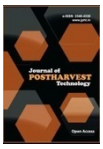

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RESEARCH ARTICLE

Effect of sodium nitroprusside on germination of *Trigonell foenum-graceum* seeds under cadmium stress

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ABSTRACT

Fenugreek (*Trigonell foenum-graceum*) is one of the oldest cultivated medicinal plants whose seeds are been found to have higher mineral, protein, carbohydrate content and antioxidant properties. Germination is one of the most important physiological events in a plant life cycle that is well protected against various stresses. The most important factors limiting germination of matured seeds are various environmental stress conditions like the presence of heavy metals in soil, lack of water etc. Metal poisoning affects the plant growth process, therefore, affecting crop yield. Cadmium is one of the most reactive heavy metal contaminants and is the most vulnerable to accumulation. Thus, fenugreek being a valuable medicinal plant demands further investigation into its response to cadmium-induced phytotoxicity during germination. The goal of this study was to investigate the effect of sodium nitroprusside (SNP) on seeds treated with cadmium chloride during germination and its antioxidant activity. SNP is known to donate nitric oxide that plays a pivotal role in seed germination and acts against oxidative stress. Phytochemical analysis of about 25 fenugreek seeds was carried out using different strategies and methodologies. Estimation of antioxidant activity and effect of SNP on $CdCl_2$treated seeds were evaluated. The phytotoxicity percentage remarkably increased with an increase in metal toxicity while seeds treated with SNP showed increased protein concentration. Germination percentage however decreased with increasing metal toxicity. Proline accumulation was found to be more in 200µM SNP and decreased with increasing SNP concentration. The scavenging activity of SNP-treated seed exposed to $CdCl_2$was found to be significant.

Keywords: Cadmium stress, fenugreek, germination, phytotoxicity, sodium nitroprusside

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INTRODUCTION

Fenugreek (*Trigonell foenum-graceum*) is a half-hardy annual dicot native to Southern Europe and Asia, and it belongs to the Leguminaceae family's Pepilionaceae subfamily. It's a popular crop in the Mediterranean, India, and China (Ahmad et al., 2005). The name "fenugreek" comes from the Latin word foenum-graceum, which means "grey hay," because the plant was once used

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to scent poor hay (Flanmang et al., 2014). Fenugreek has been used for a variety of purposes since ancient times. The plant's leaves and seeds are widely used as a spice in cooking and as a component in ethnomedicine (Syeda et al., 2008) Fenugreek is an ancient medicinal plant that has been used to cure a variety of conditions including diabetes, high cholesterol, inflammation, gastro intestinal difficulties, hypertension, sexual problems, and rheumatism. It has been as well proved as an effective antioxidant. Fenugreek also shows anti-diabetic and cholesterol-lowering properties (Nithya et al., 2014; Nandini et al., 2007). It is high in carbohydrates, proteins, fiber, and bioactive substances like diosgenin, trigonelline, and galactomannan. The seeds and leaves of fenugreek are high in minerals (iron, potassium, calcium, zinc, magnesium etc), proteins, and carbohydrate, but has lower oil content (Gad et al., 1982).

Germination is the most crucial stage in plant life that leads to successful establishment of the plant (Gorai and Neffati, 2007). Factors like temperature, light, moisture content of the soil, soil salinity influence germination at the same time. Short-term phytotoxicity in agricultural crops has been tested using germinating seeds and elongating root and shoot. (Wang and Zhou, 2005). Germination is a physiological process that is influenced by seed quality as well as environmental factors such water supply, oxygen, temperature, and light constancy (Maekawa et al., 2010).

Heavy metals are found in abundance in nature. Their numbers, on the other hand, have been steadily increasing as the world has become more industrialized. Heavy metals like cadmium (Cd), lead (Pb), and zinc (Zn) are plentiful in agricultural soils. Plant growth is hampered by metal toxicity. Metal poisoning affects crop output while also posing a threat to the food chain (Poschenrieder and Bercelo, 1999). Excessive levels of heavy metals in soil has harmed not only natural aquatic bodies but also terrestrial ecosystems. Cadmium (Cd) among the other heavy metals is the most reactive and persistent heavy metal contaminants, as well as one of the most prone to buildup due to poor agricultural practices (Gardea- Torresdey et al.,1996; Meagher, 2000). Under many agricultural systems, environmental pressures can result in significant yield losses. The pernicious issue involving both productivity and quality of economically valuable crops is a source of diverse stress (Wanger, 1993). Excessive levels of heavy metals in soils can have detrimental effects on biomass production, seed germination, root growth , morphological characteristics and architecture(Kukkola et al., 2000,Arduini et al., 1995). Heavy metal toxicity is a concern for ecological, evolutionary, and environmental factors (Nagajyoti et al., 2008). Heavy metals like lead and cadmium are extremely harmful contaminants that are released into the environment by vehicular exhaust (Lagerwerff and Specht,1970).

Cadmium is a hazardous heavy metal having a half-life of 10 to 30 years in the environment (Jan et al., 1999). Consumption of contaminated food or inhalation of tobacco smoke or polluted air exposes people to cadmium (Jarup et al., 1998). Because cadmium is integrated into the food chain mostly through plant absorption, high quantities of cadmium in soils pose potential harm to human health (Alvarez-Ayuso, 2008). Sodium nitroprusside (SNP), also known as sodium nitro ferricyanide, is a strong vasodilator used in clinical anesthetic that can donate nitric oxide (NO), cyanide, and iron (Broderick et al., 2007, Boullerne and Nedelkosk, 1999), or act through the release of iron after its light inactivation (Broderick et al., 2007, Boullerne and Nedelkosk, 1999; Cardaci et al., 2008). Nitric oxide is released using sodium nitroprusside (SNP). Nitric oxide (NO) is a gaseous molecule in the atmosphere that is water as well as lipid soluble. NO research in plants has received a lot of attention recently, and there is also a lot of evidence that this molecule plays a role in plants. NO is generated endogenously, primarily in young, actively growing tissues, in addition to its abundance in the atmosphere. It works as an intracellular and intercellular signaling plant growth regulator that primarily protects against oxidative stress. NO has been discovered to play a role in the control of a variety of plant physiological processes at low concentrations. NO, on the other hand, could be hazardous to plant systems at larger amounts (Beligni and Lamattina, 2000).

MATERIALS AND METHODS

Healthy seeds of Fenugreek (*Trigonell foenum-graceum*) were collected from a local market of Guwahati, Assam. Chemicals and glassware used, are mentioned under different methodologies.

Collection of sample

Healthy seeds of Fenugreek (*Trigonell foenum- graceum*) were collected from the market of Guwahati, Assam during the month of October. They were cleaned properly and kept in dry place in dark under room temperature before using.

Surface sterilization

Healthy seeds were collected and they were washed under tape water several times and then with distilled water. Cleaned seeds were surface sterilized with 0.1 % $HgCl_2$ for one minute and then washed with distilled water for five times to remove any traces of $HgCl_2$.

Effect of different concentration of $CdCl_2$ on seed germination

Different concentrations (50ppm, 100ppm and 200ppm) of $CdCl_2$ as a source of cadmium (Cd) were prepared from stock solution (1000ppm) in distilled water. Sterilized seeds were selected and soaked with different concentration of $CdCl_2$ for 4 hours. Seeds were also soaked in distilled water they were used as control. Twenty five seeds were placed at equidistant in each sterilized petri dish with Whatman-1 filter paper. Filter paper was moistened with distilled water. Seeds were allowed to germinate at room temperature for 48 hours.

Seeds treated with different concentrations of SNP

Sterilized seeds were taken and they were equally divided into four groups. The groups were soaked for 4 hours at 200µM, 400µM, 500µM of SNP respectively. Seeds soaked in distilled water were used as control. Twenty five seeds were placed at equidistant in each sterilized petri dish with Whatman-1 filter paper. 5ml of the respective solution was used to moistened the filter paper. Seeds were allowed to germinate at room temperature for 48 hours.

Effect of SNP on seeds exposed to $CdCl_2$

Sterilized seeds were taken and soaked at different concentrations (200µM, 400µM, 500µM) of SNP overnight. Equal number of seeds were selected and soaked at 100ppm of $CdCl_2$ for three hours. Twenty five seeds were placed at equidistant in each sterilized petri dish with Whatman-1 filter paper. 5ml of the respective solution was used to moistened the filter paper. Seeds were allowed to germinate at room temperature for 48 hours.

Germination percentage

The seedling evaluation approach in the Handbook of Association of Official Seed Analysis was used to count germination seeds (AOSA, 1983). The number of seeds that germinated were counted. For each replication of the treatment, the germination

percentage (GP) was computed using the procedure below (Tanveer et al., 2010). After 48 hours, seed germination was determined.

% Germination = Seed germination/ Total seed X 100

Phytotoxicity percentage

Phytotoxicity is the capacity of a compound (such as a plant protection product) to cause temporary or long-lasting damage to plants. The plant samples collected after 48 hours and various growth indices such as root length were measured. Phytotoxicity percentage for shoot and root of 48 hours old seedlings were calculated by the following formula (**Chau, C.H.and Lin, H.J.**).

% phytotoxicity = root length of control – root length of treated/control root length X 100

Estimation of Protein by Lowry's method

0.2 mL of sample extract was taken into a test tube and the volume was adjusted to 1mL with distilled H_2O. 5mL of reagent alkaline copper reagent was added. After incubation of 20 minutes FC reagent was added. After incubation for 30 minutes absorbance was read at 660nm spectrophotometrically against a suitable blank. BSA was taken as a standard.

Estimation of proline content

The concentration of proline in the sample was calculated using the method outlined by (Bates et al.). Seedling material (0.5 g) was homogenized in 10 ml of 3% (W/V) sulfosalicylic acid and filtered using Whatman No. 2 filter paper. The supernatant was used to calculate proline levels. 2 ml sulfosalicylic acid seed extract, 2 ml acid ninhydrin reagent, and 2 ml glacial acetic acid The test tubes holding the aforesaid mixture were heated for one hour in a boiling water bath. The reaction was stopped with a cold bath, then 4 mL of toluene was added. The upper toluene phase of the chromophase was carefully removed, and absorbance was measured at 520 nm. Proline was used as a standard.

Antioxidant activity determination by DPPH method

On the basis of the scavenging effect on the stable DPPH free radical activity, the antioxidant activity of plant extracts was investigated (Braca et al., 2002). The DPPH solution was newly produced and stored at 4°C in the dark. As a reagent, the antioxidant utilizes a radical. DPPH is lowered when it combines with an antioxidant compound. After that, the colour shift (from deep violet to pale yellow) is measured. The extract was consumed in four different concentrations (50L, 100L, 150L, and 200L). A spectrophotometer was used to measure the absorbance at 517nm. The proportion of DPPH free radical was estimated using the following formula:

Scavenging activity (%) = (A blank – A sample/ A blank) X 100

The absorbance of the control reaction, which contains all of the reagents except the extract, is referred to as "a blank." The absorbance of the extract, which is the test substance, is referred to as a "sample."

H_2O_2 radical scavenging assay

The scavenging ability of the extract for hydrogen peroxide was determined according to the method given by (**Ruch *et al*.,**1989). was prepared in phosphate buffer (pH 7.4). Seed extracts (100 µg/mL) were added to 0.6ml of hydrogen peroxide (60 mM). After 10 minutes absorbance was read at 230 against a blank containing phosphate buffer without hydrogen peroxide and compared with a reference compound, ascorbic acid.

$$H_2O_2 \text{ activity } (\%) = (\text{A sample} - \text{A control}/ \text{AA} - \text{A control}) \text{ X } 100$$

Where, 'A Control' is the absorbance of control and 'A sample' is the absorbance of the extract that is, the test compound. AA is ascorbic acid.

RESULTS

Effect of different concentration of $CdCl_2$ on germination of fenugreek seed:

Different concentrations (50ppm, 100ppm and 200ppm) of $CdCl_2$, as a source of cadmium metal were prepared from 1000 ppm of stock solution by using distilled water. 25 seeds were were surface sterilized with a 0.1% $HgCl_2$. Sterilized seeds were then soaked in different concentration of $CdCl_2$ for four hours and they were placed at equidistant in sterilized petriplate with Whatman-1 filter paper soaked in respective solutions. Seeds were allowed to germinate at room temperature for 48 hours. Maximum germination percentage was observed in case of control (seed treated with distilled water), whereas germination percentage of seed was found to be less in seeds treated with $CdCl_2$ as shown in Table:1. Likewise, the average radical length of the seeds soaked in distilled water was found to be more than the seeds treated with different concentrations of $CdCl_2$. The phytotoxicity percentage showed a marked increased with increase in metal concentration due to increased toxicity of $CdCl_2$.

Table1: Toxicity of different concentration of $CdCl_2$ on germination of fenugreek seed

Metal conc. (ppm)	Cadmium choloride ($CdCl_2$) treated		
	GP (%)	Phy (%)	Average radicle length (cm)
Control	100	0.00	2.06 ± 0.046
50	76	43.68	1.16 ± 0.041
100	92	53.39	0.96 ± 0.057
200	80	54.36	0.94 ± 0.065

Effect of different concentration of SNP on germination of fenugreek seed:

25 sterilized seeds were taken and were soaked for four hours, in different concentration SNP (200µM 400µM and 500µM); seeds soaked in distilled water was taken as control. The seeds were allowed to germinate for 48 hours. The germination percentage was calculated as shown in the table 2. and was found to be 100% in 400µM and 90% in 200 and 500µM respectively.

Likewise, the average growth of the radical increased with increased concentration of SNP but at 500mM of SNP the radicle length decreased. Phytotoxicity percentage was found to increase with increase in SNP concentration as shown in Table 2.

Table 2: Effect of different concentration of SNP on germination of fenugreek seed

SNP (µM)	GP (%)	Phy (%)	Average radicle length (cm)
Control (D. H_2O)	100	0.00	2.06 ± 0.046
200	90	0.00	2.06 ± 0.046
400	100	8.73	2.1 ± 0.024
500	90	37.86	1.88 ± 0.049

Effect of different concentrations of SNP on seed germination exposed to $CdCl_2$:

Sterilized seeds were soaked in different concentrations of SNP (200µM, 400µM, and 500 µM) overnight and these pre-treated seeds were soaked for 3 hours in 100 ppm of $CdCl_2$. After germination for 48 hours, SNP pre-treated seeds in 200µM and 400µM showed a gradual increase in the germination percentage but with 500µM, a decrease in germination percentage when exposed to $CdCl_2$ was observed. Likewise, when the seeds were exposed to 100ppm of $CdCl_2$, the average growth of the radical increased with increased concentration of SNP but at 500mM of SNP showed a gradual decrease shown in as Table 3. Similar observations were seen with phytotoxicity percentage. The findings are shown in table 3

Table 3. Effect of different concentrations of SNP on seed germination exposed to $CdCl_2$

Conc. of SNP (µM) and $CdCl_2$ (ppm)	GP (%)	Phy (%)	Average radicle length (cm)
Control (D. H_2O)	100	0	2.06 ± 0.046
200 µM SNP + 100 ppm $CdCl_2$	90	42.71	1.18 ± 0.029
400 µM SNP + 100 ppm $CdCl_2$	100	34.95	1.34 ± 0.048
500 µM SNP +100 ppm $CdCl_2$	90	64.07	0.74 ± 0.060

Effect of different concentration of $CdCl_2$, SNP, and SNP pre-treated seed exposed to $CdCl_2$ on protein and proline content:

Sterilized seeds were soaked in different concentrations (50ppm, 100 ppm and 200ppm) of $CdCl_2$ for four hours. Similarly, seeds treated with different concentrations (200µM, 400µM and 500µM) of SNP were also soaked for four hours. In the third batch, seeds were initially soaked in different concentration of SNP (200µM, 400µM and 500µM) overnight and then transferred to 100 ppm $CdCl_2$ for another three hours. After germination for 48 hours protein and proline content was measured. The concentration of protein and proline was found to be more in 50ppm $CdCl_2$ and it decreased with increase in $CdCl_2$ (100ppm and 200ppm), whereas in seed treated with SNP, the protein concentration were found to increased with the increased in SNP concentration, however the proline accumulation was found to be more in 200µM SNP and it decreased with increased in SNP concentration as shown in Table 4. Seeds pre-treated with SNP and exposed to $CdCl_2$(100ppm) showed a linear increase in protein and proline concentration as shown in Table 4.

Table 4: Effect of different concentration of $CdCl_2$, SNP, and SNP pre-treated seed exposed to $CdCl_2$ on protein and proline content

Conc.	Protein (µg/mL)	Proline (µg/mL)
Control ($D.H_2O$)	2170	598
50ppm $CdCl_2$	2120	416
100ppm $CdCl_2$	1700	295
200ppm $CdCl_2$	1020	160
200µM SNP	1030	522
400µM SNP	1980	492
500µM SNP	2740	120
200µM SNP + 100ppm $CdCl_2$	1100	185
400µM SNP + 100ppm $CdCl_2$	1610	235
500µM SNP + 100ppm $CdCl_2$	1020	151

DPPH free radical scavenging activity (%) in seeds treated with different concentration of $CdCl_2$, SNP and, SNP pre-treated seed exposed to $CdCl_2$

This assay was performed to determine the antioxidant activity in fenugreek seeds treated with different concentrations (50ppm, 100 ppm and 200ppm) of $CdCl_2$, seeds treated with different concentrations (200µM, 400µM and 500µM) of SNP and seeds pre -treated with different concentration of SNP. After 48 hours of germination, the scavenging activity was found to be more in 50 ppm $CdCl_2$, whereas the scavenging activity decreased with the increase in $CdCl_2$ concentrations (100ppm and 200ppm). The scavenging activity was found to be more in 400µM SNP treated seeds but there was linear decrease in the scavenging activity of 500µM SNP treated seeds as shown in Table 5. The scavenging activity of 200µM SNP treated seed was found to be less than 400µM SNP treated seeds. The scavenging activity ofSNP pre-treated seed exposed to $CdCl_2$ was found to be more for 400µM SNP + 100ppm $CdCl_2$ and less in 200µM SNP + 100ppm $CdCl_2$ and 500µM SNP + 100ppm $CdCl_2$ respectively as shown in Table 5.

Table 5: DPPH free radical scavenging activity (%)in seeds treated with different concentration of $CdCl_2$, SNP and, SNP pre-treated seed exposed to $CdCl_2$

Conc. (µL/mL)	Control	$CdCl_2$ (ppm)			SNP (µM)			SNP (µM) and $CdCl_2$ (ppm)		
		50	100	200	200	400	500	200 + 100	400 + 100	500 + 100
50	28.48	25.69	17.02	12.54	38.35	24.53	33.00	12.16	16.4	15.2
100	65	54.48	20.56	14.06	56.84	50.92	63.36	16.60	28.2	18.46
150	70	65.27	43.26	15.42	59.58	70.39	66.33	29.81	48.4	30.96
200	77	73.95	58.86	29.15	63.01	73.80	70.29	42.26	60.60	38.79

H_2O_2 radical scavenging assay in seeds treated with different concentration of $CdCl_2$, SNP and pre-treated seed exposed to $CdCl_2$

This assay was performed to determine the antioxidant activity in fenugreek seeds treated with different concentrations (50ppm, 100 ppm and 200ppm) of $CdCl_2$, seeds treated with different concentrations (200µM, 400µM and 500µM) of SNP and seeds pre -treated with different concentration of SNP. The scavenging activity was found to be more in 50 ppm $CdCl_2$ treated seed, and the scavenging activity decreased with the increase in $CdCl_2$ concentrations (100ppm and 200ppm). The scavenging activity was found to be more in 200µM SNP treated seeds but it decreases with the increased concentration (400µM and 500µM) as shown in Table 6. Seeds pre-treated with different concentrations (200µM,400µM and 500µM) of SNP exposed to $CdCl_2$ (100ppm) did not show any scavenging activity, as shown in Table 6.

Table 6: H_2O_2 radical scavenging assay in seeds treated with different concentration of $CdCl_2$, SNP and pre-treated seed exposed to $CdCl_2$

Conentration.	Scavenging activity (%)
Control (D.H_2O)	68.62
50ppm $CdCl_2$	71.34
100ppm $CdCl_2$	52.93
200ppm $CdCl_2$	47.27
200µM SNP	48.42
400µM SNP	24.85
500µM SNP	16.97
200µM SNP + 100ppm $CdCl_2$	-
400µM SNP + 100ppm $CdCl_2$	-
500µM SNP + 100ppm $CdCl_2$	-

CONCLUSION

In this study, about 25 fenugreek seeds were surface sterilized and taken into observation. Standardization of techniques such as estimation of germination percentage, phytotoxicity percentage, protein and proline content and antioxidant activity have been done to facilitate phytochemical analysis of the fenugreek seeds their effect on treating with the heavy metal, Cadmium chloride. Seeds pre-treated with Sodium nitroprusside(SNP) were exposed to Cadmium chloride and estimated for scavenging assay .In case of control, germination percentage of seed was found to be less, than in seeds treated with $CdCl_2$. The phytotoxicity percentage showed a marked increased with increase in metal concentration due to increased toxicity of $CdCl_2$. In seeds treated with SNP, the protein concentration were found to increase with the increased in SNP concentration, however the proline accumulation was found to be more in 200µM SNP and it decreased with increased in SNP concentration. SNP pre-treated seeds in 200µM and 400µM showed a gradual increase in the germination percentage but with 500µM, a decrease in germination percentage when exposed to $CdCl_2$ was observed. Likewise, when the seeds were exposed to 100ppm of $CdCl_2$, the average growth of the radical increased with increased concentration of SNP but at 500mM of SNP showed a gradual

decrease. Similar observations were seen with phytotoxicity percentage. In terms of antioxidant assay, methods including DPPH method and H2O2 radical scavenging method were used. In DPPH free radical scavenging method, the scavenging activity was found to be more in 400µM SNP treated seeds but there was linear decrease in the scavenging activity of 500µM SNP treated seeds. The scavenging activity of 200µM SNP treated seed was found to be less than 400µM SNP treated seeds. The scavenging activity of SNP pre-treated seed exposed to $CdCl_2$ was found to be more for 400µM SNP + 100ppm $CdCl_2$ and less in 200µM SNP + 100ppm $CdCl_2$ and 500µM SNP + 100ppm $CdCl_2$ respectively while in H2O2 radical scavenging assay, the scavenging activity was found to be more in 50 ppm $CdCl_2$ treated seed, and the scavenging activity decreased with the increase in $CdCl_2$ concentrations (100ppm and 200ppm). The scavenging activity was found to be more in 200µM SNP treated seeds but it decreases with the increased concentration (400µM and 500µM). Seeds pre-treated with different concentrations (200µM,400µM and 500µM) of SNP exposed to $CdCl_2$ (100ppm) did not show any scavenging activity.

Our observation in this study regards the possibility of increase in germination percentage and decreased level of phytotoxicity percentage in fenugreek seeds pretreated with SNP exposed to $CdCl_2$. In this experiment, we observed increase in scavenging activity of $CdCl_2$ exposed fenugreek seeds treated with SNP suggesting improvement and restoration of various factors of the seed lost due to metal toxicity.

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