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Full Length Research Paper

Drought induced changes in physiological, biochemical and phytochemical properties of *Withania somnifera* Dun.

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Drought (D) stress effects on growth, photosynthetic pigments, photosynthetic electron transport, thylakoid polypeptides and phytochemical variations of *Withania* (Solanaceae), a tropical medicinal herb were studied. Exposure of plants to D-stress led to noticeable decrease in leaf area, photosynthetic pigments, root and shoot lengths and photosynthetic activity. Well-watered plants maintained high content of total chlorophyll (Chl), root and shoot lengths, leaf area and photosynthesis. We analysed alterations at cellular level of the D-stress-associated proteins at different time intervals. A set of proteins in the range of 34 to 40 kDa showed variations in response to D-stress. Qualitative high performance thin layer chromatography (HPTLC) analysis of root extract obtained from control and D-stressed plants showed quantitative and qualitative variations. Withaferin A content increased 5% under D-stress as compared to control.

Key words: Carotenoids, chlorophyll, fluorescence, leaf area, pigments photosynthesis.

INTRODUCTION

The concentrations of various secondary plant products are strongly depending on the growing conditions and it is obvious that especially stress situations have a strong impact on the metabolic pathways responsible for the accumulation of the related natural products. The responses of plants to environmental stresses are complex and involve physiological and biochemical changes. Plants tolerate drought (D) stress by modifying their morphological and anatomical features, through physiological adaptations, or by biochemical means and molecular adjustments at the whole plant level. Biochemical adaptation may involve both primary and secondary metabolism. Among the medicinal plants, *Withania somnifera* Dun. is one of the most valued medicinal plants in Ayurveda and other traditional systems of medicine. Several studies concerning the chemistry and pharmacology (Gamoh et al., 1984), novel method to isolate withaferin A (Kannan and Kulandaivelu, 2007) and phytochemical variability in commercial herbal products (Sangwan et al., 2004) have been carried out. The biochemical response of *W. somnifera* to D-stress was studied at different day intervals. Stress effects on plants include changes in leaf pigments, altered physiology (impaired photosynthesis), ultimately poor growth, less vigor, and sometimes even death (Larcher, 1995). The yield reduction is mediated through reduced leaf growth and consequently lower photosynthetic productivity (Chen et al., 1993). Drought affects nearly all the plant growth processes depending on the intensity, rate and duration of exposure and the stage of crop growth (Brar et al., 1990). Physiological and phytochemical responses of *Withania* against D-stress have not been reported. This study is pertaining to the effects of D-stress on growth, photosynthetic pigments, photosynthetic activity, polypeptide pattern, and withanolides in *Withania*.

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MATERIALS AND METHODS

Plant and cultivation

Viable seeds of *W. somnifera* Dun. were obtained from the Foundation for Revitalisation of Local Health Traditions (FRLHT),

Madurai, India. Pre-soaked seeds were sown in 40 pots containing garden soil and were divided into four groups, each group with 10 pots. Five plants were maintained in each pot. One month-old plants were subjected to four water regimes. They were: control (C): No D was imposed and plants watered daily or D-stress and re-watering were imposed at the same time intervals, that is, 1 day (D1), 2 days (D2) or 3 days (D3).

Determination of growth parameters

Leaves were harvested unless otherwise mentioned, from the plants after adapting them at D-stress at least for a period of 30 days. Fully expanded leaves were used for various analyses. Fresh mass (FM) of the whole plant was determined immediately after harvest. Dry mass (DM) of the plant was determined after they had been dried overnight at 110°C. Leaf area was measured using a *Li-Cor 3100* leaf area meter (*Li-Cor*, USA).

Pigment analyses

Chlorophylls (Chl) and carotenoids (Car) were extracted in 80% acetone and their concentration was calculated using the coefficients of Wellburn and Lichtenthaler (1984). Anthocyanin was extracted from the leaves following the method of Mancinelli et al. (1975). Flavonoid content was determined following the method of Mirecki and Teramura (1984) and expressed as A_{315} units. Proline contents were determined following the methods of Bates et al. (1975). Amino acid and starch content was estimated as described by Mahadevan and Sridhar (1996).

Photochemical activity

Chloroplasts were isolated from leaves following the method of Reeves and Hall (1973) and the final pellet containing type II chloroplasts were suspended in a small volume of reaction buffer. Photosystems (PS) 1 and 2 activities were measured as described by Noorudeen and Kulandaivelu (1982).

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (SDS- PAGE)

Analysis of chloroplast proteins was carried out by SDS-PAGE (Laemmli, 1970) using 10% linear gel. Protein content was estimated according to Lowry et al. (1951).

High performance thin layer chromatography (HPTLC) of withaferin A

Plants at their reproductive stage were uprooted, thoroughly washed in running water, and shade dried. Root powder from control and D3 plants was accurately weighed (500 mg) and dissolved in 20 cm^3 of methanol in 25 cm^3 volumetric flask. The solution was filtered through *Whatman* filter paper No. 42 and the filtrate was made up to 25 cm^3 with the same solvent. HPTLC analysis was performed on precoated aluminium backed silica gel G HPTLC plates prewashed with methanol as described by the method of Mahadevan et al. (2003). Withaferin A (5 mg) was accurately weighed into a 50 cm^3 volumetric flask, dissolved in 25 cm^3 of methanol and the solution was made up to 50 ml with the same solvent to furnish a working standard (0.1 $\mu g\ \mu l^{-1}$ concentration). 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 $\mu g\ \mu l^{-1}$) and sample solution (withaferin A concentration is 1.5 to 2.5 $\mu g\ \mu l^{-1}$) were applied to the HPTLC plates as 8 mm bands by a *Camag Linomat IV* applicator. After development and drying of plates, evaluation of both standard and samples were performed by scanning the samples at λ 213 nm with a *Camag* TLC scanner III controlled by *CATS V.4.06* software. The peak areas were recorded for all the peaks. The amount of withaferin A of all the samples was calculated from peak areas.

RESULTS

D-stressed *Withania* plants produced significant reduction in various growth characteristics such as shoot length, root length, FM, leaf area and DM as compared to C plants (Table 1). With the decrease in water supply, shoot length, root length, and leaf area were reduced marginally in D1 and D2 plants, whereas D3 plants showed significant reduction as compared to C-plants. Table 2 shows the changes in the contents of photosynthetic pigments of *Withania* seedlings that were subjected to D-stress. The total Chl content declined in leaves subjected to D-stress. Severe reduction of Chl was observed in D3 plants, whereas the content of Car has increased under D-stress. Anthocyanin and flavonoid contents have also increased with decreased water supply. Similarly, proline and starch contents increased drastically with respect to decreased water supply (Table 3). Both PS1 and PS2 activities of chloroplasts isolated from *Withania* plants declined under D-stress (Table 4).

Prominent changes with D-stress were noticed in protein contents in the range of 34 to 40 kDa (Figure 1). In response to D-stress, 34 and 32 kDa proteins have accumulated in D2 and D3 plants after 10 and 20 days of treatment, but these changes were not noticed at the later stages. The 20 kDa protein also accumulated prominently during D-stress at all the stages.

The mobile phase resolved the withaferin A very efficiently (Figure 2A). The R_f value for withaferin A was 0.14. The wavelength 213 nm was used for detection of withaferin A in standard and samples. D3 root showed quantitative and qualitative variations as compared to control. HPTLC analysis of control revealed withaferin A at R_f 0.14, (80%) and seven other structurally unidentified phytochemicals designated as WS-1 R_f 0.16 (1.05%), WS-2 R_f 0.18 (3.25%), WS-3 R_f 0.22 (7.74%), WS-4 R_f 0.36 (2.82%), WS-5 R_f 0.50 (0.85%), WS-6 R_f 0.60 (2.32%), WS-7 R_f 0.81 (1.61%) (Figure 2B). Whereas in D3 root, withaferin A appeared at R_f 0.14 (85.13%) and five unidentified phytochemicals were designated as WS-1 R_f 0.17 (0.79%), WS-2 R_f 0.21 (2.5%), WS-3 R_f 0.23 (5.57%), WS-4 R_f 0.37 (3.03%), WS-5 R_f 0.82 (2.9%) were noticed (Figure 2C). Two unidentified compounds were absent in D3 sample.

DISCUSSION

Drought stress imposed on *Withania* plants exhibited several morphological and biochemical alterations. The

Table 1. Effect of drought stress on various growth characteristics of 30 days old *Withania*. Each value is the average of 20 samples.

Sample	Shoot length (cm^2)	Root length (cm^2)	FM (g $plant^{-1}$)	DM (g $plant^{-1}$)	Leaf area (cm^2)
10 days					
C	25.9 (100)	12.1 (100)	11.1 (100)	1.99 (100)	13.3 (100)
D1	25.1 (96)	11.5 (95)	9.3 (83)	1.39 (69)	12.7 (95)
D2	23.3 (89)	10.6 (87)	8.9 (80)	1.03 (57)	11.2 (86)
D3	22.7 (87)	09.3 (74)	8.5 (76)	1.02 (51)	11.5 (84)

Figures in parentheses are percentage values with reference to respective control (C). Values represent Mean ±SE, n = 5. FM, fresh mass; DM, dry mass; C, control; D, drought variants.

Table 2. Influence of drought (D) stress on the contents of photosynthetic chlorophyll (chl), carotenoid (car) pigments of *Withania*.

Treatment duration	Chl *a* (g kg^{-1} FM)	Chl *b* (g kg^{-1} FM)	Total Chl (g kg^{-1} FM)	Chl *a/b* (g kg^{-1} FM)	Caroteniods (g kg^{-1} FM)
10 days					
C	0.672 (100)	0.557 (100)	1.22 (100)	1.20	1.05 (100)
D1	0.638 (94)	0.483 (86)	1.12 (91)	1.32	1.32 (125)
D2	0.571 (84)	0.469 (84)	1.04 (85)	1.21	1.45 (138)
D3	0.303 (45)	0.315 (56)	0.61 (50)	0.92	1.75 (175)
20 days					
C	0.994 (100)	0.576 (100)	1.57 (100)	1.72	1.33 (100)
D1	0.598 (60)	0.330 (52)	0.90 (57)	1.97	1.83 (137)
D2	0.347 (35)	0.183 (32)	0.53 (33)	1.89	3.36 (252)
D3	0.261 (26)	0.147 (25)	0.40 (26)	1.17	4.16 (293)
30 days					
C	0.807 (100)	0.451 (100)	1.25 (100)	1.78	1.01 (100)
D1	0.504 (62)	0.286 (63)	0.79 (63)	1.76	1.13 (124)
D2	0.407 (50)	0.208 (46)	0.61 (48)	1.95	1.88 (206)
D3	0.227 (28)	0.133 (29)	0.36 (28)	1.73	2.35 (258)

Figures in parentheses are percentage values with reference to respective control (C). Mean ± SE, n = 5. FM, fresh mass; DM, dry mass; C, control; D, drought variants.

Table 3. Biochemical analysis of *Withania* grown under drought (D) stress.

Sample	Anthocyanin A/g^{-1}/A	Flavonoids (A_{315})	Amino acid [mg kg^{-1} (DM)]	Proline [mg kg^{-1} (DM)]	Starch [g kg (DM)]
C	0.25	0.43	2.99	2.39	0.519
D1	0.23	0.45	3.39	3.66	0.536
D2	0.24	0.46	7.12	7.04	0.542
D3	0.23	0.72	6.69	6.21	0.520

D, Drought variants; A, absorbance. Values represent Mean ± SE, n = 5.

growth reduction in root length and shoot length in D-treatment compared to C could be associated with decrease in the cytokinin transport from roots to shoot or the increase in the amount of phytohormone abscisic acid (ABA). The hormone imbalance leads to changes in the cell wall extensibility, and decline in the concentration of photosynthetic enzymes, which results in reduced growth.

Table 4. Influence of (D) stress on photosynthetic electron transport activities.

Treatment duration	PS1 [µmol (O_2) kg^{-1}(Chl) S^{-1}]	PS11 [µmol (O_2) kg^{-1}(Chl) S^{-1}]
10 days		
C	539 (100)	226 (100)
D1	532 (98)	163.4 (72)
D2	521 (96)	124 (55)
D3	510 (94)	123 (54)
20 days		
C	779 (100)	278 (100)
D1	789.1 (101)	259.9 (93)
D2	501.1 (64)	241 (73)
D3	182 (23)	172.8 (61)
30 days		
C	411 (100)	273 (100)
D1	319.1 (77)	127.6 (46)
D2	207.7 (50)	104.4 (38)
D3	163.6 (39)	065.4 (23)

Figures in parentheses are percentage values with reference to respective control (C). Values represent mean ± SE, n = 5.

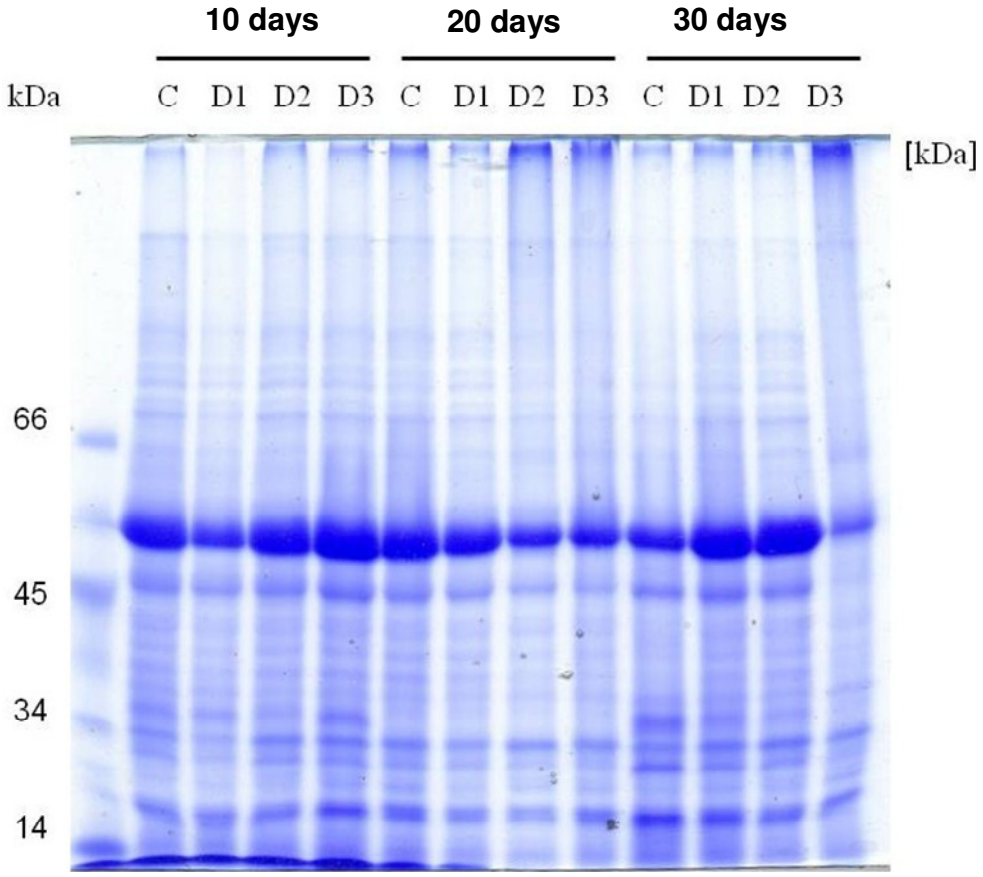

Figure 1. SDS-PAGE profiles of total chloroplast polypeptides isolated from *Withania* seedlings exposed to drought stress. Numbers on the left side indicate the position of known molecular weight marker proteins. C, Control; D, Drought variants.

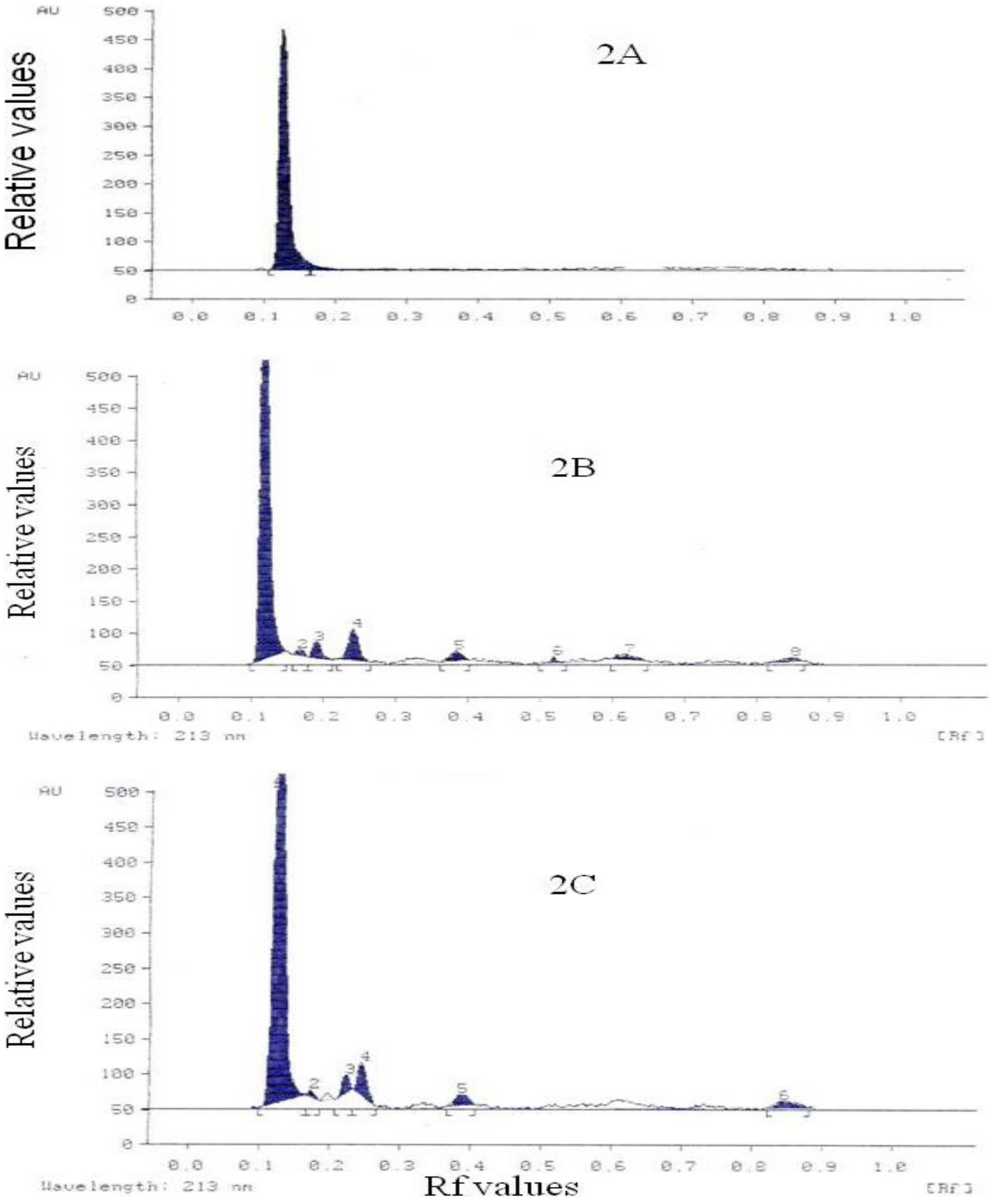

Figure 2. HPTLC analysis of root extract obtained from control (C) and drought (D) stressed *Withania* plants: (2A) Chromatogram of withaferin A; (2B) Control; (2C) D3. C, Control; D, drought variants.

The leaf area of *Withania* grown under D-stress has reduced drastically. A small drop in the water potential and turgor may be sufficient to interrupt leaf expansion. Mild water stress did not affect the leaf area, whereas in D2 and D3 leaves, it had produced large reduction. The leaves of D2 and D3 plants might have been affected due to the stress imposement on cell expansion, mitosis, and cell division resulting in reduced leaf area. The decline in growth reduced the plant demand for carbon, and hence photosynthetic activities declined to match reduced requirement for saccharides. These rapid changes induced by D-reduce plant growth and alter allocation even before there is a severe imbalance in carbon and nitrogen metabolism (Chapin, 1991).

Chl biosynthesis in *Withania* was affected by D-stress. Similar results were found in *Phaseolus vulgaris*, where both Chl and carotene together with xanthophylls decreased (Arcy-lameta et al*.,* 1996). A D-induced reduction in pigment content was previously reported in several species including pea (Moran et al., 1994). Photoinhibition and photodestruction of pigments may contribute to such changes. In addition, the photosynthetic apparatus may show acclimation responses such as changes in the relative proportion of stacked and unstacked membrane domains (Anderson and Aro, 1994).

Regarding photosynthetic activity, a significant reduction in electron transport activity was noticed in chloroplasts isolated from D-stressed plants. Earlier studies showed that the rates of photosynthesis in chickpea are markedly reduced by water deficits (Leport et al., 1999), which could be due to stomatal closure (Qifu et al., 2001). D-condition may also lead to a reduction in the proteins of light harvesting systems, thereby reducing overall efficiency of the electron transport and ATP synthesis (Demmig and Adams, 1992). Stomatal closure reduces CO_2 entry causing reduction in the intercellular CO_2 concentration and carbon fixation. This leads to an imbalance between the photochemical activity at PS2 and electron requirement for photosynthesis and ultimately to increased susceptibility to photodamage (Flagella et al., 1998).

Proline concentration increased with the decrease in water content. Accumulation of amino acids and proline is a stress response from the perspective of altered photosynthetic metabolism. Accumulation of these solutes could react with the hydroxyl radicals thereby protecting lipids, DNA, proteins, and macromolecular structure from degradative reactions leading to cell destructions during drought (Orthen et al., 1994). Various experimental methods of water-stress imposition can elicit proline accumulation in leaf tissue of young plants (Lawlor and Cornic, 2002). Proline acts as an osmoticum, a protective agent of enzyme and cellular structure and a storage compound of reducing nitrogen for rapid re-growth after stress are relieved. The results of present study are in agreement with the earlier reports on the free proline accumulation under water stress (Misra et al*.,* 2002). Several types of proteins accumulate as a result of D-stress in plants and many of them offer protection. In *Withania,* 34 to 32 kDa proteins have accumulated in stressed leaves and this is considered as an adaptation to D-stress. Pruvor et al. (1996) also reported similar increase in the contents of these proteins under D. The 32 kDa protein is located in the stroma and its synthesis is likely to be induced by a high osmolarity-related signal. The stress-induced synthesis of 34 kDa protein located in the thylakoid is mediated by an ABA-related signaling system. This protein may be involved in the reorganization of the thylakoid structure in order to tolerate the stress.

Synthesis and accumulation of secondary metabolites in plants is regulated in time and space, which is mediated by abiotic environmental factors like D, light intensity and mineral nutrition (Wink and Schimmer, 1999). HPTLC analysis of *Withania* roots indicated a high level of withaferin A was under drought stress as compared to control. The differences in R_f values may be due to the variations in enzymatic activity occurring under stress conditions thereby favouring the production of different compounds through different pathways. Non-structural carbohydrates then tend to accumulate and thus trigger the synthesis of carbon based defensive substances. The application of drought stress enhances the concentration of secondary plant products. However, it has to be taken into consideration that drought stress reduces the growth of most plants. Plants that suffer drought stress generate a high oversupply of reduction equivalents. Despite the fact that massive amounts of $NADPH + H^+$ are deoxidized by photorespiration and the xanthophylls cycle, under such stress conditions, the corresponding strong reduction power seems to enhance the synthesis of highly reduced compounds, like isoprenoids, phenols, or alkaloids. Accumulated natural products also prevent too massive generation of oxygen radicals and the corresponding damage by photo-inhibition. These results indicate that *W. somnifera* is tolerant to mild drought stress by accumulate certain proteins and secondary metabolite though overall photosynthesis is reduced.

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