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Germination and gene expression as affected by aminocyclopropane-1-carboxylic acid in deteriorated soybean seed

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ABSTRACT

Seed aging leads to reduced germination and vigor, especially in long-term storage, which can significantly reduce crop yields. Hence, identifying genetic mechanisms and providing physiological approaches to alleviate the deterioration process are needed. The current study was conducted in a series of laboratory experiments with soybean (*Glycine max* (L.) Merr.) in 2015. Accelerated aging durations (0 d, 6 d, and 10 d), priming solutions [dry seed, water, and 50 µM aminocyclopropane-1-carboxylic acid (ACC)] and priming periods (6 h and 12 h) were considered experimental factors. Total RNA was extracted from whole dry and imbibed seeds using hexadecyltrimethylammonium method. Genes and associated accessions studied were: *LIG4* (XM_003546387.3), *CTR1* (BW653506.1), *GAI1* (BW656807.1), *NCED1* (AK244131.1), and *NCED5* (AK245513.1). With increasing severity of seed deterioration, the expression of *LIG4, CTR1, GAI1*, and *NCED1* significantly increased, whereas a decreasing trend was observed for *NCED5* expression. ACC had an inhibitory effect on germination index in aged soybean seed, which was associated with expression level of *LIG4*. Priming with ACC considerably reduced the inhibitory effect of *CTR1, GAI1*, and *NCED1* in 6-day aged seeds, resulting in a positive effect on germination. It seems that positive or negative effects of ethylene priming on soybean germination depend on severity of seed deterioration.

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Ethylene; gene expression; germination; seed aging; soybean

Introduction

Seed aging is defined as deteriorative changes across time, resulting in reduced seed viability and vigor as well as in susceptibility to external factors (McDonald 2004; Seyyedi et al. 2017).

Aging is a physiologically irreversible process (McDonald 2004) that can lead to an irregular germination process or abnormal seedling production by causing damage to cellular function (Eisvand et al. 2010; Veselovsky and Veselova 2012).

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Deterioration process can be created by a range of endogenous and environmental factors (Seyyedi et al. 2015; Mohaddes Ardebili et al. 2019); it accelerates with increasing relative humidity and temperature (Eisvand et al. 2010). The membrane permeability, DNA damage (Waterworth et al. 2010), changes in enzymatic activity and lipid peroxidation (Hsu et al. 2003) are the known, important physiological consequences associated with seed deterioration. These consequences are exacerbated by the production of reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2), during seed deterioration (Bailly 2004).

The accelerated aging mechanism, especially associated with an increase in electrical conductivity (EC), can be a sign of gradual seed deterioration (Nazari et al. 2020). EC is an appropriate test for predicting soybean seed vigor, which is based on the leakage of ions and metabolites from the cell membrane during the process of seed soaking (Vieira et al. 2004). In this regard, increased electrolyte leakage is considered one of the main indicators related to membrane damage and seed deterioration (Seyyedi, Tavakkol Afshari, and Daneshmandi 2018). It has been observed that with increasing aging durations, more electrolytes are lost because of the increased damage to the membrane integrity (Eisvand et al. 2010; Hsu et al. 2003).

In general, H_2O_2, as a stable ROS, has a signaling role in balancing phytohormones, breaking seed dormancy and stimulating the germination process (Wojtyla et al. 2015). When ROS concentration is low, germination-related processes may not be fully induced, whereas at high concentrations of ROS, along with the loss of antioxidant enzymes, the mechanisms involved in seed deterioration are stimulated. Hence, whether H_2O_2 plays a crucial role in stimulating germination or it exacerbates the seed deterioration process, depends on the ROS threshold level.

The effect of H_2O_2 on the germination process is essentially related to phytohormones, such as cytokinins and ethylene (Achard et al. 2003). In soybean seeds, H_2O_2, produced in embryonic axis after seed imbibition, provokes ethylene production and increases the germination rate (Ishibashi et al. 2013). Most studies have shown that ethylene signaling has a positive regulatory effect on the germination process (Beaudoin et al. 2000; Matilla 2000; Matilla and Matilla-Vázquez 2008). El-Maarouf-Bouteau et al. (2015) reported that seed priming with ethylene in sunflower triggered H_2O_2 production in the embryonic axis and increased germination rate. According to Hajiabbasi, Tavakkol Afshari, and Abbasi (2015), ethephon increased the vigor of non-aged soybean seeds, but it decreased vigor when applied on aged seeds.

To improve the germination process in aged seeds, it is necessary to regulate ROS with antioxidants at an appropriate level, along with the DNA repair response (Donà et al. 2013). *CTR1* (constitutive triple response 1) gene is a negative regulator of the ethylene-signaling pathway (Kieber et al. 1993). The ethylene regulatory function is mediated by *DELLA* proteins, such as gibberellin insensitive (GAI) regulator, which is an inhibitor of germination (Achard et al. 2003; Cao et al. 2005).

In general, 9-cis-epoxycarotenoid dioxygenase (*NCED*) gene encodes enzymes associated with the metabolism of plant hormones and can impose crucial effects on the germination process by inducing seed dormancy. For instance, carotenoid cleavage, catalyzed by *NCED1* and *NCED5*, is considered a major process involved in the abscisic acid (ABA) regulation and biosynthesis (Frey et al. 2012). *NCED1* expression in tomato increased ABA concentration considerably, which stimulated seed dormancy. However, there is still only limited information available regarding the expression and function of *NCED1* and *NCED5* genes (Kermode 2005).

In the present study, the effects of aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene, on germination and the expression of *CTR1, GAI1, NECD1* and *NECD5* genes in the aged soybean seed were investigated. In addition, effects of ethylene on DNA repair and expression of DNA ligase IV gene (*LIG4*) were studied.

Materials and methods

Plant materials

The soybean seeds (cv. JK), produced in 2015 (standard germination = 98%), were obtained from Seed and Plant Certification and Registration Institute, Karaj, Iran. JK is adapted to the semi-arid climate of the region.

Experimental layout

The study was conducted in a series of laboratory experiments based on a completely randomized design with four replications at College of Agriculture and Natural Resource, University of Tehran, Iran, in 2015. The factors were: accelerated aging durations (0 d, 6 d, and 10 d, representing no-aging, mild aging, and severe aging, respectively), priming solutions (no priming, water and 50 μM ACC) and priming periods (6 h and 12 h).

Seed treatments

Before seed treatments, three replicates of 4 g samples were oven-dried at 103°C for 17 h to determine the seed-moisture content (ISTA 2016). Then, soybean seeds were exposed to accelerated aging process at 40°C and 91% relative humidity for target durations.

Electrical conductivity (EC)

In general, the standard germination test cannot accurately predict seedling performance, especially under adverse conditions; hence, the EC test was

conducted to assess the changes caused by the accelerated aging process. Three replicates of 50 seeds were weighed and soaked in 250 ml deionized water at 20°C under dark conditions for 24 h. Electrical conductivity (EC) of the solution was measured using an EC meter (inoLab, WTW, Germany) and expressed as μS cm^{-1} g^{-1} according to Hajiabbasi, Tavakkol Afshari, and Abbasi (2015) (see Equation (1)):

$$\mathrm{EC}\left(\mu\mathrm{Scm}^{-1}\mathrm{g}^{-1}\right) = \mathrm{EC\ for\ each\ sample}\ \left(\mu\mathrm{Scm}^{-1}\right)/\mathrm{weight\ of\ seed\ sample\ (g)}. \ldots \quad (1)$$

Germination test

Standard germination test was conducted according to ISTA (2016). Twenty-five seeds were placed on a double-layered Whatman No. 1 paper in each 10-cm Petri dish and were moistened with 15 ml of each solution (distilled water or 50 μM ACC). The treated seeds were germinated at 25°C in an incubator in the dark for 8 d. Seeds were regarded as germinated when radicles (about 2 mm or more) had protruded through the seed coat (Mahajan et al. 2011).

Quantitative polymerase chain reaction (PCR) analysis

Total RNA was extracted from seeds using the hexadecyltrimethylammonium method described by Chang, Puryear, and Cairney (1993). Twenty-five μg of RNA was treated with DNase I on Qiagen RNeasy Mini Kit for further cleanup. Then, 5 μg of total RNA was used to synthesize cDNA in a 20 μl reaction using SuperScript III (Invitrogen, Carlsbad, CA). The cDNA was diluted three times and 10 μl was used in 20 μl polymerase chain reaction (PCR) samples with Platinum Taq (Invitrogen) and SYBR® Green (Invitrogen). Reactions were run on a Corbett Rotor-Gene 6000 (Qiagen, Hilden, Germany) and data were analyzed with Rotor-Gene Q Series software using the comparative quantification tool. Three replicates were carried out for each experiment. A sample for depicting the amplification of RNA in response to the priming treatment is shown in Figure 1.

Genes and their associated accession numbers for full-length or expressed sequence tag (EST) were: *LIG4* (XM_003546387.3), *CTR1* (BW653506.1), *GAI1* (BW656807.1), *NCED1* (AK244131.1), and *NCED5* (AK245513.1). The sequence of the primers is given in Table 1. Following Li et al. (2012), *UKN2* was the housekeeping gene for soybean seed and the sequence of its primer was used.

Statistical analysis

All experimental data were subjected to analysis of variance using SAS software (SAS Institute 2011). The least significant difference (LSD) (5% level) was used to measure statistical differences between treatment means.

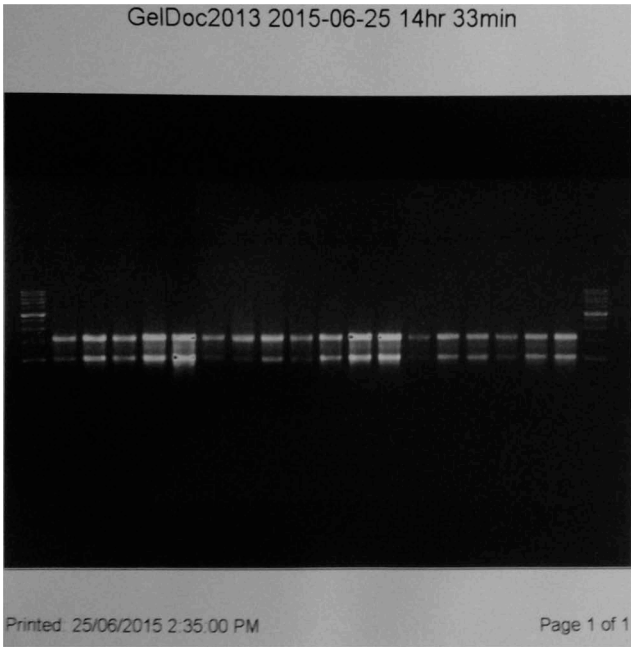

Figure 1. A sample for depicting the amplification of RNA in response to the priming treatment.

Table 1. Primer sequences used for qRT-PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Accession
LIG4	TTCTGGTGAACCTTGTTGGTC	ATTTCCAAACGACCCTTCACT	XM_003546387.3
CTR1	ATTCAACCATTCCCCCTGATACT	TTCCATCATCGCAGTGTGTTC	BW653506.1
GAI1	TGTGCGAAGTGAGGGAGATG	AGCTTGAGGTTCCTGCGTTG	BW656807.1
UKN2	GCCTCTGGATACCTGCTCAAG	ACCTCCTCCTCAAACTCCTCTG	BE330043
NCED1	AGATACGCCCCCGGAATCTAC	CAAGCTGGTCCCCAAAACCT	AK244131.1

Results and discussion

Germination and EC assay

The variation attributable to aging durations (D) and priming solutions (S) and the D × S interaction was significant for germination percentage (GP) (Table 2). However, priming periods (P), D × P, S × P, and D × S × P were not significant for GP (Table 2). The best two-way combination was 0-d aging duration with water priming, where germination was recorded as 99%.

Irrespective of seed priming, increase in aging duration significantly decreased GP, so that no seed germination was observed in the 10-d aging treatment (Tables 3 and 4). In the current study, water priming had a positive effect in that the water priming decreased seed deterioration. For example, when water priming was applied, there was no significant difference between

Table 2. Analysis of variance for aging durations (*D*), priming solutions (*S*) and priming periods (*P*) on germination percentage and electrical conductivity (EC) in soybean.

Source of variance	DF	Germination percentage	EC ($\mu Scm^{-1}\ g^{-1}$)
D	2	75.08**	46.91**
S	2	78.06**	39.97**
P	1	32.01^{NS}	33.92*
D × *S*	4	35.23**	23.42*
D × *P*	2	29.20^{NS}	19.21*
S × *P*	2	28.11^{NS}	44.54**
D × *S* × *P*	4	24.01^{NS}	29.98*
Error	54	9.93	6.82

*,** Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively; NS = non-significant.

Table 3. Effects of aging durations, priming solutions, and priming periods on germination percentage and electrical conductivity (EC) in soybean.

Experimental treatments	Germination percentage	EC ($\mu Scm^{-1}\ g^{-1}$)
Aging durations (day)		
0	94.5	25.13
6	82.5	44.17
10	0.0	54.56
LSD (5% level)	*6.21*	*1.952*
Priming solutions		
Dry seed	61.0	40.26
Water	63.8	40.72
ACC	52.2	42.88
LSD (5% level)	*6.21*	*1.952*
Priming periods (h)		
6		48.94
12		51.39
LSD (5% level)		*1.685*

Table 4. Two-way interactions (aging durations × priming solutions; priming solutions × priming periods) on germination percentage and electrical conductivity (EC) in soybean.

Two-way interactions		Germination (%)	EC ($\mu S\ cm^{-1}\ g^{-1}$)
Aging durations (day)	**Priming solutions**		
0	Dry seed	95.5	24.55
0	Water	99.0	25.80
0	ACC	89.0	25.03
6	Dry seed	87.5	40.28
6	Water	92.5	43.21
6	ACC	67.5	49.02
10	Dry seed	0.0	55.96
10	Water	0.0	53.14
10	ACC	0.0	54.59
LSD (5% level)		*8.73*	*2.387*
Priming solutions	**Priming periods (h)**		
Water	6		47.04
Water	12		50.03
ACC	6		50.84
ACC	12		52.75
LSD (5% level)			*2.013*

6-d aging duration with control (0-d aging duration) relative to GP. However, ACC priming significantly reduced GP in 6-d aged seeds, compared with water priming (Tables 3 and 4).

EC was significantly affected by D, S, and P. Moreover, D × S, D × P, S × P, and D × S × P interactions were significant for EC. There was a significant difference between aging durations relative to EC. Increasing aging duration up to 6 d increased EC significantly (Tables 3 and 4). The same trend was observed for the 10-d aging treatment in comparison with 6-d aging treatment. According to the three-way interaction, 12 h water priming on non-aged seeds was found to be the best three-way combination for EC trait (Table 5).

When the aged seeds were primed with water for 6 h or 12 h, EC was significantly lower than EC under ACC priming treatment during the same period. This decline was more pronounced under 6-d aging duration compared with 10-d aging duration. Similar to these results, Hajiabbasi, Tavakkol Afshari, and Abbasi (2015) reported that with increasing ethephon concentration, an increase in EC occurred, which decreased GP significantly in aged soybean seeds.

According to the results, ACC priming increased electrolyte leakage. Hence, the reduction in GP after 6-d aging duration might be related to defects in the function of ACC in aged seeds. Accordingly, it seems that ethylene does not have a stable effect (positive or negative) on the aged seeds. Furthermore, because of the fact that applying the accelerated aging treatment on various seeds generally results in different reactions (McDonald

Table 5. Three-way interactions (aging durations × priming solutions × priming periods) on electrical conductivity (EC) in soybean.

Three-way interactions			EC ($\mu S\ cm^{-1}\ g^{-1}$)
Aging durations (day)	**Priming solutions**	**Priming periods (h)**	
0	Dry seed	6	24.55
0	Dry seed	12	24.55
0	Water	6	25.74
0	Water	12	25.88
0	ACC	6	25.37
0	ACC	12	25.62
6	Dry seed	6	43.07
6	Dry seed	12	43.07
6	Water	6	43.14
6	Water	12	43.40
6	ACC	6	48.95
6	ACC	12	49.24
10	Dry seed	6	52.92
10	Dry seed	12	52.92
10	Water	6	52.95
10	Water	12	53.28
10	ACC	6	53.98
10	ACC	12	54.50
	LSD (5% level)		*2.615*

2004), the sensitivity of different seed parts may affect the response to hormones under different aging durations.

According to previous studies, ethylene has a positive effect on breaking seed dormancy and can also accelerate the germination process in non-dormant seeds (Matilla 2000; Matilla and Matilla-Vázquez 2008). However, the results of this study showed that ethylene had no significant effect on soybean seed germination.

Gene expression

According to the results, individual effects of D, S, and P were significant on relative expression of *LIG4, CTR1, GAI1, NCED1*, and *NCED5* (Table 6). D × P interaction was significant for expression of *LIG4, CTR1, NCED1*, and *NCED5*. In addition, S × P interaction was found to be significant for expression of *CTR1, NCED1*, and *NCED5*. However, the three-way interaction was not significant for the expression of *LIG4, CTR1, GAI1, NCED1*, and *NCED5* (Table 6).

After the 10-d aging duration, *LIG4* expression increased dramatically in comparison with 0- or 6-d aging duration (Table 7). In other words, *LIG4* increased considerably with an increase in aging duration when the seeds were exposed to high relative humidity. These changes can presumably be in response to DNA damage. Indeed, an increase in seed moisture content has been shown to accelerate DNA and RNA repair (McDonald 2004).

Regardless of aging durations, an increase in priming period significantly decreased *LIG4* expression. For example, when a 6-d aging duration was tested, 12-h priming period reduced *LIG4* expression by more than three times as compared with 6-h priming period. Moreover, water priming played a significant role in enhancing *LIG4* expression in comparison with ACC priming (Tables 7 and 8). It has been shown that DNA lesion and loss of seed

Table 6. Analysis of variance for aging durations (*D*), priming solutions (*S*) and priming periods (*P*) for expression of genes† in soybean.

Source of variance	DF	*LIG4*	*CTR1*	*GAI1*	*NCED1*	*NCED5*
D	2	5.69**	0.88**	0.92**	0.58**	4.09**
S	2	5.78**	0.79**	0.73**	0.61**	4.21**
P	1	6.01**	0.74**	0.36*	0.65**	5.83**
D × *S*	4	5.19^{NS}	0.26^{NS}	0.43^{NS}	0.28^{NS}	2.57^{NS}
D × *P*	2	4.99 **	0.59**	0.34^{NS}	0.52*	4.56*
S × *P*	2	5.59^{NS}	0.42*	0.54^{NS}	0.49*	4.89**
D × *S* × *P*	4	1.92^{NS}	0.22^{NS}	0.25^{NS}	0.19^{NS}	1.57^{NS}
Error	54	0.74	0.09	0.07	0.08	0.78

*,** Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively; NS = non-significant.

† *LIG4*: DNA ligase IV gene; *CTR1*: constitutive triple response 1; *GAI1*: gibberellin insensitive 1; *NCED1*: 9-cis-epoxycarotenoid dioxygenase1; *NCED5*: 9-cis-epoxycarotenoid dioxygenase1.

Table 7. Effects of aging durations, priming solutions, and priming periods on expression of genes† in soybean.

Experimental treatments	*LIG4*	*CTR1*	*GAI1*	*NCED1*	*NCED5*
Aging durations (day)					
0	2.62	0.03	1.64	0.039	0.83
6	2.79	0.13	2.26	0.074	0.68
10	19.70	0.50	3.73	0.139	0.48
LSD (5% level)	*1.341*	*0.063*	*0.216*	*0.0096*	*0.021*
Priming solutions					
Dry seed	7.08	0.08	1.95	0.095	0.59
Water	15.64	0.18	2.89	0.057	0.35
ACC	4.73	0.13	1.51	0.110	0.98
LSD (5% level)	*1.341*	*0.063*	*0.216*	*0.0096*	*0.021*
Priming periods (h)					
6	15.41	0.17	1.59	0.090	0.63
12	4.63	0.10	1.36	0.077	0.69
LSD (5% level)	*1.128*	*0.041*	*0.166*	*0.0074*	*0.017*

† *LIG4*: DNA ligase IV gene; *CTR1*: constitutive triple response 1; *GAI1*: gibberellin insensitive 1; *NCED1*: 9-cis-epoxycarotenoid dioxygenase1; *NCED5*: 9-cis-epoxycarotenoid dioxygenase1.

Table 8. Two-way interactions (aging durations × priming periods; priming solutions × priming periods) on expression of genes† in soybean.

Interactions		*LIG4*	*CTR1*	*NCED1*	*NCED5*
Aging durations (day)	**Priming periods (h)**				
0	6 h	4.10	0.13	0.054	0.81
0	12 h	1.15	0.08	0.023	0.85
6	6 h	24.72	0.31	0.047	0.64
6	12 h	6.58	0.19	0.100	0.72
10	6 h	5.42	0.06	0.169	0.44
10	12 h	2.66	0.03	0.109	0.51
LSD (5% level)		*1.527*	*0.098*	*0.0130*	*0.038*
Priming solutions	**Priming periods (h)**				
Water	6		0.22	0.190	0.36
Water	12		0.14	0.118	0.32
ACC	6		0.15	0.218	1.31
ACC	12		0.08	0.222	1.37
LSD (5% level)			*0.089*	*0.0126*	*0.033*

† *LIG4*: DNA ligase IV gene; *CTR1*: constitutive triple response 1; *NCED1*: 9-cis-epoxycarotenoid dioxygenase1; *NCED5*: 9-cis-epoxycarotenoid dioxygenase1.

viability occurred during aging (Elder et al. 1987). DNA repair during imbibition is essential for germination and seed longevity (Waterworth et al. 2010).

Similar to *LIG4* expression, an increase in aging duration significantly increased *CTR1* expression, whereas the increase in priming periods had a decreasing effect. (Table 7). Regardless of priming periods, water priming, compared with ACC priming, increased *CTR1* expression in aged seeds. For example, 12-h water priming increased *CTR1* expression about two times in comparison with 12-h ACC priming (Table 8).

Similar to *LIG4* and *CTR1*, prolonged aging durations caused a significant increase in *GAI1* expression. For example, *GAI1* expression under 10-d aging

treatment was found to be 65.1% higher than that under 6-d aging treatment (Tables 7 and 8).

NCED1 and *NCED5* expression exhibited different responses during the accelerated aging process. Increased aging durations significantly increased *NCED1* expression, whereas under similar conditions, *NCED5* expression was significantly reduced (Tables 7 and 8).

ACC priming increased *NCED1* and *NCED5* expression compared to water priming. In terms of *NCED1* and *NCED5* expression, the best two-way interaction was obtained under 12-h ACC priming (Table 8). In other words, the highest *NCED1* and *NCED5* expression was observed when the seeds were primed with ACC for 12-h (Table 8). Beaudoin et al. (2000) have shown that *Ctr1* mutant exhibited an increase in germination rate. Moreover, the increased *HvNCED1* expression in embryos of imbibing seed promoted seed dormancy (Gubler et al. 2008). As noted earlier, *NCED5* expression did not decrease in 6-d aged seeds primed with ACC. *NCED5* expression was 4.52 times higher than that of *NCED1* under water priming. In fact, ethylene can decrease the influence of some germination inhibitors, but it cannot improve DNA repair by *LIG4* as much as water.

Another reason for reducing GP in 6-d aged seeds primed with ethylene in comparison with water priming may be due to biochemical damage to aged seeds. In fact, ethylene has been shown to play an important role in optimal ROS production and promote germination (El-Maarouf-Bouteau et al. 2015). However, to improve germination performance, the concentration of ROS should be controlled; accelerated seed aging process is attributed to the excessive production of ROS (Bailly et al. 2004). Consequently, the effect of ethylene priming on 6-d aged seeds may be due to increased electrolyte leakage and reduced GR.

Under the 10-d aging duration, where no seed germination occurred, expression of all genes was reduced during water priming. The ethylene priming on 10-d aged seeds only caused an increase in *NCED1* and *NCED5* expression. Furthermore, when the ratio of ethylene to water priming was considered in 10-d aged seeds, the expression of all genes was found to be increased, except for *LIG4*. This indicates that severe seed aging (10-d aging duration) increases germination inhibitors.

Overall, water priming for 6 h (relative expression of *CTR1*) and ACC priming for 12 h (relative expression of *NCED1*, and *NCED5*), were considered as the best the two-way interaction.

Conclusions

In the present study, ACC priming reduced GP in 6-d aged seeds compared with water priming. Moreover, ACC priming on 6-d aged seeds considerably reduced the inhibitory effect of *CTR1, GAI1*, and *NCED1*, resulting in

increased germination. Under the 10-d aging duration, some of the germination inhibitors, including *NCED1* and *CTR1* expression, as well as the GA signaling pathway inhibitor, such as *GAI1* expression, increased. Moreover, the response levels to hormone were found to be induced during the accelerated aging durations. It seems that positive or negative effects of ethylene priming on soybean germination depend on severity of seed aging.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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