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Effect of Multi-Walled Carbon nanotubes-based Seed Priming in *Pennisetum glaucum* Seedlings Affecting the Antioxidant Activities

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Abstract

In recent years, there has been a lot of attention in the use of multi-walled carbon nanotubes (MWCNTs) in farming. This study is aimed to see the effect of MWCNTs at various concentrations such as 30, 60, 90, 120, and 150 parts per million(ppm) and their impact on antioxidant activities including CAT (Catalase), SOD (Superoxide dismutase), POD (Peroxidase), and DPPH (2,2-diphenylpicrylhydrazyl) in15 days old pearl millet seedlings. Seeds were treated to MWCNTs using priming method. In result we noted the gradual increase in CAT activity with the increasing concentration upto 90 ppm (0.71mg) H_2O_2 destroyed/sec./gmF.wt. and then it decreased with an increase in concentration. At 60ppm, POD activity was found to be the highest with 0.62µM/L/gm/sec.F.wt. and SOD activity increased till 90 ppm (0.63 unit/mg). DPPH inhibition activity was obtained 72.65% at 90 ppm to be maximum, whereas with the increase of the concentrations there was decline in DPPH activity. In conclusion the study showed MWCNTs works excellent at an optimal concentration in enhancement of antioxidant activities in developmental stage of seedling growth and shown negative results when concentrations were increased. These results suggested that the specific concentration of CNTs could help in the overall growth of the plant with the highest yield in terms of quality and quantity.

Keywords

Pearl millet, Superoxide dismutase, Peroxidase, Catalase, DPPH

Introduction

The demand for food is predicted to climb by 70% between now and 2050 as a result of rising global population, rising incomes, and changing eating habits [1]. Traditional fertilisers have been used to increase agronomic yield, but their effectiveness has pointedly decreased since the "green revolution," and overuse of chemical-fertilisers has had damaging effects on soil strength, resulting in ineffectual nutrient up-take and environmental contamination [2]. In addition, the search for new materials and skills has grown extra critical in contemporary farming due to the increased problems given by climate change, land degradation, and urbanisation [3].

In India, millets and cereals are the most common diet staples. Variations in the phenolic content make them good bases of organic antioxidants. Millets comprise phytic acid, tannins, and phenols that may help with antioxidant activity, which is crucial for preventing diseases, ageing and metabolic disorders. The most popular variety of millet is pearl millet. Regarding lipid, energy value, high-quality proteins, and minerals like iron, calcium and zinc, pearl millet is found to be greater than other chief cereals concerning nutritional status. Additionally, it

contains a significant number of micronutrients and dietary fibre [4, 5]. All naturally occurring molecules found in plants that also have the potential to have antioxidant properties are collectively referred to as "phytochemicals" or "plant chemicals." ROS, which are noxious by-products formed during consistent cell aerobic breathing, are defended against by the body's defence mechanism in effect by antioxidants [6]. Antioxidants shield foods from unfavourable alterations in flavour and nutritional value [7]. Nitrogen compounds (amines, chlorophyll derivatives, alkaloids, and amino acids) phenolic compounds (phenolic acids, flavonoids), ascorbic acid, and carotenoids are examples of antioxidants. Pearl millet (*Pennesitum glaucum*) is a member of the Paniceae section of the Poaceae family. In Africa, Asia, and the Americas, it is a significant food and fodder crop. Provided that it is suitable for agriculture's most harsh conditions, it has a lot of potentials [8].

Nearly all sectors of science are impacted by nanotechnology, and the introduction of nanotubes (NTs) into agriculture has the potential to significantly improve farming sustainability [9, 10]. One of the most logical fields today is nanotechnology, which comprises the operation of materials at the nano-scale (1 to 100 nm) in size to produce useful materials with uncommon features when equated to their bulk counterparts [11]. Due to nanotechnology's minor size, high surface area (SA), and high reactivity of nanotubes (NTs), it has demonstrated significant potential for increasing plant [12]. Carbon NTs were pragmatic to enhance crop production [13], cell division [14, 15], seed germination [16] and photosynthesis [17]. For instance, the quantity increased by double when carbon nanotubes (CNTs) were applied to the plants [13]. Since carbon NTs are made solely of carbon, they are highly stable, low in toxicity, and environmentally friendly [18].

One of the simplest methods for accelerating seedling emergence, recovery, and establishment, which will subsequently enhance the plant's growth efficiency in the field, is seed priming [19]. Because of its small size and distinctive physicochemical features, nano-priming is a more successful seed priming technique than other ones [20]. In most plants, nano priming (coating with nano-materials of micro-nutrients) enhances seed sprouting, seedling development and growth, active strength, and seedling dry weight [21]. Furthermore, nano-priming triggers exact metabolic procedures that are naturally vigorous throughout the initial phases of sprouting; this boosts the growth, productivity, and quality of the crops as well as the degree of plantlet rise, and development [22]. Also, it put its impact on how plants cooperate with their environments at the molecular and cellular levels [23]. Compared to unprime seeds, priming might increase the germination of seeds in stressful situations [24]. However, several interrelated elements include priming treatment, temperature, seed storage conditions, priming period and watering might affect priming success [25]. Because of this, the application of NTs has favourable impacts on both seed sprouting and plant development and growth [20]. Therefore, this study has been led to inspect the consequence of multi-walled carbon nanotubes (MWCNTs) on antioxidant activity of Pearl millet at different concentrations (30 ppm, 60 ppm, 90 ppm, 120 ppm, 150 ppm) due to the numerous favourable outcomes of the application of NTs in agricultural fields. These results can aid in the development of more efficient nanotubes usage techniques, particularly given the novel growth promoters for agricultural use.

Materials and Methods

Collection of plant sample

Pennisetum glaucum seeds with a variety RHB 173 were acquired from the Rajasthan Agricultural Research Institute (RARI) Durgapura, Jaipur, Rajasthan.

Dispersion of nanoparticles

Using ultrasonication, multiwalled carbon nanotubes (MWCNTs) type 10 that had been COOH functionalized were suspended after being acquired from SRL. Pvt. Ltd. in India, as previously described by Park and his team [26]. A stock of MWCNTs was diluted with autoclaved distilled water in different concentrations before use. 200 mg of nanotubes were added to 200 ml distilled water without mixing any surfactants and subjected to ultrasonic vibration [150 W, 40 KHz] for half hour in a sonicator. The samples were named Control (C_0),30 ppm (MW30), 60 ppm (MW60), 90 ppm (MW90), 120 ppm (MW120) and 150 ppm (MW150).

Preparation of nanomaterial suspensions and seed treatment

The pearl millet seeds were carefully cleaned with 20 percent Extran® (Merck, India) for 3 to 4 min. which was followed by 3 times washing with distilled water. The seeds were surface sterilized with a Laminar air flow hood fitted with UV light using a 0.1 percent $HgCl_2$ [Merck, India] solution for at least three minutes, and then rinsed three times with autoclaved distilled water. Different concentrations of carbon nanotubes (MWCNTs) including 30 ppm (MW30), 60 ppm (MW60), 90 ppm (MW90), 120 ppm (MW120) and 150 ppm (MW150) were prepared from the stock solution for the experiment. Seeds were imbibed in the prepared nano-suspension for 24 hours at 100 rpm in a shaking incubator. The experiment was done in triplicate and the seed incubated in pure DI water was used as a control denoted by C0.

Seed viability test

The blotting paper method was used to identify the seed viability test. Treated pearl millet seeds were placed (10 seeds per Petri plate) in glass Petri plates having a germination bed saturated with De-ionized (DI) water. To prepare the germination chamber, autoclaved absorbent cotton sheet of 3-5 mm thickness was placed in a petri plate, which was then covered with autoclaved filter paper cut into the same shape and autoclaved distilled water (5 mL) was put, following the instructions of the International Seed Testing Association (ISTA, 1976). This whole exercise was done under laminar airflow (LAF) to avoid unwanted growth of microorganisms on the germination bed as well as germinating seeds, which can alter the results significantly. The seedlings in these Petri plates were kept in a growth chamber for further growth at 28 ±1 °C and controlled dark and light periods (16/8 h and illumination of 24 µmol/m^2/s for 15 days. 15 days old pearl millet seedlings were used to detect the effect of MWCNTs on antioxidant activity.

Enzymatic antioxidant assays

At 15-day-old pearl millet seedlings, the antioxidant enzyme was specifically evaluated as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). All CNTs treated seedlings were crushed individually in fine powder by using mortar and pestle and used for antioxidant enzyme assay. All the experiments were performed with three replicates.

Catalase enzyme (CAT) activity

The Teranishi [27] method was used to calculate the catalase enzyme activity. 1 gram of the fresh sample was crushed in 50 mM cool/ice cold PO_4 buffer of pH 7.0, and the supernatant was used as an enzyme extract after centrifuging at 10,000 rpm (15456 g) for 10 minutes at 4 .0 ºC. The reaction with combination of 3 mL is made from 0.1 mL of enzyme abstract and 2.7 mL of 50 mM PO_4 buffer of 7.0 pH. 0.2 mL of 10 mM hydrogen peroxide was added to the reaction to initiate it. At 410 nm, the absorbance began to decrease. In terms of mg H_2O_2 destroyed/sec./gmF.wt., catalase activity was expressed.

Peroxidase enzyme (POD) activity

With the modifications listed below, the Chance and Maehly [28], method was used to measure the peroxidase activity. After being homogenised with 10 mL of phosphate buffer (pH 7.0) for 200 mg of the sample, it was centrifuged for 20 min at 10,000 rpm (15456 g). The enzyme abstract was isolated from the distinct supernatant. 2.2 mL of phosphate buffer, 0.3 mL of pyrogallol, and 0.3 mL of H_2O_2 were added. As soon as the enzyme extract (0.2 mL) was added at 420 nm absorbance was determined to govern the amount of purpurogallin formation. Analysis was done and the result was reported using a molar extinction coefficient of 26.6 $mM^{-1}cm^{-1}$, and M/L/gm/sec F.wt.

Superoxide dismutase (SOD) activity

The method explained by Beauchamp and Fridovich, [29] was used for the analysis of SOD activity. The assay was performed with a reaction mixture comprising phosphate buffer (100 mM, 7.0 pH), 0.3 mL riboflavin, 2.5 ml methionine, and 200 µl nitro blue tetrazolium (NBT), and 100 µl enzyme extract. The reaction started by exposing it to light for 15 minutes, after which absorbance at 560 nm was measured. 1 unit of superoxide dismutase (SOD) is equivalent to the quantity of enzyme required to prevent the photoreduction of NBT to blue formazan by 50 percent. In the units/mg result for SOD enzyme was expressed.

Non-enzymatic antioxidant assays

DPPH radical scavenging activity

Ghanati and Bakhtiarion, [30] method was employed for the estimation of DPPH radical scavenging activity. 1 gram of homogenised plant material was extracted using 10 mL of 50 mM PO4 buffer, followed by 15 minutes of centrifugation at 4 °C on 10,000 rpm (15456 g). The supernatant was used to assess the activity after centrifugation. 0.1 mL of sample extract and 0.1mL of the DPPH working solution were combined to complete the assay, which was then kept in the dark for 30 min. Later, the sample extract was replaced with phosphate buffer in the blank and absorbance was taken at 517 nm. To calculate the radical scavenging activity the following formula was used:

$$\% \text{ Inhibition of DPPH} = \{(A_B - A_S)/A_B\} \times 100$$

Here, A_S = absorbance of samples; A_B = absorbance of blank or reference.

Statistical analysis

The results of each experiment were performed in triplicate, and they are shown as mean ± standard deviation. One-way analysis of variance (ANOVA) performed in IBM SPSS was used to analyse the data, and Duncan's mean comparison test was used to determine the significance of differences between the mean values at $p < 0.05$.

Results and Discussion

Catalase enzyme (CAT) activity

In its capacity as an enzyme antioxidant, catalase plays a crucial part in preventing cellular oxidative harm by proficiently changing H_2O_2 into H_2O and O_2. Excellent H_2O_2-degrading antioxidant enzyme catalase can quickly reduce H_2O_2 without using up cellular energy. Catalase's enzymatic efficiency is extremely high [31]. In the present study, it was seen that carbon nanotubes treated pearl millet seed showed great variation at different concentrations. It was observed that catalase activity at MW90 (0.83 ± 0.14mg H_2O_2 destroyed/sec./gmF.wt) was significantly higher ($p < 0.05$) than those of other concentration. Then at higher concentrations (MW120 and MW150) it was found that catalase activity starts to reduce. Comparative to the control treated plant had slightly more activity of catalase enzyme **(**Table 1**).** Catalase's catalytic reaction was examined by [32], who discovered that leaves exhibit it more than roots do. Up to 150 µM of MWCNTs, an enhanced CAT reaction was seen in leaves; however, this reaction degraded as the dosage was increased after that. At 100 µM, when enzyme activity was 1.78 times ≥ in un-treated seedlings in leaves, a detectable increase in enzyme activity was observed.

Peroxidase enzyme (POD) activity

In this study, it was interpreted that the peroxidase activity augmented at initial as the concentration of carbon nanotubes was increased but it gradually decreased if the treatment at higher concentration was done and so the peroxidase enzyme activity also decreased. The carbon nanoparticle-treated

Table 1: Effects of MWCNTs on catalase enzyme activity of 15 days old *Pennisetum glaucum* seedlings at different concentrations.

Sample	Catalase content (mg H_2O_2 destroyed/sec./gmF.wt.)
C0	0.40 ± 0.07^{bc}
MW30	0.45 ± 0.03^{bc}
MW60	0.47 ± 0.08^{bc}
MW90	0.83 ± 0.14^{a}
MW120	0.56 ± 0.10^{b}
MW150	0.35 ± 0.05^{c}

All the triplicate values are present in Mean ± SD. a, b, c – Means with different superscripts within different concentration are significantly different ($p < 0.05$); F.wt: Fresh Weight.

pearl millet at MW90 showed highest POD activity (0.74 ± 0.17µM/L/gm/sec) among all concentrations, having significant difference ($p < 0.05$) as specified in table 2**.** In contrast to leaves, GOPX's catalytic response was primarily seen in roots. The increased MWCNTs dosage up to 75 µM was shown to result in better GOPX catalysis, which subsequently started to drop at 100 µM. At 150 µM MWCNTs treatment, there was a significant increase in GOPX activity (2.65 times ≥ control), which again deteriorated at high concentration [32].

Table 2: Effects of MWCNTs on peroxidase enzyme activity of 15 days old *Pennisetum glaucum* seedlings at different concentration.

Sample	Peroxidase activity (µM/L/gm/sec. F.wt.)
C0	0.39 ± 0.07^{bc}
MW30	0.41 ± 0.03^{bc}
MW60	0.48 ± 0.09^{b}
MW90	0.74 ± 0.17^{a}
MW120	0.43 ± 0.14^{bc}
MW150	0.23 ± 0.08^{c}

All the triplicate values are present in Mean ± SD. a, b, c – Means with different superscripts within different concentration are significantly different ($p < 0.05$); F.wt: Fresh Weight.

Superoxide dismutase (SOD) activity

SOD plays a crucial role in oxidation as well as the antioxidant defence mechanism against the stress of oxygen-free radicals [33]. In this study, it was analysed that carbon nanotubes treated pearl millet at different concentrations gave variation in the superoxide dismutase activity. Relatively the pearl millet treated at MW60 and MW90 concentrations had higher SOD activity with 0.72 ± 0.10 and 0.65 ± 0.07unit/mg protein, respectively having a significant difference ($p < 0.05$). SOD of the plants treated at further higher concentrations, MW120 and MW150 showed a decreasing trend **(**Table 3**).** At an initial concentration of 25 µM, carbon nanoparticles SOD activity was found to be reduced [32]. Further concentration increases led to insignificant changes in SOD activity. However, SOD activity did not alter significantly until 100 µM concentration but slowly amplified with 44% higher than 100 µM at 150 µM concentrations in roots. The improved level of reactive oxygen species that causes the augmented expression of the SOD gene may be indicated by improved superoxide dismutase catalysis, which is accompanied by an increase in CNTs dose.

Table 3: Effects of MWCNTs on superoxide dismutase enzyme activity of 15 days old *Pennisetum glaucum* seedlings at different concentration.

Sample	Superoxide dismutase activity (unit/mg protein)
C0	0.34 ± 0.03^{c}
MW30	0.47 ± 0.05^{bc}
MW60	0.72 ± 0.1^{0}a
MW90	0.65 ± 0.07^{a}
MW120	0.50 ± 0.04^{b}
MW150	0.37 ± 0.09^{c}

All the triplicate values are present in Mean ± SD. a, b, c – Means with different superscripts within different concentration are significantly different ($p < 0.05$).

DPPH radical scavenging activity

Antioxidant activity of pearl millet treated with the MWCNTs was analysed at 100 µl concentration and was seen that at MW90 radical scavenging activity was found to be best with 72.64 ± 0.08%, having significant difference ($p < 0.05$), and then it declined at higher concentrations, MW120 and MW150 showing radical scavenging activity as 49.16 ± 0.09 % and 43.53 ±0.10 %, respectively. The radical scavenging activity in treated samples was significantly higher ($p < 0.05$) as compared to the control sample having value as 40.63 ± 0.10 %. Higher radical scavenging activity showed more yellow colour, which shows the maximum % inhibition as described in table 4**.** The effect of the GO (graphene oxide) and reduced GO synthesized by *Lantana camara* leaf (LCrGO) on free radical scavenging activity (DPPH assay) was determined at different concentrations (100 µg/ml, 75 µg/ml, 50 µg/ml, and 25 µg/ml) with significant variation. The results of the comparison showed that LCrGO had a greater antioxidant effect than GO. The highest amount of DPPH inhibition activity was seen in LCrGO with 79% while GO solitary repressed 62% at 100µg/ml [34]. The existence of chemical functional moieties in rGO may be the cause of its significant radical scavenging action [35, 36]. The covering of bio-molecules like pro-anthocyanidins and phenols on the surface of rGO nano-sheets may be the cause of the improved radical scavenging effects of LCrGO [37].

Table 4: Effects of MWCNTs on DPPH activity of 15 days old *Pennisetum glaucum* seedlings at different concentration.

Sample	DPPH % Inhibition (Control-Sample/Control×100)
C0	40.63 ± 0.10^{e}
MW30	42.16 ± 0.08^{d}
MW60	43.39 ± 0.06^{c}
MW90	72.64 ± 0.08^{a}
MW120	49.16 ± 0.09^{b}
MW150	43.53 ± 0.10^{c}

All the triplicate values are present in Mean ± SD. a, b, c, d, e – Means with different superscripts within different concentration are significantly different ($p < 0.05$).

Conclusion

Carbon nanotubes could greatly improve pearl millet growth, as obvious by the increase in antioxidant activity. Multi-walled carbon nanotubes, however, produced some inhibitory effects on the plant and acted as a stress producer at higher rates that were more than 90 ppm (MW90), making it difficult for plants to grow effectively. In this study, it was found that carbon nanotubes at an intermediate concentration (30 ppm to 90 ppm) produced the greatest results for both non-enzymatic and enzymatic antioxidant activity, which led to mitigate the oxidative stress and decrease in ROS levels. The above research outcomes can be beneficial for group of scientists to focus on gene regulatory pathways and identify the key transcriptional factors responsible for MWCNTs induced gene expression changes.

Acknowledgements

None.

Conflict of Interest

None.

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