Pak. J. Bot., 48(2): 607-612, 2016.

CHANGES IN NON-ENZYMATIC ANTIOXIDANT CAPACITY AND LIPID PEROXIDATION DURING GERMINATION OF WHITE, YELLOW AND PURPLE MAIZE SEEDS

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Abstract

In this study, the changes in non-enzymatic antioxidant capacity and lipid peroxidation during the germination process of purple, yellow and white maize seeds were compared, under favorable conditions. Results showed that germination can increase non-enzymatic antioxidant capacity (evaluated with ferric reducing power and 2, 2-diphenyl-1-picryl-hydrazyl-hydrate radical scavenging capacity) and lipid peroxidation levels for all these seeds. In addition, non-enzymatic antioxidant capacity observed in the germinating seeds were in the order of purple > yellow > white. However, the highest and lowest levels of lipid peroxidation could be seen during the germination processes of the white and purple seeds, respectively. In addition, the germination rates of the seeds followed the order of white > yellow > purple. Further studies showed that H_2O_2 treatment can significantly promote seed germination, especially for purple seeds. In addition, DMTU (dimethylthiourea), a specific scavenger for H_2O_2, could slightly but significantly arrest dormancy release. Data analysis showed that a high negative correlation ($R^2 = -0.955$) existed between non-enzymatic antioxidant capacity and germination rates. However, a high positive correlation ($R^2 = 0.860$) could be detected between lipid peroxidation and germination rates. Finally, lipid peroxidation as a possible novel signaling mechanism for seed germination has been discussed under stress-free conditions.

Key words: Antioxidant capacity, Germination rate, Lipid peroxidation, Maize seeds.

Introduction

Reactive oxygen species (ROS) are derived from the reduction of oxygen which gives rise to superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) (Apel & Hirt, 2004; Gill & Tuteja, 2010). These ROS resulting in oxidative damage can be controlled by both enzymatic and non-enzymatic antioxidants. However, $OH^{\cdot}$ and $^1O_2$ only can be removed by non-enzymatic antioxidants (Gill & Tuteja, 2010).

In seeds, ROS production has been considered for a long time as being detrimental, following investigations on seed ageing and seed desiccation (Sun & Leopold, 1995; França *et al*., 2007). Recently, many studies have shown that the role of ROS in seeds is not as unfavorable as it was considered previously. On the contrary, it now appears that ROS play a key signaling role in the achievement of major events of seed life, such as seed germination (Ogawa & Iwabuchi, 2001; Oracz *et al*., 2007; Bailly *et al*., 2008; El-Maarouf-Bouteau & Bailly, 2008; Barba-Espín *et al*., 2012; Leymarie *et al*., 2012). In addition, production of H_2O_2 has been detected at the early imbibition period in seeds of maize, soybean, wheat, pea, radish, sunflower and tomato (El-Maarouf-Bouteau & Bailly, 2008).

Seed dormancy has been described as one of the least understood phenomenon in seed biology and remains confusing, despite much recent progress (Finch-Savage & Leubner-Metzger, 2006). Thus, many hypotheses have been proposed to explain the beneficial or negative roles of ROS in the regulation of seed germination and dormancy (Ogawa & Iwabuchi, 2001; Oracz *et al*., 2007; Bailly *et al*., 2008; Bazin *et al*., 2011). Interestingly, all these hypotheses were established on the basis of ROS signaling or oxidative damage.

In fact, both ROS and antioxidants are required for seed germination (Bailly, 2004; Bogdanović *et al*., 2008). Numerous studies have focused on ROS or oxidative damage on seed germination (Ogawa & Iwabuchi, 2001; Oracz *et al*., 2007; Bailly *et al*., 2008; Barba-Espín *et al*., 2012; Leymarie *et al*., 2012). However, there is less data available on the role of antioxidants, especially the non-enzymatic antioxidant capacity, during this process (Hite *et al*., 1999; Bajji *et al*., 2007). In addition, germination always increases catalase activities (Leymarie *et al*., 2012) and induces ascorbate synthesis (Tommasi *et al*., 2001).

Interestingly, much attention has been paid on the roles of protein oxidation or even RNA oxidation during seed germination (Job *et al*., 2005; Oracz *et al*., 2007; Bazin *et al*., 2011; El-Maarouf-Bouteau *et al*., 2013). Previous data also showed that lipid signaling plays a key role in plant growth and defense (Wang, 2004; van Leeuwen *et al*., 2004; Testerink & Munnik, 2005). However, there is little data showing the altering patterns of lipid oxidation during seed germination. Interestingly, lipid oxidation is seemingly not favorable for seed germination (Kumar & Knowles, 1993; Sattler *et al*., 2004).

Published data show that seeds with different colors always display different antioxidant capacities (Furuta *et al*., 2003; Takahashi *et al*., 2005; Slavin *et al*., 2009). Maize plants can produce seeds with various colors. Here, the white, yellow and purple seeds of maize plants were collected for investigation of their germination behavior. We addressed two related questions: firstly, whether these seeds with different colors exhibited the same germination behavior under favorable conditions, and secondly, if there is a changing pattern in the non-enzymatic antioxidant capacity and lipid peroxidation during germination.

Materials and Methods

Seed germination and treatment: The following maize inbred lines were used for these studies: *Bainian* (white seeds), *Jinnuo* (yellow seeds) and *Heinian* (purple seeds). The seeds were sown in petri dishes and placed in a seed germinator at 25 ± 1°C. Germination trials were conducted in 9 cm sterile petri dishes lined with Whatman No.1 filter papers and moistened with distilled water, to ensure adequate moisture for the seeds. In an attempt to remove germination inhibitors, the seeds were leached with distilled water for 60 minutes before the experiment. Seeds were then surface sterilized in aqueous solution of 0.1% $HgCl_2$ for 60 s to prevent fungal attack and rinsed with several changes of sterile water. All these experiments were performed under dark box conditions with a 25°C temperature.

The germination experiment was divided into three groups: water control group (group 1), H_2O_2 (100 mM) treated groups (group 2), and DMTU (dimethylthiourea; a H_2O_2 scavenger, 5 mM) treated groups (group 3) (Hu *et al*., 2008). All these experimental designs were completely randomized with five replicates, and each with a group of 100 seeds per treatment.

Germination assay: The seeds were examined daily and considered germinated when the radicle was visible. The germination percentage was calculated after sowing for the first 7 days. The germination experiment was performed with five repetitions with 100 seeds.

Total antioxidant capacity assay: The total antioxidant capacity (TAC) was determined by using a ferric reducing ability of plasma (FRAP) assay described by Benzie and Strain (1996), with some modifications. Briefly, the FRAP reagent containing 2.5 ml of 10 mM TPTZ (2,4,6-Tripyridyl-s-triazine) solution in 40 mM HCl, and 2.5 ml of 20 mM $FeCl_3$ in 25 ml of 0.3 M acetate buffer (pH 3.6), was freshly prepared. The extracts were dissolved in ethanol (50%) at a concentration of 1 mg ml^{-1}. An aliquot of 20 µl test solution was mixed with 180 µl of FRAP reagent. The absorption of the reaction mixture was measured at 595 nm using a spectrophotometer. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$.

Before the TAC test, the extracts were first prepared. These seeds were weighed and immediately quenched in liquid nitrogen. Samples (~1 g DW) were ground to a powder in liquid nitrogen using a mortar and pestle and transferred to 100 ml of 90% (w/v) methanol-water solution at room temperature for 24 hours in dark conditions. The filtrates of each time-point were pooled and the solvent was removed under vacuum at 45°C using a rotary evaporator. The obtained crude extracts were stored in a desiccator at 4°C for the TAC test.

DPPH radical scavenging capacity determination: The ROS scavenging capacity can be approximately evaluated with the 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging method (Brand-Williams *et al*., 1995). DPPH radicals have an absorption maximum at 515 nm, which disappears with reduction by the presence of antioxidant compounds. The DPPH solution (60 µM) in methanol was freshly prepared, and 3 ml of this solution was mixed with 100 µl of methanolic solutions of plant extracts. The samples were incubated for 20 minutes at 37°C in a water bath, and the decrease in absorbance at 515 nm was measured (A_E). A blank sample containing 100 µl methanol in the DPPH solution was also prepared and its absorbance was measured (A_B). The DPPH radical scavenging capacity was calculated using the following formula:

$$\text{Percentage of inhibition} = [(A_B - A_E)/A_B] \times 100$$

Lipid peroxidation assay: Lipid peroxidation was determined by measuring the concentration of thiobarbituric acid reactive substance (TBARS) (Heath & Packer, 1968). In brief, the germinating seeds after sowing for different times, had their germination terminated by immersion into trichloroacetic acid (5%) and collected for further assays. These collected seeds were subsequently homogenized in trichloroacetic acid (5%) and centrifuged. The supernatant was mixed with thiobarbituric acids and heated at 95°C. The mixture was then quickly cooled on ice and centrifuged again. The absorbance of the supernatant was measured at 532 nm. A correction for non-specific turbidity was performed by subtracting the absorbance value at 600 nm. The lipid peroxide content was measured as nmol TBARS g^{-1} DW using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical analysis: All data were analyzed using SPSS 13.0 software. Means followed by different letters are significantly different (Duncan's multiple range test $p<0.05$). For each stress treatment, five replicates were analyzed.

Results

Antioxidant capacity in germinating seeds: Previous data showed that seed germination is closely associated with antioxidant capacity (Bailly, 2004). In this study, the purple, yellow and white maize seeds were collected for investigating their germination behavior under favorable conditions (Fig. 1a). The non-enzymatic antioxidant capacity was evaluated using two independent methods: FRAP method (showed the total antioxidant capacity, TAC) and DPPH method (showed the ROS scavenging capacity). Here, the changing patterns in the non-enzymatic antioxidant capacity was measured during the germination process for the purple, yellow and white maize seeds (Fig. 1b,c). As shown in Fig. 1b, significant differences in TAC were detected amongst the maize seeds with different colors. The highest and lowest TAC were observed in purple and white seeds, respectively, during the first 24 hours after sowing. For example, approximately one-fold and two-fold increase in TAC could be measured in yellow and purple seeds, respectively, compared with the white ones, at the 24 hour time-point (Fig. 1b; $p<0.05$).

However, all TAC levels significantly increased with a progress in germination, compared with the zero hour time-point, especially for the white seeds. For example, about 26%, 12% and 21% increase in TAC could be monitored at 8 hour, 16 hour and 24 hour time-points, respectively, compared with the zero hour time-point in purple seed germination (Fig. 1b; $p<0.05$). As for ROS

scavenging, similar changing patterns could be observed for DPPH radical scavenging capacity in these seeds with different colors (Fig. 1c). For example, approximately 80%, 20% and 33% increase in DPPH radical inhibition percentage was measured in white seeds at the 8 hour, 16 hour and 24 hour time-points, respectively, compared with the zero hour time-point (Fig. 1c; $p<0.05$).

Germination rates of purple, yellow and white seeds: The germination rates (GR) of the purple, yellow and white seeds were investigated under favorable conditions (Fig. 2). Results showed that the GR of these seeds followed such an order: purple < yellow < white. As shown in Figure 2, approximately 13-fold and 21.5-fold increase in one-day GR and 20% and 40% increase in six-day GR were observed in yellow and white seeds, respectively, over that of the purple ones (Fig. 2; $p<0.05$). The data also suggested that the white seeds can germinate faster compared to the yellow and purple ones, under favorable conditions.

In a further study, the effects of H_2O_2 and DMTU (scavenger of H_2O_2) on germination of these seeds were investigated (Fig. 2). Reversed changes in germination patterns could be observed in all these seeds after treatment with the two reagents (Fig. 2). H_2O_2 treatment significantly increased the GR for all these seeds, compared to the controls, especially for the purple ones (Fig. 2). For example, approximately 30%, 22%, 20%, 17%,17%, 17% and 17% increase in GR were detected in the H_2O_2 treated group over the control group of yellow seeds after sowing for 1, 2, 3, 4, 5, 6 and 7 days, respectively (Fig. 2b; $p<0.05$). In addition, DMTU treatment significantly but slightly decreased these seed germination rates compared with the control group (Fig. 2). For example, approximately 16%, 12%, 10%, 10%, 10%, 10% and 10% decrease in GR for the DMTU-treated group over the control group occurred in white seeds after sowing for 1, 2, 3, 4, 5, 6 and 7 days, respectively (Fig. 2c; $p<0.05$).

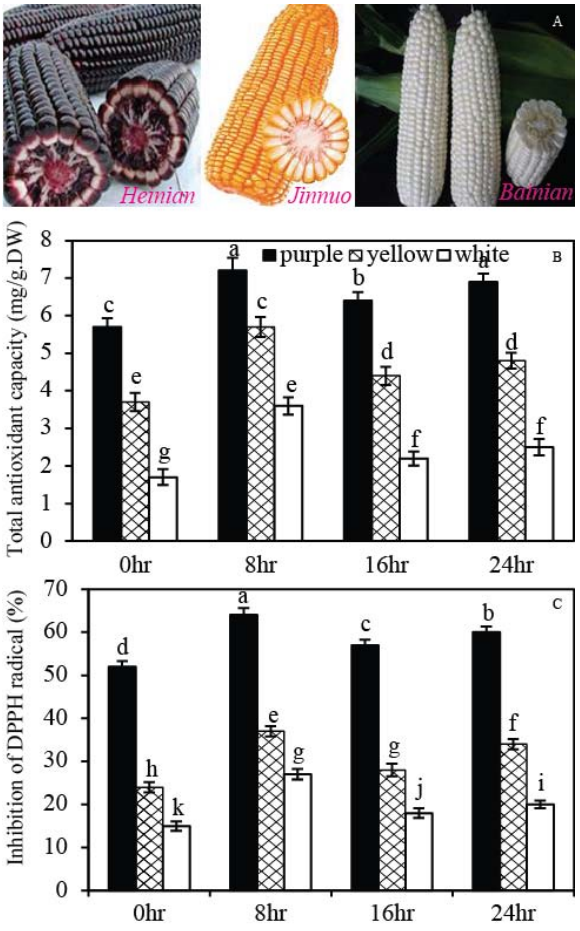

Fig. 1. Antioxidant capacity in germinating seeds.
Maize seeds used in this work (A). Changing patterns of total antioxidant capacity (B) and DPPH radical scavenging capacity (C) of the three kinds of seeds during the first 24 hours after sowing. The data are the means of five independent experiments. Means followed by different letters are significantly different (Duncan's multiple range test, $p<0.05$). Bars indicate means ± S.E. (n = 5).

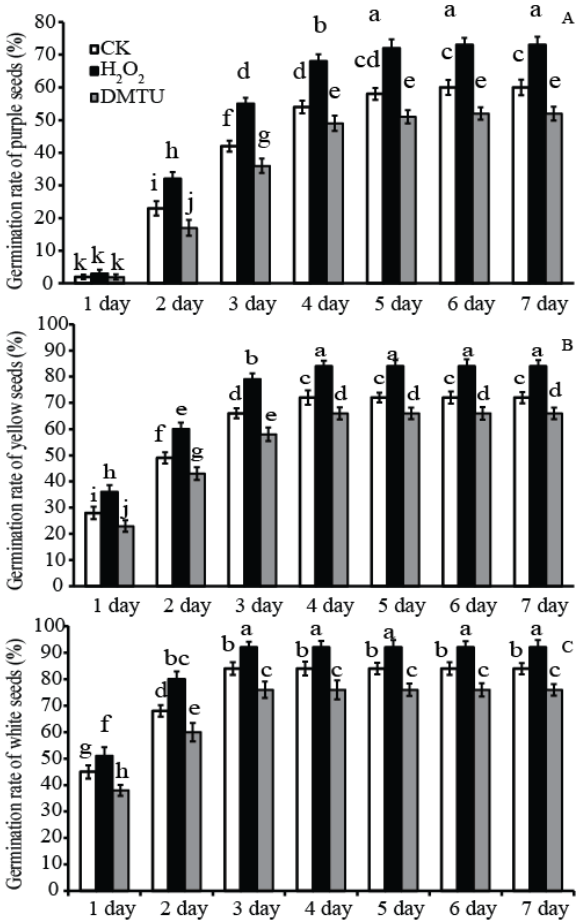

Fig. 2. Germination rates of purple, yellow and white seeds.
Effects of water (control), H_2O_2 and the H_2O_2 scavenger DMTU on germination rates was compared between the purple (A), yellow (B) and white (C) maize seeds during the first 7 days. The data are the means of five independent experiments. Means followed by different letters are significantly different (Duncan's multiple range test, $p<0.05$). Bars indicate means ± S.E. (n = 5).

Lipid peroxidation during the germination process: TBARS content was determined for evaluation of lipid peroxidation during the germination process of these seeds (Fig. 3). As shown in Figure 3, highest and lowest TBARS levels were detected in white and purple seeds, respectively. For example, about 14% and 27% increase in TBARS accumulation was monitored in yellow and white seeds, respectively, over the purple ones, 24 hours after sowing ($p<0.05$). In addition, germination stimulated the generation of TBARS, especially for the white seeds (Fig. 3). Compared with the zero hour time-point, approximately 30%, 52% and 67% increase in TBARS were detected in purple seeds after germination for 8, 16 and 24 hours, respectively ($p<0.05$). By contrast, about 50%, 60% and 80% increase in TBARS were measured in white seeds after germination for 8, 16 and 24 hours, respectively , compared with the zero hour time-point ($p<0.05$).

Discussion

Previous reports showed that seed germination is closely associated with ROS production and ROS scavenging capacity (Bailly, 2004; Oracz *et al*., 2007; Leymarie *et al*., 2012). However, there is little data showing the change in patterns of non-enzymatic antioxidant capacity during seed germination (Tommasi *et al*., 2001). Here, the non-enzymatic antioxidant capacity (evaluated as ferric reducing power ability and DPPH radical scavenging capacity) of maize seeds with different colors was measured within the first 24 hours after sowing (Fig. 1a). Results showed that germination can increase the total antioxidant capacity (TAC) for all three types of seeds to different extents (Fig. 1b). In addition, the TAC of these seeds with various colors followed such an order: purple > yellow > white. The DPPH radical scavenging capacity also showed similar changes (Fig. 1c). One plausible explanation is that the purple seeds have more secondary compounds such as anthocyanins than the other seeds. These data also support previous reports that seed germination can increase antioxidant enzyme activity and induce ascorbate synthesis (Leymarie *et al*., 2012; Tommasi *et al*., 2001). Subsequently, the germination rates amongst the purple, yellow and white maize seeds were compared. Results showed that the germination rates and germination speeds of these seeds under favorable conditions followed this order: white > yellow > purple (Fig. 2). In a further study, the effects of H_2O_2 and DMTU (a specific scavenger of H_2O_2; Hu *et al*., 2008) on germination behavior of these seeds were investigated (Fig. 2). Data showed that H_2O_2 treatment could increase germination rates for all these maize seeds, especially for the purple ones (Fig. 2a). As for DMTU, it could slightly, but significantly, decrease the germination rates for all these seeds with different antioxidant capacities (Fig. 2). These data showed that the germination rates are closely associated with the endogenous antioxidant capacity of the maize seeds.

Oxidative stress arising from ROS can impair lipids, proteins and nucleic acids in plant cells (Apel & Hirt, 2004; Gill & Tuteja, 2010). Published reports also showed that the oxidation of protein or RNA can accelerate dormancy release and stimulate seed germination (Job *et al*., 2005; Oracz *et al*., 2007; Bazin *et al*., 2011; El-Maarouf-Bouteau *et al*., 2013). However, there is less data available to learn about the altered patterns of lipid oxidation during seed germination. Here, the concentration of TBARS (for evaluation of lipid peroxidation) was measured during the germination process of the purple, yellow and white seeds (Fig. 3). Results showed that germination can increase TBARS accumulation significantly in maize seeds under favorable conditions (Fig. 3). Interestingly, the content of TBARS of these seeds followed such an order: white > yellow > purple. It showed that the white seeds can produce higher TBARS over the yellow and purple ones, under favorable conditions. This changed tendency of TBARS is in accordance with the TAC for these seeds. It suggested that seeds with different antioxidant capacities would affect the lipid peroxide production during germination.

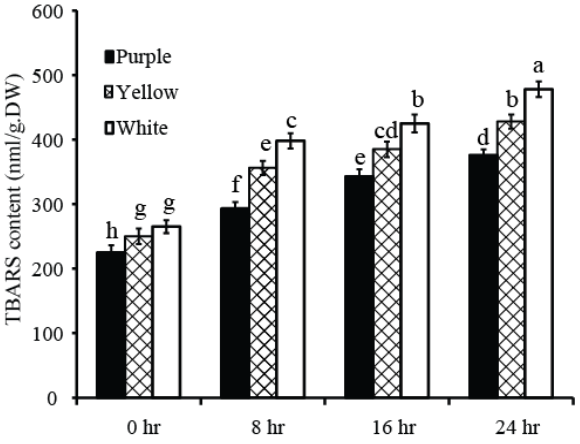

Fig. 3. Lipid peroxidation during germination process.
TBARS were measured in purple, yellow and white seeds after sowing for 0, 8, 16 and 24 hours. The data are the means of five independent experiments. Means followed by different letters are significantly different (Duncan's multiple range test, $p<0.05$). Bars indicate means ± S.E. ($n = 5$)

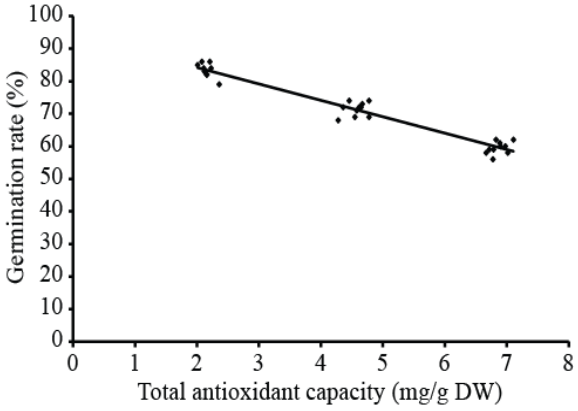

Fig. 4. Correlation between TAC and GR under favorable conditions.
Scatterplots of TAC (after sowing for 24 h) against GR (after sowing for 7 days) for maize seeds under favorable conditions. Lines represent the best-fit functions of the whole data set for each variable: $GR = 94.284 - 5.041 \times TAC$ ($R^2 = -0.955$, $p<0.05$). The X-axis data come from the TAC of the white, yellow and purple seeds. The Y-axis data come from the GR of the white, yellow and purple seeds under favorable conditions. GR, germination rate; TAC, total antioxidant capacity

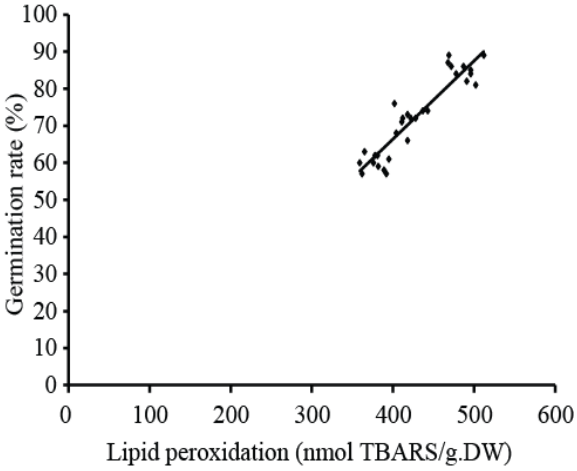

Fig. 5. Correlation between TBARS and GR under favorable conditions.
Scatterplots of TBARS (after sowing for 24 h) against GR (after sowing for 7 days) for the maize seeds under favorable conditions. Lines represent the best-fit functions of the whole data set for each variable: $GR = -18.070 + 0.211 \times TAC$ ($R^2 = 0.860$, $p<0.05$). The X-axis data come from the TAC of the white, yellow and purple seeds. The Y-axis data come from the GR of the white, yellow and purple seeds under favorable conditions. GR, germination rate; TBARS, thiobarbituric acid reactive substance

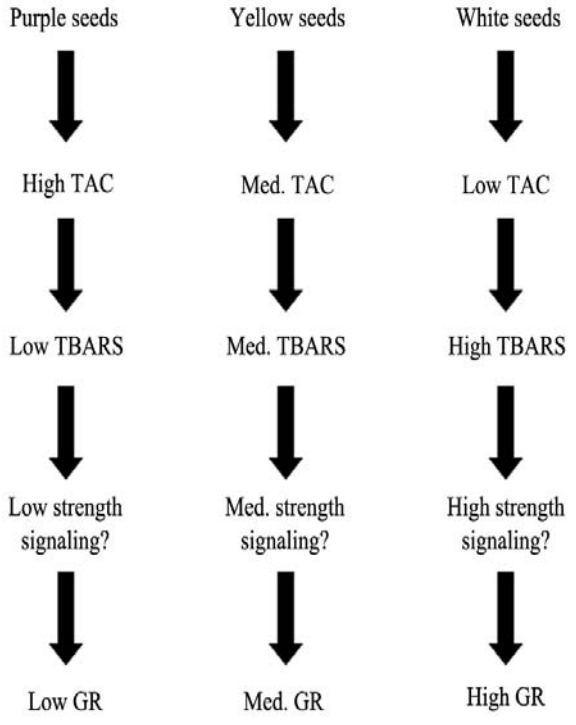

Fig. 6. A hypothetical model for germination of different colored maize seeds.
A hypothetical model based on lipid peroxidation is proposed here for illustration of the germination responses of maize seeds with different antioxidant capacities under favorable conditions. TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substance; GR, germination rate

Combining our results and previous reports (Hite *et al*., 1999; Bajji *et al*., 2007), it seems that the endogenous antioxidant capacity can profoundly affect the germination rates of these maize seeds (Figs. 1, 2). Thus, a correlation analysis between the TAC and the GR was performed (Fig. 4). Data showed that a high negative correlation ($R^2 = -0.955$) could be observed between TAC and GR. In other words, higher TAC can arrest but not promote seed germination, compared with the light colored seeds with lower TAC (Fig. 4).

In addition, the extent of lipid peroxidation appears to have a certain correlation with seed germination. Thus, the correlation between the level of TBARS and seed germination was also analyzed here (Fig. 5). As shown in Fig. 5, a high positive correlation ($R^2 = 0.860$) existed between lipid peroxidation and germination rates. In addition, higher TBARS production can be expected in the H_2O_2-treated seeds compared to the control or the DMTU-treated seeds. Accordingly, the GR increased or decreased after H_2O_2 or DMTU treatment, respectively (Fig. 2). These data suggested that lipid oxidation, in addition to protein oxidation, may contribute to seed germination at least in maize seeds. However, this conclusion is not in accordance with previous reports (Kumar & Knowles, 1993; Sattler *et al*., 2004). Kumar and Knowles (1993) found that lipid peroxidation during ageing can impair the sprouting of potato (*Solanum tuberosum*) seed tubers. In addition, Sattler *et al*. (2004) reported that tocopherol deficiency can arrest seed germination and four-fold increase of lipid hydroperoxides in the *Arabidopsis thaliana* mutant *vte2*. One plausible explanation for this contradictory evidence may be due to the differences in the extent of lipid peroxidation during seed germination. An over-production of TBARS can directly affect seed viability and thus decrease seed germination (Kumar & Knowles, 1993; Sattler *et al*., 2004). However, lipid peroxidation appeared to be at a "favorable" level (about 1-2 fold increase) during seed germination under stress-free conditions in our work (Fig. 3). Interestingly, both H_2O_2 and lipid peroxide can be categorized as peroxides. As for H_2O_2 signaling, an "oxidative window for germination" hypothesis based on H_2O_2 production has been proposed by Bailly *et al.* (2008). Similarly, it is possible that a lipid peroxide window for germination exists. Low lipid peroxidation or over-production of lipid peroxides would not favor seed germination as well as H_2O_2. In addition, protein or RNA oxidation has been reported previously to promote seed germination (Job *et al*., 2005; Oracz *et al*., 2007; Bazin *et al*., 2011; El-Maarouf-Bouteau *et al*., 2013). In fact, oxidative stress as a signaling event has recently been re-evaluated in the context of plant physiology (Foyer & Noctor, 2005). Thus, we speculate that lipid peroxidation may play a similar signaling role for triggering seed germination, as well as protein and nucleic acid oxidation (Gomes & Garcia, 2013). In other words, lipid peroxide over-production can impair seed germination, whereas slight or moderate oxidative stress resulting in lipid peroxidation may favor the release of dormancy. In addition, the importance of lipid signaling has also been receiving more and more attention (Wang, 2004; van Leeuwen *et al*., 2004; Testerink & Munnik, 2005). However, the possible mechanism underlying lipid oxidation-mediated dormancy release is still elusive. More experimental data are needed to clarify this. Here, a hypothetical model based on endogenous antioxidant capacity was proposed for illustration of the

possible signaling roles of lipid oxidation in seed germination (Fig. 6).

Some interesting conclusions can be generalized here. Firstly, germination increased the non-enzymatic antioxidant capacity of maize seeds in this order: purple > yellow > white. Secondly, the highest and lowest germination rates could be observed in white and purple seeds, respectively, under favorable conditions. Thirdly, germination can significantly increase lipid peroxidation, and it has a high positive correlation to germination rates in maize seed. In addition, the possible significance of lipid oxidation in seed germination was discussed. At last, a hypothetical model based on non-enzymatic antioxidant capacity is proposed here for illustrating the germination behavior of seeds with different levels of lipid peroxidation.

Acknowledgements

This study was supported by grants from the Initiation Foundation of Basic Science Research for Introduced Talent Person of Heilongjiang Bayi Agricultural University, to Benliang Deng.

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(Received for publication 7 March 2015)