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Fenugreek extract application improves plant performance of *Alternaria solani* infected tomato plants

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Plant extracts (PE's) has emerged as a safer alternative to manage the fungal pathogens affecting tomato productivity. The current study aimed to evaluate the antimicrobial potential of methanolic fenugreek extract against *Alternaria solani*, a causal agent of early blight disease in tomato. Fenugreek extract was used at different concentrations of 5%, 10%, 15%, 20% and 25% under *in vitro* conditions. Results concluded that 25% fenugreek extract significantly reduced the radial growth (2.5 cm) of *A. solani* under *in vitro* conditions. Based on *in vitro* results, three concentrations (5%, 15% and 25%) of fenugreek extract was examined under greenhouse and field conditions. The outcomes expressed that 5% fenugreek extract reduced the disease severity up to 30.19% under greenhouse conditions and up to 40.53% under field trials. Although, application of fenugreek extract had exhibited non-significant impact on vegetative and reproductive growth parameters. However, its application had proved better results as compared to those plants which are infected with *A. solani* but received zero application of fenugreek extract. Furthermore, the effectiveness of plant extracts was evaluated by variant photosynthetic, antioxidative, polyphenolic and hypersensitive response of *A. solani* affected tomato plants. The 25% fenugreek extract application had augmented the chlorophyll pigments along with the significant increment of superoxide dismutase (174.16 U mg^{-1} protein), peroxidase (7.61 µmol min^{-1} g^{-1} protein) and catalase activity (4.73 nmol min^{-1} g^{-1} protein). Similar outcomes were observed regarding phenolic compounds, where 5% fenugreek extract application had enhanced flavonoid levels (26.62 mg QuE g^{-1}), tannins (1.28 mg TE g^{-1} extract) and total phenolic contents (2.39 mg GAE g^{-1}) in tomato leaves demonstrating its protective effect against early blight. In dose response, 25% fenugreek extract was most effective in reducing lipid peroxidation and enhancing H_2O_2 levels. The outcomes of study exhibited the fenugreek extract as an effective strategy to be used against *A. solani* to control early blight infection in tomato plants. Thus, it can serve as suitable fungicide alternative for resource-poor agriculture areas mainly in developing countries.

Keyword: *Alternaria solani*, integrated management, antifungal activity, *Trigonella foenum-graecum*
Abbreviations: PPO: Polyphenol oxidase, MDA, Melanodialdehyde, TCA, Trichloroacetic acid, TBA, Thiobarbituric acid, H_2O_2: Hydrogen Peroxide.

INTRODUCTION

Tomato is the second most consumed vegetable after potato throughout the world. In Pakistan, it covers an area of 60.6 thousand hectare (ha) with a production of 620.1 thousand tones showing an increment of 1% over last year (GOP, 2017-18). Tomato plants are often exposed to diverse climatic conditions that cause abiotic and biotic stresses in plants. Among these, biotic stresses involving fungal and bacterial invasions, significantly affect the crop productivity and quality (Passari *et al*., 2019). Fungi, in particular, is the major threat to crop productivity due to their variant ability to withstand diverse climatic conditions (Jain *et al*., 2019). Tomato production has been prone to early blight incidence caused by *Alternaria solani* resulting in loss of 80% production (Saha and Das, 2013).

Pathogens upon infection in plants interfere with physiological functioning and lead to development of disease symptoms. Foliar pathogens such as *A. solani* infecting leaves destroys their chlorophyll thus causes reduction in photosynthesis, growth, and overall yield of plant (Pandey *et al*., 2020). Moreover, *A. solani* isolates are known to affect translocation of nutrients, water absorption, transpiration rate, and temporary enhancement of dry weight at infected sites.

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A. solani causes patches and dark concentric lesions on tomato leaves, which leads to chlorosis in plant tissues. Tomato leaves infected with *A. solani*, exhibit abnormal growth particularly in lower epidermal cells, show very small or low intercellular spaces in mesophyll cells and lacking chlorenchyma with thin cell wall (Attia *et al*., 2017). Disease attack leads to generation of reactive oxygen species which damages leaf chlorophyll, thus exhibits a marked reduction in photosynthesis (Kyselakovaa *et al*., 2011). This reduction of photosynthetic process is also ascertained to the release of inhibitor toxins of photosynthetic enzymes (Glucose-6-P dehydrogenase), or stomatal closure with increased energy consumption protecting photosystem II (Berova *et al*., 2007). Pathogen induced changes alter the primary mechanism of plants which under biotic stress demands for higher photosynthetic assimilates while the pathogen had altered the carbohydrates metabolism for its own needs (Berger *et al*., 2007).

From the recent past, much of the efforts have been done for searching and evaluating low toxic compounds with high efficacy against plant pathogens (Desaeger *et al*., 2017; Tzortzakakis and Petsas, 2003; Gerik and Hanson, 2011). Therefore, a high increment in application of plant extracts for suppressing fungal, bacterial and nematodes has been observed (Abdel-Monaim *et al*., 2011; Farooq *et al*., 2011). The application of these extracts led a significant contribution in modern agriculture thus lowering the risks of environmental pollution with negligible toxic residues (Riaz *et al*., 2010; Ibrahim and Ebady, 2014). The plant extracts play dual role in growth and development, either by enhancing plant immunity or owing to their regulatory role (Findura *et al*., 2020). Many research trials have confirmed regulatory role of plant extracts on various plant growth stages i.e., seed germination and uniformity (Khan *et al*., 2008), initial vegetative growth and biomass production (El-Rokiek *et al*., 2018), plant height (Anjum *et al*., 2011), branches/plant and leaf area index (Jeyakumar *et al*., 2008) and yield (Hussain *et al*., 2014). These stimulatory changes could be ascribed to alteration in biochemical and physiological mechanisms (Catav *et al*., 2012). These changes allow upgraded movement of nutrients and metabolites in addition to inducing systemic plant responses (Pretali *et al*., 2016; Siah *et al*., 2018) thus promoting growth and productivity (Yasmeen *et al*., 2013).

Several researchers have proved antifungal potential of plant extracts particularly in terms of inducing disease resistance (Nashwa and Abo-Elyousr, 2012; Draz *et al*., 2019). Plant extracts belonging to the *Fabaceae* family, are the most explored for controlling in terms of the biological control of plant pathogens (Santana *et al*., 2015). Up till now, little has been known regarding their efficacy against *A. solani* in tomato. Therefore, the current study was designed to examine the potential of fenugreek (*Trigonella foenum-graecum*) on *A. solani* disease suppression in tomatoes. We have summarized that fenugreek extracts could serve as suitable alternative to be used along with traditional pesticides to suppress the early blight invasion in tomatoes. The aim of the study was to quantify the phenolic compounds (flavonoid, tannins, and phenolic contents), hypersensitive response (H_2O_2 and MDA levels) and antioxidative behaviour (SOD, POD, CAT and PPO activity) of tomato plants inoculated and uninoculated with *A. solani* and received application of fenugreek extract. The outcome of the study will help to develop alternative measures that are less toxic, cheap, locally available, and easily degradable with negligible impact on the environment and farming community.

MATERIALS AND METHODS

***Collection of Alternaria solani isolates*:** Fungal isolate of *A. solani* was acquired from fungal culture bank (FCBP), University of Punjab, Lahore, Pakistan. The obtained culture was kept fresh through periodic re-culturing in potato dextrose agar (PDA) medium followed by incubation of plates at 25 $\pm$ 2°C in dark conditions (Koley and Mahapatra, 2015).

***Preparation of plant extracts*:** Dried fenugreek (*Trigonella foenum-graecum*) seeds were acquired from local vegetable market, Faisalabad. This plant material was thoroughly cleaned to remove any impurity/debris. After cleaning and washing, seeds were dried at room temperature. After drying, the plant material was grinded into fine powder using a grinder (National Grinder- WB-176) and the resultant powder was readily used for extraction. The extraction was done following the method of Raaman (2006). Briefly, 10 g powder of fenugreek was soaked in 100 ml methanol and placed in a mechanical shaker for 24 hours at room temperature. Extracts were filtered by using Whatman filter paper No 1 and then, extraction solvent was removed from filtered extract by rotary evaporator at 250 rpm.

***Determining the efficacy of plant extracts*:** The poison food technique was adopted to determine the extracts efficacy by following the methods described by Mishra and Gupta, (2012). Fenugreek extract was added in varying concentrations viz. 5%, 10%, 15%, 20% and 25% to sterilized Petri plates containing 20 ml of autoclaved Potato dextrose agar (PDA) medium. The 5 mm diameter disc of *A. solani* from seven-day old culture was dissected and placed in the center of Petri plates amended with fenugreek extract. While 1% Azoxystrobin (AZ) enriched Petri plates served as a positive control and distilled water amended Petri plates served as negative control. Petri plates were incubated at 25+3°C and results from plant extract treated plates were compared with negative control.

***Raising of tomato plantlets*:** An open pollinated tomato cultivar "Rio Grande" was purchased from Faisalabad local market. Healthy and disease-free seeds were sown in soilless growing media (peat moss) and kept under glasshouse

(25±3ºC, 12 hours photoperiod). Irrigation was done as per requirement of seedlings (usually at one-day interval). Three weeks old seedlings were transplanted into a plastic pot (7-8 inch) having 4 liters of peat moss. After transplanting, plantlets were kept in the growth room at 25±3ºC with 15-16 hours photoperiod and 65% relative humidity.

***Application of plant extracts under greenhouse and field conditions*:** Based on results obtained under *in vitro* conditions, three concentrations of plant extract viz., 5%, 15% and 25% were prepared from stock solution of plant extract. Stock solution was made by dissolving 100 mg plant extract in 1 ml ethanol and the final volume of stock was measured using $C_1V_1=C_2V_2$ equation. Where C_1 is stock concentration, V_1 is volume of stock required, C_2 stands for concentration of plant extract required and V_2 is a final volume needed. While 20 ml of plant extract was used as the final volume for each concentration to be applied to the tomato plants. The control-inoculated plants were only *A. solani* inoculated while control plants were neither treated with plant extracts nor inoculated with *A. solani*. Check was treated with Azoxystrobin @ 1 ml L^{-1} on inoculated plants (Junior *et al*., 2011). The prepared plant extracts and fungicide treatments were applied on tomato plants two days before *A. solani* inoculation.

***Inoculation of A. solani on tomato plants under greenhouse*:** One-week old *A. solani* culture plates were scraped by sterile needles after addition of 10 ml of distilled water in each plate. Freshly prepared conidial suspension was kept at mechanical shaker (40 rpm) and filtered through cheesecloth to avoid agar contamination. The resulting pure conidial suspension was adjusted to 10^6 conidia ml^{-1} using hemocytometer. Twenty ml of this conidial suspension was inoculated on each tomato plant followed by covering with an air-tight plastic bag to enhance pathogen infection by facilitating spore germination (Song *et al*., 2015).

***Transplantation of tomato nursery in open field*:** Soil was thoroughly prepared by ploughing followed by rotavator application. Thirty days old seedlings of tomato cv. "Rio Grande" were transplanted in open field plots. Three weeks after transplantation, application of plant extracts was done followed by disease inoculation after two days. The application of plant extracts and disease inoculation was done as indicated in section 2.5 and section 2.6, respectively.

***Disease severity*:** Disease severity was recorded in each treatment according to disease rating scale (0–9 scale) as suggested by Latha *et al*., (2009). The detail of scale is as, 0 = healthy; 1 = 1–5%; 2 = 6–10%; 3 = 11–25%; 5 = 26–50%, 7 = 51–75%, 9 = > 76% infected leaf area.

***Vegetative growth parameters*:** Data regarding vegetative growth parameters was recorded after three weeks of disease inoculation under both greenhouse and field conditions. Different parameters i.e., stem and root length were taken with the help of measuring scale while number of leaves were counted manually.

Fresh root and shoot weight were calculated by excising the tomato plants into root and shoot with the help of sterilized scissors and then their weight was measured with the help of electronic weighing balance.

Previously taken root and shoots from each treatment were placed in separate brown paper bags and kept in the oven for 72 hours at 70ºC then dry weight was measured using electrical weighing balance.

***Reproductive parameters*:** Different reproductive parameters like fruit weight, marketable fruit per plant (Luitel *et al.*, 2012), number of fruits per plant, yield per plant and yield per hectare (Olaniyi *et al.*, 2010) were calculated.

***Collection of diseased Samples*:** Tomato leaves having early blight symptoms were collected at 7, 14, and 21 days after inoculation (DAI) from different plants of every treatment with immediate shifting to dry ice box and stored in laboratory at -80ºC. Biochemical analysis regarding antioxidants and oxidative stress was readily done from these fresh leaves while phytochemical analysis was done from stored leaf samples.

***Measurement of Physiological parameters*:** The chlorophyll content was assessed following the protocol of Aron (1949). One gram (g) leaf sample from each treatment was taken and cut into small pieces and homogenized with 10 ml of 80% acetone (V/V) in pre-chilled mortar and pestle. The resultant solution was centrifuged at 3000 rpm for 15 minutes and supernatant was transferred into 2 ml Eppendorf tube. Absorbance of this supernatant was then taken at 645 nm and 663 nm in UV-Vis spectrophotometer (IREMCO-U2020, Germany). The values of chlorophyll 'a' and chlorophyll 'b' were determined by following the given below equations:

Chlorophyll 'a' (µg g^{-1} FW) = (12.7 x O.D.* at 663 nm) – (2.69 x O.D. at 645 nm)

Chlorophyll 'b' (µg g^{-1} FW) = (22.9 x O.D. at 645 nm) - (4.08 x O.D. at 663 nm)

Total chlorophyll (µg g^{-1} FW) = (20.2 x O.D. at 645 nm) + (8.02 x O.D. at 663 nm)

*O.D.: Optical Density

***Measurement of phenolics*:** Phenolic compounds were measured following the methods described by Singleton *et al*., (1999). Briefly, 0.5 g of leaf sample was extracted with 5 ml of extraction mixture (Methanol: Acetone: HCl 90:8:2) in sterilized pestle and mortar. The extract was collected in 1.5 ml Eppendorf tubes and vortexed for 30 seconds followed by centrifugation at 17,500 rpm for 10 minutes at 4ºC (Universal 32R, Hettich, Germany). After centrifugation, 100 µL of the supernatant was collected in Eppendorf tubes and mixed with 800 µL of 700 mM Sodium carbonate (Na_2CO_3) and 200 µL of the Folin–Ciocalteu (FC) reagent. The prepared samples were vortexed for 30 seconds and kept for one hour at room temperature. The 200 µL of the prepared sample was collected in a cuvette and absorbance was measured at 765 nm in a UV-Vis spectrophotometer (IREMCO-U2020, Germany). The results were expressed in equivalent milligrams of Gallic acid per gram of extract (mg $GAEg^{-1}$)

The standard calibration curve was made by mixing 1 ml aliquots of 50, 100, 150, 200, 250, 300 and 350 µg ml^{-1} Gallic acid in 5.0 ml of FC reagent containing 4 ml of Na_2CO_3 solution.

***Measurement of flavonoids*:** Flavonoid contents were measured by following the Dowd's method, adapted by Arvouet-Grand *et al*., (1994). For extraction, 0.1 g leaf sample was grinded with 10 ml of analytical grade methanol and centrifuged at 10,000 rpm for two minutes at 4ºC. Supernatant was collected and 100 µL of this supernatant was added with 0.3 ml of 5% Sodium nitrite ($NaNO_2$) and left for five minutes at room temperature. Subsequently, 0.3 ml of 10% Aluminium chloride ($AlCl_3$) and 0.2 ml of 1M Sodium hydroxide (NaOH) were added in Eppendorf tube followed by incubation for 30 minutes under dark conditions at room temperature. The reading of this flavonoid-aluminium colour was measured at 510 nm using UV-Vis spectrophotometer (IREMCO-U2020, Germany). While flavonoid content was expressed as milligrams of Quercetin per gram of extract (mg QuE g^{-1}).

***Measurement of tannins*:** The tannins were measured following the protocol of Amorim *et al*., (2008) using FC reagent with tannic acid as a standard. 100 µL of this supernatant solution was mixed with 7.5ml of distilled water followed by addition of 0.5 ml of FC reagent. Later, 100 µL of 35% Na_2CO_3 along with 900 µL of distilled H_2O was added. This prepared solution was incubated for half an hour at room temperature. Absorbance of blue coloured solution at 725 nm was assessed and results were expressed as milligrams of Tannic acid per gram of extract (mg TE g^{-1}).

Determination of antioxidants

***Enzyme extraction*:** One gram of fresh leaves from un-inoculated, inoculated and treated tomato plants were grinded and homogenized with 4 ml of 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 4˚C for 25 minutes at 15000×*g* and resultant supernatant was used for enzyme activity assays on immediate basis.

***Measurement of antioxidant enzymes activity*:** Superoxide dismutase (SOD) activity was determined by adopting the technique of Stagner and Ppovic (2009) by assessing 50% blockage of nitro blue tetrazolium (NBT). 100 µL of supernatant was thoroughly mixed with reaction mixture [500 µL phosphate buffer (pH 5), 200 µL methionine, 100 µL riboflavin] and later this was diluted with 800 µL of distilled water. The resultant mixture was kept under UV light for 15 minutes and readings were taken at 560 nm absorbance using microplate reader. One unit of SOD activity was expressed on the basis of protein contents as U mg^{-1} protein.

Catalase (CAT) and peroxidase (POD) activities were determined by Chance and Maehly (1955) techniques. 100 µL of enzyme extract was mixed in 50 mM of phosphate buffer (pH 5.0) and 5.9 mM H_2O_2. Changes in reaction solution were observed by absorbance at 240 nm. One unit of catalase activity was noted as change in absorbance of 0.01 units per minute. For measurement of POD activity, reaction mixture was prepared by adding 50 mM phosphate buffer (pH 5.0), 40 mM H_2O_2 and 20 mM Guaiacol in 100 µL of enzyme extract. Changes in reaction solution were detected by absorbance at wavelength of 470 nm. One unit POD activity was expressed as an absorbance change of 0.01 units per minute and exhibited on the basis of protein contents.

Polyphenol Oxidase (PPO) activity was measured following the protocol of Mahadevan and Sridhar (1992) using Catechol as a substrate. Two ml of enzyme extract was mixed in 3.0 ml of phosphate buffer (pH 6.8) and 1 ml Catechol (0.01 mM). The absorbance of the reaction mixture was observed at 495 nm in UV-Vis spectrophotometer (IREMCO-U2020, Germany) for three minutes at an interval of one minute. The enzyme activity expressed as changes in absorbance in µmol min^{-1} Fresh weight of leaves.

***Assessment of oxidative damage*:** 100 mg leaf tissue was taken and mixed with 2.0 ml of 0.1% (w/v) trichloroacetic acid (TCA) followed by centrifugation at 12,000 rpm for 15 minutes. 0.5 ml of supernatant was mixed with 10 mM Tripotassium phosphate (pH 7.0) and 1000 µL Potassium iodide (KI) solution followed by incubation for five minutes at room temperature. The absorbance of resulting solution was assessed at 390 nm (Velikova *et al*., 2000) and results were expressed as H_2O_2 $nmol^{-1}$ fresh weight of leaves (Meena *et al*., 2016).

Lipid peroxidation (MDA contents) of fresh tomato leaves collected from all treatments were assayed following the protocol of Zhou and Leul, (1998). Fresh leaves (1 g) without primary vein were extracted in pestle and mortar using 5 ml of 50 mM phosphate buffer (pH 7.0) and centrifuged at 15000 rpm. Supernatant was mixed in 2.5 ml thiobarbituric acid (TBA) containing 20% trichloroacetic acid (TCA) followed by heating at 100 °C for 15 minutes followed by rapid cooling in ice bath and centrifuged at 4800 rpm for 10 minutes. Supernatant of this reaction mixture was taken, and absorbance was measured at 450, 532 and 600 nm. The equation given below was used to calculate MDA content.

MDA Content = 6.45 (A_{532}-A_{600}) – 0.56 A_{450}

***Statistical Analysis*:** The experiment was conducted under lab, greenhouse, and field conditions. Trials were performed twice following complete randomized design (CRD) under laboratory conditions and randomized complete block design (RCBD) under greenhouse and field conditions. For statistical analysis, we used Statistix 8.1 to perform ANOVA under two factorial RCBD to evaluate the efficacy of plant extracts application with respect to days after inoculation of *A. solani* in tomato plants along with errors associated with the experiment. To determine the significant differences among treatments, a mean comparison was done by using Least Significance Difference (LSD) test ($P \leq 0.05$) while standard errors of difference between treatment means was estimated by using error mean square (Steel *et al*., 1997).

RESULTS AND DISCUSSION

***Radial Growth*:** Fenugreek extract has significant ($P \leq 0.01$) impact on radial growth of *A. solani* in both trials. The minimum radial growth (2.50 cm & 2.32 cm) was observed under 25% fenugreek extract application in 1st and 2nd trial respectively (Plate 1).

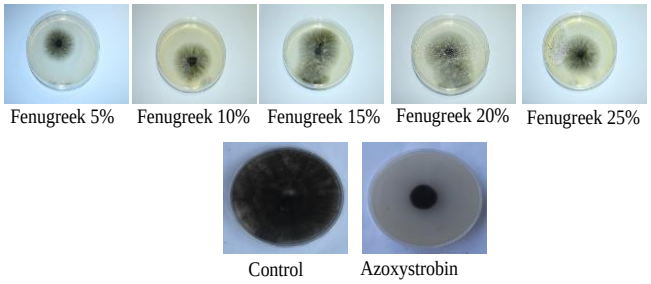

Plate 1. Radial growth of *Alternaria solani* after seven days of incubation on PDA medium amended with different concentrations of fenugreek extract in comparison to control and Azoxystrobin.

Comparatively, the check (Azoxystrobin) has led maximum control over disease progression of *A. solani* and exhibited lowest 0.72 cm and 0.86 cm radial growth (Fig. 1).

Greenhouse Studies

Disease Severity: The fenugreek extract application exhibited the significant impact in reducing disease severity ($P \leq 0.01$) as compared with inoculated-control plants. Among fenugreek extract treated tomato plants, lowest disease severity in both trials (30.19% and 30.51%) was observed in 5% fenugreek extract (Table 1). The minimum disease severity in both trials (12.81% and 13.9%) was concluded with Azoxystrobin treated tomato plants.

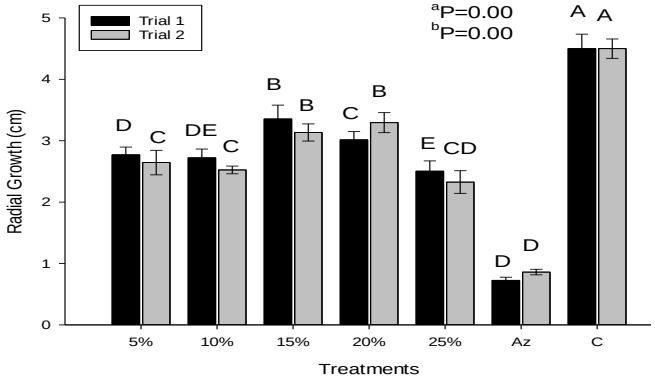

Figure 1. Radial growth of *Alternaria solani* under the influence of fenugreek extract.
Error bars represent standard error. Means with different letters are significantly different from each other. *AZ: Azoxystrobin, C: Control, aP: Significance of Trial 1, bP: Significance of Trial 2.

***Vegetative growth parameters*:** In both trials, *A. solani* inoculation have resulted in suppression of vegetative growth such as lower number of leaves (8 and 9.66), reduced stem length (8 cm and 9.2 cm) and root length (3.3 cm and 4.2 cm) in inoculated control plants (Table 2). While tomato plants treated with fenugreek extract and check (Azoxystrobin) exhibited significant ($P \leq 0.01$) enhancement of vegetative

Table 2. Effect of fenugreek extract on number of leaves, stem, and root length of *A. solani* infected tomato plants.

		No. of Leaves		Stem Length		Root Length	
	Conc.	1st Trial	2nd Trial	1st Trial	2nd Trial	1st Trial	2nd Trial
Fenugreek	5%	14.00±0.59A	17.00±1.20C	9.33±1.05C	10.00±0.85CD	3.60±0.2DE	4.66±0.31D
	15%	15.00±0.85C	17.66±0.71C	9.66±1.10C	10.30±1.10CD	4.60±0.1CD	5.40±0.20CD
	25%	14.33±1.10C	15.66±0.82C	10.66±1.20C	12.33±1.20C	5.33±0.2C	6.13±0.12C
In-C		8.00±1.12D	9.66±0.75D	8.00±0.85C	9.20±0.58D	3.33±0.1E	4.40±0.21D
Check		19.66±1.05B	23.33±1.04B	15.66±1.04B	15.66±0.80B	7.10±0.2B	8.46±0.21B
C		26.33±0.89	32.33±1.1A	25.33±1.04A	27.00±1.20A	8.51±0.2A	9.92±0.22A
LSD ($P \leq 0.05$)		0	0	0	0.001	0	0

Values given are means of three replicates ±SE; Conc. = Concentrations

Table 3. Effect of fenugreek extract on fresh and dry weight of stem and root of *A. solani* infected tomato plants.

		Stem Fresh Weight (g)		Stem Dry Weight (g)		Root Fresh Weight (g)		Root Dry Weight (g)	
	Conc.	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Fenu-	5%	3.05±0.01CD	3.23±0.09B	0.63±0.01CD	0.94±0.01B	0.39±0.01AB	0.78±0.08A	0.09±0.001AB	0.07±0.001B
greek	15%	3.12±0.01C	3.24±0.10B	0.67±0.02C	0.96±0.03B	0.25±0.01B	0.54±0.02BC	0.05±0.002B	0.09±0.002AB
	25%	3.09±0.02CD	3.26±0.10B	0.66±0.01C	0.99±0.03B	0.23±0.03AB	0.43±0.05C	0.04±0.001B	0.06±0.002B
In-C		2.90±0.10D	3.05±0.05C	0.56±0.05D	0.66±0.05C	0.15±0.01B	0.16±0.03E	0.01±0.001C	0.02±0.001D
Check		3.37±0.05B	3.38±0.10B	0.80±0.05B	1.01±0.10B	0.23±0.03AB	0.30±0.02D	0.09±0.002AB	0.05±0.003BC
C		4.55±0.10A	4.80±0.12A	1.22±0.06A	1.51±0.10A	0.61±0.01A	0.63±0.01B	0.12±0.050A	0.10±0.001A
LSD ($P \leq 0.05$)		0	0	0.01	0	0.26	0.01	0.19	0.02

Values given are means of three replicates ±SE.; Conc. = Concentrations

growth parameters. The application of 15% fenugreek extract reported the highest leaf number (15 and 17.66) whereas 25% fenugreek extract exhibited the highest stem length (10.66 cm and 12.33 cm) and root length (5.33 cm and 6.13 cm) under both trials (Table 2).

Table 1. Effect of fenugreek extract on disease severity (%) of *A. solani* under greenhouse trials

	Trial 1	Trial 2
5%	30.19 BC	30.51 BC
15%	32.32 B	33.08 B
25%	31.08 BC	31.68 BC
In-C	41.22 A	42.33 A
Check	12.81 D	13.90 D
LSD (P≤0.05)	0	0

Values given are means of three replicates ±SE

The results regarding stem fresh and dry weight exhibited maximum stem fresh (3.37 g and 3.38 g) and dry weight (0.80 g and 1.01 g) in tomato plants treated with Azoxystrobin (check) in both trials (Table 3). However, highest stem fresh and dry weight (4.80 g and 1.51 g) was assessed in control plants and minimum values (2.90 g and 0.80 g) was observed in control-inoculated plants. The outcomes of root fresh and dry weight expressed fenugreek extract application to ameliorate retention of growth parameters under *A. solani* stressed tomato plants (Table 3).

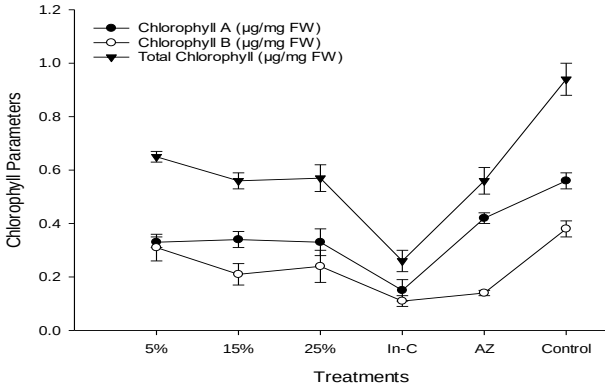

Figure 2. Effect of fenugreek extract on chlorophyll 'a', 'b' and total chlorophyll contents of *A. solani* infected tomato plants under greenhouse conditions.
Values given are means of three replicates ±SE

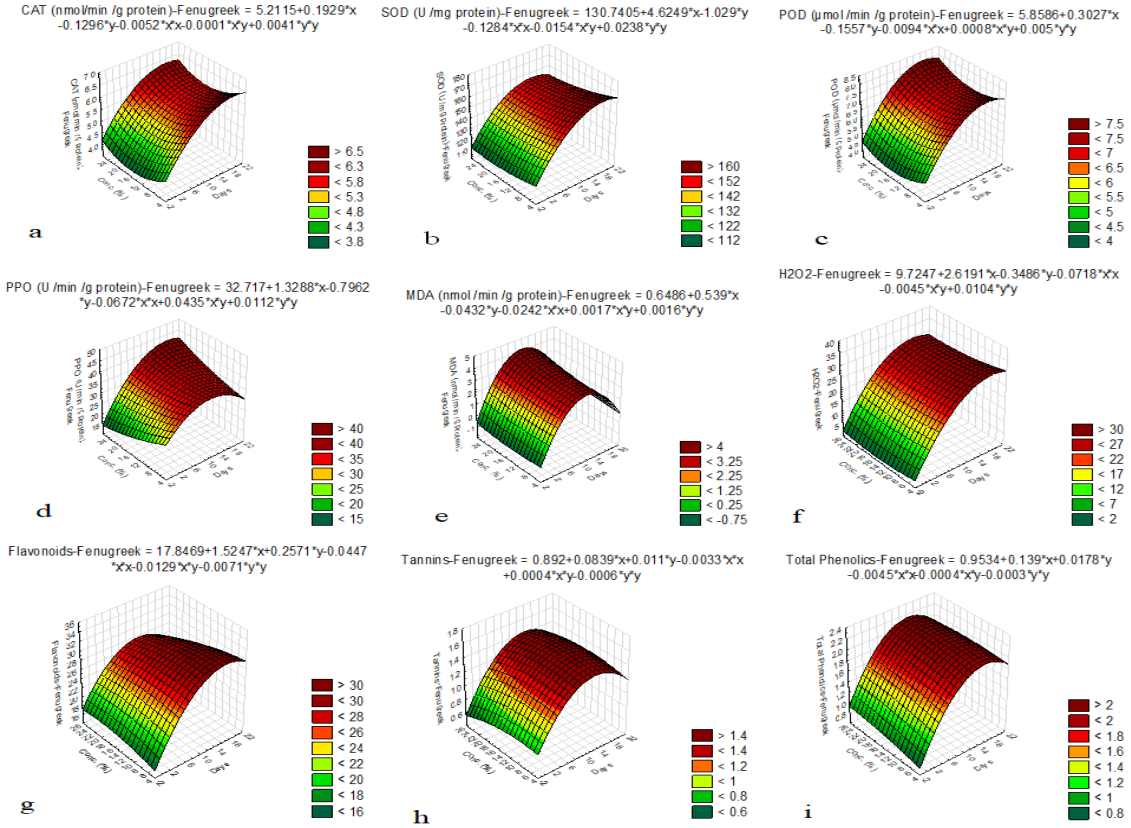

Figure 3. Response surface plots (3D) exhibiting the interactive effect of independent variables (Fenugreek concentrations & DAI*) on the antioxidant enzymes, oxidative stress and phytochemicals of tomato plants infected with *A. Solani* under greenhouse conditions: (a) CAT activity, (b) SOD activity, (c) POD activity, (d) PPO, (e) MDA, (f) H_2O_2, (g) Flavonoids, (h) Tannins and (h) TPC. **DAI: Days after Inoculation.*

Physiological Parameters: The fenugreek extract application have ameliorated chlorophyll pigments in tomato plants (Fig. 2). Among concentrations of fenugreek extract, maximum chlorophyll 'a' content (0.34 µg mg^{-1} FW) was observed in 15% fenugreek extract application while lowest 0.15 µg mg^{-1} FW chlorophyll 'a' level was obtained in only *A. solani* infected tomato plants (Fig. 2). Similar pattern was also observed in chlorophyll 'b' and total chlorophyll contents in tomato plants treated with 15% fenugreek extract.

***Induction of Antioxidative enzymes*:** Fenugreek extract application have ameliorated the biochemical responses in *A. solani* infected tomato plants. The highest CAT activity (4.73 nmol min^{-1} g^{-1} protein) was observed in control plants followed by 5% fenugreek extract treated tomato plantlets (4.50 nmol min^{-1} g^{-1} protein). The lowest CAT activity (2.98 nmol min^{-1} g^{-1} protein) was observed in control inoculated tomato plants (Fig. 3a).

Fenugreek extract application have significantly ($P \leq 0.01$) affected the superoxide dismutase activity (SOD) in *A. solani* inoculated tomato plants. The highest SOD activity (174.16 U mg^{-1} protein) was recorded in tomato plants treated with 5% fenugreek extract and the lowest SOD activity (104.01 U mg^{-1} protein) was observed in control-inoculated plants. The outcomes from control plants exhibited 116.25 U mg^{-1} protein SOD activity which was slightly lowered than Azoxystrobin treated tomato plants where 140 U mg^{-1} protein SOD activity was recorded (Fig. 3b).

A. solani inoculation has lowered the peroxidase activity (2.72 µmol min^{-1} g^{-1} protein activity) in control-inoculated plants (Fig. 3c). The 25% fenugreek extract application enhanced the peroxidase activity (7.61 µmol min^{-1} g^{-1} protein). Moreover, polyphenol oxidase activity (PPO) was also affected by the application of fenugreek extract. Notably, control-inoculated plants exhibited highest PPO activity (101.07 U min^{-1} g^{-1} protein) while 25% fenugreek extract application exhibited ameliorated control over PPO activity reporting 47.24 U min^{-1} g^{-1} protein activity. While control plants showed minimal PPO activity (20.81 U min^{-1} g^{-1} protein) among all treatments indicating stress free normal growth (Fig. 3d)**.**

***Hypersensitive response*:** Disease inoculation have expressed higher lipid peroxidation (2.53 nmol g^{-1} FW) in control inoculated plants. The application of fenugreek extracts significantly reduced ($P \leq 0.01$) the lipid peroxidation, mainly in tomato plants treated with 25% fenugreek extract, where recorded MDA contents are1.98 nmol g^{-1} FW (Fig. 3e). Moreover, outcomes have expressed that lipid peroxidation was higher in earlier days after inoculation (DAI) and decreases with plant extract application along with growing DAI.

Alike outcomes were exhibited regarding hydrogen peroxide (H_2O_2) accumulation following *A. solani* inoculation in tomato leaves. Maximum 2.5-fold increment was observed in control-inoculated plants in first week of *A. solani* inoculation (Fig. 3f). On the contrary, extract application has exhibited the controlled increment of H_2O_2 following *A. solani* inoculation throughout the trial period ($P \leq 0.01$). The 25% fenugreek extract caused higher H_2O_2 accumulation (40.31 $nmol^{-1}$ FW leaves) and control plants expressed lowest H_2O_2 accumulation (13.85 $nmol^{-1}$ FW leaves) (Fig. 3f).

***Profile of polyphenolic compounds*:** *A. solani* inoculation has suppressed the flavonoid levels in tomato plants. The 5% fenugreek extract application have enhanced the flavonoids level (26.62 mg QuE $^{-1}$g) while lowest flavonoids (14.78 mg QuE g^{-1}) were observed in *A. Solani* inoculated plants as compared to control (non-inoculated plants). In comparison, results of 25% fenugreek extract were at par with that of Azoxystrobin applied tomato plantlets (Fig. 3g).

Similar impact was observed in tannin contents of inoculated tomato leaves where fenugreek extracts have significantly incremented ($P \leq 0.01$) the tannin contents. Outcomes of trial exhibited that increment in tannin contents were observed from 7 DAI to 14 DAI whereas afterwards negative trend was followed till the end of trial period (Fig 3h). The maximum tannins (1.28 mg TE g^{-1} extract) were observed in 15% fenugreek extract treatment.

The exogenous application of fenugreek extract has increased the total phenolic compounds in *A. solani* inoculated tomato plants. Maximum phenolic contents (2.39 mg GAE g^{-1}) was observed in control plants. Among the concentrations of fenugreek extracts, the application of 5% fenugreek extract have ameliorated higher (1.84 mg GAE g^{-1}) flavonoid contents in tomato plants (Fig. 3i).

Field Studies

***Disease Severity*:** Just as compared to greenhouse study, field trial exhibited that fenugreek extract led a significant reduction ($P \leq 0.01$) in *A. solani* severity. The field trials conducted in 2018-19 and 2019-20 season exhibited that the exogenous application of fenugreek in variant concentrations efficiently suppressed the disease severity of early blight. However, in both trials, minimum disease severity (21.06% and 16.83%) was observed in check (Azoxystrobin) treated tomato plants. Among the concentrations of fenugreek extracts, less disease severity was recorded (43.10% and 40.53%) was recorded for the application of 25% fenugreek extract in both trials in both trials (Table 4).

Table 4. Effect of fenugreek extract on disease severity of *A. solani* under field trials.

	2018-19	2019-20
5%	45.13±1.35B	42.33±1.21B
15%	44.46±2.25B	43.50±1.05B
25%	43.10±1.63B	40.53±1.39B
In-C	60.40±2.25A	55.78±1.85A
Check	21.06±1.03C	16.83±1.15C
LSD ($P \leq 0.05$)	0	0

Values given are means of three replicates ±SE

Table 5. Effect of fenugreek extract on number of leaves, stem, and root length of *A. solani* infected tomato plants.

		No. of Leaves		Stem Length (cm)		Root Length (cm)	
	Conc.	2018-19	2019-20	2018-19	2019-20	2018-19	2019-20
Fenugreek	5%	23.0±1.05B	24.0±1.52B	26.3±1.20AB	28.6±0.92AB	9.96±0.54D	12.7±1.20CD
	15%	18.0±0.95C	22.0±0.82BC	23.0±1.00BC	25.0±1.10BC	11.80±1.20CD	12.8±0.80CD
	25%	24.0±1.31B	26.3±1.15B	25.0±1.40BC	26.3±1.40BC	13.44±1.10C	17.7±1.50B
In-C		15.0±1.00C	12.6±1.20D	19.3±0.85C	19.3±0.74C	9.89±0.90D	10.1±1.05D
Check		25.0±1.40B	27.0±1.50B	25.6±1.05B	24.0±0.50C	19.55±1.20B	16.8±1.20BC
C		34.7±2.10A	32.3±1.80A	32.0±1.35A	31.6±1.80A	23.10±1.30A	26.2±1.40A
LSD (P≤0.05)		0	0	0.001	0.004	0.001	0.002

Values given are means of three replicates ±SE.; Conc. = Concentrations

Table 6. Effect of fenugreek extract on fresh and dry weight of stem and root of *A. solani* infected tomato plants.

		Stem Fresh Weight (g)		Stem Dry Weight (g)		Root Fresh Weight (g)		Root Dry Weight (g)	
	Conc.	2018-19	2019-20	2018-19	2019-20	2018-19	2019-20	2018-19	2019-20
Fenugreek	5%	4.40±0.15C	5.19±0.9B	1.10±0.52B	1.20±0.04B	0.48±0.04C	0.70±0.05B	0.12±0.01A	0.11±0.01B
	15%	4.28±0.19C	5.55±1.2B	1.08±0.2B	1.23±0.1B	0.48±0.05C	0.64±0.08BC	0.11±0.02A	0.15±0.02B
	25%	5.56±0.1B	5.49±1.1B	1.09±0.3B	1.21±0.09B	0.55±0.08BC	0.58±0.07C	0.18±0.02B	0.14±0.01B
In-C		3.80±0.21D	4.17±1.05C	0.89±0.09C	0.93±0.08C	0.27±0.05D	0.38±0.02D	0.1±0.01C	0.1±0.03B
Check		4.66±0.12C	5.16±1.2B	1.14±0.3B	1.18±0.08B	0.64±0.03	0.66±0.04BC	0.12±0.01C	0.15±0.02B
C		7.14±0.24A	7.73±1.3A	1.76±0.2A	1.96±0.14A	0.88±0.09A	0.93±0.05A	0.21±0.3BC	0.18±0.01A
LSD (P≤0.05)		0	0	0.01	0	0	0.01	0	0

Values given are means of three replicates ±SE.; Conc. = Concentrations

Table 7. Effect of fenugreek extract on number of fruits, fruit weight and yield per plant in *A. solani* infected tomato plants.

	Conc.	No. of Fruits		Single Fruit Weight (g)		Fruit Yield Plant^{-1} (kg)	
		2018-19	2019-20	2018-19	2019-20	2018-19	2019-20
Fenugreek	5%	11.00±1.2C	12.66±1.0A	41.26±1.5C	43.99±1.8C	0.45±0.06C	0.64±0.02BC
	15%	9.66±0.8CD	12.66±0.9A	39.15±3.2CD	44.52±1.6C	0.37±0.02CD	0.59±0.07C
	25%	11.00±0.9C	12.00±1.1A	42.89±2.1C	41.98±1.8CD	0.47±0.05C	0.51±0.04C
In-C		7.00±0.5D	5.33±0.7B	35.33±1.8D	35.52±2.4D	0.24±0.01D	0.21±0.05D
Check		15.66±0.9B	13.00±1.2A	52.78±2.2B	53.02±2.1B	0.82±0.07B	0.74±0.05B
C		19.33±1.3A	15.66±1.3A	57.63±1.3A	60.06±1.5A	1.11±0.05A	1.02±0.08A
LSD (P≤0.05)		0	0.01	0	0.005	0.002	0.001

Values are means of three replicates ±SE.; Conc. = Concentrations

***Vegetative growth parameters*:** The results concluded significant differences in number of leaves, stem, and root length of treated, control and inoculated control tomato plants (Table 5). The maximum growth attributes were observed in control plants as compared to fenugreek extract or *A. solani* treated tomato plants. The exogenous application of 25% fenugreek extract has recorded the maximum number of leaves (24 and 26.3) in both trials and were statistically alike with that of Azoxystrobin fungicide (check). Similar trend was also observed regarding stem and root length (Table 5).

The application of fenugreek extracts greatly enhanced the stem fresh and dry weight in tomato plants as compared with the untreated and Azoxystrobin-treated plants (Table 6). Among treatments, 25% fenugreek extract exhibited the highest stem fresh (5.56 g and 5.49 g) and dry weight (1.09 g and 1.21 g) in both trials and even higher than the Azoxystrobin fungicide. Moreover, the exogenous treatment with fenugreek extracts significantly increased the root fresh weight (0.55 g and 0.70 g) and dry weight (0.18 g and 0.15 g) compared with azoxystrobin-treated fresh weight (0.64 g and 0.66 g) and dry weight (0.12 g and 0.15 g) in both trials (Table 6).

***Reproductive parameters*:** Fenugreek extract application have enhanced the number of fruits, fruit weight and yield per plant in both 2018-19 and 2019-20 field trials. The outcomes expressed that 25% fenugreek extract recorded 11 and 12 fruits per plant just lower than Azoxystrobin-treated (15.66 and 13). The maximum number of fruits (19.33 and 15.66) were obtained in control plants and minimum in inoculated-control plants (7 and 5.33), in both trials. Nevertheless, the exogenous application of fenugreek extracts has enhanced the fruit weight which ranged from 39.15 to 44.52 g, just lower than Azoxystrobin-treated fruits where its ranges from 52.78 to 53.02 g. The minimum fruit weight range (35.33 to 35.52

g) was observed in inoculated-control plants under both trials (Table 7). Similar trend was observed with yield per plant where control plants (nor disease inoculated neither extract applied) have recorded highest yield per plant (1.11 kg & 1.02 kg) followed by Azoxystrobin-treated tomato plants which exhibited 0.82 and 0.74 kg per plant in trial one and two, respectively (Table 7).

***Physiological Parameters*:** The exogenous application of 25% fenugreek extract significantly enhanced the chlorophyll 'a', 'b' and total chlorophyll.

Maximum chlorophyll 'a' content (0.54 µg mg^{-1} FW) was observed in 5% fenugreek extract treated tomato plants while regarding chlorophyll 'b' highest value (0.58 µg mg FW^{-1} was observed in Azoxystrobin-treated tomato plants followed by 25% fenugreek extract application (0.26 µg mg FW^{-1}). However, the highest chlorophyll pigments were observed in control plants expressing stress free conditions. The lowest chlorophyll pigments were observed in control-inoculated tomato plants (Fig. 4).

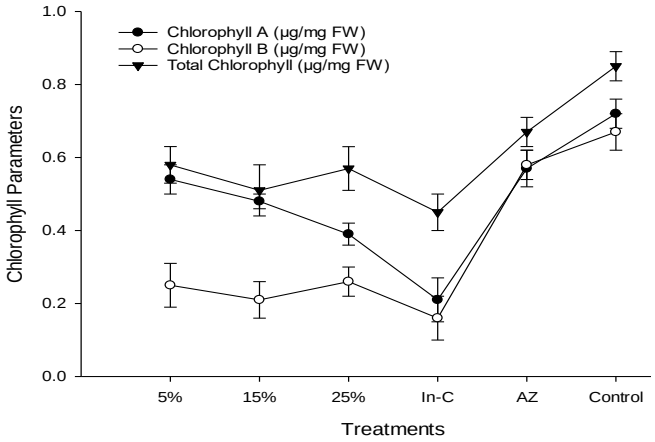

Figure 4. Effect of plant extract on chlorophyll 'a', 'b' and total chlorophyll contents of *A. solani* infected tomato plants under field conditions.
Values given are means of three replicates ±SE

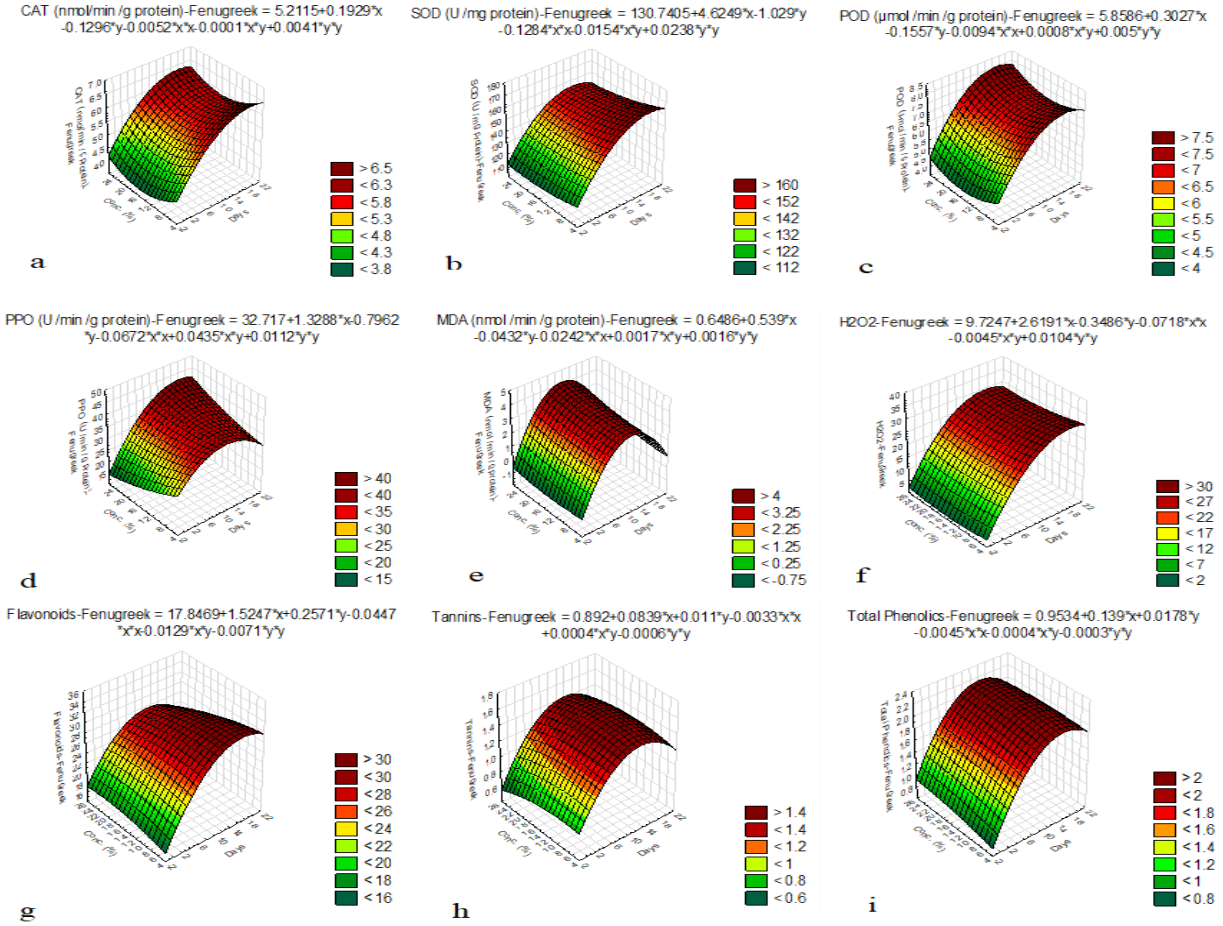

Figure 5. Response surface plots (3D) exhibiting the interactive effect of independent variables (Fenugreek concentrations & DAI) on the antioxidant enzymes, oxidative stress and phytochemicals of tomato infected with *A. Solani* under field conditions: (a) SOD activity, (b) CAT activity, (c) POD activity, (d) PPO, (e) MDA, (f) H_2O_2, (g) Flavonoids, (h) Tannins and (h) TPC. **DAI: Days after Inoculation.*

Biochemical Parameters: The outcomes from enzymatic activities expressed that treatment with fenugreek extract has incremented the superoxide dismutase activity (SOD), catalase (CAT), peroxidase (POD) and polyphenol-oxidase activity (PPO). It was noteworthy that increment was on the higher verge in initial 7 DAI with maximum activity at 14 DAI and afterwards downward trend was observed. The application of 5% fenugreek extract depicted maximum SOD (131.06 U mg^{-1} protein), CAT (4.37 $nmol^{-1}$ g^{-1} protein), and POD (5.32 $nmol^{-1}$ g^{-1} protein) activity (Fig. 5a, b and c). Likewise, PPO contents also exhibited ameliorated enzymatic activity under the influence of exogenous application of fenugreek extracts.

The incremented PPO activity (45.39 U min^{-1} g^{-1} protein) was observed in tomato plants treated with 25% fenugreek extract (Fig. 5d). Moreover, alike greenhouse studies, *A. solani* inoculation has reported higher lipid peroxidation (5.09 nmol g^{-1} FW) in control inoculated plants. Whereas fenugreek extract application has reportedly inhibited the lipid peroxidation (MDA contents) with significant reduction ($P \leq 0.01$) from 14 DAI and afterwards (Fig. 5e).

Hydrogen peroxide (H_2O_2) accumulation following *A. solani* inoculation in tomato leaves has exhibited significant differences (Fig. 5f). On the contrary, no significant differences from greenhouse studies were observed regarding flavonoids, tannins, and phenolic compounds response against *A. solani* inoculation in field (Fig. 5 g, h and i).

DISCUSSION

Biological control of early blight in tomato with the application of plant extracts is a modern, advanced, and risk-free alternative approach for disease management (Nashwa and Abo-Elyousr, 2012). In this regard, several plant extracts have been evaluated and proved significantly efficient against plant diseases (Draz *et al*., 2019). Plant extracts known to impart direct or indirect resistance against plant pathogens, either directly inhibiting the fungal progression or stimulating induced resistance in plants. Current study evaluates the efficacy of fenugreek against early blight in tomatoes caused by *A. solani*, under laboratory, greenhouse, and field conditions. Fenugreek extract @ 25% has proved to be significant for inhibiting fungal radial growth, this could be due to biologically active constituents of *T. foenum-graecum* (Haouala *et al*., 2011). The application of fenugreek extract lowered the disease severity under both greenhouse and field conditions, which could be attributed to presence of secondary metabolites i.e., phenolic compounds, flavonoids, tannins and coumarins (Gollo *et al*., 2020).

Fenugreek extract has certain allelochemicals i.e., kaempferol, kaempferol-glucoside and β-d-glucopyranoside (Singh *et al*., 2011). These metabolic compounds in turns constitute high antioxidant and antimicrobial activity, thus provides induce resistance in plants against disease attack (Attou, 2011). Kumar *et al*., (2017) also concluded the application of plant extracts in potato plants before pathogen inoculation, resulted in significant reduction in disease severity.

Results exhibited that the application of fenugreek extract have ameliorated the vegetative and reproductive growth and productivity. In addition to phenolic compounds, fenugreek extract also comprises lipids, minerals, and proteins (Wani and Kumar, 2018) whose supplementation to plants enhance their health, growth, and overall yield. Photosynthetic capacity of any plant is majorly dependent on chlorophyll pigments. Whereas, it has been observed that disease inoculation has resulted in reduction of chlorophyll pigments (Adhikari *et al*., 2017). Phenolic compounds provide the protection against pathogen's invasion generally by accumulation in higher level which led to augmentation of cell wall with increased lubrication and tissue lignification (Liyama *et al*., 1994). These phenolic compounds also induce the modifications in membranes and reduced synthesis of RNA and DNA formation (Mori *et al*., 1987).

The underlying mechanisms of disease suppression through plant extract is determined by evaluating the biochemical responses of *A. solani* stressed tomato plants. It has observed that different natural compounds offer environmentally safe approaches for inducing resistance against many plant diseases (Edogea *et al*., 2015). Following infection, plants readily signal the defense mechanism that led to an ameliorated production and accumulation of reactive oxygen species (Heyno *et al*., 2011). This reactive oxygen species leads to malfunctioning in physiological homeostasis (Srivastava and Dubey, 2011), a condition often known as oxidative burst. While oxidative burst is often scavenged by antioxidant enzymes (SOD, POD, CAT and PPO) and non-enzymatic antioxidants (tannins, flavonoids, and phenolic compounds).

Results expressed that the exogenous application of fenugreek extract led to increment of SOD and POD activity and increased accumulation of phenolic compounds i.e., tannins, flavonoids and TPC (Hassan *et al*., 2007). Similar outcomes were reported by Geetha and Shetty (2002) that bioactive compounds prevent lipid peroxidation thus help in reducing the plant disease infection (Amani *et al*., 2010). Moreover, such compounds may also reduce the disease pressure by ameliorating the antioxidant activities and enhanced H_2O_2 levels (Radwan et al., 2008).

Finally, the results suggest that fenugreek extract could be applied to manage early blight in tomatoes. Moreover, the fenugreek extracts also lowered the radial growth and disease severity while increasing chlorophyll contents and biochemical attributes. However, extract impact on growth characters was insignificant. The findings of this study concluded that plant extracts may serve as a safe and cost-effective strategy against early blight in tomato.

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