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Effect of biological and chemical elicitors on the photosynthetic pigments and antioxidant activities of *Withania somnifera*

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Abstract

Background & aim: *Withania somnifera*, popularly known as Aswagandha has an immense value in Ayurveda. In the present study we aimed to evaluate the Effect of different elicitors on the photosynthetic pigments and antioxidant activities of *Withania somnifera*. **Methods**: The seeds of *W. somnifera* were subjected to elicitors before sowing. Both the controlled as well as treated plants were subjected to various biochemical analysis using standard methods. **Results & conclusion:** The chlorophyll, carotenoid, phenol, flavonoid, proline contents were found to be higher in elicitor treated plants compared to control. Among those treated with elicitor, plants treated with *Pseudomonas flourescens* showed the great increase in the phenolic and flavonoid contents, proving it to be a better elicitor.

Keywords*:* Aswagandha; *Withania somnifera*; elicitor; antioxidants; *Pseudomonas fluorescens*; *Trichoderma*

1. Introduction

Ashwagandha (*Withania somnifera* Dunal., Solanaceae) popularly known as Indian ginseng and Winter cherry, is one of the most reputed medicinal plants of Ayurveda, the traditional medical system. Ashwagandha in Sanskrit means "horse's smell" (ashwa- horse, gandha- smell), probably originating from the odour of its root which resembles that of a sweaty horse. The species name somnifera means "sleep-inducing" in Latin[1].It grows as a short shrub (35–75 cm) with a central stem from which branches extend radially in a star pattern (stellate) and covered with a dense matte of wooly hairs (tomentose). The flowers are small and green, while the ripe fruit is orange-red and has milk-coagulating properties. The plant's long, brown, tuberous roots are used for medicinal purposes[2]. *Withania somnifera* is grown as late rainy-season (kharif) crop. Semitropical areas receiving 500 to 750 mm rainfall are suitable for its cultivation as a crop. It can tolerate a temperature range of 20 to 38°C and as low a temperature as 10°C. The plant grows from the sea level to an altitude of 1500 meters. *Withania somnifera* is cultivated in many of the dry regions of India, such as Madhya Pradesh, Sindh, and Rajasthan[2] (Mirjalili et al., 2009).

Withania somnifera (Ashwagandha) reportedly exhibit antioxidant, immunomodulatory and hematopoietic properties. The well-describe pharmacological activities of the plant include physiological and metabolic restoration, anti-arthritic, anti-aging, cognitive function improvement in geriatric states and recovery from neurodegenerative disorders[3]. Several of its traditionally proclaimed medicinal properties have been corroborated by recent molecular pharmacological investigations and have been shown to be associated with its specific secondary metabolites known as with bolides, the novel group of ergostane skeletal phytol steroids named after *W. somnifera*. Both leaves and roots of the plant are used as a source of drug and steroidal lactones. Active constituents of *W. somnifera* viz. sitoindosides VII-X and withaferin A acts as antioxidants and has been proven to increase levels of endogenous superoxide dismutase, catalase, and ascorbic acid, while decreasing lipid peroxidation[4, 5].

Secondary metabolites includes wide range of organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism and hence are generally produced at a very low concentration that does not meet the commercial demands. Due to the medicinal value, these plants are collected and used as raw material for large-scale

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medicinal industry, leading to their over exploitation and making them endangered. Continued commercial exploitation of these plants has resulted in receding the population of many species in their natural habitat[6]. Elicitation is one of the most effective ways to promote secondary metabolites production in plant cell cultures[7]. Elicitors are chemicals or biofactors from various sources that can induce physiological changes of the target living organism[8]; they possess the ability to induce physiological changes in the living organism such as plant cells[9]. So there is the need to produce more in good quantity and quality from less land, water and time to achieve enhancement in medicinal compound.

In this study the effect of both biological and chemical elicitors either singly or in combination on phenylpropanoid (phenols and flavonoids), photosynthetic pigments and the antioxidant activity of *W. somnifera*. We also evaluated the possible correlation between the total phenols, flavonoids and antioxidant defence mechanism of elicited cells.

2. *Materials and methods*

2.1 Collection of the samples and treatment with elicitors:

The seeds of *Withania somnifera* were collected from different regions of Uttarakhand Haldwani and Rishikesh, and were sown in pots of 5 kg capacity composed of soil, sand and the farmyard manure at 1:1:1 ratio. A Pot culture experiment was designed with three replicates. Plant growth promoting microorganisms *Pseudomonas fluorescens* and Trichoderma formulation were taken from Biocontrol Laboratory Department of Plant Pathology College of Agriculture G.B.P.U.A&T Pantnagar. Seed treatment was employed for giving treatment of different biological elicitors and chemical elicitor treatment. The seeds of *Withania somnifera* were treated with *Pseudomonas fluorescens* and *Trichoderma* by dissolving small amount of the formulations in the water and placing the seeds overnight in the solution and where as paclobutrazol (HIMEDIA) at a concentration of 10mg/L was used for the treatment of the seeds.

2.2 Biochemical analysis

2.2.1 Chlorophyll and carotenoid content

Chlorophyll and carotenoid content were expressed as mg/g dry weight and were measured by using the method of Machlan and Zalik[10]. 0.1 g of leaf samples were placed in 10ml of 80% acetone in a test tube and were kept overnight at 4°C. Then the leaf samples were homogenized and centrifuged at 6000xg for 15 minutes. Optical density (O.D.) of the supernatant was measured at 480, 510, 645 and 663 nm. The content of chlorophyll a and b and carotenoid were calculated using the following formula:

$$\text{Chl a content (mg/g dry leaf)} = \frac{(12.3 \times D_{663}) - (0.86 \times D_{645})}{d \times 1000 \times W} \times V$$

$$\text{Chl b content (mg/g dry leaf)} = \frac{(19.3 \times D_{645}) - (3.6 \times D_{663})}{d \times 1000 \times W} \times V$$

$$\text{Carotenoid content (mg/g dry leaf)} = \frac{(7.6 \times D_{480}) - (1.49 \times D_{510})}{d \times 1000 \times W} \times V$$

Total chlorophyll = chl a + chl b

Where, V= volume of extract (ml), d= length of light path (cm), W= dry weight of leaves taken (g)

2.2.2 Preparation of methanol extract

1 gram of leaf was taken from all the pots of treated as well as untreated plantlets and was crushed in 10 ml of methanol. The solution was then centrifuged at 6000xg for 15 minutes and the supernatant was collected in a fresh tube for the biochemical analysis.

2.2.3 Determination of total flavonoid content:

Colorimetric aluminum chloride method was used for Flavonoid determination with slight modification as per Goyal et al.[11]. Briefly, 0.5 ml solution of each plant extracts in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 m of 1 M potassium acetate, and 2.8 ml of distilled water, and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a UV/Visible spectrophotometer. Total Flavonoid contents were calculated by extrapolating on quercetin standard curve. The calibration curve was prepared by different concentrations (12.5 to 100 mg/ml) of quercetin in methanol.

2.2.4 Determination of total phenol content:

Total phenolic contents were determined by the Folin-Ciocalteau method with minor modification as per Usha et al.[12]. Briefly, the methanol extract samples (0.5 ml) were mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) for 5 min and aqueous Na_2CO_3 (4 ml, 1 M) were then added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetry at 765 nm. The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg/ml solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg/g of dry mass), which is a common reference compound[13].

2.2.5 Proline content

Proline was extracted and the content assayed spectrophotometrically according to the method of Bates et al.[14]. The leaf sample of 0.5 g was homogenized in 10 ml of 3 per cent sulphosalicylic acid. The homogenate was filtered through a double layered filter paper. A 2 ml of the filtrate was taken in a test tube to which 2 ml of

acid ninhydrin reagent (2.5 g of ninhydrin was dissolved in 40 ml of 6M orthophosphoric acid and 60 ml of glacial acetic acid), 2 ml of glacial acetic acid was added. The test tubes containing the mixture were placed in boiling water bath for one hour. The test tubes were then cooled by keeping them in an ice bath. The contents were transferred to a separating funnel and 4 ml of toluene was added and mixed vigorously. The coloured toluene fraction was separated and measured at 520 nm in a spectrophotometer. A blank was maintained with all the reactants except the leaf extract. Proline content in leaf tissue was calculated by using the formula:

$$\text{Proline } (\mu g \text{ g dry wt.}^{-1}) = \frac{34.11 \times OD520 \times V}{2 \times f}$$

Where, V = Total volume of extract, f = Grams of fresh leaf, 2 = Volume of extract taken

3. Results and discussion

3.1 Plant growth after elicitor treatment:

The seeds were taken from Haldwani (29.2200° N, 79.5200° E) and Rishikesh (30.1030° N, 78.2940° E) followed by treatment with three elicitors, *Pseudomonas flourscens*, *Trichoderma* (biological elicitors) and placlobutrazol (chemical elicitor) alone as well as in combination along with control (without any treatment). Seeds were sown in triplicates. Optimal growth was observed in the plants treated with *Pseudomonas flourscens* & *Trichoderma* alone, **Figure I**, whereas seeds treated Pseudomonas flourscens & Trichoderma in combination showed the growth equivalent to the control along with yellowing and drooping of leaves, **Figure II**. This may be due to combined effect of both the elicitors that lead to a decreased metabolism. No growth was observed when the seeds were treated with paclobutrazol alone or in combination. May be paclobutrazol was toxic to seeds as the seeds were very small with no covering.

3.2 Biochemical tests

3.2.1 Chlorophyll and carotenoid content:

Photosynthesis is the basal metabolic pathway that leads to the production of primary metabolites. These primary metabolites when present in surplus is driven towards the secondary metabolite production pathways. Thus, the rate of photosynthesis can show a relationship with the production of secondary metabolite. Chlorophyll and carotenoid content can be used to measure the photosynthetic rate. The concentration of pigments was determined using the method of Machlan and Zalik[10]. When compared to control, a significant increase in chlorophyll content was observed in the plants treated with *Pseudomonas flourscens* i.e., 3.244 mg/g dry weight in the Haldwani, India variety whereas in Rishikesh variety the maximum increment was observed in the *Trichoderma* treated plants i.e., 2.44 mg/g dry weight,

Figure I: Plants with treatment. (a) *Trichoderma,* (b) *Pseudomonas flourscens*

Figure II: Plants with treatment. (a) *Trichoderma* + *Pseudomonas flourscens*; (b) Control

Figure III.

Carotenoids are known to function as collectors of light energy for photosynthesis and as quenchers of triplet chlorophyll and O2−•. Moreover, they dissipate excess energy via the xanthophylls cycle and can act as powerful chloroplast membrane stabilizers that partition between the light-harvesting complexes (LHCs) and the lipid phase of thylakoid membranes. This reduces membrane fluidity and susceptibility to lipid peroxidation[15]. The carotenoid increment pattern was in accordance with the Chlorophyll content observed, **Figure III**.

3.2.2 Total flavonoid content

Flavonoids are well-known antioxidant constituents of plants and posses a broad spectrum of chemical and biological activities, including radical scavenging properties[16]. Therefore, the total content of flavonoids was evaluated from the regression equation of the calibration curve (R^2=0.997, Y= 0.008x -0.055) and is expressed in mg/g of leaf. The highest amount of flavonid was found in the plants treated with *Pseudomonas fluorscens* followed by *Trichoderma* treatment in both the varieties, **Figure IV**.

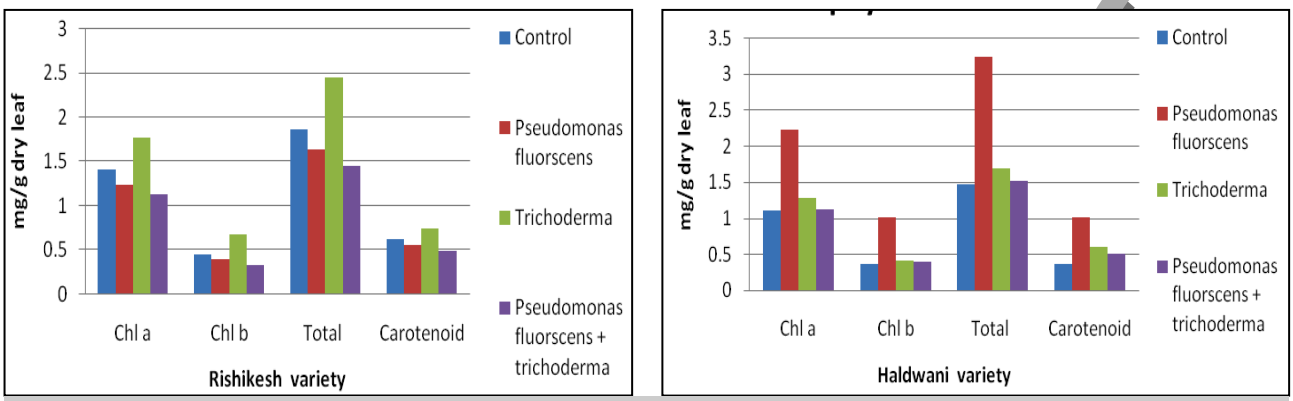

Figure III: Chlorophyll and carotenoid content in both the varieties Rishikesh (left) and Haldwani (right)

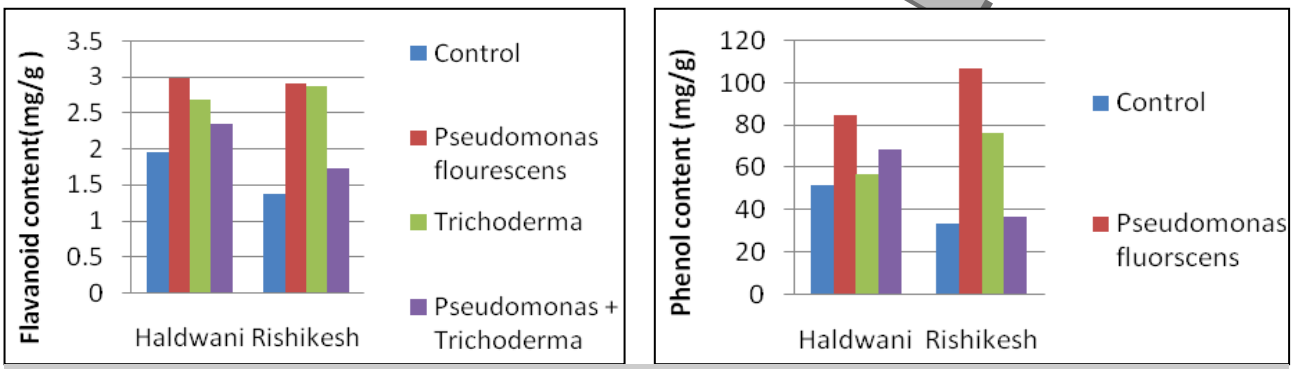

Figure IV: Total flavanoid (left) and phenol (right) content in treated and control plants of both varieties

3.2.3 Total phenol content

The phenols form a large family of low molecular weight polyphenolic compounds, which occur naturally in plant tissues. In plants, the phenols are thought to have many functions including protection against UV-B radiation, defense against pathogen attack and wounds[17]. Total phenol content in leaf, as determined by Folin-Ciocalteau method, are reported mg/gram fresh leaf by reference to a standard curve (Y= 0.005x – 0.006 R2= 0.997). In *W. somnifera*, the level of total phenols increased in the plants treated with *Pseudomonas fluorscens* in both the varieties as compared to other treatments and control, **Figure IV**.

3.2.4 Correlation between flavonoid, phenol content and antioxidant activity

Antioxidant activities are known to increase proportionally to the polyphenol content. This activity is believed to be mainly due to their redox properties[18] which play an important role in (a) adsorbing and neutralizing free radicals, (b) quenching singlet and triplet oxygen, and (c) decomposing peroxides. Also according to recent reports, a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species. Naturally occurring antioxidants such as phenols, flavonoids are well known to have very less or no side effects and hence are considered to be safe[19]. In this study, we found that the plants treated with Pseudomonas fluorscens showed the significant increase in the phenol as well as flavonoid content. Whereas, plants treated with *Trichoderma* and *Pseudomonas fluorscens* as well as *Trichoderma* alone showed very little increase in their content when compared to control, **Figure IV**. Pseudomonas fluorescens treatments have a profound effect on the antioxidant metabolism and caused an enhancement in non-enzymatic antioxidant potentials under treatments in *Catharanthus roseus*[21].

In plants, polyphenol compounds like flavonoids, which contain hydroxyl functional groups, are supposed to be responsible for the radical scavenging effect. *Withania somnifera* is known for its anti oxidant activity owing to the presence of the large number of secondary metabolites. The increase in the polyphenol and flavonoid content correlates with the increase in antioxidant activity and hence with the increase is a secondary metabolite.

3.2.5 Proline content

Several medicinal plant species react against the modifications of their environment producing secondary metabolites. These compounds protect the plant against environmental stresses. One of the measures to study stress is the determination of free proline content.

Several studies show that the levels of proline are more specifically regulated than the other solutes during adjustment to stress[22], enabling plant survival under extremely severe conditions. Proline is suggested to play

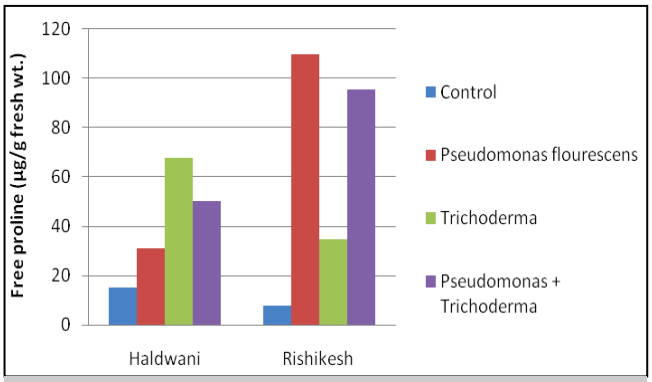

Figure V:Free proline content in response to elicitors

several roles in plants exposed to stress. It acts as the osmolyte to protect the cell turgidity under dehydration stress and prevent structural damage to macromolecules during desiccation. The high proline content is likely to depress reactive oxygen species level due to the involvement of proline in detoxification of superoxide radical.

The proline content increased drastically in Rishikesh variety when treated with *Pseudomonas fluorescens* (109.8 µg/ gm fresh weight) followed by the plant treated with *Pseudomonas fluorescens* and *Trichoderma* in combination (95.4 µg/ gm fresh weight) in Rishikesh variety. While, in Haldwani variety, plants treated with *Trichoderma* showed the maximum increase (67.78 µg/ gm fresh weight), **Figure V**. From the above result, it can be inferred that Rishikesh variety is more prone to stress as compared to Haldwani variety. Thus, the former can produce more secondary metabolites in response to stress.

4. Conclusion

Withania somnifera is a widely used medicinal crop, and its medicinal properties are due to the presence of a wide range of secondary metabolites. The metabolic pathway for these secondary metabolites involves many enzymes and genes corresponding to them. To increase the concentration of these secondary metabolites, elicitation was used. The treated plants were analyzed biochemically. In conclusion, plants treated with *Pseudomonas flourescens* showed the great increase in the phenolic and flavonoid contents, proving it to be a better elicitor. However, the plants treated with both the elicitors did not show any significant increase their content of phenols and flavonoid. Besides, two varieties of ashwagandha were also found to show the difference to cope with stress. Rishikesh variety showed a great influence of stress which was estimated using free proline content and can be used as a potent variety to show an increase in the secondary metabolite when acted upon by elicitors.

Conflict of interest

The author's declares none.

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