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Temporal Monitoring and Assessment of Inorganic Nitrogen Content of Soil due to Nitrogen Fertilizers and their Related Cytotoxic Effects

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ABSTRACT

Background: Indian economy is largely based on agriculture. Major share of agricultural investments goes into chemical fertilizers. Nitrogen (N) fertilizers are used in fields to enhance the crop yield. Most of the reports are based on growth related data, morphological and yield related data but very few reports reveal the facts about genotoxic and cytotoxic effects of these fertilizers. Therefore, the present communication is an attempt in the aforesaid direction.

Methods: In a pot experiment, mineral N content of soil in the form of ammonium-N (NH_4^+-N) and nitrate-N (NO_3^--N) were analysed at regular interval of 5 days till 30 days after treatment (DAT). On the corresponding days root tip assay was done for cytotoxic analyses and also the temporal changes in NH_4^+-N and NO_3^--N contents were observed.

Result: In the Ammonium nitrate treatments, higher mitotic index (MI%) percentages were obtained. While for the Urea, NH_4^+-N content and MI were found to have a positive correlation. Also, it was found that there is an optimum ratio of NH_4^+-N and NO_3^--N in each treatment at which the MI% was the maximum. The study gives an interesting insight for the possible cytotoxic effects of the N fertilizers.

Key words: Ammonium and nitrate ratio, Cytotoxic, Nitrogen fertilizers.

INTRODUCTION

Soil is a natural pool of nutrients and its fertility is reflected by the quantity of different nutrients and their relative ratios. Most of the soils either do not have optimum nutrient contents or get deprived due to numerous reasons like plant uptake, volatilization, leaching, soil erosion *etc*. Hence, fertilizer supplementation at regular intervals is necessary to provide essential elements and nutrients in a readily available form for the optimum plant growth. Such supplementation works only when the fertilizers are of balanced composition and fertilization is done at right time and in right amount (Bindraban *et al.,* 2015). Among various fertilizers, the global consumption of nitrogen (N) fertilizers is always high and about 70% of soil N for plants is provided by inorganic fertilizers. In the soil, inorganic N exist in two major forms, *viz*. ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N). Therefore, N fertilizers in market are either ammonium based (ammonium sulphate, urea *etc*.), or nitrate based (calcium nitrate, potassium nitrate, *etc*.) while some of these are double salt based like ammonium nitrate which provides both the inorganic forms.

Plants show selective uptake of the different N-sources and consequent varied responses which also may be affected by the interaction between soil physico-chemical properties and the environmental factors. Also, this selective uptake of ions by the plants results into different effects on the soil environment especially rhizosphere. Based on the variation in chemical properties of these two inorganic N, their initial fate in soil is different (Li *et al.,* 2012). As plants uptake NH_4^+, protons (H^+) are released which make the pH of rhizosphere acidic, whereas nitrate uptake shifts the pH towards alkalinity. In contrast to nitrate, plants cannot safely store elevated levels of ammonium in their cells which also has subsequent toxic effects (Esteban *et al*., 2016). Earlier reports certify this fact by suggesting that when NH_4^+ is used as the chief N source, most of plant species develop toxicity symptoms (Kronzucker *et al.,* 2001; Rogato *et al.,* 2010). On the other hand, nitrate toxicity occurs in plants when higher quantities of nitrates are present in soil (Britto and Kronzucker, 2005; Okushima *et al.,* 2011). There are only handfuls of reports that correlate the effect of external ammonium and nitrate contents to the toxic effects at cellular level (Qin *et al.,* 2011; Arora *et al.,* 2014; Verma *et al.,* 2016). But there are plenty of reports revealing that plant species grow well if NH_4^+-N and NO_3^--N are supplied in an appropriate ratio (Kronzucker *et al.,* 1999; Babourina *et al.,* 2007; Bar-Yosef *et al.,* 2009). In view of above cited literature, we have

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selected two chemically different N-fertilizers, *i.e.*, urea (NH_4^+-N source) and ammonium nitrate (51% NO_3^--N and 49% NH_4^+-N) and assessed the temporal changes in inorganic N-contents in soil and their effects at cellular level in plants. Various workers have earlier taken up this aspect, but their analytical approach is different and majorly based on morphological data, growth analysis and yield (Yin *et al.,* 2017; Fashaho *et al*., 2020; Kumari *et al*., 2021). To the best of our knowledge, there are very few reports suggesting the genotoxic and cytotoxic effects of fertilizers (Khaldi *et al.,* 2012; Verma and Srivastava, 2017). Hence, considering it an issue of serious concern, the present study is in continuation with our previous works on N- fertilizers (Arora *et al.,* 2014; Verma *et al.,* 2016; Verma and Srivastava, 2018a).

Allium cepa root tip bioassay is used to determine the effects of two N-fertilizers at cellular level and any evident cytotoxic risks associated with it. Earlier, this assay system was used for environmental and toxicological monitoring (Fiskesjo, 1985; Verma and Srivastava, 2018a), but in the present times it has also found application as bioindicator of genotoxicity (Firbas and Amon, 2013). This system bears the merits of providing a rapid screening for the causative pollutants, toxicants, contaminants *etc*.

MATERIALS AND METHODS

The experiment was conducted in the year 2016-2017 at In Vitro Culture and Plant Genetics Unit, Department of Botany, Lucknow University, Lucknow.

Soil sampling

Collection of soil for the experiment was done from University garden situated at 26°51′53″ N latitude and 80°56′15″ E longitude at a depth of 30 cm from an area that was not fertilized. One kg sieved soil was air-dried and placed in 6″ pots. The physico-chemical properties of the soil used are given in Table 1.

Application of fertilizers

Soil in pots was treated with selected N-fertilizers, urea and ammonium nitrate. Three treatments as control (T1) which was without any additive, soil supplemented with urea at the rate of 200 mg N kg^{-1} soil (T2) and soil supplemented with ammonium nitrate at the rate of 200 mg N kg^{-1} soil (T3) were made. Three replicates, consisting of 3 pots each were taken per treatment *i.e.* 9 pots per treatment.

Estimation of inorganic N nutrients

Temporal monitoring of inorganic N (Ammonium-N (NH_4^+-N) and nitrate-N (NO_3^--N) contents) was done on 1, 5, 10, 15, 20, 25 and 30 days after treatment (DAT). The ratio between NH_4^+-N and NO_3^--N content was calculated as NH_4^+-N per unit NO_3^--N.

Table 1: Physico-chemical properties of the soil.

Soil properties	Values
pH	7.54
Organic matter	2.40%
NH_4 +-N	18.00 mg kg^{1} soil
NO_3-N	2.34 mg kg^{1} soil

Estimation of NH_4^+-N

Nessler's method was used to analyse NH_4^+-N in soil samples (Peech *et al.,* 1947). Soil suspension was prepared by using 100 g of soil sample, taken in a conical flask and then 200 ml of acidified NaCl solution was added to it. The suspension was vigorously shaken for about 30 min. then filtered. The leachate obtained was taken and volume was made up to 100 ml using acidified NaCl. Further NH_4^+-N was determined using Nessler's method. The transmission (%) of the solution was read in a spectrophotometer at 410 nm.

Estimation of NO_3^--N

Phenoldisulphonic method (Bremner, 1965) was adopted to analyse the NO_3^--N in soil samples. For soil suspension, 50 g of soil was taken and 250 ml of extraction solution (1 part of $CuSO_4$ and 5 parts of Ag_2SO_4) was added to it. The soil suspension was shaken for 10 min and then 0.4 g $Ca(OH)_2$ was added. Further shaking of 5 min was done and then 1 g of $MgCO_3$ was added. Suspension was then filtered. Ten ml of clear filterate was taken and evaporated to dryness in evaporating dish. Three ml of phenol disulfonic acid was added and it was left for 10 min. Then 15 ml of cold water was added to it. Sodium hydroxide (12%) was added slowly to the solution until it became distinctly alkaline which was indicated by the development of yellow color. Finally, 3 ml more of sodium hydroxide solution was added to it. The transmission (%) of the solution was read in a spectrophotometer at 420 nm.

Cytotoxicity analysis

Healthy onion bulbs of medium size were properly cleaned. Their outer scales and brownish bottom plates were removed with a precaution of not damaging the ring of root primordia. The experiment was designed to run in three replicates with each replicate consisting of three pots and each pot had one onion bulb placed in the centre. The same soil was used for sowing fresh onion bulbs at 1, 5, 10, 15, 20, 25 and 30 DAT. Roots were harvested on day 3 of each sowing.

For cytological analysis, roots were fixed in Carnoy's fluid (1 part glacial acetic acid + 3 part ethanol) for 24 hrs and then preserved in 70% ethanol. Fixation and slides preparations were done by following modified protocol of Sharma and Sharma (1980). The mitotic analysis was done on temporary slides. The dividing cells were observed under microscope. Approximately 500 cells from at least 5 random root meristems per replicate were screened to determine the mitotic index percentage (MI%).

$$MI\ (\%) = \frac{\text{Total number of dividing cells}}{\text{Total number of cells observed}} \times 100$$

Statistical analysis

The data obtained was evaluated statistically with the statistical software SPSS for Windows ver. 15.0, (SPSS Inc., Chicago, Ill., USA). The data was analysed by One-way

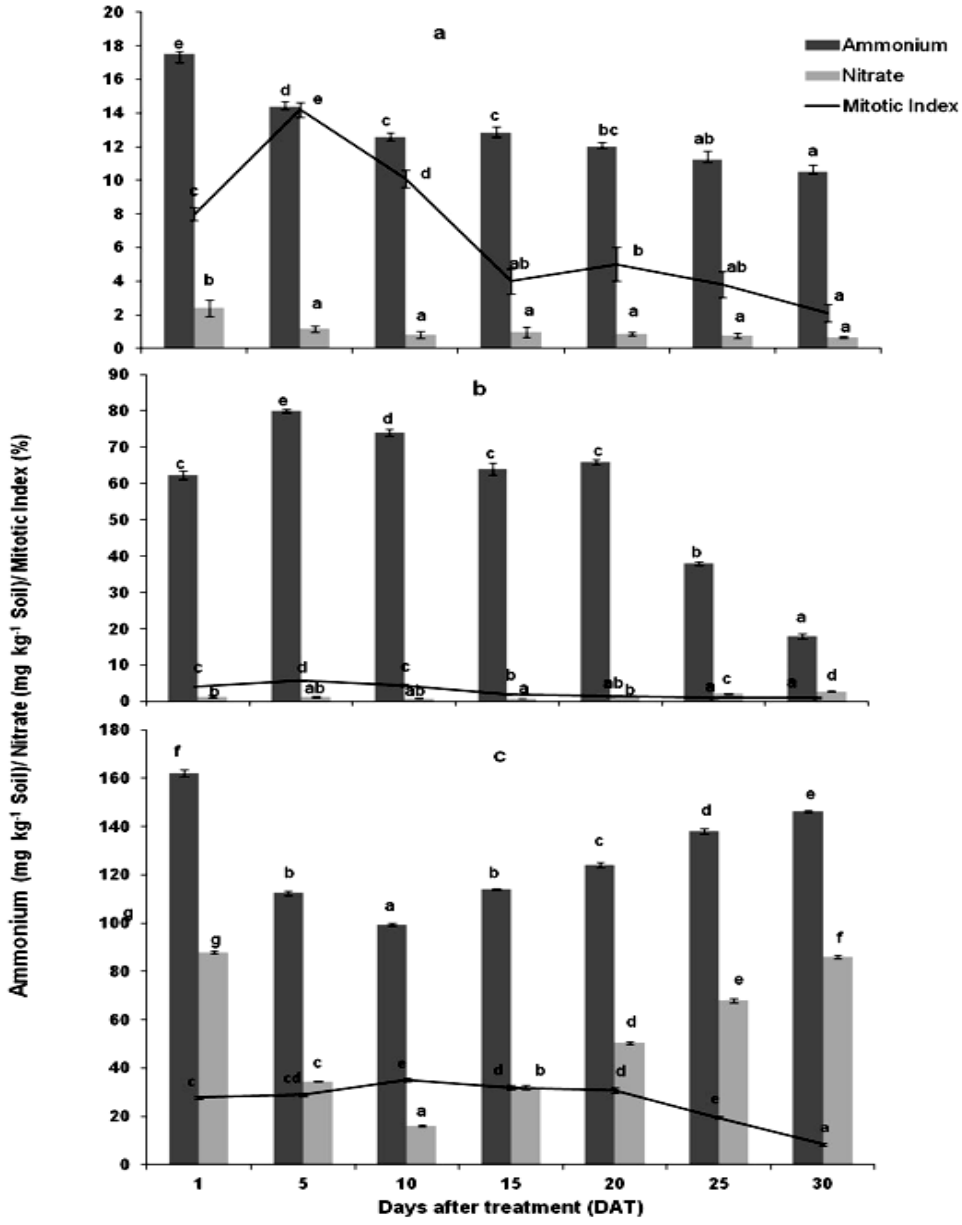
analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) and significance was measured at $p \leq 0.05$. The correlation amongst the parameters was assessed by Pearson Correlation Coefficient analysis (Zou *et al.,* 2003).

RESULTS AND DISCUSSION

The data was collected every 5 DAT till one month. Control (T1) had very low amount of NH_4^+-N and NO_3^--N as compared to other treatments (Fig 1a). Evaluation of the two treatments, T2 and T3, revealed that the overall inorganic N (NH_4^+-N and NO_3^--N) content range was higher in T3 treatment. Temporal variations in NH_4^+-N and NO_3^--N contents in individual treatments, were significantly different.

Temporal monitoring of inorganic N content

In case of T1, soil NH_4^+-N content followed a continuous decrease since 1 DAT (Fig 1a). Treatment T2 showed an increase in soil NH_4^+-N till 5 DAT and reached up to 80 mg kg^{-1}, then between 10 DAT to 30 DAT a decline from 74 mg kg^{-1} to 18 mg kg^{-1} was observed in the content (Fig 1b). In T3, periodic fluctuations were observed in the amount of NH_4^+-N *i.e.*, no continuum in the trend was observed. There



Data are means ± SE of three replicates. Bars with different letters are signiûcantly different at $p \leq 0.05$ as determined by Duncan's multiple range test.

Fig 1: Variations in mitotic indices in (a) Control (T1), (b) Urea (T2) and (c) Ammonium nitrate (T3) with NH_4^+-N and NO_3^--N contents at regular intervals of time.

was a decrease from 162.2 mg kg^{-1} on 1 DAT to 99.5 mg kg^{-1} as on 10 DAT then again, increases till 30 DAT (Fig 1c). Regarding NO_3^--N content, T1 showed a continuous decrease till 30 DAT. Urea (T2) showed a contrary result to NH_4^+-N, from 1 DAT to 15 DAT, there was a drop and then elevation in NO_3^--N content. This increase in NO_3^--N could be due to nitrification process. In case of T3, NO_3^--N content of the soil also followed similar trend as that of NH_4^+-N, that is, it was observed to be 88 mg kg^{-1} initially on 1 DAT and then a decline was obtained till 10 DAT. There was a continuous rise of nitrate amount from 15 DAT to 30 DAT. A correlation analysis showed that, NH_4^+-N and NO_3^--N contents in various treatments were almost ideally correlated with each other, that is, in T1 and T3 an ideal positive correlation with r = 0.95 and 0.98 respectively, at $p \leq 0.05$, 0.01 and 0.001 was obtained where as in T2 a negative correlation with r = -0.92 at $p \leq 0.05$ and 0.01 was obtained (Fig 2a-c).

Relation of MI with inorganic N content

Cytological analysis of root meristems revealed that in T1 and T2, there was an increase in MI from 1 DAT to 5 DAT which then decreased thereafter till 30 DAT (Fig 1a and b). In case of T3, from 1 DAT to 10 DAT, MI increased and then decreased till 30 DAT (Fig 1c). Maximum MI of 35% on 10 DAT was observed in T3, whereas in T2 treated roots maximum MI was 5.8% on 5 DAT which was even lower than T1. In case of T2, changes in NH_4^+-N content in soil was significantly correlated with MI, r = 0.76 at $p \leq 0.05$, where as in T3, NO_3^--N showed a significant negative correlation with MI, r = -0.74 at $p \leq 0.05$ and NH_4^+-N did not show any significant relation with MI.

Relation of MI with NH_4^+-N and NO_3^--N ratio

Data for ratio of NH_4^+-N to one unit of NO_3^--N was analysed in each treatment and varied trends were obtained, *viz.* in T1 ratio increased till 10 DAT then decreased till 20 DAT and again increased till 30 DAT. In T3, it initially increased till 10 DAT then kept on decreasing till 30 DAT. In urea (T2), initially the ratio increased till 15 DAT and then reduced up till 30 DAT. In the treatments, ratio at which MI% was at its peak, considered to be the optimum ratio for that particular treatment. In T1 on 5 DAT, 14.2% MI at ratio 12.52, in T2 on 5 DAT, 5.8% MI at ratio 64.5 whereas in T3, highest of all MI values that is 35% at ratio 6.22 was obtained on 10 DAT (Fig 3). These can be considered as optimum ratios because beyond these ratios lower values of MI were observed in all the treatments. No significant correlation between ratio and MI was observed in any of the treatments.

Urea undergoes hydrolysis by the action of urease and releases NH_4^+ and carbon dioxide, thereby providing NH_4^+ as sole N source for plant, whereas ammonium nitrate being the salt of strong acid undergoes ionization and releases NO_3^- and NH_4^+ ions. Final fate of NH_4^+ (from either source) is to undergo nitrification and get converted into nitrates. This fact is in consonance with our results wherein T2, temporal changes in NH_4^+-N and NO_3^--N contents are negatively correlated but in T3 a positive correlation was obtained. Both the ions after immediate ionization are available for plant in case of double salt fertilizer ammonium nitrate. This is a known fact that plants can absorb and utilize N as NH_4^+ and NO_3^- present in soil solution (Oh *et al.,* 2008), which then within plant undergoes various processes of assimilation, transformation and mobilization (Oh *et al.,* 2008). In present analysis, in case of T2 NH_4^+-N levels are significantly correlated with MI showing NH_4^+-N is the sole N-source utilized by plants initially. Among the treatments, T2 seems to cytotoxic. Initially till 5 DAT, MI is increasing but 10 DAT MI showed a decline due to higher NH_4^+-N accumulation. Also, previous studies by the same group,

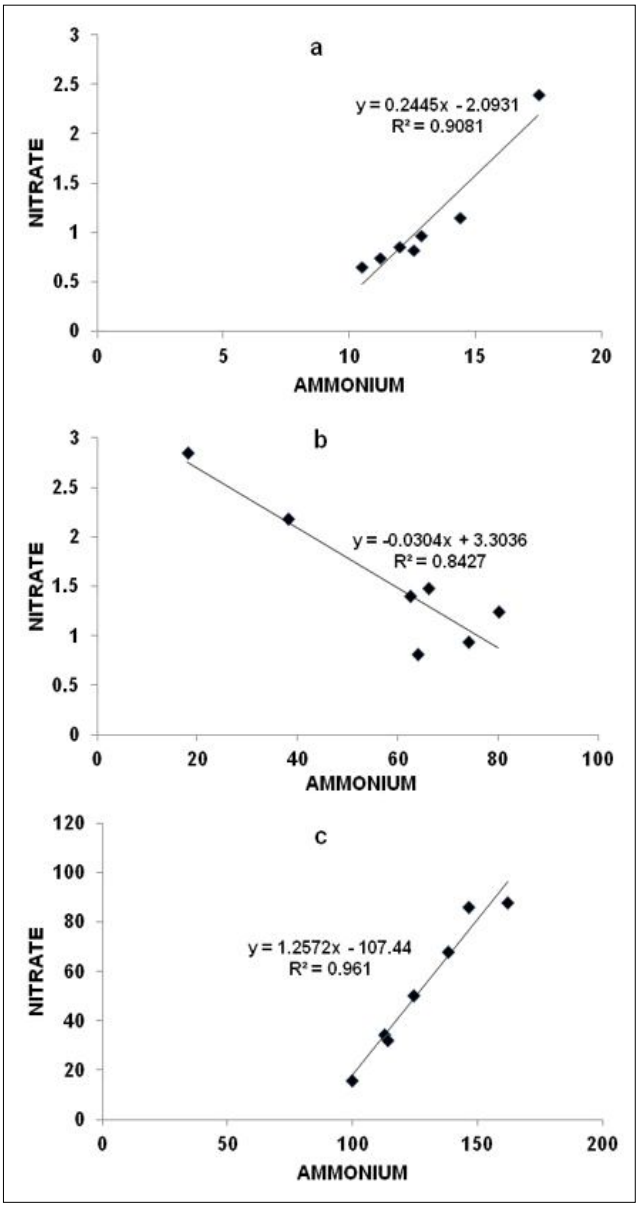
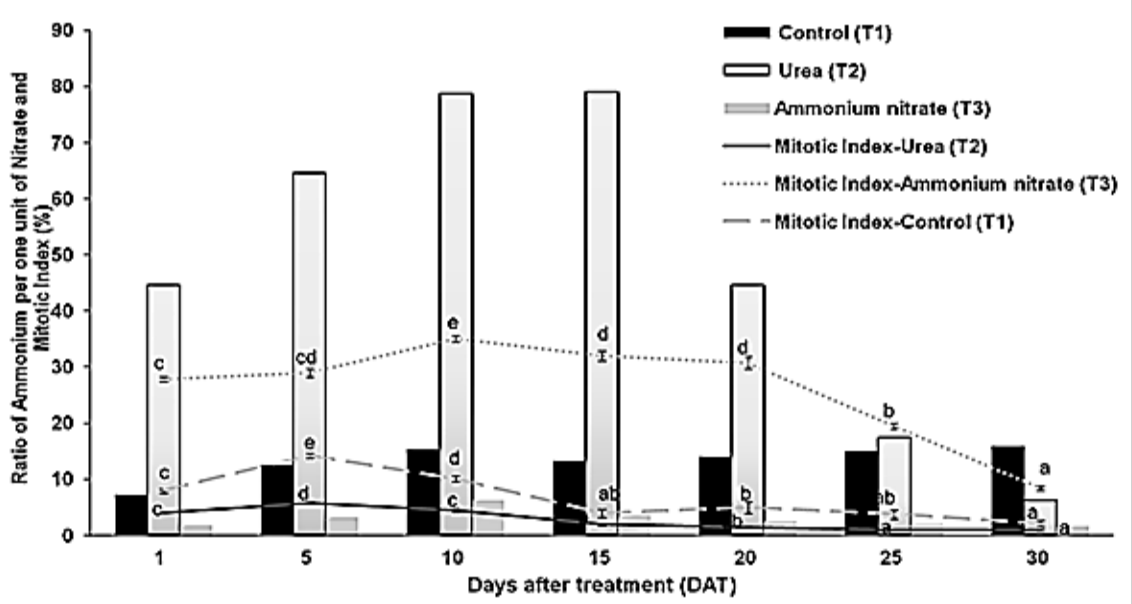


Fig 2: Relationship between NH_4^+-N and NO_3^--N contents from 1 DAT to 30 DAT in (a) Control (T1), (b) Urea (T2) and (c) Ammonium nitrate (T3).

on the fertilizer has revealed that it is not only mitodepressive but also promotes mitotic anomalies especially interphase anomalies (Verma *et al.,* 2016). The reasons may be toxicity of NH_4^+-N and its accompanied effects. Mitotic index is the measure of cell division and growth and is a potential parameter for cytotoxicity (Fiskesjo, 1985; Bianchi *et al.,* 2016), further it has also been regarded as a cytotoxicity biomarker (Verma and Srivastava, 2018b). In view of cited literature low MI in present case reflect the cytotoxic effects in plant. As NH_4^+-N is taken up by plants it releases protons in soil reducing the pH of soil which causes NH_3^+ to bind with protons and form NH_4^+-N ions, this build-up of NH_4^+-N concentration in soil hampers the uptake of other ions and induces salinity stress (Cabrera, 2001). In a recent report, it has been shown that higher levels of ammonium in rice plants, induces reactive oxygen species (ROS) mediated reactions (Yang *et al*., 2020). The excessive generation of ROS in plants is due to environmental stress like drought, salinity (Mittler, 2002) in response to ammonium toxicity. Further the results can be supported by the previous reports of Babourina *et al.* (2007), Bittsánszky *et al*. (2015), suggesting the NH_4^+-N toxicity in plants when NH_4^+-N is the sole N source. Their accumulation in soil causes acidification of soil and when they accumulate in plant cells acidification of cytosol occurs. Various morphotoxic and cytotoxic effects of NH_4^+-N in plants is well known in literature as shown by Liu *et al.* (2013) and Arora *et al.* (2014). Toxicity of NH_4^+-N caused inhibition of primary root growth by inhibiting cell elongation and division and even led to root cell death as reported by Qin *et al.* (2011). It also causes disturbed phytohormone and polyamine levels (Britto and Kronzucker, 2013).

Mitotic index seems to be higher in case of T3 as both the forms of nitrogen, can be utilized by the plants. Though in case of T3, plants get both forms of N but the peak of MI was obtained when the ratio of the two ions was optimum *i.e.*, at 10 DAT. It is possible that a lower or higher ratio between the two ions may not be effective for the root growth; therefore low values of MI were obtained. When mixed N nutrition is supplied to the plants, protons are generated during NH_4^+-N assimilation which can be utilized for NO_3^--N reduction hence regulate the intercellular pH (Li *et al*., 2012). Rhizospheric pH got neutralized when both the forms of N were supplied as reported by Hinsinger *et al*. (2003). Nitrogen as NH_4^+ can lead to release of protons which can decrease the rhizospheric pH. At low pH there is more ammonium formation as reaction proceeds in forward direction. This may lead to accumulation of ammonium in soil because of limited uptake by plant. Whereas N as NO_3^- can take up protons from the rhizosphere, which causes an increase in the rhizospheric pH. Being opposite charge ion, nitrate protects the root cells from NH_4^+ induced depolarization of plasma membrane by charge balancing (Wang *et al*., 1993). It causes decrease in internal ratio of cations and anions in plants (Britto and Kronzucker, 2002). Kronzucker *et al*., (1999) concluded in their study on NO_3^--NH_4^+ synergism in rice that presence of NO_3^- enhanced the NH_4^+ fluxes, NH_4^+ metabolism and cytosolic NH_4^+ accumulation. Whereas presence of NH_4^+ repressed the NO_3^- fluxes, accumulation and metabolism to the great extent.



Data for mitotic index are means ± SE of three replicates and bars with different letters are signiûcantly different at $p \leq 0.05$ as determined by Duncan's multiple range test.

Fig 3: Graph depicting relationship between NH_4^+-N and NO_3^--N ratio (NH_4^+-N per unit NO_3^--N) with mitotic index (%) in different treatments.

They also showed that net N-acquisition and N-translocation was enhanced when both the inorganic form of N was provided. However, it has been observed that plants show preference for the form of N available (Britto and Kronzucker, 2013) though depends on certain factors like, soil pH, aeration of soil *etc*. (Masclaux-Daubresse *et al*., 2010). Many reports have suggested the role of synergism between NH_4^+ and NO_3^- nutrition (Marschner, 2012). Lastly if we compare all the treatments, drawback/constraint with urea (T2) is that NH_4^+-N levels are very high initially till one week contributing to growth to certain limit beyond which it causes toxic effects as seen in present and previous studies. The reason is that here nitrate is not contributing to alleviate the toxic effects of its counter ion. In ammonium nitrate, the advantage is availability of both the ions for selective uptake by plants as well as counter balance of each other's ill effects and synergistic enhancement of growth.

CONCLUSION

Present study gives an idea about the cytotoxic effects of two fertilizers, urea and ammonium nitrate. Urea fertilizer provides single source of nitrogen *i.e*., NH_4^+-N which shows cytotoxic effects, whereas double salt fertilizer ammonium nitrate yields both ions on ionization, which nullify each other's negative effects. Such studies are warranted in the field of agriculture management, where input of fertilizers is meant to increase the yield.

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