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Expression Profile of Hyoscyamine Biosynthesis-related Genes in Response to UV-C Radiation in *Datura metel* Plant

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Abstract

Introduction: Tropane alkaloids as secondary metabolites are one of the most useful plant elements that are widely applied in medicinal approaches. Studies have shown that UV light led secondary metabolites to be increased. Thus, we investigated the effect of UV-C light on the expression of the main genes involved in the biosynthesis of tropane alkaloids, namely hyoscyamine 6β-hydroxylase (H6H), Putrescine N-methyltransferase (PMT), and Tropinone reductase I (TR-I).

Materials and Methods: *Datura metel* seeds cultured on MS media at 25 °C less than 12 h-12 h light-dark photoperiod. Then, they were transferred into vases and kept in the greenhouse. Three-month-old plants received 196 μWcm^{-2} UV-C light for 30 min. Afterward, the expression levels of different genes encoding H6H, PMT, and, TR-I enzymes, were measured at different post-exposure times.

Results: Our results demonstrated that UV-C increased the expression of *PMT* and *TR-I* genes after 48 h. Moreover, the rise of H6H expression was found after 24 h but its level was downregulated again after 48 h.

Conclusions: These findings indicated that UV-C light as abiotic stress could boost the formation of tropane alkaloids through upregulation of genes of enzymes catalyzing the main steps in their biosynthesis and, also, these genes are differentially affected.

Keywords: *Datura metel*, UV-C, H6H, PMT, TR-I, Expression, Hyoscyamine

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Introduction

The *Datura metel* is a plant from the *Solanaceae* family, native to northeast regions of North America that is grown as a shrub with dispersive branches and is found in wilderness, roadsides, and near residential areas mostly in temperate and subtropical regions of the Northern Hemisphere. The *Datura metel* is a medical plant that has been introduced as a sedative, anti-spasmodic, mydriatic agent.[1-4] In the past, this plant seeds and leaves have been used in pharmacy in human medicine so that its alkaloids, termed as tropane, could be used to treat spasmodic asthma, rheumatic, Lumbago, Sciatica, Scabies, Eczema, Allergy, and also for earache as external material. It has been indicated that whole parts especially Leaves, and seeds of *Datura metel* contain secondary metabolites such as tropane alkaloids including hyoscyamine, and scopolamine (Figure 1) which show therapeutic features like anti-asthmatic, anti-tussive, hypnotic, bronchodilator effects.[5] Based on recent studies, *Datura metel* especially its seed has potent painkiller and anti-hyper-allergic effects because of its muscarinic agonist activity.[6] It also needs to be implied because of the poisonous properties of this plant, there is little difference between its pharmaceutical and toxic doses and excessive doses cause dizziness, xerostomia, hallucinations, and coma. The hyoscyamine is the main tropane agent. Minor compositions include scopolamine, apoatropine, atropine, tropone, and Belladonnine.[6-8]

The tropane alkaloids are found mainly in plants of the Solanaceae family, such as *Datura*, and are categorized into two main groups. First, tropine which contains alkaloids such as hyoscyamine, atropine, and scopolamine, and second group is ecgonine which accommodates the well-known substrate cocaine.[8,9] The tropane alkaloids feature a methyl group attached to the nitrogen resembling the structure in neurotransmitter acetylcholine which is responsible for its

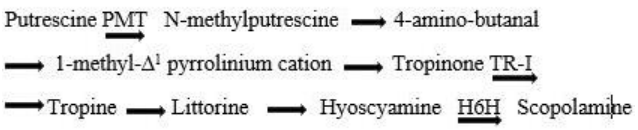

Figure1. The Biosynthetic Pathway of Main Tropane Alkaloids in the Plant Species of *Solanaceae*. PMT, TR-I, and H6H are Putrescine N-methyltransferase, Tropinone reductase I, and hyoscyamine 6β-hydroxylase, respectively.

medicinal characteristics.[10] These alkaloids are efficiently used for relieving Parkinson's symptoms, dilatation of the eye pupil, increasing heart rate, neutralizing smooth muscle weakness due to organic phosphorylated agents and reducing the secretion of sweat, saliva, and stomach acid in situations such as surgery.[11] As known, hyoscyamine, atropine, and scopolamine are anticholinergic compounds as they competitively and reversibly block the action of acetylcholine neurotransmitter during binding to muscarinic receptors, and this antagonism effect leads to sympathetic effects in tissues.[12] Furthermore, Hyoscyamine could be applied to alleviate the symptoms of various digestive disorders including gastric ulcer or spasm, irritable bowel syndrome, pancreatitis, colitis, and bladder inflammation. Moreover, scopolamine, as a potent anticholinergic hallucinogen is often prescribed for patients with schizophrenia and similar conditions. Scopolamine is the most valuable tropane alkaloid and is preferred due to its physiological activity and fewer side effects.[13] The tropane alkaloids are mainly made in young root cells and then transported to the plant's organs.[14] The root is the main source of biosynthesis of tropane alkaloids and the conversion of hyoscyamine to scopolamine also occurs in root pericycle cells. Plant alkaloids are one of the most significant groups of natural compounds that involve a wide variety of drug products.[15] Hence, researches on alkaloids have a long history and our findings are still at the beginning of its path to full knowledge of the biotechnological benefits of these compounds.[16,17] Due to the advantages of tropane alkaloids, the production of these handy compositions has been paid attention in recent years.[15] The synthesis of alkaloids is often expensive and natural resources are the only source for mass production of these compounds. So, studies on effective and affordable production pathways of these agents are necessary.[18] Recently, various species of *Datura* are grown for the production of tropane alkaloids. Besides, plant cells show physiological, chemical, and morphological responses to environmental stimulants.[19,20]

Some studies have revealed that ultraviolet light can result in higher production of plant secondary metabolites. Alkaloids as secondary metabolites are not essential factors to complement the plant's life cycle and they are assumed to act as signaling or chemical defensive molecules against stressful conditions.[18] Hence, it is thought that these compounds are increased under induced stresses as UV light increases the reactive oxygen species (ROS) that cause oxidative stress in which these hyper-active molecules react with biological molecules such as lipids, proteins, and nucleic acids leading to disruption of cell normal function. The plant cells also contain systems to stand out such conditions including antioxidant enzymes such as catalase, peroxidase, and superoxide dismutase, as well as, non-enzymatic systems including secondary metabolites like ascorbic acid, carotenoids, flavonoids, and alkaloids.[21-23] As a result, the findings indicate that UV light could trigger the plant to form larger amounts of alkaloids to protect itself against the abnormal induced situation. In this regard, we have tried to investigate whether the UV-C light can lead the plant cells to a higher yield of alkaloids So, we elucidated these alterations by observing the changes in the expression of key-stone enzymes mediating their production.

Materials and Methods

Datura metel Culture

The *Datura metel* seeds were purchased from PAKAN BAZR, Isfahan, Iran. First, the seeds were sterilized with ethanol 70% for 1 min and sodium hypochlorite 20% for 20 min. Next, to break the dormancy, the seeds were treated for 12 h in Gibberellic acid at a concentration of 200 mg/L. Then, the seeds were washed and cultured in plates containing MS media (sucrose 30% and GA3 200 mg/L), vermiculite and peat at 25 °C exposing to light (12 h) and darkness (12 h). Finally, the seedlings at a phase containing 2-3 leaves transferred into vases with a diameter of 10 centimeters.

UV Light Treatment

To assess the effect of UV-C light on tropane alkaloids production, the plants were analyzed at 3 time points, including 24, 48, and 72 h after reception of UV light. All vases received the same UV-C light with a dose of 196 μWcm^{-2} for 30 min, once. The UV-C was emitted by 2 lamps of F20T9/BL-Hitachi, Japan. To reveal the changes induced by UV-C, the plants' leaves harvested and were analyzed.

RNA Extraction and cDNA Synthesis

The RNA extraction was performed using the TaKaRa MiniBEST Plant RNA Extraction Kit. Primarily, the plants' leaves were frozen immediately and ground using the continuous presence of liquid nitrogen to form the plant leaves powder. Then, 50-100 mg of powder as well as 450 ml of buffer RL was added into a cooled (by liquid nitrogen) 1.5 RNase-free tube. The tube was centrifuged at 12,000 rpm for 5 min at 4 °C. Then, the supernatant was transferred to a new tube and 100% ethanol was added. Next, the solution was applied to the RNA Spin Column. After centrifuging at 12,000 rpm for 1 min at 4 °C, the flow-through was discarded and RNA Spin Column moved back to a new collection. The processes continued by adding the Buffer RWA and RWB after centrifuging at each step. Finally, the DEPC-treated water was applied to RNA Spin Column incubating for 5 min at RT and the final centrifuge was done at 12,000 rpm to obtain the purified RNA. In order to synthesize cDNA, the processes were followed according to manufacturer using RevertAid First Strand cDNA Synthesis Kit, thermos Fischer in which 5X Reaction Buffer (4 µl), RiboLock RNase Inhibitor (20 U/µl) (1 µl), 10 mM dNTP Mix (2 µl) and RevertAid M-MuLV RT (200 U/µl) (1 µl) were added to purified RNA as well as oligo (dt) primers

and the cDNA synthesis performed through 60 min at 42 °C and 5 min at 70 °C.

Real-time PCR

After cDNA synthesis, we measured the expression of H6H, PMT, and TR-I genes in response to UV light. The oligonucleotide primers included F: 5'-CACTTTGGCTCAT GGTTGTCA-3' and R: 5'-CCATCATAGTGTCCTCCTGA ACC-3' for H6H, F: 5'-A TTGTTCA TCTCCCACTTGG-3' and R: 5'-TCTTTTGCTGGACCAATAGG-3' for PMT and F: 5'- The house-keeping gene (Tubulin gene) as internal control for real-time PCR reaction, was amplified using specific primers (F: 5' -GGGGCGTAGGAGGAAAGCA-3' and R: 5'-GCTTTCAACAACTTCTTCAG-3'. The RT-PCR reaction was executed as following steps predenaturation (2 min at 94 °C), 35 expansion cycles (45 s at 94 °C, 45 s at 52 °C and 50 s at 72 °C) and final incubation at 72 °C for 10 min. The mathematical model of Pfaffl was used for the analysis of real-time PCR results. Expression data normalization was performed using ΔΔCt (2–ΔΔCt) method to calculate the relative fold gene expression of samples.

Statistical Analysis

The experiment and RT-PCR data were based on completely randomized three Repeats. The real-time PCR data analysis was performed using SPSS software. Means were compared with the LSD test at 95% confidence level.

Results

Our findings demonstrated that UV-C radiation altered the expression of the 3 main genes tropane in alkaloid biosynthesis. To evaluate these changes, we aimed to measure the expression of the main enzyme in the production pathway of tropane alkaloids.

The first enzyme is known as Putrescine N-methyltransferase (PMT) by which N-methyl putrescine is formed from putresine. This substrate is the primary precursor of tropane alkaloids which is subsequently converted into hyoscyamine and scopolamine, respectively. Due to data (Table 1), we observed that the PMT expression had a significant increment from 7.6711 at 24 to 9.5511 at 48 after receiving UV-C light, while its level reversed to 7.7978 at 72 h, almost to its baseline amount at 24 h. Moreover, the RT-PCR results revealed different changes for TR-I as this gene encodes the tropinone reductase I from oxidoreductase family enzymes. This protein form tropine from tropine which subsequently as well as phenyllactic acid, form littorine, the precursor of hyoscyamine and scopolamine. The real-time PCR results presented that UV light has mounted the expression of TR-I slightly in plants from 13.9707 at 24 h to 14.4240 and 17.7140 at 48 h and 72 h, respectively after exposure (Figure 2).

Table 1. The Table Shows the Mean Expression of *PMT* and *TR-I* Genes at 24, 48, and 72 h Post-Ultraviolet Exposure. As the data show the expression PMT increases and declines but in the contrary, the level of TR-I increased with a slight and vigorous intensity at 24-48 h and 48-72 h, in turn.

		Mean	Std. Deviation	Std. Error Mean	Sig. (2-tailed)
PMT	Uv24	7.6711	0.36756	0.21221	0.044
	Uv48	9.5511	0.35086	0.20257	
	Uv24	7.6711	0.36756	0.21221	0.679
	Uv72	7.7978	0.78309	0.45212	
	Uv48	9.5511	0.35086	0.20257	0.105
	Uv72	7.7978	0.78309	0.45212	
TR-I	Uv24	13.9707	0.99651	0.57534	0.573
	Uv48	14.4240	0.64490	0.37233	
	Uv24	13.9707	0.99651	0.57534	0.015
	Uv72	17.7140	0.20952	0.12097	
	Uv48	14.4240	0.64490	0.37233	0.010
	Uv72	17.7140	0.20952	0.12097	

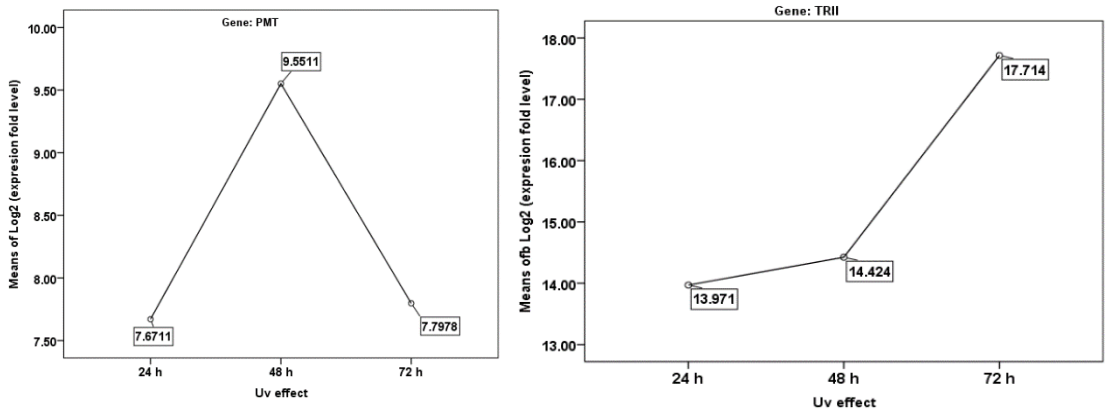

Figure 2. The Graphs Above Show the Changes in *PMT* and TR-I Genes Expression at Different Time Points Passed after Exposure to UV Light. These findings demonstrated that PMT has changed faster but transiently as its amount is seen at its baseline after 72 h. In contrast, in the *TR-I* gene, the increase has been observed gradually and stably.

Next, we measure the expression of the *H6H* gene that expresses the enzyme hyoscyamine 6β-hydroxylase that belongs to the 2-oxoglutarate-dependent dioxygenases family (Table 2).

Table 2. The Mean Level of H6H Expression at Different Time Periods has Displayed in the Table. We found that UV light triggers the plant to upregulate the H6H after 48 h without any considerable changes at 24-48 h.

		Mean	Std. Deviation	Std. Error Mean	Sig. (2-tailed)
H6H	Uv24	5.7257	.95798	.55309	0.981
	Uv48	5.7590	1.26440	.73000	
	Uv24	5.7257	.95798	.55309	0.037
	Uv72	9.0657	.51501	.29734	
	Uv48	5.7590	1.26440	.73000	0.065
	Uv72	9.0657	.51501	.29734	

This protein converts hyoscyamine into 6β-hydroxy-hyoscyamine and then into scopolamine. The data showed no alteration in the expression of H6H in 24-48 h after receiving UV-C light, but there was a significant increase at 48 h from 5.7590 to 9.0657 at 72 h. These data represented that various enzymes respond differently to UV light as they might show an increase quickly or with delay after receiving UV light. All in all, we observed that the proteins showed 3 separate patterns of changes as the PMT expression increased and then decreased in turn at 24-48 h and 48-72 h intervals. On the other hand, the TR-I showed a slight and then intense increment from 24 to 48 and 72 h, respectively (Figure 3).

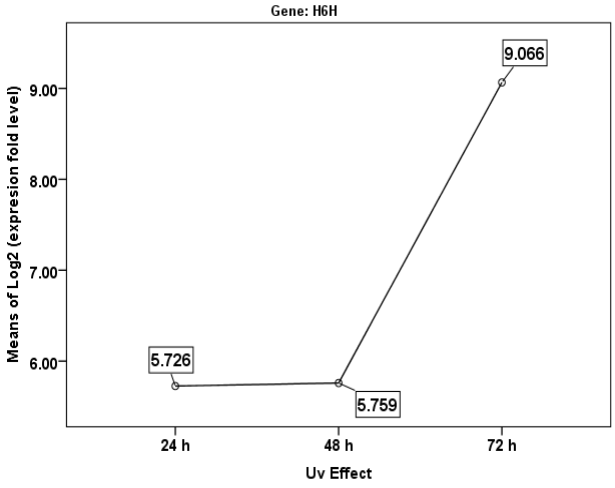

Figure 3. The Graph Shows the Mean Expression of H6H Gene which has Significantly Boosted with Delay after 48 h from Receiving UV Light.

Discussion

The *Datura metel* from the *Solanaceae* family is a widespread growing plant in tropical regions that has considerable therapeutic properties.[24] The plant traditionally was used to relieve pain and other complications such as breathlessness, asthma, and muscle cramps. On the other hand, high usage of this could lead individuals to health problems and even death as it is a vigorous hallucinogenic compound and also contributes to toxic features.[25] Studies have shown that these medicinal features of *Datura* are owed to its secondary metabolites such as alkaloids, tannins, polyphenols, steroids, saponins, and glycosides.[26,27] Alkaloids are complex compounds containing one or more nitrogen in the heterocyclic ring that originate from amino acids and have strong physiological effects. Based on the carbon skeleton, alkaloids are divided into multiple main groups.[28] The tropane alkaloids including atropine, hyoscyamine, and scopolamine, are one the most significant alkaloids presenting anticholinergic characteristics because of their structural resemblance.[29] Tropane alkaloids have been used in a variety of medicines. Scopolamine is the most valuable tropane alkaloid so that the global appeal for this alkaloid is more than hyoscyamine. In recent years, the fact is of great importance that tropane alkaloids which give pharmaceutical usefulness to these plants could be produced in mass volumes.[30] But lack of enough knowledge in the biosynthesis pathways of these molecules has limited their synthetic production as the enzymes involved in their biosynthesis are not well-known. Hence, natural production using plant resources is fairly the only way.

Recently, the potential stimuli that have the ability to mount the formation of secondary metabolites in plants are being studied and used. As known, secondary metabolites play vital roles in protecting plant in stressful conditions.[31-33] Hence, it is predicted that plants elevate the formation of these compounds in abnormal situations. Here is the point of how we can exploit this phenomenon. Some reports exist that alkaloids engage in oxidative stresses as they have free-radical scavenging activity.[34] As same as animal cells, UV light also could have detrimental effects on plant cells and damage biomolecules by forming destructive agents, ROS.[35,36] Thus, it is not unexpected that UV light ends in higher amounts of plant protective molecules such as tropane alkaloids. Consequently, we have used these dialectics to design our study. In this study, we cultured and exposed *Datura metel* to UV light and investigated the level of tropane alkaloids in response to a stress-inducing element at different time periods. So that we measured the levels of atropine, hyoscyamine and scopolamine at 24, 48, and 72 hours after exposing UV light.

Our data demonstrated that UV light results in changes in enzymes that catalyze the important steps in the production of these alkaloids so that their absence means no scopolamine,

hyoscyamine and atropine. Thus, we assessed the expression of H6H, PMT, and TR-I.

The finding showed that H6H had no increase in the time period between 24-48 h, then it elevated after 48 h so that there were significant increases at time periods including 24-72 and 48-72 h. The results for PMT revealed that a higher level of this enzyme was only seen after 24 h as its expression had reversed to baseline level after 48 h. So, a significant increase was not seen at 24-72 h and 48-72 h. The TR-I enzyme was also upregulated after 48 h so that higher level of TR-I was found at 24-72 h and 48-72 h but not at 24-48 h. Interestingly, TR-I and H6H both had increased lately after 48 hours after UV light exposure with more stability as their levels remained heightened after 72 h from UV. On the other hand, the *PMT* gene showed quicker change after 24 h but was not stable as its level lessened to the pre-treatment level after 48 h. In parallel to our study, Zeynali et al also mentioned that UV-B light could have increased the secondary metabolites including hyoscyamine and scopolamine in the *Hyoscyamus reticulatus* plant compared to control models.[37] In addition, UV irradiation increases polyphenol content and induces higher concentrations of chlorogenic acid in *Datura wrightii*.[33] Tropane alkaloids as secondary metabolites have been found to protect plant cells in stressful situations such as UV irradiation by which lethal molecules are formed in cells. This effect has been reported in water extract of *D. metel* in response to UV radiation which increased the antibacterial effect of the extract compared with higher wavelength irradiation.[28] So, it is not unexpected that UV light leads tropane alkaloids to higher levels in cells. Additionally, similar data have been reported that ultraviolet could increase the formation of tropane alkaloids.[38] The enzymes studied here, PMT, TR-I, and H6H are respectively functioning downstream in tropane alkaloids synthesis pathway and there may be a correlation as PMT expression changed faster but more transient compared to TR-I and H6H. So further studies are needed to elucidate the enzymatic systems by which pathways are led.

Conclusion

This study has shown that UV light could enhance the production of tropane alkaloids as a plant secondary metabolite in *Datura metel* through upregulation of the main enzymes involved in the biosynthesis of these compounds. As one of the most restricting issues in the production of beneficial compounds of tropane alkaloids including hyoscyamine and scopolamine, is a lack of enough knowledge about enzymatic systems in their formation, studies such as this could lead to a better understanding of the underlying mechanism by which external stimuli could boost up the yield of these compounds. Furthermore, as a higher yield of tropane alkaloids is great of importance in studying new methods of production, more studies are needed for the efficient use of UV light to obtain a higher mass yield of tropane alkaloids.

Authors' Contributions

SJD designed the experiment, wrote, and revised the manuscript. MN performed the experiment and wrote the first draft. SJD, MRK, and RK made appropriate changes to finalize the manuscript. All authors approved the final version of manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgment

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