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Table: 1, Figs : 4

Effects of light quality on light harvesting complex and withanolides in tropical medicinal plant *Withania somnifera*

N.D. KANNAN and G. KULANDAIVELU*

School of Biological Sciences,
Madurai Kamaraj University, Madurai, India-625 021
E. Mail: gkplant@yahoo.co.in

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ABSTRACT

We studied the effects of light intensities: ambient and low light on physiological and phytochemical changes in *Withania*. Low light grown plants accumulated more chlorophylls (Chl) and less carotenoids (Car), flavonoids and anthocyanins compared to ambient light. The ambient light adapted plants exhibited higher photochemical activities in isolated chloroplast than in low light plants. Thylakoid membrane protein analysis showed the presence of four proteins of light harvesting Chl-II (LHC-II) apoproteins and their level slightly increased in low light than in ambient light plants. HPTLC analysis revealed that the level of withaferin A increased by 5% in ambient compared to low light.

Keywords: D1 proteins, HPTLC, light stress, photosynthesis, thylakoid membrane, withaferin A, *Withania somnifera*

INTRODUCTION

The past decade has witnessed a tremendous resurgence in use of medicinal plant products. In the past, the medicinal plants were studied for phytochemistry, pharmacognosy and horticulture. Besides major medicinal plants physiological aspects etc. have not been investigated as done for food crops. *Withania somnifera* Dun. (Solanaceae) is Ayurvedic medicine to treat depression and inflammation and contains many phytochemicals (withaferin A, withanine, withanolides and tropine). Recently it has attracted great pharmacological research interests in chemistry (1), pharmacology (2) phytochemical variability in commercial herbal products (3), quantification of withaferin A (the main active constituent) by HPTLC (4) and novel method to isolate withaferin A from roots (5). However, studies on physiological and biochemical changes of *Withania* in response to light stress have not been reported. In plants, excess light damages the photosynthetic apparatus, hence, photosynthetic organisms adapt to changes in irradiation by altering and optimizing the abundance of specific components in photosynthetic apparatus. For instance, exposure to high light irradiances decreases the abundance of LHC-II (6) and D1 protein degrades more rapidly than other PSII proteins (7). An increased amount of LHC-II is an adaptation to shade, since it enhances the efficiency of light harvesting by the photosystems. This study aimed to determine the changes in various

*Correspondence author

photosynthetic reactions, thylakoid organization in excised leaves, and withaferin A of *Withania* plants grown under ambient and low light conditions.

MATERIALS AND METHODS

Viable seeds of *Withania somnifera* were obtained from the Foundation for Revitalisation of Local Health Traditions, Madurai. Seeds were soaked over night in running tap water and sown in pots containing field soil. One-month-old seedlings were separated into two light regimes. A set of 10 pots, each pot with 5 plants, was subjected to ambient and low light treatment. The plants which remained under full sun light (ambient) received > 400 $W.m^{-2}$ (400-700 nm) for extended periods of day and those which remained under low light condition received 40-60 $W.m^{-2}$. Visible radiation was measured using a LI–188-quantum/radiometer (Li-Cor, Inc., USA).

Chl and Car were extracted in 80% acetone and their concentration was calculated using the predetermined coefficient values (8). Anthocyanin was extracted from the leaves in acidified alcohol and its concentration was expressed as absorbance units after correction using the formula $A = A_{530}-(0.3\times A_{657})$ (9). Flavonoid content in light adapted *Withania* leaves was expressed as A_{315} units (10). Chloroplasts were isolated from light adapted leaves as per method of (11) and the final pellet was suspended in small volume of reaction buffer. Photosystem I (PSI), photosystem (PSII) and whole chain activities were measured as described earlier (12).

SDS-PAGE: Analysis of chloroplasts proteins was carried out by SDS-PAGE (13) using 10% linear gel. Protein content was estimated using BSA as a standard (14).

Thylakoid membrane isolation: For analysis of thylakoid polypeptides chloroplasts were ruptured in 30µg.ml^{-1} Na-PO_4 buffer, pH 7.6 containing 1mM DTT, 30µg.ml^{-1} chloramphenicol and 10% glycerol (15). Polypeptides were separated electrophoretically in an 8-14% gradient gel containing 6M urea and stained with Coomassie Brilliant Blue.

HPTLC analysis of withaferin A: Plants at their reproductive stage were uprooted, thoroughly washed with running tape water and shade dried. Root powder (500 mg) from ambient and low light plant was added into 25 ml volumetric flask and dissolved in 20 ml methanol. The solution was filtered through whatman filter paper No. 42 and the filtrate was made up to 25 ml with the same solvent. HPTLC analysis was performed on precoated aluminum backed silica gel G HPTLC plates as per method of (4). The sample was applied to HPTLC plates as 8 mm bands by a (Camag Linomat IV) applicator. Plates were developed with toluene: ethylacetate: formic acid (50:15:5v/v/v) in a Camag twin trough chamber. After development and drying of plates, samples were evaluated by scanning at λ 213 nm with a Camag TLC Scanner III controlled by CATS V.4.06 software. Standard withaferin A was isolated as described by the method of (5).

RESULTS

Photosynthetic and accessory pigments

Depending on the light intensity, the levels of photosynthetic pigments showed changes (Table 1). Quantitative changes occurred in these pigments in *Withania* grown under ambient and low light intensities. Total Chl level (fresh weight basis) was increased in low light grown plants than ambient light. During the early stage of adaptation, Chl a/b ratio remained same in both ambient and low light conditions and subsequently it decreased in low light condition. To check whether a similar increase also occurred in accessory pigments in low light grown plants, quantitative changes occurred in the level of carotenoids, flavonoids and anthocyanin. Unlike Chl, the level of these pigments decreased in low light than in ambient light grown plants (Table 1).

Table 1. Influence of various light intensities on level of photosynthetic pigments in *Withania* plants

Days	Chl a	Chl b	Total Chl	Chl a/b	Carotenoids	Anthocyanin	Flavonoids
	[mg/g^{-1}fr. wt]				[A/g^{-1}dr wt]		A_{315}
10 days							
Ambient	0.37 ±0.01	0.15 ±0.01	0.52	2.35	0.86 ±0.006 (100)	1.53±0.04 (100)	0.24±0.7 (100)
Lowlight	0.46 ±0.01	0.20± 0.01	0.66	2.32	0.30 ±0.01 (35)	1.30±0.02 (87)	0.19±0.02 (81)
20 days							
Ambient	0.50± 0.09	0.20 ±0.01	0.70	2.50	0.71 ±0.009 (100)	2.09±0.04 (100)	0.44±0.1 (100)
Lowlight	0.68 ±0.01	0.32 ±0.01	1.01	2.13	0.40 ±0.007 (56)	1.58±0.02 (76)	0.313±0.1 (71)
30 days							
Ambient	0.54 ±0.01	0.21 ±0.01	0.76	2.50	0.23 ±0.01 (100)	1.73±0.04 (100)	0.29±0.01 (100)
Lowlight	1.0 ±0.01	0.55 ±0.1	1.61	1.91	0.10 ±0.007 (43)	1.65±0.03 (95)	0.20±0.02 (69)

Values are expressed on unit of fresh weight and unit of dry weight. Figures in parentheses are percentage values with reference to respective control. Mean ± n= 3.

Photochemical activities

Changes in PSI and PSII catalysed electron transport activity of chloroplasts isolated from ambient and low light grown plants were studied at different stages of growth. In general PSII activity increased up to the full development stage and thereafter decreased in plants grown under both light conditions. A similar change was also found on PSI activity. There was significant reduction in PSI and PSII reactions in low light as compared to ambient light grown plants at different stages of growth (Figure 1). Maximum level of PSI and PSII activity was observed on day 20.

Chloroplast protein

Changes in level of chloroplast proteins isolated from ambient and low light

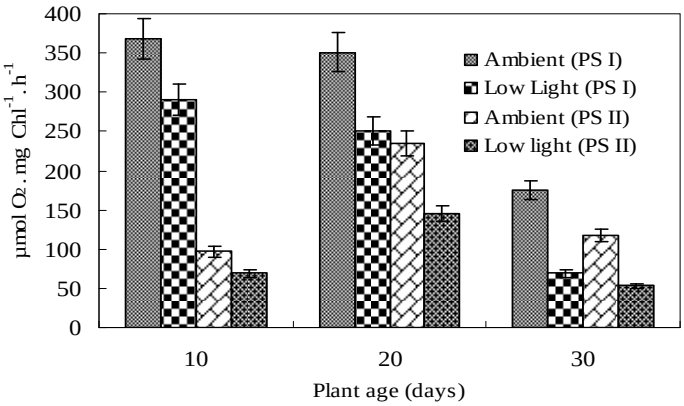

Figure 1**.** Response of PSI, PSII and whole chain activity in chloroplasts isolated from *Withania* adapted to ambient and low light treatment. Bars represent the mean of determination of three individual samples.

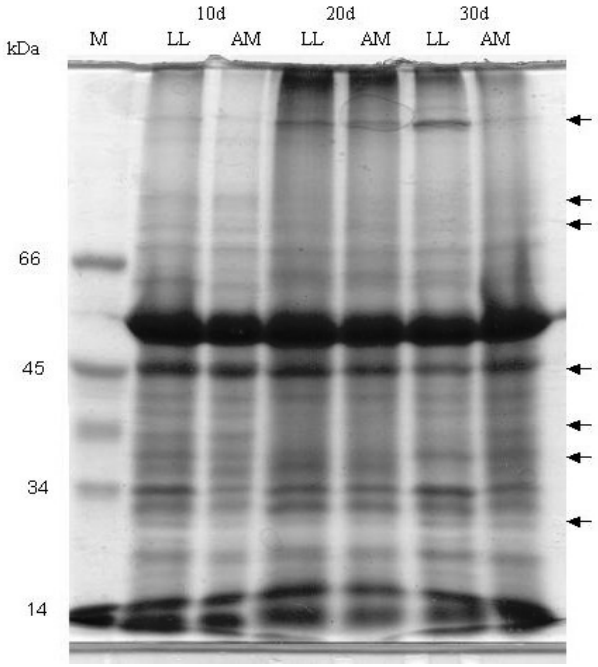

Figure 2. SDS-PAGE profiles of total chloroplast polypeptides isolated from *Withania* seedlings exposed to ambient (AM) and low light (LL) intensity. Numbers on the left side indicate the position of known molecular weight marker proteins.

adapted plants were examined by SDS–PAGE (Fig. 2). A sets of proteins in range of 90 to 70 kDa (90, 80, 75) showed variations in their level at different stages of growth. Some proteins 45, 32 and 24 kDa decreased in their level in ambient plants at all stages of growth compared to low light. Accumulation of D1 and D2 proteins was prominent in low light at all developmental period, whereas, the level of D1 and D2 proteins in ambient plants was slightly declined.

Thylakoid proteins

The profile of thylakoid membrane proteins of ambient and low light grown plants was examined by urea gel electrophoresis. A set of proteins, in the range of 60 to 45 kDa (viz, 60, 55, 50 and 45 kDa) revealed prominent accumulation in response to ambient compared to low light adapted plants (Fig. 3). In addition, there was accumulation of four distinct proteins that belongs to the LHC–II apoproteins. To facilitate presentation of the work, these polypeptides were labeled as LHC-II-I through LHC-II-4 and in urea-PAGE they migrated with apparent molecular weight of 32, 31, 30, and 28.5 kDa, respectively. Although four distinct bands of LHC were present in both ambient and low light adapted plants, the level of LHC-II declined more in ambient compared to low light grown plants. Initially all LHC-II apoproteins were synthesized with comparable rates and declined during the later stage of development.

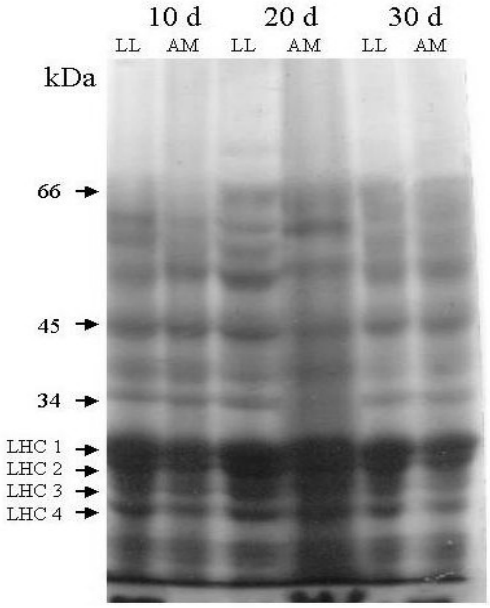

Figure 3. Urea-PAGE profiles of total chloroplast polypeptides isolated from *Withania* seedlings exposed to ambient (AM) and low light intensity (LL). Numbers on the left side indicate the position of known molecular weight marker proteins.

Chromatography

HPTLC analysis of root obtained from both ambient and low light showed significant quantitative and qualitative withanolides changes. Withaferin A had increased 5% in ambient compared to low light adapted plants. Ambient light adapted roots revealed withaferin A at R_f 0.14(88.43%) and five other structurally unidentified phytochemicals designated as withanolides WS-1 R_f 0.18(2.42%), WS-2 R_f 0.22(5.00), WS-3 R_f 0.30(1.71%), WS-4 R_f 0.37(1.32%), WS-5 R_f 0.83(1.62%) (Fig. 4). The level of withaferin A (R_f 0.14 (83.28%) decreased 5% in low light compared to ambient plants. Besides, five other phytochemicals designated as WS-1 R_f 0.16(0.79%), WS-2 R_f 0.18(2.98%), WS-3 R_f 0.22(7.34%), WS-4 R_f 0.37 (2.54%), WS-5 R_f 0.59 (3.07%) were noticed in low light (Fig . 4).

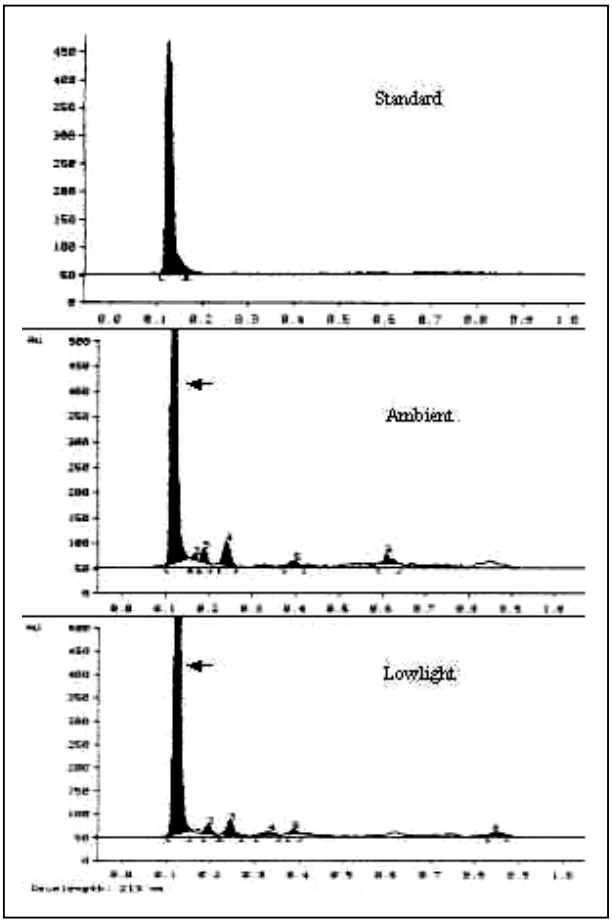

Figure 4. HPTLC analysis of root obtained from 30 days old *Withania* plants grown under ambient and low light. ⟶ Indicate withaferin A

DISCUSSION

The changes in photosynthetic pigments were typical of light adaptation. Shade and low light plants have more Chl *b* and LHC *a/b* proteins of PSI and PSII, when compared to those of strong light. Such increase in light–harvesting components occurs at the expense of electron transport, photophosphorylation and CO_2 fixation resulting in lower maximal photosynthetic rates, which saturates at low irradiance (16). This is commonly interpreted as an indication of higher ratio of core to LHCP and LHC complexes in sun leaves. Since Chl *b* is primarily a component of the LHC of PSII, it follows that a high Chl *a/b* ratio in *ambient* grown cells signifies a smaller light harvesting antenna size for this photosystem (17). In *Dunelliella tetiolecta* it was reported that the Chl *a/b* ratio of isolated LHC–II was variable and that this ratio changed in response to growth irradiance (18).

To maintain photosynthesis and the assembly of thylakoid membrane, Chl and Car are resynthesized very rapidly in ambient plants. Therefore, all components of thylakoid membrane exhibited much higher turn over in ambient plants than in low light plants. The changes in pigment composition of ambient and low light adapted *Withania* plants were very similar to those of ambient and low light adapted beech (19). Particularly high light grown plants are selectively enriched in Car involved in the xanthophyll cycle, namely violaxanthin, zeaxanthin and antheraxanthin (20). The flavonoid and anthocyanin composition can be altered by environmental factors like light and UV. Light induced production of anthocyanin and other flavonoids in the epidermis and outer tissues are considered to be a ubiquitous protective mechanism in higher plants against intense solar radiation. The induction of phenylalanine ammonia-lyase (PAL) by light is a prerequisite for the onset of light mediated anthocyanin synthesis (21). In *Rhododendron* leaves exposed to severe high light stress, the level of total flavonols was more than twice as high as in the half-shade leaves (22).

PSI and PSII activity was higher in ambient compared to low light plants. Similar results were reported in tobacco leaves (23). The photosynthetic activity of leaf is maximal, when the leaf reaches maturity and then it declines gradually during senescence (24.) The decreased activity could be associated with senescence of leaves, because of extensive degradation of both structural and functional components of chloroplasts. Conversely, ambient light plants being limited in electron transport rates, have greater amount of the cyt *b/f* complex, ATP synthase and the mobile linking electron transport compound, the PQ pool, PC and Fd and high carbon dioxide fixation enzyme to support greater rates of photosynthesis which saturates only at high light. Ambient light grown *Withania* had higher rate of photochemical activities and this might be due to plant have higher PSI/PSII ratios of 1.8-2.3 due to more PSII units each with smaller light harvesting size, while, shade and low light plants have lower PSI/PSII ratios of 1-1.3 due to lower PSII units each with larger light harvesting size evidenced by urea gel electrophoresis.

During active photosynthesis, the D1 protein and to a lesser extent D2 turn over rapidly and are replaced by newly synthesized proteins in repair cycle. Under ambient, D1 protein degraded more rapidly than under low light condition. Under excessive visible light, the D1 protein of PSII becomes photo damaged, which inhibits the PSII electron transport. Damaged D1 protein is degraded rapidly and is replaced by a newly synthesized

protein (25). Our results indicated that at all stages of growth under ambient, damaged D1 protein is replaced slowly by newly synthesized protein and maintained the normal rate of photosynthesis. Under low light conditions, there is no net loss of D1 protein content, because photo inhibition of PSII can be counteracted by D1 protein synthesis. Associated with the development of *Withania* seedlings from 10 to 30 days, a set of proteins (60 to 45 kDa) was accumulated more in ambient plants in the early stage of development compared to low light plants. Accumulation of these proteins may be associated with photosynthesis. LHC-II apoproteins accumulated much slower in ambient leaves than those of low light leaves. Stability of photosynthetic apparatus determined by the amount of Chl available to the chloroplast under the different growth conditions. The differential stabilization of LHC-II apoproteins by Chl would be one important mechanism for the coordination of Chl and apoproteins accumulation in thylakoid membrane of photosynthesis (26). These observations indicated adaptive mechanism of *Withania* for the acclimation of the photosynthetic apparatus to light stress. It suggested that under ambient conditions, LHC-II would not function efficiently due to the absence of Chl *b* from its holoprotein. When cells encounter a low light environment, however, Chl *b* could be incorporated into existing LHC-II simply by filling the vacant Chl *b* slots. (17). Levels of these proteins have declined during the senescence stage. It is probable that proteins, which were accumulated in this period, were associated with increased complexity of cellular activities (such as acquisition of enhanced photosynthetic activity, extension/growth of seedlings, etc) during development.

Environmental and climatic factors influence the production and release of secondary metabolites. In ambient plants, owing to higher photosynthesis, carbohydrates may have been produced in excess of growth demands, excess photosynthates might be used to produce withaferin A. Conversely when photosynthesis is more limiting under low light, carbohydrate contents decreased in plants as do withaferin A level. Ambient light induces production of sennosides reported in senna (27). Plants that grow under high light intensities contain higher quantities of allelochemicals and exhibit decreased susceptibility to UV-B induced damage (28).

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