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Vol. 7(42), pp. 3112-3120, 10 November, 2013
DOI: 10.5897/JMPR12.1029
ISSN 1996-0875 © 2013 Academic Journals
http://www.academicjournals.org/JMPR

Journal of Medicinal Plant Research

Full Length Research Paper

Ultraviolet-B (280-320 nm) enhanced radiation induced changes in secondary metabolites and photosystem-II of medicinal plant *Withania somnifera* Dunal.

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Accepted 14 October, 2013

This study investigated supplemental UV-B radiation effects on medicinal plant *Withania somnifera.* Photosynthetic pigments decreased upon exposure to UV-B radiation. Non photosynthetic pigments anthocyanin was enhanced by UV-B treatment. Similar changes were also noted in the photochemical activities (PS I and PS II). Photosystem II (PS II) core complex of higher plants and cyanobacteria is composed of more than 20 polypeptide subunits. This paper reports the results obtained by application of MALDI mass spectrometry directly to isolate complexes prepared from *Withania*. In plants grown under ambient light conditions, PS II core complex was not affected in which D1 and D2 proteins appeared at *m/z* 39175±8 (r775) and *m/z* 39074±9 (r1994), respectively. Three unidentified peaks were observed at *m/z* 39214±9, 38995±6 and 177798±8. Upon exposure to UV-B enhanced radiation, the degradation of D1 protein together with the other thylakoid subunits at *m/z* 15214±8 CP47 (r2311), *m/z* 39071±7 CP43 (r1441), *m/z* 15636±9 PsbJ (r2516), *m/z* 39182±6 PsbH (r1720), *m/z* 31126±8 PsbK (r2631), and *m/z* 39129±8 PsbM (r623) were observed. In addition to this, three other peaks have been detected at *m/z* 38977(r2104), 39019(r649) and 24102(r2378). Ten significantly different chromatographic peaks were found by HPTLC finger print after UV-B induction. One compound identified was withaferin A and it was increased 3% by dry weight.

Key words: *Withania somnifera*, withaferin A, UV-B, photosynthesis, HPTLC, MALDI-TOF

INTRODUCTION

The enhanced level of solar ultraviolet-B (UV-B) radiation reaching the surface of the earth has become an important issue over the past two decades. Depletion of stratospheric ozone (O_3) caused by antropogenic chloroflourocarbons has increased the amount of UV-B (280 to 315 nm) that reaches earth's surface (Ganguly et al., 2009). UV-B radiation has many direct and indirect effects on plants, including damages to DNA, proteins and induces oxidative burst and subsequently disrupts the function of the vital organelles chloroplast and mitochondria (Ganesh et al., 2013.). Light absorbing phenolic compounds as a group of phenylalanine derived aromatic secondary metabolites have been implicated in protecting plants UV-B radiation. The antioxidant potential of these

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compounds can selectively resist the free radicals generated by UV-B radiation (Yamamoto and Bassi, 1996). The total content of phenolic compounds in a tea callus culture grown under supplementary UV-B irradiation was almost 1.5 times higher than in a control culture (Strid, 1993). The effectiveness of UV-B irradiation not only increases the production of secondary metabolites, but also produces new compounds.

The theory of natural product chemistry indicates that a majority of UV-absorbing substances are active molecules produced by the secondary metabolism of plants. In other words, UVB irradiation could be utilized to promote the quality of medical plants, or even to induce some rare compounds. Plant's UV-B stress physiology has been a focus of botany and agronomy for a long time, but related research has not been reported in the area of natural medicine.

Aswagandha is widely used in various preparations of Indian System of Medicine and Homeopathy to cure several diseases including leprosy, nervous disorders, intestinal infections and rheumatism. The plant is also known as Indian ginseng for its various medicinal properties. Several studies concerning the chemistry and pharmacology (Gamoh et al., 1984), phytochemical variability in commercial herbal products (Sangwan et al., 2004), novel method to isolate withaferin A (Kannan and Kulandaivelu, 2007), light and drought stress induced changes in aswagantha (Kannan and Kulandaivelu, 2009, 2011) have been reported. The objectives of the present study are to determine UV-induced morphological and physiological responses; to identify proteomics of photosystem-II response upon UV-B stress and to detect secondary metabolites and correlations between secondary metabolites and photosynthesis during UV stress.

MATERIALS AND METHODS

Plant material and cultivation

Viable seeds of *Withania* were obtained from the Foundation of Revitalisation for Local Health Traditions (FRLHT), Madurai. Seeds were soaked overnight in running tap water and were sown in pots containing garden soil.

UV-B treatment

One-month-old seedlings were used for UV-B treatment. Seedlings were separated into two sets. One set of 10 pots, each with 5 plants, was kept under ambient solar radiation (control) and other set under 20% UV-B enhanced solar radiation. Sun lamps emitting UV-B radiation (Philips TL 20 W / 12) (Philips Gloelampenfabrieken, Holland) were mounted in the field to obtain UV-B enhanced solar radiation. UV-B treatment was given to these plants for 4 hours daily from 10 am to 2 pm and continued under ambient solar radiation. The irradiance of UV-B and visible radiation (average PAR level) were 0.2 and 450 $W.m^{-2}$, respectively. The first formed leaves were collected at different time periods for all the physiological and biochemical analysis. Radiation below 400 nm was determined using an IL 700A radiometer with SEE 400 photodiode (International Light, USA).

Isolation of chloroplast and pigment estimation

Type II chloroplasts were isolated and suspended in a buffer containing 0.4 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$ and 20 mM Tris-HCl pH 7.8. Chlorophyll (Chl) was extracted in 80% acetone. The concentration of Chl *a*, Chl *b*, total Chl and carotenoids was quantified according to Wellburn and Lichtenthaler (1984). Anthocyanin was estimated spectrophotometrically according to method of Mancinelli et al. (1975).

Activities of electron transport

The rate of O_2 uptake was continuously followed using a Hansatech O_2 electrode (Hansatech, U.K.) at 25°C. The reaction mixture in a total volume of 1.0 ml contained 20 mM Tris- HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM NaCl, 100 mM sucrose, 1 mM MV, 1 mM BQ, 50 µM DCPIP 2 mM Na ascorbate, 1 mM sodium azide, 5 mM NH_4Cl and chloroplasts equivalent to 20 µg Chl.

Isolation of thylakoid membranes

Freshly harvested leaves were homogenized in semi frozen isolation medium containing 35 mM HEPES-KOH, pH 7.4, 10 mM NaCl and 400 mM sucrose for 10 to 12 s at 50% speed in a Polytron homogeniser. Chloroplasts were ruptured in 30 mM Na-phosphate buffer pH 7.6 containing 1 mM DTT, 30 $\mu g.ml^{-1}$ chloramphenicol and 10% glycerol (Tadahiko et al. 1989).

Isolation of PS II particles

Chloroplasts were isolated in a medium containing 50 mM Na/K phosphate buffer (pH 7.4), 100 mM sucrose and 50 mM NaCl and were suspended in a medium containing 50 mm Na/K phosphate buffer (pH 6.9), 300 mM sucrose, 5 mM $MgCl_2$, 1 mM $MnCl_2$ at a Chl concentration of 1 to 2 $mgml^{-1}$. An aqueous solution of Triton X-100 (v/v) was added to the chloroplast suspension in a final Triton: Chl ratio 20:1 (w/w). After 1 min the suspension was centrifuged at 1000 g for 2 min. The supernatant was centrifuged at 35000 g for 10 min. The resulting pellet was resuspended in a medium containing 35 mM HEPES-KOH, pH 6.5 and centrifuged at 1000 g for 2 min. The pellet was discarded and the supernatant was centrifuged at 35,000 g for 10 min. The pellet was used as PS II particles and resuspended in buffer containing 25 mM MES-NaOH (pH 6.5).

Analysis of chloroplast proteins by SDS-PAGE

The chloroplast suspension was mixed with 10% ice-cold TCA and left on ice for 10 min. The solution was centrifuged at 3000 g for 5 min. Traces of TCA and pigments were removed by washing thrice

Table 1. Changes in the photosynthetic pigments of *Withania somnifera* exposed to UV-B enhanced radiation. Values are expressed on mg/g fresh weight. The estimations were made after four weeks at different day intervals. Mean ± SE, n=10.

Treatment Period (days)		Chl *a*	Chl *b*	Total Chl mg. g. fr. wt^{-1}	Chl *a/b*	Carotenoids	Anthocyanin A/g^{-1}Fr.wt
10	Ambient	1.67 (100)	0.81(100)	2.48(100)	2.06	0.66(100)	0.53(100)
	(+) UV-B	1.41 (87)	0.69(85)	2.10(85)	2.04	0.34(52)	0.64(120)
20	Ambient	2.25 (100)	1.09(100)	3.34(100)	2.06	0.88(100)	0.76(100)
	(+) UV-B	1.72 (76)	0.91(83)	2.75(83)	2.08	0.60(68)	0.95(125)
30	Ambient	2.5 (100)	1.22(100)	3.74(100)	2.04	1.37(100)	0.83(100)
	(+) UV-B	1.65 (66)	0.89(72)	2.54(72)	1.85	0.88(64)	1.18(142)

with ice-cold acetone. The final pellet was washed with cold diethyl ether and dried to powder. The powdery protein sample was thoroughly solubilised in a minimal volume of 2% SDS.

MALDI-TOF analysis of PS II core complex

Molecular weight and UV-B induced damages on intact PS II core complex proteins were studied using MALDI-TOF mass spectrometer. PS II particles were dialyzed against 50 mM ammonium bicarbonate buffer and dried under speed vacuum at 45°C and used for MALDI-TOF analysis. Kratos CFR plus mass spectrophotometer with an accelerating voltage of 20000 was used in linear mode. Saturated sinapic acid was used as matrix and the instrument was calibrated using cytochrome c (C 8857), apomyoglobin (CA897) and aldolase (A9096) (Sigma, USA) having monoisotopic masses of 12361.96, 16952.27 and 39212.28, respectively. Matrix, sinapinic acid (3, 5 dimethoxy L-hytroxycinnamic acid S 8313) was prepared as described by the manufacturer. For the analysis of PS II core complex, http//: www.matrixscience.com server was used in this study and this was further confirmed in the following web servers: http//: www.ncbi.ntm.nih.gov. http//: www.expasy.org http//: www.ebi.ac.uk http//: www.prospector.ucsf.edu/ucsfhtml4.0/msfit.htm

HPTLC analysis of withaferin A

Root powders obtained from control and UV-B treated plants were accurately weighed (500 mg) and dissolved in 20 ml of methanol in 25 ml volumetric flask. The solution was filtered through Whatman filter paper No. 42 and the filtrate was made up to 25 ml with the same solvent. Withaferin A (5 mg) was accurately weighed into a 50 ml volumetric flask, dissolved in 25 ml of methanol and finally made up to 50 ml with the same solvent to furnish a working standard (0.1µg µl^{-1} concentration). The chromatography was performed on precoated aluminium - backed silica gel G high performance thin layer chromatography (HPTLC) plates prewashed with methanol as described by the method of Mahadevan et al. (2003). Plates were developed with toluene: ethyl acetate: formic acid (50:15:5 v/v/v) in a Camag twin trough chamber. The standard solution (1 to 3 µg µl^{-1}) and sample solution (1.5 to 2.5 µg µl^{-1} of withaferin A) were applied to the HPTLC plates as 8 mm bands by a Camag Linomat IV applicator. The plate was scanned at 213 nm with a Camag TLC scanner III controlled by CATS V.4.06 software.

RESULTS

Changes on the photosynthetic pigments and non photosynthetic pigments

Changes in various photosynthetic pigments of *Withania* seedlings grown under both ambient and UV-B stressed condition at different day intervals are shown in Table 1. The level of photosynthetic pigments decreased upon exposure to UV-B enhanced radiation. As much as 32% reduction in total Chl was observed upon UV-B treatment for 10 days. Further increase in the duration of treatment decreased the level of total Chl and maximum loss was found on the 30th day. Similar trend was also found on the changes in Chl *a* and Chl *b* levels. However, the loss in Chl *a* was more than that in Chl *b*. This had resulted in low Chl *a/b* ratio after 30 days of treatment. Similar to Chl, the level of carotenoid shows a decrease at all the stages of treatment under UV-B enhanced radiation. A marked increase in the amount of anthocyanin was observed in *Withania* seedlings on exposure to UV-B enhanced radiation (Table 1). *Withania* plants grown under enhanced UV-B radiation showed significant increase in anthocyanin content. As much as 20% and 25% increase in anthocyanin was observed in 78 plants grown under UV-B enhanced radiation for 10 and 20 days, respectively. Maximum increase of 42% was observed after 30 days of UV-B treatment.

Changes on photochemical activities

The effect of UV-B treatment on the photosynthetic

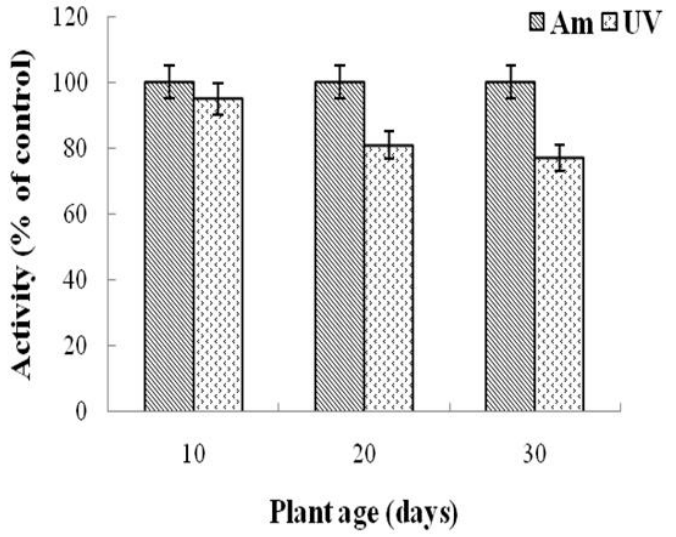

Figure 1a. The effect of UV-B enhanced radiation on photochemical activities of *Withania g*rown for different periods PS I: DCPIPH2 →MV Mean ±SE, n=3

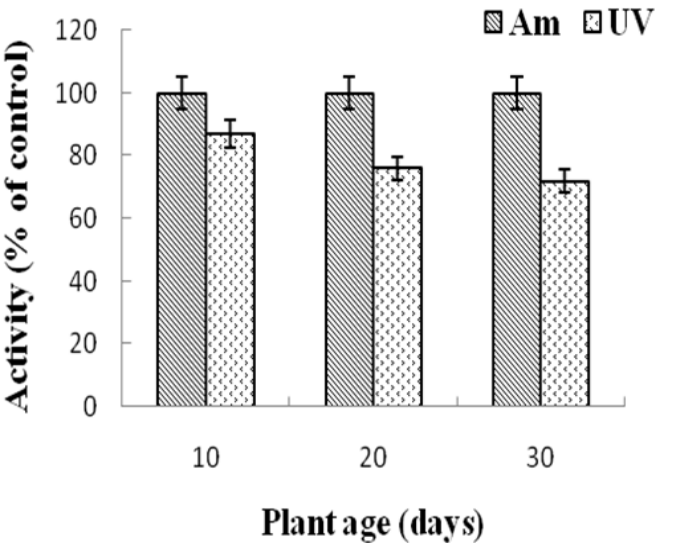

Figure 1b. The effect of UV-B enhanced radiation on photochemical activities of Withania grown for different periods PS II: H2O →BQ. Mean ±SE, n=3

apparatus of *Withania* seedlings at different periods was studied in terms of photochemical activities. Changes in the PSI and PS II rates were followed at different periods of treatment. The overall electron transport was measured in the presence of MV as an electron acceptor. A 14% decrease over the ambient control was noticed on

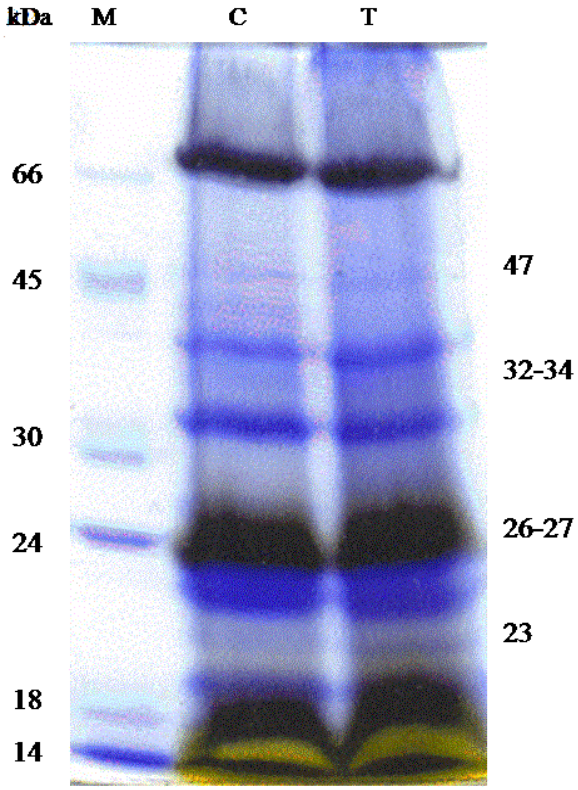

Figure 2a. Proteins profiles of PS II membranes isolated from 30 days old ambient (Am) and UV-B enhanced light (+UV-B) grown plants.

the 10th day after the initiation of UV-B treatment. Whole chain electron transport activity was reduced drastically after 20 and 30 days of UV-B treatment (not shown). PSI mediated electron transport, monitored as the rate of O_2 uptake after blocking the PS II electron transfer with DCMU, showed only marginal change after 10 days of UV-B treatment (Figure 1a). However, as much as 23% reduction was observed after 30 days of treatment. A similar change was also noticed in the level of PS II activity at different treatment periods (Figure 1b).

Effects of UV-B enhanced radiation on PS II proteins

Since UV-B enhanced radiation is known to affect strongly the PS II activity, changes in the proteins associated with this complex were analyzed. Changes in the protein pattern and composition of *Withania* PS II complex are shown in (Figure 2a). The PS II particles were prepared from both ambient and UV-B treated

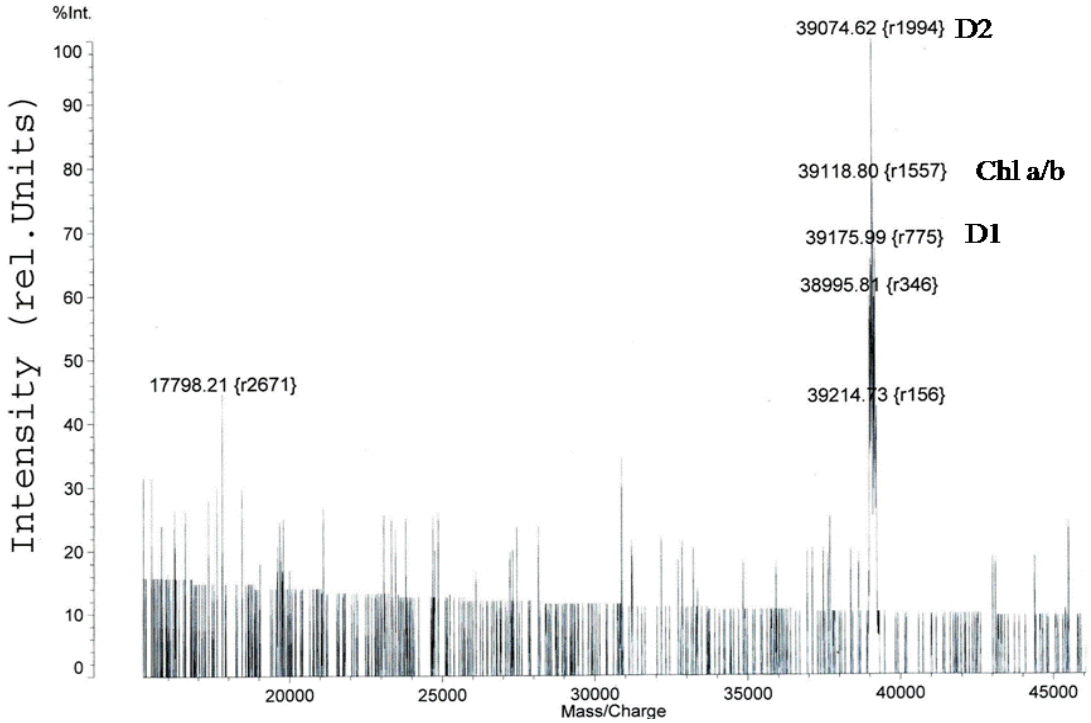

Figure 2b. MALDI spectra of PS II fraction isolated from 30 days old Withania grown ambient (Am) light.

plants 30 days after the initiation of treatment. UV-B radiation has brought about a marginal decrease in the level of 47, 34-32 kDa and 26-23 kDa proteins.

MALDI TOF analysis of PS II complex

Further characterization of the changes in the key PS II core proteins was made by MALDI-TOF analysis. The peptide masses obtained were analysed using web based peptide mass finger printing tools. The sorting out of the peptide fragments into those arising from the PS II core complex could be achieved by this analysis. The major effect of UV-B enhanced radiation on thylakoid membrane is the breakdown of the reaction centre D1 protein, as it is evidenced by loss of photosynthetic activity. The MALDI spectra of PS II complex isolated from both control and UV-B treated plants are shown in Figure 2b and Figure 2c respectively. In the *m/z* 15000 – 40000 range six main peaks were detected at *m/z* 39214±8, 39175±9, 39118±6, 39074±7, 38995±6, and 17798±9 (n=8 spectra from 4 different preparations). Each MALDI measurement was highly reproducible for the same sample and good reproducibility was observed in the *m/z* values of the peaks for samples derived from different preparations. However, the relative intensity of the peaks varied with samples, due to the presence of interfering materials such as salts and lipids. In plants grown under ambient light conditions, PS II core complex was not affected in which D1 and D2 proteins appeared at *m/z* 39175±8 (r775) and *m/z* 39074±9 (r1994), respectively. Three unidentified peaks were observed at *m/z* 39214±9, 38995±6 and 177798±8. Upon exposure to UV-B enhanced radiation, the degradation of D1 protein together with the other thylakoid subunits at *m/z* 15214±8 CP47 (r2311), *m/z* 39071±7 CP43 (r1441), *m/z* 15636±9 PsbJ (r2516), *m/z* 39182±6 PsbH (r1720), *m/z* 31126±8 PsbK (r2631), and *m/z* 39129±8 PsbM (r623) were observed. In addition to this, three other peaks have been detected at *m/z* 38977(r2104), 39019(r649) and 24102(r2378). The intensity of each fragment decreased drastically except for the subunit PsbJ. The subunit CP47, PsbJ and PsbK were free whereas, CP43, PsbH and PsbM were found to be closely associated with each other.

HPTLC analysis of *Withania* root

HPTLC analysis of root samples obtained from control

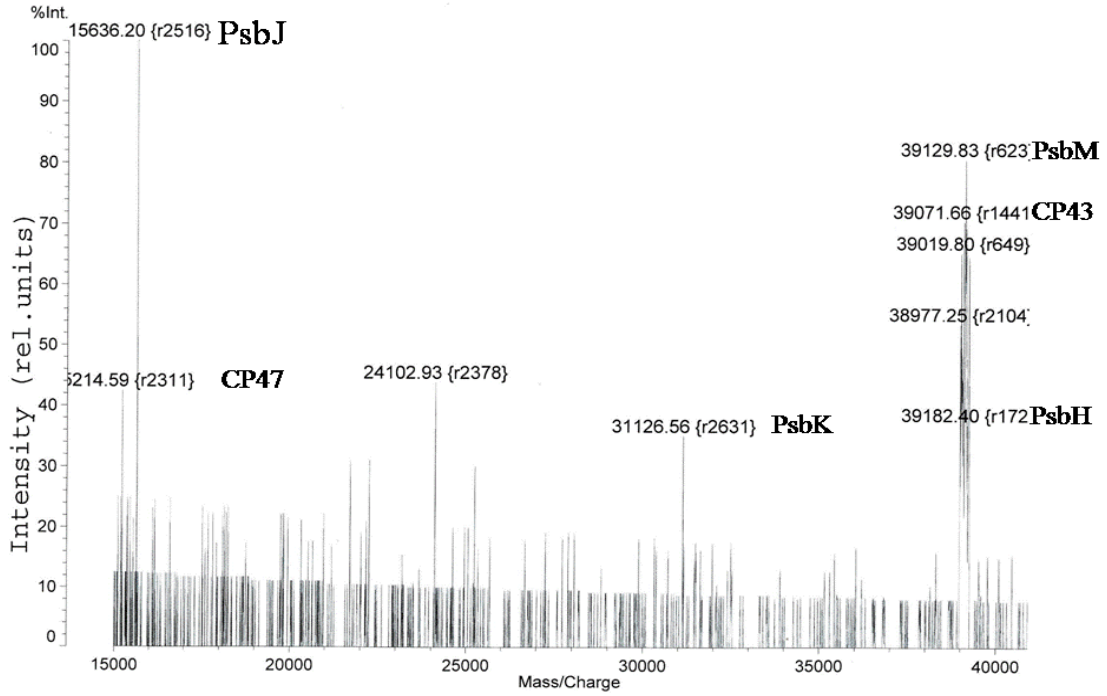

Figure 2c. MALDI spectra of PS II fraction isolated from 30 days UV-B treated plants.

and UV-B treated plants are shown in Figure 3. Withaferin A (Rf 0.14) was detected at 213 nm as described by Mahadevan et al. (2003) and it was found to increase by 3% under UV-B treatment as compared to control. In addition to withaferin A, there were other unidentified compounds detected in both UV-B and control samples. In control, no compound was detected at the Rf value 0.38 whereas in UV-B treated root, four unknown compounds of low concentration were found to have 0.59, 0.72, 0.82 and 0.85 Rf values.

DISCUSSION

Changes on photosynthetic and non-photosynthetic pigments

When the pigment analysis was done on unit fresh weight basis, the total Chl and Car content showed a reduction in UV-B treated seedlings. Similar results were obtained by (Deckmyn et al., 1994). UV-B radiation might induce non-enzymatic photooxidation of Chl and carotenoids resulting in the accumulation of their oxygenated forms. The loss of Chl could also be due to general photo-chemical degradation of the photosynthetic apparatus.

Foliar anthocyanins increased in *Withania* due to upregulation in plant tissues in response to UV-B radiation (Mendez et al., 1999). Such response is to mitigate DNA damage in UV-B irradiated cell cultures (Kootstra, 1994). Accumulation of anthocyanin under enhanced UV-B radiation indicates that they are actively involved in UV-B screening. These UV-B absorbing compounds are thought to provide a means of protection against UV-B damage and subsequent cell death by protecting DNA from dimerisation and breakage.

Changes on protein profiles

The function of energy transducing membrane depends on a precise structural arrangement of the various compounds. Hence the protein composition was analysed by SDS-PAGE to document any alterations in *Withania* seedlings grown under ambient and UV-B enhanced solar radiation. Plants grown under UV-B enhanced radiation revealed significant reduction in the levels of 55, 47, 43, 33, 27, 25 and 17 kDa proteins. Renger et al. (1989) have suggested that the catalytic site of water oxidation and especially the 33 kDa protein which contains the Mn cluster is highly sensitive to UV-B radiation. The extrinsic proteins 33, 23 and 17 kDa proteins stabilize the PS II complex and confirm the

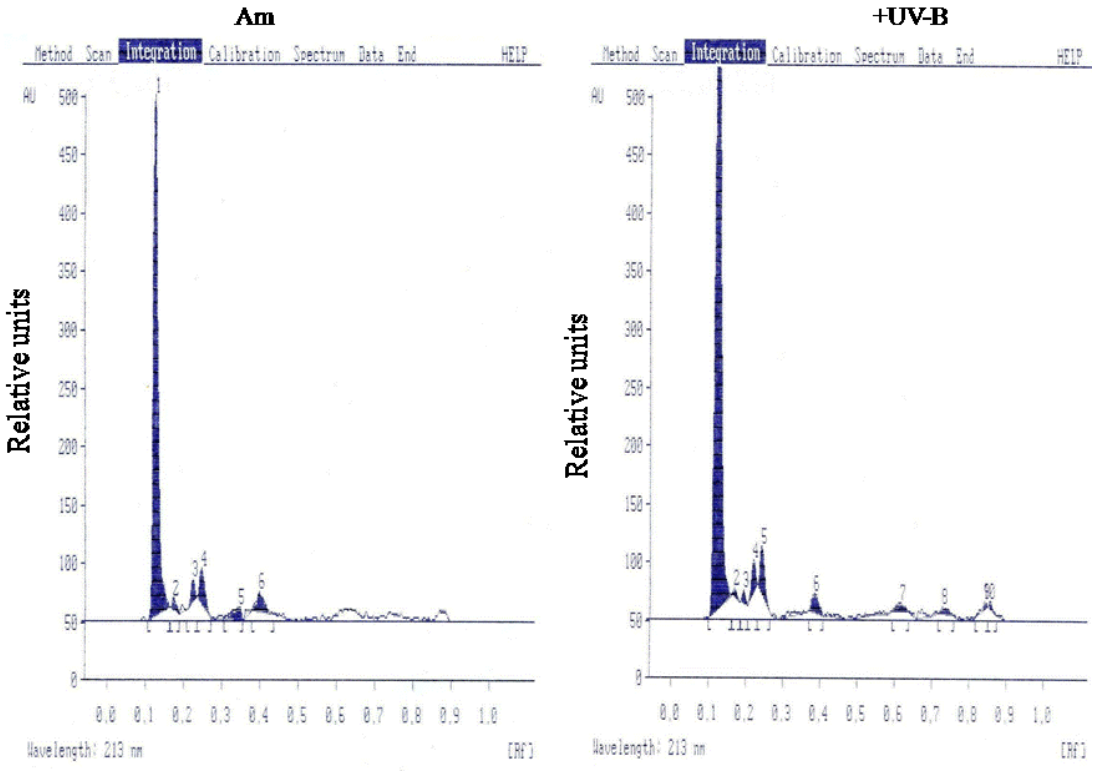

Figure 3. HPTLC analysis of roots obtained from Withania plants grown under ambient (Am) and UV-B enhanced (+UV-B) radiation.

catalytic activity during sequential oxidation of Mn atoms by the PS II reaction centre. Nedunchezhian et al (1995) have reported that UV-B radiation induced a decrease of 33, 23 and 17 kDa proteins in *Vigna* chloroplasts. Decrease in the level of these proteins or release of any of those proteins from thylakoid could result in low efficiency of O_2 evolution. When chloroplasts are isolated from UV-B treated seedlings not only the extrinsic proteins are released but also the 47 and 43 kDa proteins are lost which could be due to the disruption of the PS II complex (Kulandaivelu and Noorudeen, 1983). The reduction in the level of proteins, especially 55 and 15 kDa could be due to the change in the RuBPcase by UV-B radiation (Nedunchezhian et al., 1995). A clear reduction in the 27-25kDa of LHCP complex is prone to UV-B damage. LHCP complex of both PSI and PS II was affected under UV-B treatment.

MALDI-TOF analysis of PS II core complex

PS II core complex isolated from *Withania* grown under UV-B enhanced radiation revealed that the PS II core monomer complex collapses upon degradation of the damaged D1 protein. As a result of D1 protein damage, PS II subunits such as CP47, CP43, PsbH, PsbJ, PsbM and PsbK were found free. Accumulation of CP47 and CP43 was reported in *Arabidopsis* (Aro et al., 2005). The loss of the D1 protein observed during irradiation with UV-B (Barbato et al., 1995) is linked to an increased rate of degradation and decreased rate of synthesis. The partial loss on photosynthetic activity under UV-B might be due to the collapse of PS II core complex. Sakurai et al. (2003) have observed that high light and UV-B damage to PS II severely reduced the content of PS II dimers and conversely increased the monomer forms of PS II in *Synechocystis* thylakoid membrane. New synthesis of D1 protein occurs continuously and independently of the damage induced by UV-B radiation which had recovered slowly the rate of photosynthetic activity to some extend. It is known that the synthesis and insertion of new D1 protein depends on visible light at the level of transcription, translation and elongation of the nascent peptide (Zhang and Aro, 2002). Witt et al. (2001) reported

that PsbH, PsK, and PsbL subunits have been implicated in the stabilisation of the PS II dimers. Although most often only the D1 protein is the target for photo damage, the D2 protein and the PsbH protein are also occasionally subjected to irreversible damage (Aro et al., 2005). There are three unknown peaks at *m/z* 38977(r2104), 39019(r649) and 24102(r2378), which appeared under UV-B enhanced radiation, which may be stress-induced proteins or impurities or more probably D1 fragments associated with the PS II core. Assembly studies of the PS II centres, using various *psb* gene deletion mutants (Soursa et al., 2004) have provided evidence that the low molecular weight protein subunits, PsbH, PsbL, PsbM, PsbTc, PsbR and PsbJ associate with the PS II subassembly composed of D1/D2/Cyt *b*559/PsbI/CP47. Apparently these six protein subunits are essential for the stabilization of the D1/D2/Cyt *b*559/PsbI/CP47 sub-assembly of PS II complex and also for the structural integrity for the assembly of another set of proteins to PS II core complex. Of these protein subunits of PS II, the PabL subunit is essential for the stable association of CP43 (Suorsa et al., 2004) and the PsbJ subunit for water splitting and ligation of the oxygen evolving proteins. They are also necessary for regulation of the acceptor side activity of the PS II complex. In ambient light plants, the peak at *m/z* 39118 ± 6 (r1557) may be attributed to Chl *a/b* binding protein associated with PS II core complex. Under unstressed conditions, the full phosphorylation of PS II core proteins might have been attained and thereby maintaining the normal of photosynthetic activity during plant growth.

Changes on withaferin A

In *Withania*, root is the main medicinal part and it contains more of withaferin A than in leaves. Both quantitative and qualitative variations in withaferin A and other UV absorbing compounds were observed in *Withania* in response to UV-B stress. UV-B enhanced radiation might have triggered higher level of withaferin A synthesis than that of control which suggests a possible enhancement of the enzymes necessary for the biosynthesis of withaferin A. Along with withaferin A, some other UV absorbing compounds have also accumulated in the root. *Acer platanoids* showed differences in the quantity and quality of phenolic compounds under UV-B radiation (Zobel and Clarke, 1999). This suggests that *W. somnifera* could possess possibly more precursors for the formation of phenolic compounds or UV absorbing compounds. The question of whether the withaferin A and other compounds being synthesized in one area of of the plant but being transferred to another (shoots to root or vice versa) is not clearly understood.

ACKNOWLEDGEMENT

Authors are thankful to University Grants Commission (UGC), Government of India for financial assistance (No 42-956/2013).

ABREVIATIONS

C- control; **T-** treated**; MV,** methyl viologen; **DCPIP,** 2, 6, dichlorophenol indophenols; **BQ**, *p*- benzoquinone; DTT, dithiothretol.

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