Indian Journal of Plant Sciences ISSN: 2319-3824 (Online)
An Online International Journal Available at http://www.cibtech.org/jps.htm
2013 Vol. 2 (4) October-December, pp.54-62/Himakshi et al.
Research Article

THE SYNTHESIS AND ROLE OF BIOGENIC NANOPARTICLES IN OVERCOMING CHILLING STRESS

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ABSTRACT
Significant crop loss is observed when plant is subjected to environmental stress. One of the major problems faced in late sown varieties of wheat is low temperature stress during the months of Nov.-Dec. Because of low temperature, in these varieties more input of seeds is required and very less yield is obtained due to the poor seedling growth and its establishment in the field. The prominent reason behind this situation is the imbalance between the production of activated oxygen species and the quenching activity of antioxidants which often results in oxidative damage. Many metabolic processes produce active oxygen species. Four oxygen species [superoxide radical O^{2-}, hydrogen peroxide H_2O_2, hydroxyl radical OH and singlet oxygen species and the hydroxyl radical] are found to be most active, toxic and destructive in nature. Excess of ROS triggers phytotoxic reactions such as lipid peroxidation, protein degradation and DNA mutation. Since higher plants are immobile they cannot escape environmental stresses. Freezing induced plasma membrane disruption and considerable accumulation of different by-products like malondialdehyde and proline. Nanopriming with biogenic nanoparticles is demonstrated as one of the methods for increasing seedling vigour and improvement of germination percentage and seedling growth. Presently, biogenic nanoparticles are used as priming agents for the first time to endorse these parameters under chilling.

***Key Words*:** *Antioxidant, Catalase, Enzymes, Nanoparticles, Peroxidase, Seed Priming*

Abbreviations
$AgNO_3$ - Silver nitrate
BioNP - Biogenic nanoparticles derived from leaves of *Tridax procumbens*
TNP – Tridax nanoparticles

INTRODUCTION
Abiotic stresses adversely affect growth, productivity and trigger a series of morphological, physiological, biochemical and molecular changes in plants. Chilling temperature often affects plant growth and crop productivity, leading to significant crop losses. Generally plants from temperate regions are regarded as chilling tolerant with variable degree, and can attain cold tolerance due to biochemical and physiological changes. Some of these changes include alterations in biomembrane lipid composition, and small molecules accumulation, etc. However, plants of tropical and subtropical origins are sensitive to chilling stress and are deficient in the mechanism (s) of cold acclimation.
Conventional breeding methods have met with limited success due to complexity of stress tolerance traits, in improving the cold tolerance of important crop plants. It is imperative, therefore, to look for alternative strategies to raise cold stress tolerant crops.
In general chilling intensifies the processes concerning the production of reactive oxygen species (ROS) leading to oxidative stress and causing deleterious effects. The antioxidative system (s) within the plant include enzymatic components such as superoxide dismutatse, catalase, glutathione reducatase, ascorbate peroxidase, peroxidase and non-enzymatic molecules such as ascorbic acid, cysteine, glutathione, α-tocopherol, carotenoids, polyamines, glycinebetaine, proline, etc. The balance between the free radicals generation and free radical defense determines the survival of the system.
One of the approaches is to increase the concentrations of free radical scavengers adding them exogenously and hence causing detoxification of stress-induced free radicals. Several antioxidants added

exogenously have yielded favorable results under different stress conditions. Further, organic compounds such as amino acids (e.g. proline), quaternary and other amines (e.g. glycinebetaine and polyamines) and a variety of sugars and sugar alcohols (e.g. mannitol, trehalose and galactinol) are reported to protect the system.

In the present study biogenic nanoparticles synthesized from *Tridax procumbens* (Family: Asteraceae) are used as a priming (nanopriming) agent to boost the anti-oxidative mechanism, and investigating their effect on seed germination and seedling growth parameters.

The present is the first report where nanopriming is demonstrated to show positive response in triggering the anti-oxidative mechanism in germinating seeds.

MATERIALS AND METHODS

a). Collection of Plant Material

To perform the experiments, branches of *Tridax procumbens* L (Family: Asteraceae) were collected from the JNU, Jaipur campus, during January and February 2011, thoroughly washed with tap water, followed by distilled water and used for the preparation of extracts.

b). Preparation of the Extract

25 g of fresh leaves were weighed, thoroughly washed in distilled water and air dried. They were crushed in 100 ml triple deionized water, boiled for 15 min and then filtered through Whatman No.1 filter paper. The extract was stored at 4º C for further experiments (Parasher, 2009).

c). Nanoparticle Synthesis

The aqueous solutions of different mM concentrations of silver nitrate ($AgNO_3$) (1, 5, 10, 15 and 20) were prepared. 10 ml of each extract was added into 90 ml of aqueous solution of $AgNO_3$ individually and incubated for 1 to 5 hr at room temperature. Here the filtrate acted as a reducing and stabilizing agent for $AgNO_3$.

d). Characterization of BioNPs

Following methods were used to characterize the synthesized BioNPs

UV-Vis Spectra Analysis

The reduction of pure Ag^+ ions was monitored by measuring the UV-Vis spectrum of the reaction mixture after each hour of incubation (from 1-5 hours) at room temperature. For this small aliquot of the sample was diluted into 2 ml of triple deionized water. UV-Vis spectral analysis was done by using Gensys 10Uv spectrophotometer and the absorbance was recorded at 300- 600 nm.

SEM and XRD Analysis

The BioNPs were analyzed by SEM and XRD which revealed the size of the nanoparticles. For SEM analysis EVO-50 INCA Penta FETx 3 was used. After ultra-sonication of the sample, a small drop was taken on the stubs with carbon tape on it and extra solution was removed using a blotting paper. The sample was allowed to air dry and then subjected to analysis.

XRD analysis was done with ISO-DEBYEFLEX 2002 model consisting of Cu Kα radiation in a θ- 2 θ configuration. The crystallite domain size was calculated from the width of the XRD peaks, assuming that they were free from non-uniform strains, using the Scherrer formula.

$D = 0.94 \lambda / \beta \cos \theta$

where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the diffraction angle.

FT-IR Analysis

FT-IR measurement of sample was performed with BRUKER-VERTEX-70 Model. FT-IR spectrophotometer in a diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets, in order to find out the capping agents responsible for stabilization of synthesized BioNPs.

e). To Analyze the Role of Nanopriming in Mitigating Chilling

Seeds of late sown variety of wheat (Raj 3777) was obtained from SK Rajasthan Agricultural University, Durgapura, Jaipur (Rajasthan), India.

Seed Treatment and Growth Conditions

Wheat grains were washed with distilled water and sterilized with 5% sodium hypochlorite (NaClO) for 5 min. to avoid fungal infection and were pre-soaked in different concentrations of BioNPs (30 and 40 ppm). Thereafter, these were dried and brought to the original weight and then allowed to germinate in Petri dishes lined with blotting paper. The untreated seeds (non-primed seeds, NPS) were used as a control. 20 seeds (primed and non-primed), having uniform size were selected and sown in each Petri dish in BOD incubator set at 10°C. Petri dishes were irrigated periodically and seeds were left to germinate. The data were computed and photographed regularly up to 10 days. On 10th day uniform seedlings were selected to analyze different biochemical parameters. Each treatment was replicated thrice and data represent the average value.

Seed Germination and Seedling Growth Parameters Studied

Seven germination traits studied included percentage germination (%), speed of germination (SOG) (Maguire, 1962), coefficient of germination (COG %) (Copeland, 1976), germination rate (GR), mean of germination time (MGT) (Ellis and Roberts, 1981), germination index (GI) (Scott *et al.*, 1984) and Timson's Index (TI) (Khan and Ungar, 1984). Studied seedling parameters included root length (RL) (cm), shoot length (SL) (cm), seedling growth (SG), fresh and dry matter (g) and vigour index (VI) (Abdul-Baki and Anderson, 1973).

Enzymatic Anti-oxidative Defense Systems

The following anti-oxidative enzymes were assayed to study the effect of nanopriming on LS variety of wheat: Superoxide dismutase (SOD) (Dhindsa *et al.*, 1981), ascorbate peroxidase (APX) (Nakano and Asada, 1981), catalase (CAT) (Aebi, 1984), pyrogallol peroxidase (PPX) and polyphenol oxidase (PPO) (Kar and Mishra, 1976).

RESULTS AND DISCUSSION

Results

Biogenic Nanoparticles

Synthesis and Characterization

After observing the change in colour of the reaction mixture from green to dark brown, while incubated at room temperature, indicated the formation of nanoparticles, the pellet was collected from the reaction mixture and screened using U-V visible spectrophotometer (Shaligram *et al.*, 2009). The peaks were obtained in UV-vis spectra because the free electron of the metal nanoparticles gave surface plasmon resonance (SPR). UV-visible spectra analysis of the BioNPs indicated that the formation of BioNPs began within an hour of incubation, giving the resultant peak at 430 nm during the 5th hr (Figure 1). The broadening of the peaks revealed that the synthesized nanoparticles were polydispersed. Figure 2 shows SEM image obtianed for the synthesize nanoparticles. XRD analysis revealed that the size of synthesized BioNPs ranged from 8.27 – 18.75 nm (average 13.51 nm). Table 1 shows the results of FT-IR analysis.

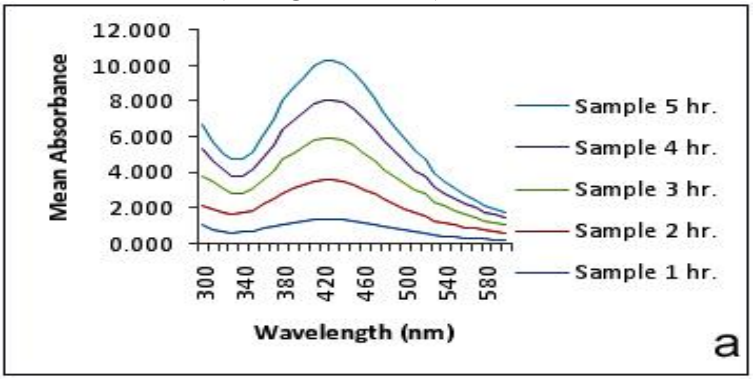

Figure 1: Shows the peak values of the synthesized TNP using $AgNO_3$ (5 mM)

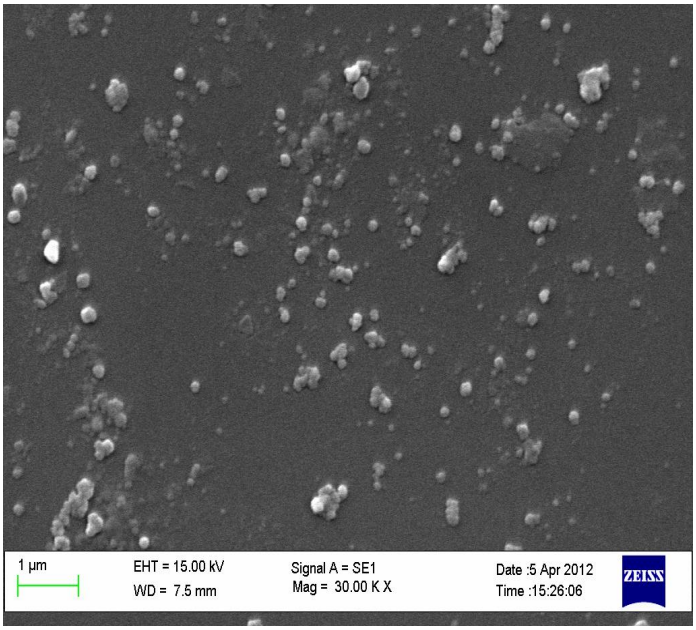

Figure 2: signifies the SEM images of TNP

Table 1: Showing FT-IR analysis of the leaf BioNPs in *T. procumbens*

Organ used (leaf) Corresponding Wave number (cm^{-1})	Type of Peak	Resultant Group
3400-3500	Broad	-OH stretching of alcohols and phenols
2900	Medium	Aldehyde stretching of alkanes and primary amines
1640-1550	Medium	N-H bends of primary and secondary amides
1400-1500	Medium	Primary amines, alkanes
1300-1400	Medium	Ketones (aromatic), aldehydes and carboxylic acid
1242- 1343.00	Medium	C-N stretching vibrations of amines or C-O stretching of alcohols, ethers, carboxylic acid, esters and anhydrides
110.40-1343	Strong	Highly symmetrical alcohols, phenols, Lactones
800-850	Medium	Aliphatic amines, alkanes

Applications

Seed Germination and Seedling Growth

The data concerning different stages of seed germination parameters in late sown wheat variety, primed with nanoparticles are shown in figure 3. Non-primed seeds were used as control. It was observed that

imbibitions began on the 2nd day, with radicle protrusion on the 3rd day. Subsequently complete seed germination was established on 6th-7th day (with plumule and radicle). Of the two concentrations of the BioNPs, with 40 ppm imbibitions was achieved on the same day of incubation, which was two days prior to NPS. In fact radicle protrusion started on the 1st day itself and complete seedling was obtained on the 4th day, with profound growth of plumule and radicle occurring on 6th -7th day. The whole analysis revealed that BioNPs showed highly significant positive results compared to other concentration (30 ppm) and NPS.

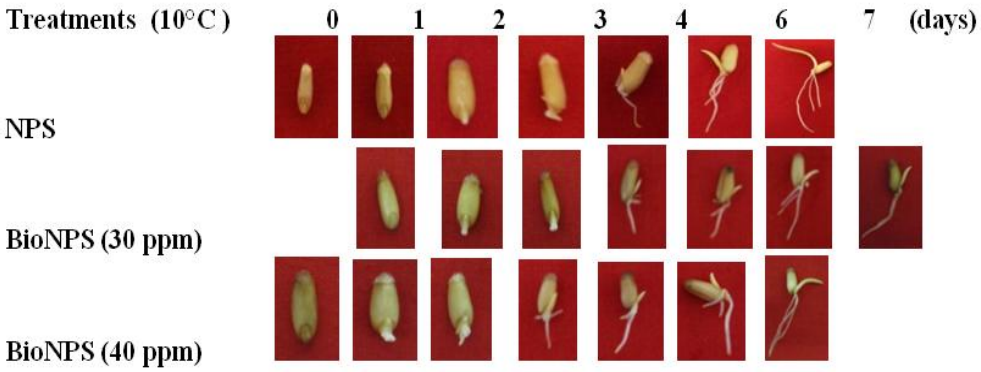

Figure 3: Stages of seed germination of nanoprimed seeds

Table 2 displays data regarding effect of BioNPs on different seed germination parameters

Temp. (°C)	Treatment	GP (%)	SOG	COG (%)	GR	MGT	GI	TI
10	**NPS**	90 ± 2	1.78±0.02	18.6 ± 0.27	1.07± 0.01	0.94 ± 0.02	4.3 ± 1.2	30.5 ± 2.8
	PS (ppm)							
	BioNP (30)	90 ± 2	2.05± 0.04	17.27 ±0.42	1.4 ± 0.05	0.71± 0.01	13.9± 2.2	43.69 ± 1.5
	BioNP (40)	**100**	**2.31** ± 0.02	**17.31**± 0.3	**1.6**± 0.02	**0.63** ± 0.01	**15.6**± 1.9	**49.29** ± 2.0

(GP-Germination percentage; SOG-Speed of germination; COG-Coeffecient of germination; GR-Growth rate; MGT-Mean germination time; GI-Germination index and TI-Timson's index)

Effect of BioNP s on Seedling Growth Parameters

Seedling growth parameters such as shoot and root length and fresh and dry matter in LS were studied in seeds primed with BioNPs, and compared with non-primed seeds as controls.

Table 3: Shows mean values of various seedling traits after 10 days of germination carried out at 10°C. Effect of BioNPs is also evident

Temp. (°C)	Treatment	SL (cm)	RL (cm)	SGL (cm)	SL/RL	RL/SL	Shoot (g) FM	Shoot (g) DM	Root (g) FM	Root (g) DM
10	**NPS**	2.8±0.11	2.46±0.2	5.26	1.138	0.87	0.252	0.025	0.047	0.02
	PS (ppm)									
	BioNP(30)	2.97±0.2	3.2±0.1	6.17	0.928	1.077	0.268	0.029	0.04	0.029
	BioNP(40)	3.5±0.22	2.87±0.11	6.37	1.21	0.82	0.286	0.029	0.059	0.032

(SL-Seed length; RL-Root length; SGL-Seedling growth length; FM-Fresh matter; DM-Dry matter)

Table 4: Shows mean values of vigor index (VI) and seedling growth (SG) after 10 days of germination carried out at 10°C. Effect of BioNPs is also shown

Temp. (°C)	Treatment	VI	SG (cm)
10	**NPS**	473.4	0.75
	PS (ppm)		
	BioNP (30)	555.3	0.68
	BioNP (40)	637	0.64

Seedling growth parameters of non-hardened and BioNPs (30 and 40 ppm) were analyzed. Tables 3 and 4 shows the trend of increase in seedling growth parameters.

Effect of BioNPs on Enzymatic Defensive Mechanism

The activities of key antioxidant enzymes (SOD, CAT, APX, PPX and PPO) in germinating seeds primed with BioNPs were examined at chilling temperature. At 40 ppm, the activities of most of these enzymes enhanced significantly. In brief, it can be inferred from figure 4 (A-C), the activities of catalase and superoxide dismutase increased markedly following priming.

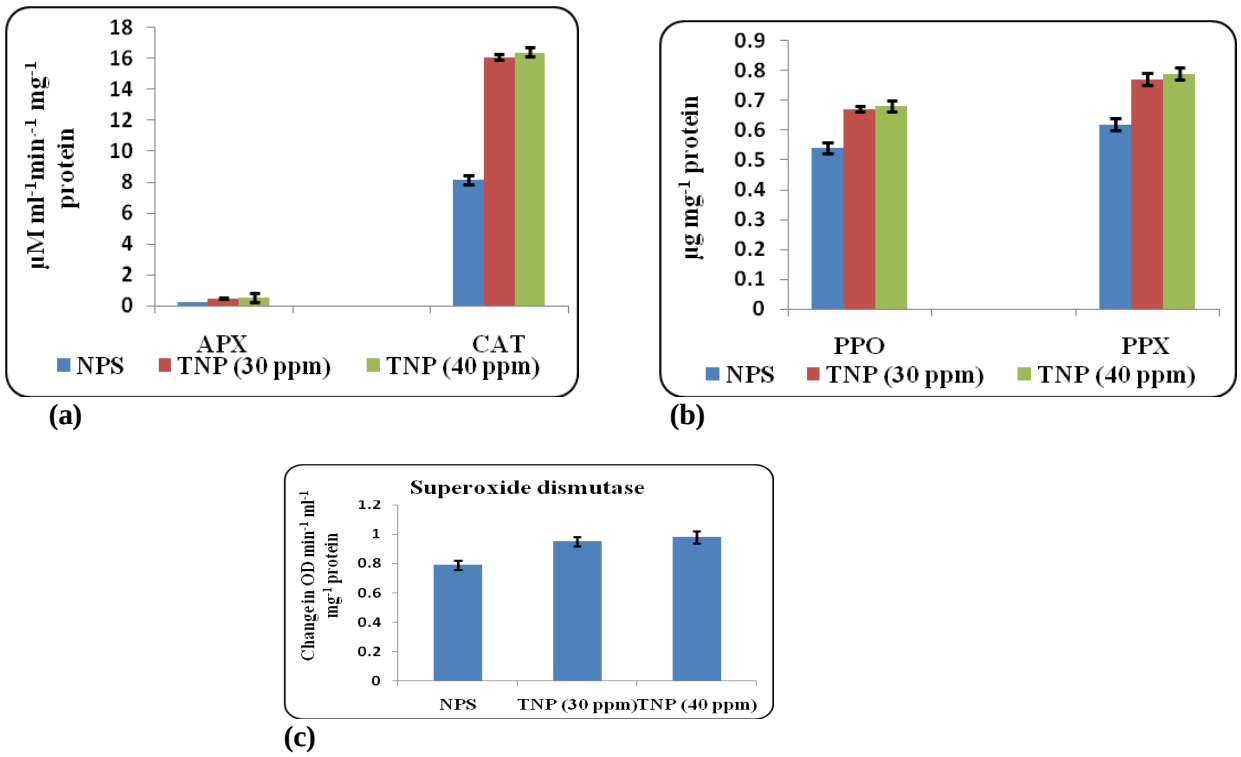

Figure 4 (A-C): Shows pattern of anti-oxidant enzyme activities incubated at chilling temperature. Priming with BioNPs ( ) caused marked increase in the activities of all the enzymes

Discussion

Wheat is an important cereal crop mainly sown in end October, and late varieties sown in December, in generally irrigated lands. Late sown varieties experience chilling stress which disturbs the balance between the free radical generation and free radical defense jeopardizing the survival of the seedlings.

Exogenous application of antioxidants has given favorable results under different environmental stress conditions. Different antioxidants, putrascine, ascorbic acid, glycinebetane, proline, cysteine, phenols are applied to mitigate stresses.

In the present investigation an attempt was made to mark out the causes of chilling stress in relation to antioxidant defense systems in late sown wheat variety, and also exploring the possibility of using biogenic nanoparticles in boosting the antioxidative defense mechanism (s).

The germination phase is of prime importance in the growth cycle of plants as it determines the standard establishment and final yield of the crop. The ambient temperature during periods of water availability is known to be an important cure for seed germination, and the interactive effects of temperature and moisture availability at seed germination substantially contribute to promoting germination during conditions that enhance the survival of the seedling growth (Karimian and Inallou, 2012). Seed priming improves germination or seedling growth in a wide range of agro-climatic conditions and decreases sensitivity to external factors. Briefly, the performance of various crops could be improved by using various growth regulators as well as BioNPs (as shown in the present studies) as priming agents (Ashraf and Foolad, 2005). Hence we initiated the study to evaluate the response of seed and seedlings to chilling at various time intervals using BioNPs as safer alternative.

When $AgNO_3$ was dissolved in triple deionized water gets split into Ag^+ and NO_3^-. The metabolites present in the stem and leaves extract acts as e^- donor and reduce Ag^+ ions into Ag. The nanoparticles formed exhibit yellowish brown color in aqueous solution due to excitation of surface plasmon vibrations (Shankar et al. 2004). On addition of extract in the aqueous solution of the silver ion complex, they start to change the color due to reduction of silver ion; which indicated the formation of biogenic nanoparticles. This occurs due to the surface plasmon vibration of the synthesized nanoparticles (Cappuccino and Sherman, 2006). The synthesis of nanoparticles was characterized by UV-visible spectroscopy. Further, crystalline size and structure of the biogenic nanoparticles was carried out through XRD images revealed characteristic peaks. FT-IR measurements were done to identify the potential functional groups of the biomolecules in the *Tridax procumbens* leaf. Table 1 gives the identified functional groups and some are responsible for the reduction of the silver ions. These functional molecules are associated with biogenic nanoparticles. From the FT-IR spectra it can be made out the prevalence of NH group of amines, secondary alcohols, nitro groups, phenols and aromatic amines. Some of these especially phenolic groups, amides, secondary alcohols may act as reducing agents for the synthesis of biogenic nanoparticles. These techniques have been used in several studies to characterize nanoparticles, and also identify the occurrence of potential functional groups.

In our studies chilling temperature appears to be one of the very important factors controlling seed germination, affecting both the rate and final percentage of germination. It influences the percentage of germination, affecting both the speed of water absorption and the biochemical reactions. To us it appears that chilling during germination seems to be associated with the imbibitions phase, which is considered to be the most sensitive phase. Abbas (2011) has made similar inferences in tomato and cucumber. Presently, emergence percentage decreased under the stressful temperature because of impenetrability of water uptake.

Seed priming is an important method associated with the process of seed germination and is widely used to synchronize the germination of individual seeds under stress conditions. Priming allows some of the metabolic processes necessary for germination to occur without actual germination taking place (Ghobadi *et al.*, 2012).

In general the germination performance of a seed lot is characterized mainly by three parameters; time of onset of germination, speed of germination and extent or capacity of germination (cumulative germination percentage at the end of the testing period).

Thus, germination parameters are useful in estimating the switch over from seeds to seedlings and, thus, signify the suitability of a seed lot for commercial crop establishment. These parameters are also helpful

in determining the type of seed pre-treatment (s) needed to attain a high level of germination (Kolotelo *et al.*, 2001).

Concomitantly, we also examined different germination parameters e.g. percentage germination (GP), speed of germination (SOG), coefficient of germination (COG), germination rate (GR), mean germination time (MGT) and Timson Index (TI) to differentiate the effect of chilling on the selected variety of wheat which were primed with BioNPs in order to overcome chilling and accomplishing rapid speed of germination.

Interestingly, speed of germination became fastest at 10°C with priming which also improved SOG, COG, GR and GI. Furthermore, priming also lessened MGT such that the required time was faster in primed seed compared with normal seeds (see Table 2).

We are tempted to agree with Alaei *et al.*, (2010) who demonstrated that priming of seed with osmotic solution improved germination through metabolic activation involving the synthesis of nucleic acids, proteins and enzymes, and increasing respiratory activity and energy utilization.

We also compared different phases of seed germination for e.g. imbibitions, radicle protrusion, plumule protrusion and then complete germination followed by seedling growth in primed and non primed seeds. Thus, the imbibitions phase occurred after 17 hours of incubation though, seeds primed with BioNPs (30 and 40 ppm) revealed rapid speed germination as priming with BioNPs have profound effects on initial stages of germination and thereby also effects the seedling growth parameters. The novelty of the present study is that the BioNPs appeared to mitigate the deleterious effect of chilling.

Table 5: Shows enzymatic antioxidants in leaves of late sown variety under chilling

Variety	Treatments (ppm)	SOD (Change in O.D. min.$^{-1}$ml^{-1}mg^{-1} protein)	CAT (µMml^{-1}min.$^{-1}$mg^{-1} protein)	APX (µM ml^{-1}min.$^{-1}$mg^{-1}protein)	PPX (µg mg^{-1} protein)	PPO (µg mg^{-1} protein)
LS (Raj 3777)	**NPS**	0.79	8.13	0.24	0.62	0.54
	BioNP (40)	0.98	16.4	0.5	0.79	0.68

Seemingly, low temperature causes oxidative stress probably through indirect mechanism e.g. interaction with antioxidative defence system, lipid peroxidation, etc., so in another set of experiment, the effect of BioNPs on enzymatic antioxidative defence mechanism was also analyzed. It was interesting to observe that the activities of superoxide dismutase, catalase and peroxidase increased significantly in the primed seeds compared with non primed seeds. In order to confirm the role of BioNPs we estimated the activities of various oxidases to assess whether they could be attributed to antioxidant properties.

Enhanced activities of catalase and peroxidase are reported to scavenge H_2O_2 by breaking it down into water and oxygen.

BioNPs significantly enhanced the SOD activity and also other oxidases e.g. catalase, peroxidase. The excessive levels of H_2O_2 are reported to be reduced through the activities of catalase and APX. Present studies demonstrate that the activities of oxidases increased markedly with BioNPs.

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