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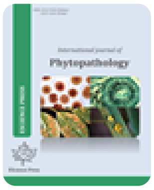

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UNVEILING THE ANTIBACTERIAL POTENTIAL OF PLANT DEFENCE ACTIVATORS IN CONJUNCTION WITH METEOROLOGICAL VARIABLES AGAINST BACTERIAL LEAF SPOT IN SPINACH

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ABSTRACT

Spinach is susceptible to various fungal, bacterial, and viral pathogens, with bacterial leaf spot being a particularly significant threat, often leading to substantial reductions in both yield and quality. Recent research evaluated different plant defense activators for their effectiveness against bacterial leaf spot in spinach. Among the tested activators, Salicylic acid achieved the lowest disease severity and incidence, at 24.76% and 27.73% respectively, followed by Citric acid (27.43% and 32.76%), Benzoic acid (29.57% and 38.32%), and K_2HPO_4 (33.31% and 38.38%) under both greenhouse and field conditions compared to the control. Several epidemiological factors—including minimum and maximum temperature, relative humidity, rainfall, and wind speed—were assessed for their impact on disease progression in three local spinach varieties: Dasi Palak, Lahori Palak, and Swiss Chard Palak. The results showed a considerable increase in disease severity when temperatures ranged from 26-28°C, relative humidity was between 69-73%, rainfall measured 0.5-1 mm, and wind speeds reached 1.5-2 km/h. All environmental factors positively correlated with bacterial leaf spot development in spinach. Greenhouse and field trials were conducted using a Complete Randomized Design (CRD) and a Randomized Complete Block Design (RCBD), respectively. Based on the findings, Salicylic acid application is recommended as an effective and ecologically sustainable strategy for managing bacterial leaf spot in spinach.

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INTRODUCTION

Spinach (*Spinacia oleraceae* L.) is a green vegetable crop grown all over the world that belongs to *Chenopodiaceae* family (Yamamoto *et al.*, 2014). It contains vitamin C, K, antioxidants, and flavonoids (Miano, 2016). Spinach leaves are rich in alpha-lipoic acid, a pivotal antioxidant that is advantageous for diabetic patients (Ibrahimpasic, 2013). Globally, it is cultivated on an area of 921.2 thousand hectares with a production of 3.2 million tonnes. While, in Pakistan it is cultivated on an area of 6.75 thousand hectares with a production of 83.34 thousand tonnes (FAO, 2020). China is the leading producer of spinach contributing 93% to the world total production, followed by USA, Turkey, Japan and Kenya in 2020 (FAO, 2020).

Spinach production is facing different biotic (Damping off, downy mildew, asters yellows, spinach mosaic, and spinach blight) and abiotic (drought, injury, sunscald, temperature, and salinity) stresses causing severe production and quality losses. Among all constraints, bacterial leaf spot of spinach caused by seed-borne bacterium *Pseudomonas syringae* pv. *spinacae* is the most destructive one, which infects young seedlings and causes 5 to 50% yield losses (Shila *et al.*, 2013). *P. syringae* is an aerobic, gram-negative and rod-shaped bacterium containing 1 to 5 polar flagella (1.55 × 0.7-1.2 μm) (Baltrus *et al.*, 2017). Bacterium penetrates plants via wounds or natural openings such as stomata, hydathodes, and lenticels and employs the type III secretion (T3S) mechanism to produce toxins, which contributes to its pathogenicity and disrupts the plant defence system (Kennelly *et al.*, 2007). Water-soaked lesions, irregular brown spots, dark brown necrotic lesions on petioles, and wilting are the typical symptoms induced by pathogen (Koike *et al.*, 2002). The most appropriate environmental conditions required for the establishment of bacterial leaf spot in spinach includes 24-28°C temperature, relative humidity 85% and pH 6-7.5 (Hansen, 2009).

There are different management strategies including the use of resistant cultivars, synthetic chemicals, biological agents and cultural practices are being employed to combat bacterial leaf spot of spinach. No doubt cultivation of resistant cultivars is the most suitable and significant way to manage the disease, but due to sudden climate change and mutation in pathogen, available resistant varieties are becoming susceptible towards bacterial leaf spot of spinach. Moreover, due to the unavailability of vast resistant sources of spinach and variability in the virulence of bacterium it is utmost requirement to find out another eco-friendly management approach to manage this disease (Scortichini *et al.*, 2013). On the other hand, chemotherapy is the second most prominent approach to control plant diseases due to their quicker action and easier availability (Shaheen *et al.*, 2023). However, their residual effects are making them highly dangerous for humans, animals and non-targeted microorganisms (Usman *et al.*, 2024; Chhipa, 2019). That's why it is the need of hour to look for alternatives of synthetic chemicals to prevent the hazardous residual effects on the environment and human health. Plant defence activators are one of the prominent alternates that are being employed to enhance plant resistance against pathogens (Sun *et al.*, 2015). These are the specialized chemicals that activate the defence-related genes of host plants including chitinase and phytoalexins (Shad *et al.*, 2023), which trigger induced systematic resistance (ISR) by generating signals via the signal transduction pathway, providing long-term protection against a wide range of pathogens (Malik *et al.*, 2023; Ahmad *et al.*, 2010). SAR inducers are chemical compounds that activate the signaling pathways such as the salicylic acid pathway to induce plant resistance (Achuo *et al.*, 2004). Keeping in view the severity of the disease, the current study was designed to evaluate plant defence activators towards *Pseudomonas syringae* pv. *spinacae* causing bacterial leaf spot of spinach.

Environmental factors including temperature and humidity are the main influencers for biotic disease development (Atiq *et al.*, 2024). Forecasting of infection prior to disease development enables the creation of disease prediction models, empowering farmers and researchers to promptly implement protective measures to manage pathogenic diseases and prevent yield losses (McGuffie and Henderson-Sellers, 2014). The key objective of studying environmental factors is to provide farmers and researchers with valuable insights into the ideal conditions that contribute to the occurrence of epidemic diseases.

MATERIALS AND METHODS

Collection of Diseased Samples

Different localities of district Faisalabad (AARI (31°25′0″N 73°5′28″E), Samundri (31°03'45"N 72°57'15"E) and Jaranwala (31°20′0″N 73°25′0″E)) were surveyed for the collection of diseased samples. Samples were taken in brown paper bags (13" × 9.5") and labelled with the name, date, and place of the collection site. Samples were brought to the Molecular Phyto-bacteriology Laboratory, Department of Plant Pathology, UAF. The samples were washed, air-dried and placed at 4°C in the refrigerator (PL6500) for further analysis.

Regression Analysis

The least significant difference (LSD) test was used to detect disease incidence on spinach varieties/advanced lines. All environmental data (maximum and minimum temperature (°C), rainfall (mm), relative humidity (%), and wind velocity (Km/h), as well as disease incidence

(%) data collected from several districts in Punjab, were subjected to variance and difference analysis. By using correlation, it was determined how these environmental factors affected the bacterial leaf spot on spinach (Steel *et al.*, 1997). Regression analysis was used to identify the varieties/advanced lines where significant impacts were plotted, as well as the most conducive environmental conditions for disease development. By comparing real sickness with the values predicted by various regression models, it was possible to identify how these circumstances affected the development of the bacterial leaf spot of spinach.

Data collection of Environmental Factors

Environmental data including maximum and minimum temperature (°C), wind speed (Km/h), relative humidity (%), and rainfall (mm) was obtained from the meteorological station, Agronomy Department, UAF. Data was measured from October 2023 to December 2023, and weekly averages were calculated. Simple correlation and regression analysis were used to estimate the impact of each variable (maximum and minimum air temperature, rainfall, relative humidity, and wind velocity) on disease development.

Isolation, Purification and Identification of Pathogen

Nutrient agar (NA) media was used for the isolation of the bacterium. Media was prepared by adding 28g of synthetic NA powder in 1000 mL distilled water and autoclaved at 121°C at 15 PSI for 15 minutes. Diseased samples were thoroughly washed with tap water to remove dirt particles. After cutting in small pieces (3-5 mm), samples were surface sterilized using 1% sodium hypochlorite solution for 30 seconds and again washed with distilled water and kept for drying on filter paper (Singh *et al.*, 2014). After that, samples were placed on petri plates (9 Cm) containing solidified NA media, with the help of sterilized forceps. After wrapping with paraffin film, petri plates were labelled and kept in an incubator at 25°C, bacterial growth was regularly observed. To avoid contamination all the work was performed in a hygienic atmosphere under a laminar airflow chamber (RTVL-1312).

After the appearance of bacterial growth in mother plates, purification was done by streaking of single colony with the help of a sterilized loop on fresh NA media plates and incubating at 25°C temperature (Ranjan *et al.*, 2021). The isolated bacterium was identified on the basis of biochemical (KOH, Gram staining and catalase tests) and morphological (colour, shape and growth pattern) characterization (Sharma and Singh, 2019).

Pathogenicity Test

By following Koch's Postulates, a pathogenicity test was conducted for the confirmation of the pathogen. The bacterial suspension was prepared @ 1×10^8 CFU mL^{-1} with the help of a spectrophotometer set to OD 600=0.2 (Hitachi U-2001, model 121003) (Tahat *et al.*, 2010). Spinach was grown in earthen pots (28×14 Cm) containing sandy loam soil (2 kg). Spinach plants of 40-45 days of age were artificially inoculated using prepared bacterial suspension early in the morning (when a maximum number of stomata were opened) by using the spray method (Planas-Marquès *et al.*, 2018). After the appearance of symptoms, the pathogen was re-isolated from the diseased plant and was compared with the mother culture for confirmation.

Evaluation of Plant Defence Activator Against Bacterial Leaf Spot of Spinach Under Greenhouse Conditions

A pot experiment was conducted to assess the efficacy of plant activators against bacterial leaf spot of spinach under greenhouse conditions using earthen pots having 26×14 Cm size, contained sandy loam soil (2 kg/pot). Seeds of desi spinach were brought from the local seed market of Faisalabad and grown at a glasshouse of Department of Plant Pathology, UAF. 40 days old spinach plants were artificially inoculated using bacterial suspension 1×10^8 CFU mL^{-1} with the help of hand sprayer (Planas-Marquès *et al.*, 2018). Four different plant defence activators like Salicylic acid, Benzoic acid, Citric acid, and Dipotassium phosphate (K_2HPO_4) were collected from Citrus Pathology Lab, Department of Plant Pathology, UAF. Three different concentrations i.e. 0.25, 0.50 and 0.75% of each treatment were prepared by mixing 0.25, 0.50 and 0.75g powder in conical flask contained 100mL of distilled water respectively. After appearance of typical leaf spot symptoms, all the treatments were applied using the spray method, where distilled water was used as the control treatment. The experiment was conducted under CRD by maintaining three replications of each treatment. Data regarding disease incidence (%) was calculated after 5, 10 and 15 days of the interval through the following formula:

$$\text{Disease incidence } \% = \frac{\text{Total number of infected plants}}{\text{Total number of observed plants}} \times 100$$

In vivo Evaluation of Plant Defence Activator Against Bacterial Leaf Spot of Spinach

Spinach (Desi variety) was grown in the field at the Research Area, Department of Plant Pathology, UAF. After 40 days of germination, the crop was artificially inoculated using bacterial suspension (1×10^8 CFU mL^{-1}), prepared from fresh bacterial culture by using spectrophotometer (Hitachi U-2001, model 121003). Bacterial suspension was applied early in the morning (when maximum number of stomata was opened) through spray method (Planas-Marquès *et al.*, 2018). After 5-7 days, disease symptoms appeared and the most effective concentration (0.75%) of each treatment were applied. The experiment was conducted under RCBD with three replicates of each treatment. Data regarding disease incidence (DI) was noted after 5, 10 and 15 days of application.

Epidemiological Factors Conducive to Disease Development

Environmental factors such as relative humidity (%), rainfall (mm), temperature (°C), and wind speed (km/h) were observed under field conditions. Data regarding environmental factors were collected from the meteorological station, Agronomy Department, University of Agriculture, Faisalabad. Data and infection period were monitored based on weekly intervals and evaluated by using the Disease Rating scale. The impact of epidemiological factors on bacterial leaf spots of spinach was correlated by correlation and regression analysis. R square and Mean Square Error (MSE) were selected for developing the best model. The data obtained from correlation and regression analysis was fit into the model and the goodness of the data determined. A disease predictive model developed based on environmental conditions having a significant influence on bacterial leaf spot of spinach development through stepwise regression.

Data Analysis

Greenhouse and *in vivo* experiments were performed under a Completely Randomized Design (CRD) and Randomized Complete Block Design (RCBD) by using SAS software (Littell *et al.*, 1998). The recorded data was analysed by using analysis of variance (ANOVA).

RESULTS

Evaluation of Plant Defense Activators Against Bacterial Leaf Spot of Spinach under Greenhouse Conditions

Among all treatments, minimum disease severity was expressed by Salicylic acid (24.761%) followed by Citric acid (27.430%), Benzoic acid (29.579%) and K_2HPO_4 (33.316%) as compared to control (Figure 1). The interaction between treatment and concentration (T×C) showed that minimum disease severity was exhibited by Salicylic acid (21.348, 25.662 and 27.274%) followed by Citric acid (24.596, 28.088, and 29.606%), Benzoic acid (27.430, 30.001 and 31.307%) and K_2HPO_4 (31.117, 33.438, and 35.394) at 0.25%, 0.50%, and 0.75% respectively as compared to control (Figure 2). Interaction between treatment and day (T×D) expressed that maximum disease severity was exhibited by K_2HPO_4 (36.206, 32.873, and 30.870%) followed by Benzoic acid (32.183, 29.471, and 27.083%), Citric acid (30.134, 27.386, and 24.769%) and Salicylic acid (21.990, 24.301, and 27.993%) after 5, 10 and 15 days individually as compared to control (Figure 3).

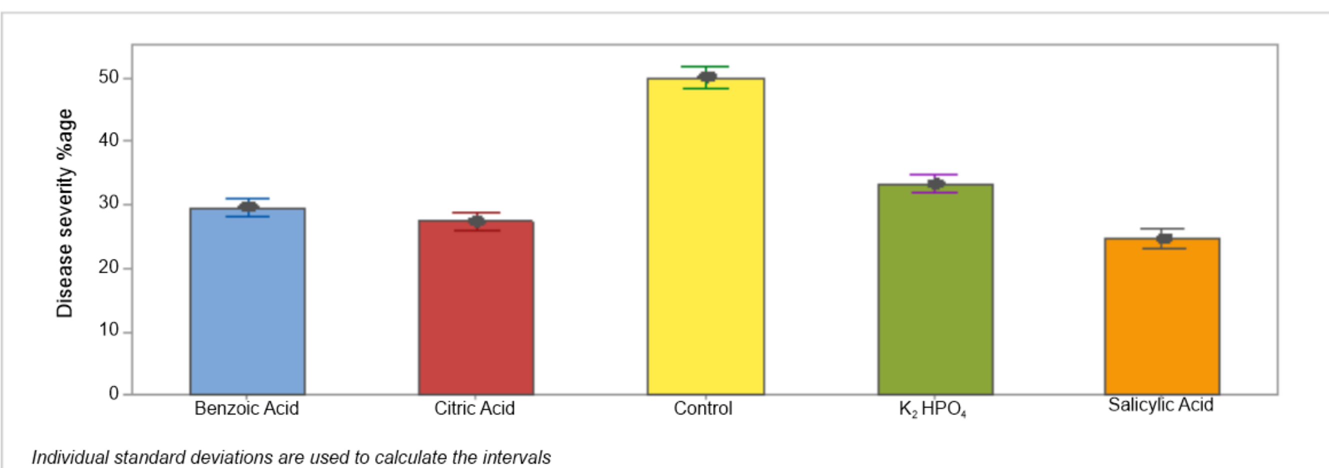


Figure 1. Evaluation of plant defence activators against bacterial leaf spot of spinach under greenhouse conditions.

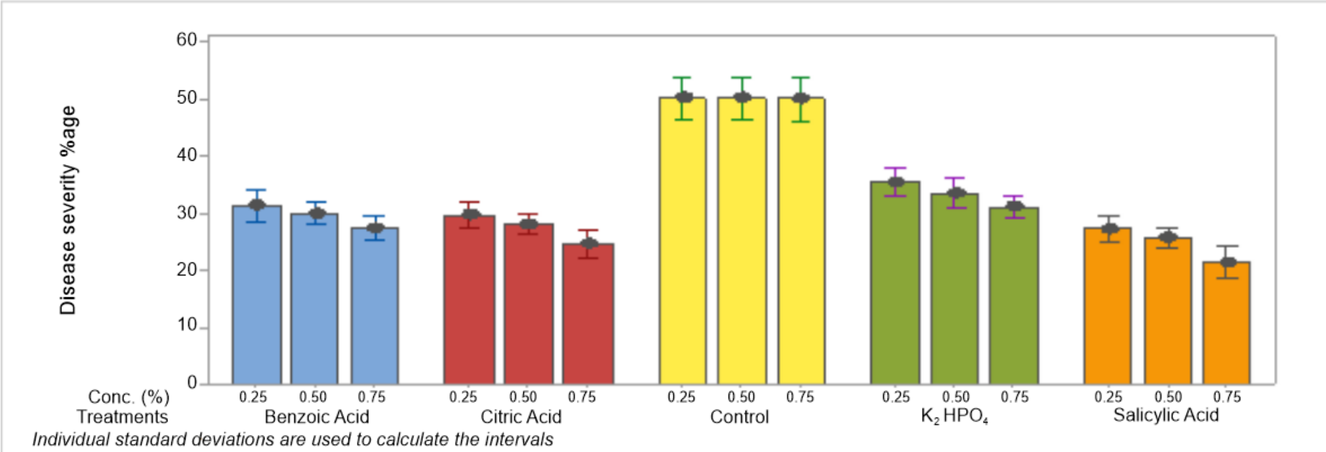


Figure 2. Impact of interaction between treatments and concentrations (T×C) towards bacterial leaf spot of spinach under greenhouse conditions.

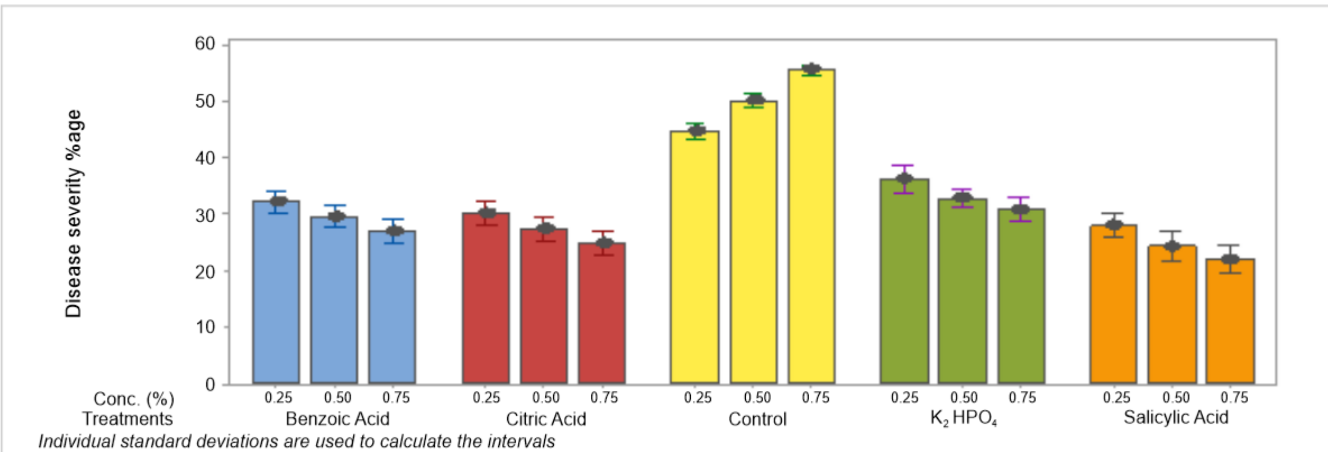


Figure 3. Effect of interaction between treatments and days (T×D) against bacterial leaf spot of spinach under greenhouse conditions.

Evaluation of Plant Defense Activators against Bacterial Leaf Spot of Spinach Under *in vivo* Conditions

Among all treatments, minimum disease incidence was expressed by Salicylic acid (27.733%) followed by Citric acid (32.767%), Benzoic acid (38.322%) and K_2HPO_4 (38.389%) as compared to control (Figure 4). The interaction between treatments and days (T×D) expressed that minimum disease incidence was exhibited by Salicylic acid (24.167%, 27.000, and 32.033%) followed by Citric acid (37.500, 32.333, and 28.467%), Benzoic acid (41.933, 38.267, and 34.767%) and K_2HPO_4 (42.500, 38.667, and 34.000%) after 5, 10 and 15 days of application respectively (Figure 5).

Development and Evaluation of Bacterial Leaf Spot Predictive Model Based on One-Year Data

The multiple regression equation of bacterial leaf spots predictive model for one year: $Y=194 -2.32 X_1 + 1.14 X_2 + 0.774 X_3 - 7.81 X_4 -0.827 X_5$ (Y= Disease incidence X_1 = Maximum temperature, X_2 = Minimum temperature, X_3 = Rain fall, X_4 = Wind speed and X_5 = Relative humidity). The R^2 value 86.5 % expressed that model was statistically appropriate under prescribed environmental conditions. Some data points deviated from the reference line according to the normal probability graph (Figure 6), while most values were scattered equally around the residual line in case of residual vs. fit model which showed better fit of regression model (Figure 6). Few data points were little far from the line of reference i.e., near to zero; -3.5 to + 4 primarily exhibited as an error in the regression model. Model was designed according to (Chattefuee and Haddi, 2006).

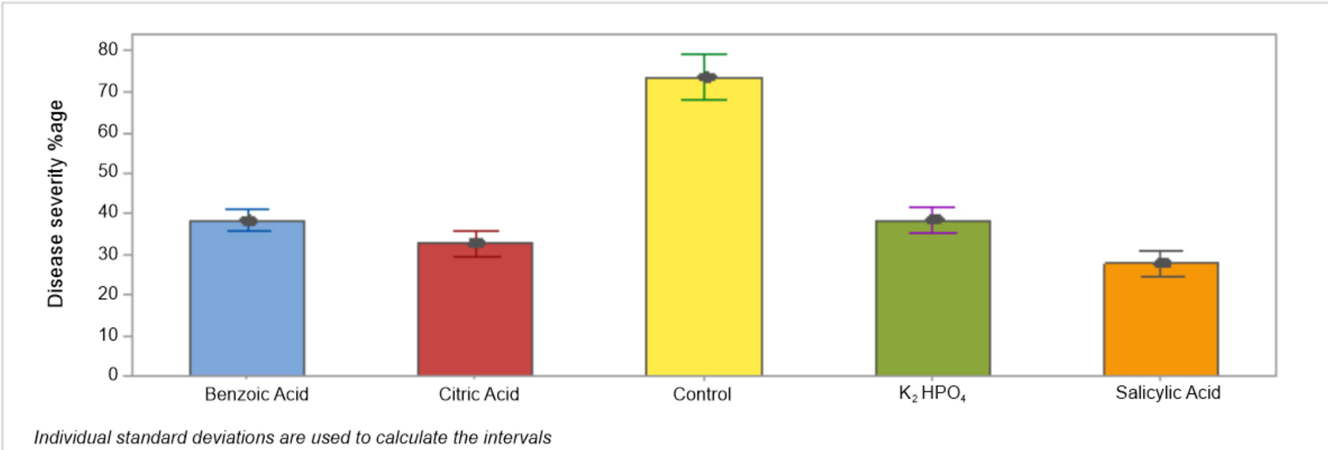


Figure 4. Evaluation of plant defence activator against bacterial leaf spot of spinach under field conditions.

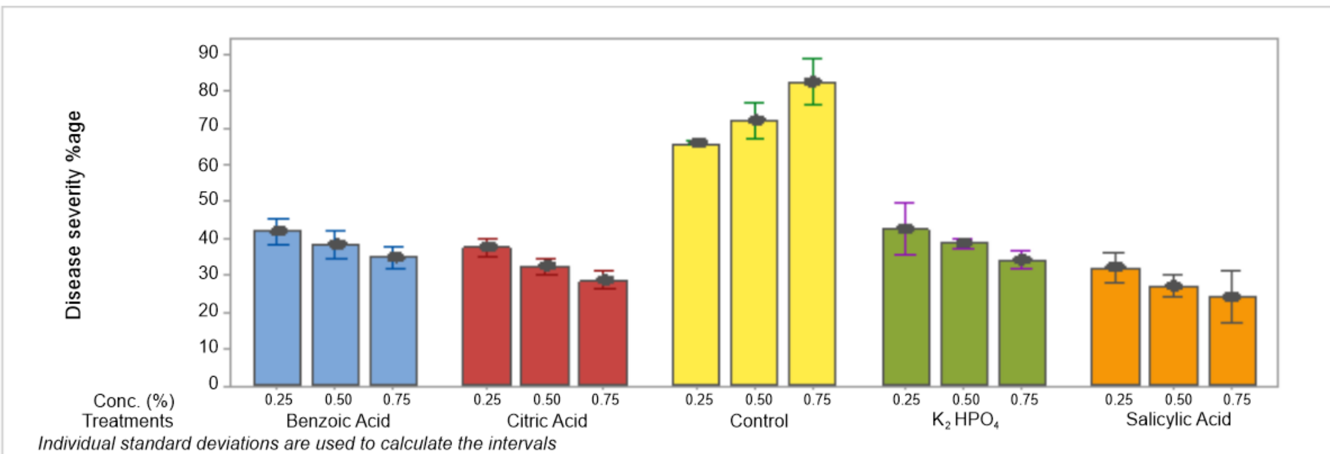


Figure 5. Impact of interaction between treatments and days (T×D) against bacterial leaf spot of spinach under field conditions.

Estimation of model on the basis of predicted and observed values

For assessing the reliability of model value differences of observed and predicted data points were estimated. Among the observed values five data-points were beyond reference line (standard error= 2.32001) and created an error in experiment. According to the graphs, maximum prediction (out of 30 values, 10) values have differences (less than 5) were consolidated between 95 % interval of confidence (C.I) and 95 % interval of predictive (P.I) which showed that there was a good fit in between predictive and observed values (Figure 7).

Correlation and characterization of environmental factors required for the development of Bacterial leaf spot of spinach

Three spinach varieties viz. Desi Palak, Lahori Palak and Swiss chard Palak were used for the determination of environmental factors (maximum temperature, minimum temperature rainfall, relative humidity and wind speed) which are conducive for bacterial leaf spot development. The relationship of maximum temperature with the disease severity of cultivars Desi Palak, Lahori Palak and Swiss chard Palak was noticed as extremely strong but with negative sign as the correlation coefficients for their inter- relationship were -0.93, -0.92 and -0.90 respectively. The relationship between disease incidence and maximum temperature, maximum disease was recorded at 26-28°C temperature range (Figure 8). The relationship of minimum temperature and the disease severity was negative and showed highly significant results on all varieties except two. The relationship between minimum temperature and disease incidence, maximum disease was recorded between temperature ranges of 10-15 °C (Figure 8). For both factors (maximum temperatures and minimum temperatures) the correlation values of temperature and disease indicated that when one variable was increased at the same time the other variable was decreased. In case of Relative Humidity, it exhibited positive non-significant results and in relationship with disease incidence, maximum disease was recorded at 69-73% R.H (Figure 9). The rainfall showed positive non-significant results and in relationship between disease incidence and rainfall, maximum disease was recorded between 0.5-1 mm rainfall (Figure 9). Wind speed also had statistically positive non- significant results with all varieties and in relationship with disease incidence, maximum disease was recorded at 1.5-2 km/h wind speed (Figure 9). The results showed that there was a positive relationship between the disease severity and environmental factors like relative humidity, rainfall and wind speed. As these factors increased severity of disease also increased.

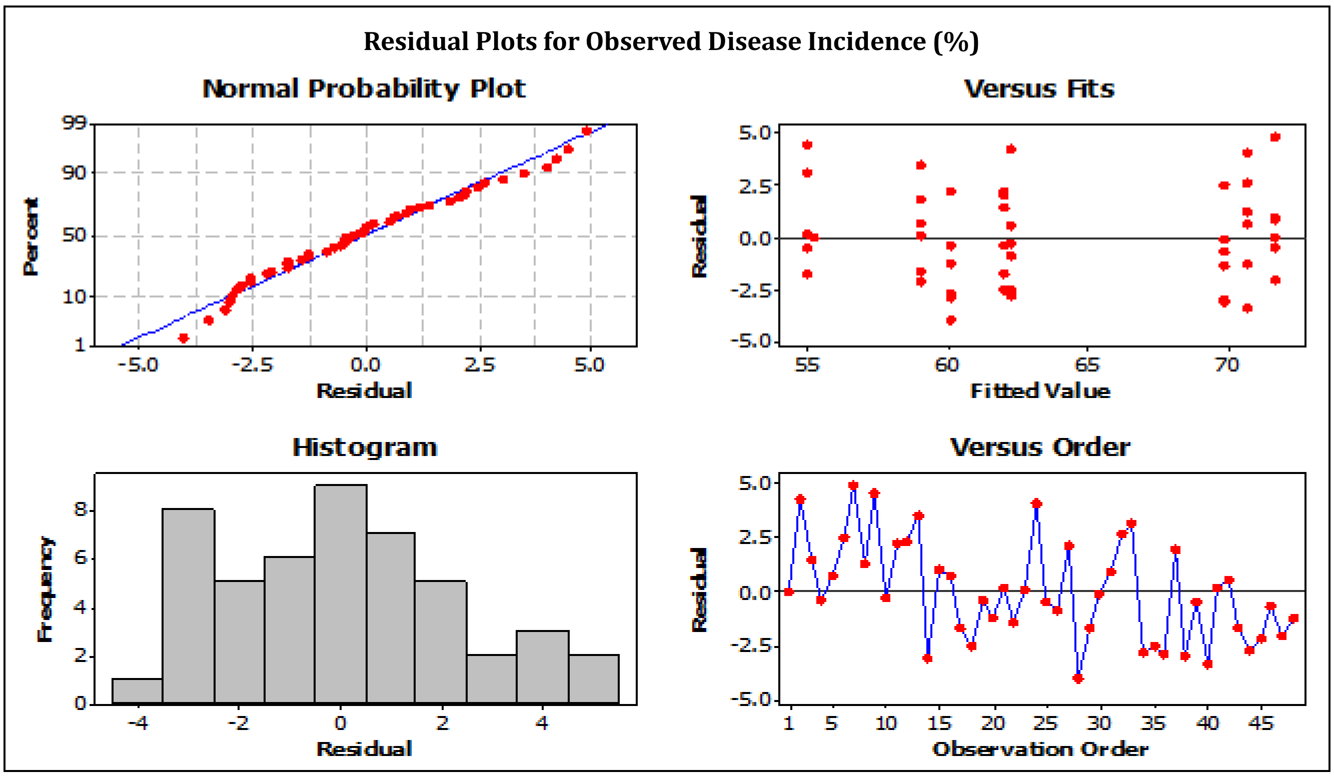


Figure 6. Residual plots for observed disease incidence for bacterial leaf spot predictive model based on one-year data.

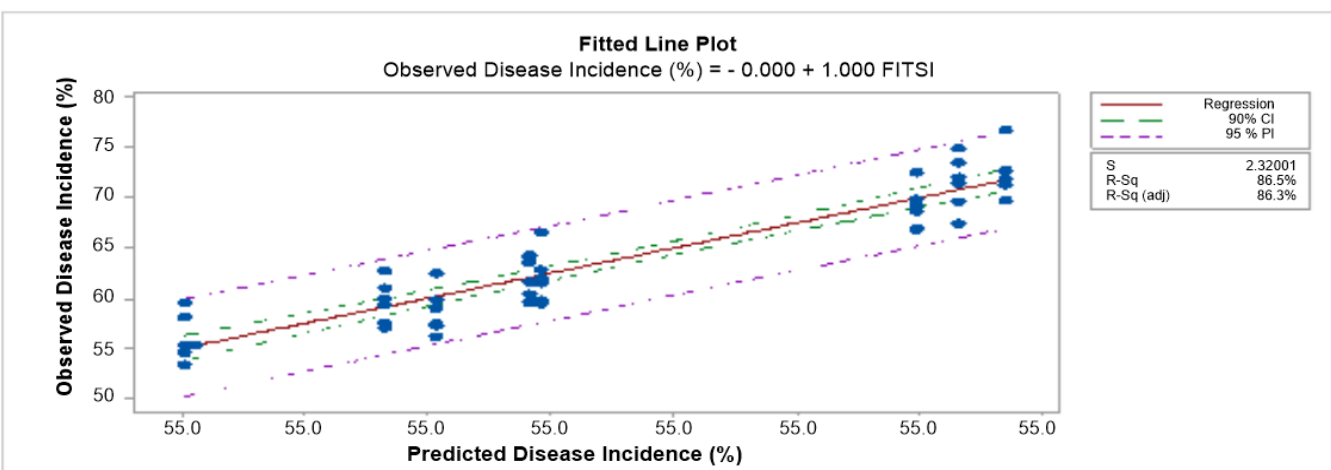


Figure 7. A fitted line plot for bacterial leaf spot of spinach with observed and predicted data points.

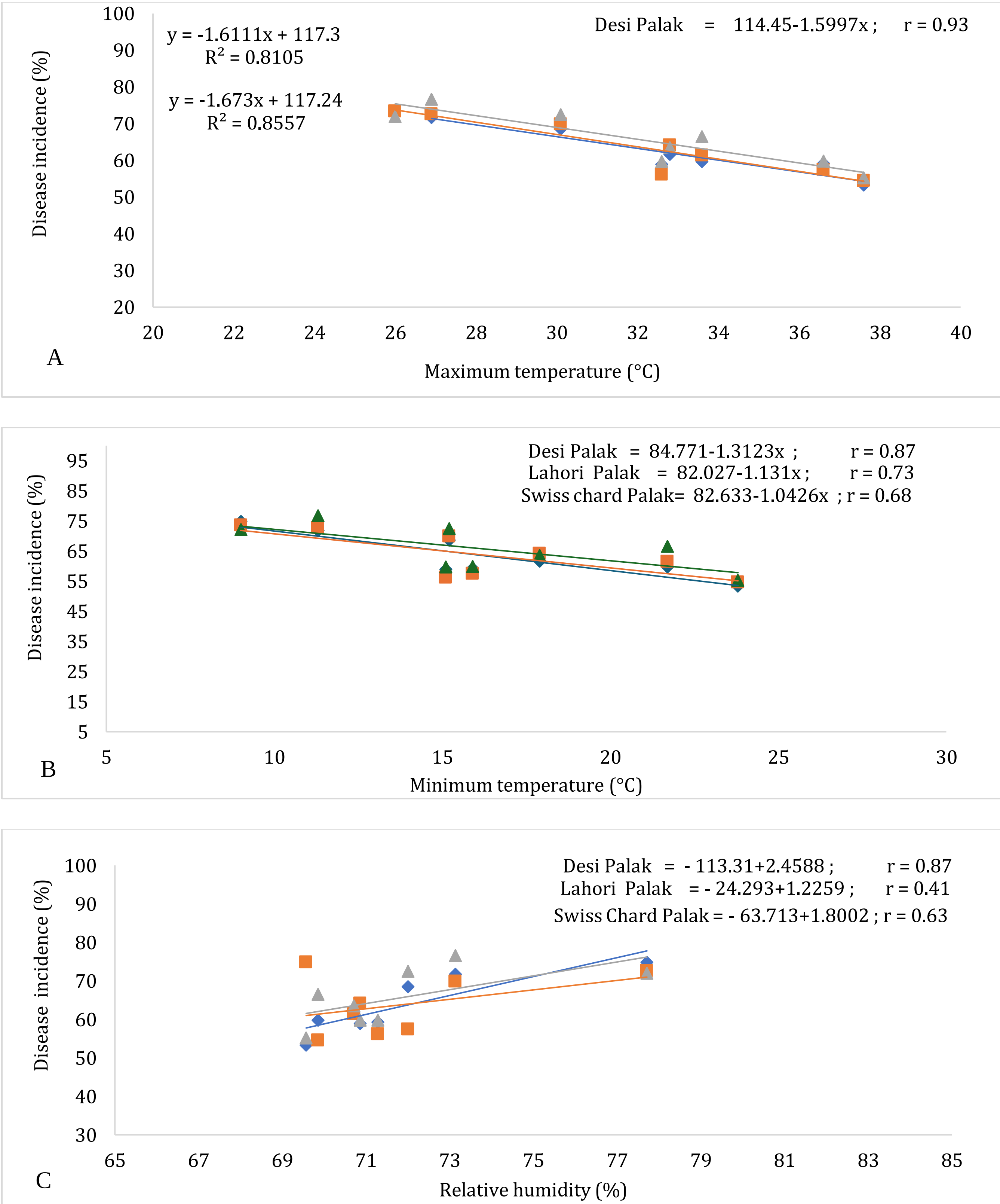
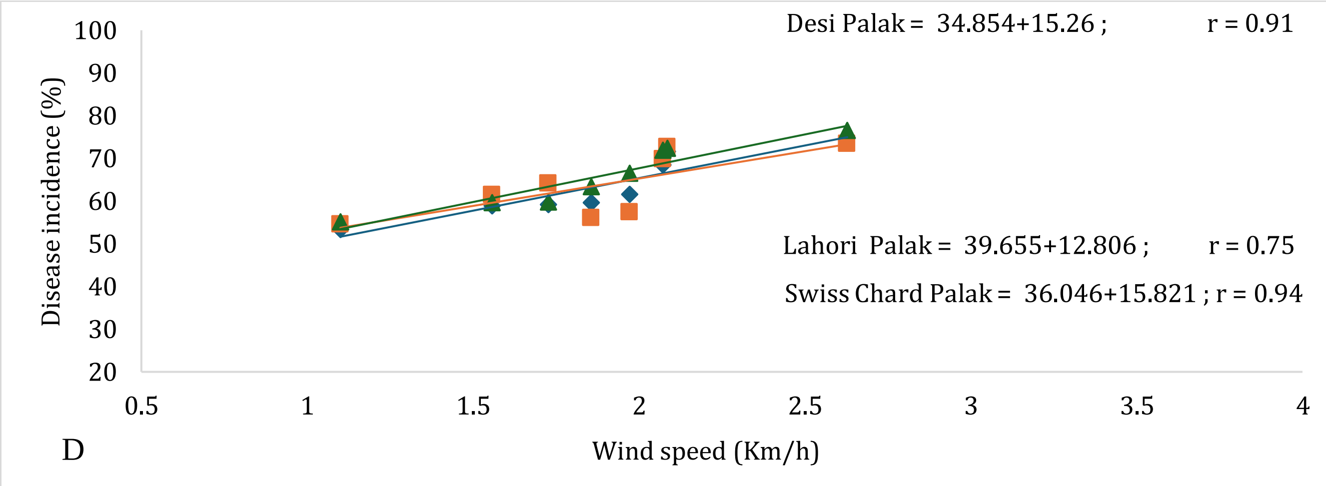


Figure 8. Effect of Maximum (A), Minimum (B) Temperature and Relative Humidity (C) on the disease incidence of bacterial leaf spot of spinach.



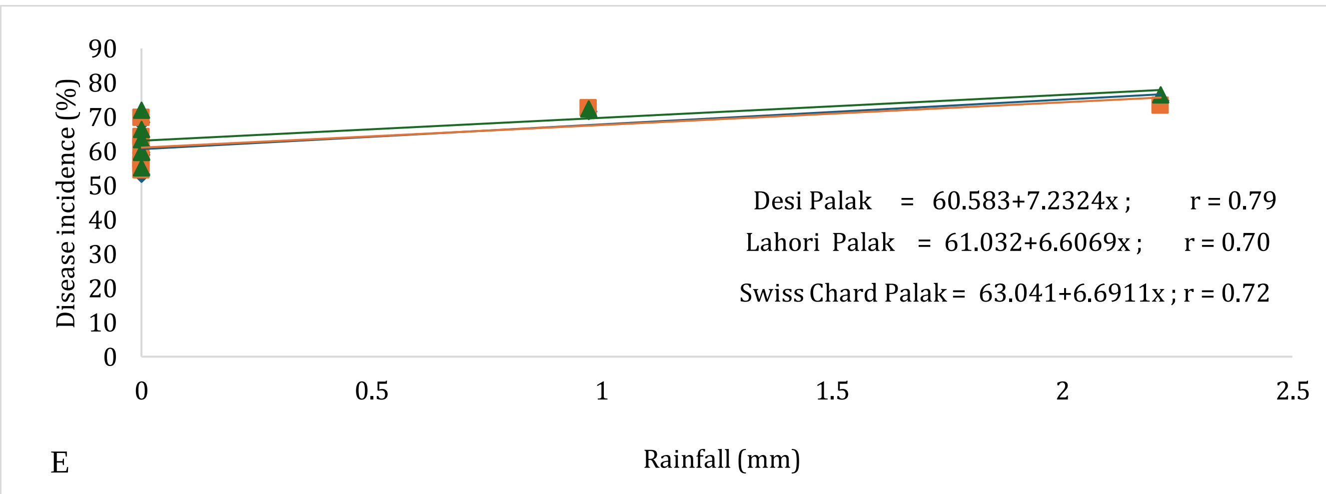


Figure 9. Impact of Wind Speed (D) and Rainfall (E) on the disease incidence of bacterial leaf spot of spinach.

Table 1. Correlation and characterization of environmental factors conducive for the development of bacterial leaf spot of spinach.

Varieties	Max. Temp. (°C)	Min.Temp.(°C)	R.H (%)	RF (mm)	WS (km/h)
Swiss chard	0.900**	0.687	0.110	0.293	0.343
	0.002	0.060 NS	0.796NS	0.481 NS	0.406 NS
Lahori spinach	0.944**	0.874**	0.090	0.006	0.383
	0.000	0.005	0.832 NS	0.989 NS	0.349 NS
Multan Selection	0.958**	0.873**	0.010	0.007	0.453
	0.000	0.005	0.981 NS	0.987 NS	0.260 NS
Smooth spinach	0.925**	0.737*	0.033	0.087	0.332
	0.001	0.037	0.938 NS	0.837 NS	0.422 NS
Kandiari spinach	0.821**	0.737*	0.185	0.048	0.401
	0.012	0.037	0.661 NS	0.910 NS	0.325 NS
Desi palak	0.911**	0.679	0.089	0.210	0.309
	0.002	0.064 NS	0.835 NS	0.618 NS	0.457 NS

Assessment of disease predictive model by comparing the dependent variables with regression coefficient through physical theory

Analysis of variance of regression articulated that T (max. & min.), RH, RF and WS significant contribution towards development of disease. The R2 value 86.5 expressed the model to be statistically suitable under the given environmental conditions. Variables coefficients of regression model for spinach are given in (Table 2).

Table 2. Regression model's coefficients of variables for bacterial leaf spot of spinach.

Parameter	Coefficient	Standard Error	t-Stat	P-value
Intercept	193.55	20.32	9.53	0.000*
Max. temp. (°C)	2.3218	0.2970	7.82	0.000*
Min. temp. (°C)	1.1384	0.3155	3.61	0.001*
Rain fall (mm)	0.7739	0.8979	0.86	0.394^{NS}
Wind Speed (km/h)	7.805	1.638	4.76	0.000*
Relative Humidity (%)	0.8274	0.2488	3.33	0.002*

* = Significant NS = Non-Significant P > 0.05

DISCUSSION

One of the most ravaging biotic stresses to spinach is bacterial leaf spot which is caused by *Pseudomonas syringae* pv. *spinacae*, Several control measures were previously used to overcome the incidence of the pathogen. Farmer's community is preferably using synthetic chemicals due to their quick action and avaialabiltiy, but these chemicals are highly hazardous to humans and animals. So, it is the most demanding need to use environmently friendly approaches to manage bacterial blight of spinach. That's current study was carried out to evaluate various plant defense activators to manage BBS. Among all plant defence activators, Salicylic acid expressed minimum disease incidence under field and greenhouse conditions.

Results of the present research are supported by the findings of Li *et al.* (2020) who concluded that plant defence activators enhanced plant resistance against *Xanthomonas oryzae* pv. *oryzae*. Similarly, Sun *et al.* (2015) also evaluated different plant defence activators and their findings proved the strong antibacterial activity against *Pseudomonas syringae*. Present outcomes are also in line with the findings of Choi *et al.* (2014) who boosted the host resistance through plant defense activators against bacterial leafspot of pepper. Plant defense activators are the essential compounds that activate host plants defense by the production of various defence related enzyme i.e. superoxide dismutase, catalase, peroxidase, hydrogen peroxide, which results in triggering induced resistance (IR) by initiating signals via signal transduction pathway and provide prolonged defense against a variety of pathogens (Ahmad *et al.*, 2010). Salicylic acid is an essential element for regulating plant growth and development by its specific action to mediate the oxidative burst during biotic stress in plants. It has also been reported that salicylic acid increased H_2O_2 levels in plant cells and potentially induce antioxidant enzyme expression, thereby improving plant resistance to biotic and abiotic stress (Tahir *et al.*, 2023).

Plant defense activators stimulate plant defense against invading pathogens by the production of various enzymes. After application, salicylic acid causes the production of hydrogen peroxide, a major component of reactive oxygen species. This newly produced hydrogen peroxide have antifungal abilities with inhibition of fungal spore germination. Lipid peroxidation is triggered by reactive oxygen species (ROS), leading to membrane breakdown, cell necrosis, and the activation of lipoxygenases (LOXs). These LOXs stimulate the production of phytoalexins. Additionally, the metabolites of LOXs alter the gene expression related to the fungal defense system. In conclusion, these plant defense activators are key regulators of plant defense during oxidative stress. There is a very significant role of epidemiological factors in the outbreak of disease. To foresee the epidemics of disease, there is the requirement to find out the relationship between bacterial blight of spinach (BBS) development and epidemiological factors, so that the yield losses may be reduced. In the current study, great intention was given to find out the epidemiological factors which have great impact on the BBS development. It was noticed that the BBS was significantly influenced by some epidemiological factors including minimum temperature, maximum temperature, wind speed, rain fall and relative humidity. The correlation between BBS development and these epidemiological factors was observed positive which means that the disease will increase by the increase of these factors. Environmental factors that have been linked to bacterial leaf spot include maximum temperature, minimum temperature, rainfall, relative humidity, and wind speed. Maximum and minimum temperatures, wind direction, amount of precipitation, relative humidity, and the prevalence of disease, all have a significant ($p<0.05$) but positive correlation (Pohronezny *et al.*, 1990) examined wind and the windstorm sand were investigated for the contribution in bacterial leaf spot of spinach. Diab *et al.* (1982) discovered that the growth of leaf spots was significantly altered by variations in relative humidity (RH). The infection was aided by extended periods of high relative humidity and the free moisture on the leaves. When inoculated plants were exposed to high RH (>85%) for a few hours over the course of one or two days, pathogens could result in disease symptoms.

CONCLUSION

Present exploration has drawn conclusion that salicylic acid is the most fruitful treatment among all tested plant defence activators. Under greenhouse and field conditions, minimum disease incidence was exhibited by salicylic acid followed by citric acid, benzoic acid and K_2HPO_4. Environmental factors like relative humidity, rainfall and wind speed exhibited positive correlation with the bacterial leaf spot of spinach.

CONFLICT OF INTEREST

There is no conflict of interest among the authors.

AUTHORS CONTRIBUTION

All the authors contributed equally to this work.

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