Ammonium toxicity on germination and early seedling growth of *Lolium multiflorum* L. (Italian ryegrass): oxidative stress biomarkers and potential mitigation strategy based on antioxidative defence

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology in the School of Biological Sciences

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Abstract

Ammonium (NH_4^+) toxicity is a major issue to plant growth as well as of economic importance. Various ammonium nitrate (NH_4NO_3) concentrations were applied to *Lolium multiflorum* L. (Italian ryegrass) seeds to determine the threshold ammonium toxicity to this plant. It was also revealed that the toxicity of NH_4NO_3 to ryegrass seedlings, particularly root growth, was largely attributed to NH_4^+ and not the nitrate ion. Histochemical detection of superoxide and hydrogen peroxide, and biochemical assays (peroxidase activity and lipid peroxidation) were also carried out to investigate the possibility that NH_4^+ toxicity, like many other abiotic stress conditions, is linked to oxidative stress. Seeds treated with NH_4NO_3 were found to exhibit higher levels of oxidative stress (superoxide, hydrogen peroxide, peroxidase activity and lipid peroxidation). It was of interest to apply seed priming technique and antioxidant treatments to assist plants in overcoming ammonium toxicity. Casein hydrolysate as the primary seed priming agent did not assist seedlings against NH_4^+ toxicity. It was thought that 10 and 20 mM of antioxidant treatments (L-ascorbic acid) were too probably high and inhibited germination of *L. multiflorum* L. seeds. Exogenous application of potassium iodide (KI) at 10 mM to Italian ryegrass seeds incubated in 1 mM ammonium sulphate [$(NH4)_2SO4$] seemed to have longer roots compared to those incubated in 1 mM $(NH_4)_2SO_4$ alone.

Introduction & Literature Review

1.1 *Lolium multiflorum*

Lolium is a genus of tuft grasses in the Pooideae subfamily of the Poaceae family (Wheeler et al., 2002). There are several species of *Lolium* that are often called ryegrasses and the species chosen for this study was *Lolium multiflorum* (also commonly known as Italian ryegrass). Italian ryegrass can be distinguished from perennial ryegrass (*L. perenne*) by the rolled emerging leaf. Italian ryegrass is one of the most common ryegrasses used in many pastures in New Zealand (Lamp et al., 2001). Italian ryegrass along with hybrid ryegrasses are used by dairy farmers in cooler seasons where the farmers might need extra quality herbage (Lamp et al., 2001). Having rotations of silage corn with annual Italian ryegrass can be considered an effective method to improve foraging production on dairy cattle farms (Lloveras-Vilamanya, 1987). Besides assisting in forage production, Italian ryegrass can also be part of phytoremediation solution. It was proven in the study that Italian ryegrass are able to remove the herbicide from the hydroponic water efficiently (Mimmo et al., 2015).

It was reported that the dry yield of Italian ryegrass on testing sites showed a fast initial N response followed by an asymptote being reached at high N levels and concluded that 300-350kg N/ha/yr exceeded the Italian ryegrass N requirements to have optimum yield (Eckard, 1989). The study was testing the effects of applied nitrogen on Italian ryegrass with additional added nutrition such as calcium, magnesium, potassium, zinc and phosphorus. Other factors that need to take into account are the temperature, weather as well as N mineralization potential. Other studies reported that best seed yield were obtained by applying the lowest rate (50-60kg $ha^{-1}N$) of N (Choi et al., 2002; Ahrens and Oliveira, 1997). These studies also included various additional of nutrients as well as environmental factors. Current study differs from the previous studies by testing the effects of N fertilizers on Italian ryegrass in a controlled environment.

1.2 Nitrogen nutrition in plants and ammonium toxicity

Nitrogen (N), which is one of the essential factors that promotes the yield and growth of plants. Nitrogen absorption by plants involve three main steps which are grouped into assimilation, uptake and remobilization (Han et al., 2016). Synthetic fertilizers like NH_4NO_3 and $(NH4)_2SO4$ or microbes in soil can provide bioavailable nitrogen which can be absorbed by plants as ammonium (NH_4^+) or nitrate (NO_3^-) (Stitt et al., 2002). Then, the plants are able to utilize the supplied nitrogen for signalling, synthesising amino acids and storage of various molecules, not excluding other many metabolic processes (Stitt et al., 2002). It has been estimated that around 1% of the world's annual energy supply is required to produce majority of the N in commercial fertilizers through the Haber-Bosch process (Smith, 2002). It has been reported that applied N fertilizer absorption were less than

50% by majority crop plants (Tilman et al., 2002). Excess N fertilizer unabsorbed by plants can flow freely and contaminate the surrounding air and water which in turn can cause serious impacts to the environment (Wuebbles, 2009).

A major concern of applying nitrogen fertilizer is NH_4^+ as one of the major N sources. $NH4^+$Toxicity symptoms can be found in many plants even though ammonium is an intermediate in many metabolic reactions (Joy, 1988). Plants fed with NH_4^+ nutrients absorb higher ratio of cations to anions even though the presence of NH_4^+ generally causing the reduction of inorganic cations uptake (Kirkby, 1968; Clark, 1982; van Beusichem et al., 1988). To compensate for the charge imbalance, it was suggested that plants may undergo proton efflux and this normally acidify the external surroundings (Schubert and Yan, 1997). Different plant species have different threshold of NH_4^+ concentration at which toxicity symptoms start to show. It was reported that concentration of NH_4^+ (10 mmol/L) could inhibit development of primary roots of barley seedlings (Britto and Kronzucker, 2002). Some plants can tolerate higher NH_4^+ concentrations. For example, *Lotus japonicas* was reported to exhibit significant repressed root growth in response to 20 mM NH_4^+ (Rogato et al., 2010).

1.3 Seed priming

Seed priming is a procedure in which seeds are first hydrated before drying. During seed priming, seed germination processes are initiated but radicle emergence is not permitted (Giri and Schillinger, 2003). Priming mechanisms can include the changes in epigenetics as well as accumulation of inactive forms of signalling proteins and various transcription factors (Ibrahim, 2016). Based on previous studies, seed priming technique has been reported to help seedling growth under abiotic stress conditions (Farooq et al., 2006; Hussain et al., 2016). An example is improvement of salt tolerance in wheat (Jafar et al., 2011). The accumulation of osmolytes can also be enhanced by altering the metabolic processes through seed priming (Delavari et al., 2008). Following seed priming, there is a reduction in lipid peroxidation in seeds while the antioxidant activities were elevated (Afzal et al., 2008; Jafar et al., 2011). Enhancing antioxidant activities is crucial as plants are exposed to environmental stress, reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) increases its production which can cause significant damages to plant cells (Hussain et al., 2016). Antioxidative enzymes like peroxidase (POD; EC 1.11.1.7) play a vital role in plants as a defence mechanism against ROS (Hussain et al., 2016). It was reported that ascorbic acid has the ability to reduce H_2O_2 to water through the reaction of ascorbate peroxidase as well as directly scavenge superoxide, singlet oxygen and hydroxyl radicals (Noctor and Foyer, 1998). Through seed priming technique and antioxidant treatments, it is hypothesized that NH_4^+ toxicity in *L. multiflorum* L. can be reduced by enhancing antioxidant defence system.

Aim and Objectives

The aim of this research was to obtain a better understanding of NH_4^+ toxicity in *L. multiflorum* L. during seed germination and early post-germinative seedling growth which is a critical stage in the successful establishment of the productive pasture plants. There is a lack of information on this potential problem to *L. multiflorum* L. in the literature. There was a time limitation for this thesis to be completed within 12 months from conducting literature review, concept and hypothesis development, writing up research proposal, conducting preliminary experiments, taking time for some chemicals such as nitro blue tetrazolium to arrive, doing the key experiments, and finally writing up the thesis. Hence, the non-dormant, fast germinating Italian ryegrass was chosen for this work.

The objectives of this research were to follow up on an initial experiment in the lab showing that root growth of Italian ryegrass seedlings seemed to be inhibited when the seedlings were incubated in ammonium nitrate. These include the following:

1. It was of interest to determine if it was ammonium ion or nitrate ion that was toxic to Italian ryegrass seedlings. It was also of interest to investigate the possibility that the different effects of different concentrations of ammonium nitrate on Italian ryegrass seedlings might be related to different pH of the ammonium nitrate solutions.

2. Different abiotic stress conditions including salinity stress, drought stress, heavy metal stress, etc. have often been linked to elevated oxidative stress (Cruz de Carvalho, 2008; AbdElgawad, 2016; Clijsters, 1999). Arguably, NH_4^+ toxicity is a form of abiotic stress. It would, therefore, reasonable to hypothesise that oxidative stress could also be associated with NH_4^+ toxicity in plants, although there is a lack of information on this in the literature. To investigate this hypothesis, some biomarkers of oxidative stress and an antioxidative enzyme (peroxidase) will be investigated in Italian ryegrass seeds incubated in water and in ammonium nitrate.

3. Seed priming is a practical pre-sowing treatment. It has been shown that when seedlings were grown under abiotic stress, for example, elevated heavy metal concentration, those that developed after seed priming would be protected against the stress compared to those that were not in the priming treatment (Espanany, 2016). It has been suggested that the protective effect of seed priming might be mediated through enhanced antioxidant defence (Xu et al., 2011; Khaliq, et al., 2015; Hussain et al., 2016). It was investigated to see if seed priming which could enhance antioxidative defence would be useful for protection of Italian ryegrass seedlings in the presence of ammonium nitrate.

4. If elevated oxidative stress was involved in NH_4^+ toxicity in plants or ryegrass seedlings in particular, exogenous application of antioxidants could counteract NH_4^+ toxicity. This possibility was also investigated in the present study.

Materials and Methods

2.1 Germination time

L. multiflorum seeds were used in this experiment. Twenty seeds each were placed into 3 plastic Petri dishes (15 cm x 9 cm diameter). Fifteen ml of deionised water were added to each Petri dish. The petri dishes were placed in an incubator at 22^0c and with continuous lighting. The seeds were observed until they showed visible signs of germination (radicle emergence). Days required for germination were recorded. The arrangement of Petri dishes was randomised in the incubator with every new set-up. Three trials were repeated to determine the average days required for germination.

2.2 Observation and measurement of seedlings length

The seedlings from each Petri dish were placed on a paper towel. The length of the seedling parts (root and shoot) were determined from their digital images taken with a cell phone and then measured using the Photoshop Measurement Software. A measurement scale bar was included in each image of the seedlings that will help to set a specified number of pixels in the image equal to a number of scale units. This will allow the measurement of areas and make calculations to obtain results in the selected scale units. Lastly, average root and coleoptile lengths were calculated.

2.3 Determine the different concentration effects of ammonium nitrate on root growth

Different concentrations (1, 10 and 25 mM) of ammonium nitrate (NH_4NO_3) were prepared and dissolved in deionised water. Four groups of Petri dishes were set up (1 control and 3 concentrations of (NH_4NO_3). Each group had 3 petri dishes and each with 20 seeds. Fifteen ml of deionised water were added to each petri dish of the control group. Steps were repeated for each respective concentration of NH_4NO_3. The dishes were placed in the incubator for 4 days. Root and coleoptile lengths were measured after day 4.

2.4 Comparing the effects of NH_4^+ and NO_3^- on root growth

Potassium nitrate (KNO_3) and ammonium sulphate [$(NH_4)_2SO_4$] were used as chemical treatments for this part of the experiment. Different concentrations of both KNO^3 and $(NH_4)_2SO_4$ (0.5, 1, 5, 10 and 25 mM) were used. The required amounts of both chemicals at each concentration were calculated and dissolved in deionised water. Six groups of Petri dishes were set up for both treatments (1 control and 5 concentrations of chemical agent). In each group, there were 3 petri dishes, each with twenty seeds. Fifteen ml of deionised water were added to each petri dish of the control group. Steps were repeated for each respective concentration of both chemical agents. The seeds were incubated for 4 days and root and coleoptile lengths were measured at day 4.

2.5 Testing the effects of NH_4NO_3 solutions at the same initial pH on root growth

Different concentrations (1, 3 and 5mM) of NH_4NO_3 were prepared and dissolved in deionised water. The pH for each concentration of NH_4NO_3 solution was adjusted to roughly 6.1 pH using 0.1M hydrochloric acid (HCl) and 0.1M sodium hydroxide (NaOH). The pH adjustment also applied to deionised water for the control group. The Petri dishes set up were similar as discussed above. Seeds were incubated and measurements of root and coleoptile lengths were taken at day 4.

2.6 Histochemical detection of superoxide (O_2^-) and hydrogen peroxide (H_2O_2)

A modified version of histochemical assays in Kumar et al. (2014) was used for detection of H_2O_2 and superoxide in the root tips of 4-day-old *L. multiflorum* seedlings. Nitro blue tetrazolium chloride (NBT) solution was prepared for the detection of O_2^- free radical. NBT (0.5 mM) was prepared in 10 mM citric acid buffer with pH 6.0 and stored in the dark. Three roots were randomly chosen from 3 different replicate dishes in each group (control, 1, 3 and 5 mM of NH_4NO_3). The following procedure was performed on a single root tip at a time to prevent measurement error. Roughly 5 mm of the root from the tip was excised and placed into a glass Petri dish. Twenty µl of NBT solution was added to cover the excised root tips completely. A stopwatch timer was started the moment the root tip was in contact with the NBT solution. The root tip was observed under a stereo microscope. The timer was stopped when the visible sign of purple staining of the root tip was observed and the result was recorded. The above steps were repeated for individual roots from each group of treatments.

For hydrogen peroxide staining, 50 mg benzidine dihydrochloride ($C_{12}H_{12}N_2 \cdot 2HCl$) was dissolved in forty ml deionised water. The solution was adjusted to pH 6.0 using 0.1M HCl and NaOH and the final volume was brought up to fifty ml. The mixture was covered with aluminium foil to prevent light exposure and left stirring. Sodium phosphate (2.5 ml of 200 mM Na_2HPO_4) was added to the stirring solution. The mixture was left stirring for additional thirty minutes. The staining and measurement protocol for superoxide was repeated for detection of hydrogen peroxide by using twenty µl of benzidine dihydrochloride ($C_{12}H_{12}N_2 \cdot 2HCl$) solution instead of NBT solution.

2.7 Peroxidase extraction and assay

A modified method of Fielding and Hall (1978) for assaying guaiacol peroxidase (GPOX; EC 1.11.1.7) activity was used. In this experiment, 4- and 6-day-old *L. multiflorum* seedlings were used. The seed germination procedure was repeated according to the method discussed at section 2.5. All the Eppendorf tubes, pestle and mortar, buffers and the other required solutions were kept on ice unless specified.

Potassium phosphate buffer (KPO_4, 0.1 M of 200 ml of pH7.0) was prepared with a mixture of dipotassium phosphate (K_2HPO_4) and monopotassium phosphate (KH_2PO_4). Deionised water (160 ml) was added into a glass flask to dissolve 1.869g of K_2HPO_4 and 1.262g of KH_2PO_4. Deionised water was added until the final volume was brought to 200 ml. Five root tips were randomly chosen from individual dishes from each group (control, 1, 3 and 5 mM). The root tips were then grounded in a

mortar and pestle with a total of 1.2 ml of KPO_4 buffer. The extraction method was repeated for 5 root tips from each replicate Petri dish within a treatment.

The Eppendorf tubes containing the homogenates of the root tips were centrifuged at 4^0c for 5 minutes at 10,000 rpm. The supernatant (enzyme extract) was removed from each tube and diluted 1:3 using KPO_4 buffer to a final volume of 400 µl into another set of Eppendorf tubes. H_2O_2 (10%, v/v) was prepared by mixing 0.3 ml 30% H_2O_2 and 0.6 ml deionised water. The boiled enzyme control was prepared by placing, 100 µl a diluted enzyme extract in a boiling water bath at 100^0C for 5 minutes. The composition of the reaction mixtures for peroxidase activity assay is shown below in the Table 1.

Table 1: Materials required for peroxidase assay.

Substances	**Tube 1 (enzyme control)**	**Tube 2 (enzyme reaction) [2 replicate tubes for each enzyme extract]**
KPO_4	943 µl	943 µl
10% H_2O_2	5 µl	5 µl
Guaiacol	2 µl	2 µl
Supernatant (enzyme extract)	50 µl (boiled diluted supernatant)	50 µl (diluted supernatant)
	Total of 12 tubes	Total of 24 tubes (six water treatment, six 1 mM, six 3 mM and six 5 mM NH_4NO_3 treatment of the seedlings)

All the analysis except the control tubes were done at roughly 2 minutes mark. The spectrophotometer was blanked with distilled water and tested with all 12 control tubes for the base reading. Proceeding onto the treatment tubes, the tube containing KPO_4, 10% H_2O_2 and guaiacol was removed from ice after 50 µl a diluted supernatant was added and timed instantly. The tube was left on the lab bench at 22^0c (room temperature) for 2 minutes. Before the 2 minutes mark, the mixture was poured into a cuvette and then the absorbance was read at 470 nm at the 2 minutes mark. The peroxidase activities in the extracts of 4- and 6-day-old Italian ryegrass seedlings were determined using the procedure described above. The final absorbance coefficient (A/min/root tip) was calculated using the formula below:

a: values obtained from spectrophotometer

$\frac{(a*4\ dilution\ factor*1200ul)}{50ul}$ = B

$\frac{B}{2\ minutes}$ = C

$\frac{C}{5\ root\ tips\ per\ dish}$ = final absorbance coefficient (A/min/root tip)

2.8 Lipid peroxidation assay

The analysis was carried out using a modified version of methods by Heath and Packer (1968). In this experiment, 6-day old seedlings were used. The seed germination procedure was as described in Section 2.5, but there were 10 Petri dishes in a treatment. All tubes and solutions were kept on ice unless specified otherwise.

A few solutions were prepared before starting the lipid peroxidation assay: 100ml of 0.1% (w/v), 20% (w/v) trichloroacetic acid (TCA) and 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA). Roots were excised from 150 seedlings in each group of treatments and the fresh weight of the roots were determined using an analytical balance. The roots were homogenised in a total of 1.2 ml ice-cold 0.1% (w/v) TCA in a mortar and pestle. The homogenate was centrifuged at 10,000 r.p.m. for 5 minutes at 4^0C. An aliquot of the supernatant (0.5 ml) was mixed with 1 ml of 20% (w/v) TCA containing 0.5% (w/v) TBA in a test tube. For the blank, 0.5 ml of supernatant was mixed only with 20% (w/v) TCA. The test tubes were then vortexed. The tubes were placed in a hot water bath and heated at 90^0c for 30 min. After 30 min of heating, the reaction products and blank were quickly cooled in an ice bath subsequently and centrifuged at 10,000 r.p.m. for 10 min at 4^0c. The absorbance of the supernatant was read at 532nm and corrected by subtracting the absorbance at 600nm. The final absorbance value was calculated using the formula below.

a: value obtained at 532nm
b: value obtained at 600nm

$$(\mathrm{a}-\mathrm{b}) * \frac{1200\ ul}{500ul} = c$$

$$\frac{c}{fresh\ weight\ of\ 150\ root\ tips\ (g)}$$ = Final absorbance value (A/gram)

2.9 Seed priming

The seed priming agent used was casein hydrolysate (CH) which was also used in another seed priming study of Italian ryegrass seeds for protection against salinity stress in the Biotechnology Lab, University of Canterbury. The enzymatic hydrolysed casein was purchased from Sigma (now Merck). According to the manufacturer, CH may be used as a source of reduced nitrogen as it contains a mixture of up to 18 different amino acids including a relative large amount of glutamine. In addition, CH may contain calcium, phosphate, other microelements and vitamins. Three different concentrations of CH were used for preparation of the seed priming solution (100, 300 and 500 mg per litre). CH was dissolved in deionised water. Seeds were divided into 1 control group and 4 priming treatment groups. The seeds in 3 priming groups were soaked in 100, 300 and 500 mg/L casein hydrolysate solution. The seeds in the final priming group was soaked in deionised water and this treatment is called hydropriming. All seeds were soaked for 24 hours. The seeds were then left to air dry for a day. For germination, there were 18 Petri dishes each containing 20 seeds (6 Petri dishes with seeds that were soaked before or control, 6 Petri dishes with seeds from the hydropriming treatment and 6 Petri dishes with seeds that were soaked in casein hydrolysate at a chosen concentration). The seeds from the treatments were placed onto the petri dishes as shown in Figure 2. Deionised water and 1 mM NH_4NO_3 were adjusted to roughly pH 6.1. Root and coleoptile lengths were measured at day 4.

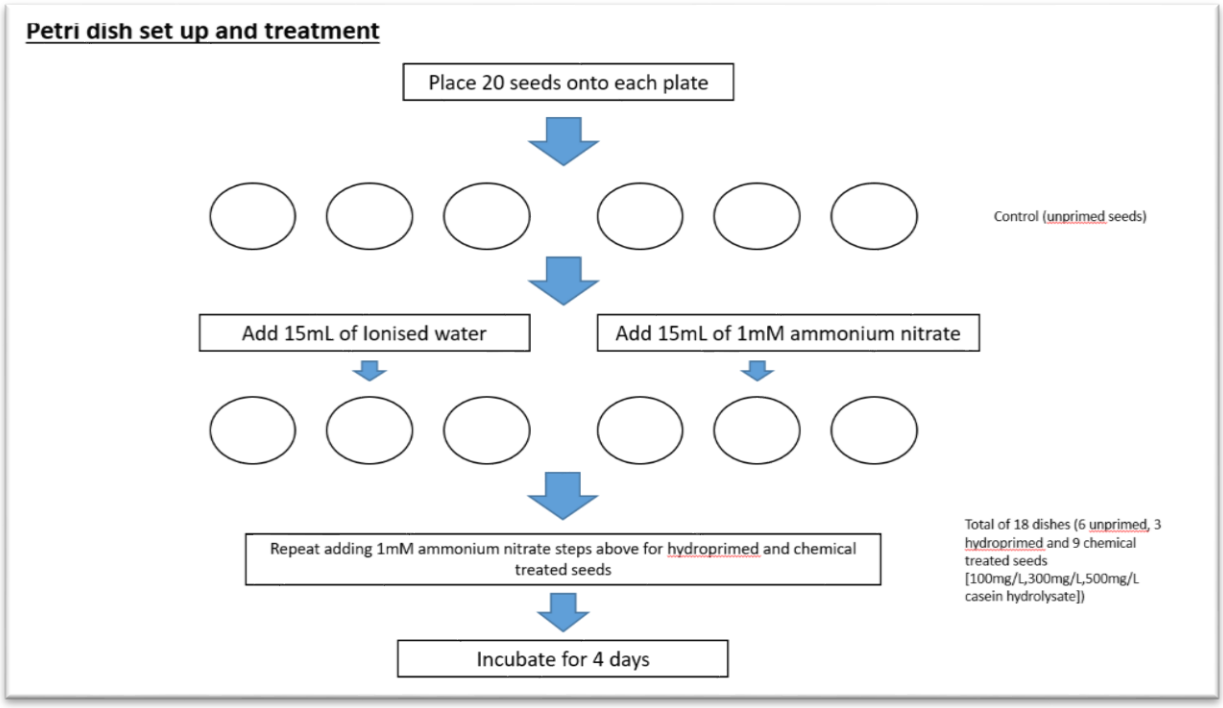

Figure 1: Petri dish set up and treatment.

2.10 Antioxidant treatments

Two known antioxidants, potassium iodide (KI) and L-ascorbic acid ($C_6H_8O_6$), were used in this antioxidant treatment. Italian ryegrass seeds were germinated as described in Section 2.5, but there were 5 Petri dishes in a treatment group. In the 10 mM antioxidant treatment, the groups were as below:

(i) The deionised water control,

(ii) 1 mM $(NH_4)_2SO_4$ only,

(iii) 10 mM KI only,

(iv) 10 mM KI + 1mM $(NH_4)_2SO_4$,

(v) 10 mM L-ascorbic acid only, and

(vi) 10mM L-ascorbic acid + 1mM $(NH_4)_2SO_4$.

The above experimental setup was repeated for the 20 mM antioxidant treatment. The seeds were incubated for 4 days and length of seedlings were measured at day 4.

2.11 Statistical analysis

Statistics were performed using IBM SPSS Software version. 25 and Statistical Analysis Software version. 9.4 (SAS). All data were analysed using one-way ANOVA and Tukey test was used for comparison of means. Values are means ± standard error (SE) of three replicates and those do not share the same letter are significantly different ($P<0.05$).

Results

3.1 NH_4^+ hinders germination and the root growth of Italian ryegrass seedlings

Italian ryegrass seedlings started to germinate in deionised water after about 2 days from sowing in the present study. Germination of Italian ryegrass seeds was inhibited with increasing concentrations of NH_4NO_3 (Table 2). The seeds incubated in 25 mM NH_4NO_3 did not germinate after 4 days from sowing (Table 2).

Table 2: Effects of ammonium nitrate concentration (mM) on Italian ryegrass seed germination rate (%)

Treatment	Germination rate (%)
Control	76.67±4.41[a]
1 mM	63.33±3.33[b]
10 mM	33.33±7.64[c]
25 mM	0[d]
Coefficient variance (CV)	14.132
Pr > F	<0.0001

No significant differences were observed between control treatment (incubation of seeds in deionised water) with 1 mM NH_4NO_3 treatment ($p=0.86$) or 10 mM NH_4NO_3 treatment ($p=0.34$) in terms of average shoot length. As for the root length, significant differences were found when comparing control with the different NH_4NO_3 treatments ($p<0.05$). This shows that NH_4NO_3 concentration as low as 1 mM hindered the growth of *L. multiflorum roots* (Fig. 2).

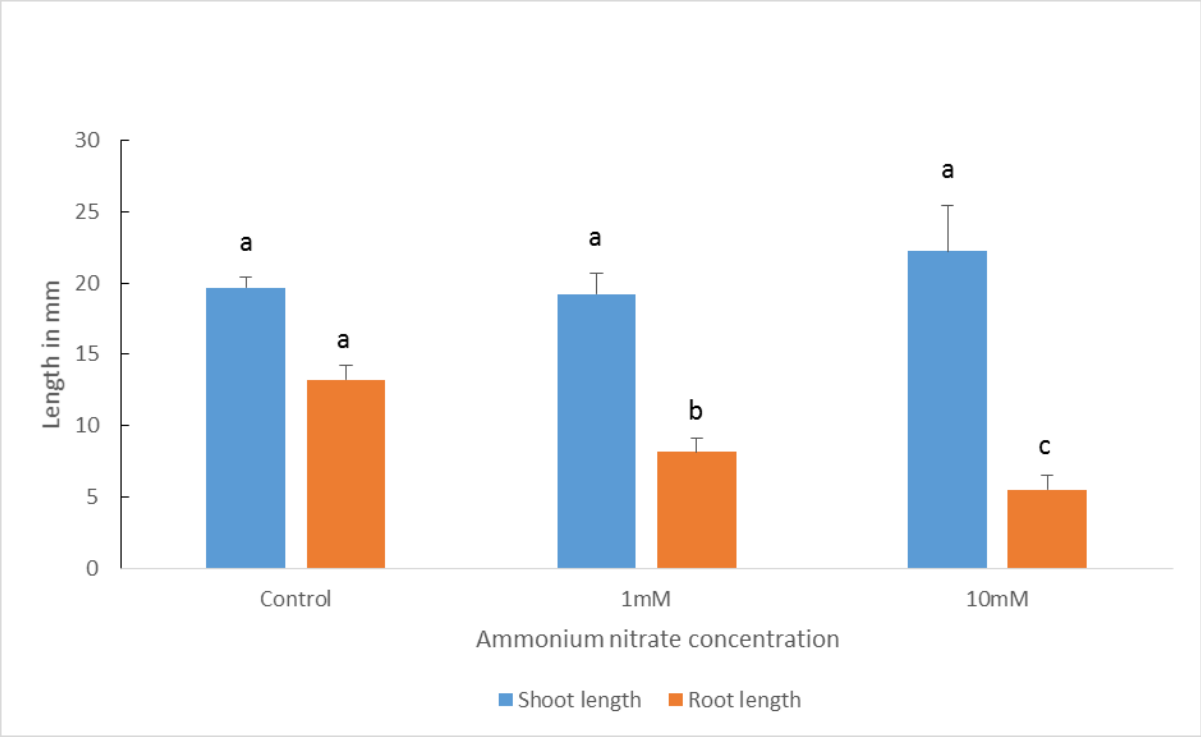

Figure 2: Effects of ammonium nitrate concentration (mM) on seedlings' shoot and root length (mm) after 4 days of incubation. Means having the same letter are not significantly different by Tukey test (P<0.05).

KNO_3 and $(NH_4)_2SO_4$ were used to help to determine which ions in (NH_4NO_3) that was affecting the root growth of Italian ryegrass seedlings. KNO_3 as the source of NO_3^- and $(NH_4)_2SO_4$) as the source of NH_4^+. All *L. multiflorum* seeds germinated under $(NH_4)_2SO_4$ showed significantly reduced root length compared to KNO_3-treated seeds and control (Fig. 3). Statistical analysis showed that all seeds germinated under $(NH_4)_2SO_4$ had significantly shorter root length ($P<0.05$) compared to control and KNO_3-treated seeds. The difference was at least 2 fold.

As for the shoot length, seeds germinated in KNO_3 (with possible exception at 10 mM) showed no significant difference compared to control (Fig. 4). When comparing the effects of same respective concentrations, 10 mM and 25 mM $(NH_4)_2SO_4$ showed significant differences on shoot length ($p=0.001$ & $p=0.29$) when compared to 10 mM and 25 mM KNO_3. Low concentrations of $(NH_4)_2SO_4$ did not seem to not hinder shoot growth. However, root growth was severely affected even at a low concentration (0.5mM) of $(NH_4)_2SO_4$. This suggests that NH_4^+ could potentially hinder Italian ryegrass seedling shoot and root growth. It can be suggested that early post germinative seedling growth was particularly sensitive to NH_4^+.

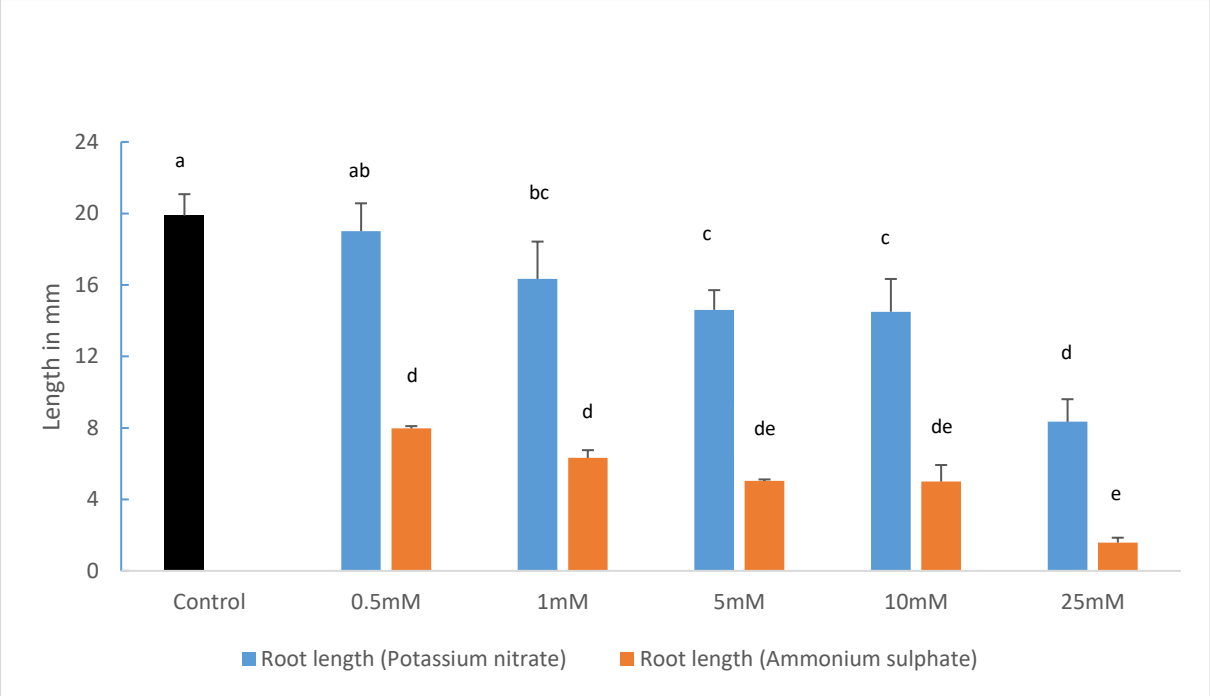

Figure 3: Effects of potassium nitrate and ammonium sulphate (mM) on root length (mm) after 4 days of incubation. Means having the same letter are not significantly different by Tukey test (P<0.05).

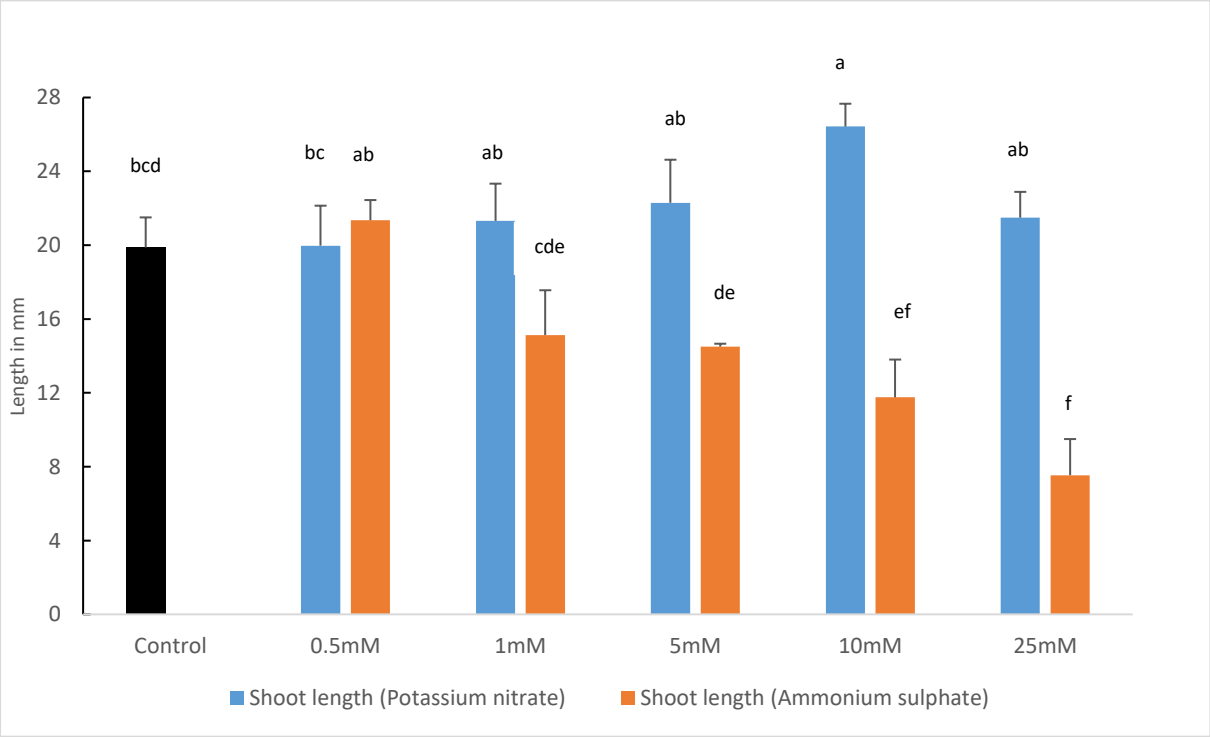

Figure 4: Effects of potassium nitrate and ammonium sulphate (mM) on shoot length (mm) after 4 days of incubation. Means having the same letter are not significantly different by Tukey test (P<0.05).

3.2 Effects of NH_4NO_3 concentrations with the same initial pH on seedlings growth

Different concentrations (1, 3 and 5 mM) of NH_4NO_3 of about the same initial pH (6.1) were applied to *L. multiflorum* seeds. In this experiment, 10 and 25 mM of NH_4NO_3 were thought to be too high of concentrations for seed germination and seedling growth. No significant differences were found between control and all NH_4NO_3 treatments ($p>0.05$) for the shoot length. Presence of NH_4NO_3 halved the root length of seedlings when compared to control seedlings (Fig. 5). Significant root length differences were found between control and all NH_4NO_3 treatments ($p<0.05$) but no significant differences were found among the NH_4NO_3 treatments ($p>0.05$).

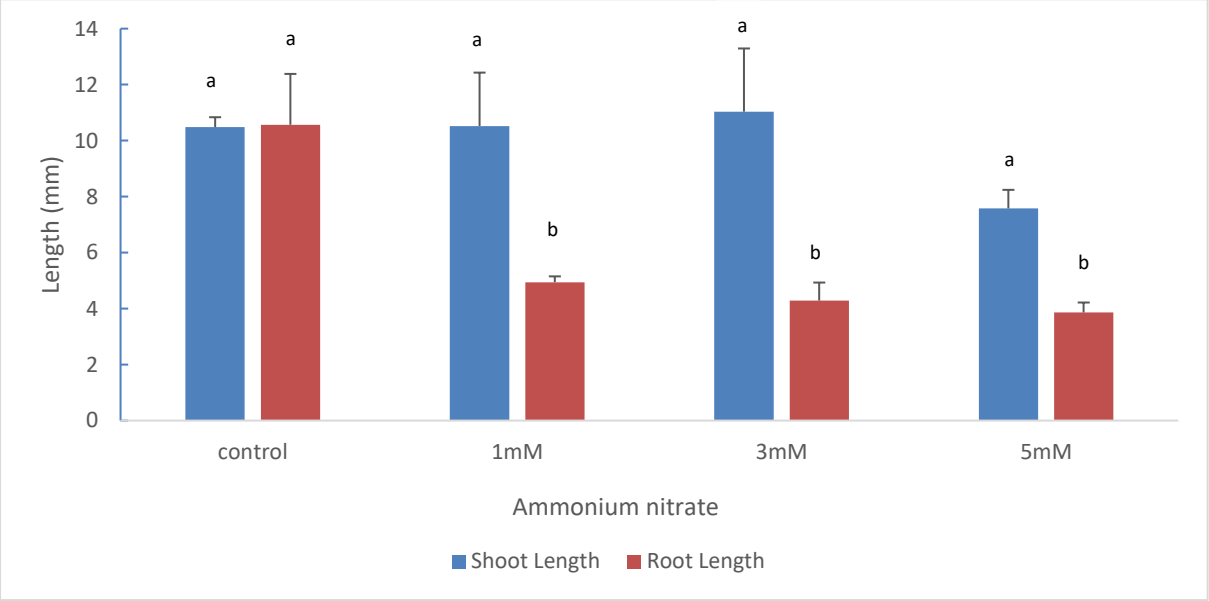

Figure 5: Effects of ammonium nitrate concentration (mM) at the same initial pH (6.1) on seedlings' shoot and root length (mm) after 4 days of incubation. Means having the same letter are not significantly different by Tukey test (P<0.05).

3.3 Detection of superoxide and hydrogen peroxide

In this experiment, average time taken was recorded at the first visible signs of the specific colour changes indicating reaction between NBT and benzidine dihydrochloride with superoxide and hydrogen peroxide, respectively, of the root tips observed under a stereo-microscope (Table 3). Starting with NBT staining, significant time differences were observed when comparing control with all NH_4NO_3 treatments ($P<0.05$). Time taken for dark blue stain to appear on root tip was shortened with increasing NH_4NO_3 concentrations. This suggests that seeds treated with NH_4NO_3 had higher concentrations of O_2^- free radicals in the root cells. As for H_2O_2 staining, similar results were observed. Significant time differences were observed between control groups and NH_4NO_3 treatment groups ($P<0.05$), suggesting higher concentration of H_2O_2 in seedlings roots treated with NH_4NO_3. Overall, average time of staining taken for seeds treated with NH_4NO_3 were shorter in both NBT and H_2O_2 staining.

Table 3: Average time taken (s) for NBT and H_2O_2 colorimetric assay. Means within a column having the same letter are not significantly different by Tukey test ($p<0.05$).

NBT staining		**H_2O_2 staining**	
Treatment	Average time taken (s)	Treatment	Average time taken (s)
Control	60.44±4.08[a]	Control	84±2.22[a]
1 mM NH_4NO_3	47.89±2.44[b]	1 mM NH_4NO_3	57.78±2.98[b]
3 mM NH_4NO_3	28.22±1.95[c]	3 mM NH_4NO_3	35.56±2.01[c]
5 mM NH_4NO_3	24.89±2.71[c]	5 mM NH_4NO_3	25.44±2.45[d]
Coefficient variance	12.459	Coefficient variance	8.343
Pr > F	<.0001	Pr > F	<.0001

3.4 Biochemical analysis

Seedlings after 4 and 6 days from sowing seeds were used for peroxidase activity assay. Higher peroxidase activities (A/min/root tip) were observed in seedlings treated with 1, 3 and 5 mM NH_4NO_3 in both 4- and 6-day-old seedlings (Fig. 6). In the 4-day-old seedlings, significantly different peroxidase activities were observed between control and all NH_4NO_3 treatments ($p<0.05$), but there were no significant differences among NH_4NO_3 treatment groups ($P>0.05$). Similar results were obtained in 6-day-old seedlings. Significantly different peroxidase activities were observed between control and all NH_4NO_3 treatment groups. An interesting observation in 6-day-old seedlings is that the seedlings treated with 5 mM NH_4NO_3 showed significant peroxidase activity compared to those incubated in 1 mM NH_4NO_3 ($P=0.001$) and 3 mM NH_4NO_3 ($P=0.018$). This suggests that the seedlings continue to increase peroxidase production over time to counteract the presence of high concentrations of H_2O_2. Presence of NH_4NO_3 at least doubled the peroxidase activity compared to the control group.

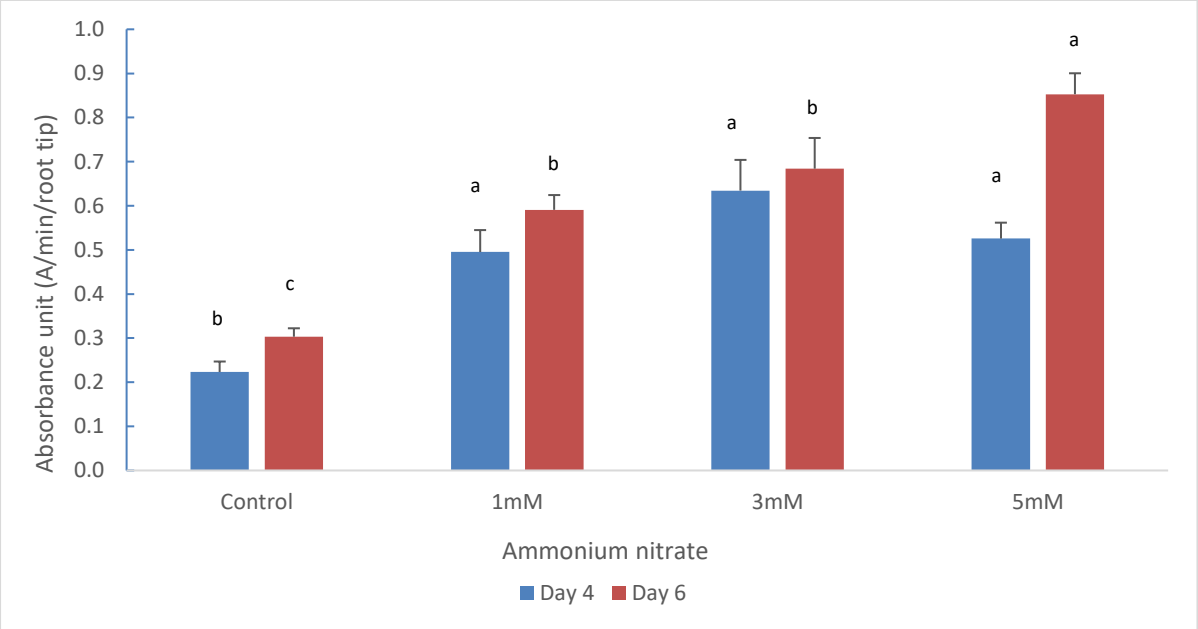

Figure 6: Average peroxidase activity (A/min/root tip) of seedlings treated with water (control) and 1, 3, 5 mM ammonium sulphate after day 4 and 6 incubation. Means having the same letter are not significantly different by Tukey test (P<0.05).

In this study, lipid peroxidation was assayed as a biochemical marker of oxidative stress that might be triggered in the Italian ryegrass roots that were sensitive to NH_4 toxicity. Significant absorbance value (A/gram) differences were observed when comparing control groups with all NH_4NO_3 treatment groups (Fig. 7). The highest level of lipid peroxidation was observed in seedling roots treated with 5 mM NH_4NO_3. There were, however, no significant differences between 1 and 3 mM NH_4NO_3 treatment groups. This suggests that higher increased lipid peroxidation can be resulted in the roots of Italian ryegrass seedlings treated with NH_4NO_3.

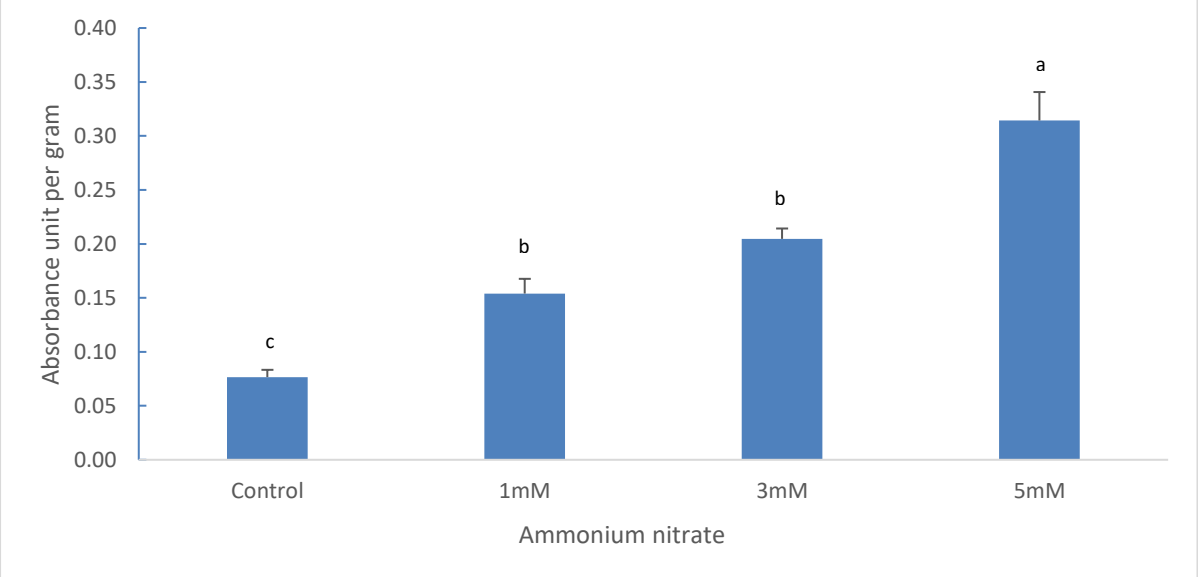

Figure 7: Average final absorbance value for lipid peroxidation assay (A/gram) of seedlings treated with water (control) and 1mM, 3mM, 5mM ammonium nitrate after day 6 incubation. Means having the same letter are not significantly different by Tukey test (P<0.05).

3.5 Effects of seed priming and antioxidant application on seedlings

Seed priming technique was applied to see if there would be some protective effects on the Italian ryegrass seedlings incubated in toxic NH_4NO_3 solutions. It seems that hydroprimed seeds showed highest shoot length compared to control and seeds treated with casein hydrolysate but statistical analysis showed no significant differences in shoot length among all groups (Fig. 8). As for the root length, seed priming did not protect seedlings against the presence of NH_4^+. The root length was significantly higher in the control ($p<0.05$), but there was no differences among the seed priming treatments.

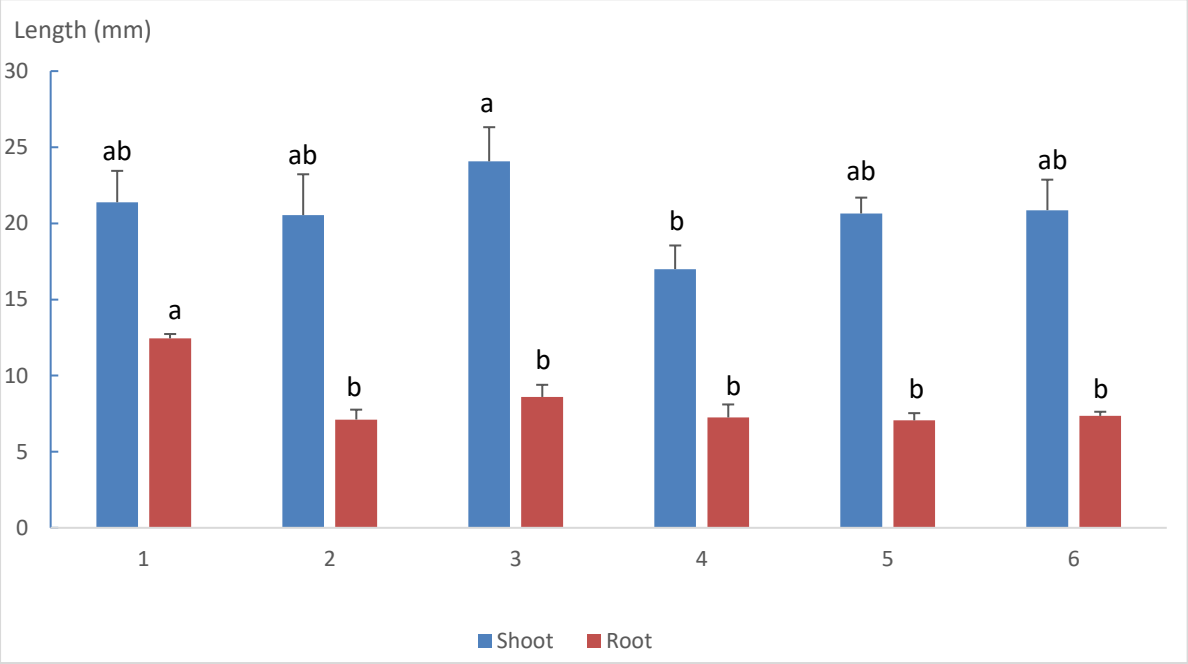

Figure 8: Average shoot and root length (mm) of seedlings with seed priming treatment. Means having the same letter are not significantly different by Tukey test ($P<0.05$) [1:Unprimed-water; 2:Unprimed-NH_4NO_3; 3:Hydroprimed- NH_4NO_3; 4: 100 mg/L casein hydrolysate- NH_4NO_3; 5: 300 mg/L casein hydrolysate- NH_4NO_3; 6: 500 mg/L casein hydrolysate- NH_4NO_3].

Antioxidants were applied to seeds as radical chain-breaking substances. In the application of both 10 mM and 20 mM of L-ascorbic acid, the seeds did not germinate. In the 10 mM KI treatment (Table 4), seeds germinated under 1 mM $(NH_4)_2SO_4$ + 10 mM KI and there were significant differences in shoot length compared to seeds germinated in only 1 mM $(NH_4)_2SO_4$ (p=0.035). However, there were no significant differences compared to the control group. As for the root length, 1 mM $(NH_4)_2SO_4$ + 10 mM KI group showed significant differences when compared to the control group and also 1 mM $(NH_4)_2SO_4$ group.

Table 4: Effects of 10mM antioxidant on length and germination rate of seedlings. Means having the same letter are not significantly different by Tukey test (P<0.05).

Treatment	Shoot length (mm)	Root length (mm)	Germination rate (%)
Control	9.76±0.98^{ab}	12.09±1.18^{a}	85±1.58^{b}
1 mM Ammonium sulphate	8.13±0.24^{b}	6.93±0.69^{c}	44±1.87^{d}
10 mM Potassium Iodide	8.68±0.88^{b}	6.13±0.41^{c}	72±2.55^{c}
10 mM L-ascorbic acid	0^{c}	0^{d}	0^{e}
1 mM Ammonium sulphate + 10 mM potassium Iodide	11.53±1.2^{a}	9.08±1.32^{b}	90±1.58^{a}
1 mM Ammonium sulphate + 10 mM L-ascorbic acid	0^{c}	0^{d}	0^{e}
Coefficient variance	24.946	24.77	7.289
Pr > F	<0.0001	<0.0001	<0.0001

Almost similar results were observed in the 20 mM antioxidant treatments (Table 5), seeds germinated under 1 mM $(NH_4)_2SO_4$ + 20 mM KI showed significant shoot differences when compared to seeds germinated in only 1 mM $(NH_4)_2SO_4$, but no significant root length differences. This suggests that KI could potentially assist seedlings against the presence of NH_4^+ in terms of shoot growth. Seeds germinated in KI along seems to worsen the growth (Tables 4 & 5) and *L. multiflorum* might be sensitive to high concentrations of L-ascorbic acid.

Table 5: Effects of 20mM antioxidant on length and germination rate of seedlings. Means having the same letter are not significantly different by Tukey test (P<0.05).

Treatment	Shoot length (mm)	Root length (mm)	Germination rate (%)
Control	9.38± 0.88^a	10.33± 0.97^a	83±2^a
1 mM Ammonium sulphate	6.28± 0.82^b	4.90± 0.28^b	46±2.92^c
20 mM Potassium iodide	4.39± 1.27^b	5.13± 0.62^b	39±2.92^d
20 mM L-ascorbic acid	0^c	0^c	0^e
1 mM Ammonium sulphate + 20 mM Potassium Iodide	9.92± 0.82^a	6.13± 0.45^b	73±3.39^b
1 mM Ammonium sulphate + 20 mM L-ascorbic acid	0^c	0^c	0^e

Discussion

Based on the findings in this study, $(NH_4)_2SO_4$ and NH_4NO_3 was found to have severe inhibitory effects on *L. multiflorum* root growth compared to KNO_3 even at low concentrations. It was reported that plants in the presence of NH_4^+ greater than 0.1 mmol/L showed NH_4^+ toxicity-like symptoms in general (Schenk and Wehrmann, 1979; Peckol and Rivers, 1995; van Katwijk et al., 1997). NH_4^+ toxicity symptoms can include lower root to shoot ratios (Wang and Below, 1996; Bauer and Berntson, 2001) and decrease in fine to coarse root ratio (Haynes and Goh, 1978; Boxman et al., 1991). Plants fed with NH_4^+ nutrients absorb higher ratio of cations to anions even though the presence of NH_4^+ generally cause the reduction of inorganic cations uptake (Kirkby, 1968; Clark, 1982; van Beusichem et al., 1988). To compensate for the charge imbalance, it was suggested that plants may undergo proton efflux and this normally acidify the external surroundings (Schubert and Yan, 1997). Further studies could be conducted to examine the surrounding pH of the seedlings after day 4 to determine whether low pH surrounding might be a factor for short root growth.

Although no significant differences in both shoot and root length (except 25mM KNO_3) were found in the KNO_3 treatment compared to control group, the presence of K^+ in KNO_3 might benefit the seedlings. Potassium ions have quite important roles in plant growth which can include enzyme activation, stomatal activity, photosynthesis, transportation of sugar and nutrients, protein and starch synthesis (Prajapati, 2012). Presence of K^+ ions can help to stabilize pH suitable for a majority of enzyme reactions (pH 7 to 8) by neutralizing many different organic anions and various compounds in the plant (Prajapati, 2012). It was also reported that a lack of K^+ in plants will hinder nitrate reductase activity, an enzyme that is important in the synthesis of proteins (Patil, 2011). It was found that a low concentration of NH_4NO_3, or 1mM, was able to affect the root growth of *L. multiflorum* L.. This suggests that *L. multiflorum* L. might be one of the NH_4^+ sensitive plants. Several domesticated plants had been reported to be sensitive to NH_4^+ toxicity, for example, potatoes (Cao and Tibbits, 1998), citrus species (Dou et al., 1999), barley (Lewis et al. 1986), strawberry (Claussen and Lenz 1999) and many others.

It was found that seeds germinated in 1, 3 and 5 mM NH_4NO_3 showed significantly shorter time for staining to appear in both superoxide and H_2O_2 staining assays. Moreover, seedlings showed higher peroxidase activity and lipid peroxidation in the biochemical assays. Taken together these findings suggest that Italian ryegrass seeds treated with NH_4NO_3 have higher levels of reactive oxygen species (ROS) like superoxide anion radical (O_2^-) as well as H_2O_2 in the roots. ROS could damage several cellular components including nuclei acids, membrane lipids and proteins (Sharma et al., 2012). To

counteract the damage by ROS, plant cells are able to increase the activities of metaloenymes such as peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.16), superoxide dismutase (SOD; EC 1.15.1.1) and other non-enzymatic antioxidant substances (Halliwell and Gutteridge, 1987). It was reported that POD is responsible in decomposing H_2O_2 through co-substrate oxidation (Mittler, 2002). It can be suggested that seedlings are continuously producing peroxidase to counteract the high concentration of H_2O_2 in cells. As mentioned above, seeds treated with NH_4NO_3 had a higher concentration of ROS. It was reported that normal cellular functions can be affected through elevated lipid peroxidation taking place in both cellular and organellar membranes when the ROS level is beyond the suitable level for plant cells (Sharma et al., 2012). Plants growing under environmental stresses can lead to a lipid peroxidation elevation (Han et al., 2009; Tanou et al., 2009). One of several final products of unsaturated fatty acids peroxidation in phospholipids is malondialdehyde (MDA) which is responsible for damaging the membrane of cells (Halliwell and Gutteridge, 1985). It was also reported that DNA damage can be caused by ROS (Imlay and Linn, 1988). Malfunctions or deactivation of the encoded proteins can be resulted from changes in encoded proteins through oxidative damage on DNA by ROS (Sharma et al., 2012). This could possibility explain as why seeds treated with NH_4NO_3 had shorter roots due to prevention of root cells elongation.

Based on the results of seed priming and antioxidant treatments, casein hydrolysate used as our seed priming solution did not improve the growth of the seedlings in the NH_4NO_3 treatment. Even though it was reported that seed priming may assist plants in reducing lipid peroxidation and elevating antioxidant activities (Afzal et al., 2008; Jafar et al., 2011). This does not necessarily mean that we are dismissing the possibility of seed priming as only one seed priming agent was used in this experiment. There were studies that reported using calcium chloride ($CaCl_2$) as their priming agent and aerating deionised water for hydropriming (Farooq et al., 2017; Tabassum et al., 2017). One study also reported using polyamines (PAs) as seed priming agent (Paul and Roychoudhury, 2017). It was reported that ascorbate (vitamin C) was able to react with ROS like H_2O_2, $O_2^{\cdot-}$, OH^- and lipid peroxides (Smirnoff, 1996; Noctor and Foyer, 1998). It was also reported that tocopherol (vitamin E) may be able to assist plants by preventing lipid peroxidation through ending of free radical reactions (Burton et al., 1983). A lower concentration of ascorbate than 10 mM used in the present study and other phenolic compounds as antioxidants such astannins, hydroxycinnamate esters and lignin could be investigated further in future studies on the effects of antioxidant defence system to counteract NH_4 toxicity in ryegrass seedlings (Blokhina et al., 2003).

Conclusion

The growth of *L. multiflorum* L. seemed to be sensitive to low concentrations of NH_4^+. NH_4^+ toxicity in ryegrass seedlings seems to be linked to oxidative stress. Protection of seedlings against NH_4^+ toxicity based on antioxidative defence manipulation may be worthy of further investigations.

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