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Comparative Evaluation of Changes in Biochemical Compounds in Tomato Plant (*Solanum lycopersicum* L.) Infected by *Phytophthora infestans* and in Response to Antifungal Plant Extracts

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Late blight, caused by the oomycete's fungi *Phytophthora infestans,* is one of the most destructive diseases affecting tomato (*Solanum lycopersicum* L.) crops worldwide. Herbal-based foliar sprays derived from plant extracts offer an eco-friendly, biodegradable alternative to chemical fungicides for managing fungal diseases in crops like *Solanum lycopersicum* (tomato). This study examines significant alterations in carbohydrates, proteins, total free amino acids, total free fatty acids, phenolic content, and chlorophyll levels in tomato plants subjected to late blight infection and subsequent treatments with antifungal plant extracts as foliar spray. About five novel formulations using aqueous leaf extracts of *Carica papaya, Lantana camara* and *Syzygium cumini* were tested against *Phytophthora infestans*-infected tomato plants. By the results, it showed an increase in the level of protein content, Phenolic content and chlorophyll content. This research underscores the potential of antifungal foliar sprays in modulating biochemical pathways to bolster tomato plant defenses against late blight, offering a sustainable approach to disease management.

Keywords: Solanum lycopersicum L.; Phytophthora infestans; antifungal foliar spray; biochemical alternations.

1. INTRODUCTION

The Late Blight disease was caused by the Oomycetes fungi *Phytophthora infestans* (Mont.) de Bary and it is one of the serious diseases which cause loss in yield of tomatoes. Late blight earned notoriety due to its historical role in the Irish Potato Famine of the 19th century, where it caused widespread crop failures and famine. Late blight continues to pose a significant threat to global food security and agricultural economies. Additionally, ongoing research into safer, more eco-friendly fungicides and alternative disease management strategies is crucial for addressing these challenges and ensuring the long-term sustainability of agricultural systems. And bio-control would be one of the best solutions for it. Bio-control agents are typically less harmful to the environment compared to chemical fungicides. They do not leave harmful residues in soil or water bodies, reducing the risk of environmental contamination and harm to non-target organisms.

Plant-derived antifungal compounds offer a promising and eco-friendly alternative to conventional chemical fungicides. Numerous studies have highlighted the ability of certain plant extracts to inhibit fungal growth, spore germination, and pathogenicity mechanisms in *P. infestans* (Yanar et al., 2011). Plants possess innate immune systems and produce secondary metabolites such as alkaloids, terpenoids, flavonoids, tannins, and saponins that serve as defense chemicals against microbial pathogens. These natural compounds often work through multiple modes of action, which makes it harder for pathogens to develop resistance.

The antifungal mechanisms of plant-derived compounds vary based on their chemical structure. Flavonoids, for example, have been shown to disrupt fungal cell walls and membranes, leading to leakage of cellular contents. Phenolic compounds can inhibit enzymes required for fungal metabolism and reproduction. Alkaloids and saponins often target ergosterol synthesis, impairing the integrity of fungal plasma membranes (Borges et al., 2018). Importantly, these compounds can work synergistically when present as complex mixtures in whole-plant extracts, often producing enhanced or broad-spectrum effects. Furthermore, some plant extracts also induce systemic resistance in host plants, stimulating the production of pathogenesis-related proteins and defensive enzymes such as chitinases and glucanases (Hernández-Soto et al., 2024). This dual activity—direct pathogen inhibition and host resistance activation makes plant-based extracts particularly attractive for integrated disease management (IDM).

The present study emphasis on the efficiency of prepared aqueous herbal formulation and shows the significant biochemical changes takes placein the late blight disease infected plants and the herbal foliar spray treated tomato plants at field conditions.

2. MATERIALS AND METHODS

2.1 Selection and Collection of Plant Materials

The present work was designed to prepare a non-toxic, cost efficient and eco-friendly herbal

consortia for controlling the late blight disease in tomato plants. For that, the plants are selected which are commonly available and have high anti-fungal activity.

Based on the literature the above mentioned plants and its parts have been selected and used for the preparation of the anti-fungal herbal consortia to inhibit the growth of *Phytophthora infestans* which cause late blight disease in tomato plant. From the selected plant material which is mentioned above, mature and healthy leaves have been collected.

2.2 Preparation of Herbal Consortia (Bhupendra, 1999)

The collected plant materials (leaves) were washed with running tap water inorder to remove the dirt and the prominent mid veins in the leaves has been removed using the scissors. Then the leaves are chopped into small pieces by using the knife and dropped into the mixer jar. To that chopped leaves, 30 ml of water and 2 ice cubes (to prevent from denaturing of phytochemicals or secondary metabolites) was added and finely grounded using the mixer. Then the homogenate was filtered by using the 3 to 4 layers of muslin cloth. Then the aqueous extract or the filtrate was stored in glass bottle and refrigerated at 4^0C till have to be used.

2.3 Field Experimental Plot

The experiment plot for tomato has been done in 1 acre of land at Chinnappampalayam and the commercial variety of tomato “SHIVAM” were used for the present study. The spacing provided between each row of the tomato plant is 4 feet and between each plant is 2 feet. There are 12 columns in the field and each column of the plot contains 15 plants. For single formulation, 2 rows of 30 plants were selected to test and for five formulation 150 plants in 10 rows have tested. About 2 rows with 30 plants treated as control. Three times foliar herbal spray was sprayed in the interval of 10 days. The results had been noted and tabulated.

2.4 Proximate Analysis of Some Important Biochemicals

2.4.1 Estimation of chlorophyll (Arnon, 1949)

The content of chlorophyll a, b and total chlorophyll were estimated on the healthy, diseased and treated fresh plant samples and it is expressed in mg g-1. About 5g of fresh leaves were taken and the mid rib was removed and it is made into fine paste using mortar and pestle with 80% acetone. The mixture was centrifuged at 5000 rpm for 5 minutes and the supernatant was poured into a conical flask. Then the process was repeated for many times till the residue become colourless. Makeup the volume to 100 ml by adding 80% acetone to the conical flask. 80% acetone serves as the blank. Shake the supernatant in the conical flask properly and read the absorbance at 645 nm and 663 nm against solvent blank. The chlorophyll content has been calculated by using the below formula and the obtained results were tabulated.

Calculation:

Chlorophyll a = (12.7 x A663) - (2.69 x A645) x V/1000*W mg g-1
Chlorophyll b = (22.9 x A645) - (4.68 x A663) x V/1000*W mg g-1
Total Chlorophyll = (20.2 x A645) + (8.02 x A663) x V/1000*W mg g-1

2.4.2 Estimation of carbohydrate (Yemm and Willis, 1954)

The total content of carbohydrate was estimated on the healthy, diseased and treated fresh plant samples and it is expressed in mg g-1. About 5g of fresh leaves were taken and hydrolyzed by keeping it in a boiling water bath for about one and half an hour with 10 ml of 2.5 N Hydrochloric acid and cooled. Then make the sample into fine paste using mortar and pestle. Centrifuge it and collect the supernatant.

Into a series of test tubes, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml of working standard solution was poured into respective test tubes and the volume was made upto 1 ml by adding distilled water to it. 0.5 ml of the supernatant was added to another test tube and made upto 1 ml by adding distilled water. 4 ml of Anthrone reagent was added and heated for exactly 8 minutes in boiling water bath. 4 ml of Anthrone reagent along with 1 ml of distilled water serves as a blank. After heating, the mixture was cooled rapidly and the intensity of green colour was read at 630 nm. A standard graph was drawn by taking concentration of glucose on x - axis and optical density on y - axis. From the graph the amount of carbohydrate present in the sample was calculated and the results were tabulated.

Table 1. Lists of used plants

S.No.	Name of the plant used	Plant parts used
1	*Carica papaya*	Leaves
2	*Lantana camara*	Leaves
3	*Syzygium cumini*	Leaves

Calculation:

Amount of carbohydrate present in the sample,

$$\text{100 ml of the sample contains} = \frac{\text{g of glucose} \times \text{100 mg of glucose}}{\text{Volume of the sample}}$$

2.4.3 Estimation of protein (Lowry et al., 1951)

The total content of protein was estimated by Lowry method on the healthy, diseased and treated fresh plant samples and it is expressed in mg g-1. About 5g of fresh leaves were taken and the mid rib was removed and it is made into fine paste using mortar and pestle with 10 ml of buffer. Centrifuge for 5 minutes and collect the supernatant.

Into a series of test tubes, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml of working standard solution was poured into respective test tubes and the volume was made upto 1 ml by adding distilled water to it. 0.5 ml of the supernatant was added to another test tube and made upto 1 ml by adding distilled water. Add 5 ml of the reagent C to each test tube and allow it to stand for 10 minutes. Then add 0.5 ml of Folin's ciocalteau reagent, mix it well and incubate at room temperature in dark for 30 minutes. The blue colour was developed and its intensity was read at 660 nm. Reagent C, Folin's ciocalteau reagent along with 1 ml of distilled water serves as a blank. A standard graph was drawn by taking concentration of protein on x - axis and optical density on y - axis. From the graph the amount of protein present in the sample was calculated and the results were tabulated.

Calculation:

Amount of protein present in the sample,

$$\text{100 ml of the sample contains} = \frac{\text{g of protein} \times \text{100 mg of protein}}{\text{Volume of the sample}}$$

2.4.4 Estimation of total Free amino acids (Mahesha, 2019)

The content of total free aminoacid were estimated on the healthy, diseased and treated fresh plant samples and it is expressed in mg g-1. About 5g of fresh leaves were taken and the mid rib was removed and it is made into fine paste using mortar and pestle with 80% ethanol and acid - washed sand. Then filter, centrifuge and save the supernatant. Repeat the process twice, pool all the supernatant and reduce the volume if needed by evaporation and use the extract for qualitative estimation.

Into a series of test tubes, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml of working standard solution was poured into respective test tubes and the volume was made upto 1 ml by adding distilled water to it. 0.5 ml of the supernatant was added to another test tube and made upto 1 ml by adding distilled water. Then add 1 ml of Ninhydrin reagent to all the test tubes and kept it in the boiling water bath for exactly 20 minutes. Cool the test test tubes in cold water and add 5 ml of diluent solution to all the test tubes and kept for 15 minutes undistrubed. Purple colour was developed and the intensity was measured at 570 nm. 80% ethanol, Ninhydrin and distilled water serves as a blank. A standard graph was drawn by taking concentration of total free amino acid on x - axis and optical density on y - axis. From the graph the amount of total free amino acid present in the sample was calculated and the results were tabulated.

Calculation:

Amount of total free amino acid present in the sample,

$$\text{100 ml of the sample contains} = \frac{\text{g of amino acid} \times \text{100 mg of amino acid}}{\text{Volume of the sample}}$$

2.4.5 Estimation of total Free fatty acids (Mahesar et al., 2014)

The content of total free fatty acid was estimated on the healthy, diseased and treated fresh plant samples and it is expressed in mg g-1. About 5g of fresh leaves were taken and the mid rib was removed and it is made into fine paste using mortar and pestle with 80% ethanol. The mixture was centrifuged and the supernatant was collected. Neutral solvent was prepared and 25 ml of the solvent was poured into the conical

flask. To that few drops of phenolphthalein and 0.5 ml of supernatant was added and mixed thoroughly. It was titrated against the 0.1 N of potassium hydroxide until a pink colour which persists for 15 seconds is obtained. The titrate value was noted and the free fatty acid value was obtained using the below formula and the results were tabulated.

Calculation:

$$\text{Acid value (mg KOH/g)} = \frac{\text{Titrate value x Normality of KOH} \quad \text{x 56.1 mg/g of free fatty acid}}{\text{Weight of the sample}}$$

2.4.6 Estimation of phenolics (Samidha et al., 2014)

The total content of phenolics were estimated on the healthy, diseased and treated fresh plant samples and it is expressed in mg g-1. About 5 g of fresh leaves were taken and the mid rib was removed and it is made into fine paste using mortar and pestle with 10-time volume of 80% ethanol. The mixture was centrifuged at 10,000 rpm for 20 minutes and the supernatant was collected and the process is repeated for 5 times with 80% ethanol. Then the supernatant was evaporated and the residue was taken and dissolve in 5 ml of distilled water, which serves as the sample.

Into a series of test tubes, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 ml of working standard solution was poured into respective test tubes and the volume was made upto 2 ml by adding distilled water to it. 0.5 ml of the supernatant was added to another test tube and made upto 2 ml by adding distilled water. Add 0.5 ml of Folin's ciocalteau reagent to all the test tubes and kept undistrubed for 3 minutes. Then add 2 ml of 20% sodium carbonate solution to all the test tubes and mix thoroughly. Then place all the test tubes in the boiling water bath for exactly 1 minute and cool it rapidly. Next read the absorbance at 650 nm where, 80% ethanol, 20% sodium carbonate solution, Folin's ciocalteau reagent along with distilled water serves as a blank. A standard graph was drawn by taking concentration of phenolics on x - axis and optical density on y - axis. From the graph the amount of phenolics present in the sample was calculated and the results were tabulated.

Calculation:

Amount of phenolics present in the sample,

$$\text{100 ml of the sample contains} = \frac{\text{g of phenolics x 100 mg of phenolics}}{\text{Volume of the sample}}$$

2.5 Qualitative Phytochemical Analysis

Phytochemical analysis was carried out to identify the presence or absence of phytochemicals or secondary metabolites in plant samples. The plant sample was prepared by drying the fresh plant in shade for few days and after it gets dried it is powdered using mixer and sieved. Then the powder was dissolved in water, filtered and the filtrate which was made is used for analysis.

2.5.1 Test for alkaloids (Prashant et al., 2011)

To a few drops of the plant sample filtrate, 2 ml of Mayer's reagent was added. The formation of yellow colour precipitate indicates the presence of alkaloids.

2.5.2 Test for flavonoids (Kumar et al., 2014 and Pradeep et al., 2014)

To a few drops of the plant sample filtrate, 1 mL of 10% lead acetate solution was added. The formation of yellow or white precipitate indicates the presence of flavonoids.

2.5.3 Test for Terpenoids (Pradeep et al., 2014)

To a few drops of the plant sample filtrate, 1 ml of ethanol, acetic anhydride and few drops of concentrated sulphuric acid was added. The formation of pink to violet colour indicates the presence of terpenoids.

2.5.4 Test for tannins (Harsha et al., 2013)

To a few drops of the plant sample filtrate, few drops of 1% lead acetate solution was added. The formation of yellowish precipitate indicates the presence of tannins.

2.5.5 Test for glycosides (Harsha et al., 2013)

To a few drops of the plant sample filtrate, add few drops of dilute sulphuric acid, boil it and filter the solution. To the solution add few drops of benzene and shake it well. Decant the organic layer formed into the test tube and add few drops of dilute ammonia solution. The ammonia layer will turn into pink colour which indicates the presence of glycosides.

2.5.6 Test for saponins (Kagbo and Ejebe, 2009)

To a few drops of the plant sample filtrate, 20 ml of distilled water was added and shaken vigorously. The formation of froth which persist indicates the presence of saponins.

2.5.7 Test for steroids (Harsha et al., 2013)

To a few drops of the plant sample filtrate, 2 ml of chloroform and sulphuric acid was added and shake it well. The formation of red chloroform layer and greenish-yellow fluorescence indicates the presence of steroids.

2.5.8 Test for resins (Kumar et al., 2019)

To a few drops of the plant sample filtrate, few drops of acetic anhydride solution and 1ml of concentrated sulphuric acid was added. The formation of orange to yellow coloration indicates the presence of resins.

2.5.9 Test for volatile oils (Harsha et al., 2013)

To a few drops of the plant sample filtrate, 0.1 ml of dilute sodium hydroxide and few drops of dilute hydrochloric acid was added. The formation of white precipitate indicates the presence of volatile oils.

3. RESULTS AND DISCUSSION

The proximal studies showed that total protein content of the diseased plant showed that 144 mg/g while the healthy plant was recorded as 469.37 mg/g, which is higher than the diseased plant. The T3 and T5 formulation was well responded and increased the protein content to 458.24 mg/g and 448.6mg/g respectively (Table 2). This finding is similar with the reports of some workers. Pareek and Varma (2015) observed increase in protein content in healthy plant parts of cluster bean than in the *Fusarium solani* infected plant.

The total free aminoacids in control plant is 87.87 mg/g while diseased was increased to 118.44 mg/g which has a steep increase in total free aminoacids. The T3 and T5 formulation shows decrease in free aminoacid content to 90.4 mg/g and 93.37 mg/g respectively (Table 3) compared to free aminoacid content in infected plant. This finding is similar with the reports of some workers. Sutha et. al., (1998) reported that the increase in total free amino acid content in diseased plant of tomato affected by tospovirus. Ramiah (1970) also reported that there was an increased in total free aminoacid content in bhindi leaves infected with bhindi yellow vein mosaic virus.

The total carbohydrate was was recorded as 40.4 mg/g in control plant while diseased plant has evidenced in increase in content carbohydrate as 44.07 mg/g when compared to it. The T3 and T5 formulation shows decrease in free carbohydrate content to 37.04 mg/g and 35.57 mg/g respectively (Table 4) compared to carbohydrate content in infected plant. This finding is similar with the reports of some workers. Junqueira et al., (2004) through their findings reported that the carbohydrate (reducing sugar) content has increased in the maize bushy stunt phytoplasma infected corn plants. Lobato et. al., (2009) reported that the carbohydrate content has increased in the *Colletotrichum lindemuthianum* infected common bean cultivar plants.

The total phenolic content of the diseased plant showed that 22.3 mg/g while the healthy plant was recorded as 42.44 mg/g, which is higher than the diseased plant. The highest total phenolics content was found in T3 treatment with 30.6 mg/g whereas only 22.3 mg/g was found in diseased plant (Table 5). This finding is similar with the reports of some workers. Adandonon et al., (2017) by the results they recorded that tolerant cultivar of cowpea shows highest phenolic content than the susceptible cultivar when it gets infected by the *Sclerotium rolfsii*.

Table 2. Estimation of total protein content of control, diseased and treated plants

S.No	Treatments	Protein content (mg/g)
1	Control	469.37 $\pm$ 0.42
2	Diseased	144.04 $\pm$ 0.21
3	F1	393.8 $\pm$ 0.2
4	F2	426.27 $\pm$ 0.21
5	F3	458.24 $\pm$ 0.15
6	F4	415.4 $\pm$ 0.2
7	F5	448.6 $\pm$ 0.1

Table 3. Estimation of total free amino acid content of control, diseased and treated plants

S.No	Treatments	Total free amino acid content (mg/g)
1	Control	87.87 ± 0.45
2	Diseased	118.44 ± 0.30
3	F1	102.4 ± 0.3
4	F2	102.64 ± 0.20
5	F3	90.4 ± 0.3
6	F4	101.7 ± 0.2
7	F5	93.37 ± 0.15

Table 4. Estimation of total carbohydrate content of control, diseased and treated plants

S.No	Treatments	Carbohydrate content (mg/g)
1	Control	40.4 ± 0.3
2	Diseased	44.07 ± 0.20
3	F1	34.7 ± 0.2
4	F2	35.67 ± 0.15
5	F3	37.04 ± 0.15
6	F4	34.2 ± 0.26
7	F5	35.57 ± 0.15

Table 5. Estimation of secondary metabolites phenolics of control, diseased and treated plants

S.No	Treatments	Phenolics content (mg/g)
1	Control	42.44 ± 0.25
2	Diseased	22.3 ± 0.3
3	F1	23.04 ± 0.35
4	F2	28.17 ± 0.3
5	F3	30.6 ± 0.34
6	F4	27.37 ± 0.28
7	F5	28.64 ± 0.25

The total free fatty acids in control plant is 0.5 mg/g while diseased was increased to 0.7 mg/g which has increase in total free fattyacids. The total free fatty acid was recorded very low in nature as 0.7 in diseased crops and other treated crop more or less very much equal and recorded in the range between 0.3 to 1.5 mg/g (Table 6). This finding is similar with the reports of some workers. Losel and Lewis (1974) recorded that the free fatty acids (lipid) content was three times higher in the Puccinia poarum infected Tussilago farfara leaves than the healthy leaves of the plant.

The chlorophyll content also showed considerable variation among the treated and diseased crop plants. The chlorophyll content a, b and total chlorophyll was estimated separately. The chlorophyll a, b, total chlorophyll content of the diseased plant showed that 3.30, 6.22, 5.53 mg/g while the healthy plant was recorded as 4.74, 7.42, 6.62mg/g which is higher than the diseased plant. The T3 formulation treated crops considerably responded and crops showed that about 4.64, 7.09 and 6.29 respectively, while diseased plant recorded as 3.30, 6.22 and 5.53 mg/g respectively (Table 7). This finding is similar with the reports of some workers. Arpita and Ghosh (2008) by their findings reported that the chlorophyll content in the mesta plants infected with yellow vein mosaic disease has decreased compared to the healthy plant of mesta.

Secondary metabolites are the important parameter, a phytochemical, which is produced only the plant which undergoes stress condition and it is proved to present in the results of tabulation. The more phytochemicals were recorded in the diseased plant than control plant and the results are displayed in the Table 8. The more variety of phytochemicals was found in the diseased plant because theses secondary metabolites are produced to overcome the fungal pathogen infection and to suppress its growth.

Table 6. Estimation of total free fatty acid content of control, diseased and treated plants

S.No	Treatments	Free fatty acid content (mg/g)
1	Control	0.5 ± 0.057
2	Diseased	0.7 ± 0.057
3	F1	1.5 ± 0.1
4	F2	0.3 ± 0.057
5	F3	0.3 ± 0.057
6	F4	0.4 ± 0.057
7	F5	0.3 ± 0.057

Table 7. Estimation of total chlorophyll content of control, diseased and treated plants

S.No	Treatments	Chlorophyll a content (mg/g)	Chlorophyl b content (mg/g)	Total chlorophyll content (mg/g)
1	Control	4.74 ± 0.004	7.42 ± 0.008	6.62 ± 0.050
2	Diseased	3.30 ± 0.002	6.22 ± 0.011	5.53 ± 0.005
3	F1	3.56 ± 0.005	6.37 ± 0.005	5.64 ± 0.007
4	F2	3.94 ± 0.005	6.69 ± 0.007	5.93 ± 0.006
5	F3	4.64 ± 0.05	7.09 ± 0.012	6.29 ± 0.008
6	F4	3.68 ± 0.003	6.46 ± 0.006	5.73 ± 0.011
7	F5	4.51 ± 0.006	6.96 ± 0.011	6.17 ± 0.008

Table 8. Qualitative phytochemical analysis of control and diseased plant

S.No	Phytochemical	Control plant	Diseased plant
1	Alkaloids	-	-
2	Flavonoids	-	+
3	Terpenoids	-	+
4	Tannins	-	-
5	Glycosides	-	-
6	Saponins	+	+
7	Steroids	-	+
8	Resins	-	-
9	Volatile oils	-	-

" + " = Present; " - " = Absent

4. CONCLUSION

The study demonstrates significant biochemical changes in diseased plants compared to healthy ones, with treatments using formulations T3 and T5 showing promising effects in restoring plant health. Diseased plants exhibited a sharp decline in protein content (144.04 mg/g) compared to the healthy control (469.37 mg/g), but treatments with T3 (458.24 mg/g) and T5 (448.6 mg/g) increased protein levels. Free amino acids were elevated in diseased plants (118.44 mg/g) but were reduced in treated plants (T3: 90.4 mg/g, T5: 93.37 mg/g), indicating a positive treatment response. Carbohydrate content was also higher in diseased plants (44.07 mg/g), with T3 and T5 treatments reducing the levels (T3: 37.04 mg/g, T5: 35.57 mg/g). Phenolic content was significantly lower in diseased plants (22.3 mg/g), but T3 treatment increased it to 30.6 mg/g, enhancing the plant's defense mechanism. Chlorophyll content was reduced in the diseased plants, but T3 treatment showed improvement in chlorophyll a, b and total chlorophyll levels, suggesting enhanced photosynthetic activity. Phytochemical analysis revealed the presence of stress-related metabolites like flavonoids, terpenoids, and saponins in diseased plants, further supporting the plant's response to infection. Overall, T3 and T5 formulations significantly mitigated the biochemical stress caused by disease, offering a potential strategy for improving plant resilience and health.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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