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Dynamics Of Reactive Oxygen Species And Antioxidant Enzyme Activity In Tomato Plants Infected With Fusarium Oxysporum F. Sp. Lycopersici

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Abstract:
Objective: *Tomato (Solanum lycopersicum) is one of the most widely cultivated and consumed vegetable crops globally, recognized for its high nutritional value and economic importance. However, its productivity is significantly constrained by various biotic stresses, with Fusarium wilt being one of the most destructive diseases. This vascular wilt is caused by the soil-borne fungal pathogen Fusarium oxysporum f. sp. lycopersici (FOL), which disrupts water and nutrient transport within the plant. The objective of this study was to examine the associated biochemical changes occurring in tomato plants in response to infection, in order to better understand host-pathogen interactions and the biochemical basis of disease progression.*
Scope: *The study was carried out under controlled pot culture conditions during the period of January to April 2025 at SRM College of Agricultural Sciences, Baburayanpettai, Chengalpattu District, Tamil Nadu. Ten isolates of FOL, obtained from distinct geographic and symptomatic tomato fields, were used to inoculate healthy tomato seedlings. Observations were made to assess disease development, and plant tissue samples (including leaves, stems, and vascular regions) were collected on the 40th day of post-inoculation for biochemical analysis. Key physiological and biochemical parameters were measured, such as chlorophyll content, soluble protein concentration, superoxide radical levels, oxalic acid accumulation, and the activity of defence-related enzymes like polyphenol oxidase and peroxidase. This comprehensive approach aimed to correlate pathogen virulence with the extent of host metabolic disruption.*
Result: *Among the ten isolates tested, isolate F7 was identified as the most virulent, inducing early and severe disease symptoms characterized by yellowing of leaves, stunted growth, and vascular browning as early as the 20th day of post-inoculation. Plants infected with F7 displayed pronounced biochemical alterations. There was a significant reduction in chlorophyll content (0.30 mg/g), indicating impaired photosynthetic activity. In contrast, the levels of soluble proteins (151.03 mg/g), superoxide radicals (6.20 nmol g ⁻¹ FW), and oxalic acid (0.600 mg/g ⁻¹) were considerably elevated, reflecting heightened stress and cellular damage. Furthermore, the activity of defence-related enzymes such as polyphenol oxidase (222.00 U/g of protein) and peroxidase (58.843 U/g of leaf tissue) was notably suppressed in F7-infected plants compared to others. These findings establish a clear correlation between the virulence of FOL isolates and the intensity of host biochemical responses, offering valuable insights into the mechanisms of disease development and plant defence suppression.*
Keywords: *Fusarium wilt, Fusarium oxysporum f. sp. lycopersici, biochemical changes, antioxidants.*

INTRODUCTION

Among the vegetables, tomato is the major sources of vitamins and minerals and occupies number one position in its nutrient contribution to human diet. Tomato ranks third in production in the world. In Tamil Nadu, tomato is grown in an area of 22,433 ha, with a production of 2,82,912 tonnes and a productivity of 12,611 kg/ha. It is affected by several diseases, reflecting negatively on plant growth and the produced yield. Tomato crop is damaged by the pathogens viz., fungi, bacteria, viruses, and nematode at various stages of growth, among them wilt caused by Fusarium oxysporum f. sp lycopersici (FOL) is one of the major diseases which causes a severe yield losses (Barone and Frusciante, 2007).

Various biochemical assays were employed to evaluate the health status and disease resistance of tomato plants. These included measurements of defence-related enzymes such as peroxidase (POX) and polyphenol oxidase (PPO), which typically showed increased activity in response to pathogen attack. Chlorophyll content was analysed to assess photosynthetic performance, while total soluble protein levels were measured to gain insights into both plant growth and defence-related protein synthesis. The study aimed to investigate biochemical responses such as chlorophyll content, soluble protein, enzymatic activities, hydrogen peroxide, oxalic acid and superoxide radicals. Observed changes in these biochemical parameters reflected the aggressive nature of the F. oxysporum f. sp. lycopersici isolates.

MATERIALS AND METHODS

Collection of Fusarium Isolates from Different Tomato Growing Areas of Tamil Nadu:
Tomato plants displaying typical symptoms of Fusarium wilt were collected from major tomato-growing regions of Tamil Nadu during a field survey (August - September 2024) and used for pathogen isolation. A total of ten isolates of F. oxysporum f. sp. lycopersici were obtained from four districts in Tamil Nadu. Specifically, isolates were collected from Dharmapuri (F1, F2, F3), Coimbatore (F4, F5, F6), Krishnagiri (F7), Dindigul (F8), and Theni (F9, F10). This sampling pattern highlights the prevalence of Fusarium wilt across these regions during the study period.

Pathogenicity test:

Pot Culture Experiment:
Pot culture experiment was conducted during January – May 2025 at SRM College of Agricultural Sciences, Baburayanpettai, Chengalpattu Dist., Tamil Nadu to test the pathogenicity of ten isolates of F. oxysporum f. sp lycopersici collected from different districts.

Soil Inoculation of Pathogen
Healthy tomato seedling of PKM-1variety was planted in pots filled with sterilized soil and maintained under controlled greenhouse conditions. A sand maize medium was prepared by mixing sand and broken maize in a 19:1 ratio (1900 g of sand and 100 g of maize powder). This mixture was sterilized by autoclaving at 1.4 kg/cm^2 for two hours on consecutive days. After sterilization, the medium was inoculated with 90 mm disc (eight discs per bag) of actively growing (seven days old) F. oxysporum f. sp lycopersici cultures and incubated at room temperature (28 ± 2°C) for three weeks to allow for optimal pathogen growth. The fully colonized inoculum was then used for pathogenicity testing. The sand maize inoculum was applied to the soil in potted plants when they reached 25 days of planting and observed the Fusarium isolates (10 isolates individually) inoculated plants for the appearance of symptoms. Variation in severity of wilt symptoms observed from 20 to 40 days after inoculation with the virulent nature of Fusarium isolates.

Biochemical Analysis:
Samples exhibiting symptoms of Fusarium wilt, including leaves, stems, and vascular tissues, were collected from each Fusarium isolate inoculated tomato plants on the 40th day of inoculation. These samples were used to analyse changes of biochemicals such as chlorophyll content, soluble protein levels, activities of defence-related enzymes (polyphenol oxidase and peroxidase), and the accumulation of hydrogen peroxide (H_2O_2), oxalic acid, and superoxide radicals.

1. Estimation of Chlorophyll Content:
Tomato tissue infected with F. oxysporum f. sp. lycopersici (0.5 g) was homogenized by using 10 mL of acetone to analyse pigment content. The resulting homogenate was then centrifuged at 3000 rpm for 10 min. The absorbance of the clear supernatant was recorded at four specific wavelengths 480, 510, 645, and 663 nm using a spectrophotometer, as described by Hiscox et al. (1979). Based on these readings, the levels of chlorophyll A, chlorophyll B, total chlorophyll, the chlorophyll A/B ratio, and carotenoids were calculated using standard formulas.

Formula:

1. Chlorophyll A = (12.7 × OD at 663 - 2.69 × OD at 645) × V/1000 × W
2. Chlorophyll B= (22.9 × OD at 645 - 4.68 × OD at 663) × V/1000 × W
3. Total chlorophyll = (20.2 X OD at 645 - 8.02 x OD at 663) × V/1000 × W
4. Carotenoids = (7.6 X OD at 480 + 1.49 x DO at 510) × V/1000 × W
5. Chlorophyll A/B ratio= Chlorophyll A / Chlorophyll B

2. Estimation of hydrogen peroxide (H_2O_2):

Five mL of 0.1% trichloroacetic acid (TCA) was used to estimate hydrogen peroxide content by macerating 0.5 g of diseased tomato leaves. The homogenate was then mixed with 2 mL of 1 M potassium iodide and 0.5 mL of phosphate buffer. The mixture was left to incubate at room temperature for one hour. Following incubation, the absorbance was measured at 390 nm using a spectrophotometer, as per the method described by Wojtaszek et al. (1997).

The hydrogen peroxide content was then calculated using the formula:

$\mu mol\ H_2O_2\ g^{-1}\ FW = ([H_2O_2] \times V) / W$,

where $[H_2O_2]$ is the concentration determined from the standard curve, V is the volume of the extract (in mL), and W is the fresh weight of the sample (in g).

3. Estimation of Superoxide Radicals:

A 65 mM phosphate buffer was used to homogenize 0.1 g of infected leaf and sheath tissues in 3 mL of the buffer solution. To this homogenate, 1 mL of 3 mM naphthylamine and a drop of safranine were added, and the mixture was incubated for 30 min. Following incubation, the optical density was recorded at 530 nm using a spectrophotometer.

Preparation of Phosphate Buffer:

Sodium phosphate dibasic heptahydrate 20.214g and sodium phosphate monobasic heptahydrate 3.394 g were dissolved in distilled water to prepare 800 mL of buffer (Beauchamp et al.,1971). Superoxide was quantified with the formula:

Superoxide generation=Extinction coefficient×path length (cm)ΔA×Volume

4. Estimation Of Soluble Test

A 0.25 g infected leaves sample was macerated in 10 mL of phosphate buffer and centrifuged at 3000 rpm for 10 min. One mL of the supernatant was added to 5 mL of alkaline copper reagent and incubated at room temperature for 30 min. for colour development. Then, 0.5 mL of phenol reagent was added and measured for absorbance at 660 nm. Soluble protein content was calculated as mg $g^{-1}$of leaf tissue Lenka et al. (2018).

Preparation of alkaline copper tartrate reagent:

Solution A: 2% sodium carbonate in 0.1 N sodium hydroxide

Solution B: 0.5% copper sulphate in 1% sodium potassium tartrate

Solutions A and B were combined in the ratio 50 mL:1 mL just prior to use

*Folin Ciocalteau was diluted 1:2 ratio with water for use.

mg sugar g−1 FW=([C]×$V_{extract}$)/W

5. Estimation of Polyphenol Oxidase:

A sample of infected plant leaves weighing 0.25 g was homogenized in 5 mL of 0.2 M phosphate buffer and centrifuged at 3000 rpm for 15 min. To the phosphate buffer (2.5 mL) in a test tube, 0.3 mL of 0.1 M catechol and 0.2 mL of enzyme extract were added. The absorbance change was read at 410 nm (Thimmaiah, 2006). PPO activity was determined using the formula:

PPO activity (U/mL) = 0.001ΔA410/min

6. Estimation of Peroxidase:

In the same way, 0.25 g of infected material was homogenized in 5 mL of 0.2 M phosphate buffer and centrifuged at 3000 rpm for 15 min. In a test tube, 2.5 mL of phosphate buffer, 0.3 mL of 0.1 M hydrogen peroxide, and 0.2 mL of enzyme extract were taken. The absorbance was recorded at 410 nm (Costa et al., 2002). Peroxidase activity was estimated as:

Peroxidase activity (U/mL) = Extinction CoefficientΔA410/min × Total Reaction Volume ÷ Sample Volume

7. Oxalic Acid Estimation:

Infected leaf samples were boiled in distilled water and filtered. Ten mL of the filtrate was taken in a conical flask, and 10 mL of 4% sulphuric acid was added. The solution was heated at 60°C for 10 min. and then titrated with 0.1 N potassium permanganate (KMnO4) until a persistent pale pink colour appeared (Jayaraj et al., 2010). Oxalic acid content was calculated using the formula:

Oxalic acid= Vol. of KMnO4 used (ml) x 0.0045g

Statistical Analysis:

The pot culture and laboratory experiments were carried out using a Completely Randomized Design (CRD). Statistical analysis of the experimental data was performed using GRAPES (General R-shiny based Analysis Platform Empowered by Statistics), a web-based application designed for agricultural data analysis, following the methodology proposed by Gopinath et al. (2020).

RESULT AND DISCUSSION:

Isolation and Identification of the Pathogen:

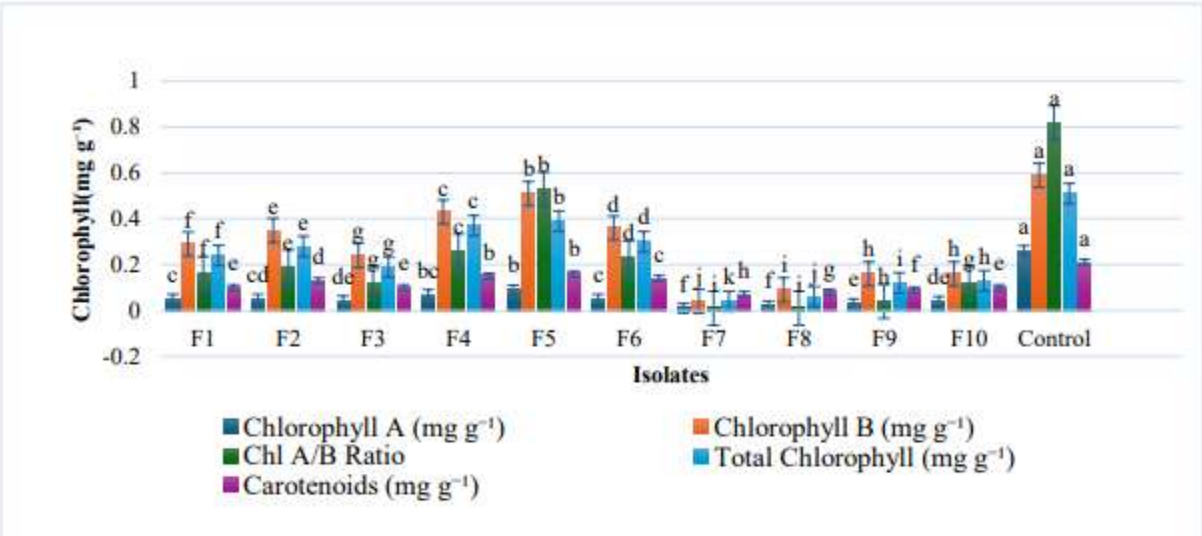
The pathogen F. oxysporum f. sp lycopersici was isolated from the diseased tomato plants collected from various places and purified in the laboratory as per the standard procedure. These ten isolates were maintained on PDA in Petri plates. The isolates of pathogen were identified as F. oxysporum f. sp lycopersici based on morphological and cultural characters described by Sehim et al. (2023). Variation among the isolates showed differences in host reaction, i.e., level of virulence and aggressiveness.

Assessment of chlorophyll content variation in tomato plants infected with F. oxysporum isolates under in vitro condition

Tomato plants inoculated with ten different Fusarium oxysporum f. sp. lycopersici isolates (F1–F10) showed varying levels of chlorophyll reduction on 40th day of post-inoculation. The lowest total chlorophyll content (0.04 mg/g) was observed in plants infected with the highly virulent F7 isolate (Fig. 1). Chlorophyll levels generally decreased with increasing isolate virulence, following the order: F7 < F8 < F9 < F10 < F3 < F1 < F2 < F6 < F4 < F5 with the percentage increase ranging from 92.16 to 23.53. Control plants maintained higher chlorophyll levels (0.51 mg/g). These significant variations indicated that chlorophyll degradation was closely associated with the pathogenicity of the FOL isolates.

Carotenoid content was also measured in the infected samples. The most virulent isolate, F7, caused a notable reduction in carotenoid content, dropping to 0.07 mg/g (fresh weight) in infected leaf tissue. In contrast, the control plants had the highest carotenoid level at 0.21 mg/g, followed by 0.15 mg/g in plants infected with the F5 isolate, which exhibited the mildest symptoms and lowest virulence.

Fig 1: Changes in chlorophyll content in tomato plants infected with F. oxysporum f. sp. lycopersici isolates

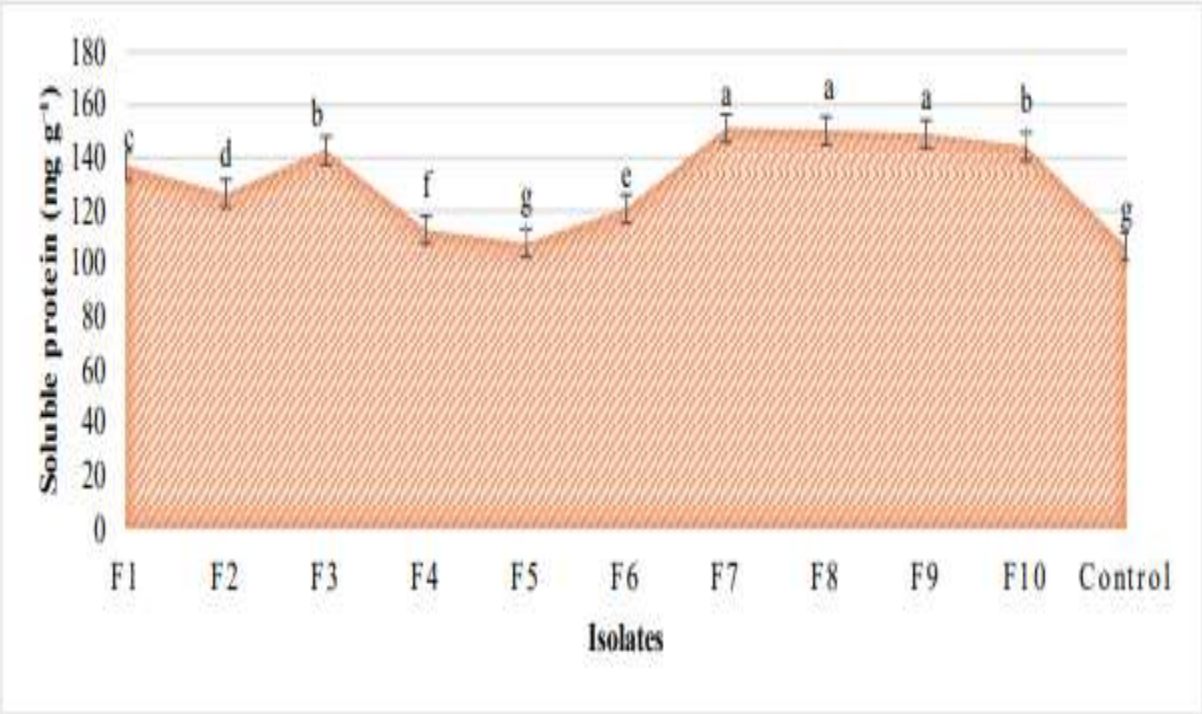


In the present study, the infected plants showed a marked reduction in total chlorophyll, particularly in isolates F7, F8, F9, and F10, compared to control plants. This reduction correlated with the intensity of FOL virulence and systemic colonization of the xylem, which ultimately impaired photosynthetic capacity Maqsood et al. (2020). Chlorophyll degradation under FOL infection can be attributed to multiple biochemical alterations, including the accumulation of hydrogen peroxide and malondialdehyde, which damage chloroplast membranes (Abass et al., 2025), triggered the synthesis of defence-related phytohormones like salicylic acid and jasmonic acid, and downregulated chlorophyll biosynthesis (Iqbal et al., 2024).

Assessment of soluble protein content in tomato plants following infection with F. oxysporum f. sp. lycopersici isolates under in vitro conditions

Soluble protein content varied significantly among tomato plants infected with different F. oxysporum f. sp. lycopersici (FOL) isolates. The highest level (151.03 mg/g) was observed in plants infected with the most virulent isolate, F7, followed closely by F8, F9, and F10. In contrast, less virulent isolates showed lower protein levels, ranging from 107.66 to 151.03 mg/g. Control plants had a protein content of 106.26 mg/g. The increasing trend in soluble protein content correlated with isolate virulence, likely due to the breakdown of inhibitory proteins that intensified disease severity.

Fig 2: Soluble protein accumulation in tomato plants in response to F. oxysporum f. sp. lycopersici infection



The findings indicated a significant decline in soluble protein concentration in FOL-infected plants compared to the healthy control, suggesting that pathogen stress leads to disruption in protein biosynthesis and/or enhanced proteolytic activity. Similar reductions in protein content in tomato plants under Fusarium stress were reported by Pandey et al. (2024), who observed a marked decline in total soluble proteins, coincided with symptom progression. The loss in protein content has been attributed to oxidative stress-induced degradation and inhibition of metabolic enzymes, as noted in the proteomic study by Maqsood et al. (2020), plants with compromised protein content exhibited weaker defense responses and were more susceptible to pathogen invasion. Reduced protein levels also imply a lower concentration of defense-related enzymes such as PR-proteins (Li et al., 2023).

Modulation of polyphenol oxidase (PPO) and peroxidase activities in tomato plants challenged with F. oxysporum f. sp. lycopersici isolates under in vitro conditions

Polyphenol oxidase (PPO) and peroxidase (PO) activities were measured in tomato plants infected with different F. oxysporum f. sp. lycopersici isolates. The lowest enzyme activities were recorded in F7-infected plants (PPO: 221.33 U/g protein, PO: 58.84 U/g tissue), which showed severe wilt symptoms. F7 and F8, the most virulent isolates, significantly suppressed PPO and PO activity compared to other treatments. Control plants, though uninoculated, had lower baseline levels (PPO: 211.66 U/g, PO: 52.93 U/g). Overall, enzyme activity declined with increasing disease severity, indicating weakened host defence responses in plants infected by highly virulent isolates.

Fig 3: Variations in polyphenol oxidase concentrations in F. oxysporum f. sp lycopersici infected tomato plants

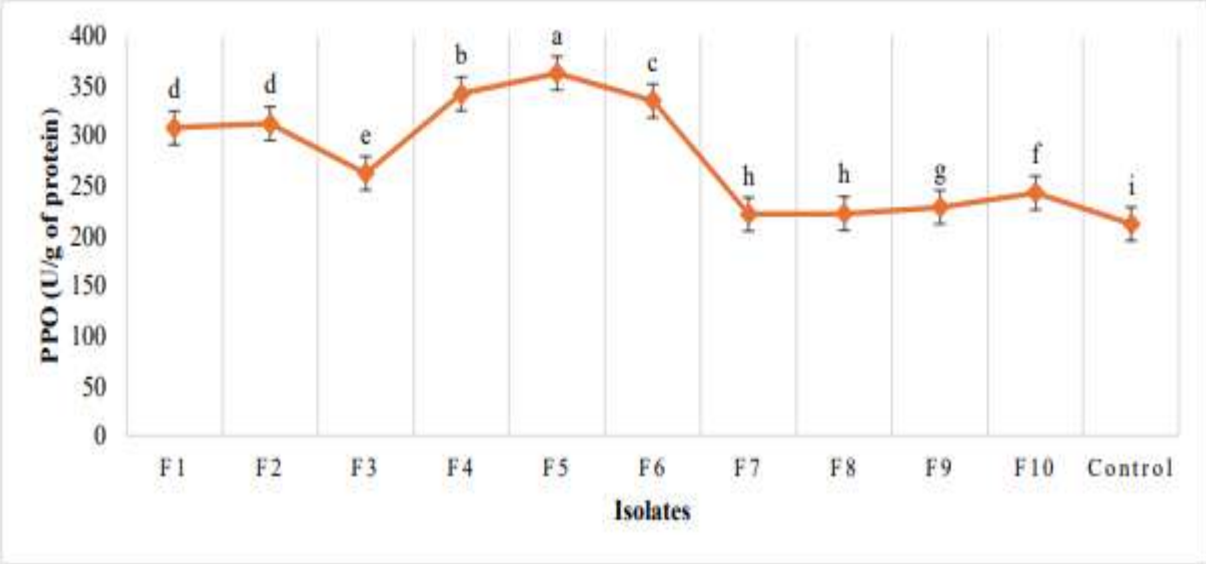
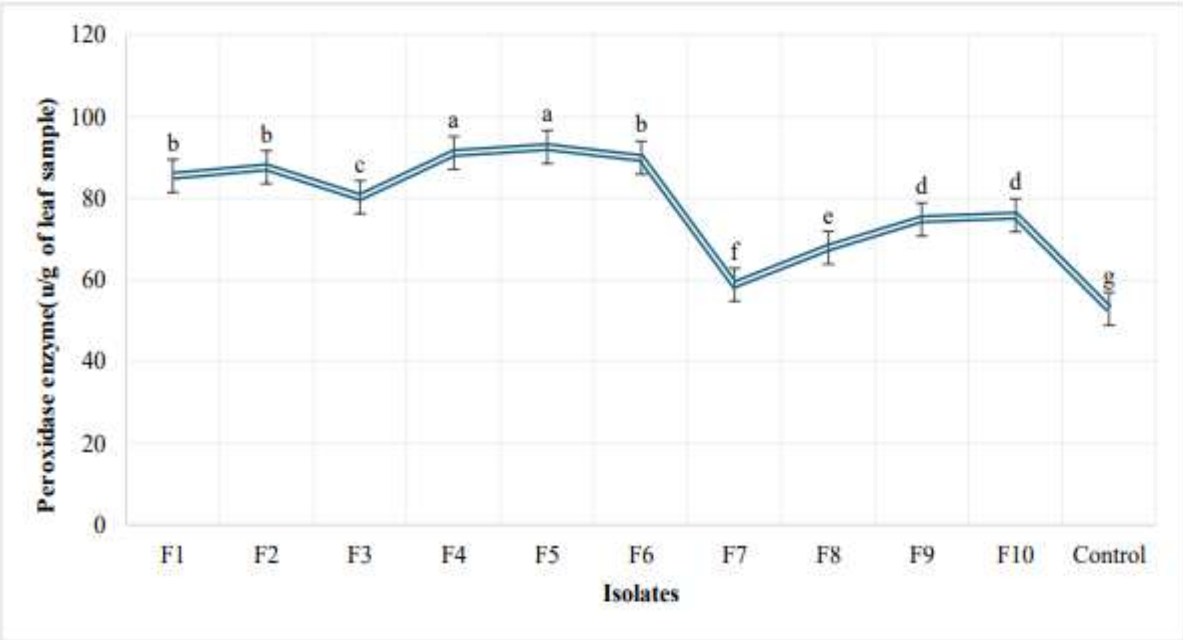


Fig 4: Changes in peroxidase enzyme levels in tomato plants infected with F. oxysporum f. sp. lycopersici

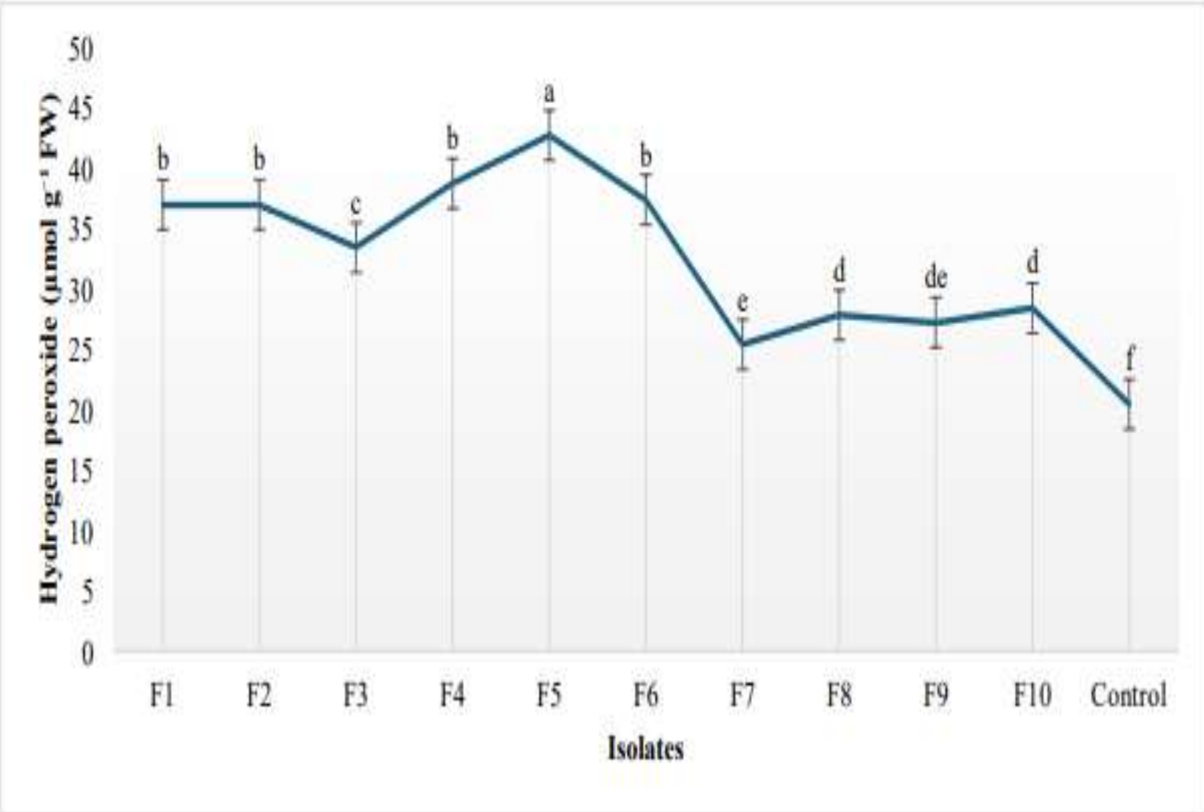


Different isolates of F. oxysporum f. sp. lycopersici inoculated tomato plant samples showed variation in PPO and PO contents and coincided with the results of Attia et al. (2022). They reported the role of PPO in plant disease resistance; the polyphenol oxidase activity assayed in the leaf samples of the resistant as well as susceptible cultivars of tomato. Higher PPO activity was recorded in the resistant cultivars viz., FEB-2, FEB-4, Flora Dade and NF-31 as compared to the susceptible (Sel-7, Sel-18 and Punjab Chhuhara). The peroxidase (PO) (EC1.11.1.7; donor: hydrogen peroxidase oxido-reductase) activity was assayed in the leaf samples of all the seven cultivars and it was noticed that the PO activity was significantly higher in the resistant cultivars as compared to the susceptible ones (Hashem et al., 2022). The maximum peroxidase (PO) activity was recorded in the resistant cultivar Flora Dade (2.073 units/g) and minimum in the susceptible cultivar Sel-18(0.241 units/g). In this experiment high virulent isolate inoculated plant exhibited severe wilting with lesser content of PPO and PO.

Variation in hydrogen peroxide levels in tomato plants infected with F. oxysporum f. sp. lycopersici isolates under in vitro conditions

Hydrogen peroxide (H_2O_2) levels in tomato plants infected with different F. oxysporum f. sp. lycopersici isolates varied significantly, reflecting oxidative stress responses. The lowest H_2O_2 level (25.37 µmol/g FW) was recorded in plants infected with the highly virulent F7 isolate, close to the control (20.43 µmol/g FW). This suggested the suppressed ROS accumulation due to rapid pathogen colonization. In contrast, higher H_2O_2 levels were found in plants infected with less virulent isolates, particularly F5. The increasing trend across isolates (F7 < F8 < F9 < … < F5) indicates that oxidative stress is more pronounced in moderately affected plants, correlating with the virulence levels of the isolates.

Fig 5: Impact of F. oxysporum f. sp lycopersici isolate infection on hydrogen peroxide accumulation in tomato plants



Plants utilize reactive oxygen species (ROS), especially H_2O_2, not only as direct antimicrobial agents but also as secondary messengers that activate downstream defense genes. Elevated H_2O_2 levels in infected plants, as seen in our results, are consistent with recent studies where H_2O_2 was significantly restricting pathogen spread (Ali et al., 2018). This oxidative burst facilitates cross-linking of cell wall proteins, lignin formation, and the induction of pathogenesis-related proteins. Pathogenesis occurs due to a disruption in the balance between oxidants and antioxidant enzymes within the cellular system. During Fusarium infection, hydrogen peroxide (H_2O_2) was rapidly produced and accumulated, that damaged the macromolecules and contributed disease development in the leaves and root tissues of Fusarium-infected seedlings (Sahu et al., 2023). As indicated Dey et al. (2020), hydrogen peroxide fight against some fungal diseases showed that these stimuli reduced the severity of the infection at higher concentration in tomato plant with vascular wilt caused by Fusarium oxysporum.

Alterations in superoxide radical levels and oxalic acid content in tomato plants infected with F. oxysporum f. sp. lycopersici isolates under in vitro conditions

Superoxide radical and oxalic acid levels were highest in tomato plants infected with the highly virulent F7 isolate (6.20 nmol/g FW and 0.60 mg/g, respectively), aligning with severe wilt symptoms. In contrast, control plants showed much lower values (4.20 nmol/g FW and 0.13 mg/g). Among infected samples, the lowest concentrations were recorded in F5-infected plants, indicating lower virulence. The results

showed a clear positive correlation between increased levels of these biochemical markers and the pathogenicity of the
F. oxysporum f. sp. lycopersici isolates.

Fig 6: Effect of infection by F. oxysporum f. sp. lycopersici isolates on superoxide radical buildup in tomato plants

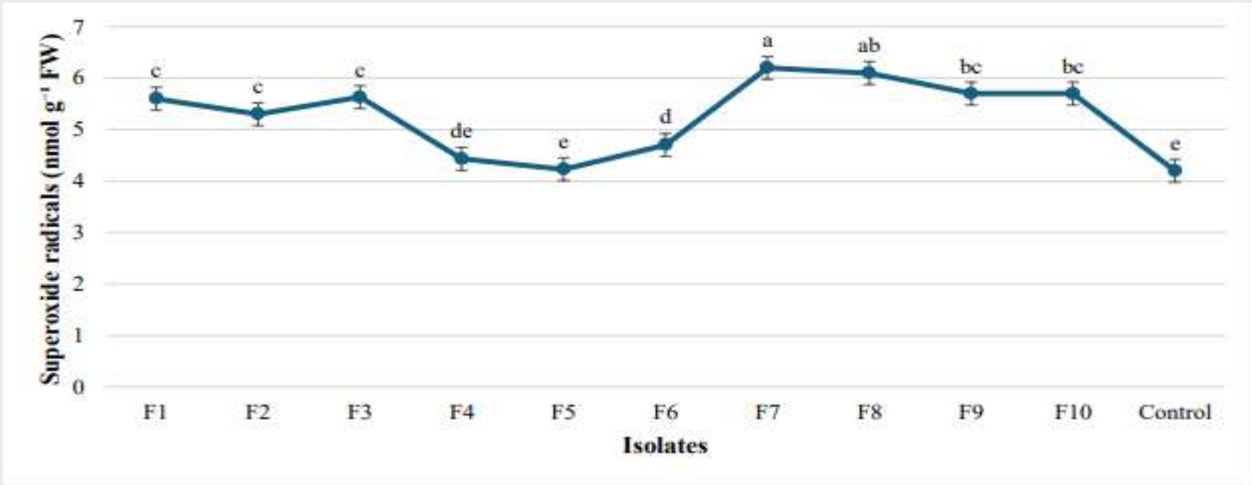
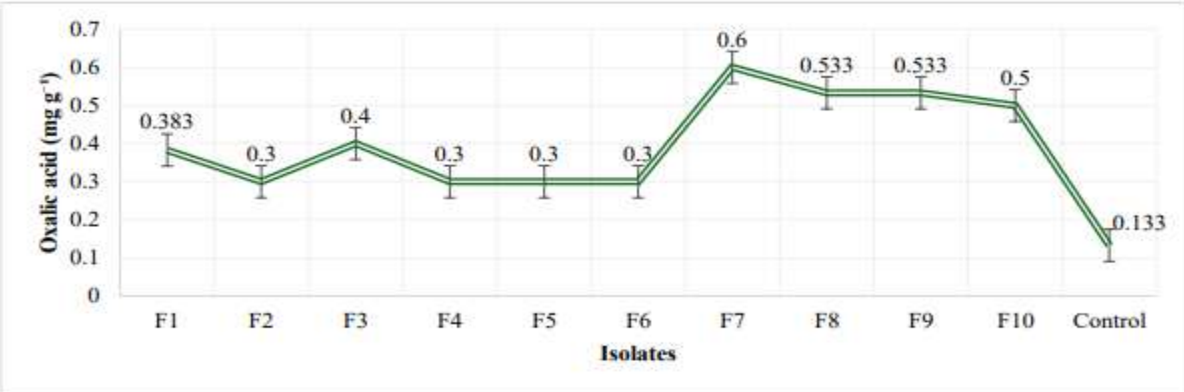


Fig 7: Oxalic acid content in tomato plants as affected by different F. oxysporum f. sp. lycopersici isolates



The findings align with that of Hao et al., (2023). Superoxide radicals (O_2^-) were generated in response to pathogen attack, acting as signaling molecules to activate downstream defense. Our findings are in line with recent reports (Iqbal et al., 2023) that showed enhanced O_2^- generation in plants infected with FOL, during early stages of infection. The excessive production of O_2^- may result in cellular damage by enzymes such as superoxide dismutase (SOD), leading to programmed cell death, a characteristic symptom of Fusarium wilt. Simultaneously, the oxalic acid content in infected tomato tissues was significantly elevated as pathogenicity factor plays multiple roles during infection. It acidifies the host environment, chelates calcium from cell walls, and facilitates pectin degradation, thereby enhancing fungal penetration and colonization. The increased oxalic acid levels recorded in this study corroborate previous findings (Jiao et al., 2024 and Al-Askar et al., 2021) which noted that high oxalic acid concentration correlated with increased disease severity in high virulent isolate inoculated tomato plants.

CONCLUSION

Isolates of F. oxysporum f. sp. lycopersici (FOL) causing Fusarium wilt in tomato were collected from various locations and tested for their virulence (pathogenicity) under in vitro conditions. Biochemical changes in tomato plants, including alterations in the stem and leaf tissues, were observed following the expression of wilt symptoms. The plants inoculated with the highly virulent F7 isolate exhibited a marked decrease in chlorophyll content (including chlorophyll A, chlorophyll B, A/B ratio, and total chlorophyll). In contrast, these plants showed an increase in soluble protein (F7, F8, and F9 isolates), superoxide radicals (F7 isolate), and oxalic acid (F7, F8 and F9 isolates). These changes suggested that the infection triggered oxidative stress and altered cellular metabolism. Additionally, defence-related enzymes such as polyphenol oxidase (PPO) and peroxidase (PO) were found to be reduced in the F7-inoculated plants, highlighting the isolate's ability to suppress plant defence mechanisms as part of its virulent action. Among all the isolates, F7 demonstrated significant virulence, as evidenced by symptoms expression and corresponding biochemical changes.

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Conflict of interest: None

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