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EFFECTS OF EXOGENOUS NITRIC OXIDE ON GERMINATION AND CARBOHYDRATES MOBILIZATION IN ALFALFA SEEDLINGS UNDER CADMIUM STRESS

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Abstract: Alfalfa is widely used as cattle food and as ornamental plant, it is also known to be a bio-accumulator and extremely resistant to heavy metal stress; however there has been a little information about the physiological and biochemical aspects involved in its germination once submitted to Cadmium stress. The aim of this study was to investigate the methods of improving stress resistance and ability of alfalfa seedlings germinated under Cadmium (Cd) stress by applying exogenous Sodium Nitroprusside (SNP) as Nitric oxide donor. Physiological indexes of alfalfa seeds treated by SNP under Cd stress like germination percentage, germination index, shoot length and root length were measured. Mobilization of carbohydrate reserves have been assessed through quantification of soluble proteins, soluble starch, as well as activity of α and β-amylases. Germination parameters of alfalfa seeds treated under 30µmol Cd stress underwent a drastic inhibition. After treatment with different concentrations of SNP every germination indexes were all increased and the seeds that were treated with Cd+200µmol SNP had the most significantly increase ($p \leq 0.05$) in every germination parameter as well as carbohydrate mobilization. SNP 200µmol/L could significantly alleviate damages to seeds and seedlings of alfalfa under Cd 30µmol/L stress and promote Cd-resistance of seeds and seedlings during germination.

Keywords: Alfalfa, Germination, Nitric Oxide, Carbohydrate mobilization, Cadmium stress.

Abbreviations: SNP (Sodium Nitroprusside); Cd (cadmium); NO (Nitric oxide); ANOVA (Analysis of variance); Ck (Control).

Introduction

Seed germination and seedling growth are the most important phases in lifecycle of Kingdom Plantae. Germination is the strongest period and the basis of life activity in all life periods of plant formation where the metabolism of fats, proteins and carbohydrates provide substance and energy to seedlings growth [1].

Seeds as well as seedlings are extremely vulnerable to both biotic and abiotic stresses. Among these environment stresses Cadmium (Cd) has been reported to impair morphological and physiological changes in plant [2]. Germinating seedlings exposed to Cd stress causes the disorder in carbohydrate mobilization [3]. Cd in germinating seedlings has been reported to

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delay the germination, induce membrane damage, impair food reserve mobilization and enhance electrical conductivity [4, 5]. Germinating seedlings exposed to Cd stress showed a deleterious effect on root and shoot growth as well as on germination energy [6].

Nitric Oxide (NO) is a gaseous, small, reactive molecule that readily diffuses the cell and interacts with different cellular compounds [7]. NO is an important endogenous plant bioactive signaling molecule having a key function in various processes of plant growth and development including seed dormancy, seed germination , primary and lateral root growth, floral transition, flowering, stomatal movement, photosynthesis, mitochondrial functionality, senescence, plant metabolism and cell death as well as stress response [8]. Sodium Nitroprusside (SNP) as NO donor regulates the growth of germinating mug submitted to Arsenic stress by improving plasma membrane integrity, reducing arsenic uptake and improving activity of some hydrolytic enzymes [9]. At low concentrations, NO alleviates toxicity effects of Cd by increasing macro and micro nutrients, decreasing root-to- shoot translocation of Cd and by protecting the plant from oxidative stress. But at higher concentration of NO there is no significant effect [10].

Applying plants such as METICAGO SATIVA L. (Alfalfa) has been recently seen as one of environmental friendly technique for heavy metal rehabilitation [11, 12]. Alfalfa has been reported to be extremely resistant to contaminants as well as bio-accumulator[13, 14], the role of exogenous NO on alleviating heavy metal toxicity has been elucidated in recent studies, however the influence of exogenous NO on germination of alfalfa seedlings under Cd stress has not been understood yet.

In this study we provided the evidences that exogenous NO could significantly influence MEDICAGO SATIVA L (Alfalfa) seed germination under Cd stress. Also, preliminary mechanisms were investigated.

Materials and Methods

Plant materials and growth conditions

Alfalfa seeds were surface sterilized with 2% Sodium Hypochlorite for 5 minutes, and then thoroughly rinsed with distilled water. Seeds were germinated in 10-cm petri dishes on sheets of filter papers and moistened according to the following experimental design [Control, with only distilled water], [30µmol Cd, indicated by Cd], [Cd+25µmol SNP], [Cd+50µmol SNP], [Cd+100µmol SNP], [Cd+200µmol SNP], [Cd+300µmol SNP], [Cd+400µmol SNP], [Cd+500µmol SNP]. Treatments were replicated 3 times and the nutrients were renewed every day. The germination was carried out at 30^{o}C with 16 hours light and 8 hours dark [15],

and every 24 hours the germinated seeds in petri dishes were removed from incubator for various analyses. Non-germinated or unevenly germinated seeds were discarded.30µmol Cd was taken after pre-experiments where at this concentration, germinated seeds were at least half compared with seeds in control.

Germination parameters

Germination percentage (GP) was defined as the number of germinated seeds divided by the total number of sown seeds multiplied by one hundred percent [16].GP= $\frac{Germinated\ seeds\ X\ 100}{Total\ number\ of\ seeds}\%$

Seedling Vigor index (SVI) was determined as seedling length multiplied by germination percentage [17]. $SVI = Seedling\ length\ X\ GP.$

Germination Index (G.I) was calculated by using the formula proposed by Gupta [18]. G.I is obtained by dividing the number of seedlings emerging on day divided by day after plant.

G.I= $\sum_{i=1}^{n} Gt/\Delta t$

Determination of α-amylase and β-amylase

Amylase activity was analyzed according to Xiangnan Li [19]. In brief, 1 g of germinating seeds was macerated in 10 ml ice-cold distilled water at 4^{o}C in a pre-chilled mortal. The extract was ten centrifuged at 15,009rmp for 30 min at 4^{o}C.The supernatant was collected to estimate α and β-amylase activities.

Activity of α-amylase was assayed after inactivating β-amylase activity at high temperature. In brief, 5ml of enzyme extract was mixed with 3ml of 3mM $CaCl_2$ and incubated at 70^{o}C for 5 min. Then, 2 ml reaction mixture containing 0.1g mM citrate buffer (pH=5), 2% soluble starch solution and 0.7 ml hot enzyme extract was incubated at 30^{o}C for 5 min and the reaction was stopped by adding 2m of color reagent. The mixture was heated at 50^{o}C for 5 min and the final volume was made up to 10 ml with distilled water. The activity was determined by using spectrophotometer at 540nm. (Color reagent was obtained by dissolving 1 g 3,5-dinitrosalicylic acid in 20 ml of 2M NaOH and 30 g Potassium Sodium tartrate , and then the volume was adjusted to 100 ml with distilled water).

Activity of β-amylase was estimated after inactivating α-amylase at low pH of 3.4 with 0.1 M EDTA. 2ml reaction solution contained 0.1mM citrate buffer (pH= 3.4), 2% soluble starch and 0.7 ml EDTA treated enzyme extract. The mixture was incubated for 5 min at 30^{o}C. To stop the reaction, 2ml of color reagent were added and activity of β-amylase was then assayed following the same method of α-amylase activity analysis described above.

Determination of Starch content and soluble proteins

Starch content was assayed by using anthrone reagent as described by Pavan in 2013 with some modifications[20]. Briefly 50 mg of seedling powder were homogenized in 5ml 80% ethanol then centrifuged at 4000rpm for 20 min. The supernatant was removed for other treatments. The residues were re-extracted in the same way as before. The residues left were suspended with 5ml distilled water and 6.5ml 52%cold perchloric acid (4^{o}C) for 12 hours. After, they were centrifuged at 7000rpm for 20 min at 4^{o}C. The supernatant was collected in 10ml volumetric flask. Extraction was repeated with 2.5ml of distilled water and 6.5ml of 52% cold perchloric acid at 4^{o}C and centrifuged as above. Both supernatants were combined and the final volume was made up to 100 ml. To 0.5 ml of aliquot 10ml of anthrone reagent was added to each test tube, allowing the reagents to run down the side of the test tube. Mixed well and placed in boiling water bath for exactly 8min after which it was cooled to room temperature with ice water. The absorbance was measured spectrophotometrically at 630nm. The amount of starch was calculated using a standard curve prepared from glucose. Soluble proteins were assayed after Bradford method [21].

Data analysis

All the data presented here are the mean values of three independent experiments with three replicates. Statistical analyses were performed by analysis of variance (ANOVA) and means are compared by Duncan's multiple range test ($p \leq 0.05$) using Microsoft excel.

Hypocotyl length, Root length and Germination percentage

Compared with control, the data presented in table 2 indicate that for Cd treated seedlings there is a significant decrease ($p \leq 0.05$) in root length, hypocotyl length. After treatment with different concentrations of SNP, the increase in root and hypocotyl length was dose-dependent where the optimum increase was observed at seedlings treated with Cd+200µmol SNP. In these conditions, roots and hypocotyls increased at 52.44% and 62.8%respectively. For the concentrations above optimum, there was a non-significant decrease ($p \leq 0.05$).

Germinated seedlings treated with only Cd had significantly inhibitory effect in germination percentage ($p \leq 0.05$) with 24.09% inhibitory effect compared with seedlings in the control. Application of SNP improved significantly ($p \leq 0.05$) seed germination in dose-dependent manner, where the highest germination percentage was increased to 17.07% for seedlings treated with Cd+200µmol SNP compared with the one treated with only cd. The concentrations above 200µmol SNP showed a non-significant decrease in germination percentage ($p \leq 0.05$).

	Root length (mm)	Hypocotyl length (mm)
	Day 10	Day 10
Ck	68.00*±2.00	26.00*±1.73
30µmol Cd	5.66±0.57	10.66±1.15
Cd+25 µmol SNP	15.33*±1.52	12.33±0.57
Cd+50 µmol SNP	16.00*±4.00	13.66±1.52
Cd+100 µmol SNP	31.66*±1.52	13.66±1.52
Cd+200 µmol SNP	35.66*[a]±0.57	18.00*[a]±1.00
Cd+300 µmol SNP	32.33*±1.54	13.66±1.52
Cd+400 µmol SNP	29.33*±2.08	13.33±1.52
Cd+500 µmol SNP	28.66*±1.54	13.00±1.73

Effect of NO on root and shoot length (mm); Data are means ± SD (n=3), and post-test analysis are done by Duncan's multiple range test (P≤0.05) using Microsoft Excel. *indicate that there is a statistical significant difference compared with seedlings treated with only Cd, a means there is no statistical significance compared with Ck.

Seedling Vigor Index (SVI)

In comparison with seedlings germinated in control (Ck), there was a significant decrease ($p \leq 0.05$) of seedling vigor index for seedlings treated with Cd; we observed the reduction of 86.4%. Application of different concentrations of SNP the seedlings showed a dose-dependent significant increase in SVI. The optimal value was recorded at seedlings treated with 200µmol SNP with increment of 38.8% compared to seedlings treated with only Cd. For the concentrations above 200µmol SNP there was a non-significant decrease in SVI ($p \leq 0.05$).

Germination Index

In Cd+ SNP treated seedlings, the results revealed that there was a linear and positive correlation between germinated seedlings and germination period. Germination Index increased with increasing concentration of SNP up to 200µmol where we observed a non-significant decrease at ($p \leq 0.05$).

	G.P	**SVI**	**G.I**
	Day 10	Day 10	Count days : 1,2,3,4
Ck	92.22±6.93	2602.00*±222.99	43.30*±3.25
30µmol Cd	70.00±11.54	342.33±50.83	25.44±3.84
Cd+25 µmol SNP	74.44±5.09	617.00*±46.89	29.22±3.13
Cd+50 µmol SNP	80.00*±0.00	712.00*±113.41	31.72*±4.76

Cd+100 µmol SNP	82.22*±3.84	1117.33*±53.26	33.00*±3.46
Cd+200 µmol SNP	86.66*[a]±3.33	1352.00*±68.03	39.11*[a]±4.45
Cd+300 µmol SNP	82.22*±3.84	1136.00*±108.22	36.72*±2.43
Cd+400 µmol SNP	80.66*±0.00	1027.33*±153.14	35.88*±2.47
Cd+500 µmol SNP	78.88*±5.09	988.33*±124.93	34.52*±1.50

*Effect of Nitric Oxide on Germination Percentage (G.P), Seedling Vigor Index (SVI) and Germination Index (G.I). Data are means ± SD (n=3); and post-test analysis are done by Duncan's multiple range test (P≤0.05) using Microsoft Excel; *indicate that there is a statistical significant difference compared with seedlings treated with only Cd, the letter "a" means there is no statistical significance compared with Ck..*

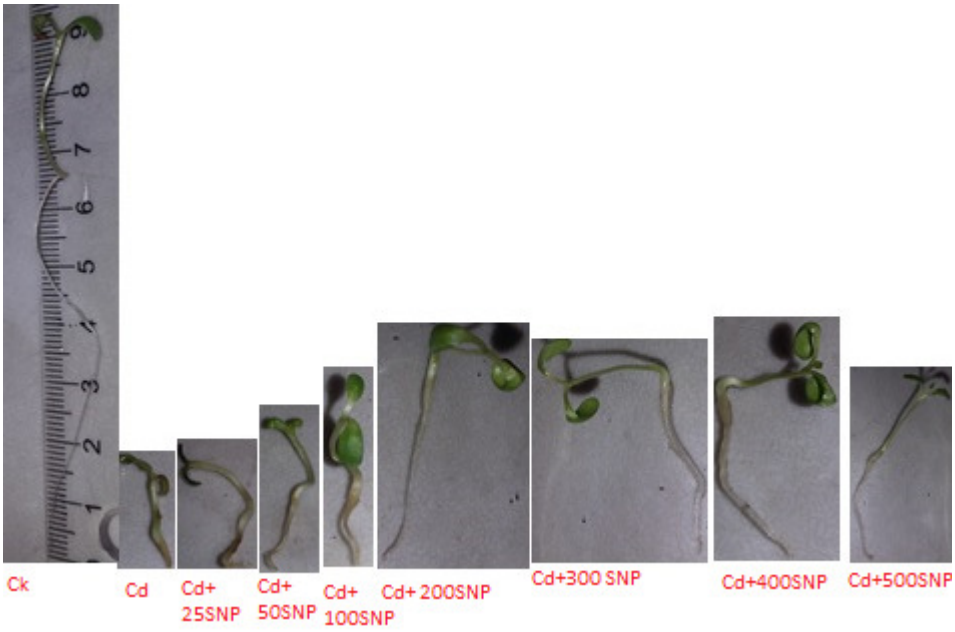

Figure1. Effect of different concentrations of SNP on germination and seedling growth of alfalfa under 30µmol Cd stress (10 days seedlings).

Soluble proteins content

The mobilization of soluble proteins gets the maximum value on the 2nd day after imbibition for all treatments, but however declines during the following days. Again for seedlings treated with Cd alone, soluble proteins were significantly less mobilized ($p \leq 0.05$) whereas for those treated with Cd+ different concentrations of SNP the mobilization of soluble proteins was in dose-dependent manner. As compared with Ck, the mobilization of proteins in seedlings treated with Cd alone declined 46.08% and it went increasingly in seedlings treated with Cd+ SNP. The optimum mobilization was observed on 2nd day in seedlings treated with Cd+200µmolSNP at 86.93% as compared with Ck.

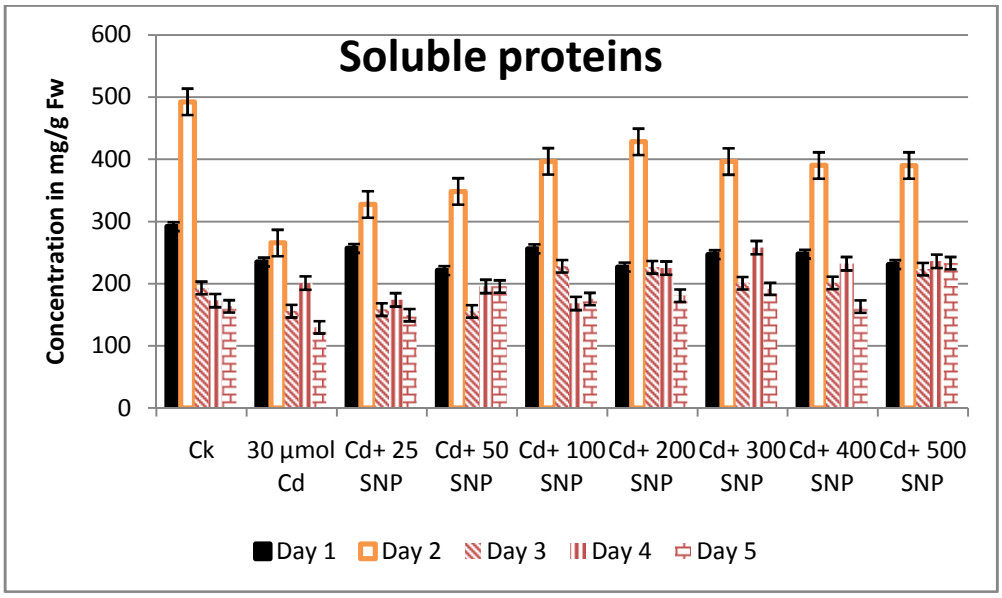

Fig.2. Effect of Nitric Oxide on soluble proteins; data are means ± SD (n=3); and post-test analysis were done by Duncan's multiple range test ($p \leq 0.05$) using Microsoft Excel.

Content of starch soluble

Generally there was no significant difference ($p \leq 0.05$) in mobilization rates of starch during germination and seedling growth for all treatments. The highest mobilization was observed on 2nd day after imbibition except for all treated seedlings, the least mobilization was observed in seedlings treated with only Cd. Seedlings treated with only Cd declined 13.6% on the first day and 14.33% on the second daycompared with seedlings in Ck. Application of SNP increased starch soluble in dose-dependent manner where the optimum values were obtained among seedlings treated with Cd+200µmol SNP. At this concentration we observed 11.55% and 13.92% increment as compared with seedlings treated only with Cd.

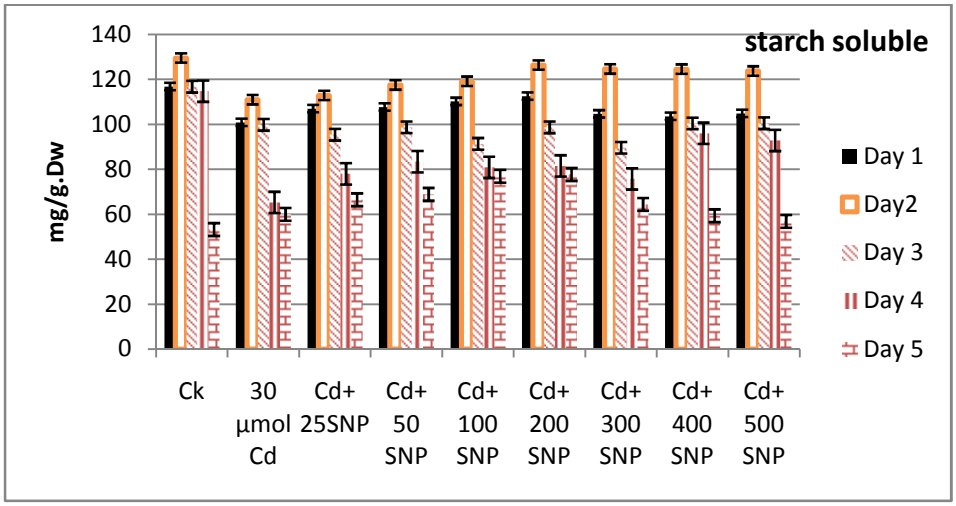

Fig.3. Effect of Nitric Oxide on the content of starch soluble; data are means ± SD (n=3); and post-test analysis were done by Duncan's multiple range test ($p \leq 0.05$) using Microsoft Excel.

Amylase activity

Amylase activity is shown in figures 4 and 5. For seedlings treated with only Cd, amylase activities were lower than all other treatments and the significant difference ($p \leq 0.05$) was observed on the 4th day of treatment. Generally for α-amylases, their activities were continuously increasing up to the 4th day after which a slight decrease on 5th day of treatment was observed. For β-amylases, we observed increment and decline during days of treatments. On the 2nd day there was an increase in all treatments but the activity in seedlings treated with only cd declined by 26.07% as compared with Ck. On this 2nd day, seedlings treated with Cd+ SNP had no significant effect on β-amylase activity compared with seedlings treated with only Cd. On 4th day of treatment, both α and β-amylase activities increased. The highest activity was observed in seedlings treated with Cd+200µmol SNP where they were 15.32% for α-amylase and 20.66% for β-amylase greater than seedlings in Ck.

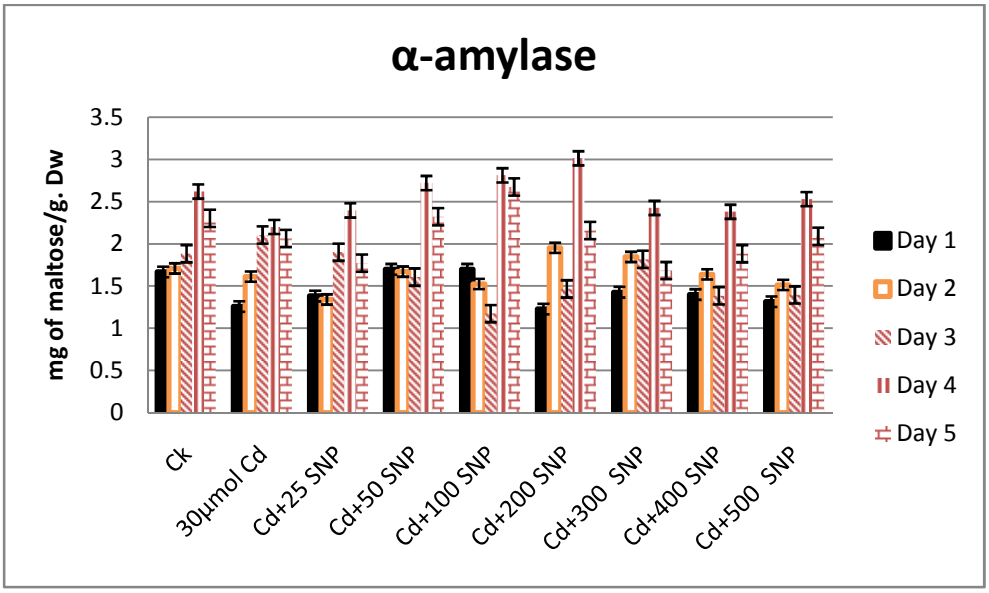

Fig.4. *Effect of Nitric Oxide on α-amylase activity. Data are means ± SD (n=3); post-test analysis are done by Duncan's multiple range test ($p \leq 0.05$) using Microsoft Excel.*

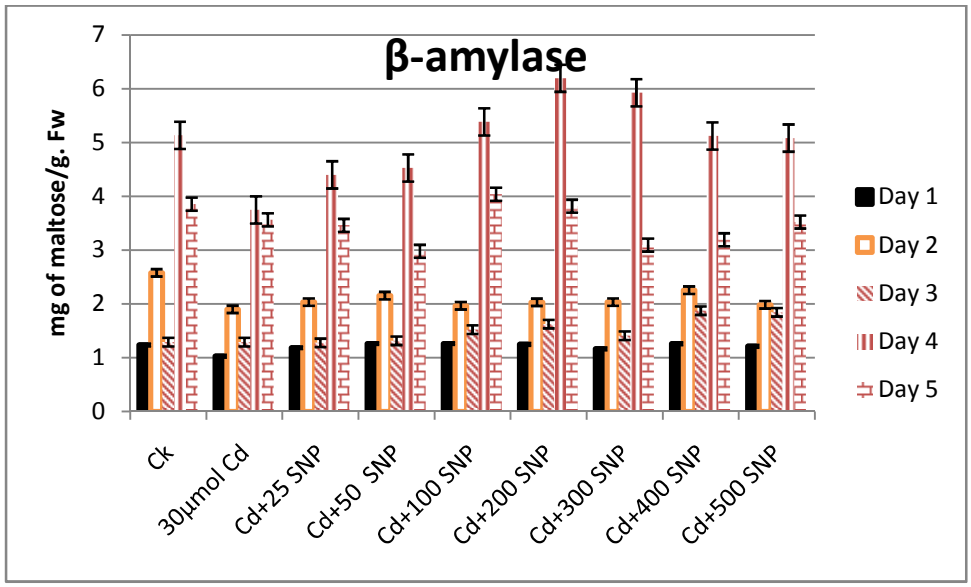

Fig.5. Effect of Nitric Oxide on β-amylase activity. Data are means ± SD (n=3); post-test analysis are done by Duncan's multiple range test ($p \leq 0.05$) using Microsoft Excel.

Discussion

Effect of exogenous NO on germination percentage, germination Index, seedling vigor, and root &shoot length of alfalfa seedling under Cd stress

Germination and early seedling development are more likely to be adversely affected by heavy metals.

In the present investigation, Cadmium treatment decreased significantly the process of seed germination, seedling growth, germination index and seedling vigor index. The decrease in germination parameters can be attributed to the accelerated breakdown of stored food materials in seeds and to the selection permeability properties of cell membrane [22]. During our experiments, a decrease of seed germination, seedling growth, seedling vigor index and germination index due to Cd treatment was in conformity with previous findings. [23], [24], [25].

However the detoxifying effect of exogenous NO has been observed after seedlings under 30µmol Cd stress were subjected to various concentrations of SNP. A dose-dependent application of SNP as a NO donor alleviated the inhibitory effects caused by Cd stress. The optimal concentration of SNP was observed at 200µmol/l. This was described as ability of exogenous NO to counteract the inhibitory effect in germination index, vigor index, shoot length and hypocotyl length caused by Cd stress. (Figure 1, Table 1&2). This was in the conformity with previous researches on the role of exogenous NO as a signal molecule and its ability to counteract the inhibitory effect of abiotic stress in plant [26]. NO attenuated the

inhibition effect of germination and seedling growth of rice (Oryza sativa .L) submitted to Cd stress [27], in sesame seedlings (Sesamumindicum L), NO increased germination percentage, vigor index and the growth of seedlings submitted to Cd stress [28]. Similar roles of SNP, a NO donor have been elucidated in reduced metal uptake and membrane injury upon application of SNP in Cd stressed rice seedlings [29], in germination and antioxidase activities of Perillafrutencens seedlings under NaCl stress[30], and on germination of Senna macranthera seeds under salt stress [31].

Effect of Exogenous NO on mobilization of soluble starch, soluble proteins and amylase activity in alfalfa seedlings under Cd stress

Germination process is characterized by reserves mobilization such as carbohydrates and proteins by hydrolytic enzymes where the newly formed products are used in formation of new structures [32].

These reserve materials are made available by hydrolytic enzymes to deliver fuel for respiration and various anabolic reactions in the form of anabolites [33]. Enzymes accountable for starch and proteins breakdown and mobilization of these reserves materials are α and β-amylases [34].

It has been reported that Cd causes a restriction in reserve mobilization [5]. This is in accordance with our study where the average total α and β-amylase activities were significantly depressed by only Cd 30µmol/L. In these conditions, the quantities of soluble proteins and starch also have reduced drastically after the 2nd day of treatment. This is the major factor in the depression of seed germination.

However, our study revealed that application of exogenous NO had a strong stimulating effect on germination of alfalfa seedlings under 30µmol Cd stress **[Fig 1].** Activity of amylases, soluble proteins and starch were highly significant as compared with the one in seedlings treated with only Cd. Our findings were compatible with recent studies made on impact of exogenous NO on activity of amylases in germinating pea seeds submitted to Cd stress [35]. After application of exogenous NO, activity of both α and β-amylases were significantly enhanced **[Fig 4&5];** on the 2nd day of treatment, the content of both soluble starch and proteins were higher than the one found in seedlings treated with only Cd. This benefited and ameliorated the germination of alfalfa seedlings despite being submitted to Cd stress.

Conclusion

In a nutshell, the detoxifying effect of exogenous NO on germination of alfalfa seedlings submitted to Cd 30µmol/L stress. The study revealed that SNP at200µmol/L germination conditions were significantly ameliorated, α-amylase and β-amylase activities were enhanced and carbohydrates were moderately degraded. Further researches are recommended to elucidate genetic and proteomic analyses and additional physiological approaches to understand the details of exogenous nitric Oxide in metabolic functions in plant. This will provide further understanding on this multifaceted compound once applied to different species of plants submitted to abiotic stresses.

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Bibliography

[1] Zhou Li, Y.P., Xin-Quan Zhang, Xiao Ma, Lin-Kai Huang and Yan-Hong Yan, *Exogenous Spermidine Improves Seed Germination of WhiteClover under Water Stress via Involvement in Starch Metabolism, Antioxidant Defenses and Relevant Gene Expression.* Molecules, 2014, 19, 18003-18024; doi:10.3390/molecules191118003. **19**: p. 18003-18024.

[2] B. Ali1, X.D., X. Hu 2, R. A. Gill 1, S. Ali 3, S. Wang 4, and W. Zhou 1,2, *Deteriorative Effects of Cadmium Stress on Antioxidant System and Cellular Structure in Germinating Seeds of Brassica napus L.* . J. Agr. Sci. Tech. , 2015. **17**: p. J. Agr. Sci. Tech. (2015) Vol. 17: 63-74.

[3] S. Rahoui, A.C., and E. El Ferjani, *RESERVE MOBILIZATION DISORDER IN GERMINATING SEEDS OF VICIA FABA L. EXPOSED TO CADMIUM.* Journal of Plant Nutrition, 2010. **33**: p. 809–817,.

[4] Amira Sfaxi-Bousbih, A., EzzedineElFerjani n, *Cadmium impairs mineral and carbohydrate mobilization during the germination of bean seeds.* Ecotoxicology andEnvironmentalSafety, 2010. **73**: p. 1123–1129.

[5] Sondès Rahoui, A.C., Ezzeddine El Ferjani∗, *Membrane damage and solute leakage from germinating pea seed under cadmium stress.* Journal of Hazardous Materials, 2010. **178**: p. 1128–1131.

[6] IFTIKHAR AHMAD1*, M.J.A., ZAHIR AHMAD ZAHIR1 AND AMER JAMIL2, *EFFECT OF CADMIUM ON SEED GERMINATION AND SEEDLING GROWTH OF FOUR WHEAT (TRITICUM AESTIVUM L.) CULTIVARS.* Pak. J. Bot., 2012. **44(5)**: p. 569-1574.

[7] Fernanda S. Farnese1*, P.E.M.-S., Grasielle S. Gusman2 and and J.A. Oliveira3, *When Bad Guys Become Good Ones: The Key Role of Reactive Oxygen Species and Nitric Oxide in the Plant Responses to Abiotic Stress.* FrontiersinPlantScience | www.frontiersin.org, 2016. **7**.

[8] Parvaiz Ahmad, A.A.A.L., Abeer Hashem, Elsayed F. Abd_Allah, and Salih Gucel and Lam-Son P. Tran, *Nitric Oxide Mitigates Salt Stress by Regulating Levels of Osmolytes and Antioxidant Enzymes in Chickpea.* Frontiers in Plant Science | www.frontiersin.org, 2016. **7**.

[9] Ismai, G.S.M., *Protective role of nitric oxide against arsenic-induced damages in germinating mung bean seeds.* Acta Physiol Plant, 2012. **34**: p. 1303–1311.

[10] •, Q.W.X.L.Y.D., L.X.X.Z.J.H. •, and Z. Fan, *Effects of exogenous nitric oxide on cadmium toxicity, element contents and antioxidative system in perennial ryegrass.* Plant Growth Regul, 2013. **69**: p. 11–20.

[11] LAI LA M.H. ABUSRIWIL, H.E.A.F., BAYOUMI HAMUDA, ALAELDDIN A, ELFO UGHI, *Seed germination , growth and metal uptake of Meticago Sativa L. gown in heavy metal contaminated clay loam brown forest soil.* Tájökológiai Lapok 2011. **89**: p. 111–125.

[12] Karimi, N., *Comparative Phytoremediation of Chromium-Contaminated Soils by Alfalfa (Medicago sativa) and Sorghum bicolor (L) Moench.* International Journal of Scientific Research in Environmental Sciences (IJSRES), 2013. **1(3)**: p. 44-49.

[13] Fu-qiang Wang, Yu-juan Li, Qian Zhang, Jiao Qu, *Phytoremediation of Cadmium , Lead and Zinc by MEDICAGO SATIVA (Alfalfa) : A study of different period.* Bulgarian Chemical Communications, 2015. **47**: p. 167 - 172.

[14] Gao Zhanwu, Z.H., Gao Jicai, Yang Chunwu1, Mu Chunsheng and Wang Deli, *Germination responses of Alfalfa (Medicago sativa L.) seeds to various salt–alkaline mixed stress.* African Journal of Agricultural Research, 2011. **6(16)**: p. 3793-3803.

[15] Chekuboyina Ravi Kiran, D.B.R., Nagala Sirisha, Tamanam Raghava Rao, *Impact of Germination on Biochemical and Antioxidant Enzymes of Ceiba pentandra (Kapok) Seeds.* American Journal of Plant Sciences, 2012, 3, 1187-1192. **3**: p. 1187-1192.

[16] Dugald C. Closea, Stephen J. Wilsonb, *Provenance effects on pre-germination treatments for Eucalyptus regnans and E. delegatensis seed.* Forest Ecology and Management, 2002. **170**: p. 299–305.

[17] Abdul Baki, A.a.J.D.A., *Vigor determination in soybean seed by multiple criteria.* Crop Sci, 1973. **13**: p. 630-633.

[18] et.al, G., *Insect cuticle and yeast extract effects on germination, growth, and production of hydrolytic enzymes by Nomuraea rileyi.* Mycopathologia, 1993. **122(3)**: p. 143-147.

[19] Jiang, X.L.H.J.F.L.J.C.T.D.W.C.D., *Induction of chilling tolerance in wheat during germination by pre-soaking seed with nitric oxide and gibberellin.* Plant Growth Regul, 2013. **71**: p. 31-40.

[20] Pavan, *DETERMINATION OF TOTAL PROTEIN CONTENT AND SOLUBLE SUGAR IN WHEAT CROP.* IJRST, 2013. **2**.

[21] BRADFORD, M.M., *A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding.* ANALYTICAL BIOCHEMISTRY, 1976. **72**: p. 248-254.

[22] 1Muhammad, S.I., M. Zafar; 2Mohammad, Athar, *Effect of Lead and Cadmium on Germination and Seedling Growth of Leucaena leucocephala.* Journal of Applied Sciences and Environmental Management, 2008. **12**: p. 61-66.

[23] IFTIKHAR AHMAD, M.J.A., ZAHIR AHMAD ZAHIR1 AND AMER JAMIL2, *EFFECT OF CADMIUM ON SEED GERMINATION AND SEEDLING GROWTH OF FOUR WHEAT (TRITICUM AESTIVUM L.) CULTIVARS.* Pak. J. Bot., 2012. **44(5)**: p. 1569-1574.

[24] HE Jun-yu, R.Y.-f., ZHU Cheng, JIANG De-an, *Effects of Cadmium Stress on Seed Germination, Seedling Growth and Seed Amylase Activities in Rice (Oryza sativa).* Rice Science, 2008, 15(4): 319–325, 2008. **15(4)**: p. 319–325.

[25] M. Keerthi Kumari, D.V., D. Narasimham, K. Paramesh and T. Chandrasekhar, *IMPACTS OF CADMIUM AND MANGANESE ON IN VITRO SEED GERMINATION AND SEEDLING GROWTH OF HORSEGRAM.* Indian Journal of Plant Sciences, 2016. **5(1)**: p. 2319–3824.

[26] Nurun Nahar Fancy, A.-K.B., Gary J. Loake, *Nitric oxide function in plant abiotic stress.* Plant, Cell and Environment, 2016.

[27] JunyuHe n, Y., Xiulan Chen, HuiChen, *Protective roles of nitricoxide on seed germination and seedling growth of rice (Oryza sativa L.) undercadmiumstress.* EcotoxicologyandEnvironmentalSafety, 2014. **108**: p. 114-119.

[28] Raquel Maria de Oliveira Pires, G.A.d.S., Amanda Ávila Cardoso, Denise Cunha Fernandes dos Santos Dias, Eduardo Euclydes de Lima e Borges5, *Action of nitric oxide in*

sesame seeds (Sesamum indicum L.) submitted to stress by cadmium1. Journal of Seed Science, 2016. **38(1)**: p. 022-029.

[29] Prerna Singh a, K.S.b., *, *Evidences for reduced metal-uptake and membrane injury upon application of nitric oxide donor in cadmium stressed rice seedlings.* Plant Physiology and Biochemistry, 2014. **83**: p. 180-184.

[30] Zhang CP, H.P., Du DD, Wei PX, Yu ZL, Xie YZ, Liu HY., *[Effect of exogenous nitric oxide donor SNP on seed germination and antioxidase activities of Perilla frutescens seedlings under NaCl stress.* China journal of chinese Materia Medica, 2011. **34(5)**: p. 665-9.

[31] Aparecida Leonir da Silva, D.C.F.d.S.D., Eduardo Euclydes de Lima e Borges, Dimas Mendes Ribeiro, Laércio Junio da Silva, *Effect of sodium nitroprusside (SNP) on the germination of Senna macranthera seeds (DC. ex Collad.) H. S. Irwin & Baneby under salt stress1*. Journal of Seed Science, 2015. **37(4)**: p. 236-243.

[32] NARA L.M. ALENCAR, RENATO INNECCO, ENÉAS GOMES-FILHO, MARIA IZABEL GALLÃO, JUAN C. ALVAREZ-PIZARRO, JOSÉ T. PRISCO and ALEXANDRE B. DE OLIVEIRA, *Seed reserve composition and mobilization during germination and early seedling establishment of Cereus jamacaru D.C. ssp. jamacaru (Cactaceae).* Annals of the Brazilian Academy of Sciences, 2011. **84(3)**: p. -832.

[33] BorgesII, G.d.M.A.E.E.d.L.e. and A.V. FloresIII, *Enzymatic activity in braúna seeds subjected to thermal stress.* Ciência Rural, Santa Maria, 2016. **4(6)**: p. 1044-1049.

[34] al., S.e., *Exogenous Application of Ethylenediaminetetraacetic Acid and Oxalic Acid Improve the Seed Germination and Enzymes Activities of Sunflower (Helianthus annuus) Under Cadmium Stress.* INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY, 2015. **17(6)**: p. 1117–1124.

[35] Sawhney, L.K.C.S.K., *EFFECT OF CADMIUM ON GERMINATION, AMYLASES AND RATE OF RESPIRATION OF GERMINATING PEA SEEDS.* Environmental Pollution, 1996. **92 (1)**: p. 1 5,.