



An Investigation into the Effects of Cadmium and Nitric Oxide on Induced Secondary Metabolism and Antioxidant System in *Pimpinella anisum* L. Through Transcriptional Upregulations in AIS1, PAL, SOD, R2R3-MYB, and bZIP Genes

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Abstract

This study was aimed to evaluate the cytoprotective role of nitric oxide (NO) against cadmium (Cd) through the potential transcriptional modifications in the expression of genes. *Pimpinella anisum* seedlings were treated with Cd (0 and 1 mM) and/or NO (0 and 25 μ M). The Cd-associated decreases in shoot and root biomass were mitigated by the exogenously applied NO. Proline concentrations were increased in response to NO and/or Cd. Cd and NO treatments caused a considerable increase in peroxidase activities in comparison to the control. The individual Cd treatment led to the slight significant ($p \leq 0.001$) up-regulation in expression of R2R3-MYB transcription factor by 2.5 folds, while the simultaneous exposure to NO and Cd stimulated this gene by 7.2 folds ($p \leq 0.0001$). With a similar trend, NO significantly ($p \leq 0.0001$) upregulated bZIP transcription factor by 5.9 folds in Cd-treated seedlings. Likewise, upregulation (5.2 folds) in the significant ($p \leq 0.0001$) increase in expression of the phenylalanine ammonia-lyase (PAL) gene resulted from the NO+Cd application. Moreover, t-anol/isoeugenol synthase 1 (AIS1) gene was slightly stimulated ($p \leq 0.05$) in response to individual NO treatment. However, the Cd treatment resulted in a drastic significant ($p \leq 0.0001$) increase in AIS1 expression by 42 folds which was partly declined in the Cd+NO group and reached 22 folds ($p \leq 0.0001$). Moreover, the NO+Cd treatment caused significant ($p \leq 0.0001$) induction in the transcription of the superoxide dismutase (SOD) gene by 11.3 folds. The correlation observed among expression patterns of mentioned genes, therefore, highlights the close interplay between the transcription factors, secondary metabolism, and antioxidant system.

Keywords: Gene expression, Heavy metal, *Pimpinella anisum*, Secondary metabolism, Stress, Transcription factor

Introduction

Throughout the life cycle, most plants face a plethora of challenges among which heavy metal stress is one of the most important factors worldwide limiting plant growth and productivity [1-5]. High consumption of heavy metals in industrial activities (mining, disposal of municipal waste, pesticides, etc.) widely contaminate soil and water and increases the likelihood of entering these metal contaminants, especially cadmium (Cd) to plants (initiating living organism of the food chain) [2,4,6]. Among various heavy metal contaminants, Cd owing to its high mobility and bioavailability impairs cellular metabolism and function [5-6].

It is well known that nitric oxide (NO) as a gas-transmitter signaling molecule dependent on dose can govern plant responses to environmental factors [3, 7]. At the accurate concentrations, NO presents a cytoprotective role against oxidative stress [8-10]. It has been observed that the exogenously applied NO associated with improving plant protection against diverse stresses, like drought [11], salinity [12], heavy metals [13], and nanoparticles [3,9,14]. Data on the potential NO-mediated changes at transcriptional levels are rare and more scientific studies are, therefore, required [15].

Apart from constitutively-expressed genes whose expression is almost constant; most genes exhibit stimulus/signal-inducible expression [1]. In this regard,

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signal-mediated variations in concentrations of transcription factors, their posttranslational modifications, their subcellular localization, and subsequent interference with promoters are key regulatory mechanisms by which genes are transcriptionally regulated at promoter levels [10]. The family of MYBs is responsible for producing proteins with functioning as transcription factors containing MYB DNA-binding domains [16]. As key components in regulatory signaling networks, these transcription factors via interfering at promoter level are involved in signal transduction and regulation of plenty of important processes, thereby modulating plant reactions to internal and/or external cues [16, 10]. In addition to MYBs, the bZIP family of transcription factors is another vital transcriptional regulator that effectively contributes to the modulation of the plethora of biological processes from developmental reactions to stress responses [10,17-18].

Anise (*P. anisum*) belongs to the Apiaceae family. In addition to its function as a spice, this aromatic plant widely exploited in the modern pharmaceutical industry, traditional medicines, and food industries [19]. The nutritional and medicinal value of *P. anisum* is due to its great phytochemistry. Taking its chemical composition into account, anethole, limonene, p-anisaldehyde, estragole, acetophenone, and pinene are the most significant compounds. Various scientific studies in *P. anisum* have confirmed its enormous significant medicinal properties among which antidiabetic, anti-inflammatory, antispasmodic, antioxidant, antihysterical, expectorant, antidepressant, and antimicrobial are of critical importance in pharmaceutical and food industries [19].

Numerous studies imply that exogenous utilization of NO can enhance plant resistance to stress conditions. However, further molecular evidence is still needed to identify its action mechanism. Considering the

importance of transcription factors, we, therefore, attempted to monitor the possible transcriptional changes in bZIP and MYB following the Cd and/or NO treatments. Taking the secondary metabolism and antioxidant system into account, this study investigated the potential effects of NO and Cd through monitoring expression pattern of PAL, SOD, and AIS1 along with R2R3-MYB and bZIP genes in *P. anisum*. This study was aimed to evaluate the role of nitric oxide (NO) against cadmium (Cd) stress through the potential transcriptional modifications in the secondary metabolism and antioxidant system.

Material and Methods

Seeds of anise (*P. anisum*) were planted in the pot containing vermiculite and perlite (1:1). Seedlings were watered with Hoagland nutrient solution and water every other day. Sodium nitroprusside and cadmium nitrate were applied as sources of NO and Cd, respectively. One-month-old seedlings were treated with different doses of Cd (0 and 1 mM) and/or NO (0 and 25 μ M). Seedlings were sprayed with NO twice a week for two weeks. To treat seedlings with Cd, seedlings were irrigated with Hoagland solution supplemented with Cd at 0 and 1 mM (100 ml per pot) twice a week for two weeks. Seven days after the last treatments, plants were harvested and subjected to experimental analysis.

Monitoring Expression Patterns of Target Genes

To evaluate the transcriptions of target genes, RNA was extracted from the leaves by application of Trizol and its purity was estimated using Nanodrop (Thermo Scientific, 2000c). Next, complementary DNA (cDNA) was synthesized with PCR (PEQLAB, 96Grad). Three biological replicates of cDNA were used as the template for amplification.

Table 1 The primers of the target genes, including PAL, AIS1, bZIP, R2R3-MYB, SOD, and 18srRNA

Primer name	Seq 5'-3'	Tm	Amplicon (bp)
PAL-F1	GACATCTCCTCAATGGCTTG	57	120
PAL-R1	GCTTTGTTCCCTGGACACATC	57	
AIS1-F	CCGTTTCAAGAAGGGGTCTG	59	136
AIS1-R	CATCAGAGGGACGAAGCAAG	59	
bZIP-F	GGAAGCTGAAGTGGCAAAGC	59	157
bZIP-R	CCAAGGTCCAGTCACTGTCC	59	
MYB-F	GTTCTTGATTTCATTGTCTGTTCG	59	134
MYB-R	GACCTGATATCAAGAGAGGTAACAT	59	
18s-F	GGAGTATGGTCGCAAGGCTGAAAC	59	133
18s-R	CTCAATCTGTCAATCCTCACTATGTCTGG	60	
SOD-F	GAGAACCCTCATGCTGGTGA	60	200
SOD-R	CCTCCACCAGCATTTCAGT	60	

The forward and reverse sequences of primers for phenylalanine ammonia-lyase (PAL), t-anol/isoeugenol synthase 1 (AIS1), bZIP, R2R3-MYB, SOD, and 18S rRNA (housekeeping) genes are presented in Table 1.

Then, the gene transcription rates were determined according to the real-time quantitative PCR procedure (Applied Biosystem, Step One). The PCR conditions were 94 °C for 2 min followed by cycles at 94 °C for 30 s and 56 °C for 30 s and 72 °C for 20 s. The expression of the 18S rRNA gene was used as an internal control (housekeeping gene). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression. ΔCt is the difference of Ct between the target genes and housekeeping gene; i.e., $\Delta Ct = Ct (\text{target gene}) - Ct (\text{housekeeping gene})$.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using GraphPad software. The mean and standard error values of three independent replications for each treatment group were compared based on Tukey's test. The P value at ≤ 0.05 was considered significant.

Results and Discussion

The individual application of NO increased the shoot and root fresh mass when compared to the control (Fig. 1a, b). Shoot and root fresh mass declined in the Cd- or NO+Cd-treated plants. However, the Cd-associated decrease in biomass was mitigated by NO (Fig. 1a, b). In line with our results, other studies have been reported that Cd affects adversely plant growth and development [13, 20]. Such a decrease in growth following Cd stress has been attributed to a reduction in water content, disturbances in nutrient uptake, and changes in the cell cycle [20]. Dong *et al.* [21] also indicated that Cd increased reactive oxygen species (ROS) production in cytosol resulting in decreasing photosynthetic pigments, thereby suppressing plant growth. Exogenously-applied NO has been observed to diminish plant growth suppression by Cd. Mostofa *et al.* [22], Ahmad *et al.* [20], Basalah *et al.* [23], Nazerieh *et al.* [9], Zhao *et al.* [24] have reported mitigation of metal stress by application of NO in rice, tomato, wheat, peppermint, and *Typha angustifolia*, respectively. The application of NO alleviated the negative effects of Cd toxicity on growth which was similar to the studies of Laspina *et al.* [25]; Xu *et al.* [26]; Dong *et al.* [21]. The reasons may be NO help cell enlargement and plant growth by protecting the phospholipid bilayer membrane. Another reason might be that NO improves cell osmotic pressure and cytoplasm viscosity [9,20]. Proline (compatible osmolyte and protectant agent) was observed to increase in NO, Cd, and NO+Cd-treated plants (Fig. 1d). The Cd-mediated

accumulation of osmotic constituents has been reported in *Brassica juncea* [27] and *Typha angustifolia* [24].

The enhanced accumulation of glycine betaine and proline by exogenous NO has been reported in several studies [9,18, 20,22,24,] indicating the protective role of NO against heavy metals especially Cd. Exogenous NO causes greater accumulation of osmolytes, including proline and glycine betaine, and contributes to stress mitigation by the maintenance of cell water content [20]. Such osmotic constituents play a vital role in stress tolerance, osmoregulation, and membrane stability in plants [9]. It has been also indicated that high accumulation of glycine betaine and proline contribute to the restoration of enzyme activity through enzyme hydration [20].

Cd treatment caused a considerable increase in peroxidase activity relative to the control (Fig. 1c). Enhancement of peroxidase activity as a result of Cd stress has been observed in several studies [28-30]. NO-induced peroxidase activity in Cd-exposed plants is consistent with the results of [20- 24,31] under cadmium stress. Findings of He *et al.* [31] indicated that the regulation of endogenous auxins and gibberellins by exogenous NO may be involved in improved growth and seed germination of rice. Exogenously sourced NO resulted in a decrease in Cd-induced increase of MDA levels and H₂O₂ in rice. [31]. Similar to our results, the protective role of NO on improving antioxidant defense and reduction of heavy metal-induced ROS have been recorded [31, 9, 26, 32].

The individual application of Cd or NO increased R2R3MYB expression by 2.7 and 5 folds respectively in comparison to the control (Fig. 2a). While the simultaneous treatment of NO and Cd synergistically stimulated the expression of R2R3MYB by 7.2 folds relative to the control (Fig. 2a). With a similar trend, the Cd, NO, and Cd+NO treatment groups exhibited the higher transcription rates of bZIP by 3.1, 2.2, and 5.9 folds over the control (Fig. 2b). The exogenously applied NO also up-regulated the expression of the PAL gene by 5.2 folds in the Cd-treated seedlings (Fig. 2c). However, Cd treatment individually induced expression of the PAL gene only by 2.6 folds (Fig. 2c). Likewise, the AIS1 gene was slightly upregulated in response to individual NO treatment (Fig. 2d). However, the Cd treatment resulted in a drastic increase (42 folds) in AIS1 expression which was partly declined in the Cd+NO group and reached 22 folds (Fig. 2d). These results confirmed that NO application was associated with upregulation in the expression of bZIP and R2R3-MYB transcription factors which may be considered as a key mechanism conferring higher tolerance against Cd stress. Similarly, NO-mediated stimulation in the expression of PAL and AIS1 implying potential induction in secondary metabolism.

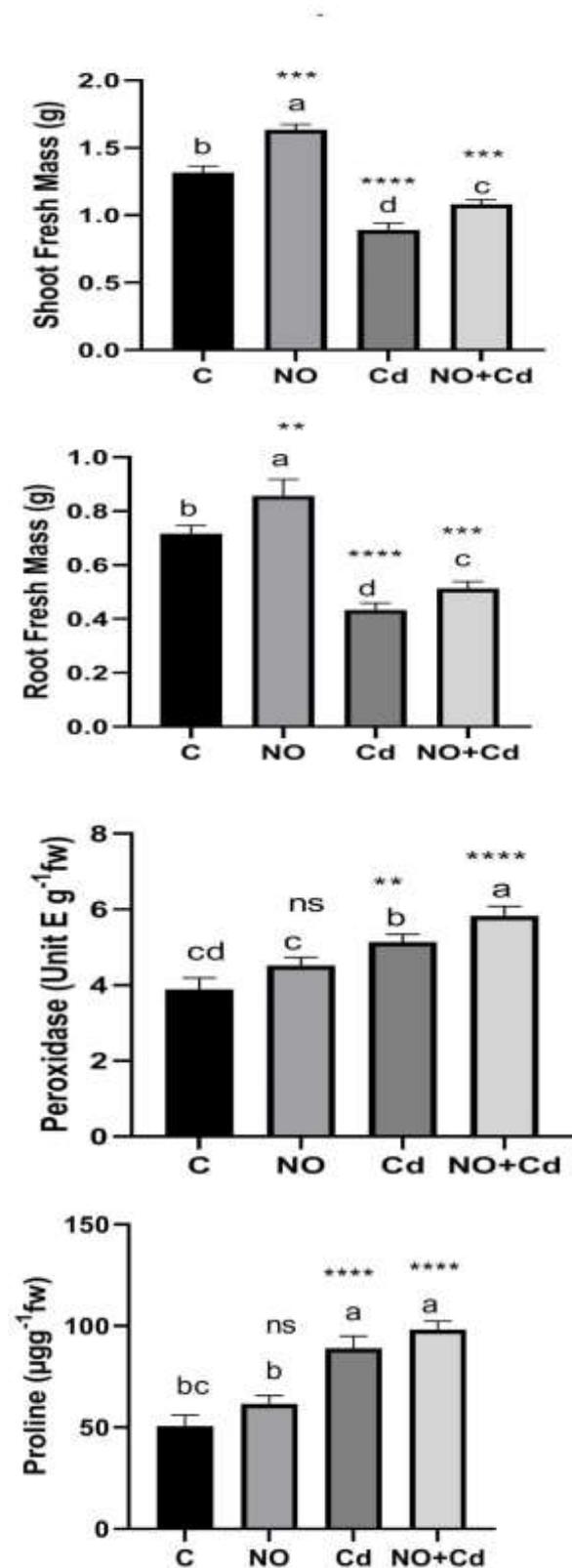


Fig. 1 the effects of NO and Cd alone as well as combined treatments on the shoot and root biomass, peroxidase activity, and proline concentration. Data show as Mean ± SD (n = 3). Different letters on each bar indicate significant differences among treatments according to the Tukey's test. ns: non-significant; *: 0.01 < p ≤ 0.05; **: 0.001 < p ≤ 0.01; ***: 0.0001 < p ≤ 0.001; ****: p ≤ 0.0001. The asterisk on each column shows a p-value level for comparing the mean of each group with the control group.

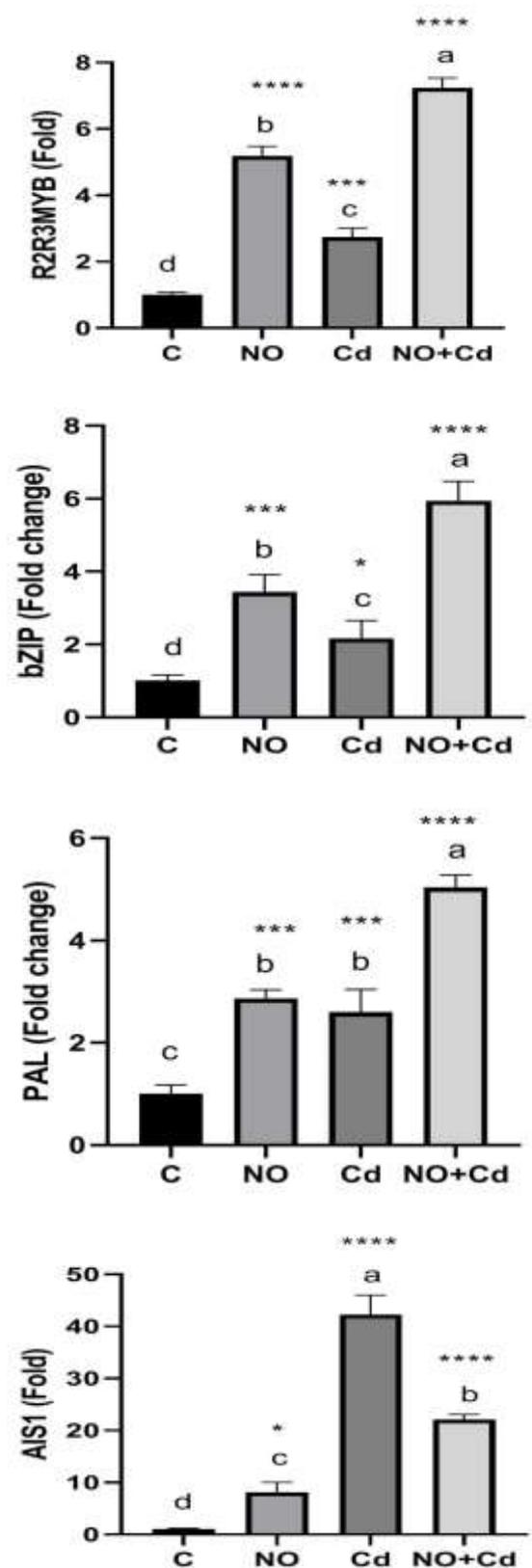


Fig. 2 The effects of NO and Cd alone as well as combined treatments on the expression patterns of R2R3MYB, bZIP, PAL, and AIS1 genes. Data show as Mean ± SD (n = 3). Different letters on each bar indicate significant differences among treatments according to the Tukey's test. ns: non-significant; *: 0.01 < p ≤ 0.05; **: 0.001 < p ≤ 0.01; ***: 0.0001 < p ≤ 0.001; ****: p ≤ 0.0001. The asterisk on each column shows a p-value level for comparing the mean of each group with the control group.

Scientific reports highlight this hypothesis that R2R3MYB genes are implicated in the modulation of secondary metabolism [33-35]. The regulatory role of bZIPs towards secondary metabolism is also supported by several recent studies [36-37]. In plants, the TGA transcription factor (belongs to bZIPs) modulates the salicylic acid signaling route which is responsible for activation of the defense system [17].

Moreover, NO and Cd alone as well as combined treatments caused significant up-regulation in the transcription of the SOD gene by 4, 4.8, and 11.3 folds, respectively when compared to the control (Fig. 3). Taking antioxidant machinery into account, the cytoprotective effect of NO against oxidative stress has been underlined [1,6,7,9,10,13].

Taken collectively, this experiment provides convincing evidence on the NO-associated molecular modifications in expressions of transcription factors as a key mechanism by which exogenous application of NO may confer improved tolerance to an abiotic stress condition, like heavy metals. Moreover, the correlation observed among expression patterns of bZIP, MYB, PAL, AIS1, and SOD genes may indirectly highlight this idea that these transcription factors are involved in the control of secondary metabolism and antioxidant system.

In conclusion, the provided molecular evidence can improve our knowledge of the potential advantages of NO application, thereby improving plant tolerance and stimulating secondary metabolism, especially in medicinal plants.

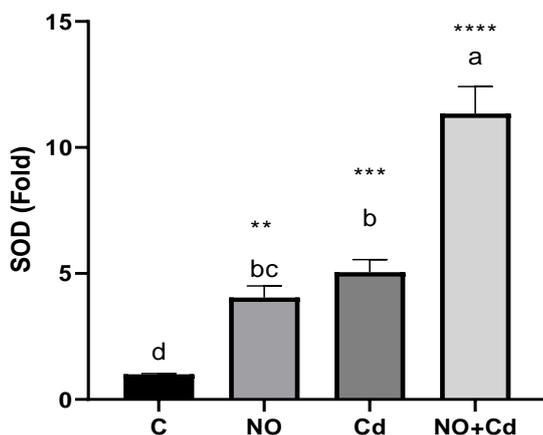


Fig. 3 Changes in the expression of SOD gene in response to individual and mixed treatments of No and Cd. Data show as Mean \pm SD (n = 3). Different letters on each bar indicate significant differences among treatments according to the Tukey's test. ns: non-significant; *: 0.01 < p \leq 0.05; **: 0.001 < p \leq 0.01; ***: 0.0001 < p \leq 0.001; ****: p \leq 0.0001. The asterisk on each column shows a p-value level for comparing the mean of each group with the control group.

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