The Effect of Antioxidant Vitamins on Mustard Plants in a Hydrogen Peroxide-Induced Injury Model

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Summary

Vitamins A, C and E are known as antioxidants, but their role in various biological systems is not well understood. We assessed the antioxidant properties of these vitamins in a plant model. A model of free radical–induced growth inhibition was created and then used to test the effect of the vitamins. The model was first validated by demonstrating that hydrogen peroxide (H_2O_2) can slow the growth of mustard seeds. A concentration of H_2O_2 that caused definite but non-lethal inhibition of growth was selected using data from the model validation study. Following this, various groups of mustard seeds were treated with H_2O_2 and vitamins in varying concentrations. The plant number and mean height for each vitamin group were compared to controls treated only with distilled water (DW) or H_2O_2. The group treated with only DW had significantly (p<0.001) better growth than other groups. H_2O_2 caused a significant reduction in plant growth compared to DW (p<0.001). Vitamin A caused a dose-dependent partial reversal of the effect of H_2O_2. Vitamin C caused dose-dependent worsening. Vitamin E had no significant effect. A separate study demonstrated that the vitamins alone did not significantly affect plant growth in the absence of H_2O_2-mediated damage. In conclusion, this study demonstrated that, among three vitamins that are considered to have antioxidant properties, only vitamin A exhibited such an effect in a plant model of oxidative damage. Vitamin C worsened the effect of H_2O_2, whereas the effect of vitamin E treatment was neutral.

Introduction

A vitamin is defined as a substance that is essential in small quantities to the human body, but not able to be synthesized by the body. The importance of vitamins has been established by scientists over many centuries of study, generally by observation of the negative health consequences of not consuming any given vitamin. One important function of vitamins is to act as antioxidants. All living organisms are subject to damage mediated by free radicals, the creation of which are promoted by oxygen and peroxides (1). Free radicals are electron-deficient, and they can "steal" electrons from other substances. This leads to chemical chain reactions that can be deleterious to key molecules like DNA. Antioxidants work in part by reducing free radicals. Vitamins A, C, and E are three well-known antioxidant vitamins (1, 2).

Like other living organisms, plants are exposed to many different stresses, both abiotic and biotic. Typical examples of abiotic stresses are drought and soil salinity (3), whereas a common biotic stress is exposure to bacteria or fungi. Extensive experimental data show that almost all biotic and abiotic stresses induce oxidative injury to some degree (4). Therefore, the ability of plants to control oxidant levels is an important predictor of stress response. Investigators have demonstrated the important role of antioxidant pathways in plant cells (3–5). Surprisingly, however, the potential beneficial effects of exogenous antioxidants have not been well studied.

This project sought to assess the antioxidant properties of exogenous vitamins A, C and E in a plant model. In particular, the question was whether one vitamin was better than the others in preventing oxidative injury. We used hydrogen peroxide (H_2O_2) in the model, because this chemical agent is known to cause oxidative stress in plants (3,4). Studies using plant models have direct applicability to gardening and agriculture. A better understanding of plant growth can lead to improved methods of growing plants, whether in the garden or on the farm. Simple models such as ours can be useful for the preliminary study of biological issues because of their low cost and ease of analyzing multiple variables. The results found in such models can be used to design focused larger studies of plant growth (6).

For this project, it was necessary to first develop and validate a plant model of free radical–mediated growth inhibition. To this end, mustard seeds were watered with various concentrations of H_2O_2. The strategy was to identify the lowest dose of H_2O_2 that would definitively inhibit the growth of mustard plants without causing lethal damage. It was hypothesized that this dose of H_2O_2 could be used to test whether antioxidant vitamins reversed free radical–mediated damage.

In the second phase of the study, plants were
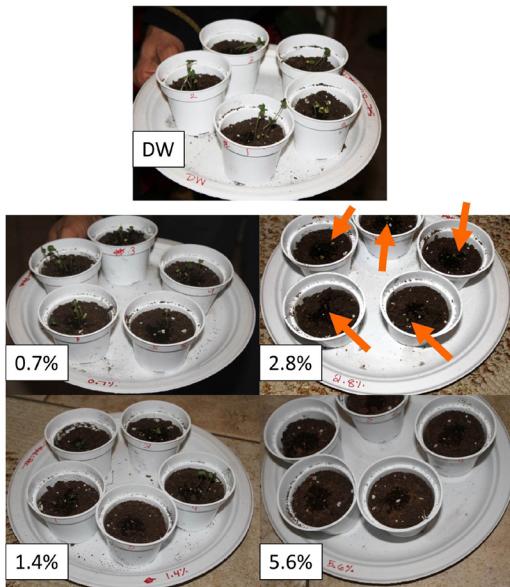
Figure 1: Mustard Plant Growth. At 5 days of being watered daily with distilled water (DW) or with DW containing H_2O_2 at various concentrations. The figure shows marked, but non-lethal, suppression of growth with 2.8% H_2O_2 when compared to DW alone. The orange arrows show the tiny sprouts that grew under 2.8% H_2O_2. Minimal or inconsistent suppression was seen with 0.7% or 1.4% H_2O_2, whereas no growth at all was seen with 5.6% H_2O_2.

watered with H_2O_2 supplemented with vitamins in varying concentrations. Two control groups (distilled water only and H_2O_2 only) were used. This strategy allowed for the identification of dose-related effects of vitamins on H_2O_2-induced growth inhibition.

Results
Development and Validation of Plant Model

Mustard plants were grown for 5 days, during which they were watered with distilled water containing varying concentrations of H_2O_2 (0% to 5.6%). At the end of the fifth day, inspection showed that H_2O_2 at 0.7% led to no significant growth inhibition, while 1.4% H_2O_2 led to inconsistent growth suppression (**Figure 1**). At 2.8% H_2O_2, inhibition was observed in every pot. Finally, at 5.6%, no growth was seen, indicating a lethal level of H_2O_2. Based on these data, 2.8% was selected as the optimal H_2O_2 concentration for consistent inhibition of growth in a non-lethal manner.

Testing Effect of Vitamins

Mustard plants were grown under various conditions, as summarized in **Table 1** and as described in detail in the Methods. **Figure 2** displays the number of sprouts for each group, totaled across all three pots, as a function of time. We observed visible growth on days 4 and 5. As expected, the DW group had the largest number

of sprouts. H_2O_2 caused marked suppression of the number of sprouts.

Vitamin A partially reversed the deleterious effect of H_2O_2 at the high (HI) and middle (MID) concentrations (**Figure 2B**), and the effect was dose-dependent. However, vitamin C exacerbated the deleterious effect of H_2O_2 in a dose-dependent manner (**Figure 2C**). Vitamin E had no clear effect on the number of sprouts (**Figure 2D**).

The mean plant height at the end of 5 days of growth is shown in **Figure 3**. These data confirm that the DW group had significantly better growth than all other groups ($p \ll 0.0001$). H_2O_2 caused significant ($p \ll 0.0001$) suppression of mean height. Vitamin A affected mean height in a dose-dependent fashion. The A-HI group had significantly taller plants than the H_2O_2 group ($p=0.0085$). The A low (LO) and MID groups exhibited a non-significant trend toward greater height compared to treatment with H_2O_2 alone ($p=0.08$ and $p=0.07$, respectively).

Vitamin C also affected mean height in a dose-dependent fashion, but its effect was deleterious. The C-HI group, with virtually no growth at all, had a significantly ($p=0.0005$) lower mean height than H_2O_2 alone. The C-MID and C-LO groups were significantly taller than the C-HI group ($p=0.0013$ and $p=0.0001$, respectively), but their heights were not significantly different from the H_2O_2-treated groups.

The heights of the vitamin E groups were not significantly different from H_2O_2-treated groups, and there was no discernible dose effect of vitamin E on height.

The effects of vitamins A, C, and E were also assessed on mustard plant growth independent of H_2O_2-mediated injury. The mean height of the plants at 5 days for each condition is shown in **Figure 4**. There was no significant difference in height for any group compared to those treated with DW alone.

Group	Water	Vitamin	Concentration (mg/ml)	# of Pots	Vitamin Levels in Plasma (mg/ml)
0	Distilled	None	n/a	3	
1	2.8% H_2O_2	None	n/a	3	
A-HI	2.8% H_2O_2	A	45	3	Vit. A 0.34
A-MID	2.8% H_2O_2	A	4.5	3	
A-LO	2.8% H_2O_2	A	0.45	3	
C-HI	2.8% H_2O_2	C	3	3	Vit. C: 0.014
C-MID	2.8% H_2O_2	C	0.3	3	
C-LO	2.8% H_2O_2	C	0.03	3	
E-HI	2.8% H_2O_2	E	2.7	3	Vit. E: 0.01
E-MID	2.8% H_2O_2	E	0.27	3	
E-LO	2.8% H_2O_2	E	0.027	3	

Table 1: Summary of treatment conditions used in study.

Figure 2: Sprout Number. Number of sprouts as a function of days after planting totaled over all three replicates for each condition. A) Curves for distilled water (DW) and H_2O_2. Other panels add curves for B) vitamin A; C) vitamin C; and D) vitamin E groups
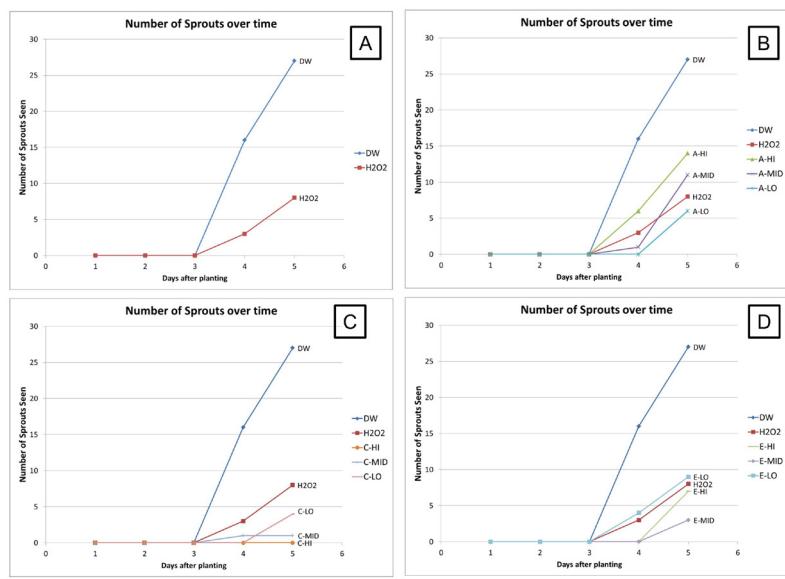
Discussion

This project examined the possibility that oxidative injury to mustard plants might be reversed by vitamins known to have an antioxidant effect. A model of oxidative injury was developed by treating mustard plants with H_2O_2. Daily watering with 2.8% H_2O_2 caused non-lethal growth inhibition compared to pure distilled water.

In the second phase of the study, vitamins A, C and E were administered daily watering at different concentrations. Both daily sprout number and final mean plant height were measured. None of the vitamin-treated groups could fully reverse the damaging effect of 2.8% H_2O_2. Vitamin A led to a dose-dependent partial reversal of the deleterious effect of H_2O_2 on both growth parameters. Surprisingly, vitamin C caused a dose-dependent worsening of the deleterious effect of both parameters. Finally, vitamin E had no discernible effect in this model. The data from a follow-up experiment (**Figure 4**) demonstrate that when vitamins were used to treat the plants independent of H_2O_2-mediated injury, they had no significant effect on growth.

Oxidative injury to plants by H_2O_2 has been previously studied (3,4,7). Paradoxically, H_2O_2 is generated in very low concentrations as an essential metabolite, and it has important signaling functions. Exogenously administered H_2O_2 can even improve seed germination in some models (3). However, with the concentrations used in the current model, H_2O_2 had either no effect or a deleterious effect (**Figure 1**).

The role of innate antioxidant pathways has been well studied in plants. A recent review highlighted the importance of glutathione, ascorbate, tocopherol, proline, betaine, and other intrinsic agents (5). It is notable that ascorbate and tocopherol are vitamins C and E, respectively. However, the possibility of reversing oxidative damage to plants using exogenous antioxidant vitamins has not been well studied. In view of these known intrinsic pathways, our findings – that exogenous vitamin C is deleterious and vitamin E is neutral - are somewhat unexpected.

One potential weakness of the current project is that the results may not be generalizable to other plants, animals, or humans. Moreover, it is possible that the chosen dose of 2.8% H_2O_2 may have caused more damage than desired. This may explain why none of the vitamins were able to fully reverse the damaging effect of H_2O_2. It may be useful to repeat the study using 1.4% H_2O_2, a concentration at which some growth inhibition was observed (**Figure 1**). Finally, the lowest concentrations of vitamins were selected to be roughly equal to normal plasma levels in humans. This strategy was necessary because there are no data available regarding the levels of these agents in plant cells. It is not known what relevance the human plasma levels have for mustard plant growth. However, this weakness was partially mitigated by the fact that concentrations 10- and 100-fold higher were used in addition to the lowest concentrations.

The present findings are interesting in that each vitamin had a discernibly different impact on plant growth. The data show that substances classified as "antioxidant" do not always behave in the same manner, even when used in a model of oxidative injury. Future study will be necessary to understand why each of the antioxidant vitamins influenced growth differently. In particular, it may be useful to determine why vitamin C had a deleterious impact on mustard plant growth. At this stage, we can offer only speculation. It is possible that the concentrations of vitamin C in our study, chosen based on normal human plasma concentrations, were too high for oxidatively injured mustard plants. Plants, unlike humans, may be more sensitive to excessive amounts of vitamin exposure because they lack active
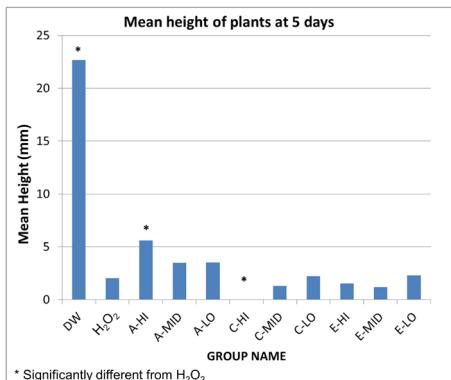
Figure 3: Effect of Vitamins on H$_2$O$_2$-Injured Plants. Mean height of mustard plants are shown after 5 days of growth, separated by group. All groups except DW were treated with 2.8% H$_2$O$_2$. The nine vitamin groups were also treated with the indicated vitamin at the final concentration shown in **Table I**. An asterisk (*) indicates that the group mean was significantly different from the H$_2$O$_2$ group (p<0.05). Error bars represent standard deviation of each group.

Figure 4: Vitamin Control Experiment. Mean height of mustard plants after 5 days of growth are shown, separated by group. Groups were treated either with DW or DW with vitamins at concentrations described in **Table I**. None of the groups were treated with H$_2$O$_2$.

elimination pathways like the human kidney. Another potential explanation is that the deleterious agent in our study (vitamin C) is water-soluble, while the other vitamins are fat-soluble.

This study demonstrated that among three vitamins that are considered to have antioxidant properties, only vitamin A exhibited such an effect in a plant model of oxidative damage. Vitamin C worsened the effect of H$_2$O$_2$, whereas the effect of vitamin E was neutral.
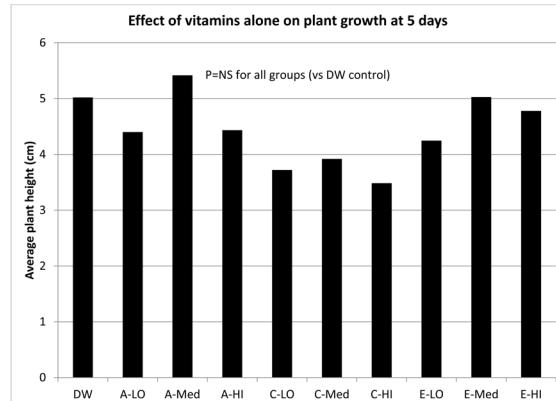
Methods
Phase 1: Development and Validation of Plant Model

Twenty-five plastic pots having a top opening diameter of 7.5 cm and height of 7 cm were divided into five groups of five pots each. Each pot was filled with 158 mL of potting soil (Miracle-Gro Potting Mix) and 20 mustard seeds. The seeds were covered with an additional 30 mL of potting soil. All pots were placed in a single room with constant temperature (22°C) and uniform lighting. The room has a large window to admit sunlight, and all plants were kept at the same distance from the window to avoid any variation in sunlight exposure. A solution of 5.6% H$_2$O$_2$ was created by diluting 60 mL of 35% hydrogen peroxide (Food Grade hydrogen peroxide, The One Minute Miracle, Inc.) in 315 mL of distilled water. Serial 2-fold dilutions were used to obtain 2.8%, 1.4%, and 0.7% solutions. Each group of pots was watered daily for 5 days with one of these four solutions or with plain distilled water.

Testing Effect of Vitamins

Thirty-three plastic pots with the same dimensions as in phase 1 were divided into eleven groups of three

pots each. All pots were filled with soil and seeds and were maintained under the same conditions as in phase 1. Two control groups (Groups 0 and 1) were watered daily for 5 days with only distilled water or only 2.8% H$_2$O$_2$, respectively. Vitamin A (Dry A 10,000 IU tablets; Vitamin Shoppe brand) was added to 2.8% H$_2$O$_2$ solution at three different final concentrations (A-HI 0.45 mg/mL, A-MID 4.5 mg/mL, and A-LO 45 mg/mL). Each of these vitamin A solutions was used to water three separate groups of plants on a daily basis for 5 days. Similarly, vitamin C (Liquid C; TwinLab brand) was added to 2.8% H$_2$O$_2$ at three different final concentrations (C-LO 0.03 mg/mL; C-MID 0.3 mg/mL; C-HI 3 mg/mL). Finally, vitamin E (Liquid Vitamin E; Solaray brand) was added to 2.8% H$_2$O$_2$ at three final concentrations (E-LO 0.027 mg/mL; E-MID 0.27 mg/mL; E-HI 2.7 mg/mL). For each vitamin, the concentration in normal human plasma was determined using published data (8,9). This "LO" vitamin concentration was selected to roughly match this value for each vitamin; the other two concentrations (MID and HI) were 10- and 100-fold higher. The group treatments are displayed in **Table 1**.

Vitamin Control: Testing Effect of Vitamins Independent of H$_2$O$_2$-Mediated Injury

Thirty plastic pots with the same dimensions as in phase 1 were divided into ten groups of three pots each. All pots were filled with soil and seeds and were maintained under the same conditions as in phase 1. One control group (DW) was watered daily for 5 days with only distilled water. Vitamin A (Dry A 10,000 IU tablets; Vitamin Shoppe brand) was added to DW solution at three different final concentrations (A-HI 0.45 mg/mL, A-MID 4.5 mg/mL, and A-LO 45 mg/mL). Similarly,

vitamin C (Liquid C; TwinLab brand) was added to DW at three different final concentrations (C-LO 0.03 mg/mL; C-MID 0.3 mg/mL; C-HI 3 mg/mL). Finally, vitamin E (Liquid Vitamin E; Solaray brand) was added to DW at three final concentrations (E-LO 0.027 mg/mL; E-MID 0.27 mg/mL; E-HI 2.7 mg/mL). Each of these nine vitamin solutions was used to water the respective groups of plants on a daily basis for 5 days.

Data Analysis

The number of visible sprouts was counted in each pot at the end of each day. For analysis, the total number of sprouts for each group was used. At the end of the fifth day, the height of each mustard plant in every pot was measured. Seeds that failed to sprout were considered to have a height of "0." Groups were compared to one another using the Student's T-test.

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