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EFFECT OF COPPER NANOPARTICLES ON *IN-VITRO* SEED GERMINATION OF WHEAT (*TRITICUM AESTIVUM* L.) VARIETIES

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ABSTRACT: The comparative study of *in-vitro* seed germination was conducted to determine the influence of different concentrations of chemically synthesized copper nanoparticles (CuNPs) on the *in-vitro* seed germination and growth of two different varieties of wheat- PBW677 and PBW725 on MS medium. For all growth parameters taken under the present study, PBW 725 variety of wheat performed better in the basal MS media having different concentration of CuNPs than PBW 677. 100% germination percentage of PBW 725 variety of wheat is achieved on Basal MS medium supplemented with 5 ppm CuNPs. This variety has better seed vigour and tolerance index. It indicates that a lower concentration of CuNPs is potential enhancer of seed germination of this particular variety. But the higher concentration of the nanoparticles proved to be detrimental and resulted into poor seed germination and decreased morphological development of the plants of both varieties.

INTRODUCTION: Wheat is a globally consumed cereal crop, which is a staple food in most part of Asia, Africa, and other parts of the world. Approximately 215 million hectares of land is utilized for wheat cultivation all over the world out of which around 30 million hectares of land is used in India. Wheat is commonly used as a ground, powdery form called wheat flour. It is also used in the preparation of a large variety of food products, including bread, biscuits, cakes, pies, fermented beverages like beer, breakfast cereal, *etc*. The crop belongs to the family of Poaceae and contains great benefits - carbohydrate 70% and protein 3% and inorganic ions 1.5-2.0%, fat 1.5-2.0%, and vitamins such as B complex and E[1]. Being the most important staple food crop of India, a wide number of agricultural research programs are involved in increasing the growth and yield of this particular crop.

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To increase productivity of important agricultural crops in a resourceful manner, agriculture needs to be integrated with innovating science-based technologies, and the rapid increase in the use of nanoparticles in every discipline, had brought focus on their potential to increase food production to match with the ever-growing population [2]. The field of plant tissue culture and nanobiotechnology has revolutionized the entire scenario of crop improvement of wheat [3]. Nanotechnology refers to the field of science that deals with matter having dimensions on the order of magnitude of 10^{-9} and is a rapidly developing discipline substantially influencing every field of science and biology [4, 5]. Nanoparticles of gold, silver, copper, zinc, aluminium, silica, zinc oxide, caesium oxide, titanium dioxide, and magnetized iron are extensively used in agriculture [6].

Several factors like size, shape, concentration, composition *etc*., are responsible for nanoparticles' reactivity with biomolecules, which enables their penetration, translocation or inhibition across the plasma membrane of the plant cell, which may leads to different responses of plants to the same nanoparticle [7, 8]. Copper nanoparticles are easily synthesized in the laboratory and appear as dark brownish-red powdery substances [9]. The uptake and accumulation of copper nanoparticles have a direct relation with the concentration used [10, 11, 12]. They have shown both positive as well as toxic responses on plant morphology, germination and quality of produce, and transpiration, *etc*.

So, we have taken up the present study of the effect of copper nanoparticles (CuNPs) on *in-vitro* seed germination of two varieties of wheat PBW725 and PBW677 grown in the Punjab region, India. This study is the first to report the effect of CuNPs on *in-vitro* seed culture and can be more broadly used in other cereal crops.

MATERIALS AND METHODS:

Synthesis of Copper Nanoparticles (CuNPs): Synthesis of copper nanoparticles (CuNPs) was achieved by using methods of Kathad and Gajera [13]. The presence of brownish blue particles shows the formation of copper nanoparticles. The nanoparticles are washed with ethanol and dried in hot air oven at 60 °C, and different concentrations (1 ppm, 2ppm, 5ppm, 10ppm, and 100ppm) of the nanoparticles were prepared. We have characterized these nanoparticles by using UV–Visible spectrophotometer.

In-vitro Seed germination of Wheat:

Source of Explants: Seeds of two varieties of wheat (PBW725 and PBW677) were collected from Punjab agricultural university, Ludhiana, Punjab. The seeds were separately washed under running tap water for few minutes so as to remove the dust particles adhered to the surface. Seeds were treated with fungicide (15% carbendazim) for 15 min and then rinsed with distilled water three to four times [14]. The surface-sterilized was done by using 0.1% of mercuric chloride for 3 min followed by washing with autoclaved distilled water two to three times to remove the residual effects of the sterilant. Surface sterilization was performed under laminar airflow conditions.

Preparation of MS Media: Different Basal MS media supplemented with vitamins, 3% sucrose and different concentration of the nanoparticles *i.e.*, 1 ppm, 2 ppm, 5 ppm, 10 ppm and 100 ppm were prepared and Basal MS medium having all other ingredients except any nanoparticles was designated as control. pH of all the media was adjusted to 5.6-5.8 by either 1 M NaOH or HCl. The media are solidified by using agar 8% and autoclaving is done at 121 °C and 15 psi for 15 min.

***In-vitro* Inoculation of Seeds:** Surface sterilized seeds of both the varieties were placed singly on the control (basal MS media) and basal MS media having different concentration of nanoparticles using autoclaved forceps in a LAF chamber. The cultures were maintained at 25±2 °C under 16 h photoperiod with a light intensity of 2000 lux provided through white fluorescent lights.

Data Collection and Statistical Analysis: The *in-vitro* germination and growth of the plants were observed on control media and media having different concentrations of CuNPs for two wheat varieties. The emergence of radicles of length 2mm are considered as germinated seeds. The germination percentage was calculated after the emergence of radicals. Percent seed germination was calculated as the number of seeds germinated/Total number of seedx100. After 15 days of culture, the data for shoot length and Root length was recorded in centimeter (cm). Seed vigour index and tolerance indices for both the varieties were also calculated [15].

The experiment was carried out in a completely randomized design with ten replicates, and each individual treatment was repeated three times. Data is statistically analyzed by analysis of variance (ANOVA) tested for significance using Fisher's protected Least Significant Difference (LSD) at $p \leq 0.05$ [16].

RESULTS AND DISCUSSION:

Characterization of Copper Nanoparticles (CuNPs): To study the stability of the copper nanoparticles colloidal solution, the absorption of CuNPs was measured by UV-visible spectroscopy. The spectra of CuNPs in water for different concentrations were recorded immediately after the synthesis of particles.

For the CuNPs, no absorption in the visible range was observed, while distinct broad peaks were observed at approximately 300, 320, and 350nm, relatively same peaks at all the concentrations of CuNPs. With the addition of CTAB as a stabilizing agent, the UV spectra were shifted to lower wavelengths (300-350nm). This shift is probably due to the slight reduction of particle size by the action of CTAB. CTAB acts as a size controller, preventing aggregation of metal nuclei. Polyethylene glycol (PEG) as size controlling agent as well as the capping agent whose concentration is crucial to control the particle size distribution of nanoparticles [17]. Some literature confirms the peak in the range of 500-600nm [18]. The absorption peak was around 588nm, without using any stabilizing agent [19].

Effect of Copper Nanoparticles on Seed Germination of PBW 677 and PBW 725 Varieties: The germination percentage of the wheat variety PBW 677 decreased with an increase in the copper nanoparticle concentration in the medium. At lower concentrations (1ppm and 2 ppm) the germination percentage did not decrease with respect to the control, but as the concentration increases, the germination percentage shows a drop, with only 16.66 % at 100 ppm concentration. The results obtained did not reveal a clear cut increasing or decreasing effect of copper nanoparticles on the wheat seed germination but only revealed that an increasing concentration of CuNPs negatively affect the seed vigour index, whereas this study revealed clearly that by increasing the concentration, germination percentage decreases [20]. Very low concentration (0.2-0.4ppm) of CuNPs did not affect wheat seed germination, but as the concentration was increased to 0.8ppm to 1 ppm, detrimental effects on the seed germination and vigour were visible [2]. Another study revealed that although green synthesized copper nanoparticles are less toxic, their effect on the seed germination of *Citrus reticulata* [21] tends to be negative. The same effects of silver nanoparticles were reported on wheat [22].

In the case of the variety PBW 725, the germination percentage varied with the varying concentration of the nanoparticles. At lower concentrations, the germination percentage did not vary significantly with respect to the control **Table 1**. This result is similar to the results observed in which only seed vigour was affected while seed germination did not [15]. However, in the current study, a remarkable difference was observed for PBW 725 at 5ppm concentration. At this concentration, the seed germination was 100% which indicates its positive effect on the seed germination. Similar to PBW 677, at 100 ppm concentration, the seed germination declined abruptly with as low as 16.66 % seed germination. The CuO nanoparticles have also shown toxicity toward seed germination thus, reducing biomass in crops like alfalfa, mung bean, wheat, kidney bean, onion, rice, *etc*. [10] Nonetheless, careless use of copper nanoparticles may cause environmental pollution and health effects [23]. Copper(II) oxide nanoparticles have been regarded as more toxic nanoparticles as compared with copper nanoparticles [24].

TABLE 1: *IN-VITRO* SEED GERMINATION OF WHEAT VARIETIES (PBW 677 AND PBW 725) ON BASAL MS MEDIA (CONTROL) AND MS MEDIA HAVING DIFFERENT CONCENTRATION OF COPPER NANOPARTICLES

Concentration of CuNPs	Germination percentage (%) for PBW 677	Germination percentage (%) for PBW 725
Control	83.33	83.33
1 ppm	83.33	83.33
2 ppm	83.33	50.00
5 ppm	66.66	100
10 ppm	66.66	83.33
100 ppm	16.66	16.66

Effect of Copper Nanoparticles on Shoot and Root Morphology of PBW 677 and PBW 725 Varieties: An inhibitory effect of the copper nanoparticles on the shoot length and root length on both PBW 677 and PBW 725 was observed. With an increasing concentration of CuNPs both root length and shoot length were affected. A dose-dependent inhibitory effect of CuNPs on wheat root and shoot length has been evaluated [25], which supports the results of the current study. The reduction in root and shoot length may be due to changes in nitrate regulated auxin transport and cell death, resulting in the reduction of root development as well as shoot development [20]. With the increasing concentration of copper nanoparticles, the root length decreases significantly. Although, in the case of PBW 725, lower concentration treatments (1ppm and 2 ppm) show an increase in the root and shoot growth as compared to the control as the concentration is increased further, the roots get affected adversely, and shoot length also decreases **Table 2**. Copper nanoparticles are proven as enhancers of seed germination and growth for some plants at lower concentrations, whereas high concentrations have given negative impacts like retarded growth [23]. Higher concentration changes the physiology of the germinated plant, such that roots fail to develop at all. These results were similar to the results obtained in wheat and mungbean [26,] and in *Glycine* and *Cicer arietinum* [27], *Brassica rapa* [28] and *Brassica pekinensis* L. [29] Copper nanoparticles are transported to the plant tissues when roots absorb moisture from the soft agar gel containing CuNPs and negatively affects rooting systems of both the plants. The current study reveals that low concentrations of copper nanoparticles can show positive effects on the root and shoot development of PBW 725 in contrast to high Copper nanoparticles concentration, which elucidates negative impacts on the plant morphology and growth. Copper nanoparticles considerably affect the morphology of wheat roots, possibly due to changes in nitrate regulated auxin transport and cell death, resulting in the reduction of root development as well as shoot development [25].

TABLE 2: EFFECT OF DIFFERENT CONCENTRATION OF COPPER NANOPARTICLES ON SHOOT AND ROOT LENGTH OF BOTH VARIETIES OF WHEAT (PBW 677 AND PBW 725)

Concentration of CuNPs	For PBW 677 Mean shoot length (cm) ± SD	Mean root Length (cm) ± SD	For PBW 725 Mean shoot length (cm) ± SD	Mean root Length (cm) ± SD
Control	2.65 ± 1.34	3.16 ± 1.80	0.70 ± 1.09	0.86 ± 1.39
1 ppm	2.23 ± 1.11	2.50 ± 2.11	2.16 ± 1.12	2.67 ± 1.33
2 ppm	1.95 ± 1.00	1.87 ± 1.55	1.21 ± 1.37	0.64 ± 0.76
5 ppm	1.86 ± 1.07	1.93 ± 1.36	2.21 ± 0.41	1.21 ± 0.70
10 ppm	1.78 ± 0.91	1.63 ± 0.95	1.81 ± 0.89	1.36 ± 0.86
100 ppm	0.56 ± 0.88	0.37 ± 0.61	0.36 ± 0.89	0.03 ± 0.09

For the above given table, data given is mean ±standard deviation. the value of the $p < 0.05$, so the data is statistically significant

Seed Vigour Index and Tolerance Index: The seed vigour index of PBW 677 decreased significantly with increasing copper nanoparticle concentrations. The seed vigour index of PBW 725 was independent of the copper nanoparticle concentrations, as the Seed vigour index was variable at lower concentrations. It was found to be maximum at 5ppm concentration of CuNPs. However, the Seed vigour index was reduced to a much lower value at high copper nanoparticle treatment (100 ppm), which was observed for both the varieties **Fig. 1**. Our findings are in line with Hussain *et al.* [30] Root length, shoot length, and Seed vigour index was also significantly reduced after 35 days in *Artemisia absinthium* seeds treated with AuNPs, whereas *Green* gram seeds nano primed with MgO (100 mg/L) showed significantly enhanced seed germination (%) and seedling vigour compared to conventional hydropriming method [31, 32].

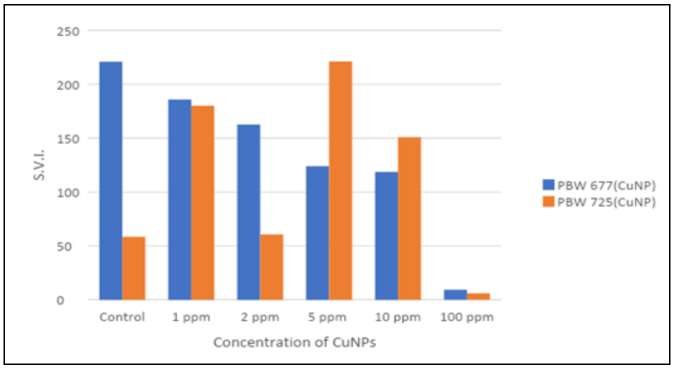

FIG. 1: SEED VIGOUR INDEX OF PBW 677 AND PBW 725 WITH RESPECT TO COPPER NANOPARTICLE TREATMENT

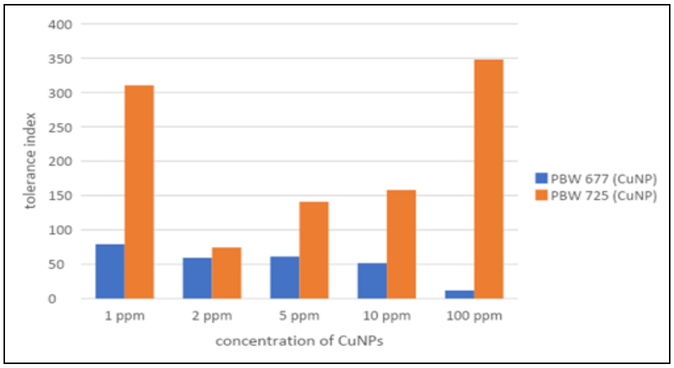

FIG. 2: TOLERANCE INDICES OF PBW 677 AND PBW 725 WITH RESPECT TO COPPER NANOPARTICLE TREATMENT

In the case of copper nanoparticle treatment, a decrease in the tolerance index (T.I.) of PBW 677 was observed with the increase of concentration.

On the other hand, at 1 ppm treatment, the T.I. was higher as compared to 2 ppm treatment, but increasing the concentration beyond 2 ppm significantly increased the tolerance index of PBW 725. At 100 ppm, the T.I. of PBW 677 was lowest whereas the T.I. of PBW 725 was highest, as indicated in **Fig. 2**. This indicates that tolerance of PBW 725 against the toxicity generated by the high concentration of nanoparticles is better as compared to PBW 677.

Under *in-vitro* conditions, 50–500 mg/l copper oxide nanoparticles reduced the overall growth of *Glycine max* [33]. It has been reported that metal and metal oxide nanostructures tend to damage the genetic material of a living cell or, simply, they can behave as mediators of DNA damage in living organisms [34]. A recent systems biology approach, including omics data from tobacco, rice, rocket salad, wheat, and kidney beans, confirmed that metal NMs provoke a generalized stress response, with the prevalence of oxidative stress components [35]. Generally, the toxicity of these nanoparticles depends on the size, surface charge, and pH of the environment [36]. In *Arabidopsis thaliana*, a concentration of 0.2 mg/L of CuO NP does not affect the genetic expression of genes that elicit oxidative stress responses, sulphur assimilation, glutathione, and proline biosynthesis; however, an upregulation of the expression of such genes was observed as the concentration approached a higher scale [37]. The mitotic index of actively dividing cells in *Allium cepa* increases at a lower concentration of copper nanoparticles and declines significantly as the concentration is increased [38]. An RAPD analysis has been carried out on *Cucumis sativus* revealed the presence of new bands that may be exhibited due to alterations in the genetic material [39].

CONCLUSION: The present study explored the influence of chemically synthesized copper nanoparticles on *in-vitro* germination and growth of two different varieties of wheat. Higher concentration (100 ppm) affected the plant adversely, inhibiting both root and shoot development. The germination percentage of PBW 725 did not reveal an increasing or decreasing trend in response to the varying concentrations of copper nanoparticles. However, the medium supplemented with 5 ppm CuNPs, resulted into 100% germination percentage of PBW 725, which indicates that lower concentration of copper nanoparticles can be potential enhancers of seed germination. Also, the growth of seedlings was enhanced at a lower concentration with respect to the control. It can be concluded that copper nanoparticles can exhibit different effects on different varieties of the same species.

Therefore, an intense experimental study is required to completely understand the effects of metal nanoparticles like CuNPs on plants, especially on agriculturally important crops and their varieties. Furthermore, molecular mechanisms of NPs on different cultivars can be investigated that can help in understanding how these nanoparticles bring a change in the physiology and morphology of the plants.

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