Discussion

NPs due to their unique physical and chemical properties are being used in the agricultural sciences. A large number of studies have been conducted to explore mechanism by which NPs influence plant growth and development. Application of biosynthesized NPs in agricultural field leads us for sustainable development.

Synthesis and Characterization

Flower extract of *Thymus serpyllum* L. has been used as reducing and surface stabilizing agents for the synthesis of ZnO and CuO NPs. Biological synthesis of NPs provides an improved platform as compared with other synthesis methods. In this method, ZnO and CuO formed due to the reaction between Zn and Cu ions from their respective precursor i.e. zinc acetate dihydrate and copper acetate monohydrate and reducing agents i.e. flower extract. The presence of biomolecules as characterized by FTIR spectrum reveals that phytochemicals viz. flavonoids, polysaccharide, carboxylic acid, etc. are present on the surface of ZnO and CuO NPs. Biomolecules might act as stabilizing or growth terminating agent during the synthesis of NPs as well as they might act as a linker molecule between two or more NPs in making self assembly (Singh *et al*., 2016). SEM image of NPs shows the presence of some large particles, which can be credited to aggregation or overlapping of smaller particles while TEM image shows a well dispersed and spherical shape of NPs with a 20–50 nm of size range. The size of NPs can show variation to some extent with various characterization methods as they measure the different diameter of the same particle (Singh *et al*., 2016).
Germination and seedling growth

Seed germination is an initial, most significant and sensitive phase in the growth cycle of plants as it governs the plant establishment and crop yield. The comparative impact of green and chemically synthesized ZnO NPs on the seed germination is well generalized in the present study. The seed germination was found to be affected by both green and chemically synthesized ZnO NPs. The seeds treated with lower concentration of both NPs revealed maximum germination but were suppressed at higher concentration. The level of germination and seedling growth of tomato seedlings were higher in green synthesized ZnO NPs as compared to the chemically synthesized NPs. Conventional chemical reduction method involves a variety of toxic chemicals for the synthesis of NPs which can later be a reason for various issues due to their toxicity, while green synthesis method is an eco-friendly approach to produce NPs. A similar study revealed that the seeds treated with low concentrations showed 100% germination on the third day of germination whereas the seeds with higher concentrations showed 60-80% germination for both the green synthesized and chemically synthesized Ag NPs treated seeds (Thuesombat et al., 2014). The length of radicle and plumule growth by lower concentration of ZnO NPs was found to be high in the green synthesized NPs than chemically synthesized NPs but the result was quite insignificant. Increase in germination can be attributed with the tendency of NPs to penetrate plant seed coat and enhanced seed germination and growth (Khodakovskaya et al., 2012). Previous study has shown that effect of ZnO NPs is species specific and also dependant on size of the NPs (Singh et al., 2016).

Majority of major crop plant species belongs to glycophyte category. They are vulnerable to salt stress, the most serious environmental stress that effect crop productivity worldwide (Hillel, 2000). In this concern nanotechnology proves to be promising solution; hence the present study has evaluated the positive aspects of NPs in mitigating salt stress. It is well established that NaCl treatment affects
germination and seedling growth in tomato seedlings. Salinity widely influences imbibitions process of seeds due to lower osmotic potential of germination media (Katembe et al., 1998). It poses toxicity in progression of germination which alters the enzymatic activities involved in nucleic acid metabolism (Gomes-Filho, 2008). Zn is an essential element for growth and development of plant (Pathak et al., 2012) as it helps building of natural auxin (IAA) and subsequently activating cell division (Ali and Mahmoud, 2013). The seed-priming with ZnO NPs positively affects the growth traits in NaCl-stressed seed.

**Growth parameters**

The lower concentration of green synthesized ZnO NPs caused increase in shoot and root length and fresh and dry weight but on the other hand at the similar concentration of chemically synthesized NPs the increase in shoot length, root length, fresh weight and dry weight was quite insignificant. At the highest concentration of chemically synthesized NPs the reduction in shoot length, fresh and dry weight was reported while the toxic effect of green synthesized NPs at same concentration was lesser as compared to the chemically synthesized NPs. Zn plays a vital role in synthesis of natural auxin (IAA), activation of cell division and cell enlargement (Ali and Mahmoud, 2013) and improvement in protein synthesis (Ebrahimian and Bybordi, 2011). Further, growth parameter of tomato plants growing under salinity treated with different concentration of ZnO NPs showed mitigation of salt stress on plant growth. Application of various concentrations of ZnO NPs increased growth and diminished the harmful effects of salt stress. Our finding is compatible with the results on onion (Laware and Raskar, 2014), green pea (Pisum sativum) (Mukerjee et al., 2014), cotton (Gossypium hirsutum L.) (Rezaei and Abbasi, 2014) and moringa (Moringa peregrina) (Soliman et al., 2015). The most common impact of salt pressure on plant physiology is a depression in the growth which is necessary for the survival
of a plant exposed to this pressure. In this study, salinity stress caused depression in plant growth by lessening growth attributes due to water deficit which leads to abnormal changes in plant morphology (Jiang et al., 2014), osmotic stress, nutritional disorders, and physiological and biochemical imbalance (Soliman et al., 2015; Ahmad et al., 2016).

The reduction rate of root length and fresh weight biomass of plant is higher in root exposure medium than foliar exposure medium as compared with root exposure treatment. It is possible that higher concentration of CuO NPs given by root exposure treatment could have reached toxic level in root that reduced the root length. Our results were in agreement with earlier work done by Lidon and Henriques (1998) in which they reported that Cu at 0.25 and 1.25 mg/L caused a reduction in rice shoot length. Foliar application of CuO NPs may cause toxicity in most plant which induced stunned shoot growth and chlorosis. The reduction in shoot growth at higher doses of NPs may be credited to toxic level of NPs (Bahadar et al., 2016). The Cu NPs may release cupric ions during sonication process and aqueous Cu species dissolved from Cu NPs can be toxic to plants at high concentrations (Singh et al., 2017). Cu NPs also caused a reduction in the seedling length of Phaseolus radiatus and Triticum aestivum by 65 and 75%, respectively (Lee et al., 2008). Similarly, CuO NPs reduced root length in Cucurbita pepo at 1000 mg L$^{-1}$ concentration (Stampoulis et al., 2009).

Accumulation

The increase of over 25 folds in the Cu content was observed in roots at the highest concentration of CuO NPs by root exposure medium. However, increase was only 2 folds in roots treated with foliar spray method as compared to their controls. The greater accumulation of Cu NPs in the root might be due to its greater surface area as it bears numerous thin walled roots, therefore, more NPs can penetrate and accumulate in the cells. The AAS analyses revealed that
accumulation of NPs in the roots greatly increased when compared to their shoots. It depicted that traces of NPs were transported from the roots to the shoots; thus, it led to major toxic effect in the roots. The higher metal content in roots than shoots shows an exclusion mechanism to withstand toxicity; such plants accumulate metals in roots and check their transport to shoots (Wang et al., 2004). Due to the complex structure of shoot only traces of Cu content were absorbed by the foliar application.

**Pigment content, sugar content and nitrate reductase activity**

Photosynthesis is the basic function determining the productivity of green plants. Pigment content along with sugar and protein amount was measured as an indicator of health and growth of the plant. Amount of total Chl and carotenoids registered maximum value at lower concentration of green synthesized ZnO NPs. These results are consistent with earlier reports. Raliya et al. (2013) reported that relative Chl content increased significantly by both TiO$_2$ and ZnO NPs. They also found that foliar application of NPs enhanced the absorption of PAR in tomato plant leaves. This signified that the NPs could help raising the rate of photosynthesis. Zn has potential to increase the biosynthesis of Chl and carotenoids and can improve the photosynthetic machinery of the plant (Aravind and Prasad, 2004). Chlorophyll content was not affected or even decreased by NPs treatment in some of the studies. Thus, the original mechanism of the NPs impact over photosynthetic machinery is still obscure (Raliya et al., 2013).

High concentration of salt adversely affected the pigment content. The decrease in Chl amount under salt stress is a commonly stated phenomenon. The Chl concentration was used as a sensitive indicator of the cellular metabolic state (Saha et al., 2010). The destruction of chloroplast structures and inactivation of biosynthetic machinery of Chl lead to reduction in pigment content under saline environment (Rockenfeller and Madeo 2008). The detrimental effect of salinity on
pigment content can be alleviated by application of suitable concentration of ZnO NPs. It is reported that at salt concentration of 9000 ppm there was a reduction of 48% in chl content of *Moringa* leaves. Salt stress on *Vigna radiata* decreased the levels of total Chl (31%), Chl *a* (22%), Chl *b* (45%), carotenoid (14%) and xanthophylls (19%), a typical 16% loss of the intensity of Chl fluorescence was also accompanied with decreased pigment content (Saha *et al.*, 2010). The decrease in pigment contents under salt stress might be due to the production of high level of ROS and the activities of antioxidant enzymes were not stimulated to the optimum level and the damage caused by free radicals was not counteracted and more pigment damage was caused. The enhancement of pigment contents was due to stimulation in efficiency of photosynthetic machinery. Zinc only at specific concentrations are required for structural and catalytic components of proteins and enzymes as cofactors which are essential for normal growth and development of plants (Srivastava *et al.*, 2014). Seed-priming with ZnO NPs positively affects the growth traits in NaCl-stressed plant as Zn plays a vital role in improvement in protein synthesis (Ebrahimian and Bybordi, 2011) and translocation of nutrients from the aged cells to newborn cells (Rockenfeller and Madeo, 2008; Jiang *et al.*, 2014). Zn probably keeps on Chl synthesis through the protection of the sulffydryl group, a function primarily associated with Zn (Cakmak, 2000; Weisany *et al.*, 2011). Moreover, it shares in Chl synthesis (Li *et al.*, 2006; Weisany *et al.*, 2011).

Data clearly depict that foliar exposure have more negative impact on the reduction of Chl content. Our findings are in agreement with the previous study where Chl content of *Lemna minor* decreased with the increase in concentration of CuO NPs, 200 mg/L (Song *et al.*, 2016). However, low Cu concentrations proved to stimulate photosynthesis, with its rate increased to 130–140% of the control level (*p* = 0.05). A significant inhibitory effect was observed only at concentrations of 1 and 5 mg Cu/L (*U* = 0.025, *p* = 0.05). This can be attributed
directly to oxidative stress (Burzynski et al., 2004) or to inactivation of ribulose biphosphate (RuBP) carboxylase, a key enzyme in photosynthetic CO$_2$ fixation.

Stimulation in efficiency of photosynthetic machinery might be the strong reason for enhancement of pigment, protein and sugar contents. Sugar content improved significantly at low concentration in both chemically and green synthesized NPs. Salt stress resulted in significant decrease in soluble sugar, however, sugar content was positively affected in combined treatments with ZnO NPs and NaCl. Zn also plays a main role in sugar formation and enzyme structure involved in the biosynthesis of amino acids (Soliman et al., 2015). These findings are in agreement with Soliman et al., (2015). The accumulation of organic solutes and total phenols due to priming with ZnO NPs might boost salt tolerance of cells through osmotic adjustment, which consequently improved plant growth. Same pattern was depicted in the treatment with lower concentration of CuO NPs i.e. soluble sugar decreased in the plants linearly with the increased concentration of CuO NPs.

Nitrate is one of the major N sources for higher plants and promotes vegetative development and yield. NR, an enzyme is found mostly in higher plants and appears to be a key regulator of nitrate assimilation as a result of enzyme induction by nitrate. NR activity declined significantly at the highest concentrations and increased at lower concentration of chemically and green synthesised ZnO NPs in tomato. Higher concentration of NaCl caused suppression in NR activity. Though, ZnO NPs treatment under salt stress significantly increased the NR activity. Singh et al. (2013) reported that NR activity was modified in plants response to ZnO NPs. The NPs favoured the absorption and assimilation of nitrate. NR activity increased under higher concentrations of ZnO NPs. Seedlings treated with 4.5 $\mu$M ZnO NPs shows maximum NR activity with about 81.29% and 65.23% enhancement over those treated with bulk ZnO and control respectively. The NR activity decreased upon exposure to the highest
concentration of CuO NPs. NR is the only substrate induced enzyme. The decrease in activity of NR may be due to low rate of absorption of NO$_3^-$ under the influence of CuO NPs.

**Electrolyte leakage, hydrogen peroxide and lipid peroxidation**

A major indicator of plant stress is modification in cellular membrane which either leads to improper functioning or complete dysfunctioning of biological membranes. This membrane dysfunctioning is expressed as increased permeability and efflux of ions which can be measured by the leakage of electrolyte (Hasanuzzaman *et al.*, 2011). Oxidative stress biomarkers viz. EL, H$_2$O$_2$ and LP cause oxidative damage to plant macromolecules and cell structures, and relatively long-lived H$_2$O$_2$ acts as a central player in stress signal transduction pathways (Hossain *et al.*, 2015). MDA is the decomposition product of polyunsaturated fatty acids of biomembranes, and its significant accumulation results stimulation under stress and also is an indirect reflection of the extent of cell damage (Wang *et al.*, 2011). It was observed that exposure of both green and chemically synthesized ZnO NPs at higher concentration showed a significant rise in EL, H$_2$O$_2$ and MDA content. The elevation was more in the case of chemically synthesized NPs than green synthesized NPs as compared to the control. Further, under salt stress H$_2$O$_2$, EL and MDA contents increased significantly. The present study showed that there was high accumulation in MDA content under saline conditions, suggesting that salt stress could destroy the integrity of the cellular membrane, as well as cellular compounds, like proteins and lipids. ZnO NPs application reduced MDA content, thus ameliorating the injury normally induced by salinity stress. This is consistent with the results of Burman *et al.* (2013) who reported that Zn NPs induced defensive impacts on biomembranes versus alternations of membrane permeability and oxidative stress in chickpea seedlings. Application of ZnO NPs considerably lowered salt induced increase in H$_2$O$_2$, EL
and MDA. Seed-priming with ZnO NPs positively affects the growth traits in NaCl-stressed plant as Zn plays a vital role in maintenance of the structural integrity of biomembranes (Weisany et al., 2012), phospholipids accumulation (Jiang et al., 2014), scavenging free oxygen radicals (Jiang et al., 2014), and decreasing the uptake of excess of Na\(^+\) and Cl\(^-\) (Weisany et al., 2012). Our results coincide with the previous study of Bettger and O’Dell (1981) who suggested that Zn is necessary for stabilization and maintenance of biomembrane against various stresses.

On application of CuO NPs by foliar and root exposure method, there is significant rise in EL, H\(_2\)O\(_2\) and MDA content with the increasing concentration of CuO NPs. The increase was more prominent in foliar spray method. Increased level of MDA and EL may be explained by the noxious action of Cu on proteins with SH bonds which bind this metal or during the Fenton reaction, i.e., production of hydroxyl radicals catalyzed by the metal (Esra et al., 2012). An increased rate of LP has also been reported from plants and other biological models under CuO NPs stress (Wang et al., 2012). The plants might have produced more free radicals under the NPs stress and the activities of antioxidants were not stimulated to the optimum level. Thus the damage caused by free radicals was not counteracted which lead to the production of more MDA content and EL which trigger the LP or damage of membrane in the seedlings. The MDA content of *Lemna minor* also increased with the increase of CuO NPs in culture media (Song et al., 2016). The MDA content of *Lemna minor* cultured in media was relatively higher as compared with that of the bulk CuO. The decrease in MDA content and EL at optimum concentration is in agreement with the previous reports on ROS scavenging effect of NPs (Hussain et al., 2017).

**Antioxidant enzymes activities**

The activities of antioxidant enzymes are stimulated by oxidative stress (Devi and Prasad, 2005). The production of active oxygen species is a biochemical
change that possibly occurs when plants are subjected to harmful stress conditions. The chloroplasts and mitochondria of plant cells are important intracellular generators of ROS. SOD functions at the early stages of oxidative reactions, changing the O$_2^-$ into H$_2$O$_2$, thus maintaining intracellular O$_2^-$ level (Sharma et al., 2012). The increased expressions of enzymatic antioxidant, SOD with the increasing concentration of ZnO NPs and CuO NPs, portray a protective process against the development of oxidative stress in the plants. SOD and APX are the key enzymes involved in metabolizing ROS (Caverzan et al., 2012). CAT and POX catalyze the conversion of H$_2$O$_2$ to H$_2$O. CAT activity was found maximum in tomato seedlings treated with chemically synthesized ZnO NPs. This can be clarified by enzyme inactivation specifically, due to the replacement of Fe$^{2+}$ ion in its active center with Cu$^{2+}$ ion (Hou et al., 2007). Contrary to our results Hong et al. (2016) reported a sharp decrease in the CAT and APX activities in cucumber treated with 200 mg L$^{-1}$ of CuO NPs when compared with the control. The antioxidant enzymes were activated in response to higher concentrations of both green and chemically synthesized NPs. The activities of SOD and CAT increased significantly in case of chemically synthesized NPs, and decreased in response to the lowest concentrations of both NPs. The salt increases SOD and CAT activity significantly while plants treated with ZnO NPs decreased SOD and CAT enzymatic activities. Activity of SOD increased in low concentration of ZnO NPs which showed that plants have ability to scavenge free radicals. Variation in activity of SOD is not surprising as in some studies SOD activity increased under excess Zn (Rao and Sresty, 2000) while in some other studies it is reported to be decreased (Panda and Khan, 2004).

Plants can cope up with the toxic effect of ROS by inducing the defence system up to certain level which maybe energy consuming process. The production of ROS may be suppressed on exposure to lower concentration of NPs which induced cellular defence system against exogenous oxidative stress. It has
been reported in many studies that exposure to salt stress could induce ROS formation causing increased activity of antioxidative enzymes as a defense system (Weisany et al., 2012; Soliman et al., 2015; Ahmad et al., 2016). This is harmonious with our results that showed that growing lupine in saline conditions led to a marked increase in the antioxidant enzymes. The effect of different concentrations of CuO NPs through different exposure media on antioxidant enzyme activities in cauliflower plants shows an increase in the SOD and CAT activities on foliar and root exposure. Several studies have shown that higher amount of Cu increases the expression of APX, SOD, and other enzymatic antioxidants in plants (Yoshimura et al., 2000). SOD performs a pivotal function in combating oxidative stress in plants, and a marked increase in SOD activity has been demonstrated to occur upon exposure to oxidative stress (Jalali et al., 2011). CAT often shows the same trend as that of SOD when an organism is under stress. A study showed that SOD increases and CAT decreases when an organism is exposed to stress (Cui and Zhao, 2011). However, some reports show that SOD decreases and CAT increases in organisms exposed to stress (Li et al., 2001; Sai et al., 2010). The activities of antioxidant defence enzymes are unstable and change with culture time. It is difficult to demonstrate if the plant can protect itself from environmental stress by using several antioxidant enzymes at a certain time. However, the activities of antioxidant defence enzymes often increase when the organism is under stress.

**In vivo detection of reactive oxygen species and hydrogen peroxide in leaves**

The dark blue spots by NBT staining on leaves of seedlings exposed to different concentrations of ZnO NPs shows that $O_2^-$ production is comparatively more in the leaves treated with the highest concentration of chemically synthesized NPs as compared to green synthesized NPs and control. The leaves
stained with DAB show a gradual increase in the formation of dark brown coloration in leaves of plants indicating the accumulation of \( \text{H}_2\text{O}_2 \) in leaves and it follows the same trend as \( \text{O}_2^- \). Chemically synthesized ZnO NPs pose toxic chemicals in its synthesis process resulting in the higher generation of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \). It is observed that the petiole exhibits intense staining which is associated with \( \text{O}_2^- \) accumulation resulting from NADPH oxidase activity, a key effector of the oxidative burst in the wound response (Mehdy et al., 1996). The intensity of stain in petiole region is more prominent in the veinal tissue, from where the leaves were detached from the plant. The strength of stain reduces gradually on moving away from the junction point. This may be associated with the systemic wounding signal that stimulates the production of \( \text{O}_2^- \) that circulated along the veins from the wound. On the basis of the strength of NBT stain in leaves of both plants, it seems that ROS production is comparatively more in tomato than cauliflower. Further, DAB reacts with \( \text{H}_2\text{O}_2 \) in presence of POX led to the production of brown polymerization product. \( \text{H}_2\text{O}_2 \) was detected significantly more in the tip region of the leaf. Some interesting and novel insights into the spatial organization of oxidant biochemistry occurring in response to metal stress can be suggested by the accumulation of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) in intact leaves. Visualization of \( \text{H}_2\text{O}_2 \) in vascular tissue of wounded leaves has been stated and it is assumed to be associated with systemic octadecanoid pathway (Orozco-Cárdenas and Ryan, 1999).