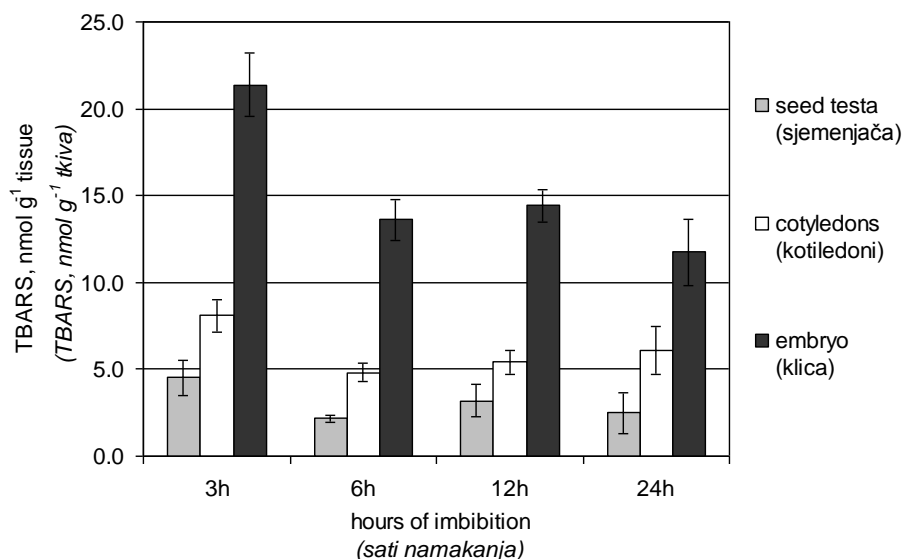
Soybean cultivar Podravka 95 - seed old 18 months
(*kultivar soje Podravka 95 - sjeme staro 18 mjeseci*)



Graph 1. Dynamics of lipid peroxidation (TBARS) in seed testa, cotyledons and embryo of 18 months old seed of soybean cultivar Podravka 95, imbibed in deionized water up to 24 h (bars are means of four replicates ± standard deviation, with 50 grains per replicate submerged in 250 mL water)

Grafikon 1. Dinamika lipidne peroksidacije (TBARS) u sjemenjači, kotiledonima i klici sjemena soje kultivara Podravka 95 starog 18 mjeseci, pri namakanju u deioniziranoj vodi tijekom 24 h (stupci su srednje vrijednosti 4 ponavljanja ± standardna devijacija, s po 50 zrna po ponavljanju potopljenih u 250 mL vode)

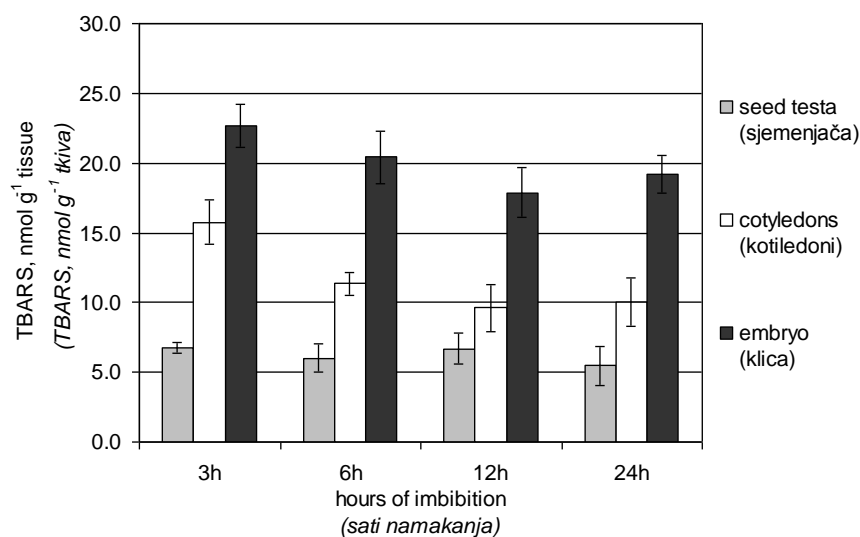
Soybean cultivar Podravka 95 - seed old 6 months
(*kultivar soje Podravka 95 - sjeme staro 6 mjeseci*)



Graph 2. Dynamics of lipid peroxidation (TBARS) in seed testa, cotyledons and embryo of 6 months old seed of soybean cultivar Podravka 95, imbibed in deionized water up to 24 h (bars are means of four replicates ± standard deviation, with 50 grains per replicate submerged in 250 mL water)

Grafikon 2. Dinamika lipidne peroksidacije (TBARS) u sjemenjači, kotiledonima i klici sjemena soje kultivara Podravka 95 starog 6 mjeseci, pri namakanju u deioniziranoj vodi tijekom 24 h (stupci su srednje vrijednosti 4 ponavljanja ± standardna devijacija, s po 50 zrna po ponavljanju potopljenih u 250 mL vode)

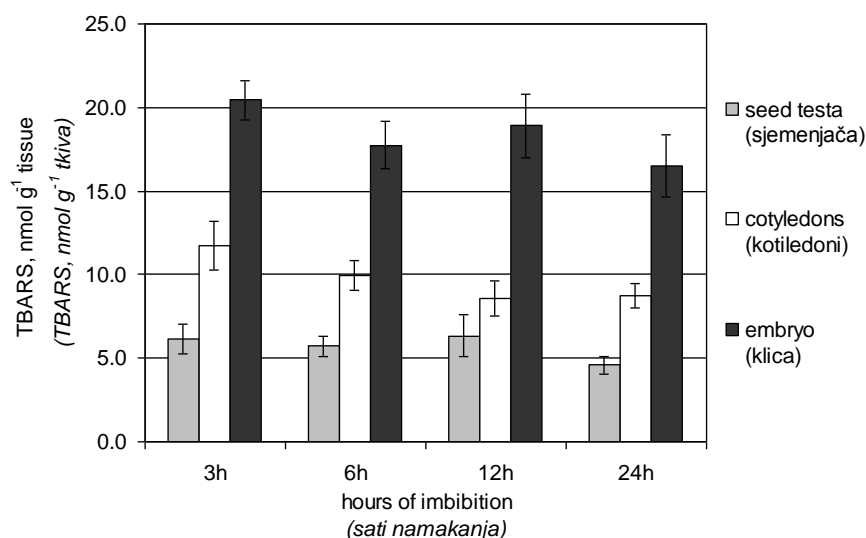
Soybean cultivar Vita - seed old 18 months
(*kultivar soje Vita - sjeme staro 18 mjeseci*)



Graph 3. Dynamics of lipid peroxidation (TBARS) in seed testa, cotyledons and embryo of 18 months old seed of soybean cultivar Vita, imbibed in deionized water up to 24 h (bars are means of four replicates ± standard deviation, with 50 grains per replicate submerged in 250 mL water)

Grafikon 3. Dinamika lipidne peroksidacije (TBARS) u sjemenjači, kotiledonima i klici sjemena soje kultivara Vita starog 18 mjeseci, pri namakanju u deioniziranoj vodi tijekom 24 h (stupci su srednje vrijednosti 4 ponavljanja ± standardna devijacija, s po 50 zrna po ponavljanju potopljenih u 250 mL vode)

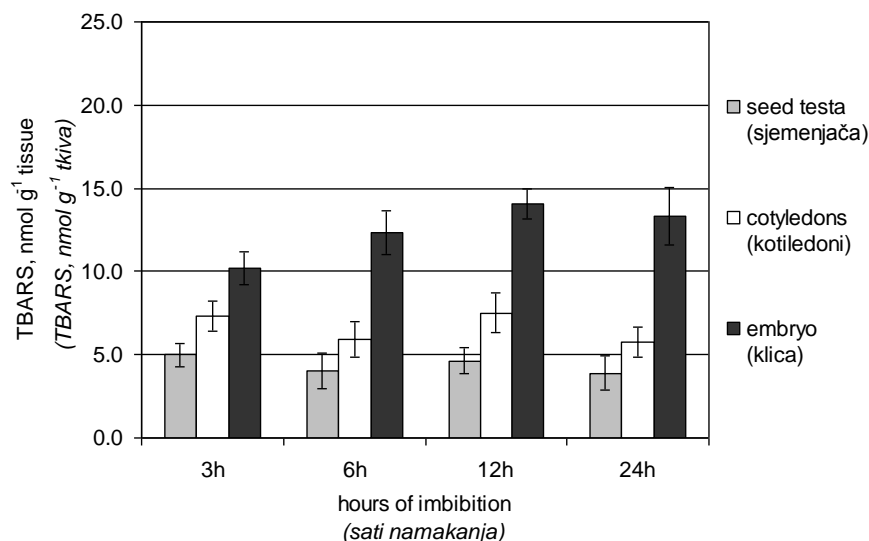
Soybean cultivar Vita - seed old 6 months
(*kultivar soje Vita - sjeme staro 6 mjeseci*)



Graph 4. Dynamics of lipid peroxidation (TBARS) in seed testa, cotyledons and embryo of 6 months old seed of soybean cultivar Vita, imbibed in deionized water up to 24 h (bars are means of four replicates ± standard deviation, with 50 grains per replicate submerged in 250 mL water)

Grafikon 4. Dinamika lipidne peroksidacije (TBARS) u sjemenjači, kotiledonima i klici sjemena soje kultivara Vita starog 6 mjeseci, pri namakanju u deioniziranoj vodi tijekom 24 h (stupci su srednje vrijednosti 4 ponavljanja ± standardna devijacija, s po 50 zrna po ponavljanju potopljenih u 250 mL vode)

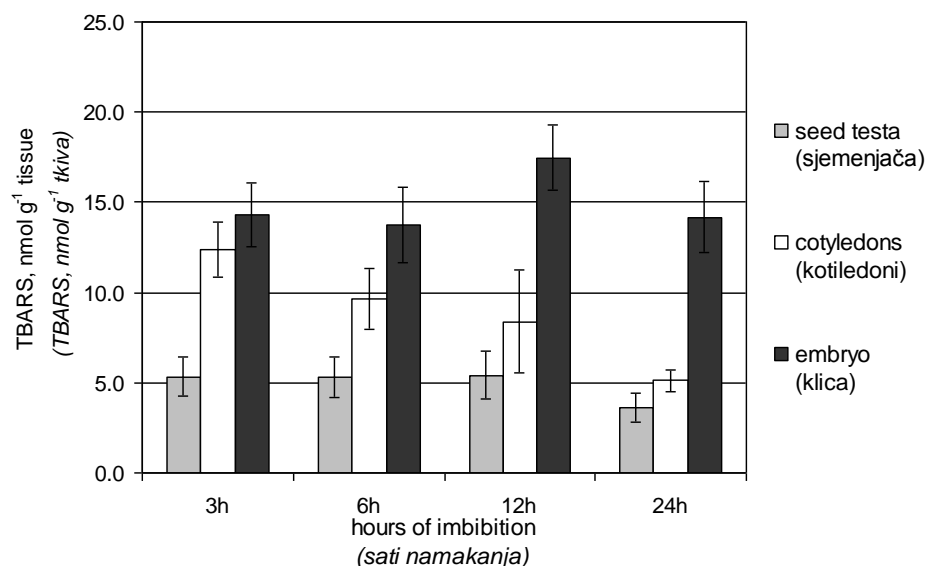
Soybean cultivar Tisa - seed old 18 months
(*kultivar soje Tisa - sjeme staro 18 mjeseci*)



Graph 5. Dynamics of lipid peroxidation (TBARS) in seed testa, cotyledons and embryo of 18 months old seed of soybean cultivar Tisa, imbibed in deionized water up to 24 h (bars are means of four replicates ± standard deviation, with 50 grains per replicate submerged in 250 mL water)

Grafikon 5. Dinamika lipidne peroksidacije (TBARS) u sjemenjači, kotiledonima i klici sjemena soje kultivara Tisa starog 18 mjeseci, pri namakanju u deioniziranoj vodi tijekom 24 h (stupci su srednje vrijednosti 4 ponavljanja ± standardna devijacija, s po 50 zrna po ponavljanju potopljenih u 250 mL vode)

Soybean cultivar Tisa - seed old 6 months
(*kultivar soje Tisa - sjeme staro 6 mjeseci*)



Graph 6. Dynamics of lipid peroxidation (TBARS) in seed testa, cotyledons and embryo of 6 months old seed of soybean cultivar Tisa, imbibed in deionized water up to 24 h (bars are means of four replicates ± standard deviation, with 50 grains per replicate submerged in 250 mL water)

Grafikon 6. Dinamika lipidne peroksidacije (TBARS) u sjemenjači, kotiledonima i klici sjemena soje kultivara Tisa starog 6 mjeseci, pri namakanju u deioniziranoj vodi tijekom 24 h (stupci su srednje vrijednosti 4 ponavljanja ± standardna devijacija, s po 50 zrna po ponavljanju potopljenih u 250 mL vode)

Wojtyla et al. (2006) stated that the observed changes in free radical levels, antioxidant content and associated enzyme activities in seed embryo axes and cotyledons of pea appear to be more closely related to metabolic and developmental processes associated with the preparation for germination and do not correspond directly to the hydration of the tissues. Meyer et al. (2007) recognise that seed imbibition occurs in two stages. In the relatively slow first step the seed testa becomes hydrated. In the second stage this is followed by rapid water uptake as a result of cotyledon hydration. If these steps of seed hydration are followed by ROS production and subsequent lipid peroxidation, then the peroxidation levels seen here, - high levels in the first few hours with a subsequent decline in intensity thereafter, would be expected. A detailed study of the water uptake dynamics during soybean seed imbibition and the role of the seed testa in preventing imbibitional damage was reported by Koizumi et al. (2008). They stated that slow and controlled hydration is essential for the reactivation of metabolic processes in the dry seed if this is to lead to efficient germination and subsequent growth.

The older soybean seeds showed higher lipid peroxidation levels than younger seeds in two of three cultivars tested (Table 1). One could conclude that more pronounced seed membrane damage developed during longer seed storage, which in turn may have contributed to a lower antioxidative potential in older seeds during conditions of imbibition stress. Regardless of seed age and cultivar, lipid peroxidation was the highest in embryo and the lowest in seed testa (Table 1). Hence, it is possible that lipid peroxidation products may participate in cell signalling systems, especially during the rapid increase in the metabolic activity shown by the germinating embryo post-imbibition. However, when considering the time of imbibition, the highest levels of lipid peroxidation were observed after 3 h of soaking, again in cultivars Podravka 95 and Vita. This suggests that intense oxidative stress is an early event, resulting in high levels of lipid peroxidation in all the seed tissues tested (Graphs 1-4). Liu et al. (2009) demonstrated that superoxide, generated during early imbibition, is an excellent marker for evaluating seed vigour. High lipid peroxidation levels were seen after only 3 h of imbibition and significantly decreased thereafter, especially in younger seeds, which may imply better functioning of membrane repair and/or anti-oxidative mechanisms in younger than in older seeds. However, the possibility of increased lipid peroxidation levels as a component of increased metabolic activity and signalling prior to embryonic axis growth during germination should also be considered.

CONCLUSION

Significant differences among tested soybean genotypes in the lipid peroxidation level were observed in specific seed tissues, during 24-hours of seed imbibition in water. Seed age and imbibition duration were important factors that determined lipid peroxidation dynamics in seed testa, cotyledons and embryo. It appears that soybean embryos were the most sensitive of the tested seed tissues to oxidative stress under hypoxia, showing the highest lipid peroxidation levels. However, it remains unclear if the established lipid peroxidation levels were a consequence of imbibitional damage or a result of increased metabolic activity at the onset of seed germination.

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RAZINE LIPIDNE PEROKSIDACIJE U DIJELOVIMA SJEMENA SOJE (*Glycine max* (L.) Merr.) KAO POSLJEDICA STRESA PRI USVAJANJU VODE

SAŽETAK

Intenzivne oborine i intenzivno usvajanje vode suhoga sjemena nakon sjetve u polju mogu rezultirati takozvanim imbibicijskim oštećenjem sjemena. U ovom istraživanju analiziran je intenzitet lipidne peroksidacije u sjemenjači, klici i kotiledonima sjemena tri sorte soje (Podravka 95, Tisa i Vita), nakon 3, 6, 12 i 24 imbibicije u vodi pri 20°C. U cjelini, lipidna peroksidacija bila je povećana u klici soje, a najslabije izražena u sjemenjači. S obzirom na dužinu imbibicije, najveći intenzitet lipidne peroksidacije utvrđen je nakon 3 h imbibicije, nakon čega se smanjivao kod sorata Podravka 95 i Vita, uz sličan trend, s obzirom na sjeme iste starosti.

Ključne riječi: *Glycine max* (L.) Merr., imbibicijsko oštećenje, lipidna peroksidacija, vigor sjemena, soja, sorta, TBARS

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