

## **Effects of Germination Time on Antioxidant Contents and Enzymatic Antioxidant Activities in the Grains of Different Rice Varieties**

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Tocopherols, tocotrienols and  $\gamma$ -oryzanol are potent antioxidants of rice grains, and they may play an important role in the germination and growth of rice plants. In this study, the objective was to examine the effects of germination time on contents of Toc, T3, GO and ascorbate, as well as enzymatic antioxidant activities in the grains of two different rice varieties, namely TN71 and KS139. Samplings were conducted at 0, 3, 6 and 9 days after imbibition. The results showed that T3 and GO contents, but not Toc increased during seedling emergence. Toc content showed a trend of decrease from 0 DAI to 6 DAI. Contrasting to KS139, the AsA content in the grains of TN71 increased with increasing DAI. KS139 showed a time-dependent increase in the dehydroascorbate level, while that of TN71 remains unchanged at all times. TN71 showed significant increases in superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase activities in the late germination stages (9 DAI); with the exception of APX, KS139 exhibited a relatively constant enzymatic activities throughout the germination period. The changes in the malondialdehyde and  $H_2O_2$  levels were minimum before 6 DAI, however a significant increase was noted at 9 DAI. This study indicates that besides the enzymatic antioxidants, the increase in T3 and GO contents may play a role in countering the oxidative stress during rice grain germination.

**Keywords:** rice grain, germination time, seedling emergence, tocopherols, enzymatic antioxidants

**Abbreviations:** Toc: tocopherols; T3: tocotrienols; GO:  $\gamma$ -oryzanol; AsA: ascorbate; TAsA: total ascorbate; DHA: dehydroascorbate; DAI: days after imbibition; APX: ascorbate peroxidase; SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase; GR: glutathione reductase; PUFAs: polyunsaturated fatty acids; ROS: reactive oxygen species.

### **Introduction**

In plants, the seeds contain significant higher amount of lipids than other tissues, and a large proportion of these lipids are PUFAs, which are susceptible to attack by  $^1O_2$  and  $HO\cdot$  in generating the ROS; this phenomenon is relatively obvious in seed germination

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(Schopfer et al. 2001). Germination is known to cause drastic change in the chemical profile and oxidative stress status in cereal grains. Toc, T3 and GO are important lipophilic antioxidants in the rice grains, their levels were shown to enhance during germination (Moongngarm and Saetung 2010; Ng et al. 2013). Both Toc and T3 are collectively called tocopherols, tocopherols or vitamin E that comprises four analogs ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ) of Toc and T3. Unlike Toc that are widely seen in all plants, T3 exist only in a small number of plant species, and are mainly accumulated in the seeds and fruits (Ivanov and Aitzemuller 1995), whereas GO is rarely found in common crops and vegetables (Yoshie et al. 2009).

ROS in high concentrations are known to cause harmful effects to seeds, however at low concentrations they are beneficial for seed germination and seedling growth. Oxidative stress arises from an imbalance between generation and elimination of ROS. ROS scavenging systems such as SOD, CAT, GPX, AsA,  $\alpha$ -Toc, and others can counteract the harmful effects of ROS, so that the seed can resistant to oxidative damage and have minimal damage on cells. During germination, with increasing moisture content of seeds, the biochemical reactions occur may lead to the increased production of ROS. Hence, the biological balance of oxidative stress and antioxidant systems is considered to be the crucial factors in dictating if the plants can be successfully germinated and grown (Gill and Tuteja 2010).

Previous studies have shown that Toc plays an important role in resisting lipid peroxidation and maintaining vitality, as well as participating in the antioxidant and signal transduction systems during germination (Sattler et al. 2004). Although T3 and GO are potent antioxidant in rice grains, their changes in contents and relation to oxidative stress during rice grain germination remain unclear. In this study, our aim was to examine the effects of germination time on contents of Toc, T3, GO and AsA, and activities of enzymatic antioxidants in the grains of two different rice varieties.

## Materials and Methods

### *Chemicals*

Standards of tocopherols ( $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -Toc),  $\gamma$ -oryzanol, ascorbate and sodium hypochlorite were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Tocotrienols ( $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -T3) were obtained from Davos Life Science Pte. Ltd. (Helios, Singapore). All other chemicals used were of analytical grade.

### *Rice samples*

The rice species (*Oryza sativa* L.) used in this study was Tainung 71 (TN71) and Kaohsiung 139 (KS139), which are rice varieties popularly cultivated in Taiwan. TN71 is a fragrant rice that is resistant to cold stress, whereas KS139 is a high yield variety that requires good soil and water quality for cultivation.

### *Seed germination induction*

Rice seeds were sterilized with 2% sodium hypochlorite for 30 min and then washed extensively with distilled water. They were pregerminated in Petri dishes with wetted filter paper at 37 °C under dark conditions, with 80 seeds in each dish and a total of four dishes (4×). After 3 days of incubation, germinated seeds were transferred to a 500 mL beaker containing half-strength Kimura B solution as described previously (Hsu and Kao 2008). The hydroponically cultivated seedlings were grown in a beaker covered with aluminum foil barrier to prevent light, and placed in a 25 °C growth chamber with photoperiod of 16 h light and 8 h darkness for 6 days. Water was added every day to maintain the water level, while the nutrient solutions were replaced every 3 days.

Twenty germinated grains at each developmental stage were randomly collected from four independent replicates for all analyses. After removing the moisture, the portion outside the germinated grain was removed with a razor blade, while retained the radicle and germ that were inside the grain. Half of the fresh samples were taken for enzymatic assays, whereas the other half was subjected to drying in an oven at 80 °C for 72 h. The dried samples were used for Toc, T3, GO, AsA and total AsA analysis.

### *Quantification of tocopherols, tocotrienols and $\gamma$ -oryzanol contents*

The contents of Toc, T3 and GO in germinated rice grains was determined by reverse phase HPLC as described previously (Ng et al. 2013).

### *Determination of ascorbate, total ascorbate and dehydroascorbate contents*

The AsA, TAsA and DHA contents were measured according to the methods as described by Pullman et al. (2009). The concentration of DHA was estimated from the difference between TAsA and AsA.

### *Measurement of activities of enzymatic antioxidants, and contents of malondialdehyde and hydrogen peroxide*

Germinated grains were taken and frozen in liquid nitrogen, followed by grinding with 0.1 M phosphate buffer (pH 6.8, containing 0.5 mM AsA and 1 mM phenylmethanesulfonyl fluoride), and then centrifuged at 12,000×g for 20 min at 4 °C. The supernatant was used to measure the activities of SOD, CAT, APX and GR. SOD activity was measured using the method described by Beauchamp and Fridovich (1971). APX activity was determined according to Nakano and Asada (1981). The activity of CAT was measured according to Kato and Shimizu (1987). GR activity was measured according to the method described by Sgherri et al. (1994). Malondialdehyde (MDA), as a lipid peroxidation marker, was measured by the thiobarbituric acid-reactive method according to Verma and Dubey (2003). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was measured colorimetrically as described by Mukherjee and Choudhuri (1983).

### Statistical analysis

Values are expressed as mean  $\pm$  standard deviation. The significance of differences was determined by one-way ANOVA, followed by Duncan's multiple range tests. A  $P$  value  $< 0.05$  was considered statistically significant.

## Results

### Weight changes during rice seed germination

Results showed that the fresh weight of rice grains reached the plateau at 3 days after imbibition (3 DAI), followed by a slow decline (Table S1\*), whereas the highest dry weight was noted at the beginning of the experiment (0 DAI). A similar trend of changes in fresh and dry weight was noted on both tested rice varieties.

### Changes of tocopherols, tocotrienols and $\gamma$ -oryzanol contents

Results showed that the total vitamin E, T3 and GO contents in both TN71 and KS139 increased with increasing time of germination, and reached the highest at 9 DAI (Table 1). The level of Toc was the lowest at 6 DAI for both TN71 and KS139.

Among the eight vitamin E analogs, only  $\alpha$ -Toc,  $\alpha$ -T3 and  $\gamma$ -T3 were detected at all stages of germination (Table S2). The highest  $\alpha$ -Toc level was noted at 0 DAI, and was reduced with increasing time of germination. The  $\gamma$ -Toc was only detected at 9 DAI. In contrast to  $\alpha$ -Toc, the levels of both  $\alpha$ -T3 and  $\gamma$ -T3 increased with increasing germination time, and reached the highest at 9 DAI. Compared with the level at 0 DAI, the  $\alpha$ -Toc

Table 1. Total vitamin E, tocopherols, tocotrienols and  $\gamma$ -oryzanol contents in TN71 and KS139 rice grains during seedling emergence

	DAI	Contents (mg kg <sup>-1</sup> )			
		Total vitamin E	Tocopherols	Tocotrienols	$\gamma$ -Oryzanol
TN71	0	21.1 $\pm$ 3.7b	10.1 $\pm$ 1.4a	11.0 $\pm$ 2.4c	53.7 $\pm$ 10.1c
	3	28.6 $\pm$ 12.0ab	8.0 $\pm$ 1.6a	11.6 $\pm$ 1.8c	130.1 $\pm$ 60.8b
	6	23.3 $\pm$ 2.3b	6.8 $\pm$ 1.1b	16.4 $\pm$ 1.2b	141.7 $\pm$ 6.2b
	9	31.0 $\pm$ 2.4a	9.0 $\pm$ 2.0ab	21.9 $\pm$ 2.2a	240.8 $\pm$ 99.8a
KS139	0	23.5 $\pm$ 3.4b	10.8 $\pm$ 1.8a	12.7 $\pm$ 1.7c	137.9 $\pm$ 35.9c
	3	22.6 $\pm$ 0.8b	6.7 $\pm$ 0.3b	15.9 $\pm$ 0.8b	238.5 $\pm$ 50.6bc
	6	19.6 $\pm$ 5.0b	3.5 $\pm$ 1.3c	16.1 $\pm$ 3.8bc	338.0 $\pm$ 91.9ab
	9	35.4 $\pm$ 1.6a	13.7 $\pm$ 1.8a	21.4 $\pm$ 1.5a	507.2 $\pm$ 107.1a

Values are mean  $\pm$  SD. Averages followed by the same letter in the same column are not significantly different at  $P < 0.05$  as analyzed by Duncan's multiple range tests. DAI: days after imbibition.

\*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

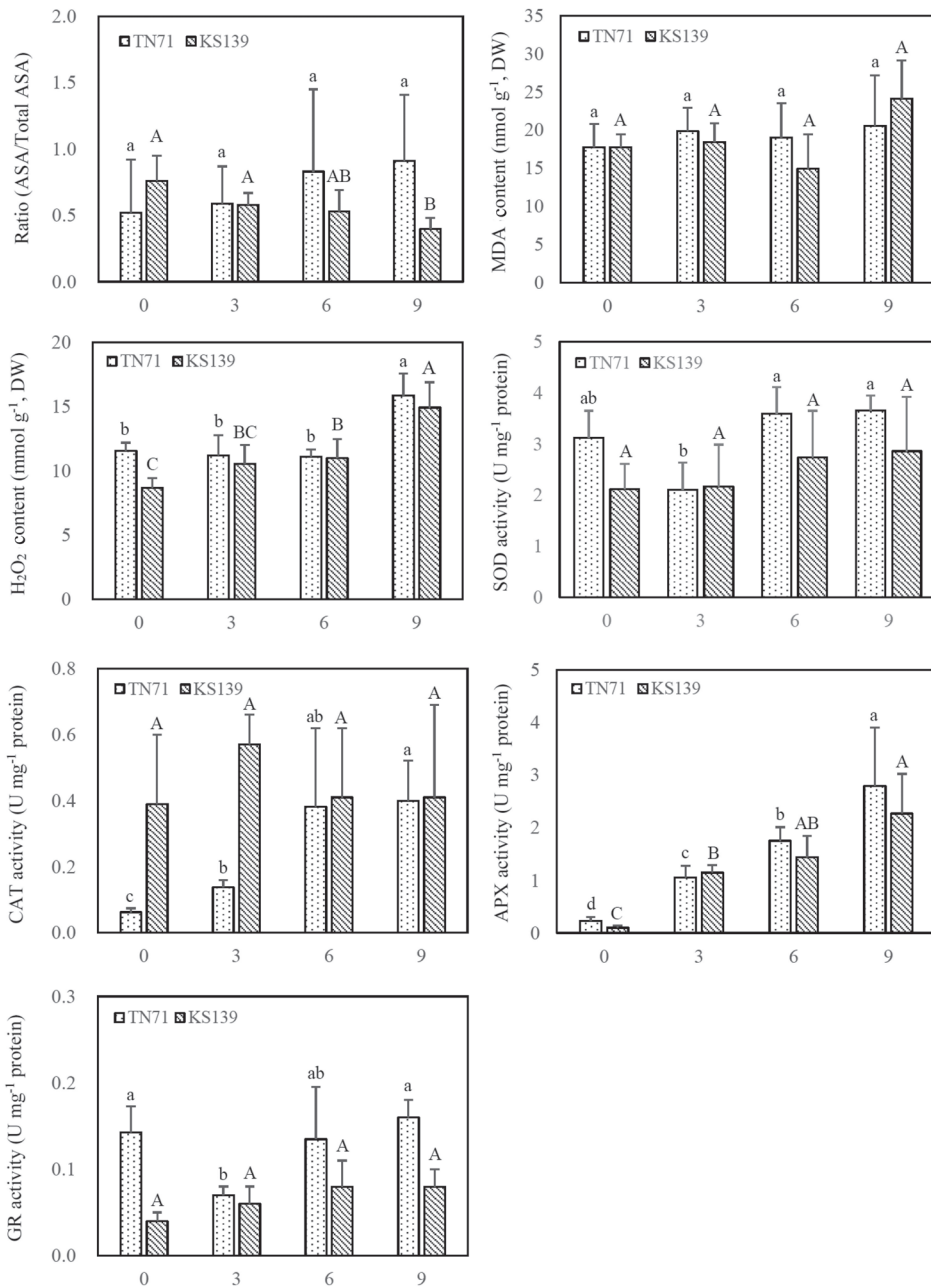


Figure 1. The redox state, contents of malondialdehyde (MDA) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) in rice grains during seedling emergence. Bars with different lower case letters indicate significant different within the TN71 variety, whereas bars with different capital letters indicate significant different within the KS139 variety (Duncan's multiple range tests;  $P < 0.05$ ). DAI: days after imbibition. Redox state was calculated as the ratio of reduced ascorbate/total ascorbate

level was noted to decrease by 1.97-fold, while the levels of  $\alpha$ -T3 and  $\gamma$ -T3 increased by 1.94- and 2.02-fold, respectively, in the grains of TN71. In the grains of KS139, the  $\alpha$ -Toc level decreased by 3.29-fold, while the levels of  $\alpha$ -T3 and  $\gamma$ -T3 increased by 1.53- and 1.79-fold, respectively, as compared with that at 0 DAI.

#### *Changes of ascorbate, total ascorbate and dehydroascorbate contents*

Results showed that there was no significant change in the TAsA content in the seeds of both TN71 and KS139 during the early stage of germination. However, an obvious increase in the TAsA level was noted at 9 DAI in TN71 but not in KS139 (Fig. S1a and b). After imbibition, the AsA level in the grains of TN71 increased with increasing germination time, while that of the KS139 appears to decrease and reached the lowest at 9 DAI. From 0 DAI to 9 DAI, there was no significant change in the DHA concentration in TN71 grains, however a trend of increase was noted in the grains of KS139. The redox state (defined as the ratio of AsA to TAsA) of TN71 was not significantly changed, however, there is a trend of decrease in the redox state in KS139 with increasing germination time (Fig. 1).

#### *Changes in activities of enzymatic antioxidants*

After imbibition, the CAT and APX activities in TN71 increased with increasing time of germination and reached the maximum at 9 DAI, while no difference was noted in the SOD activity between 0 DAI, 6 DAI and 9 DAI (Fig. 1); significant lower SOD and GR activities were observed at 3 DAI, however, GR activity appears to increase from 3 DAI to 9 DAI. Although a time-dependent increase in APX activity was observed during the seedling emergence of KS139, its SOD, CAT and GR activities were no difference between the different stages of germination.

#### *Changes in oxidative stress indicators*

Results showed that the change of MDA and  $H_2O_2$  levels during the early phase of germination (0–6 DAI) in TN71 was small, however, at 9 DAI the  $H_2O_2$  level was significantly higher than at other germination periods (Fig. 1). Although KS139 appears to have the highest MDA level at 9 DAI, there was not statistically difference between the different stages of germination; however its  $H_2O_2$  level was shown to increase with increasing time of germination, and reached the highest at 9 DAI.

## **Discussion**

A complete germination of seeds (from early imbibition to radicle protrusion) requires both enzymatic and non-enzymatic antioxidants to protect against ROS that are produced during the different stages of germination. Contrasting to other plant species (Hall and Laidman 1968; Henryk and Halina 2003), a dramatic decrease in the Toc content with

increasing germination time (from 0 DAI to 6 DAI) was noted in both TN71 and KS139; in germinated grains of KS139, this phenomenon could be due to the significant reduction in the content of AsA, which was not sufficient to reduce Toc radicals, and hence resulting in the decrease of Toc content. The other reason is that rice grains contain high level of T3, which was reported to be a better antioxidant than Toc (Wong and Radhakrishnan 2012), and may have contributed to the lowering of oxidative stress. Besides, it is also possible that at the later part of rice grain germination (at 9 DAI), the seeds may have entered into a new physiological stage, where different forms of tocols are produced for the smooth growth of the seedling, as demonstrated by a significant increase in the accumulation of T3 with increasing germination time in both TN71 and KS139, this observation suggests that T3 may play an important role as antioxidants in the removal of ROS. Consistent to the observation of Andarwulan et al. (1999), this study also showed that the accumulation rate of different tocol analogs vary at different stages of seed germination.

GO is another important antioxidant in rice grains; its function is known to scavenge free radicals and to stabilize the membrane structure (Kim et al. 1995). It was reported to increase significantly after 24 h of germination (Moongngarm and Saetung 2010). In this study, the level of GO dramatically increased at early stage of germination, and maintained at high levels even at the later stage of germination; this observation suggests that GO may also play a role in antioxidation during rice grain germination.

AsA is one of the most important hydrophilic antioxidants in plants, it can directly reduce  $O_2^{\bullet-}$ ,  $H_2O_2$  and Toc radicals (Noctor and Foyer 1998); it can remove  $H_2O_2$  through the AsA-GSH cycle (Pinto et al. 2003). AsA was reported to increase gradually with increasing time of germination (Tommasi et al. 2001; Wojtyla et al. 2006). In TN71 grains, the TAsA and AsA contents were found to increase significantly at the later stage of germination; this could be due to the increase in  $H_2O_2$  level. As for KS139 grains, AsA content decline with increasing time of germination, whereas the DHA content was higher than the AsA content at 9 DAI, suggesting a slower AsA regeneration ability during the early stage of germination, hence resulting in the lowest TAsA content at 9 DAI. This phenomenon explains that in KS139 grains, AsA may act as the main antioxidant at the initial stage of germination, hence there is a drastic change after germination; it is also possible that the AsA level is associated with a low GR activity. In KS139 grains, increasing germination time resulted in an increased oxidizing AsA redox state; it could be due to a loss of total AsA pool and accumulation of oxidized AsA. However, a relative stable redox status was noted in TN71 grains; this observation could reflect a limitation in the transport of DHA from the apoplast to cytoplasm where DHA is reduced to AsA by the ascorbate-glutathione cycle (Noctor and Foyer 1998).

In the process of seed germination, enzymatic antioxidants have been reported to play a vital role in the regulation of ROS for the smooth seed germination (Tommasi et al. 2001). SOD is the first line of defense against oxidative damage through converting  $O_2^{\bullet-}$  to  $H_2O_2$ , which is further breakdown to  $H_2O$  through the action of CAT and APX. In this study, besides at 3 DAI, SOD activity was not significantly changed at different stages of germination in both TN71 and KS139 grains; this could be the response towards the increased production of free radicals during seed germination (Wojtyla et al. 2006). The

H<sub>2</sub>O<sub>2</sub> formed by the action of SOD is directly reacted with CAT to form H<sub>2</sub>O, or accompanied by action of APX and GR AsA-GSH cycle to produce H<sub>2</sub>O. This study also showed that both CAT and APX activities in the grains of TN71 increased markedly during the germination process, whereas the APX activity in the KS139 grains increased gradually with increasing germination time. The increase in these enzymatic antioxidant activities could be an indication of the presence of elevated ROS levels. The main function of GR is to reduce GSSG to GSH as to maintain the normal operation of AsA-GSH cycle. In TN71 grains, the GR activity at 6 DAI and 9 DAI was significantly higher than at 3 DAI, this observation may be related to the high APX activity and AsA-GSH cycle. Surprisingly, germination appears to have lesser effects on the SOD, CAT and GR activities in the KS139 grains. The differences in SOD, CAT, APX and GR activities suggests that they could play a different role in the different stages of seed germination.

An increase in lipid peroxidation had been reported to take place during seed germination (Cai et al. 2011), which is one of the important indicators for assessing the degree of oxidative stress. In this study, although the H<sub>2</sub>O<sub>2</sub> level at 9 DAI was significantly higher than at other earlier stages of germination, the MDA content in both TN71 and KS139 germinated grains remain relatively constant from 0 DAI to 6 DAI; this suggests that the situation of lipid peroxidation in the grains during germination was under control and the oxidation of PUFAs was protected by Toc, T3 and GO. In other words, ROS content in the grains are well regulated during germination, hence the grains can germinate and grow successfully (El-Maarouf-Bouteau and Bailly 2008).

In conclusion, this study demonstrates that the contents of T3 and GO were steadily increased over the course of rice grain germination, whereas the content of Toc progression was sigmoidal. At 9 DAI, the germinated rice grains may have entered into a different stage of growth, at which a rapid increase in activities of enzymatic antioxidants and contents of T3 and GO in order to meet the physiological needs. This study also indicates that besides the variety difference in antioxidant system, the rice grains also accumulate different forms of tocopherols at different stages of germination, suggesting that they may have different functions in rice grain germination.

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### Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at <http://www.akademai.com/content/120427/>

Electronic Supplementary *Table S1*. Weight of seeds during seedling emergence

Electronic Supplementary *Table S2*. Contents of eight vitamin E analogs ( $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocopherols and tocotrienols) in TN71 and KS139 rice grains during seedling emergence

Electronic Supplementary *Figure S1*. Total ascorbate (TAsA), ascorbate (AsA) and dehydroascorbate (DHA) contents in rice grains during seedling emergence. a. TN71 and b. KS139. Error bars represent standard deviation and bars with the same letter are not significantly different at  $P < 0.05$  as analyzed by Duncan's multiple range tests. DAI: days after imbibition