Journal of Animal & Plant Sciences, 36(1): 2026, Page: 193-202
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2026.1.0016

INDUCTION OF RESISTANCE AGAINST *Fusarium equiseti* IN *Spinacia oleraceae* BY *Catharanthus roseus* AND EXPRESSION ANALYSIS OF STRESS-RELATED GENES

S. Shafique[1*], S. Shafique[1], A. Mubarak[2], R. Rafique[2] and M. F. Irfan[1]

[1]Department of Plant Pathology, Faculty of Agricultural Sciences, University of the Punjab, Lahore 54590, Pakistan
[2]Department of Botany, University of Education, Township Campus, Lahore, Pakistan.
[*]Corresponding Author's Email: shazia.iags@pu.edu.pk

ABSTRACT

Spinacia oleraceae L. is a significant food vegetable, and it is vital to enhance its yield against the stress of many devastating pathogens such as *Fusarium* spp. The manipulation of plant extracts may mitigate the detrimental effects of disease-causing fungi. The antifungal activity of *Catharanthus roseus* methanolic extracts was investigated against the *Fusarium equiseti* pathogen, which causes leaf spot disease in spinach. Moreover, the elicited expression of the stress genes of *C. roseus* was also explored. Antifungal bioassays revealed that increased concentration of leaf methanolic extracts significantly suppressed pathogen development. However, leaf extract exhibited maximum inhibition (approx. 95%) in fungal biomass production. The methanolic extract of *C. roseus* utilized in pot trials for biocontrol assays offered superior plant defense against pathogens, delivering the highest level of disease protection. Subsequently, RT-PCR was performed using *ETR1*, *ETR2*, and *ESR1* coding genes to examine variations in the expression profiles of ethylene-related genes under pathogenic stress in *C. roseus*. The results concluded that either individual or synergistic higher expression of the *ETR2* gene may suppress the growth of the pathogen, demonstrating its efficacy in controlling the disease.

Keywords: *Catharanthus roseus,* Biocontrol potential, *Fusarium equiseti,* Methanolic extract, stress genes, RT-PCR, Spinach.

Published first online December 05, 2025 Published final January 20, 2026

INTRODUCTION

Spinach (*Spinacia oleracea* L.) is one of the most widely consumed nutrient-rich vegetables, often eaten in both fresh and processed forms or incorporated into various culinary preparations (Roberts and Moreaua, 2016; Siddique *et al*., 2021). The cultivation of spinach predominantly occurs during the midwinter months in numerous regions worldwide, including Pakistan, China, and India. Globally, approximately 253,800 hectares are dedicated to spinach cultivation, with an average yield of 250,000 kg per hectare. Notably, China accounts for nearly 90% of global spinach production (FAOSTAT, 2015; Rashid *et al*., 2020; Hussain *et al*., 2023).

In Pakistan, spinach production has shown considerable growth, contributing to the country's improving agricultural economy. Currently, Pakistan ranks among the top 10 countries globally for spinach production. Spinach has garnered increased attention in the country, owing to its significant nutritional benefits. For example, consuming just 20 grams of spinach provides only about 5 to 6 calories, making it a highly nutritious, low-calorie food option (Patricia, 2014). Additionally, spinach is known to support health in various ways, including the management of iron deficiency-related anaemia, which can lead to osteoporosis (Miano, 2016; Zeb and Jamil, 2025).

Despite its nutritional value, spinach is highly susceptible to numerous fungal and viral diseases (Sunil and Yadav, 2020; Liu *et al*., 2021; Singh *et al*., 2025). Among these, anthracnose is a particularly prevalent infection that significantly reduces crop yield (Correll *et al*., 1994; Shova *et al*., 2020; Shafique *et al*., 2022). Fungal leaf spots are considered the most damaging and widespread pathogens affecting spinach crops globally. One of the most destructive pathogens is *Fusarium oxysporum*, which is responsible for significant losses in spinach production worldwide (Larsson and Gerhardson, 1992; Correll *et al*., 1994; Shafique *et al*., 2022; Wariebi, 2025). This disease is notoriously difficult to manage under field conditions and can lead to complete crop failure, with up to 100% destruction reported (Beckman, 1987). Current disease management practices rely heavily on chemical fungicides, which are often environmentally hazardous, economically unsustainable, and ineffective in the long term.

In this context, plant innate resistance emerges

as a viable and cost-effective approach to managing fungal pathogens. It is generally favoured over conventional disease control methods (Liu *et al*., 2010; Javaid *et al*., 2018; Shafique *et al*., 2019). Furthermore, systemic immunity can be activated by applying biological agents to the plant surface. Plant extracts have been extensively studied for their role in inducing resistance and suppressing disease pathogenesis (Kaushik and Dhiman, 2002; Javaid *et al*., 2008; Shafique *et al*., 2018, 2019), particularly for their potential as biocontrol agents.

Among such plants, *Catharanthus roseus* (L.)—commonly known as bright eyes, Madagascar periwinkle, or rose periwinkle—is renowned for its potent antifungal (Carew and Patterson, 1970; Zahari *et al*., 2018) and antibacterial properties (Jaleel *et al*., 2007; Balaabirami and Patharajan, 2012; Rajashekara *et al*., 2022). *Catharanthus roseus* is highly valued in traditional and modern medicine for its alkaloid content, which has potent anticancer and antidiabetic properties. The plant contains over 130 alkaloids, but the most significant bioactive compounds are vincristine and vinblastine. Kumari and Gupta (2013) reported the antagonistic effects of ethanolic leaf extracts of *C. roseus* against *Fusarium moniliforme*. Similarly, Rajashekara *et al*. (2022) characterized methanolic leaf extracts (CrPLE) using phytochemical estimation, Thin-Layer Chromatography (TLC), and High-Performance Liquid Chromatography (HPLC), revealing notable antimicrobial activity of CrPLE metabolites.

Plant infections—caused by viruses or fungi—trigger the production of pathogenesis-related (PR) proteins, which are regulated by various phytohormones. Among them, ethylene plays a vital role in plant defense, as its production increases in response to wounds, flooding, cold, nutrient stress, and pathogen attacks (Lin *et al*., 2009; Iqbal *et al*., 2013). Pathogen invasion accelerates ethylene synthesis, activating downstream signaling pathways and inducing the expression of PR genes (Kitajima and Sato, 1999).

Local spinach cultivars remain underexplored in terms of their defense responses and potential resistance induction by natural plant extracts. Furthermore, limited studies have connected the biochemical defense markers (e.g., ethylene signaling and PR protein induction) with disease suppression in field-relevant pathogens affecting spinach in Asia. There remains a significant gap in the literature specific to Pakistan regarding the use of *C. roseus* extracts for enhancing systemic resistance in spinach, particularly under stress from *Fusarium equiseti*.

Given the increasing impact of *Fusarium* infections on spinach crops, it is essential to explore the genetic and biochemical mechanisms underlying plant defense. This study was designed to investigate the potential of *C. roseus* leaf extracts in inducing resistance in spinach against *Fusarium equiseti*. Understanding this interaction not only contributes to the biological control of spinach diseases but also aligns with sustainable agricultural practices by reducing reliance on synthetic fungicides.

MATERIALS AND METHODS

Selection of Fungal Pathogen: In a previous report by Shafique *et al*. (2022), a field survey was conducted in the fields of the Institute of Agricultural Sciences (IAGS), University of the Punjab, Lahore, at Depalpur and Okara, during August-September 2018-2019. Infected spinach samples were collected to record disease severity. Through morphological and molecular analysis, *Fusarium equiseti var. bullatum* (Sherb) was identified as the fungal pathogen causing leaf spot disease in infected spinach samples. The pathogenicity trials, conducted using the detached leaf method and in pot trials, confirmed *Fusarium equiseti* as the most destructive pathogen in spinach. The identified pathogen was assigned the code *F. equiseti* FB-107 (Shafique *et al*., 2022) and utilized for current research work.

Antifungal Potential of Metabolites

***In vitro* Antifungal Bioassays:** *Catharanthus roseus* was used for the biocontrol of the pathogen. The plant was identified by Dr. Abdul Rehman Khan Niazi (Associate Professor, Institute of Botany, University of the Punjab, Lahore, Pakistan), assigned voucher no. LAH # 269023 and was deposited in the Virtual Herbarium, Institute of Botany, University of the Punjab, Lahore, Pakistan. The latitude of the sampling place is 31.4° N, and the longitude is 74.35° E. Plant materials (leaves and stems) were collected from various locations in Lahore. The collected samples were thoroughly washed with distilled water to remove dust and debris, and then oven-dried at a controlled temperature to eliminate moisture content. Once dried, the leaves and stems were separately ground into a fine powder using a mechanical grinder. For extraction, 100 grams of the powdered plant material from each part (leaves and stems) were soaked in 1000 mL of absolute methanol in separate containers. The mixtures were left to macerate at room temperature for 30 days with occasional shaking to facilitate extraction. After the maceration period, the extracts were filtered and stored for further analysis. The filtrates were incubated in a hot air oven at 40 °C until they became a thick paste-like material to obtain crude methanolic extracts of *C. roseus* (20 g). To make the stock solution (10%), 5 mL of DMSO (Dimethyl sulfoxide) was added to dissolve the residues, and subsequently, the required amount of distilled water was added. Eight concentrations were created using residues ranging from 0.5% to 4.0% obtained from the stock solution. The control treatment chosen for comparing antifungal activity results was a 0% concentration. Subsequently, 1 mL of spore suspension

(5×10^5 spores mL^{-1}) was inoculated in each flask, applied via micropipette in aseptic conditions, and incubated at 28±2 °C for one week. The amount of fungal biomass was measured on filter paper that was previously weighed and then dried in an oven at 55-60 °C for 24 hours. Then, the dry weight of fungal pathogens was noted. The impact of different proportions of methanolic leaf and stem extracts from *C. roseus* on the pathogen was assessed by determining the percentage of inhibition in fungal biomass (Shafique *et al*., 2020).

$$\text{Growth increase/decrease (\%)} = \frac{\text{Growth in control - Growth in treatment}}{\text{Growth in control}} \times 100$$

Percentage inhibition constants were calculated using regression analysis based on the reduction in fungal biomass caused by various concentrations of metabolite extracts from different regions of *C. roseus.*

***In vivo* Antifungal Bioassays:** To evaluate the in vivo antifungal efficacy of the plant extract against *Fusarium equiseti*, spinach plants were cultivated in pots under controlled greenhouse conditions (28 ± 2 °C and 70% relative humidity). A total of 27 pots were prepared, and 3–5 spinach seeds were sown in each. At 20 days post-sowing, seedlings were thinned to one plant per pot. The plants were then treated with 5 mL of *Catharanthus roseus* leaf methanolic extract at concentrations of 1.0%, 2.0%, 3.0%, 4.0%, 5.0%, 6.0%, 7.0%, and 8.0%, following the methodology of Tice *et al*. (2000), which justifies the use of higher concentrations for in vivo bioassays. Control pots received only distilled water (0% extract).

Two days after extract application, each plant was inoculated with 3 mL of a *F. equiseti* spore suspension (5×10^5 spores mL^{-1}). To maintain humidity, plants were covered with polythene bags and watered as needed with tap water. After a one-week incubation period, disease symptoms were assessed, and the percentage of disease control was calculated. The experiment followed a completely randomized design (CRD) with three replicates per treatment and was repeated the following year to validate the results.

Stress Gene Activation: The analysis of gene expression was performed using the RT-PCR technique. Fresh leaves of *C. roseus* were used to extract RNA using the RiboEx" of "biomol" (Geneall) kit to analyze gene expression. The cDNA was prepared from the extracted RNA by a commercially available first-strand cDNA kit (Enzymomics). In the reaction, a mixture of cDNA was used as a template. RT-PCR was performed on target genes *ETR1*, *ETR2*, and *ESR1* using selected primers, with actin as the internal control housekeeping gene. The Primers were designed based on the GenBank database information. The specificity of primers was checked by running a PCR with plant DNA (extracted by the CTAB method) as a template, and the primers' temperature was optimized. The detail of primers is given in Table 1. The sequence of primers used to study the expression of antifungal genes in plants was: *ETR1* (F) 5′-GCT CAA ACA CAG TCT TTA GCG AC-3′, *ETR1* (R) 5′- ATC ACA CTA AAC CTC GCA CCA G- 3′, *ETR2* (F) 5′-GGT TTC GGT TTA CGG TTG ATG C- 3′, *ETR2* (R) 5′-CTG TTC CAT GGA CTG ATA TGG AC-3′, *ESR1* (F) 5′- CTA TAG GCG ATG AGA AAC GTC TG-3′, *ESR1* (R) 5′- GTG ATT TGG CTG CAA GAC GTA GC-3′, *ACT1* (F) 5′- GCT ATC CAA GCT GTC CTC TC-3′ and *ACT1* (R) 5′- GAC AGG TTC TCC TTG ATG TC-3′. The PCR cycle conditions are: 1 cycle of initial denaturation at 95 °C for 4 min., 36 cycles of denaturation at 95 °C for 1 min., annealing at 40-55 °C for 1 min., elongation at 72 °C for 1.5 min., 1 cycle of final extension at 72 °C for 15 min., and 1 cycle of hold at 4 °C for 60 min. Agarose gel (1% w/v) electrophoresis was used to determine the products of RT-PCR due to unequal amplification of the genes.

Table 1: Details of primers and antifungal genes used to study the expression of genes in plants.

Sr no.	Primers	Primers Sequences	T_m
1	*ETR1* (F)	5'-GCT CAA ACA CAG TCT TTA GCG AC-3'	57 ºC
	ETR1 (R)	5'- ATC ACA CTA AAC CTC GCA CCA G- 3'	
2	*ETR2* (F)	5'-GGT TTC GGT TTA CGG TTG ATG C- 3'	57 ºC
	ETR2 (R)	5'-CTG TTC CAT GGA CTG ATA TGG AC-3'	
3	*ESR1* (F)	5'- CTA TAG GCG ATG AGA AAC GTC TG-3'	58 ºC
	ESR1 (R)	5'- GTG ATT TGG CTG CAA GAC GTA GC-3'	
4	*ACT1* (F)	5'- GCT ATC CAA GCT GTC CTC TC-3'	55 ºC
	ACT1 (R)	5'-GAC AGG TTC TCC TTG ATG TC-3'	

Statistical Analysis: The *in vitro* experiments were conducted in six replicates, while the pot trials were performed in triplicate. Data from the pot trials were analyzed using Statistix 8.1 software. All data underwent analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) to identify significant

differences among treatments (Steel *et al*., 1996). Kinetic constants were calculated to determine the concentration at which biomass was reduced by 50%. Expression levels from RT-PCR bands were quantified using ImageJ software.

RESULTS

***In vitro* Evaluation of Metabolite Potential of *Catharanthus roseus*:** The influence of different levels of concentrations of leaf and stem extracts of *C. roseus* (with maximum antifungal potential) was assessed for their influence on *F. equiseti* growth in laboratory trials.

Antifungal Evaluation of *Catharanthus roseus* Leaf Extract: The increase in dry biomass production of *F. equiseti* was monitored when exposed to the methanolic leaf extract of *C. roseus.* A nonlinear relationship was observed between fungal biomass and concentration, with an R-squared value of 0.991. The findings showed that every concentration of leaf extract was successful in causing a substantial reduction in the biomass production of *F. equiseti.* The growth of *F. equiseti* was inhibited by a 0.5% extract concentration, resulting in approximately 20% less fungal biomass. The highest concentration (4%) resulted in 85-95% suppression in *F. equiseti* biomass (Fig.1).

Antifungal Analysis of *Catharanthus roseus* Stem Extract: The assessment of the effectiveness of *C. roseus* stem extracts against *F. equiseti* showed a significant decrease in *F. equiseti* biomass production with increasing extract concentration. Nevertheless, it was found that the antifungal effectiveness of stem extracts was lower compared to that of leaf extracts. The relationship between concentrations and biomass production was nonlinear, showing an R^2 value of 0.9789. The findings implied that a 0.5% concentration was relatively ineffective, with only a 15% inhibition in the biomass of the test fungus. Concentrations ranging from 3-4% resulted in the most significant decrease in *F. equiseti* biomass production, reaching around 75-90% reduction (Fig. 2).

Comparative Analysis of the Antifungal Efficacy of *Catharanthus roseus* Stem and Leaf Extract: The antifungal activity of plant leaf and stem extracts against *Fusarium equiseti* was evaluated through a clustered heatmap analysis (Fig. 3), which illustrated fungal biomass levels at extract concentrations ranging from 0% to 4%. The results showed a clear concentration-dependent reduction in fungal biomass for both extracts. At lower concentrations (0–1.5%), fungal biomass remained high, and differences between leaf and stem treatments were minimal, indicating limited antifungal activity. However, beginning at 2%, the leaf extract exhibited a more rapid and consistent decrease in fungal biomass compared to the stem extract. This trend was evident in the heatmap through a faster transition from red to blue hues in the leaf extract column, reflecting stronger inhibition. At concentrations of 3% and 4%, the leaf extract reduced fungal biomass to near-zero levels, while the stem extract, although inhibitory, showed higher residual biomass. Hierarchical clustering further supported these observations by grouping higher concentrations (especially 3.5% and 4%) into distinct clusters associated with low fungal biomass, particularly in the leaf extract samples. The stem extract treatments, while also grouped by concentration, exhibited greater variability and less potent inhibition. Overall, the findings demonstrated that both plant parts possess antifungal properties, but the leaf extract was significantly more effective. Therefore, the leaf extract exhibited higher potential as a natural source of antifungal agents that could be used as a biocontrol agent against the *Fusarium equiseti* pathogen in Fig. 3.

Validation of Kinetic Constant of Pathogen Growth Inhibition: The methanolic extracts from the leaves and stems of *C. roseus* exhibited great potency in combating *F. equiseti*. Regression analysis was performed to investigate the relationship between fungal biomass and concentrations of leaf and stem extracts. The specific concentration was determined using the regression equation, which decreased fungal biomass up to 50% compared to the control. The determined Ki values for the pathogen are shown in Table 1. The results for the kinetics constants from treatments using leaf and stem extracts were 14.84 and 16.84. Nonetheless, the leaf extract fraction exhibited a lower value compared to the stem extract fraction, indicating that the leaf fractions were the most efficient at inhibiting the growth of fungal pathogens. A comparison of Ki values revealed that stem extracts showed lower efficacy in inhibiting the growth of *F. equiseti* (Table 1).

In Vivo Evaluation of the Resistance Potential of *Catharanthus roseus* Methanolic Leaf Extract Against *Fusarium equiseti* Over Two Cropping Seasons: Field bioassays were conducted to assess the antifungal defense potential *of Catharanthus roseus*, specifically evaluating the efficacy of its methanolic leaf extract against *Fusarium equiseti*, a known fungal pathogen of *Spinacia oleracea*. The results demonstrated a concentration-dependent increase in disease control across both growing seasons (Table 2). Even at the lowest concentration (1%), the extract achieved 10% and 12% disease control in Seasons 1 and 2, respectively, indicating measurable baseline activity. Subsequent increases in concentration led to significant improvements in disease suppression. At concentrations of 2–4%, disease control increased steadily from 22% to 48% in Season 1 and from 24% to 50% in Season 2, representing a notable improvement of 13% to 41% over

the baseline of 1%. Higher concentrations (5–8%) resulted in statistically significant levels of protection, with control values ranging from 60% to 95% in Season 1 and 62% to 98% in Season 2. The highest tested concentration (8%) provided the maximum level of protection, with disease control ranging from 95% to 98%, effectively neutralizing the pathogen's impact on treated plants. These findings confirm the strong antifungal efficacy of the methanolic leaf extract of *C. roseus*, particularly at higher concentrations, and support its potential use as a biological control agent for managing *F. equiseti*-induced diseases in spinach.

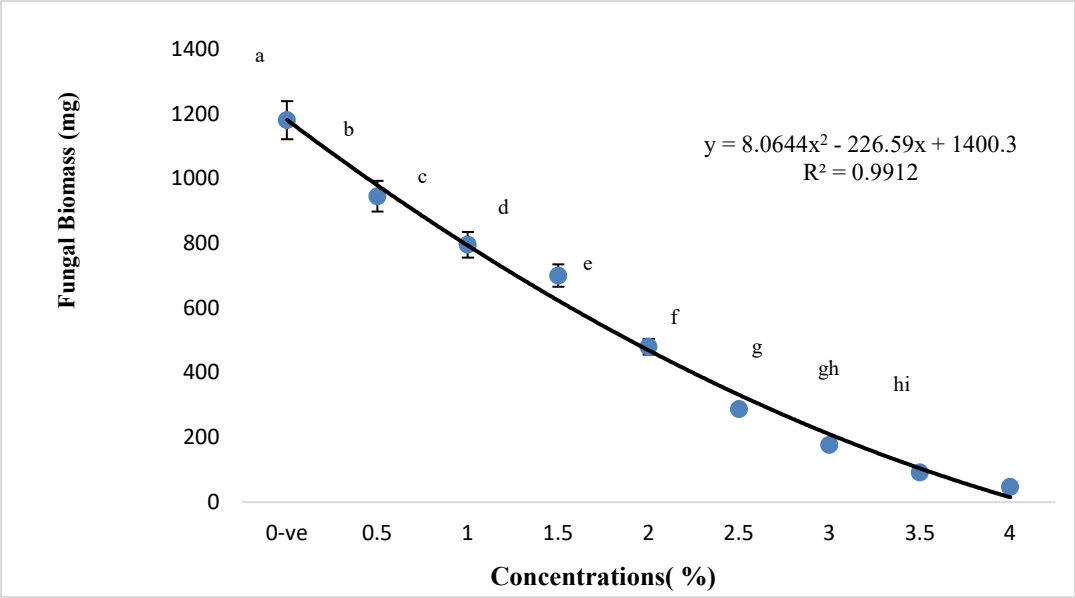

Fig. 1: Effect of various concentrations of *Catharanthus roseus* leaf extract on the biomass production of *Fusarium equiseti*. Vertical bars show the standard errors of the mean from six replicate samples. Values with different letters show a significant difference by ANOVA, as determined by Statistix 8.1 software at $p \leq 0.05$.

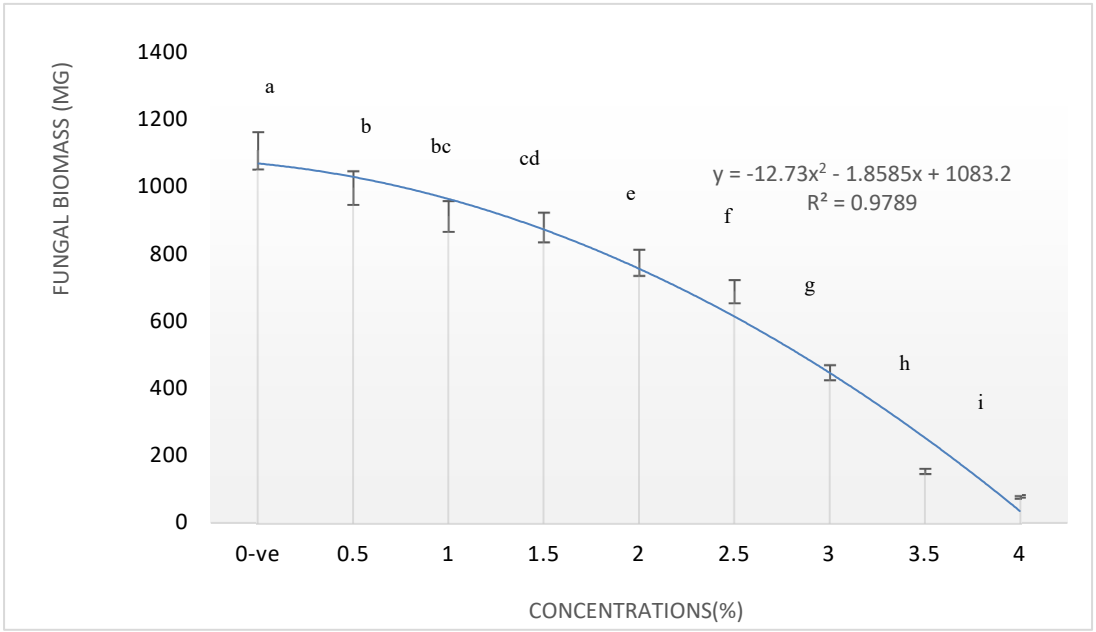

Fig. 2: Effect of various concentrations of *Catharanthus roseus* stem extract on the biomass production of *Fusarium equiseti*. Vertical bars show the standard errors of the mean from 6 six replicate samples. Values with different letters show a significant difference by ANOVA, as determined by Statistix 8.1 software at $p \leq 0.05$.

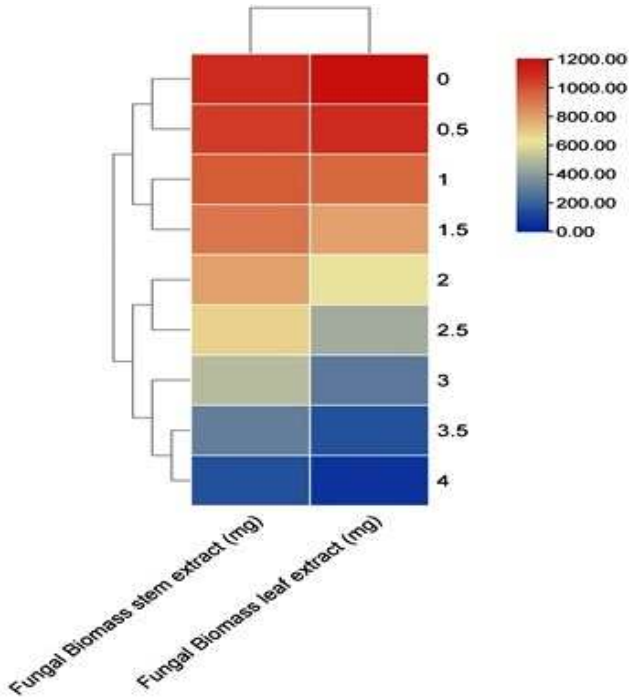

Fig. 3: Comparative analysis of the antifungal efficacy of *Catharanthus roseus* Stem and Leaf Extract through Heatmap visualization.

Table 1: Kinetic constant for fungal biomass inhibition by *Catharanthus roseus* leaf and stem methanolic extracts.

Species	Fraction	Ki	SE
F. equiseti	Stem	16.84	±0.19
	Leaf	14.84	±0.29

Table 2: Defensive potential of methanolic leaf extract of *Catharanthus roseus* for percentage disease control of *Spinacia oleraceae* over two cropping seasons.

Concentration of methanolic leaf extract of *C. roseus* (%)	Season 1	Season 2
	Disease Control of *S. oleraceae* (%)	Disease Control of *S. oleraceae* (%)
1	10 ± 0.92 a	12 ± 2.11 a
2	22 ± 1.04 b	24 ± 1.05 b
3	35 ± 0.65 c	34 ± 0.86 c
4	48 ± 1.16 d	50 ± 1.17 d
5	60 ± 1.22 e	62 ± 0.74 e
6	72 ± 2.0 f	74 ± 1.63 f
7	85 ± 0.85 g	87 ± 2.09 g
8	95 ± 0.90 h	98 ± 1.47 h

Values are presented as mean ± SE. Means with different letters in a column indicate a significant difference ($p \leq 0.05$).

Comparative Analysis of the Antifungal Efficacy of *Catharanthus roseus* over Two Cropping Seasons: Heatmap analysis (Fig. 4) provided a comprehensive graphical display of the concentration-dependent antifungal effect of *C. roseus* methanolic leaf extract on *F. equiseti* over two cropping seasons. At the lowest concentration (1%), the levels of disease control were low, with values of 12% in Season 2 and 10% in Season 1, as indicated by the dark blue colour on the heatmap, which denoted a low level of antifungal efficacy. With rising concentration up to 2 and 3%, a progressive improvement in disease suppression was noted, with the colour scale harmonized on bluish-grey, and disease control increased to 22-35% in the first season and 24-34% in the second season. At 4%, yellow colourations appeared, indicating an intermediate period of modest antifungal performance, with control ratios of 48% and 50% in Seasons 1 and 2, respectively. Specifically, the most significant disease control was observed at 5 to 8%, as represented by deep orange to red colors in the heat map. Disease control rates for these treatments ranged between 60-95% in Season 1 and 62-98% in Season 2, with an 8% concentration almost completely suppressing the disease. Red colour saturation and the uniform agglomeration of highly concentrated programs certify their excellent and repeatable antifungal performance.

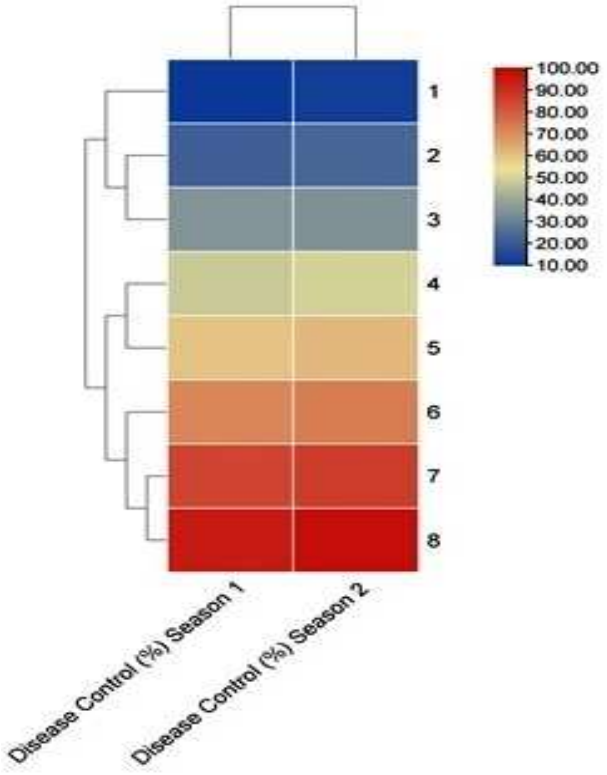

Fig. 4: Heat map visualization for the Comparative analysis of the antifungal efficacy of *Catharanthus roseus* in two seasons

Expression Analysis of Stress Genes: Real-time PCR was conducted to validate the activation of stress genes in the host plant that demonstrated immunity to the disease-causing fungus. All three stress genes were amplified and verified through gel analysis to confirm their presence. The top thickness of the band indicated the greatest expression of the transcript, which is the *ETR2* gene, while *ESR1* showed lower expression. *ETR1* had the least intense band and therefore had the minimum gene quantity displayed (Fig. 5). Decreased disease susceptibility to the soilborne pathogen *F. equiseti* in *C. roseus* was linked to a decrease in the expression of the ethylene receptor *ETR1* gene, thus validating the plant's defense response. The bands underwent analysis through the ImageJ software, resulting in the formation of bands (Fig. 6). The existence of the three genes suggests that *C. roseus* could potentially regulate the entry of pathogenic fungi. Either the *ETR2* gene alone or all three stress genes could collaborate to hinder the growth of the pathogenic fungus's biomass.

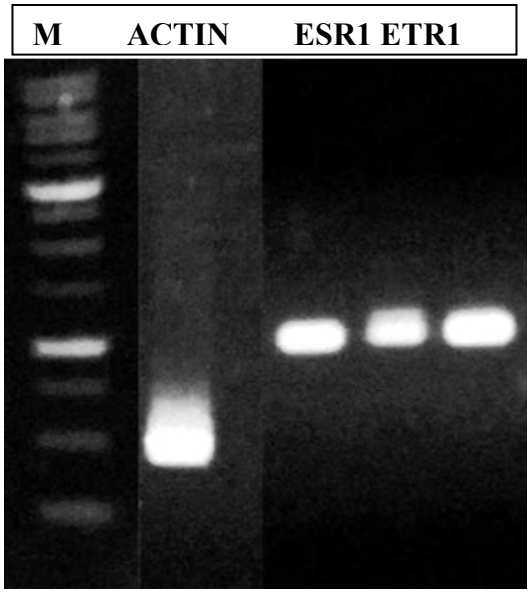

Fig 5: Bands showing expressed genes from *Catharanthus rose*us cDNA on Agarose gel electrophoresis.

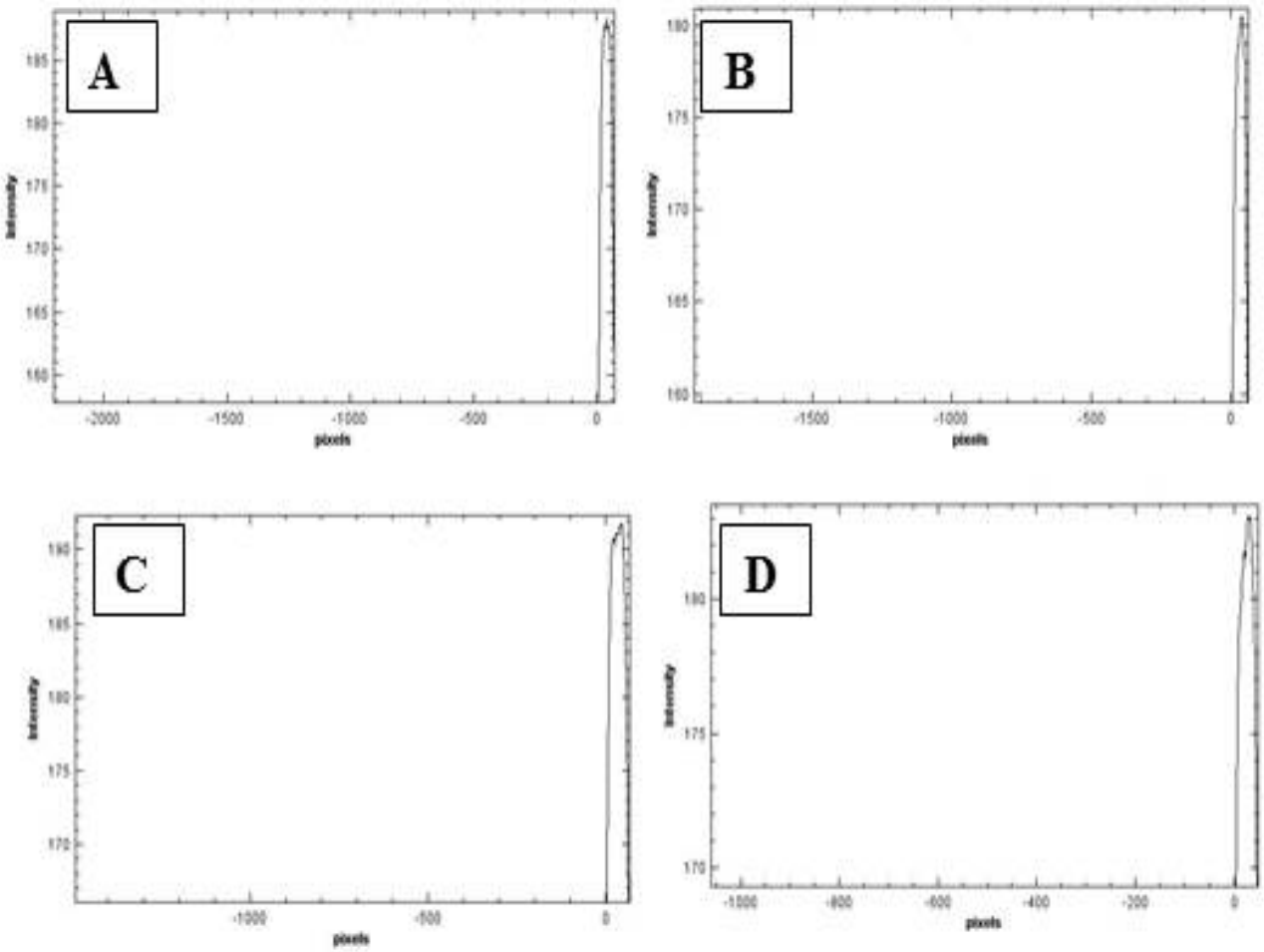

Fig. 6: Genes' expression profile of Bands' intensity-based graph of the genes of *Catharanthus roseus.* (A): ACTIN, (B): *ESR1*, (C): *ETR1* and (D): *ETR2*.

DISCUSSION

C. roseus triggered immunity in spinach plants against fungal diseases, supporting the research by Balaabirami and Patharajan (2012), who used *C. roseus* for plant disease control against fungi like *Aspergillus fumigatus*, *Candida albicans, Pythium aphanidermatum, Penicillium chrysogenum,* and *Aspergillus niger*. This research focuses on developing horizontal resistance in spinach plants as a strategy for effective management of fungal pathogens that affect spinach crops. The antifungal potential of methanolic extracts from the leaves and stems of *C. roseus* was tested against the isolated pathogen *F. equiseti* in this study. The leaf extracts were more effective in inhibiting the pathogen's biomass compared to the stem extract. The differences in the antifungal effectiveness of extracts might be due to the existence of various antifungal compounds in different regions of the plant species being tested. Previous research also confirms that the organic compounds found in the leaves of *C. roseus* are effective in controlling the pathogen due to their antifungal properties (Parekh and Chanda, 2007). In another study, Shafique *et al*. (2015) investigated the control of Fusarium wilt in chili plants using methanolic extracts from the fruit, bark, and leaves of *Eucalyptus citriodora* (Hook). The findings revealed that the leaf extracts were highly effective against the pathogens, resulting in a significant reduction in fungal biomass, up to 98%. Currently, the antifungal tests exhibited substantial inhibition of fungal growth at every concentration of extract used, but the highest concentrations of *C. roseus* extract suppressed biomass production by 90 to 95%. In a previous study, Kumari and Gupta (2013) found that *C. roseus* leaf and stem extracts (Ethanol, Acetone, and Aqueous extract) exhibited antifungal properties against important fungal strains (*Candida albicans, Aspergillus fumigatus, Aspergillus niger, Fusarium moniliforme*). Organic extract from the leaves of *C. roseus* was more effective in inhibiting fungal pathogens compared to the extracts from the stems when used at higher concentrations. *Catharanthus roseus* L. is used as a prophylactic agent against certain human pathogens by previous researchers (Gomaa *et al*., 2019). It is an important plant for the development of novel antimicrobial drugs, as it contains active compounds responsible for its bioactivity. Additionally, it provides insight into the genetic regulation of alkaloids present in the extracts, which serve as potential antimicrobial agents. A similar pattern of defense response enhancement in *C. roseus* was observed in pot trials when spinach plants were treated with various concentrations of *C. roseus* extracts before pathogen inoculation. Another study conducted by Shafique *et al*. (2019) further affirms these findings, showing that using *Eucalyptus citriodora* improved the defense mechanism in *Capsicum annuum L*. and decreased Fusarium Wilt infection caused *by Fusarium oxysporum*. The basic mechanism of induced resistance involves the heightened activation of defense genes to impede pathogen entry, or the reduced activity of certain genes to lower the host plant's vulnerability to pathogens. In this study, the detection of defense-related genes in *C. roseus* was conducted using RT-PCR. Reverse transcriptase PCR has emerged as an effective method for examining gene expression because of its precision and specificity (Bustin, 2002; Le *et al*., 2024). The findings indicated that the *ETR2* gene showed the highest level of expression. The increased levels of *ETR2* and *ESR1* genes and decreased levels of the *ETR1* gene indicate that *C. roseus* is capable of suppressing fungal pathogens. These genes may work together or independently to inhibit the growth of pathogenic biomass. Scientists highlighted the significant contribution of *ETR1*, *ETR2*, and *ESR1* in immune responses to pathogens (Fluhr, 1998; Hoffman *et al*., 1999; Tjamos *et al*., 2005; Pantelides *et al*., 2013). According to the literature, it is clear that Fusarium takes control of *ETR1*-mediated ethylene signalling to advance disease progression in plants (Pantelides *et al*., 2013). In the current study, the reduced expression of *ETR1* may be attributed to the influence of *Catharanthus roseus*, which, at higher concentrations, suppressed pathogen invasion in the host plants, thereby reducing disease incidence.

Conclusion: The study concludes that methanolic extracts of the leaves and stems of *C. roseus* demonstrated strong antifungal activity against *F. equiseti.* The upregulation of stress genes, viz., *ETR2* and *ESR1,* as well as reduced expression of *ETR1* in *C. roseus,* is attributed to reducing pathogen penetration, henceforth reducing the disease incidence.

Acknowledgments: The fungal biotechnology research laboratory at the Department of Plant Pathology, Faculty of Agricultural Sciences, University of the Punjab, Lahore, provided the facilities needed for this investigation.

REFERENCES

Balaabirami, S. and S. Patharajan (2012). *In-vitro* antimicrobial & antifungal activity of *Catharanthus roseus* leaves extract against important pathogenic organisms. Acta Sci. 4: 1–4.

Beckman, C.H. (1987). The nature of wilt diseases of plants. American Phytopathological Society Press, St Paul, MN, 21 (3): 175–183.

Bustin, S.A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. 29 (1): 23–39.

Carew, D.P. and B.D. Patterson (1970). The effect of antibiotics on the growth of *Catharanthus roseus* tissue cultures. Lloydia. 33: 275–277.

Correll, J.C., T.E. Morelock, M.C. Black, S.T. Koike, L.P. Brandenberger and F.J. Dainello (1994). Economically important diseases of spinach. Pl. Dis. 78: 653–660.

FAOSTA. (2015). FAOSATAT online database.

Fluhr, R. (1998). Ethylene perception: from two-component signal transducers to gene induction. 3 (4): 141–146. https://DOI.org/10.1016/S1360-1385(98)01226-6.

Gomaa, S., M. Yahayu, M. Nurjayadi, D.D. Joe and E.E. Hesham (2019). Antimicrobial Compounds from *Catharanthus roseus*-A review. Int. J. Sci. Tech. Res. 8: 113–121. https://mail.ijstr.org/final-print/oct2019/Antimicrobial-Compounds-From-Catharanthus-Roseus-A-Review.pdf

Hoffman, T., J.S. Schmid, X. Zheng and Bent (1999). Isolation of Ethylene-Insensitive Soybean Mutants That Are Altered in Pathogen Susceptibility and Gene-for-Gene Disease Resistance. Am. Soc. Pl. Physiol. 119(3):935–950. DOI: 10.1104/pp.119.3.935.

Hussain, N., H. Ali, G. Mustafa, M.S. Khan, B. Ali, S. Ameer, S. Zamir, R. Iqbal, B. Ali and M.N. Khan (2023). *In Vitro* Plant Regeneration from Petioles of Spinach (*Spinacia oleracea* L.). ACS Agri. Sci. Technol. 4 (1): 57–62. DOI: 10.1021/acsagscitech.3c00283.

Iqbal, N., A. Trivellini, A. Masood, A. Ferrante and N.A. Khan (2013). Current understanding on ethylene signaling in plants: the influence of nutrient availability. Pl. Physiol. Biochem. 73: 128–138. https://DOI.org/10.1016/j.plaphy.2013.09.011.

Jaleel, C.A., P. Manivannan and B. Sankar (2007). Induction of drought stress tolerance by ketoconazole in *Catharanthus roseus* is mediated by enhanced antioxidant potentials and secondary metabolite accumulation. Coll. Surf. B. Biointerfaces. 60: 201–206. DOI: 10.1016/j.colsurfb.2007.06.010.

Javaid, A., G. Shahzad, N. Akhtar and D. Ahmed (2018). First report of Alternaria leaf spots of broccoli in Pakistan and management of Van Loonpathogen by leaf extract of *Syzigium cumini*. Pakistan J. Bot. 5 (4): 1607–1614.

Javaid, A., S. Shafique, S. Shafique and T. Riaz (2008). Effect of rice extracts and residue incorporation on *Parthenium hysterophorus* management. Allelopath. J. 22: 353–362.

Kaushik, P. and A.K. Dhiman (2002). Medicinal Plants & Raw Drugs of India. P. XII+623. http://www.vedicbooks.net/medicinal-plants-and-raw-drugs-of-india-p-13886.html.

Kitajima, S. and F. Sato (1999). Plant Pathogenesis-Related Proteins: Molecular Mechanisms of Gene Expression and Protein Function. J. Biochem. 125: 1–8. DOI: 10.1093/oxford journals.jbchem.a022244.

Kumari, K. and S. Gupta (2013). Phytopotential of *Catharanthus roseus* L. (G.) Don Var Rosea & Alba. Int. J. Res. Pure App. Microbiol. 3 (3): 77–82.

Larsson, M. and B. Gerhardson (1992). Disease progression and yield losses from root diseases caused by soilborne pathogens of spinach. Phytopathol. 82: 403–406. DOI: 10.1094/Phyto-82-403.

Le, A.T., M. Shakiba, I. Ardekani and W.H. Abdulla (2024). Optimizing Plant Disease Classification with Hybrid Convolutional Neural Network–Recurrent Neural Network and Liquid Time-Constant Network. Appl. Sci. 14: 9118. https://DOI.org/10.3390/app14199118.

Lin, Z., S. Zhong and D. Grierson (2009). Recent advances in ethylene research. J. Exp. Bot. 60: 3311–3336. DOI: 10.1093/jxb/erp204. Epub 2009 Jun 30.

Liu, B., L. Stein, K. Cochran, L.J.D. Toit, C. Feng and J.C. Correll (2021). Three New Fungal Leaf Spot Diseases of Spinach in the United States and the Evaluation of Fungicide Efficacy for Disease Management. Pl. Dis. 105 (2): DOI.org/10.1094/PDIS-04-20-0918-RE.

Liu, F., Y.Y. Zhuge, C.Y. Yang, S.X. Jin, J. Chen, H. Li and G.H. Dal (2010). Control effect of some plant extracts against powdery mildew (*Sphaerotheca fuligunea)* and their stability study. Eur. J. Horticul. Sci. 75 (4): 147–152. https://DOI.org/10.1079/ejhs.2010/1725667.

Miano, T.F. (2016). Nutritional Value of *Spinacia oleraecea* Spinach-An Overview. Int. J. Life Sci. Rev. 2 (12): 172–174. DOI: 10.13040/IJPSR.0975-8232.IJLSR.2(12).172-74.

Pantelides, I.S., S.E. Tjamo, S. Pappa, M. Kargakis and E.J. Paplomatas (2013). The ethylene receptor ETR1 is required for *Fusarium oxysporum* pathogenicity. Pl. Pathol. 62: 1302–1309. https://doi.org/10.1111/ppa.12042.

Parekh, J. and S. Chanda (2007). *In vitro* antimicrobial activity & phytochemical analysis of some Indian medicinal plants. Tur. J. Biol. 31: 53–58. DOI: https://journals.tubitak.gov. tr/biology/ vol31/iss1/9.

Patricia, O. (2014). Proximate composition & nutritive value of leafy vegetables consumed in northern côte d'ivoire. Eur. Sci. J. 10 (6): 1857. DOI: 10.19044/ESJ.2014.V10N6P.

Rajashekara, S., D. Reena and M.V. Mainavi (2022). Biological isolation and characterization of *Catharanthus roseus* (L.) G. Don methanolic leaves extracts and their assessment for antimicrobial, cytotoxic, and apoptotic activities. BMC Comp. Med. Ther. 22: 328. https://DOI.org/10.1186/s12906-022-03810-y.

Rashid, M., Z. Yousaf, M.N. Ullah, M. Munawar, N. Riaz, A. Younas, A. Aftab and B. Shamsheer (2020). Genetic variability assessment of worldwide spinach accessions by agro-morphological traits. J. Taibah Uni. Sci. 14 (1): 1637–1650. DOI: 10.1080/16583655.2020.1853920.

Roberts, J.L. and R. Moreaua (2016). Functional properties of spinach (*Spinacia oleracea* L.) phytochemicals and bioactives. Food Funct. 7 (8): 3337–3353. DOI: 10.1039/c6fo00051g.

Shafique, S., A. Mubarak, S. Shafique, A. Hussain, A. Javed and R. Rafique (2022). Isolation and identification of novel leaf spot pathogen *Fusarium equiseti* from *Spinacia oleraceae*. Pakistan J. Bot. 54 (3): 1139–1145. DOI: 10.30848/PJB2022-3(21).

Shafique, S., S. Shafique and S. Sahar (2020). *Thymus serpyllum* – A Novel Biocontrol Agent against Leaf Spot Pathogens of *Solanum melongena.* Pakistan J. Weed Sci. Res. 26 (4): 469–479.

Shafique, S., A. Muhammad and S. Shafique (2015). Management of *Fusarium oxysporum F. Sp*. Capsici by leaf extract of *Euclyptus citriodora*. Pakistan J. Bot. 47 (3): 1177–1182.

Shafique, S., A. Naseer, N. Akhtar and S. Shafique (2018). Biological control of leaf spot causing fungal pathogens in red-edge dracaena and sow thistle. Pakistan J. Agri. Sci. 55: 667–674. DOI: 10.21162/PAKJAS/18.5019.

Shafique, S., S. Shafique, M. Zameer and M. Asif (2019). Plant defence system activated in chili plants by using extracts from *Eucalyptus citriodora.* Biocont. Sci. 24: 137–144. DOI: 10.4265/bio.24.137.

Shova, N.J., S. Shamsi and M.A. Bashar (2020). Prevalence and Pathogenic Potentiality of Fungi Associated with Leaf Spot of *Basella alba* and *B. rubra.* Dhaka Uni. J. Biol. Sci. 29 (2): 183–189. DOI: 10.3329/dujbs.v29i2.48761.

Siddique, W., M.U. Hasan, M.S. Shah, M.M. Ali, F. Hayat and A. Mehmood (2021). Impact of blanching and packaging materials on postharvest quality and storability of fresh spinach. J. Horti. Sci. Technol., 4 (1), 7–12. https://doi.org/10.46653/jhst2141007.

Singh, B., S. Tripathi, S. Tyagi, A. Tripathi, P. Dubey and S. Kumar (2025). Phytostimulation and Induction of Systemic Resistance by Plant Growth Promoting Fungi. In: Plant Microbiome and Biological Control: Emerging Trends and Applications (pp. 221–238). Springer.

Steel, R. G. D., J. H. Torrie and D. A. Dickey (1996). Principles and Procedures of Statistics: A Biometrical Approach. 3rd Edition McGraw-Hill Book Co., Inc, New York, USA.

Sunil, P. and L.P. Yadav (2020). Isolation of fungal pathogens from seeds of selected vegetables and study of their impact on germination and seedling growth. Ind. J. Sci. Res. 11 (1): 139–145. DOI:10.32606/IJSR.V11.I1.00022.

Tice, R.R., E. Agurell, D. Anderson and B. Burlinson (2000). Single-cell gel/Comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. Env. Mol. Mutagen. 35 (3): 206–21. DOI: 10.1002/(sici)1098-2280(2000)35:3<206::aid-em8>3.0.co;2-j.

Tjamos, S.E., E. Flemetakis, E.J. Paplomatas and P. Katinakis (2005). Induction of Resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the Biocontrol Agent K-165 and Pathogenesis-Related Proteins Gene Expression. Mol. Plant Microbe Interact. 18 (6): 555–561. DOI: 10.1094/MPMI-18-0555. PMID: 15986925.

Wariebi, A.B. (2025). Morphological and Molecular Characterization of Fungal Species Associated with *Fusarium oxysporum f. sp. cubenese* (Foc) in Gatundu North, Kenya. Int. J. Horti. Sci. Technol. 12 (1): 281–296. DOI: 10.22059/ijhst.2024.373885.802.

Zahari, R., N. Halimoon, M.F. Ahmad and S.K. Ling (2018). Antifungal Compound Isolated from *Catharanthus roseus* L. (Pink) for Biological Control of Root Rot Rubber Diseases. Int. J. Anal. Chem. 5: 8150610. DOI: 10.1155/2018/8150610.

Zeb, A. and M. Jamil (2025). Spinach ameliorates dietary oxidized tallow-induced NAFLD by regulating inflammatory cytokines, peroxisome proliferator-activated receptors, and antioxidant systems. Phytomed. Plus. 5 (1): 100722. DOI: https://doi.org/10.1016/j.phyplu.2024.100722.