Studies on leaf spot disease of *Withania somnifera* and its impact on secondary metabolites

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Abstract During an investigation of the disease profile of *Withania somnifera*, it was observed that leaf spot is the most prevalent disease. Repeated isolations from infected leaf tissues and pathogenicity tests showed the association of fungal pathogen identified as *Alternaria alternata* (Fr.) Keissler. Scanning electron microscopy showed various histological changes in the leaf tissues of infected plants. A decrease in total content of reducing sugars (20%) and chlorophyll (26.5%) was observed in diseased leaves whereas an increase was noticed in proline (25%), free amino acids (3%) and proteins (74.3%). High performance thin layer chromatography (HPTLC) analysis of secondary metabolites viz. withanolides, withaferin-A and total alkaloids of the diseased leaves *vis-à-vis* control revealed reduction in withaferin-A and withanolides contents by 15.4% and 76.3% respectively, in contrast to an increase in total alkaloids by 49.3%, information hitherto unreported in *W. somnifera*.

Keywords *Alternaria alternata* · Biochemical changes · HPTLC · Scanning electron microscopy · Microbial deterioration

Introduction

Withania somnifera (L.) Dunal commonly known as ‘ash-wagandha’ or ‘asgandh’ has a high repute in traditional Indian medicine, and is one of the most extensively used plant in Ayurveda and Unani medicines [1]. It is widespread in Africa, Mediterranean region and the Middle East [2, 3]. The plant roots are a major source of alkaloids including tropine, pseudotropine and somniferine [4]. Besides alkaloids, *W. somnifera* possesses other secondary metabolites, viz. withaferin-A, withanolides, flavonol glycosides, glycowithanolides, sterols and phenolics [5, 6]. It possesses immense therapeutic value against a large number of ailments such as mental diseases, asthma, inflammation, arthritis, rheumatism, tuberculosis and a variety of other diseases including cancer [7, 8]. Due to its rejuvenating properties it is also called ‘Indian Ginseng’. *W. somnifera* is prone to several diseases and pests both under wild and cultivated conditions [9, 10]. Under field conditions, the crop is damaged by fungal pathogen or by frequent infestation of mites and other insects. There are many reports on the biodeterioration of the nutritive content of cereals [11], pulses [12], oil seeds and vegetables and fruits [13] due to diseases. However in medicinal plants such reports are scanty and so far, no study on microbial deterioration of the biochemical and secondary metabolites of *W. somnifera* has been reported. In the present investigation, we report the occurrence of leaf spot disease of *W. somnifera* and its impact on the histology of the plant and secondary metabolites.

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Materials and methods

Plant material

Withania somnifera grown in the herbal garden of the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, India was used in the present study. Healthy plants raised from seeds in green house conditions served as control. A voucher specimen (PKP-W1) of the plant is deposited at the departmental herbarium.

Disease etiology

Disease sample collected from naturally infected plants of *W. somnifera* were used to isolate the pathogen through tissue segment method. Preliminary observations of infected leaf tissue under microscope showed the presence of fungus *Alternaria* sp. Isolation of the associated pathogen was done by taking small segments of necrotic leaf tissue along with healthy portions. The infected bits were surface disinfected with 1% NaOCl (v/v) for 1 min and washed thrice in sterile distilled water (SDW), placed onto potato-dextrose-agar (PDA) plates and incubated at $25 \pm 2^\circ\text{C}$ for fungal growth. The hyphal tips of fungal colonies growing around the infected leaf tissues were transferred to fresh PDA slants for further study. The purified fungus culture was used to study the morphological characters to identify the pathogen associated with leaf spot disease [14]. Observations regarding disease incidence were recorded on the basis of infected leaves on each plant and graded on a scale of 5 [15].

Pathogenicity test

Pathogenicity tests were conducted on detached leaves and whole plant of *W. somnifera*. Leaves obtained from healthy plants grown under glass house conditions were surface sterilized with 1% NaOCl (v/v) solution for 1 min, washed thoroughly with SDW and placed on glass slides kept in sterile petri plates lined with moist blotting paper. The leaves were injured gently with a sterile needle at two sites and 8 mm fungal discs were placed at the injured sites with fungal culture facing the leaf surface. These plates were incubated at $25 \pm 2^\circ\text{C}$ and observed after 5–7 days for the appearance of symptoms. Pathogen was re-isolated from the test leaves and its identity was confirmed. For *in vivo* pathogenicity, the inoculum consisting of spore suspension was prepared from 7–10 days old fungal colonies

in SDW, filtered through two layers of sterile cheesecloth and concentration of spores was adjusted to 3×10^5 spores/ml by using a hemocytometer. The spore suspension was sprayed on the leaves of healthy plants with the help of an atomizer and pots were kept in moist chamber (100% relative humidity [RH]) and maintained at 25°C for 10 days. Leaves sprayed with distilled water served as controls. Leaf spot symptoms were recorded after 7–10 days of incubation. The fungus was re-isolated and its identity was confirmed from morphological and cultural characteristics.

Scanning electron microscopy

To study the histological changes in healthy and diseased leaf surface of *W. somnifera*, scanning electron microscopy (SEM) [16] was conducted using a scanning electron microscope (JSM 6100, JEOL, Japan). Photographs were taken using a Canon camera with Ilford Pan 100 black and white film.

Biochemical analysis of diseased and healthy leaves

Biochemical studies on the amount of proline, reducing sugars, free amino acids, total proteins and chlorophyll in both healthy and diseased leaves of *W. somnifera* were carried out. Total free amino acids in clarified ethanol extracts of both healthy and diseased leaves were estimated. The amount of proline in clarified extract of aqueous sulphosalicylic acid in the leaf tissue was determined according to Sadasivam and Manickam [17]. Estimation of reducing sugar was carried out according to the method of Nelson [18], total protein content by Lowry's method [19] and chlorophyll content following the method of Trivedi et al. [20].

Analysis of pharmaceutically important constituents

Preparation of crude extract

Air-dried and finely powdered leaves (0.2 gm each of healthy and diseased leaves) of *W. somnifera* were subjected to methanol (3–10 ml) extraction and the methanol extracts were distilled and vacuum dried. The samples were dissolved in 2 ml of AR grade methanol and were used for quantitative analysis of withaferin-A by HPTLC. Stock solution of withaferin-A (1 mg/ml) was prepared in methanol and different amounts (250 ng to 1.25 µg) of these were loaded on a thin layer chromatography (TLC plate), using ATS 4 for preparing calibration curves.

High performance thin layer chromatography

A Camag HPTLC system equipped with an automatic TLC sampler ATS 4, TLC scanner 3 and integrated software Win CATS version 1.2.3 was used for the analysis. HPTLC was performed on a pre-coated silica gel HPTLC 60F254 (20 × 20 cm) plate with layer thickness of 0.2 mm. Samples and standards were applied to the plate as 6 mm wide bands with an automatic TLC sampler (ATS 4) under a flow of N₂ gas.

Estimation of withaferin-A

The linear ascending development was carried out in a CAMAG twin trough chamber (20 × 20 cm) which was pre-saturated with 25 ml mobile phase chloroform:methanol (95:05, v/v) for 30 min at room temperature (25 ± 2°C) and 50% ± 5 RH. The length of chromatogram run was 8 cm. Subsequent to the development, TLC plates were dried in a current of air with the help of an air dryer in a wooden chamber with adequate ventilation. The flow of air in the laboratory was maintained unidirectional (laminar flow, towards exhaust). Quantitative evaluation of the plate was performed in the absorption reflection mode at 222 nm, using a slit width of 6 × 0.4 mm, data resolution 100 µm/step and scanning speed 20 mm/s with a computerized CAMAG TLC Scanner-3, Win CATS software version 1.2.3. The source of radiation utilized was a deuterium lamp emitting a continuous UV spectrum of 190 nm and 400 nm. Determination of the content of withaferin-A in extracts was performed by the external standard method, using pure withaferin-A as standard. Each sample was carried out in triplicate.

Calibration curve prepared from standard withaferin-A was used for determining the contents in test samples using standard procedures.

Extraction of total alkaloids and withanolides

Methanol leaf extract (1 gm) was defatted with hexane (10 ml × 3) and then extracted with 1% HCl (5 ml × 3), basified with ammonia, extracted with CHCl₃ (10 ml × 3), dried over anhydrous Na₂SO₄, filtered, evaporated and weighed for total alkaloid contents. The HCl insoluble fraction was extracted with diethyl ether (10 ml × 3), dried over anhydrous Na₂SO₄, filtered, evaporated and weighed for total withanolide contents.

Results and discussion

Disease incidence and etiology

Leaf spot was found to be the most prevalent disease in *W. somnifera*. In our study, initially the disease incidence was

found to be 20–30% with infected leaves having brown to black spots of 2–9 mm in diameter surrounded by a yellow halo. The brownish black spots were apparent on both the dorsal and ventral surfaces of the infected leaves, though more pronounced on the ventral surface. In the later stage of infection, the leaf spots enlarged and 80–90% leaves on a single plant were severely infected. Such leaves often dehisced prematurely. Under moist conditions (70–80% RH), fungal growth was often observed on the leaves. Irregular necrotic spots expanded along the veins on the ventral surface of the leaves. Similar symptoms have also been observed in peach [21] and citrus [22]. The symptoms described above could be attributed to the toxins secreted by *A. alternata* [23]. Based on the cultural and morphological descriptions by Barnett and Hunter [14], the test fungus found to be associated with *Withania* leaf spot disease resembled *A. alternata* (Fr.) Keissler. The pathogenicity test on detached leaves and whole plant established its pathogenic nature as the test fungus induced formation of similar spots as were noticed on naturally infected plants. No disease symptoms were observed on healthy controls.

Histological studies

Examination of diseased and healthy leaves under scanning electron microscope showed remarkable changes on the leaf surface. The SEM pictures of healthy and diseased leaves indicate a series of changes during the process of infection (Fig. 1a–c). In the disease initiation stage, an apparent protuberance was observed on the surface of the infected leaf whereas, no such symptoms were observed in the healthy leaves (Fig. 1a). A hyphal structure (marked with arrow) was also observed in the diseased leaf unlike the healthy one (Fig. 1b). Further, in contrast to healthy leaves, accumulation of crystal-like substances near the opening of stomata was also observed in diseased leaves (Fig. 1c). This could be the result of a localized interaction between the pathogen and the host. During the initiation of the disease, a swelling on the surface of the leaf was noticed (Fig. 1a). Such protuberance could be because of the presence of a hyphal mat that accumulates beneath the epidermis [24].

Biochemical analysis

The results of biochemical analysis indicated a considerable decrease in the total content of reducing sugar (20%) and chlorophyll (26.5%) in diseased leaves. However, a significant increase in proline (25%), free amino acids (3%) and proteins (74.3%) were found in infected

Fig. 1 a–c. Histopathology of healthy and diseased leaves of *Withania somnifera* under a scanning electron microscope. (a) Protuberances on the surface of the diseased leaf depicting disease initiation whereas no such symptoms are observed in healthy leaves. (b) Hyphal structures (arrow) on the diseased leaf section. (c) Accumulation of crystal-like substances near the opening of stomata in case of diseased leaf section unlike healthy leaf section.

Table 1 Analysis of biochemical and pharmaceutically important constituents of healthy and diseased leaves of *Withania somnifera*

Different parameters	Healthy leaves	Diseased leaves	% Loss (↓) or gain (↑) in diseased plant
Biochemical constituents (μg/g)			
Proline	70.0	87.5	25.0 (↑)
Reducing sugar	60.0	48.0	20.0 (↓)
Free amino acids	82.0	84.5	3.0 (↑)
Proteins	56.8	99.0	74.3 (↑)
Chlorophyll	16.43	12.08	26.5 (↓)
Pharmaceutically important constituents (%)			
Total withanolides	3.75	0.89	76.3 (↓)
Withaferin-A	0.13	0.11	15.4 (↓)
Total alkaloids	2.10	4.14	49.3 (↑)

leaves as compared to healthy leaves (Table 1). During host/pathogen interaction, amino acids may act as substrate for the pathogen [25] or they may have a fungistatic effect through their involvement in metabolic reactions associated with disease resistance, e.g. the synthesis of specific proteins related to the infection [26]. In the present investigation, it was observed that the total content of reducing sugar decreased more rapidly in the infected leaves than the healthy ones. This may be attributed to their utilization by the pathogen for its growth. These results are in agreement with those reported for *Botryosphaeria* apple rot [27] and for *Phytophthora* blight of pepper [28]. Besides carbohydrates, a considerable decrease in the chlorophyll content was observed in infected leaves which could be the consequence of disorganization of the chloroplast membrane system and breakdown of the chloroplast envelope during infection as demonstrated by earlier researchers [29].

Microbial deterioration of withanolides and withaferin-A

The results of HPTLC analysis of healthy and diseased leaves presented in Table 1 revealed that infection caused considerable reduction in total withanolides and withaferin-A contents; 76.3% decline in total withanolides was observed in diseased leaves. The results also showed a reasonable reduction in withaferin-A contents (15.4%). Such microbial deterioration of withaferin-A and withanolides in diseased sample could be the consequence of extremely complex interaction that exists between plant pathogenic fungi and their host plants. In contrast to the above, a two-fold increase in the total alkaloids in the diseased leaves was observed (Table 1). The total alkaloid was higher in the diseased leaves (49.3%) as compared to the healthy leaves. This difference could be linked to biotic stress

as a result of host pathogen interaction [30]. *Withania somnifera* is used in the manufacture of herbal drugs. Hence, microbial infection of *W. somnifera* plants and the consequent deterioration of withanolides and withaferin-A contents are of significant economic importance. Besides the control of the disease for improved production of withaferin-A and withanolides, emphasis should also be given on the identification of disease-resistant clones.

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