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ZnO nanoparticle Induced expression of hyoscyamine biosynthesis related genes in *Datura metel* plant leaves

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Abstract

Background:Medicinal herbs have long been used for human and veterinary ailments. Secondary metabolites, like tropane alkaloids (TAs) such as hyoscyamine, scopolamine, and atropine, are key to their therapeutic effects. Datura metel contains these compounds, which serve as protective molecules under stress. Recent efforts focus on mass-producing TAs. This study investigates the impact of ZnO nanoparticles on TA production by assessing key enzymes in their biosynthesis: Putrescine N-methyltransferase (PMT), tropinone reductase 1 (TR-I), and hyoscyamine 6β-hydroxylase (H6H).

Method & Material: Datura metel seeds were sourced from PAKAN BAZR, Isfahan, Iran. After sterilization with 70% ethanol and 20% sodium hypochlorite, they were cultured in MS media with vermiculite and peat at room temperature. Seedlings were transferred to vases and treated with ZnO nanoparticles from Diptronic, Tehran, sprayed on leaves. RNA was extracted from leaf powder using liquid nitrogen, and enzyme expressions were measured via Real-Time PCR after cDNA synthesis.

Result: ZnO nanoparticles enhanced the expression of enzymes involved in hyoscyamine and scopolamine biosynthesis. Significant increases in PMT and TR-I were observed at 15 mg/ml, with more than a 4-fold increase for PMT and a 3-fold increase for TR-I. H6H expression increased over 2 and 3 times at 5 and 15 mg/ml, respectively.

Conclusion:Inducing stress in Datura metel cells via ZnO nanoparticles upregulated genes responsible for TA biosynthesis, suggesting that oxidative stress enhances the antioxidant defense system and promotes protective molecule synthesis.

Keywords: ZnO Nanoparticles, Hyoscyamine Biosynthesis, Datura metel

1.Introduction

The plants have been paid attention as potential therapeutical from long past and have applied to many diseases in various human cultures, in east Asia, India, for instance, but has originated from America (1) (2). These characteristics come from chemical compounds many of which are known as herbal secondary metabolites producing by plants including flavonoids, alkaloids, tannins, saponins, phenolic compounds and essential oils (3). *Datura metel*, also called thorn apple, devil's apple, is one the most studied herbs that had been widely used in medicinal approaches. Datura could grow in tropical and subtropical regions and belongs to *Solanaceae* family that includes, also known, nigh-shades plants such tomato,

2. Chemical and Physical properties

The chemical structure of organophosphate pesticides (OPs) is a critical determinant of their reactivity, stability, and toxicity. At the core of these compounds lies the phosphate moiety, typically substituted with O,O-dialkyl groups, which forms ester bonds with organic substituents.

These ester bonds are prone to hydrolysis, a key degradation pathway for OPs in environmental systems. A defining feature of OPs is the presence of a phosphoryl group (P=O) or its sulfur analog, the thiophosphoryl group (P=S). Compounds containing the latter, such as malathion, are categorized as organothiophosphates but are often grouped with OPs due to their analogous biological activities. The diverse physical and chemical properties of OPs influence their environmental behavior and toxicity potential. Most OPs exhibit low water solubility and high oil-water partition coefficients (log Kow), reflecting their lipophilic nature. This characteristic facilitates their penetration through biological membranes and accumulation in lipid-rich tissues. Vapor pressures among OPs vary, with the majority demonstrating low volatility under ambient conditions, except for dichlorvos, which is significantly more volatile. These properties influence their potential for atmospheric dispersion and inhalation exposure. The stability of OPs is highly dependent on environmental pH. Many OPs are stable in slightly acidic to neutral conditions, as exemplified by parathion, which remains stable at pH levels below 7.5. Hydrolysis represents a primary degradation mechanism, converting OPs into water-soluble products that are generally less toxic at practical concentrations [13]. However, hydrolysis rates vary widely among different OP compounds; for instance, dichlorvos degrades within hours under neutral pH, whereas parathion can persist for several weeks [14]. In acidic soils (pH 4–5), these half-lives are further extended. Furthermore, the hydrophobicity of OPs spans a broad spectrum, with log Kow values ranging from -0.98 to 10.6, influencing their potential for bioaccumulation in aquatic ecosystems and environmental persistence [15]. Understanding the chemical structure and intrinsic properties of organophosphate pesticides is fundamental for evaluating their environmental impact, toxicity, and potential risks to ecosystems and human health. Additionally, this knowledge underpins the development of targeted degradation strategies, addressing the challenges posed by the persistence, mobility, and bioavailability of OPs in diverse environmental contexts.

3. Chemical Degradation Methods

In chemical degradation methods, pesticides are typically organic compounds containing specific functional groups that can undergo various chemical transformations. Incineration is a common yet crude method for the disposal of residual pesticides in wastewater. At high temperatures (around 1000 degrees Celsius) and in the presence of adequate oxygen, non-volatile pesticides like methomyl can be broken down effectively [16-17]. However, this process also leads to the release of organic compounds and carbon dioxide when volatile pesticides are incinerated, resulting in significant environmental impactsneration is not regarded as a suitable method for comprehensive pesticide degradation due to these adverse effects. Nevertheless, it can still serve as a cost-effective approach for wastewater treatment within the pesticide manufacturing industry.

3-1- Hydrolysis Mechanisms for Organophosphate Degradation

Hydrolysis is a pivotal process in the degradation of organophosphate pesticides, serving as an effective method for their breakdown under various environmental conditions. This process involves the cleavage of chemical bonds through the addition of water molecules, resulting in the formation of less toxic byproducts. The hydrolysis of organophosphates can proceed via different mechanisms, each with its own specific characteristics and efficiency.

3-2- Alkaline hydrolysis

Alkaline hydrolysis represents a powerful method for the decomposition of organophosphate pesticides. In this process, hydroxide ions act as nucleophiles in a bimolecular nucleophilic substitution reaction. The alkaline hydrolysis mechanism can follow two distinct pathways depending on the orientation of the attacking nucleophile. In one pathway, the reaction progresses through a multi-step process characterized by the formation of a pentavalent intermediate. Alternatively, a one-step mechanism can occur, involving a single transition state.

mass production for their usage in pharmacy industry (25).These ATs are widely implemented from very long past till now as therapeutical substrates for a wide range of medical approaches including pupil dilation, motion sickness, cardiac and respiratory issues, intestinal cramping. It has also been reported that smoking of leaves containing hyoscyamine and scopolamine could attenuate the asthma (26, 27). Furthermore, studies have shown helpful advantages of atropine in arthritis and that of hyoscyamine and scopolamine in GI-related problems such bowel syndrome (28). Thus, the mass production of these valuable compounds, have been focused in recent years and the natural biosynthesis of these ATs is still the only possible way and usage of external stimuli presumably could be efficient inducers to higher yields of these compounds, especially those who are able to put the plants into stressful situation, so here, we have aimed to investigate the effects of ZnO on production well-known ATs; hyoscyamine, scopolamine and atropine through assessment of expression of key-stone enzymes in pathways of biosynthesis of given ATs including hyoscyamine 6β-hydroxylase (H6H), Putrescine N-methyltransferase (PMT) and Tropinone reductase 1 (TR-I).

Materials and Methods

Datura metel culture

Primarily, the seeds of Datura metel were seeds form PAKAN BAZR co., Isfahan, Iran. In order to sterilize the seeds, those were washed using ethanol 70% and sodium hypochlorite 20% for 1 and for 20 mins, respectively. In the following, the dormancy of *Datura metel* seeds was ceased through treatment of seeds in Gibberellic acid at a concentration of 200 mg/L for 12 hours. The culture of seeds was performed in plates containing MS media (sucrose 30% and GA3 200 mg/L), vermiculite and peat at 25° C in which the cultures experienced light and darkness, each for 12 h. At final step, the seeds were washed and cultured in exposing to. Finally, the seedlings having 2-3 leave, were moved into vases with a diameter of 10 centimeters.

The ZnO nanoparticles

The ZnO nanoparticles were purchased from Diptronic Company, Tehran, Iran. The X-ray diffraction (XRD) analysis was performed by Manufacturer Company. The data showed that the nanoparticles were in an appropriate nanoscale range and peak intensity including 30.64°, 32.62°, 35.31°, 39.63°, 46.77°, 51.66° and 58.43°, position and width, full-width at half maximum of prepared material were determined. The mean size of nanoparticles was reported 16,43 nm using Debye-Scherrer formula. Furthermore, the SEM/TEM executed by Diptronic reveal the formation, spherical shape with sizes less than 10 nm of nanoparticles and also the SAED pattern showed preferential orientation of nanoparticles based on existing definite bright rings. Moreover, the hexagonal plane of ZnO nanoparticles was confirmed by transmission electron microscope (TEM).

The *Datura metel* treatment

The *Datura metel* herbs were categorized into 4 groups each of which included 3 vases. The treatments of plants were conducted through administration of ZnO nanoparticles at concentrations 3 (group 1), 5 (group 2) and 15 (group 3) mg/ml. Primarily, the prepared material containing nanoparticles was dispersed by ultrasonic dispersion manner using Hielscher UIP1000hdT and then sprayed onto *Datura metel* leaves. The group 4 was considered as control which did not receive any

nanoparticles.

RNA Extraction and cDNA synthesis

For measurements of enzymes (H6H, PMT and TR-I) at 24H post-treatment, the RNA was extracted based of manufacturer instructions using TaKaRa MiniBEST Plant RNA Extraction Kit. For this purpose, the powder of plants leaves was prepared through immediate freezing and grinding of leave via the continuous presence of liquid nitrogen. Next, 50-100 mg of powder and 450 ml of buffer RL were added respectively into a 1.5 RNase-free tube cooled by liquid nitrogen. After, centrifuge at 12,000 rpm for 5 mins at 4°C, the supernatant was moved to a new tube which followed by addition of 100% ethanol added. Then, the solution was transferred to RNA Spin Column and centrifuged for 1 min at 4°C at 12,000 rpm. The RNA Spin Column was back to a new collection after discarding flow-through. Afterward, the Buffers RWA and RWB were added to RNA Spin Column when centrifuge was done at each step with same condition. Eventually, the RNA Spin Column hosted DEPC treated water and incubated for 5 mins at RT. The DEPC water containing purified RNA isolated via centrifuge at 12,000 rpm.

cDNA synthesis

The cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit, thermos Fischer. In these processes, firstly 5X Reaction Buffer (4 µl), RiboLock RNase Inhibitor (20 U/µl) (1 µl), 10 mM dNTP Mix (2 µl), RevertAid M-MuLV RT (200 U/µl) (1 µl) and nuclease-free water were mixed and added to purified RNA as well as oligo (dt) primers. The steps followed as 60 min at 42 °C and 5 min at 70 °C.

Real-Time PCR

The expression of genes H6H, PMT and TR-II were measured after ZnO treatment. The oligonucleotide primers used for amplification of genes included 1) H6H; F: 5¢-CACTTTGGCTCATGGTTGTCA-3¢ and R: 5¢-CCATCATAGTGTCCTCCTGAACC-3¢, 2) PMT; F: 5¢-ATTGTTCATCTCCCACTTGG-3¢ and R: 5¢-TCTTTTGCTGGACCAATAGG-3¢ and 3) TR-I and finally 4) The house-keeping gene (Tubulin gene) as internal control; F:5¢-GGGGCGTAGGAGGAAAGCA-3¢ and R:5¢-GCTTTCAACAACTTCTTCAG -3¢. The RT-PCR reactions proceeded as phases 2 min at 94° C, 35 expansion cycles of 45 s at 94° C, 45s at 52° C and 50s at 72° C and final incubation at 72° C for 10 min. The Real-Time PCR results were analyzed by the mathematical model of Pfaffl.

Statistical analysis

The analysis of data from Real-Time PCR were done according to thoroughly randomized three Repeats. The Real Time-PCR results were via SPSS software and means were compared with LSD test at 95% confidence level.

Results

The results indicated that the expression of the given genes mediating biosynthesis of tropane alkaloid, have altered in response to ZnO nanoparticles. In order to reveal these alterations, we measured the level of enzymes forming the tropane alkaloids or their precursors. Firstly, the expression of enzyme Putrescine N-methyltransferase (PMT) by which N-methylputrescine is formed from putrescine, were measured in response to different doses of ZnO nanoparticles. The N-methylputrescine is subsequently converted into tropinone. . In the following, the tropine as the precursor of hyoscyamine and scopolamine,

is catalyzed by TR-I enzyme from tropinone. The PMT gene expression showed changes as its level increased significantly about 4-fold at 24H only when received high concentration of ZnO. Notably, the expression of certain genes in all treated groups were compared to control group based on their DCTs (Delta cycle threshold), so that the expression of PMT was considered as 1 and PMT expression in other group were calculated comparatively to 1. Moreover, the level of PMT also increased at doses 3 and 5 mg/ml of ZnO, though statistically insignificant almost 0.9 and 1.4-fold, respectively. The Real Time-PCR results revealed different changes for TR-I gene. This gene encodes the tropinone reductase 1 that belongs to oxidoreductase family enzymes. The enzyme TR-I, as said before catalyzes the formation of tropine from tropinone. Afterward, tropine and phenyllactic acid are subsequently converted into hyoscyamine. Our data demonstrated that the expression of TR-I had significant increment about 3 times, similarly to PMT, at dose 15 mg/ml of ZnO 24H after exposure, in spite of ignorable increase at lower concentrations.

Eventually, we measure the expression of H6H gene encoding the hyoscyamine 6β-hydroxylase enzyme, a member from 2-oxoglutarate-dependent dioxygenases family. The H6H leads the reactions by which 6b-hydroxyhyoscyamine and scopolamine are formed from hyoscyamine, respectively. The data showed that the mean expression of H6H has displayed a different pattern of alterations as it level had raised significantly more than 2 and 3 times at both doses 5 and 15 mg/ml of ZnO, respectively. The results indicated that H6H has more sensitively changed to stress inducing stimuli as its level increased even at low doses of ZnO. The mechanisms

Table 1. The mean expression of PMT and TR-I genes at different groups are shown here after exposing to ZnO nanoparticles. The alterations patterns for given genes are same for these 2 gene as their expressions have significantly increased more than 2 times fold at dose 15 mg/ml (group 3). Nevertheless, ignorable and statistically inconsiderable increase have been observed for both at lower doses 3 (0.7) and 5 (1.3) mg/ml.

Groups	**DCT (control)**	**DCT (Treated)**	**Difference between**	**p.value**	**Fold change**
PMT					
Group 1 (3 mg/ml)	3.05 ± 0.9352	1.713 ± 0.8918	-1.337 ± 1.292	0.3594 (ns)	0.979
Group 2 (5 mg/ml)		-0.6183 ± 2.725	-3.668 ± 2.881	0.2718 (ns)	1.422
Group 3 (15 mg/ml)		-6.303 ± 0.5325	-9.353 ± 1.076	0.0010 (***)	4.1926
TR-I					
Group 1 (3 mg/ml)	4.04 ± 0.6979	2.167 ± 0.2422	-1.873 ± 0.7387	0.0643 (ns)	0.989
Group 2 (5 mg/ml)		1.67 ± 0.6819	-2.37 ± 0.9757	0.0721 (ns)	1.539
Group 3 (15 mg/ml)		-5.277 ± 0.4971	-9.317 ± 0.8568	0.0004 (***)	3.254

by how these changes are induced, are not completely understood but it could be suggested that ZnO firstly may put the cells in an oxidative stress by which the TAs expression raise as protective molecules. Hence, these compounds presumably are able to have direct anti-oxidant properties or could initiate or enhance the anti-oxidant defense system. Furthermore, as the PMT and TR-I represented similar alterations, there could be probable correlation between them.

Discussion

The herbal therapies were almost, the only way for disease in the past and recently they have been paid attention because of their side-effect-free features. As said before, the secondary metabolites are main products of plants which act as multitask factors (29, 30). These chemicals function for health maintenance and hemostasis of herbs as they act as protective molecules in stressful conditions or induce the excretion of toxic and unusable substrates

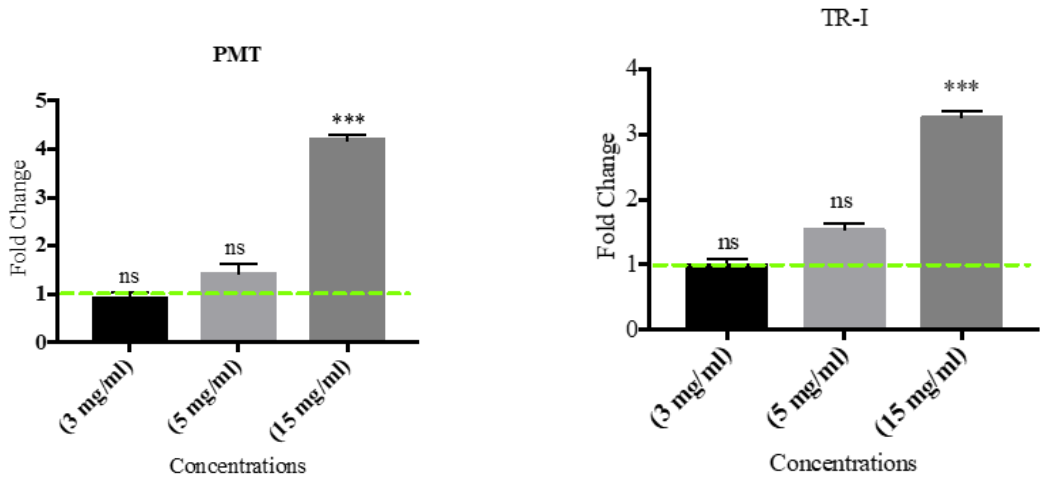

Figure 1. Due to graphs above, the changes of PMT and TR-I genes expression are significant only at dose of 15 mg/ml ZnO nanoparticles. The green line is control level that other groups are compared to.

Table 2. The mean levels of H6H expression in different groups received various doses of ZnO nanoparticles are shown at 24H. The table presents, ZnO has triggered changes of H6H separately from that of PMT and TR-I as lower dose of nanoparticles also augmented the expression of H6H

H6H					
Groups	DCT (Control)	DTC (Treated)	Difference between means	p.value	Fold change
Group 1 (3 mg/ml)	5.19 ± 0.6994	2.097 ± 1.115	-3.093 ± 1.316	0.0784 (ns)	1.101
Group 2 (5 mg/ml)		-1.98 ± 0.5948	-7.17 ± 0.9181	0.0015 (**)	2.113
Group 3 (15 mg/ml)		-5.413 ± 0.8695	-10.6 ± 1.116	0.0007 (***)	3.660

from plants. Many studies have also shown the antioxidant, anti-inflammatory, antitumor, diuretic anti-cholinergic, anti-tumor, diuretic, antiviral, antimicrobial, antihypertensive, hypno-analgesic, antidepressant and anti-tussigen effects of this compounds (31-34). TAs exist abundantly in plant families including *Solanaceae*, *Erythroxylaceae*, *Convolvulaceae* and *Brassicaceae*. The *Datura metel* plant belongs to *Solanaceae* family and has been proven to be effective in medical applications. Its main therapeutical agents have consisted of TAs including hyoscyamine, scopolamine and atropine (35, 36). These TAs are valuable chemicals that are on focus today and mass production of them is great of importance. The researchers have been trying to increase secondary metabolites yields. Here, our results demonstrated that ZnO nanoparticles could upregulate the expression of genes which include main enzymes in ATs biosynthesis pathway. The enzyme PMT is prior than TR-I and H6H enzymes in this pathway which that synthesize the basic substrate, N-methyl-putrescine. This compound passes through various reactions to form tropinone. Subsequently, tropinone is converted into tropine by enzyme TR-I. The tropine and phenyllactic acid forms the hyoscyamine which in the following, the enzyme H6H converts hyoscyamine (atropine) to 6b-hydroxy-hyoscyamine and scopolamine, respectively. Due to our results, we showed that all enzymes upregulate at any doses of ZnO nanoparticles, though statistically significant increases are only observed at 15 mg/ml for PMT and TR-I but at 5 and 15 mg/ml for H6H gene. The underlying mechanisms why the H6H show changes even to lower doses of ZnO, are not found but it could be suggested that it benefits more sensitive regulation system such as more powerful promotor because of it key role for final steps by which hyoscyamine and scopolamine are produced. In accordance to our results, baifu et al. reported that UV-B stressed hairy cell of *Anisodus luridus* have higher expression of genes *PMT*, *TR-I*, *H6H* and *CYP80F1 compared to control cells (37). The* baifu has also revealed that stress could upregulate the expression of genes involved in TAs biosynthesis through induction of oxidative stress as the basic reason. In this situation, the plant recognizes that it must heighten its protective secondary metabolite to lessen the damages. Moreover, this fact could be seen in karimi et al study who showed that *Datura innoxia* exposed to $AlCl_3$, had higher level of hyoscyamine and scopolamine and higher activity of anti-oxidant enzymes (38). Manorma et al also implies that exposure to stress could eventuate to higher yield of secondary metabolites. Due to current study, the data indicated that ZnO nanoparticles also could intensify the production of TAs as well as other secondary metabolites, probably by induction of oxidative stress. Though, more studies are needed for reveal other possible underlying mechanisms(39).

It is well know that in Solanaceae TA are synthesized in the roots and then are transferred

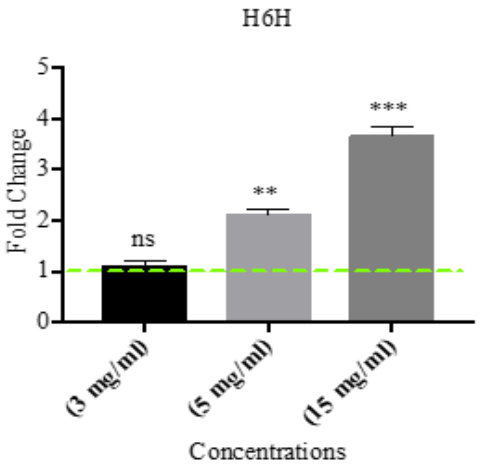

Figure 2. The graph above indicates significant increase of H6H gene at doses of 5 and 15 mg/ml of ZnO. These data could suggest that this enzyme acts or response to stressful condition more sensitively compared to other enzymes PMT and TR-I.

to the leaves where alkaloids are accumulated(40) but there are evidences that reported main hyoscyamine biosynthesis related genes expression in Solanaceae plant leaves (41-43). Environmental and abiotic stresses increase TA biosynthesis related genes at transcriptomic level by affecting promoters of these genes which has been reported for pmt gene expression in shoots and roots of Nicotiana benthamiana and Atropa belladonna under methyl jasmonate, UV radiation and wounding treatments. Their results displays spatial difference of expression profile in root and leaves(41), However, Sachan et al., confirmed induced upregulation of pmt gene in leaves in response to mechanical wounding but low expression level in unwounded leaves and also lack of responsiveness in exposed leaves. The differences may arise from plant species, time of expression assessment and type of stress. Two pmt cDNA clones were reported from Anisodus acutangulus, AaPMT1 and AaPMT2 show constitutive expression template with higher level in roots than leaves and higher expression levels of AaPMT1 and AaPMT2, respectively. Their upregulation in response to methyl jasmonate follows similar trends between root and leaves and these two genes after 24H(42) Our results in accordance with former ones confirm that TA biosynthesis related gens have spatial and temporal expression profile in absence and presence of abiotic stresses and gene expression level depends on the type of stress, time of measurement and where the gene locates in the biosynthetic pathway.

Conclusion. The Tropane alkaloids are widely used in medicinal approaches these days. In spite of massive need, natural production via given plants is still chief rout for these compounds. Recently, many efforts have been done by researches for mass production of TAs such as stimuli that put the herbal cells in stress condition like oxidative stress-inducing agents as mentioned above. Many studies have divulged that in stress condition, TAs production raises that is partly related to their biological roles including anti-oxidant activity. Eventually, in this study, we have shown that ZnO nanoparticles could rise the production of TAs through upregulation of enzymes leading their biosynthesis. Hence, it has been demonstrated that stress inducing stimuli are able to enhance production of TAs, though the underlying mechanisms need to be elucidated in future.

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