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# Hydrogen Peroxide Alleviates Hypoxia during Imbibition and Germination of Bean Seeds (*Phaseolus vulgaris* L.)

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## Abstract

**Bean seeds (*Phaseolus vulgaris* L. cv. Tendergreen) were imbibed in water to examine the effects of hypoxia during imbibition and subsequent germination. Hypoxic conditions occurred when seeds were imbibed in water for 24 h or longer and resulted in severe reduction of hypocotyl elongation and stem growth during subsequent germination under non-limiting oxygen conditions. Under continued hypoxic conditions, bean seeds failed to germinate, however, this was reversed in the presence of hydrogen peroxide (20 mM) in the medium. Furthermore, imbibition of seeds in the presence of hydrogen peroxide overcame the adverse hypoxic effects on hypocotyl elongation and stem growth. Exogenous hydrogen peroxide increased the dissolved oxygen levels in the germinating medium, and catalase and transition metal ions such as $Fe^{2+}$, $Cu^{2+}$ and $Mn^{2}$ helped to facilitate the production of oxygen from hydrogen peroxide. In these catalysts, catalase played a major role in the decomposition of hydrogen peroxide as demonstrated by the use of a catalase inhibitor, 3-amino-1, 2, 4-triazole, which reversed the positive effects produced by hydrogen peroxide on germination of seeds under hypoxic conditions. The results show that imbibition is sensitive to oxygen deficits which affect subsequent hypocotyl growth and seedling performance. The adverse effects of hypoxia on germination of bean seeds can be overcome by exogenous hydrogen peroxide.**

## Keywords

**Aminotriazole, Anoxia, Catalase, Hydrogen Peroxide, Hypoxia, *Phaseolus vulgaris*, Seed Germination**

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## 1. Introduction

Hypoxia in water-logged soils caused by transient flooding is a common occurrence for most crop plants and can adversely affect their establishment, growth and productivity. In addition, oxygen deficiency can readily occur in heavy, compacted or poorly drained soils on a regular basis, affecting various plant functions [1] [2]. Adverse effects of hypoxia and possible mechanisms to overcome them have long been studied in seedlings and mature plants [1] [3] [4]. The primary consequence of oxygen deficiency in the roots is the anaerobic metabolism resulting in decreased energy status and accumulation of ethanol and other toxic metabolites, which can lead to injury and eventual death of plants [1] [5]. The seed germination is particularly sensitive to oxygen deficiency. The period of natural hypoxic conditions for seed germination can last from a few hours to several days in poorly drained soils. The oxygen requirement for seeds typically varies depending on the species and their dormancy status. Although many aquatic plants germinate well under reduced oxygen concentrations, seeds of most species either fail to germinate or at least, show severely restricted seedling growth when deprived of oxygen [6]. Even if germination were to occur under hypoxic conditions, seedlings do not establish well, resulting in poor growth and development. Green beans (*Phaseolus vulgaris* L.), also known as common beans, snap beans or string beans are a warm season vegetable crop which does well in well-drained, aerated soils as it is sensitive to oxygen deficits. It is grown for both fresh market use and for processing in most crop growing regions of the world with the U.S. being the largest commercial producer [7].

The role of oxygen during germination is rather complex. In addition to being an essential factor in meeting the demands of increasing respiration rates during germination process, oxygen may act as an oxidizing agent to help break seed dormancy [8]. As early as 1926, Morinaga [9] examined seed germination of numerous species and found that a great majority of seeds either completely failed to germinate under water or showed poor germination. Bean seeds (*Phaseolus* sp.) typically do not germinate under water and their germination is sensitive to low oxygen levels which may affect the respiratory process, rather than the water uptake by seeds during imbibition [6]. Although the effects of hypoxia in plants have been investigated by a number of studies [1] [2], they are not clearly understood particularly during seed imbibition, germination, and subsequent seedling growth. It is not clear if transient hypoxia during seed imbibition will have an impact on subsequent germination and seedling growth.

In this study we examine exogenous hydrogen peroxide as a source of oxygen during germination of bean seeds under hypoxia. Hydrogen peroxide is a natural substance produced by most plant tissues and particularly, at elevated levels, in response to a host of biotic and abiotic stresses [10] [11]. Furthermore, oxygen deprivation, like other abiotic stresses, is known to induce hydrogen peroxide and other reactive oxygen species in both hypoxia-tolerant and hypoxia-sensitive species [12]-[14]. The induced hydrogen peroxide is known to be involved in signaling plants' responses to various stresses which are considered to be part of their defense mechanism and may play an important role in plant adaptation [10] [11] [13] [15]. In addition, hydrogen peroxide is readily decomposed to free oxygen and water by catalase (CAT) and many transition metals such as copper and manganese and their compounds [14] [15]. Although plants produce CAT during normal metabolic processes, they tend to accumulate at much higher levels under stressful conditions [11] [16]. In this study, we characterize the effects of hypoxia during imbibition of seeds on subsequent germination and seedling growth and examine the possible use of exogenous hydrogen peroxide and the role of catalase and transition metal ions in alleviating hypoxia in germinating bean seeds.

## 2. Materials and Methods

### 2.1. Hypoxia during Imbibition and Germination

The main objective of this study was to examine if hypoxia during imbibition has any effects on subsequent germination and seedling growth. Thus, the effects of hypoxic conditions were tested during two stages of germination, first, by imposing hypoxia only during seed imbibition and monitoring subsequent germination and seedling growth under normal aerated conditions (normoxia) and second, by imposing hypoxia during the whole process of germination in beans. Bean seeds (*Phaseolus vulgaris* L. cv. Tendergreen) were purchased from Chesmore seed company (St. Joseph, MO). Batches of seeds each containing 30 seeds of uniform size were submersed in one liter of distilled water to induce hypoxia, or 20 mM of hydrogen peroxide solution contained in 1.5 L beaker in the dark at 25˚C in a growth chamber for 0 h, 6 h or 24 h of imbibition. After the imbibition treatment, seeds were placed on a 0.8% agar medium contained in a covered Petri dish for germination (under

normoxia). Control (*i.e.*, 0 h) consisted of seeds directly placed on the agar medium for germination. The hypocotyls elongation was measured up to 96 h of planting. To test the effect of hypoxia during imbibition on the subsequent seedling growth, 20 mM of hydrogen peroxide was used as the germination results were the best at this concentration of hydrogen peroxide. To impose hypoxic conditions, bean seeds were submersed in distilled water or 20 mM of hydrogen peroxide solution for 24 h for imbibition and were subsequently planted in pots (60 × 60 mm) containing soil (Metromix-360, The Scotts Co, Marysville, OH) at 3 cm depth. Stem elongation in seedlings was measured after 4 and 5 days of planting. The experiment was conducted on a completely randomized design with 10 replications.

To test how seeds germinate and grow under hypoxic conditions, bean seeds of uniform size were placed in 20 ml of distilled water, 5 mM or 20 mM of hydrogen peroxide solutions contained in unsealed test tubes [23 mm (L) × 14 mm (W)] for germination. Each tube contained one seed and each treatment had 6 replications. The test tubes were placed in a growth chamber at 25˚C in the dark to observe for hypocotyl growth. Control consisted of 35 seeds, placed in aerated water in a one-liter beaker at 25˚C and constant aeration was provided by circulating the tap water at a flow rate of 15 mL/sec. Hypocotyl growth of beans in the hydrogen peroxide solutions or water was measured periodically until 72 h. Oxygen and hydrogen peroxide concentrations in the medium were monitored during imbibition and germination.

## 2.2. Hydrogen Peroxide and Oxygen Concentrations in Solutions

Oxygen concentrations in solutions were determined polarographically using YSI-oxygen electrode (YSI, Yellow Springs, OH). A 3 mL aliquot from the each solution was sampled every 12 or 24 h to quantify the dissolved oxygen.

Hydrogen peroxide concentration in the incubation solutions was determined spectrophotometrically [17]. An aliquot of 0.1 ml of the incubation solution was collected and diluted 20 times. The diluted solution was mixed with 0.1 ml phenol red (0.28 mM) and 0.8 ml potassium phosphate-sodium phosphate buffer (12.5 mM, pH 7.0) containing type II horseradish peroxidase (62.5 g/ml). The reaction mixture was incubated at 25˚C for 30 minutes with mild shaking. The reaction was stopped by centrifugation at 8000 g and by raising the pH of the reaction mixture with 3 M NaOH. Hydrogen peroxide was quantified by the measuring absorbance at 610 nm using a spectrophotometer (Gilford Systems, Oberlin, OH).

## 2.3. CAT Activity

CAT activity was determined in bean seeds submersed in water, 5 mM of hydrogen peroxide or 20 mM of hydrogen peroxide. Control consisted of bean seeds in aerated water and aeration was provided by circulation of fresh tap water at a flow rate of 15 mL/s. Incubated bean seeds were homogenized with a mortar and pestle in 10 ml of sodium phosphate buffer (pH 7.0) containing 0.2 mM EDTA and 1% (w/v) polyvinylpyrrolidone in an ice bath. The homogenate was filtered through four layers of cheesecloth and centrifuged at 4˚C for 10 minutes at 15,000 g. The supernatant was used to determine the enzyme activities. Total proteins from the enzyme extract were quantified using Bradford method [17]. Bradford reagent was made by 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid. The extract (0.1 ml) was added to 5 ml Bradford reagent and incubated for 15 min. The absorbance was measured at 595 nm against a reagent blank. The total protein in the extract was derived from a standard curve obtained with bovine serum albumin (Sigma Chemical Co., St. Louis, MO).

The reaction mixture (3 ml) for CAT assay consisted of 50 mM of phosphate buffer (pH 7.0), 15 mM of hydrogen peroxide, and 0.1 ml of enzyme extract. The enzyme activity was assayed in triplicate samples. The reduction of hydrogen peroxide was monitored by the decline in absorbance at 240 nm for l min [19] [20]. One unit of enzyme corresponded to a decrease of 0.01 absorbance/min caused by the enzyme aliquot [19].

## 2.4. Aminotriazole (3-AT) Treatment

To determine if seed catalase activity has any role in reducing the adverse hypoxic effects on germination in presence of hydrogen peroxide, bean seeds were incubated in 20 mM of hydrogen peroxide with 3-AT (Sigma Chemical Co., St. Louis, MO), a specific inhibitor of CAT activity. Aminotriazole solutions at 0.1, 1, 10, and 100 mM were prepared in 20 mM hydrogen peroxide. For imbibition, bean seeds of uniform size were sub-

mersed in 20 ml of solution containing 20 mM hydrogen peroxide and various concentrations of 3-AT in test tubes [23 mm (L) × 14 mm (W)]. Each test tube contained one seed and each treatment was replicated 6 times. After 48 h incubation, the length of hypocotyls was measured.

### 2.5. Oxygen Evolution from Hydrogen Peroxide by Catalysts

The efficiency of various transition metal catalysts ($Fe^{2+}$, $Cu^{2+}$, $Mn^{2+}$) and CAT in breaking down hydrogen peroxide to generate oxygen was tested. Various concentrations of catalysts were prepared using $FeSO_4 \cdot 7H_2O_2$, $CuSO_4 \cdot 5H_2O$ and $MnSO_4 \cdot H_2O$, and CAT (Sigma Chem. Co., St. Louis, MO). A 1.5 ml of the aliquot of these solutions was mixed with 1.5 ml of 40 mM of hydrogen peroxide in the cuvette of YSI-oxygen polarograph. Oxygen evolution was measured for 15 min as the reaction mixture was constantly stirred. The mean rate of oxygen evolution was computed over the first 10 min of the reaction.

The seed content of these transition metals was also determined by digesting 0.25 g of ground seed (0.5 mm) with nitric acid and perchloric acid [21]. The concentrations of metal catalysts in the digest were determined using the atomic absorption spectrophotometry (Perkin Elmer 3110, Norwich, CT) and were expressed as ppm based on dry weight of seeds. Each treatment consisted of 3 replications.

### 2.6. Data Analyses

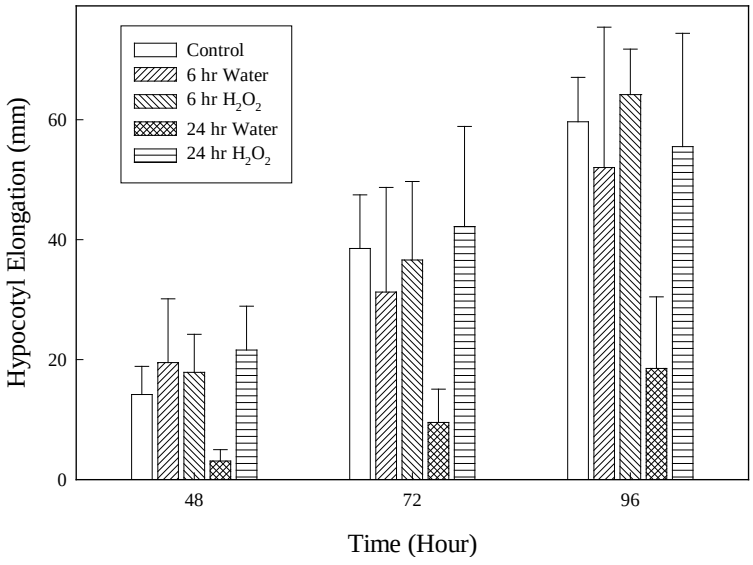
The experiments were conducted on a completely randomized design with variable replications as indicated above for each study, and the data were analyzed to compare treatment means with the control, and the means were separated for significance at 99% confidence level ($\alpha = 0.01$) using t-statistics.

## 3. Results

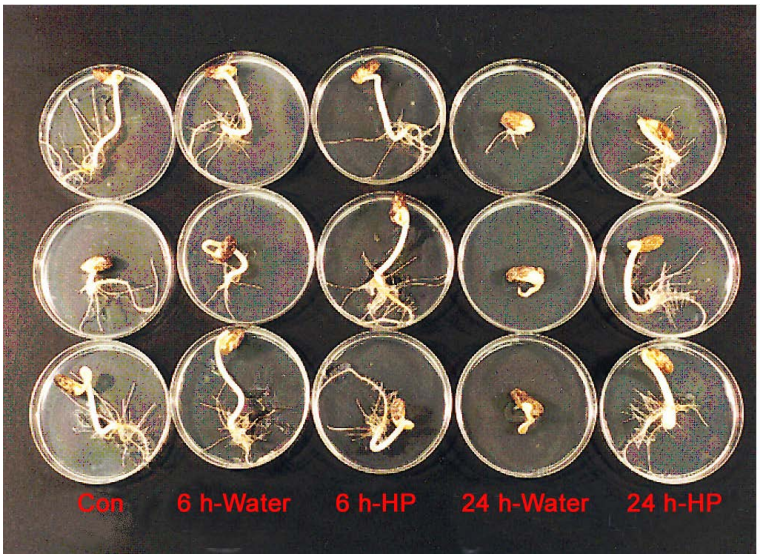
### 3.1. Hypoxia during Imbibition

Bean seeds subjected to hypoxic conditions during imbibition had significant effect on their subsequent germination under normoxia. Bean seeds exposed to hypoxic conditions only during imbibition up to 24 h were planted on 0.8% agar for germination under normoxia. All seeds treated with hypoxia during imbibition showed germination under normoxia, meaning that they were capable of producing radicle growth. When bean seeds were imbibed under water for 6 h and then allowed to germinate on agar, there was no significant difference in germination or hypocotyl elongation compared to seeds that were directly seeded on agar without the imbibition treatment or those imbibed either in hydrogen peroxide or in water with aeration (**Figure 1**). Nonetheless, direct-seeded seedlings produced more root mass and secondary roots and root hairs than the ones treated with hypoxia for 6 h during imbibition (data not shown).

Seeds were also subjected to hypoxia during imbibition for 24 h and then planted on agar for seedling growth under normoxia. The hypocotyl elongation after 96 h was characteristically slow and was only about 30% of that in the direct-seeded plants. The germinating seeds showed poor radicle growth with restricted elongation and with little or no secondary roots after 96 h of germination, suggesting that longer hypoxic conditions during imbibition can have an adverse effect on the subsequent growth of germinating bean seeds. The reduction in hypocotyl elongation in seeds submersed in water is the result of decreasing oxygen content in the water during seed imbibition and more than 25% of dissolved oxygen was depleted by germinating seeds within 24 h (see **Figure 4**). However, imbibition of seeds with 20 mM hydrogen peroxide overcame the reduction of hypocotyl elongation caused by hypoxia during imbibition. Seeds imbibed for 24 in hydrogen peroxide solution increased hypocotyl length to roughly 3 times of that observed in seeds imbibed in water under hypoxic conditions, a response similar to that observed in the direct-seeded plants. However, the hydrogen peroxide-treated seeds produced more secondary roots than did the direct-seeded plants. To determine the long term effects of transient hypoxia imposed during seed imbibition on the seedling growth, treated seeds were also planted in a soil medium (**Figure 2**). Consistent with above observations, plants from seeds that were submersed in water were severely stunted and showed very little stem growth compared to the direct-seeded control plants or plants from seeds imbibed in hydrogen peroxide solution (20 mM). After 5 days of planting, stem elongation in seeds treated with hydrogen peroxide was 5 times as large as that from seeds submersed in water. These results clearly suggest that bean seeds are sensitive to hypoxia during imbibition and the resulting poor hypocotyl elongation and seedling growth can be overcome by treating seeds with hydrogen peroxide during imbibition.




(a)




(b)

**Figure 1.** Hypocotyl elongation in beans following imbibition under hypoxia (a). Bean seeds were imbibed in water or in solution containing 20 mM of hydrogen peroxide for 6 h and 24 h. The imbibed seeds were placed on agar medium and allowed to germinate for up to 96 h. Control seeds were direct-seeded on agar without imbibition. Values represent means (n = 10) with S.D. Hypocotyl elongation for all the treatments including control was significantly different (t-test, $\alpha$ = 0.01) from that with seeds submersed in water for 24 h. The photograph shows the hypocotyl elongation of germinating seeds on agar after 96 h of seeding (b). The seeds were pretreated by submersing them in either water or 20 mM hydrogen peroxide (HP) solution.

## 3.2. Hypoxia during Germination

To determine the effects of hypoxia on the germination process (including imbibition and hypocotyl elongation), bean seeds were allowed to germinate submersed in water, 5 mM or 20 mM of hydrogen peroxide for up to 72 hours (**Figure 3**). There was no germination of seeds submersed under water. On the other hand, 12 h of incubation in 20 mM hydrogen peroxide produced hypocotyl growth, yielding nearly a linear hypocotyl elongation up

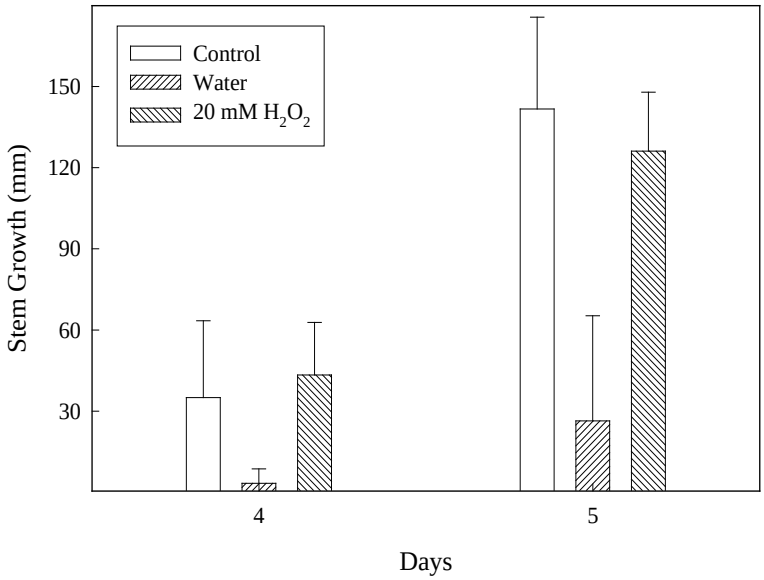


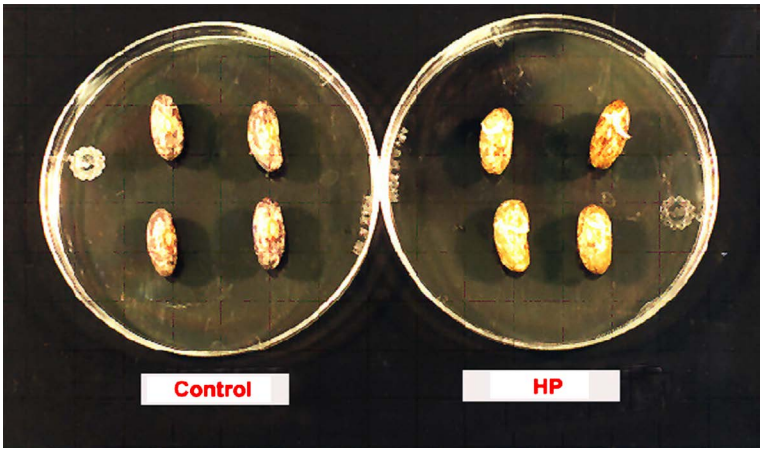

**Figure 2.** Plant heights of seedlings following imbibition under hypoxia. Bean seeds were imbibed in water or solution containing 20 mM hydrogen peroxide for 24 h and planted in soil-mix. Control consisted of seeds directly planted in soil-mix. Plant height was measured after 4 and 5 days. Values represent means (n = 10) with S.D. Stem growth for seedlings from seeds imbibed in aerated water or 20 mM hydrogen peroxide was significantly higher than seeds submersed in water without aeration ($\alpha$ = 0.01).

to 48 h. Seeds imbibed under water with aeration also showed a similar response. With aeration, the hypocotyl growth was more or less linear up to 72 h of incubation, whereas in 20 mM hydrogen peroxide, the hypocotyl elongation was similar to aerated seeds but leveled off after 48 h of incubation indicating perhaps the decreasing availability of oxygen in the medium with time. In addition, the seeds in 20 mM hydrogen peroxide solution appeared to imbibe water (and hydrogen peroxide) more readily than those in water as evidenced by their rapid swelling (**Figure 3**). This suggests that even imbibition of water in bean seeds is dependent on oxygen levels in the medium. Hydrogen peroxide at 5 mM showed slow germination and little hypocotyl elongation and was not much better than the seeds submersed in water.

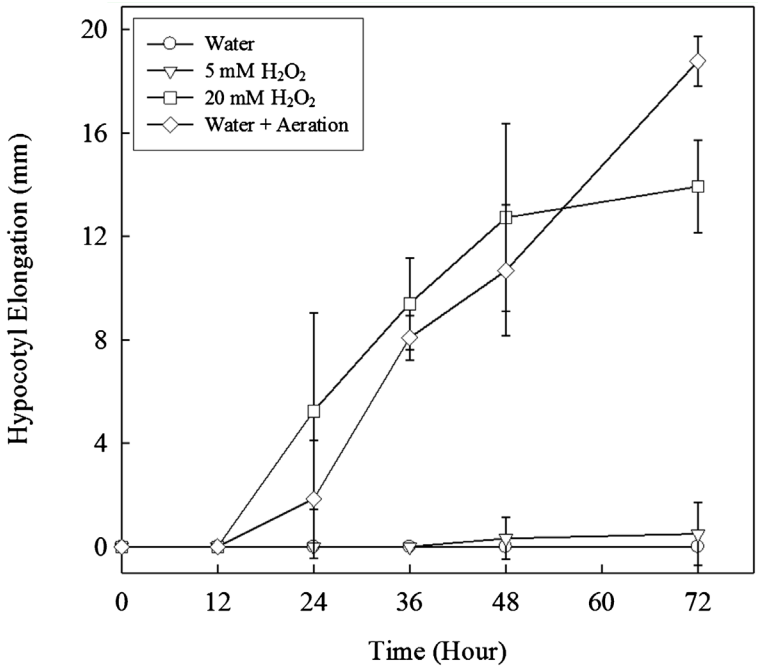
### 3.3. Hydrogen Peroxide and Dissolved Oxygen

Dissolved oxygen levels in water and 5 mM and 20 mM hydrogen peroxide solutions containing bean seeds were monitored during imbibition and germination (**Figure 4**). When seeds were submersed in water (hypoxic condition), there was rapid decline in oxygen levels, more than 50% of dissolved oxygen in water was depleted within 48 h while in aerated water, dissolved oxygen level remained constant at 250 µM. Also, the dissolved oxygen levels in water or hydrogen peroxide solution without seeds remained constant (data not shown). Significant oxygen consumption typically occurred only after 12 hours of imbibition and appeared to level off after 48 h. Oxygen consumption by germinating bean seeds was the greatest during the initial 48 h with a marked decline following this period. Addition of hydrogen peroxide increased the dissolved oxygen levels in the solution. Consistently, higher dissolved oxygen levels were observed with 20 mM hydrogen peroxide throughout the experimental period up to 72 h than with 5 mM hydrogen peroxide. After incubating seeds for 72 h, the medium with 20 mM hydrogen peroxide had nearly twice as much dissolved oxygen remaining as did the water. In addition with 20 mM hydrogen peroxide solution, there was sharp increase in dissolved oxygen immediately after incubating the seeds, approximately a 40% increase over a 12 h period.

The depletion of hydrogen peroxide from the medium depended on its concentration; higher the concentration, greater was its rate of breakdown, as indicated by oxygen levels in this medium. During the initial 36 h of germination, the average rate of hydrogen peroxide breakdown in 20 mM hydrogen peroxide was approximately 4 times of that in 5 mM hydrogen peroxide.




(a)




(b)

**Figure 3.** Hypocotyl elongation in beans germinated under hypoxia (b). Bean seeds were germinated in water, 5 mM hydrogen peroxide and 20 mM hydrogen peroxide for up to 72 h. Seeds were also germinated in water with aeration, provided by a constant circulation of tap water at a flow rate of 15 ml/sec. Values are means (n = 6) with S.D. Hypocotyl elongation for seeds in 20 mM hydrogen peroxide or in water with aeration was significantly higher (t-test, $\alpha$ = 0.01) than that seeds submersed either in water or 5 mM hydrogen peroxide. The photograph shows seed swelling and hypocotyl growth in seeds in water and 20 mM hydrogen peroxide (HP) solution after 48 h (a).

### 3.4. Catalase Activity and Transition Metal Catalysts

CAT activity in germinating seeds was investigated as it is one of the catalysts involved in producing oxygen from hydrogen peroxide. CAT activity was higher in seeds incubated in water after 12 h compared to those in hydrogen peroxide or aerated water (**Figure 5**). However, after 24 h of incubation, no difference in CAT activity in seeds was observed among the treatments. When a CAT inhibitor, 3-AT at 0.1 mM was added to the incubating solutions containing 20 mM hydrogen peroxide, a sharp reduction in hypocotyl elongation was observed,

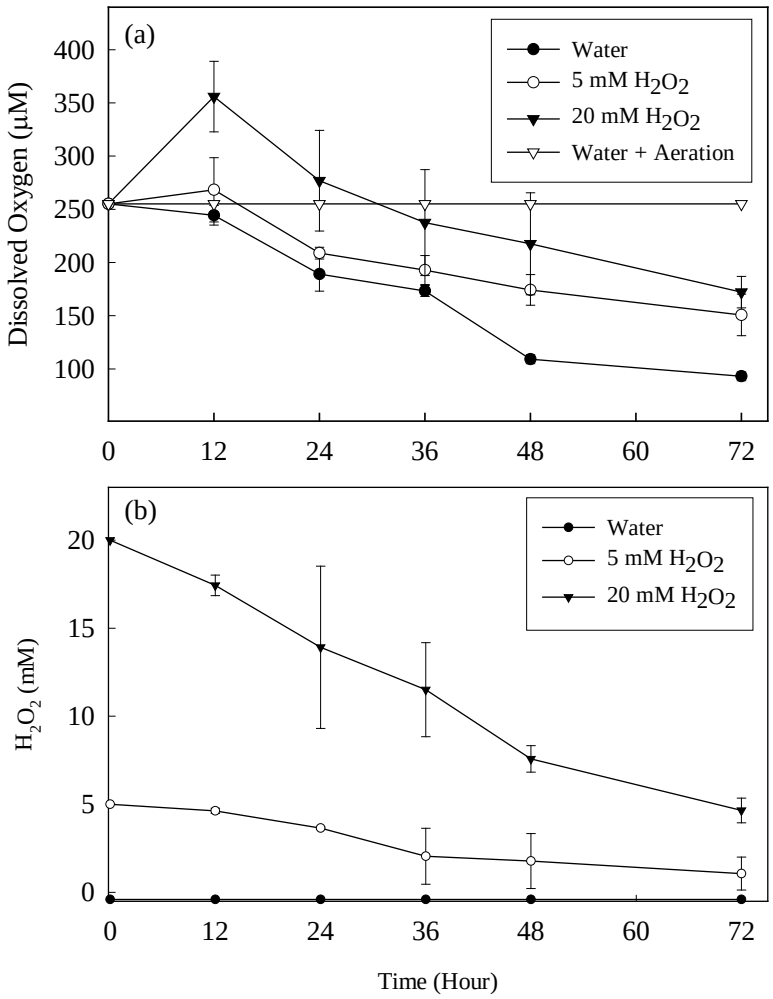



**Figure 4.** Dissolved oxygen and hydrogen peroxide levels in the germinating media. Dissolved oxygen levels in water, 5 mM hydrogen peroxide and 20 mM hydrogen peroxide containing seeds were measured polarographically over a 72 h period (a). Oxygen levels were also monitored in water containing seeds which was aerated with a constant circulation of tap water at a flow rate of 15 ml/sec. Changes in hydrogen peroxide levels were also monitored in the above media (b). Values represent means (n = 4) with S.D.

which was less than 20% of that observed in the medium without 3-AT. In addition, higher concentrations of 3-AT appear to be toxic as they produced deformed hypocotyls, resulting in eventual seedling death. It is important to note that hypocotyl elongation in 20 mM hydrogen peroxide in the presence of 3-AT (0.1 M) was similar to that observed in seeds imbibed under hypoxic conditions (**Figure 6**). Thus, these results show that CAT plays an important role in breakdown of hydrogen peroxide more than the metal catalysts.

We also examined the catalytic role of transition metal catalysts such as $Cu^{2+}$, $Fe^{2+}$ and $Mn^{2+}$ in producing oxygen from hydrogen peroxide during germination of bean seeds. The catalytic activities of these ions at various concentrations are shown in **Table 1**. While all these cations have high catalytic activity, $Fe^{2+}$ (0.001 M) appeared to be as effective as CAT at 10.5 units/mL in generating oxygen from 20 mM hydrogen peroxide solution. In addition, the transition metal contents of bean seeds were analyzed to determine if they played a role in the disproportionation of hydrogen peroxide (**Figure 7**). Iron content was by far the highest in bean seeds and its levels were about five-fold greater than those of either copper or manganese suggesting that these ions in addition CAT can actively decompose hydrogen peroxide in the medium containing the germinating seeds to overcome hypoxia.

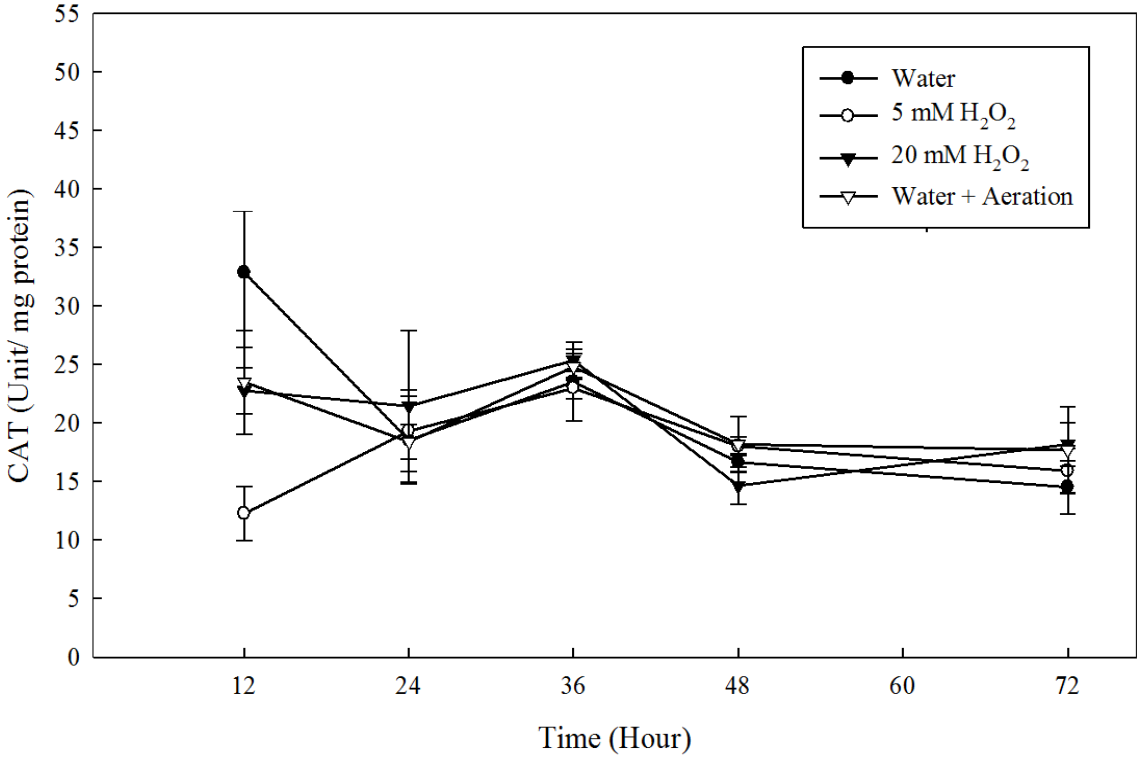



**Figure 5.** Changes in CAT activity in germinating bean seeds. Bean seeds were incubated in water, 5 mM hydrogen peroxide, 20 mM hydrogen peroxide and water with aeration for up to 72 h. Aeration was provided by a constant circulation of tap water at a flow rate of 15 ml/sec. Values represent means (n = 3) with S.D.

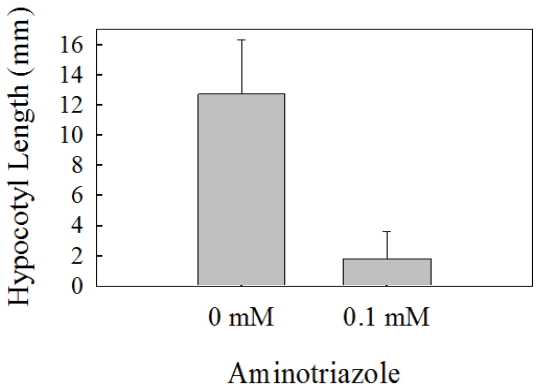



**Figure 6.** Hypocotyl elongation in bean seed in hydrogen peroxide with aminotriazole. Bean seeds were incubated in hydrogen peroxide solution containing either 0 or 0.1 mM aminotriazole for 48 h. Values represent means (n = 3) with S.D.

## 4. Discussion

The study focuses on hypoxia in seeds during imbibition and germination of bean seeds. When seeds were imbibed by submersing in water, oxygen limitation is likely to occur after 24 h. Thus, seed imbibition through submersion in water for 6 hours did not significantly affect the germination and subsequent seedling growth in beans. However, it appears to adversely affect the radical growth and reduce the formation of secondary roots even after these germinating seeds were transferred to normal aerated conditions. On the other hand, when seeds were imbibed for 24 h through submersion in water, hypoxic conditions occur as the dissolved oxygen level in water dropped below 75% of its original level. It led to a drastic reduction in hypocotyl elongation and subsequent poor seedling growth when plants were grown on agar or in soil under normoxia. It should be noted that seeds

**Table 1.** Dissolved oxygen was measured polarographically in 20 mM hydrogen peroxide produced by catalysts. Metal catalysts, $Cu^{2+}$, $Fe^{2+}$ and $Mn^{2+}$, and catalase were added to the hydrogen peroxide solution and dissolved oxygen levels were averaged over the initial 10 min.

| Catalyst | Concentration | Oxygen nM/min |
|---|---|---|
| $Cu^{2+}$ | 0.0001 M | 573.8 |
| | 0.001 | 828.8 |
| $Fe^{2+}$ | 0.0001 M | 637.5 |
| | 0.0001 | 3187.5 |
| | 0.001 | $>1250 \times 10^3$ |
| $Mn^{2+}$ | 0.00001 M | 446.3 |
| | 0.0001 | 653.4 |
| CAT | 10.5 unit/mL | $>1250 \times 10^3$ |

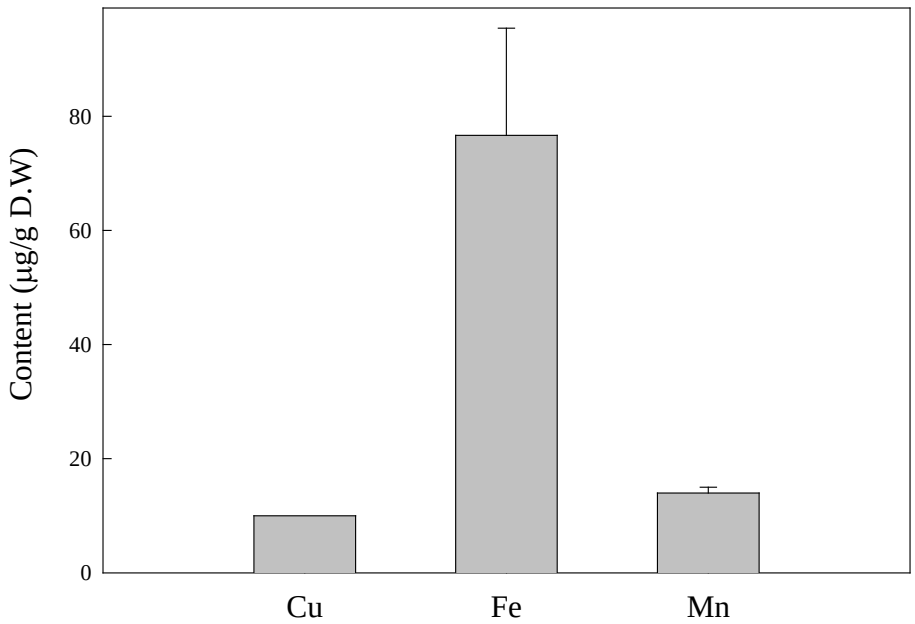



**Figure 7.** Seed composition of catalytic metals, copper, iron and manganese in beans. Amount of catalytic metals in bean seeds was determined. Values represent means (n = 3) with S. D.

consume oxygen at slower rates during early imbibition but at a much higher rate during radicle and hypocotyl growth. Thus, oxygen limitation during imbibition can produce adverse effects which may last during early seedling growth and development under normoxia. Typically, root growth during germination appears to be more sensitive to limiting oxygen conditions which can result in poor germination and subsequent seedling growth [22] [23]. Even roots of mature plants are sensitive to hypoxia and the transient exposure of plants to hypoxia is known to have a long lasting effect on plant growth [3]. Our results show that oxygen limitations can occur when seeds are submerged in water for a relatively short period of time (24 h). Lehle *et al*. [24] found that even a brief period of hypoxia (for 2 hours) in cotton seeds could drastically reduce the radicle growth. They found that cotton seeds to be sensitive to hypoxia during germination and could readily accumulate ethanol and acetaldehyde in response to hypoxia during not only germination but also imbibition. Similar results were noted in maize by Cobb *et al*. [25].

When hypoxic conditions were extended to cover the whole germination process, bean seeds failed to germinate, indicating that adequate oxygen is essential during imbibition, germination and seedling growth. These

findings are consistent with those of Morinaga [8] who showed that bean seeds failed to germinate in water. Our results show that bean seeds germinated when brief hypoxic conditions were imposed during imbibition, provided that they were subsequently allowed to germinate under aerated conditions. On the other hand, extended hypoxic conditions during imbibition in bean seeds resulted in poor growth of hypocotyl and a failure to germinate.

We used exogenous hydrogen peroxide to mitigate the adverse effects of hypoxia both during imbibition and germination as it can generate free oxygen and water by CAT and other catalysts including many transition metal ions present in the seeds [16]. In the presence of germinating seeds, 20 mM hydrogen peroxide produced oxygen rapidly to maintain higher oxygen levels in the medium. The oxygen levels in the medium containing hydrogen peroxide remained higher than those in well-aerated water during the initial 30 h of seed imbibition and germination. Thus, the adverse effects of hypoxia during imbibition on subsequent hypocotyl elongation and seedling growth were overcome by including 20 mM of hydrogen peroxide in the water. In fact, the seeds which were imbibed in 20 mM hydrogen peroxide produced rapid hypocotyl growth and more secondary roots as the radicle developed than those grown under non-hypoxic conditions on agar or soil.

When seeds were submersed in water, they failed to germinate. However in well-aerated water, bean seeds germinated and their hypocotyl elongation was roughly linear with time as was in water containing hydrogen peroxide, although the hypocotyls elongation in hydrogen peroxide solution (20 mM) appeared to level off after 48 h perhaps due to rapid depletion of hydrogen peroxide. Hydrogen peroxide was rapidly degraded in the presence of seeds, more than 60% was broken down within 48 h. This was also reflected in the elevated dissolved oxygen levels in the medium containing hydrogen peroxide. Thus, these results suggest that hydrogen peroxide can provide oxygen under hypoxic conditions for bean seeds during imbibition and germination and can thus overcome adverse effects of hypoxia. Previous studies have shown that hydrogen peroxide can improve germination of aged seeds [26]. Similarly, hydrogen peroxide was shown to improve the germination in rice, but its role was primarily thought to be in breaking seed dormancy [9].

Oxygen is produced from decomposition of hydrogen peroxide only in the presence of seeds, which suggests that source of catalysts for the breakdown of hydrogen peroxide comes from the seeds. Some primary catalytic agents that can breakdown hydrogen peroxide are CAT and many metal cations such as $Fe^{2+}$, $Cu^{2+}$ and $Mn^{2+}$ found in seeds. Many transition metals and their oxides including iron, copper, manganese and nickel have long been known to act as catalysts in decomposition of hydrogen peroxide [27]. We found that both CAT and metal ions were found to be very efficient in degrading hydrogen peroxide. However, use of CAT inhibitor, 3-AT, suggests that CAT plays a key role in the breakdown of hydrogen peroxide. CAT inhibitor at 0.1 mM was effective in retarding the hypocotyls elongation, due to its ability to block CAT activity which produces free oxygen from hydrogen peroxide and thus, overcomes hypoxia. It is important to note that inhibition of CAT activity by 3-AT completely reversed the hypocotyl elongation produced by hydrogen peroxide, suggesting that CAT plays an important role in the breakdown of hydrogen peroxide, more than the metal catalysts, in bean seeds. However, higher concentrations of 3-AT did not further restrict the hypocotyl elongation, but rather caused injury to the seedlings. CAT activity in the seeds during imbibition and germination remained more or less constant and the treatment with hydrogen peroxide did not affect the CAT activity. In contrast in maize seedlings, Prasad *et al*. [11] found that hydrogen peroxide can activate CAT (CAT3) gene and was associated with their chilling tolerance. CAT and other antioxidants levels appear to increase following hypoxia in a number of species and furthermore, elevated superoxide dismutase activity may even play a role in inducing tolerance to hypoxia [28]-[30].

In addition to CAT, other catalysts namely, transition metals also play an important role in decomposition of hydrogen peroxide. Bean seeds contained abundant amount of iron, about 5-fold higher than either copper or manganese, all of which could facilitate the production of oxygen in the seeds. But their role in producing free oxygen in bean seeds appears to be limited.

It is interesting to note that high levels of oxygen were detected in the medium while hydrogen peroxide is absorbed by the seeds where primarily CAT can facilitate its degradation to release oxygen [31]. Similarly, oxygen from the root tissues can diffuse from plant tissues into the medium, which has been shown to play a key role in oxidizing the reduced $Fe^{2+}$ to detoxify the rhizosphere in flooded soils [1]. This suggests that the oxygen levels in the seeds and tissues are likely to be higher than those determined in the medium containing hydrogen peroxide. The results also show that CAT more than the metal catalysts in the seeds plays an important role in providing oxygen to germinating seeds under hypoxia in the presence of hydrogen peroxide. However, one

would expect that in soils typically containing many of these transition metal catalysts at much higher concentrations, decomposition of hydrogen peroxide will be more rapid, thus, resulting in greater a release of oxygen into soil water. It is likely that the transition metal catalysts could play a more significant role in the breakdown of hydrogen peroxide in soils, but their role in yet to be determined.

## 5. Conclusion

Keeping bean seed submersed in water for 24 h during imbibition can cause hypoxia resulting in subsequent adverse effect on hypocotyl elongation and seedling growth. However, hypoxia for longer periods during imbibition can prevent germination. Hydrogen peroxide (20 mM) can overcome hypoxia during imbibition and germination of bean seeds as it provides oxygen during germination process. While catalase plays a major role other catalytic agents including transition metal ions like $Fe^{2+}$, $Cu^{2+}$ and $Mn^{2+}$ are also effective in breaking down hydrogen peroxide to release oxygen.

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