Synergistic Effect of Bio-Stimulants and *Trichoderma* Spp. on Physio-Biochemical Parameters of Cucumber (*Cucumis sativus* L.)

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THESIS

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF

Master of Science (Agriculture)
in
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Submitted by

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ID. No. 20412MPP018 **2022** **Enrolment No. 383067**

DEDICATED TO:

My Parents

Shri. Ashok Kumar Paswan

&

Smt. Neelam Devi

and

My Beloved ones

काशी हिन्दू विश्वविद्यालय BANARAS HINDU UNIVERSITY

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CERTIFICATE

To,
The Registrar (Academic)
Banaras Hindu University
Varanasi-221005 (India)

Through The Head, Department of Mycology and Plant Pathology,
Institute of Agricultural Sciences,
BHU, Varanasi, 221005

Dear Sir,

I have great pleasure in forwarding the thesis entitled "Synergistic effect of bio-stimulants and *Trichoderma* spp. On physio-biochemical parameters of cucumber (*Cucumis sativus* L.)" submitted by **Mr. Suman Jee, I.D. No. 20412MPP018, Enrolment No. 383067** in partial fulfillment of the requirements for the award of the degree of **Master of Science (Agriculture) in Plant Pathology**, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi (U.P.) and placing on record that he has completed the requisite residential requirements as contained in the statutes of the University.

I certify that the entire scheme of investigation presented herein was planned and carried out solely by the candidate under my guidance and supervision. The data presented in the thesis, to the best of my knowledge and belief, are genuine and original.

Thanking you

Yours faithfully

Forwarded

Dr. Satyendra Pratap Singh
Supervisor

Head

Synergistic effect of bio-stimulants and *Trichoderma* spp. On physio-biochemical parameters of cucumber (*Cucumis sativus* L.)

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Date: /   /2021
Place**: Varanasi* ***(Suman Jee)

CONTENT

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
/	:	Per
cm	:	centimeter
et al.	:	(et albeit) and others
etc.	:	Et cetera
g	:	gram
mg	:	milligram
µl	:	Microliter
µM	:	Micro molar
i.e.	:	Id est (that is)
mM	:	milliMolar
ltr	:	Litre
dai	:	Days after inoculation
f.sp.	:	*Formae specialis*
spp.	:	Species
subsp.	:	subspecies
D.W.	:	Distilled Water
PDA	:	Potato Dextrose Agar
BOD	:	Biological Oxygen Demand
PSB	:	Phosphate Solubilizing Bacteria
MT	:	Metric tonnes
Vit.	:	Vitamin
USD	:	United States Dollar
SAR	:	Systemic Acquired Resistance
ISR	:	Induced Systemic Resistance
PGPR	:	Plant Growth Promoting Rhizobacteria

No.	:	Number
^{o}C	:	Degree Centigrade
pH	:	Puissance de Hydrogen
TPC	:	Total phenol content
PAL	:	Phenylalanine ammonia-lyase
PO	:	Peroxidase
PPO	:	Polyphenol Oxidase
CAT	:	Catalase
min	:	Minutes
ml	:	Millilitre
CRD	:	Completely Randomized Design
SA	:	Salicylic Acid
MT	:	Melatonin
TH	:	*Trichoderma harzianum*
TV	:	*Trichoderma viride*
FW	:	Fresh Weight
cfu	:	Colony-forming unit

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Chapter-I

INTRODUCTION

Cucumber (*Cucumis sativus* L.), popularly known as khira, is a major vegetable crop that belongs to the Cucurbitaceae family and is one of the oldest cultivated vegetables, dating back over 5,000 years (Wehner and Guner, 2004). After cabbage, onion, and tomatoes, it is the world's fourth most important grown vegetable crop. China, Russia, Turkey, the United States, Ukraine, Egypt, Spain, and India are the top cucumber producers. In India, cucumber production is 1665.00 thousand MT with an area of 118.00 thousand hectares and productivity of 14.11 MT /hectare (Anonymous, 2020). Haryana, Madhya Pradesh, Uttar Pradesh, Karnataka, Andhra Pradesh, Punjab, Assam, Telangana, Maharashtra, and Kerala are the main producers. India is becoming the world's top exporter of gherkins. From April to October (2020-21), India exported 1,23,846 metric tonnes of cucumber and gherkins worth USD 114 million (Anonymous, 2022).

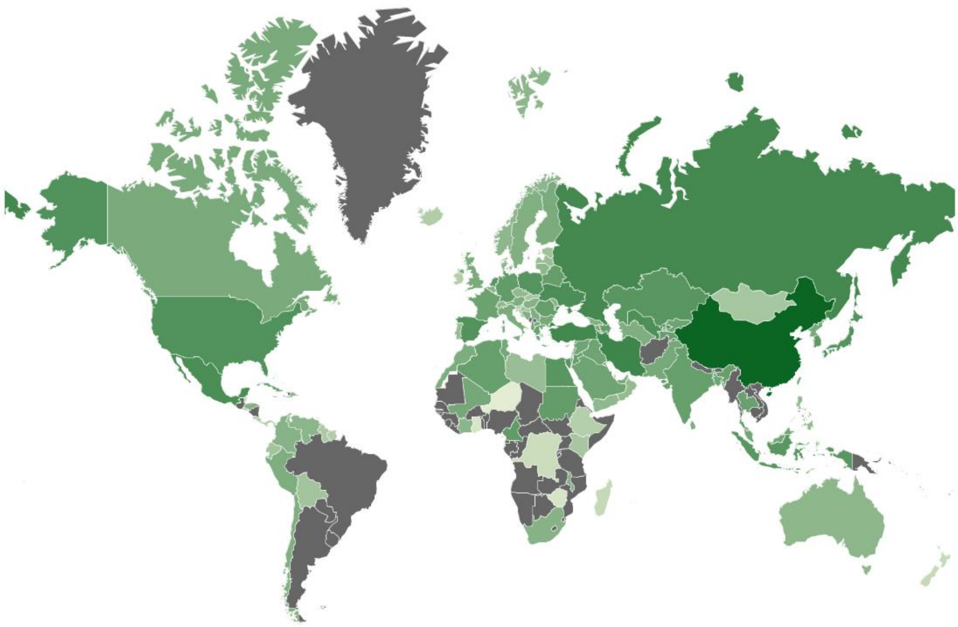

Figure.1.1 ■ Top cucumber producing countries (Anonymous, 2020).

Cucumber is cultivated for its tender fruits, which are mostly preferred to be consumed directly or preserved as pickled cucumbers marinated in vinegar, salt, dill, or other spices. Cucumber fruit is supposed to provide a cooling effect, prevent constipation, and alleviate jaundice and indigestion (Uthpala *et al*., 2020). Cucumber seeds and raw fruits are also employed in Ayurveda and cosmetic formulations. The oil derived from the seed is said to be beneficial to the brain and body. From a nutritive point of view, a 100-gram edible amount of cucumber includes 96.3 g of water,2.5 g of carbohydrates,0.4 g of protein, 0.4 g fat, 0.3 g of minerals,10.0 mg of calcium,0.4 mg of fiber,1.5 mg of iron, and 2.0 mg of vitamin C (Rai and Yadav, 2005).

Phytopathogens, on the other hand, are a major constraint in cucumber cultivation, resulting in lower yields. Pathogens reduce yield by reducing the quality and quantity of the crop. Cucumber is affected by a variety of fungi, bacteria, viruses, and nematodes. The diseases like downy mildew (*Pseudoperonospora cubensis*), powdery mildew (*Erysiphe cichoracearum*), gray mold *(Botrytis cinerea*), root rot (*Phomopsis sclerotioides*), white mold (*Sclerotinia sclerotiorum*), gummy stem blight and black rot (*Didymella bryoniae*), anthracnose (*Colletotrichum orbiculare*) and Fusarium wilt (*Fusarium oxysporum*) cause severe impact on the cucumber plant. Cucumber is more susceptible to downy mildew than other cucurbits.

Downy mildew of cucumber caused by the oomycetes *Pseudoperonospora cubensis*. Cucumber downy mildew causes major losses under favourable environmental conditions. The disease is the principal limiting factor for cucumber production in many humid climates. Angular, chlorotic lesions on the foliage are typical signs. Because they are joined by leaf veins, these lesions seem angular. The underside of the leaf exposes grey-brown to purplish-black 'down' in humid conditions. Leaves eventually get necrotic and curl upwards (Chand *et al*., 2014).

The fungus *Erysiphe cichoracearum* DC and *Sphaerotheca fuliginea* (Schlecht. ex Fr.) Poll produce powdery mildew on cucumbers. White powdery development on the top surfaces of leaves and stems of infected plants are common signs. Infected portions are often stunted and distorted, and they may fall off the plant prematurely. Fruits are rarely directly harmed, although their size and development may be impeded (Singh and Sahore, 2002; Morsy *et al*., 2009; Chand *et al*., 2014).

Pythium sp., Fusarium sp., and *Rhizoctonia* sp. are fungi that dwell in soil and attack cucumber seedlings. Seedlings are attacked before or after they emerge, causing gaps and uneven stands. Young plants begin to wilt and fall over. Only a few days before the seedling dies, water-soaked sores appear on stems at the soil line. Cucumber mosaic virus, Pumpkin mosaic virus, and Watermelon mosaic virus are prevalent viruses that can harm cucumbers. Plants infected with the virus may be stunted or have mottled, crinkled, or light green leaves. Fruits might be warty, speckled, or irregularly shaped. Viruses are transmitted by several insects (Zitikaite, 2002; Chand *et al*., 2014).

Although proper agricultural practices and growing reasonably resistant plants can lower the average amount of damage caused by these pathogens but the disease's widespread breakout needs the use of chemical compounds. Earlier, Chemicals were utilized for the management of plant diseases, resulting in yearly expenses of $15-$16 billion on fungicides for pathogen management (Lucía, 2021). Chemical fungicides are used heavily in the management of downy mildew and powdery mildew infections. Foliar sprays of chemical fungicides such as Copper oxychloride, Metalaxyl + Mancozeb, Chlorothalonil, Cymoxanil + Mancozeb, and Fosetyl-Al have proved useful in combating phytopathogens.

In the recent past, several more novel fungicides with systemic and curative action that have proven effective against downy mildew on diverse crops have been discovered as combination products. Ridomil MZ (0.25 percent) is the fungicide with the longest persistence (8 days) and has the best eradicative activity among the fungicides, which has proven effective against downy mildew disease (Thind *et al*., 1991). Similarly, Indofil M-45 is found to be efficient against downy mildew in cucumber leaves, providing 9 days of protection (Bains and Jhooty 1978; Maharshi and Siradhana 1988). The number of fruits per plant, length of the fruits, and the production of the fruits are all considerably higher when these treatments are used. However, the phytotoxicity of such fungicides, along with their prolonged persistence in tissues and massive residue deposition in soil, has had disastrous environmental and human health implications. Chemical fungicides have a residual effect, which has caused an impact on human health resulting in cancer, sterility, brain damage, birth anomalies, respiratory diseases, organ failure, and skin irritation. Also, pathogen resistance to the relevant chemical agents may develop as a result of these substances.

In response to environmental and health concerns, there is a lot of interest in finding alternative management approaches for crop diseases, such as integrated disease management strategies. It appears inevitable that fewer pesticides will be used in the future and greater reliance will be placed on biological technology which includes microorganisms and bio-stimulants that act as antagonists.

Trichoderma, an endophytic fungus, is well-known and frequently employed as a biocontrol agent for its ability to promote plant growth in the face of biotic and abiotic stress. Several *Trichoderma* strains have been identified, including *T. harzianum*, *T. asperellum*, *T. atroviridae*, *T. virens*, and *T. viride*. Its evolution adds to our understanding of the adaptable behaviour required to survive in plants. *Trichoderma* strengthens the plant's overall defence by colonizing the rhizosphere and triggering systemic resistance, so providing a barrier to pathogen invasion and boosting plant nutrition intake through solubilization of phosphates and micronutrients. (Patel *et al*., 2019).

Many species of them can produce enzymes such as hemicellulose and cellulose, making them commercially and industrially valuable. *Trichoderma* looks to be a potential alternative to pesticides and chemical fertilizers for enhancing crop productivity and growth, as well as other beneficial benefits of rhizosphere activity (Singh *et al*., 2014). It also produces antibiotics and antifungal metabolites that may be utilized to fight a wide range of plant pathogens. *Trichoderma* spp. and/or their secondary metabolites have shown potential in the management of plant diseases, including fungal infections, bacteria, and viruses. Resistance mechanisms akin to the hypersensitive response (HR), systemic acquired resistance (SAR), and induced systemic resistance (ISR) in plants are induced as a result of this (Harman *et al*., 2004).

Salicylic acid (SA; 2-hydroxybenzoic acid) is one of several phenolic compounds produced by plants (described as compounds with a benzene ring having one or more hydroxyl groups). It is a plant hormone that is involved in basal resistance against plant pathogens. Salicylic acid (SA) has been identified as a key signal molecule in the modulation of plant responses to stress (Bergmann *et al*., 1994; Breusegem *et al*., 2001). Exogenous application SA may trigger a range of plant processes, including seed germination, stomatal closure, ion absorption and transport, membrane permeability, photosynthetic and growth rate (Korkmaz, 2005).

Salicylic acid (SA) in addition to stimulating plant growth, has also been reported to play a function in alleviating the negative impacts of various environmental stresses, such as low temperature on peppers, high temperature, heavy metals, phytopathogens, and drought on various crops viz; cucumber, bean, tomato and wheat (Shi *et al*., 2006). Pathogen-induced systemic acquired resistance (SAR) is based on salicylic acid (SA) signalling and leads to the production of pathogenesis-related proteins (PR) and phytoalexins, which may protect against later attacks (Sticher *et al*., 1997).

Melatonin (N-acetyl-5-methoxytryptamine), an animal hormone identified in the pineal gland of a cow in 1958 (Lerner *et al*., 1958) which has a wide range of roles in humans and animals. In 1995 and it has been discovered in plants, where it has a wide range of regulatory functions (Hattori *et al*., 1995). Melatonin is a pleiotropic molecule that has a wide range of cellular and physiological effects in a plant. In plants, Melatonin is found in different organs like seeds, roots, stems, shoots, leaves, flowers, bulbs and fruits. Its amount varies from picograms and micrograms. Melatonin has been shown to have a role in a variety of physiological reactions to environmental stress, rerouting plant growth and development, and shielding developing tissues from harm in stressful situations. Melatonin is a potent antioxidant that protects the plant against reactive oxygen and nitrogen species (ROS/RNS). The functions of melatonin in plants as a protective agent against various stress situations are the most studied area among researchers.

Melatonin accumulation in plants increases resistance to foliar diseases like powdery mildew and soil-borne oomycetes in watermelon and other cucurbits by affecting the expression of genes involved in ETI- and PAMP-mediated defences (Mandal *et al*., 2018).

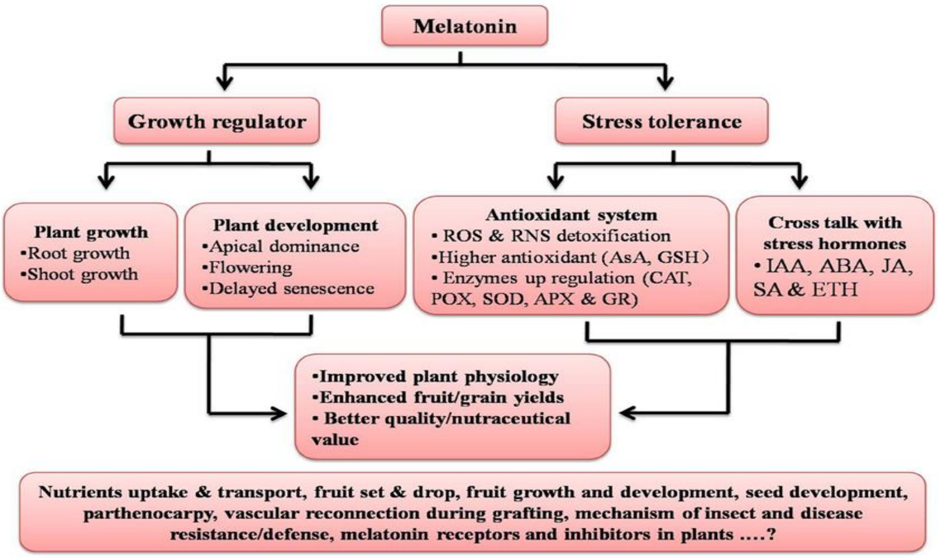

Figure 1.2. Role of Melatonin in plant. ROS, Reactive oxygen species; RNS, Reactive nitrogen species; AsA, Ascorbic acid; GSH, Glutathione; CAT, Catalase; POX, Peroxidase; SOD, Superoxide dismutase; APX, Ascorbate peroxidase; GR, Glutathione reductase; IAA, Indole-3- acetic acid; ABA, Abscisic acid; JA, Jasmonic acid; SA, Salicylic acid; and ETH, Ethylene (Nawaz *et al*., 2016)

Considering all these factors, it seems that *Trichoderma*, salicylic acid, and melatonin is an environmentally friendly and may represent an economical alternative strategy to induce plant protection against biotic and abiotic stress and at the same time promote better plant growth and development that's why in the present study I have selected following objectives: -

- To evaluate the efficacy of *Trichoderma* spp. on different phytopathogens (*Alternaria solani*, *Curvularia oryzae*, *Phomopsis vexans*, *Colletotrichum capsici*, *Fusarium oxysporum* f.sp. *lycopersici, and Bipolaris sorokiniana*).
- To investigate the impact of salicylic acid, melatonin and *Trichoderma* spp. on physio-biochemical parameters of cucumber (*Cucumis sativus* L.)

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Chapter-II

REVIEW OF LITERATURE

This chapter focuses on the research work conducted by different researchers with a similar background related to the application of salicylic acid, Melatonin, and *Trichoderma harzianum* by both foliar and drenching method and their effect to induce resistance against both biotic and abiotic stress and also enhancing growth and development on cucumber (*Cucumis sativus* L.*)*. The biochemical test like TPC, PAL, PO, PPO, catalase, proline content, chlorophyll content, and protein content is discussed. The research work related to antimicrobial properties of *Trichoderma* spp. against different pathogens is also focused.

2.1 Application of Salicylic acid and its effect on the plant

Metraux *et al*., (1990) identified the Changes in the amount of phloem sap in cucumber plants treated with either tobacco necrosis virus or the fungal pathogen *Colletotrichum lagenarium* were studied in an attempt to discover the signal molecule that induces systemic acquired resistance (SAR). After inoculation, the concentration of a fluorescent metabolite increased transiently, reaching a peak before SAR was identified. The chemical was isolated and identified as salicylic acid, a recognized exogenous inducer of resistance, using gas chromatography-mass spectrometry. The findings show that salicylic acid may have a role in SAR transmission in cucumber as an endogenous signal.

Gaffney *et al*., (1993) examined the role of salicylic acid in systemic acquired resistance in transgenic tobacco plants containing a bacterial gene expressing salicylate hydroxylase, which converts salicylic acid to catechol. Transgenic plants expressing salicylate hydroxylase produced very little or no salicylic acid and were unable to develop acquired resistance to the tobacco mosaic virus. As a result, salicylic acid is required for the establishment of tobacco systemic acquired resistance.

Delaney *et al*., (1994) reviewed that salicylic acid accumulation is required for the manifestation of several forms of plant disease resistance.

Segarra *et al*., (2006) reported that the level of endogenous salicylic acid (SA) in cotyledons and roots was 30.96 and 8.31 ng g^{-1} fresh weight, respectively in noninfested control whereas plants infected with *Rhizoctonia solani* showed up to 2-fold higher concentration of SA than noninfested cucumber plant which induced basal resistance against plant pathogen. Also, the levels of SA in plants treated with the biological control agent *Trichoderma asperellum* strain T-34 were similar to those in control plants.

Shi and Zhu (2007) studied the effects of salicylic acid (SA) on manganese toxicity in cucumber by investigating the symptoms, antioxidative enzymes, antioxidants, lipid peroxidation, and plant growth. The exogenous application of salicylic acid slowed down the movement of Mn from roots to shoots, checked Ca, Mg and Zn absorption induced by excess Mn, decreased the toxicity symptoms, and promoted the growth and development of the cucumber plant. ROS and lipid peroxidation levels were dramatically reduced when SA was added. Catalase (CAT) and ascorbate peroxidase (APX) activities were decreased by SA, but superoxide dismutase (SOD), peroxidase (POD), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) activities were elevated in cucumber leaves exposed to high Mn. SA treatment significantly increased the levels of essential antioxidants, ascorbate, and glutathione in cucumber leaves exposed to excess Mn.

Daw *et al*., (2008) examined the effects of two methods of exogenous applications (foliar and drenching) of salicylic acid (SA) on host resistance and biochemistry of near-isogenic lines (4) of rice against blast disease (*Magnoporthe grisea)*. There was enhanced suppression of blast lesion with the foliar application of SA at 8 mM concentration in two susceptible isogenic lines. All isogenic lines showed an increase in peroxidase and polyphenol enzyme activity with exogenous SA treatment. A higher concentration of four rice phytoalexins (oryzalexin A, C, and F, and momilactone) was detected in crude leaf extract when the chemical analysis was performed in root treated plant. Inducible conjugate antifungal compounds were also found. These findings show that exogenous SA treatment of rice can boost the synthesis of enzymes involved in oxidative stress resistance and antifungal chemicals, both of which appear to play a role in rice resistance to blast fungal pathogen invasion.

Zhang *et al*., (2010) reviewed that salicylic acid is a defensive hormone found in plants that has a function in both local and systemic acquired resistance.

War *et al*., (2011) reported the biochemical response at different concentrations of salicylic acid ((1.0, 1.5, and 2 mM). The response of the plant at 1.5 mM was very fast. It showed higher induction of peroxidase (POD) and polyphenol oxidase (PPO) activities, along with the higher accumulation of phenols, H_20_2, and proteins which can be utilized for the induction of plant defence.

Kazemi (2013) studied the effect of foliar application of salicylic acid at three concentrations (0.25, 0.5, and 0.75 mM) on the growth, yield, and yield components of strawberry plants. He found that at 0.25 mM of SA, the vegetative and reproductive growth was affected significantly. In low salicylic acid and calcium chloride concentrations, mean comparisons showed that strawberry plant yield and quality increased. Finally, the use of salicylic acid and calcium chloride can aid increase yields and prevent them from declining.

Yildirim *et al*., (2013) researched the effect of foliar applications of salicylic acid (SA) at different concentrations (0.0, 0.25, 0.50, and 1.00 mM) on the growth, mineral and chlorophyll content of cucumber grown under salt stress. Cucumber growth, chlorophyll content, and mineral absorption were all significantly impacted by salt stress. Foliar applications of SA, on the other hand, resulted in increased shoot fresh weight and dry weight, root fresh weight and dry weight, as well as more plants under salt stress. The number of leaves per plant was also increased. The best result was obtained with 1.00 mM SA treatment in which the greatest chlorophyll content and lower values of electrolyte leakage were observed both in saline and non-saline conditions.

Kunzniak *et al*., (2015) suggested in their findings that angular leaf spot disease caused by *Pseudomonas syringae* pv *lachrymans* was lowered due to arbutin induce suppression which could be mediated by salicylic acid-based signalling.

Randjelovic *et al*., (2015) reviewed that salicylic acid is a phenolic compound found in a variety of plants, where it plays an important role in pathogen defence.

Abbasi *et al*., (2020) investigated the effect of different concentrations of salicylic acid i.e (0.00, 0.001, 0.01, 0.10, and 1.00 mM), and two methods of its application, one was foliar and another one was seed priming, on morphological and physiological traits of cucumber in greenhouse condition. One-time foliar treatment in seedlings at the four-leaf stage and seed priming in salicylic acid solutions for 24 hours were used as the application methods. They found that the concentration of 0.01 mM was showing the highest values for measured traits (stem length, number of fruits per plant, number of leaves fresh and dry weight of stems, number of branches fruit diameter, and dry weight of leaves and fruits) and also disease incidence was not observed by seed priming method. Foliar application at the concentration of 0.10 and 1.00 mM showed the presence of the highest percentage of potassium and phosphorus in the leaf samples.

Abdelhameed *et al*., (2021) investigated the effect of foliar spray of salicylic acid (0.5 mM) on the physiological features of fenugreek (*Trigonellafoenum-graecum* L.) plants growing under three salt concentrations (0, 75, and 150 mM NaCl). SA treatment resulted in an increase in proline, total phenolics, and flavonoids. Furthermore, foliar application of SA resulted in higher levels of total free amino acids and shikimic acid in both control and salt-treated plants. With SA application, similar findings were achieved for ascorbate peroxidase, peroxidase, polyphenol oxidase, and catalase. The content of chlorophyll was also boosted by the foliar application of SA. As a result, we concluded that foliar application of SA reduces salinity and is a growth regulator that increases fenugreek plant resistance to salt stress.

Galviz *et al*., (2021) studied the effect of two methods of application (seed priming and drenching) of salicylic acid (SA) on the vegetative growth of tomato (*Solanum lycopersicum* L.) during water deficit condition. Under drought circumstances, seed priming with SA caused increases in leaf area, root dry weight, and root/shoot ratio. In well-irrigated plants, soil drenched with SA improved CO2 assimilation rate and stomatal conductance. Even though the activity of antioxidant enzymes was not increased in general by the treatment of SA, cellular redox homeostasis was maintained due to increases in proline and total soluble sugars resulting from starch degradation.

2.2 Application of melatonin and its effect on the plant

Tekbas *et al*., (2007) studied the antibacterial effects of melatonin against bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Acinetobacter baumannii* in vitro. The best results were noticed after 48 hr of incubation at a lower dose concentration (0.13-0.53 mM) and more potent effects were found on gram-negative bacteria. Hence, melatonin had antibacterial effects by checking intracellular substrates.

Park and Back (2012) investigated that melatonin helped the growth of roots in transgenic rice after germination and also encouraged the growth of seminal roots in the wild type at the concentration of 0.5 and 1.0 μM. Hence, it regulated both root growth and seminal root length after germination in transgenic rice plants.

Lihua Yin *et al*., (2013) showed that exogenous application of melatonin on apple enhanced the resistance against Marssonina apple blotch (*Diplocarpon mali).* They investigated that melatonin was able to regulate the levels of hydrogen peroxide, activities of antioxidant enzymes, and PR protein at the time of plant-pathogen interactions. Among the concentrations of melatonin (0.05, 0.1, 0.5 mM), 0.1 and 0.5 mM showed better results in which incidence of disease declined by 54% and 51% respectively.

H.Y. Lee *et al*., (2014) suggested that melatonin is a novel molecule that acts as defence signalling in plant-pathogen interactions. They found that the melatonin-treated *Arabidopsis* plant induced greater resistance against the virulent bacteria *Pseudomonas syringae* DC3000 which was 10 times the normal plants. At 10 μM concentration, it induced various PR genes (pathogenesis-related), as well as many defence genes which were activated by salicylic acid and ethylene. Hence, it helps in plant innate immune response.

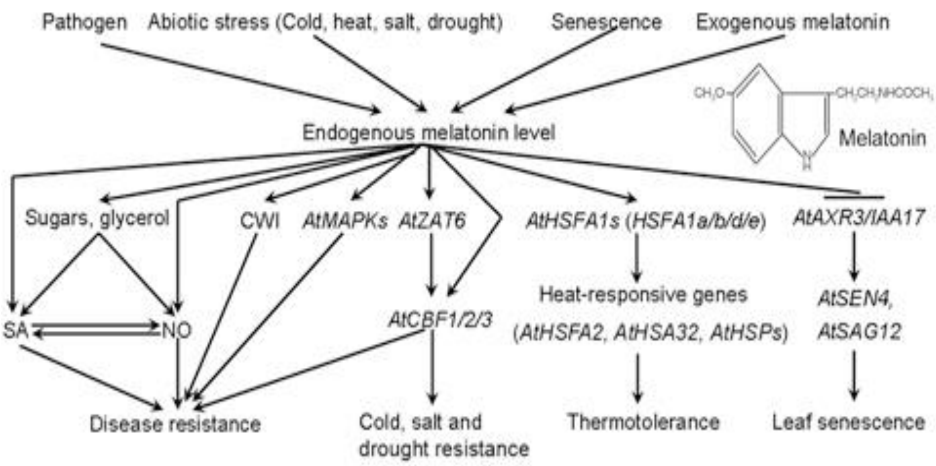

Figure 2.1. Hypothetical model explaining melatonin-mediated stress responses in Arabidopsis (Shi *et al*., 2015)

Byeon *et al*., (2014) suggested that melatonin is a novel molecule that acts as defence signalling in plant-pathogen interactions. They found that the melatonin-treated tobacco plant induced greater resistance against the virulent bacteria *Pseudomonas syringae* which was 10 times the normal plants. At 10 μM concentration, it induced various PR genes (pathogenesis-related), as well as many defence genes which were activated by salicylic acid and ethylene. Hence, it helps in plant innate immune response.

Shi *et al*., (2015) investigated the level of melatonin and nitric acid were induced upon the infection of bacterial pathogen *Pseudomonas syringe* pv. tomato in *Arabidopsis*. It was found that melatonin was responsible for the induction of endogenous nitric oxide production which was responsible for innate immunity in Arabidopsis against the infection of *Pseudomonas syringe* pv. tomato.

Liu *et al*., (2015) reported that different species of *Trichoderma* (*T. koningii*, *T. harzianum, T. asperellum*, *T. longibrachiatum*, and *T. viride*) were capable of producing endogenous melatonin. Mycelia mats, culture filtrates, and HPLC chromatogram were used to detect the presence of melatonin in these species.

Hasan *et al*., (2015) reported that the exogenous application of melatonin gradually decreased the deleterious effect of cadmium (Cd) in tomato (*Solanum lycopersicum* L). They found that level of melatonin was also increased in those plant which was treated with cadmium but stable in control. Among different concentrations of melatonin, 100 µM had a greater ameliorative effect as compared with plants only treated with cadmium.

Wang *et al*., (2016) showed that the photosynthetic capacity of cucumber was improved due to the exogenous application of melatonin (50-150 µM) under salt-stressed conditions. Melatonin pre-treated plant enhanced the concentrations of the antioxidant (ascorbic acid and glutathione) and antioxidant enzyme (superoxide dismutase, peroxides, catalase) activity.

Zhang *et al*., (2017) studied that pre-treatment of melatonin had a greater effect on the growth, mineral nutrition, and nitrogen metabolism in cucumber under nitrate stress. The growth of plants and reduced level of effect due to high level of nitrate was seen at 0.1 mM of melatonin application.

Mihir *et al*., (2018) studied the role of melatonin as an environmental-friendly immune inducer in watermelon (*Citrullus lanatus*) and other cucurbits which helps in boosting immunity against the pathogens. They found that the level of melatonin had increased which provided resistance against foliar pathogen powdery mildew (*Podosphaaera xanthii*), and soil-borne pathogen (*Phytophthora capsica*) in watermelon as well as in other cucurbits. It had also been suggested that the expression of those genes involved in pathogen-associated molecular pattern (PAMP) and effector-triggered immunity (ETI) mediated defences got altered with a 1mM concentration of melatonin sprayed on watermelon leaves.

Yunkuo *et al*., (2019) analysed that when the melatonin was given as pre-treatment, it acted as the resistance to downy mildew of cucumber. The application of 100 $\mu ML^{-}1$ melatonin followed by inoculation showed an increased level of antioxidant ability. The disease incidence was also reduced after pre-treatment. Along with these, photosynthetic efficiency and chlorophyll content had also been shown to increase which means that melatonin helps in benefiting fruit yields as well as resistance to downy mildew.

Chen *et al*., (2019) investigated the infection caused by *Xanthomonas oryzae* pv. *oryzicola* in susceptible rice (IR24) was reduced significantly by 17% after the application of melatonin. It was found that the lesion length was more than 23% smaller in the pre-treated melatonin (200 µg ML^{-1}) plant than in the control one. The activities like biofilm formation, extracellular protease, and swimming motility of *Xanthomonas oryzae* pv. *oryzicola* was also sharply reduced under melatonin treatment. Hence, melatonin seemed to be effective in reducing the pathogenicity of *Xanthomonas oryzae* pv. *oryzicola* towards this susceptible rice (IR24).

Zhang *et al*., (2020) studied the effect of exogenous application of melatonin (50, 100, 200, 300 µML^{-}1) in mollifying salt stress in cucumber. The pretreatment of exogenous melatonin of concentration 100 µML-1 showed the best result in alleviating salt stress. When compared to salt exposure alone, exogenous melatonin pre-treatment improved cell viability, protected photosynthesis, increased antioxidant enzyme activity, inhibited active oxygen explosion, reduced malondialdehyde (MDA) content, and reduced relative conductivity in cucumber seedlings.

Brengi *et al*., (2021) suggested that with the foliar application of 100 µML^{-1} of melatonin, the tolerance of cucumber plants to salt stress conditions got enhanced along with the increase in protein, catalase activity, and essential nutrients like nitrogen, phosphorus, potassium, and calcium.

2.3 Application of *Trichoderma* and its effect on the plant

Lorito *et al*., (1993) reported that three types of siderophores i.e a trimer disdepsipeptide (copragen), a monohydroxamate (cis- and trans-fusarinines), and a dipeptide of trans- fusarinine (dimerum acid) was produced by T. *virens*. Microbial membrane receptor proteins (identify and take up the siderophores-Fe-complex, which was chelated by siderophores. As a result, rhizosphere bacteria, including plant diseases, created fewer or different siderophores with lower binding coefficients, rendering iron accessible to them. As a consequence, pathogen infection was reduced and biological control was improved.

Nelson and Harman (1997) demonstrated that the combination of granule broadcast treatments of *Trichoderma harzianum* followed by foliar applications of its conidia minimized root and foliar disease damage.

Yedidia *et al*., (2003) investigated that the drenching of *T. asperellum* in the cucumber induced the systemic response against angular leaf spots. They also noted that plant defence response was initiated when *T.* harzianum T- 203 spores were inoculated to old seedlings of cucumber (7 days) in the aseptic hydroponic system.

Lee *et al*., (2006) investigated that foliar and potting mixing + foliar application of *Trichoderma* (*Trichoderma harzianum* YC459) greatly reduced the severity of gray mold (*Botrytis cinerea*) in cucumber and tomato. Potting mix with granular formulation followed by foliar application had better results as compared to leaf spray and granular formulation alone.

Segarra *et al*., (2007) investigated that Plants treated with *Trichoderma* became even more resistant to pathogen attacks as a response to changes in the expression of a series of defense-oriented proteins that can either directly protect the plant or shift the metabolism to a defensive mode.

T. N Ha, (2010) studied that the strains of *Trichoderma* were able to reduce the incidence of many soil-borne fungal diseases such as *Rhizoctonia solani*, *Fusarium* spp., *Sclerotium rolfsii*, *Phytophthora palmivora,* and *pythium* spp of several crops like peanut, tomato, cucumber, and durian, higher than fungicides along with this the efficacy was longer. Thus, it is helpful not only in controlling plant disease but also in reducing health risks to the grower and the consumer besides this it lowers the cost and environmental damages. Moreover, they noted the better and higher yield in those crops that were treated with *Trichoderma* than the normal ones.

John *et al*., (2010) reported enhanced root and shoot growth as well as fruit yield in infected soybean plants treated with *Trichoderma* as compared to the control plant. The fruit yield was near about 20% higher in *Trichoderma* treated plants. Similarly, the height of *Trichoderma* treated plants but infected with Pythium and Fusarium spp was ~194% and 141% higher than plants with pathogen alone.

Zaidi and Singh (2013) reported *Trichoderma* to have plant growth-promoting, antifungal, antinematode, and plant defense-inducing activities. Hence, it has been widely used as a biocontrol agent.

Singh *et al*., (2016) studied the seed germination and development and defense response of bioprimed seeds with different doses (10^2 to 10^8 spores ml^{-1}) of *Trichoderma asperellum* BHUT8 on six vegetable crops (brinjal, tomato, ridge gourd, chili, guar, and okra). At 10^3, 10^4 and 10^6 spores ml^{-1}, seed germination, and radical length were more in tomato, brinjal and okra, chili and gaur respectively. Seed germination was also found to be enhanced with a greater percentage value in these crops. Along with this phenylpropanoid activities and lignification in tomato bioprimed seedlings were detected which induced defense response in it. Hence, it is clear from this study that *Trichoderma asperellum* helps in growth and development as well as triggers defense responses in crops.

Mei *et al*., (2019) demonstrated that three *Trichoderma* strains (T. *asperellum* 525, T. *harzianum* 610, and T. *pseudokoningii* 866) exhibited excellent growth and disease prevention effects on cucumber. *Trichoderma* 866 was the most successful of the three strains, with a disease control efficiency of 78.64 percent and a 33 percent increase in cucumber yield. Superoxide dismutase (SOD), peroxidase (POD), polyphenol oxidase (PPO), catalase (CAT), and ascorbate oxidase (AAO) like stress-resistance enzyme activities significantly increased along with chlorophyll content, nitric nitrogen content, plant height, stem diameter, above and underground fresh weight, and leaf area, in *Trichoderma* inoculated seedlings. Their findings imply that inoculating cucumbers with *T. asperellum* 525, *T. harzianum* 610, and *T.* pseudokoningii 886 inhibits F. oxysporum infection, promotes cucumber metabolism, and boosts stress-resistance enzyme activity, all of which increase cucumber plant growth, and enhance cucumber yield and quality.

Palacios-Torres *et al*., (2019) studied how foliar treatment of *Trichoderma* affected the quality of tomato fruits at different cluster levels on the same plant in two commercial hybrids (Ramses and Cid) grown in a greenhouse on three different substrates (river sand, tezontle, and tepezil). Six foliar treatments of a liquid biological preparation of *Trichoderma* (T. *asperelloides* + T. *koningiopsis*) were applied every 15 days following transplanting at a dosage of 4 mL L^{-1} (1×10^8 conidia per ml). Titratable acidity of fruits, pH, and electrical conductivity was all improved by *Trichoderma*. The addition of *Trichoderma* to the Cid hybrid growing in the tezontle substrate enhanced the ratio of total soluble solids to titratable acidity. Sand enhanced the percentage of juice in the fruits at the same time. *Trichoderma* foliar treatment improved the quality of tomato fruits and should be regarded as a crop management tool. Hence, *Trichoderma* are biostimulants that have the ability to promote plant growth and development, as well as fruit quality.

Manzar *et al*., (2020) studied the management of anthracnose of sorghum (*Colletotrichum graminicola*) with the different isolates of *Trichoderma asperellum* (T3, T4, T15, AND T19) and *Trichoderma harzianum* (T6) by a different mode of application (seed biopriming, soil application and combination of both). They found that as compared to individual seed biopriming and soil application of different isolates of *Trichoderma*, the additive effect of soil enriched with FYM + seed biopriming + foliar spraying was found to be highly effective for reducing the percent disease index (PDI) and increasing grain yield.

2.4 Biochemical assay

Induce Systemic Response (ISR) has a crucial role in plant defence mechanisms. It reprogrammes and mobilizes defence-related enzymes such as pathogenesis-related (PR) proteins, peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL) and superoxide dismutase (SOD), as well as induce proline and phenol (Jain *et al*., 2012). According to Yukie Momose *et al*., (2003) oxidative stress leads to a decline in catalase activity in many plant species, which is linked to the build-up of salicylic acid in oxidatively stressed plants. According to Yasmin *et al*., (2016) phenolic compound production in plants in response to pathogen challenge is linked to resistance, and its higher accumulation is attributable to PAL activity providing protection to the plants. Rashid *et al*., (2011) reported the biochemical response induces peroxidase (POD) and polyphenol oxidase (PPO) activities, along with the higher accumulation of phenols, H_20_2, proteins, and chlorophyll which can be utilized for the induction of plant defence along with better growth and development in cucumber.

2.4.1 Phenylalanine Ammonia Lyase (PAL) assay

According to Vanitha *et al*., (2009) when *Ralstonia solanacearum* was inoculated on resistance, susceptible, and highly susceptible, the activities of PAL were maximum in resistance cultivars as compared to both other cultivars which triggered host resistance.

Patel *et al*., (2017) reported that the activity of PAL had increased nearly two-fold when pea seed was treated with *Trichoderma asperellum* and *Pseudomonas fluorescens* (OKC) were challenged with powdery disease (*Erysiphe pisi*).

Several other studies reported that PAL activity is associated with the reduction of incidence of disease and the development of resistance to plant pathogens (Chandrasekaran *et al*., 2017)

Singh *et al*., (2020) reported that *Trichoderma asperellum* and *Ochrobactrum* spp. when bioprimed on tomato seeds, the activities of PAL had significantly enhanced upto 72 hrs during the attack of wilt pathogen (*F. oxysporum* f.sp. *lycopersici*).

2.4.2 Peroxidase (PO) assay

Yasmin *et al*., (2016) reported that rice plants inoculated with *Pseudomonas* sp. Rh323, PO activity was maximum after 48 hrs of inoculation of *Xanthomonas oryzae* pv. *oryzae* in comparison to control.

Patel *et al*., (2017) observed that in comparison to non-challenged plants, *E. pisi* challenged plants had a greater H_2O_2 accumulation pattern. At 48 hours, the accumulation of H_2O_2 was constant in pathogen-challenged and *T. asperellum* (T42) and *P. fluorescens* (OKC) infected plants, but the level of H_2O_2 was dramatically decreased in just pathogen-challenged plants without microbial treatment.

Patel *et al*., (2017) reported that the activity of PO had increased nearly two-fold when pea seed was treated with *Trichoderma asperellum* and *Pseudomonas fluorescens* (OKC) were challenged with powdery disease (*Erysiphe pisi*).

Singh *et al*., (2020) reported that *Trichoderma asperellum* and *Ochrobactrum* spp. when bioprimed on tomato seeds, the activities of PO had significantly enhanced upto 72 hrs during the attack of wilt pathogen (*F. oxysporum* f.sp. *lycopersici*).

2.4.3 Polyphenol oxidase (PPO) assay

J. C. Steffens (2002) investigated that PPO activity had increased many times (upto 30-fold) in transgenic tomato plant (*Solanum tuberosum* L.) which led to enhancing resistance to *Pseudomonas syringae* pv. *Tomato.* The severity of disease symptoms and lesions had reduced to 15-fold in the infected plant as compared to the control one.

Akhtar and Mahmood (2017) reported that the seed of *Arabidopsis thaliana* which has *oryza sativa* Polyphenol oxidase gene when exposed to drought and salt stress, the activity of PPO get increased and make the plant tolerant to these stresses.

Mei *et al*., (2019) demonstrated that three *Trichoderma* strains (T. *asperellum* 525, T. *harzianum* 610, and T. *pseudokoningii* 866) exhibited excellent growth and disease prevention effects on cucumber. *Trichoderma* 866 was the most successful of the three strains. Polyphenol oxidase (PPO) like stress-resistance enzyme activity significantly increased along with chlorophyll content, nitric nitrogen content, plant height, stem diameter, above and underground fresh weight, and leaf area, in *Trichoderma* inoculated seedlings.

2.4.4 Total Phenolic Content (TPC) assay

Vanitha *et al*., (2009), reported that total phenol content was increased in all tomato cultivars (highly resistant, resistant, susceptible, and highly susceptible) after 12 hours of *Ralstonia solanacearum* inoculation as compared to their respective controls. After pathogen inoculation, the resistant cultivar also showed the maximum phenolic compound activity when compared to the other cultivars

According to Jain *et al*., (2012) at 48 hours after inoculation with Sclerotinia sclaerotina, the pea seeds treated with Pseudomonas aeruginosa, *T. harzianum*, and Bacillus subtilis showed the maximum increase of total phenolics. When untreated plants challenged or unchallenged with S. sclerotiorum were investigated, the phenol accumulation was shown to be greater in plants treated with microbial agents (P. aeruginosa, *T. harzianum*, and B. subtilis) as compared to previous one.

According to Datta and Lal (2012), wilt resistant cultivars of pigeonpea root showed considerable increases in phenolics, but the rise in susceptible cultivars was negligible at the advent of infection by F. *udum* relative to before the onset. Pigeonpea vulnerable cultivars had lower levels of phenolics and were unable to acquire resistance to wilt disease. However, resistant pigeonpea cultivars had more phenolics and were thus capable of resisting F. *udum*.

Jain *et al*., (2014) reported that seeds and pericarp of pea when treated with a consortium of B. subtilis, P. aeruginosa and *T. harzianum* had maximum TPC content.

Patel *et al*., (2017) reported that the activity of PO had increased nearly two-fold when pea seed was treated with *Trichoderma asperellum* and *Pseudomonas fluorescens* (OKC) were challenged with powdery disease (*Erysiphe pisi*).

Singh *et al*., (2020) reported that *Trichoderma asperellum* and *Ochrobactrum* spp. when bioprimed on tomato seeds, the activities of TPC had significantly enhanced upto 72 hrs during the attack of wilt pathogen (*F. oxysporum* f.sp. *lycopersici*).

2.4.5 Catalase (CAT) assay

Vanitha *et al*., (2009), reported that CAT activity was increased in all tomato cultivars (highly resistant, resistant, susceptible, and highly susceptible) after 12 hours of *Ralstonia solanacearum* inoculation as compared to their respective controls. After pathogen inoculation, the resistant cultivar also showed the maximum phenolic compound activity when compared to the other cultivars

According to Jain *et al*., (2012) at 48 hours after inoculation with Sclerotinia sclaerotina, the pea seeds treated with Pseudomonas aeruginosa, *T. harzianum*, and Bacillus subtilis showed the maximum increase of CAT. When untreated plants challenged or unchallenged with *S. sclerotiorum* were investigated, the CAT accumulation was shown to be greater in plants treated with microbial agents *(P. aeruginosa, T. harzianum, and B. subtilis*) as compared to previous one.

Yasmin *et al*., (2016) reported that rice plants inoculated with *Pseudomonas* sp. Rh323, CAT activity was maximum after 48 hrs of inoculation of *Xanthomonas oryzae* pv. *oryzae* in comparison to control.

Patel *et al*., (2017) reported that the activity of CAT had increased nearly two-fold when pea seed was treated with *Trichoderma asperellum* and *Pseudomonas fluorescens* (OKC) were challenged with powdery disease (*Erysiphe pisi*).

Several other studies reported that CAT activity is associated with the reduction of incidence of disease and the development of resistance to plant pathogens (Chandrasekaran *et al*., 2017)

According to Wu et al., (2019), the CAT activity was highest 3 days after inoculation of *Bacillus subtilis* SL-44 in pepper, which was 15.8% higher than the control. In addition, the CAT enzyme showed the first increase and subsequently a drop in response to varied times of *Rhizoctonia solani* inoculation.

Singh *et al*., (2020) reported that *Trichoderma asperellum* and *Ochrobactrum* spp. when bioprimed on tomato seeds, the activities of CAT had significantly enhanced upto 72 hrs during the attack of wilt pathogen (*F. oxysporum* f.sp. *lycopersici*).

2.4.6 Proline assay

Two-day barley plants' shoot axils and excised root systems, as well as portions of the lamina and leaf sheath of the first leaf, were incubated in aerated polyethylene glycol solution (osmotic potential -20 bars) or distilled water. The amino acid proline accumulated quickly in leaf lamina sections subjected to osmotic stress, but only slowly in those floating on the water after a 16-hour delay (TN Singh *et al.,* (1997).

Yildirim *et al*., (2013) reported that the foliar applications of salicylic acid (SA) at different concentrations (0.0, 0.25, 0.50, and 1.00 mM) resulted in an increase in proline content. The best result was obtained with 1.00 mM SA treatment in which the greatest proline content and lower values of electrolyte leakage were observed both in saline and non-saline conditions

According to Vives-Peris *et al*., (2017) when plants were exposed to salt or heat stress, more proline was released into the rhizosphere. The quantity of this amino acid was greater in exudates collected from plants tolerant to different stress conditions. Furthermore, stress conditions and each genotype's tolerance to the particular stress have a considerable quantitative and qualitative impact on the exudation pattern.

Manzer H. Siddiqui *et al*., (2018) reported that the application of melatonin on tomato seedlings improved the physio-biochemical parameters like proline content in the leaves. The 20 and 50 μM concentration was found to be best in this response. Moreover, proline greatly helped in making plant tolerance to salt stress conditions.

Abdelhameed *et al*., (2021) investigated the effect of foliar spray of salicylic acid (0.5 mM) on the physiological features of fenugreek (*Trigonellafoenum-graecum* L.) plants growing under three salt concentrations (0, 75, and 150 mM NaCl). SA treatment resulted in an increase in proline in both control and salt-treated plants.

2.4.7 Chlorophyll and Protein assay

Rashid war *et al.*, (2011) reported the biochemical response at different concentrations of salicylic acid ((1.0, 1.5, and 2 mM). The response of the plant at 1.5 mM was very fast. It showed higher induction of chlorophyll and protein content which can be utilized for the induction of cucumber plant defence and growth and development.

Yildirim *et al*., (2013) researched the effect of foliar applications of salicylic acid (SA) at different concentrations (0.0, 0.25, 0.50, and 1.00 mM) on the growth, mineral, protein, and chlorophyll content of cucumber grown under salt stress. Cucumber growth, chlorophyll content, and mineral absorption were all significantly impacted by salt stress. Foliar applications of SA, on the other hand, resulted in increased shoot fresh weight and dry weight, root fresh weight and dry weight, as well as more plants under salt stress. The number of leaves per plant was also increased. The best result was obtained with 1.00 mM SA treatment in which the greatest chlorophyll content and lower values of electrolyte leakage were observed both in saline and non-saline conditions.

Mei *et al*., (2019) demonstrated that three *Trichoderma* strains (T. *asperellum* 525, T. *harzianum* 610, and T. *pseudokoningii* 866) exhibited excellent growth and disease prevention effects on cucumber. *Trichoderma* 866 was the most successful of the three strains, with a disease control efficiency of 78.64 percent and a 33 percent increase in cucumber yield. Chlorophyll content, nitric nitrogen content, plant height, stem diameter, above and underground fresh weight, and leaf area, in *Trichoderma* inoculated seedlings had increased to sufficient value.

Sun *et al*., 2019 analysed that when the melatonin was given as pre-treatment, it acted as the resistance to downy mildew of cucumber. The application of 100 μML^{-1} melatonin followed by inoculation showed an increased level of photosynthetic efficiency and chlorophyll content had also been shown to increase which means that melatonin helps in benefiting fruit yields as well as resistance to downy mildew.

2.5 Antimicrobial assay

Antibiosis is the mechanism that involves the production of extracellular compounds by *Trichoderma* spp (biocontrol agents) which restrict the growth of pathogens present in its vicinity. *Trichoderma viride* produces the compounds like viridin, viridiol, pyrone, etc. similarly *Trichoderma harzianum* secretes harzianopyridone, harzianic acid, harzianolide, azaphilones, and some other steroids which inhibit in vitro the growth of a wide range of plant pathogens including fungal and both gram-positive and negative bacterial (Francesco et al., 2014).

According to Scarselletti and Faull (1994) *Trichoderma harzianum* acted as antagonistic to *Fusarium oxysporum* f.sp. *lycopersicum* in vitro. The growth of pathogen reduced by 31.7% just in 2 days.

According to Sumangala *et al*., (2008) in vitro study shows that the radial growth of the fungus *Curvularia lunata* was inhibited by 96.44% and 93.50 % by *Trichoderma viride* and *Trichoderma harzianum* respectively.

Sanjeev Choubey and R.K Patil (2009) found that the maximum inhibition of mycelial growth of *Phomopsis phyllanthi* which were 67.52% and 66.65% was observed in dual culture with *Trichoderma harzianum* and *Trichoderma viride* respectively.

Abhiram and Masih (2018) studied that when the dual culture of *Trichoderma viride* is done against the different strains of *Fusarium oxysporum*, growth is checked at over 71 %. It shows that *T. viride* has greater potential as a biocontrol agent.

SC *et al*., (2020) reported that when the in vitro biocontrol efficacy of *Trichoderma harzianum* and *Trichoderma viride* was checked against *Alternaria solani*, the maximum inhibition of radial growth was 85.13% and 80.67% respectively.

Sutarman *et al*., (2020) investigated that *Trichoderma harzianum* was an effective biocontrol against anthracnose of chili (*Colletotrichum capsici* and *Colletotrichum gloeosporioides*). In vitro dual culture of *T. harzianum* inhibited the mycelial growth of *C. capsica* and *C. gloeosporioides* upto 67% and 68% respectively.

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Chapter-III

MATERIALS AND METHODS

The present study entitled **“Synergistic effect of bio-stimulants and *Trichoderma* spp. on physio-biochemical parameters of cucumber (*Cucumis sativus* L.)”** was conducted in the year 2022. In this chapter, the materials used, methods adopted, and techniques followed are benevolently described below.

3.1 Experimental site and Planting materials

3.1.1 Location

The present investigation in respect of the Studies on “Synergistic effect of bio-stimulants and *Trichoderma* spp. on physio-biochemical parameters of cucumber (*Cucumis sativus* L.)” was carried out at the experimental site (25°15'49.7"N 82°59'29.3"E) of Department Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh (221005) while the in vitro experiment was carried out in Plant Health Clinic lab and DST-FIST lab of the same department.

3.1.2 Layout and Design

The experiments were conducted in Completely Randomized Design (CRD) with three replications of each eight treatments and two methods of exogenous applications of treatments (foliar and drenching).

Details of the layout (Table 3.1)

Design	**:** CRD
Treatments	**:** Eight (8)
Replications	**:** Three (3)
Variety	**:** Kashi Nootan
Sowing date	**:** 9 March 2022
Applications	**:** Foliar and Drenching

3.1.3 Plant culture

The mud pots (size) were filled with a potting mixture containing loam soil, sand, and vermicompost in the ratio of 2:1:1. The soil was sandy loam rich in organic matter with good drainage and P^H range from 6.8-7.5. Seeds were sown at the rate of 1-2 seeds/pot and then pots were placed at the experimental site of the Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu (IAS) University, Varanasi (BHU), Uttar Pradesh (221005).

3.1.4 Seed source

Pure and viable cucumber seeds of Variety Kashi Nootan were obtained from ICAR-Indian Institute of Vegetable Research, Jakhini (Shahanshapur), Varanasi, Uttar Pradesh (221305).

3.2 Treatments

The following eight treatments were taken for experimental studies. The treatments consisted of either alone or in a combination of two compounds i.e salicylic acid (SA) and melatonin (MT), and a biocontrol agent *Trichoderma harzianum* (T.H). The treatments were applied exogenously by adopting two methods one was foliar spraying on the leaves and another was drenching at the root zone of cucumber plants. The total volume of application was 500 ml.

Treatments	Details
Foliar spray application (F)	
T1F	Control
T2F	SA (50 mg L^{-1})
T3F	MT (50 µM)
T4F	T.H (1× 10^8 CFU ml^{-1})
T5F	SA (50 mg L^{-1}) + MT (50 µM)
T6F	T.H (1× 10^8 CFU ml^{-1}) + SA (50 mg L^{-1})
T7F	T.H (1× 10^8 CFU ml^{-1}) + MT (50 µM)
T8F	T.H (1× 10^8 CFU ml^{-1}) + SA (50 mg L^{-1}) + MT (50 µM)

Soil drenching application (D)	
T1D	Control
T2D	SA (50 mg L^{-1})
T3D	MT (50 µM)
T4D	T.H (1× 10^8 CFU ml^{-1})
T5D	SA (50 mg L^{-1}) + MT (50 µM)
T6D	T.H (1× 10^8 CFU ml^{-1}) + SA (50 mg L^{-1})
T7D	T.H (1× 10^8 CFU ml^{-1}) + MT (50 µM)
T8D	T.H (1× 10^8 CFU ml^{-1}) + SA (50 mg L^{-1}) + MT (50 µM)

(Table.3. 2) Note: SA= Salicylic acid, MT= Melatonin, T.H= *Trichoderma harzianum*

3.3 Exogenous Melatonin treatment

Melatonin (MT, extra pure 99%) was obtained from Sisco research laboratories Pvt. Ltd., India. In a conical flask, the required quantity of MT was dissolved in 25 % methanol to create a stock solution of MT (50 M), then after final volume of 750 ml of was prepared. Then 250ml of MT solution to each replication was drenched to the soil and foliar sprayed onto the cucumber plants according to treatments.

3.4 Exogenous Salicylic acid treatment

The concentration of all these bio-stimulants was standardized based on the previous preliminary studies. Salicylic acid (SA) was procured from sigma Aldrich Chemicals Pvt. Ltd., India. A solution of SA (50 mg/L) was prepared by dissolving required quantity of SA in 5% ethanol in a conical flask, and the final volume was made to 750 ml. Then 250ml of SA solution to each replication was drenched to the soil and foliar sprayed onto the cucumber plants according to treatments

3.5 Exogenous *Trichoderma harzianum* treatment

In addition, to prepare a treatment solution of *Trichoderma spp.,* two strains of *Trichoderma* viz. *Trichoderma viride* and *Trichoderma harzianum* were previously assayed *in vitro* against six different fungal pathogens (*Fusarium oxysporum f.sp.*

lycopersici, *Colletotrichum capsici*, *Curvularia oryzae*, *Phomopsis vexans*, *Bipolaris sorokiniana* and *Alternaria solani*). Among these two *Trichoderma* spp. tested, *Trichoderma harzianum* showed higher antifungal efficacy, hence this strain was further mass cultured in the potato dextrose broth (PDB) (PDA; Himedia M096) at 25±2^0 C for 2 weeks. Mycelial mat containing spores was taken out of flasks, mixed, and filtered through three layers of muslin cloth. The spore concentration of *Trichoderma harzianum suspension* in the final solution was adjusted to 1×10^8 cfu ml^{-1}. Then 250 ml of *Trichoderma* spore suspension to each replication was then drenched to the soil and foliar sprayed onto the cucumber plants according to treatments.

3.6 Miscellaneous

In vivo experimental material viz sprayer, weighing balance, white labels tag, marker, polythene bag, fertilizers, pots, threads, scale, etc. were utilized from the Department of Mycology and Plant Pathology, IAS, BHU, Varanasi.

In vitro experimental works

The materials, methods, and techniques followed during in vitro experimental work are briefly described below.

3.7 Materials used (Table.3.3)

1. Agar-Agar	2. Atomizer
3. Autoclave	4. Beaker
5. BOD	6. Centrifuge
7. Centrifuge tube	8. Compound microscope
9. Conical flask	10. Dextrose
11. Filter paper	12. Forceps
13. Hot air oven	14. Ice packs
15. Inoculation needle	16. Laminar airflow

17. Measuring cylinders
18. Micropipette
19. Mortar and pestle
20. Needles
21. Petri plates
22. Potatoes
23. Spirit lamp
24. Test tubes
25. Tissue paper
26. UV-VIS Spectrophotometer
27. Vortex shaker
28. Wash bottle
29. Water Bath
30. Weighing balance etc.

3.8 Methods

3.8.1 Cleaning and sterilization of materials

Before use, glass wares were cleaned with detergent powder and washed with tap or distilled water as per the requirement of the experiment. The dried glass wares were sterilized in a hot air oven at 160°C for 2 hours. The forceps, inoculation needle, and other metallic instruments were sterilized by dipping them in alcohol and heating them over the flame of a spirit lamp before use. The medium and distilled water employed were sterilized in an autoclave at 15lbs p.s.i at 121.6°C for 20 minutes.

3.8.2 Culture media

Following media were used during laboratory studies on *Trichoderma* spp. **Table.3.4**

Name of Medium	Compositions	Quantities
	Potato peeled and sliced	200 g
Potato Agar Dextrose	Dextrose	20 g
	Agar	20 g
	Distilled water	1000 ml
Potato Dextrose broth	Peeled and sliced potato	200 g
	Dextrose	20 g
	Distilled water	1000 ml

3.8.3 Potato Dextrose agar

The required amount of peeled potato was cut into fine pieces. It was boiled in 500 ml of distilled water for 30 minutes and filtered through a muslin cloth. Thereafter, 20 g of dextrose and 20 g of Agar-agar were dissolved in 500 ml boiling water. Potato extract was added to the boiling mixture and mixed thoroughly by stirring with a glass rod. After a few minutes of boiling, it was transferred to, about 200 ml in each, 500 ml. capacity flask and plugged with non-absorbent cotton. The pH of the medium was adjusted to 7.0 ± 0.2 in the same way as mentioned above and autoclaved at 15 lbs p.s.i. at 121.6°C for 15 minutes.

3.8.4 Potato Dextrose broth

The required amount of peeled potato was cut into fine pieces. It was boiled in 500 ml of distilled water for 30 minutes and filtered through a muslin cloth. Thereafter, 20 g of dextrose was dissolved in 500 ml boiling water. Potato extract was added to the boiling mixture and mixed thoroughly by stirring with a glass rod. After a few minutes of boiling, it was transferred to, about 200 ml in each, 500 ml capacity flask and plugged with non-absorbent cotton. The pH of the medium was adjusted to 7.0 ± 0.2 in the same way as mentioned above and autoclaved at 15 lbs p.s.i. at 121.6°C for 15 minutes.

3.8.5 Slant preparation and PDA plating

The melted potato dextrose agar (PDA) medium was transferred @ 5 ml per culture tube. While transferring care was taken that the medium should not touch the inner wall of culture tubes. The culture tubes were sterilized at 15 lbs p.s.i. at 121.6°C for 15 minutes. After sterilization, it was allowed to solidify in a slanting position and then stored in a refrigerator for further use. Similarly, the sterilized and melted medium was poured aseptically in sterilized Petri plates @ 20 ml per Petri plate.

3.8.6 Pure cultures of *Trichoderma*

Procurement of known pure culture of *Trichoderma* spp. from Plant Health Clinic lab, Department of Mycology and Plant Pathology, BHU. Two species of *Trichoderma* viz. *T. harzianum, T. viride*. from old slant of *Trichoderma* spp. were picked up and purified by single hyphal tip method, which was transferred to Potato

Dextrose Agar media plates with help of sterilized inoculating needle and the pure cultures so obtained were stored in an incubator at 250C for further use.

3.8.7 Subculturing of *Trichoderma* spp.

Subculturing was done on the PDA medium. These Petri plates were incubated at 27°C for seven days with periodic observation for the development of colonies of *Trichoderma* spp. The green colour colonies of *Trichoderma* were identified by key based on the branching of conidiophores, the shape of phialides, the emergence of phialides, and spore characters.

3.8.8 Radial growth

For measuring the radial growth rate, all the isolates of *Trichoderma* were inoculated in four replications at the centre of 90 mm PDA plates. The inoculum was in the form of 5 mm mycelial discs taken from the margin of colonies grown on PDA plates. The plates were incubated at 27°C and the radial growth was measured (in mm) 1 day, 2 days, 3 days, 4 days and 5 days post inoculation.

3.8.9 Inoculation of liquid media

The five-day-old culture was used for inoculation. Mycelial discs of 5mm diameter were cut from the margins of the colonies by a sterilized 5mm metal cork borer. A set of three such discs was transferred to each flask of potato dextrose broth by using a sterilized inoculation needle. The inoculated flasks were incubated for 5 days at 24 ± 2ºC

3.8.10 Isolation of plant pathogenic fungi

In the present study, a set of six plant pathogenic fungi were used. These were isolated from their targeted infected crop from diseased part samples. A list of plant pathogenic fungi used and their targeted affected crop with isolated parts has been given in table3.5.

Table 3.5: Plant pathogenic fungi isolated from different crop plants

Crop	Disease	Causal organism	Plant parts Used for isolation
Tomato	Early Blight	*Alternaria solani*	Leaf
Paddy	Leaf Blight	*Curvularia oryzae*	Leaf
Brinjal	Fruit Rot	*Phomopsis vexans*	Stem
Chili	Anthracnose	*Colletotrichum capsici*	Fruit
Tomato	Wilt	*Fusarium oxysporum* f.sp. *lycopersici*	Fruit
Wheat	Spot blotch	*Bipolaris sorokiniana*	Leaf

3.8.11 Isolation procedure for fungi

The diseased samples showing typical symptoms were collected from the agricultural research farm of the Institute of Agricultural Sciences, BHU, and taken to the laboratory. The samples having typical symptoms were thoroughly washed repeatedly in tap water. Thereafter, small pieces measuring about 5 mm were cut using a sterilized blade, for isolation. Care was taken to ensure that each cut piece had healthy parts as well. The pieces were then surface sterilized in mercuric chloride ($HgCl_2$) solution (1:1000) for 20- 30 sec. followed by three thorough rinsing in sterilized distilled water. The surface-sterilized pieces were then aseptically transferred separately to the slants/plates containing Potato dextrose agar medium and then incubated at 24 ± 2°C. After 48-72 h of incubation, the growing mycelium from the margin of apparently distinct colonies was sub cultured on fresh PDA slants. In this way, the cultures of different plant pathogenic fungi were maintained.

3.8.12 Confrontation assay (Dual culture)

Antagonistic activity of *Trichoderma* spp. (*T. harzianum and T. viride*) was assayed against *Alternaria solani, Curvularia oryzae, Phomopsis vexans, Colletotrichum capsici, Fusarium oxysporum* f.sp. *lycopersici,* and *Bipolaris sorokiniana* by using dual culture inoculation technique. The dual culture plate technique (Dennis and Webster, 1971) was used to study the antagonistic effects of *Trichoderma* spp on Pathogens. All antagonistic pathogen combinations were examined on 20ml of PDA in 9-cm Petri plates, with Three replicate plates per treatment. For the dual culture technique, a mycelial plug (0.5cm in diameter), was taken from an actively growing 3 days old culture of Pathogens and *Trichoderma* isolates placed 8 cm apart from each other on the PDA. For control treatments, a plug of pathogens was placed on the PDA medium. The plates were incubated at 280C. Observations on the antagonistic activities of *Trichoderma* spp on pathogens were recorded after every 24hr for 4 days the and inhibition percentage was calculated using the following formula given by (Vincent, 1947).

$$\text{Per cent Growth inhibition} = \frac{C - T}{C} \times 100$$

Where,

C = Mycelial growth (mm) in control plate

T = Mycelial growth (mm) in treatment plate

Biochemical Parameters:

The sample of cucumber leaves from differently treated plants were collected. Nearly four to five leaves were taken randomly from each treatment. Samples from each treatment were kept separately in a plastic zip pouch bag and were labelled carefully. All the collected samples were immediately placed in a cold chamber which was having ice packs, as soon as they were harvested. These samples were then taken to the laboratory from the field and transferred inside the deep freezer (-20°C) until the biochemical parameters assay performed. The followings were the biochemical parameters performed for this study.

3.9.1 Estimation of Total Phenolic Content (TPC): -

The total phenol contents (TPC) were assessed using the Folin-Ciocalteu reagent and Gallic acid as standard, as described by Zheng and Shetty (2000).

Principle:

The Folin-Ciocalteau reagent oxidizes all phenolic compounds found in plant extracts. This reagent is made from a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$), which following the phenol oxidation, is reduced to a mixture of blue oxides of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}). The resulting blue coloration has a maximum absorption in the wavelength of 730nm and is directly proportional to the amount of phenolic compounds present. The resulting blue coloration has a maximum absorption in the wavelength of 730nm and is directly proportional to the amount of phenolic compounds present.

Reagent used: Methanol (50%)
Folin-Ciocalteau reagent (1N)
Sodium carbonates (20%)
Gallic acid

The estimation of total phenolic content was done by using Folin-Ciocalteau's phenol reagent (Ragazzi and Veronese, 1973) with some alteration. First of all, one gram of leaf samples was homogenized in 10 ml of 50% methanol. Homogenized sample was then collected in a centrifuge tube and left for one hour. The homogenized was taken to centrifugation and the supernatant was used for the assay. For the assay,100 μL of sample was taken in a test tube and then made up to the volume of 1.0 ml by adding 900 μL distilled water to it. 500 μL of Folin-Ciocalteau's phenol reagent (1N), and 1.0 ml Na_2CO_3 (20%) were added in the same manner as it is given. The whole mixture was then vortexed and kept at room temperature for 15 minutes to allow to proceed the reaction. Then, 10 ml of distilled water was added to the mixture for its dilution and vortexed thoroughly. The absorbance was measured at 725 nm. The blank was made with methanol in place of the sample. The results were expressed in µg gallic acid equivalent (GAE) g^{-1} fresh weight (FW).

3.9.2 Estimation of Phenylalanine ammonia-lyase (PAL): -

Phenylalanine ammonia-lyase (PAL) was estimated as per the protocol given by Brueske (1980) with some necessary modifications.

Principle:

Phenylalanine ammonia-lyase (PAL) is an important plant enzyme that eliminates ammonia from phenylalanine to form trans-cinnamic acid, precursor of lignins, flavonoids, and coumarins.

Reagent used: Potassium phosphate buffer (0.1 M; pH-8.7; 4^0 C)
β- mercaptoethanol (0.4 mM)
Potassium phosphate buffer (0.2 M; pH-8.7; 4^0 C)
Trichloroacetic acid (TCA, 1M)
L- phenylalanine (0.1 M)

Quantitative estimation of PAL was carried out by following the protocol given by Brueske (1980) with minor modifications. First of all, 0.3 g of leaves sample was homogenized in 3 ml of 0.1M ice-cold phosphate buffer (K_2HPO_4; pH 7.0) containing 2-3 drops of β- mercaptoethanol in mortar placed in a plastic tray filled with ice. Homogenized was then collected in a centrifuge tube and taken for centrifugation at 16000 x g at 4°C for 15 minutes. 200 μL enzyme extract was taken in a test tube and added 500 μL of 0.2M phosphate buffer (K_2HPO_4; pH 8.7), 1.3 ml of distilled water, and 1 ml of 0.1M L-phenylalanine (pH 8.7) in it. Then, the reaction mixture was incubated for 30 minutes at 32°C. The reaction was terminated by adding 0.5 ml of 1M trichloroacetic acid (TCA). Finally, the absorbance was recorded at 290 nm. The amount of t-CA (trans-cinnamic) formed was calculated and was expressed in μM t-CA mg^{-1} FW.

3.9.3 Estimation of Peroxidase (PO): -

Principle:

The enzyme peroxidase catalyzes the oxidation of substrate by oxygen generated from the decomposition of hydrogen peroxide.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Reagent used: Potassium phosphate buffer (K_2HPO_4; 0.1M; pH 7.0)
Pyrogallol (0.05 M)
Hydrogen peroxide (H_2O_2; 1 % V/V)

Peroxidase was assayed following the protocol given by Hammerschmidt and Co-workers (1982) with minor modification. First of all, 0.1 g of leaves sample was homogenized in 3ml of 0.1M ice-cold potassium phosphate buffer (K_2HPO_4; pH 7.0) in mortar placed in a plastic tray filled with ice. Homogenized was then collected in a centrifuge tube and taken for centrifugation at 16000 x g at 4°C for 15 minutes. 50 μL enzyme extract was taken in a test tube and added 1.5 ml of 0.05M pyrogallol, and 500 μL of H_20_2 (1% v/v). Finally, the change in the absorbance was recorded at 420 nm at 30 seconds intervals for 3 minutes. The enzyme activity was read as a change in the O.D(optical density) $min^{-1}g^{-1}$FW.

3.9.4 Estimation of Polyphenol oxidase (PPO): -

Polyphenol oxidase (PPO) assay was conducted as per the protocol given by Mayer and Co-workers (1994) with some necessary modifications.

Principle:

A class of copper-containing enzymes known as polyphenol oxidases (PPOs) catalyzes the o-hydroxylation of monophenols into o-diphenols and the oxidation of o-diphenols into quinones when oxygen is present (Araji *et al*. 2014). Several crops have shown increased PPO activity against biotic and abiotic stresses.

Reagent used: Potassium phosphate buffer (K_2HPO_4; 0.1 M; pH 6.5)
Catechol (0.01 M)

Polyphenol oxidase was assayed following the protocol given by Mayer and Co-workers (1994) with minor modification. First of all, 0.1 g of leaves sample was homogenized in 3ml of 0.1M ice-cold potassium phosphate buffer (K_2HPO_4; pH 6.5) in mortar placed in a plastic tray filled with ice. Homogenized was then collected in a centrifuge tube and taken for centrifugation at 16000 x g at 4°C for 15 minutes. 100 μL enzyme extract was taken in a test tube and added 1.5 ml 0.1M potassium phosphate buffer (K_2HPO_4; pH 6.5). Then added 0.2 ml of 0.01M catechol in it to make the reaction mixture. Finally, the change in the absorbance was read at 495 nm at 30 seconds intervals for 3 minutes. The enzyme activity was read as a change in the O.D(optical density) $min^{-1}g^{-1}$FW.

3.9.5 Estimation of Proline: -

Proline content estimation was carried out as per the protocol given by Bates *et al*. 1973 with some necessary modifications.

Reagents:

(i) 3 % aqueous sulphosalicylic acid (w/v)

(ii) Acid ninhydrin (prepared by dissolving 2.5 g ninhydrin in 60 ml glacial acetic acid and 40 ml 6.0 M o-Phosphoric acid until dissolved)

(iii) Toluene

The determination of proline was done by following the protocol given by Bates and Co-workers (1973). First of all, 0.5 g of frozen leaves samples were homogenized in 5 ml of 3% (w/v) aqueous sulphosalicylic acid. Homogenized were collected in the centrifuged tube and taken for centrifugation at 10000 g for 10 minutes. 2 ml of supernatant were taken into a test tube, and then 2 ml of acid-ninhydrin and 2 ml of glacial acetic acid were added to it. The test tube containing the mixture was transferred into the water bath at 100°C for 30 minutes to start the reaction. After 30 minutes the test tube was suddenly transferred to the ice bath to terminate the reaction. 2 ml of toluene was added, mixed vigorously, and left at room temperature for 30 minutes until the separation of the two phases. The upper phase of separated phases which was chromophore-containing toluene, was used to take absorbance at 520 nm. Toluene was used for the blank. The concentration of proline was estimated from the standard curve using D-Proline.

3.9.6 Estimation of Catalase (CAT) activity: -

The catalase activity assay was conducted by following the protocol given by Aebi (1984) with some necessary modifications.

Principle:

Catalase catalyzes the reduction of hydrogen peroxide to water and molecular oxygen. It is regarded as less effective H_2O_2 scavenging system than ascorbate peroxidase due to its greater K_m value for H_2O_2 than APX. It is also found in mitochondria and peroxisomes, but not in chloroplasts, which are important sites of H2O2 production.

$$2H_2O_2 \xrightarrow[\text{Catalase}]{} 2\ H_2O + O_2$$

Catalase assay is based on the absorbance of H_2O_2 at 240 nm (UV-range). A decrease in the absorbance is recorded over a time period.

Assay solutions:

Solution A: Sodium phosphate buffer (100 mM, pH 7.0)

Solution B: 30 mM H2O2 solution

Procedure:

a. Solution A 1.5 ml
b. Enzyme extract Up to 0.1 ml
c. 30 mM H2O2 1 ml
d. Distill water 0.4 ml

Total volume 3 ml

The determination of catalase activity was done by following the protocol given by Aebi (1984). First of all, 0.5 g of leaves samples were homogenized in 5 ml of ice-cold 50 mM Sodium phosphate buffer (pH 7.0) containing 2 mM EDTA, 5 mM β - mercaptoethanol (β - ME), and 4% PVP-40. Homogenized were collected in the centrifuged tube and taken for centrifugation at 10000 g for 10 minutes at 4°C.1.5 ml of sodium phosphate buffer (100 mM, pH 7.0) was added to a test tube, and then 100 μL of enzyme extract, 1 ml of 30 mM H_2O_2, and 0.4 ml of distilled water respectively were added in it to make 3 ml of total volume. The reaction was initiated by the addition of 1 ml of 30 mM H_2O_2. The decrease in absorbance was read for 1 minute at 20 second interval at 240 nm. 2.5 ml of sodium phosphate buffer (100 mM, pH 7.0) with 500 μL of distilled water were used as blank.

3.9.7 Estimation of Chlorophyll content: -

The chlorophyll content estimation was conducted by following the protocol given by Arnon (1949) with some necessary modifications.

Reagent used: Acetone (80 %)

The assay of chlorophyll was done by following the protocol given by Arnon (1949). First of all, fresh leaves were harvested 25 days after the application of treatments. 1.0 g of fresh leaves were crushed well in 10 ml of 80% of acetone with the help of a mortar and pestle and collected in the centrifuge tube. Then, the samples were taken for centrifugation at 10000 rpm for 10 minutes in dark. The supernatant was used for the assay. The absorbance was read at 647 nm and 664 nm to determine the content of chlorophyll and was expressed in mg chlorophyll per gram FW (fresh weight). 80% acetone (v/v) was used as blank.

3.9.8 Estimation of protein content: -

The protein content in cucumber leaves was estimated as per the protocol given by Lowry and co-workers (1951).

Reagent used:

Solution A: 2 % Sodium carbonate in 0.1 N Sodium hydroxide
Solution B: 1 % Copper sulphate solution
Solution C: 2 % Sodium –Potassium tartarate solution
Solution D: (Alkaline copper reagent) 1ml of reagent (B) and 1 ml of reagent (C) in 100 ml of reagent (A)
Solution E: 1N Folin – Ciocalteu reagent

Extraction buffer: Potassium phosphate buffer (K_2HPO_4; 0.1M; pH 7.0)

Protein content in leaves was estimated by following the protocol given by Lowry and co-workers (1951). First of all, 500 mg of leaves sample was homogenized in 5ml of potassium phosphate buffer (K_2HPO_4; pH 7.0) by using a mortar and pestle. Homogenized was then collected in a centrifuge tube which was further taken for centrifugation at 10000 rpm for 10 minutes. 200 μL of supernatant was taken in the test tube and then made up the volume to 1.0 ml by adding 800 μL of distilled water. Further added 5.0 ml of alkaline reagent and 0.5 ml of Folin-Ciocalteau's phenol reagent (FCR). Then, the mixture was incubated for 30 minutes at room temperature. Finally, the absorbance was recorded at 660 nm and was expressed as μg g^{-1} FW through a standard curve obtained using bovine serum albumin.

3.10. Statistical Analysis

The Values from different experiments shown in the figures are mean ± standard deviation (SD) of at least three replications of each of the experiments. All the data collected in this study were subjected to analysis of variance (ANOVA). The treatment mean values were compared by Duncan's multiple range test at the $P \leq 0.05$ significance level. The software used for analysis was SPSS ver. 16 (SPSS, Inc., Chicago, IL, USA).

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Chapter-IV

RESULTS AND DISCUSSION

The present study is based upon the **"Synergistic effect of bio-stimulants and *Trichoderma* spp. on physio-biochemical parameters of cucumber (*Cucumis sativus* L.)"**. The confrontation assay (Dual culture) of two *Trichoderma* spp (*Trichoderma harzianum and Trichoderma viride*) was tested against *Alternaria solani, Curvularia oryzae, Phomopsis vexans, Colletotrichum capsici, Fusarium oxysporum* f.sp. *lycopersici, and Bipolaris sorokiniana*. The *Trichoderma harzianum* that performed well against the pathogens was then taken for further study. The synergistic effect of melatonin, salicylic acid, and *Trichoderma spp* on cucumber had been observed by applying these two bio-stimulants and *Trichoderma harzianum* into different combinations and by two exogenous methods (Foliar and Soil Drenching).

Biochemical Estimation

4.1 Total Phenolic Content (TPC)

Total Phenolic Content (TPC) activity in cucumber was noted 14 days after applications of treatments. TPC activity was significantly higher in cucumber leaves in all the treatments and in both the methods of applications i.e foliar and drenching. However, the best results were seen in the case of the foliar one. The following amount of total phenol was observed during the assay.

(A) Foliar Application.

It was observed that TPC was highest in T8F which was 2530.00 µg GAE g^{-1} FW. The lowest TPC value was noticed in control one, T1F, i.e, 1721.50 µg GAE g^{-1} FW. The average value of TPC was seen highest in T8F followed by T7F, T6F, T5F, T4F, and T3F as shown in fig.4.1. The results showed that TPC was higher when the biostimulants were given in combinations as compared to the condition when they were given alone.

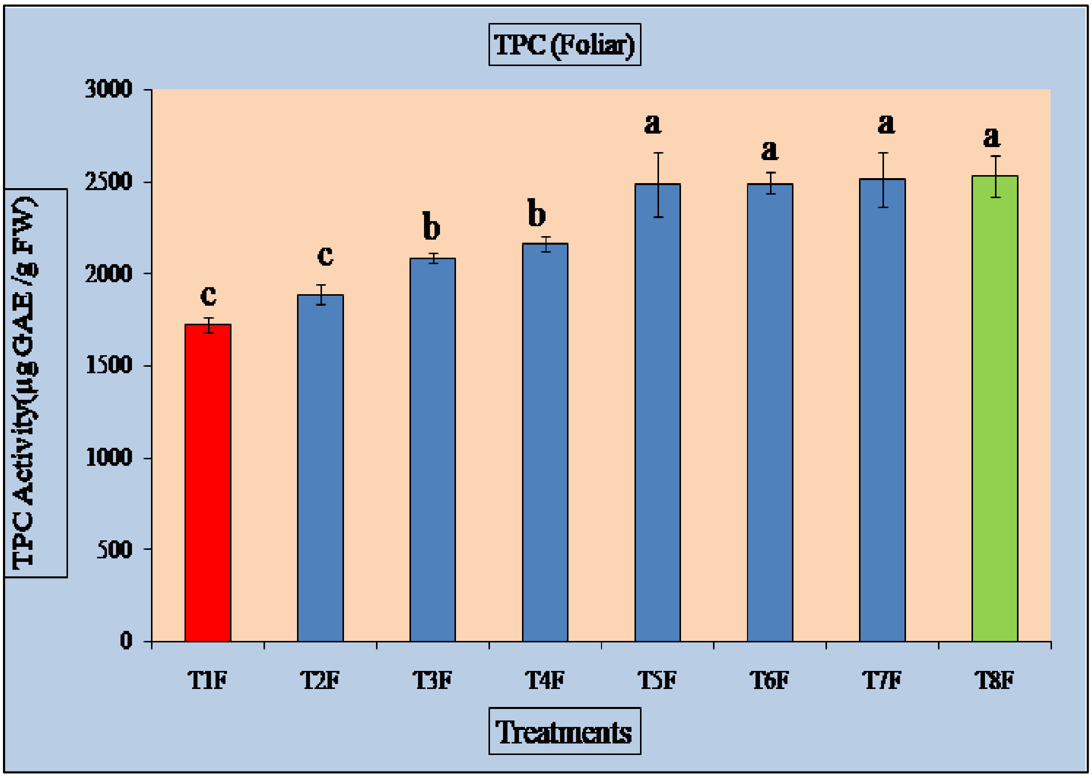


Figure.4.1. Total phenolic content in cucumber in Foliar application of treatments. Green colour denotes maximum TPC content whereas red colour denotes minimum TPC content. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at $p \leq 0.05$

(B) Soil drenching

The results of the application of salicylic acid, melatonin, and *Trichoderma spp*. alone and in different combinations by soil drenching method, were slightly different from the foliar method. In this case, the best result was also found in the same concomitant. T8D had the highest total phenolic content, i.e 2337.50 µg GAE g^{-1} FW, followed by T6D, T5D, and T7D while the lowest value was observed in T1D, which was the control one (fig.4.2).

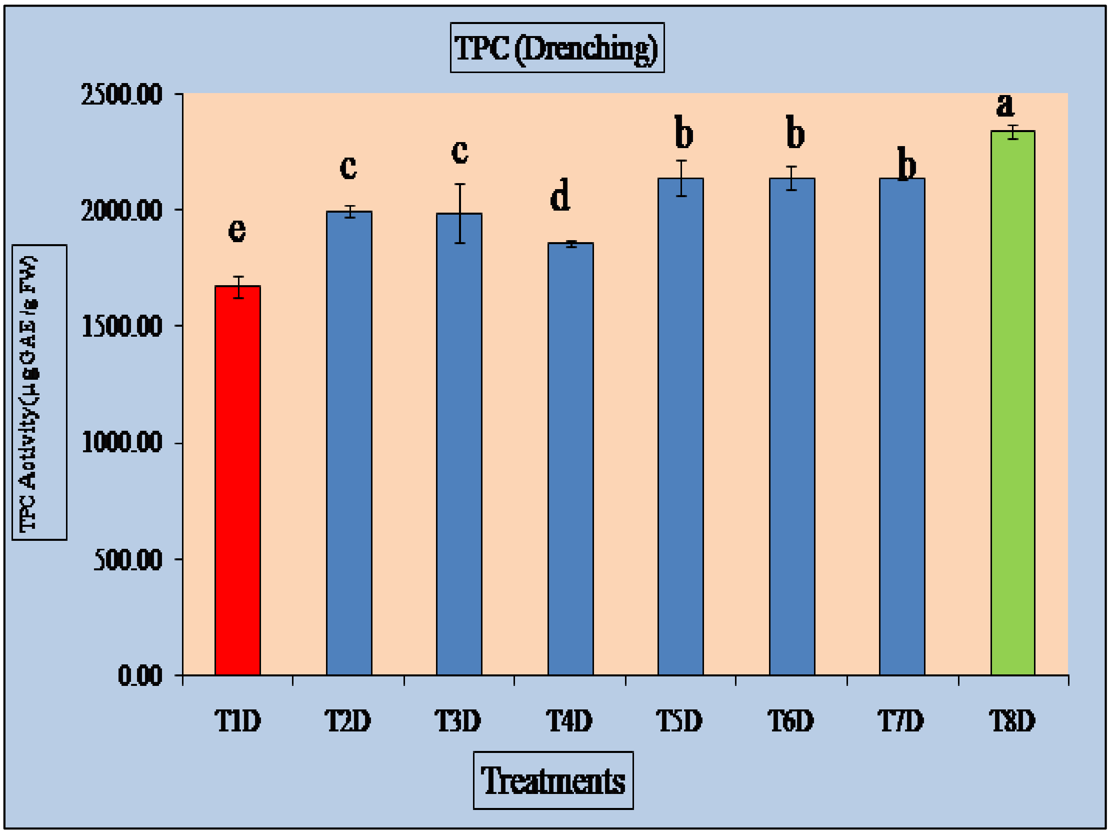


Figure. 4.2. Total phenolic content in cucumber in Soil drenching application of treatments. Green colour denotes maximum TPC content whereas red colour denotes minimum TPC content Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test a *p*≤ 0.05.

Phenolic compounds are a crucial precursor for the production of lignin. Plants communicate their defensive reaction when they become infected by pathogens by depositing phenolic chemicals into their cell walls. The phenolic compound deposition may be used to either increase the local wall's strength or use the cell's own hypersensitive response (Datta and Lal, 2012; Nicholson and Hammerschmidt, 1992). Since PAL catalyses phenol formation and is a key component of plants' antimicrobial defence system, its activity and phenol accumulation are directly correlated (Jain *et al*., 2012).

Glucose is produced as a result of the multi-step metabolic process that breaks down carbohydrates, followed by dehydroshikimic acid and gallic acid (Kumar and Goel, 2019). According to Nguyen *et al*. (2013), gallic acid is intended to produce the enzyme chitinase, which is crucial in the breakdown of the chitin in pathogenic fungi's cell walls and protects plants against disease.

The effect of foliar spray of salicylic acid (0.5 mM) on the physiological features of fenugreek (*Trigonellafoenum-graecum* L.) plants growing under three salt concentrations resulted in an increase in proline, total phenolics, and flavonoids. Furthermore, foliar application of SA resulted in higher levels of total free amino acids and shikimic acid in both control and salt-treated plants (Abdelhameed *et al*., 2021)

Sarrou *et al*., (2015) came up with the same finding as observed in this experiment. The total leaf phenolic content was increased after the application of salicylic acid and melatonin alone or in combination on one-year-old bitter orange leaves.

In the present study, results show the increase in total phenol is more or less in all treatments as compared to the control. The phenolic content was high in almost all combined treatment in which combination of salicylic acid, melatonin and *Trichoderma* showed maximum phenolic content. This result confirms the finding of all other previous reports.

4.2 Phenylalanine ammonia-lyase (PAL)

Phenylalanine ammonia-lyase (PAL) activity in cucumber was noted 14 days after application of treatments. PAL activity was significantly higher in cucumber leaves in all the treatments and in both the methods of applications i.e foliar and drenching. However, the best results were seen in the case of the foliar. The following amount of PAL was observed during the assay.

(A) Foliar Application

In the case of foliar application, T8F had a 1.5 folds increase over T1F (control). The highest value of PAL was 2602.47 µM transcinnamic acid g^{-1} FW in T8F followed by 2577.85 µM transcinnamic acid g^{-1} FW in T5F and 2574.78 µM transcinnamic acid g^{-1} FW in T7F while the lowest value 1698.87 µM transcinnamic acid g^{-1} FW in control(T1F) followed by 1921.70 µM transcinnamic acid g^{-1} FW in T4F and 2152.83 µM transcinnamic acid g^{-1} FW in T2F was noticed. (Fig no 4.3)

(**B) Soil Drenching**

After investigating the results of PAL activity, it was concluded that when soil drenching methods of application were taken over the foliar one, the effects of *Trichoderma* on the plant eventually got increased as compared to the foliar application. All the combinations having *Trichoderma* as one of their components showed better results when applied to the soil directly. The best result was observed in T8D which was 1.2 times higher than the normal control plant (T1D). The maximum average value for PAL activity was 2551.08 µM transcinnamic acid g^{-1} FW (T8D), followed by 2423.05 µM transcinnamic acid g^{-1} FW (T4D) and 2329.79 µM transcinnamic acid g^{-1} FW (T7D) whereas the lowest value was 2061.11 µM transcinnamic acid g^{-1} FW (T1D) lagging by 2150.06 µM transcinnamic acid g^{-1} FW (T3D) and 2229.06 µM transcinnamic acid g^{-1} FW (T5D). (Fig no 4.4)

PAL is a crucial enzyme in the phenylpropanoid biosynthesis pathway that results in the production of phytoalexins or phenolic compounds, which serve as defensive chemicals in plants and have antimicrobial properties as well as in the production of other signaling molecules like salicylic acid (Wen *et al*., 2005). In the present study maximum PAL activity was detected in cucumber plants from treatments combination of salicylic acid + melatonin + *Trichoderma* spp in both the method of application (Foliar and soil drenching).

Several other studies reported that PAL activity is associated with the reduction of incidence of disease and the development of resistance to plant pathogens (Chandrasekaran *et al*., 2017)

Our results are supported by findings of Ahmed *et al.*, 2021 where the foliar application of melatonin and salicylic acid was done on infected eggplants, resulted in significantly increase in PAL activity. This finding is matching with the results obtained.

Foliar applications of SA, on the other hand, resulted in increased shoot fresh weight and dry weight, root fresh weight, and dry weight. The number of leaves per plant was also increased. Induce Systemic Response (ISR) has a crucial role in plant defence mechanisms. It reprogrammes and mobilizes defence-related enzymes such as pathogenesis-related (PR) proteins, peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL) and superoxide dismutase (SOD), as well as induce proline and phenol (Jain *et al*., 2012).

The healthy and R. *solani*-inoculated tomato plants grown in the media containing the spores of all three examined strains of *Trichoderma* showed increases in phenylalanine ammonia-lyase (PAL) activity as well as improved production of total phenolics and orthodihydroxyphenolics. It could mean that *Trichoderma* strains cause the tested plants' immune systems to respond defensively. This is demonstrated by the fact that in response to such biotic stress, one of the primary-secondary metabolic pathway characteristics of plant defence systems, i.e PAL is catalysed (Juszczuk *et al*.2004).

The activity of PAL had increased nearly two-fold when pea seed was treated with *Trichoderma asperellum* and *Pseudomonas fluorescens* (OKC) were challenged with powdery disease (*Erysiphe pisi*) (Patel *et al*., 2017).

When the salicylic acid and melatonin were applied to the cucumber plants, an increase in PAL activity was observed. The increase had been estimated about 1.5 times higher than the control. The same effect had been observed in our results. Thus, increased PAL activity in the current study may be associated with improved defensive mechanisms by the helpful microorganisms.

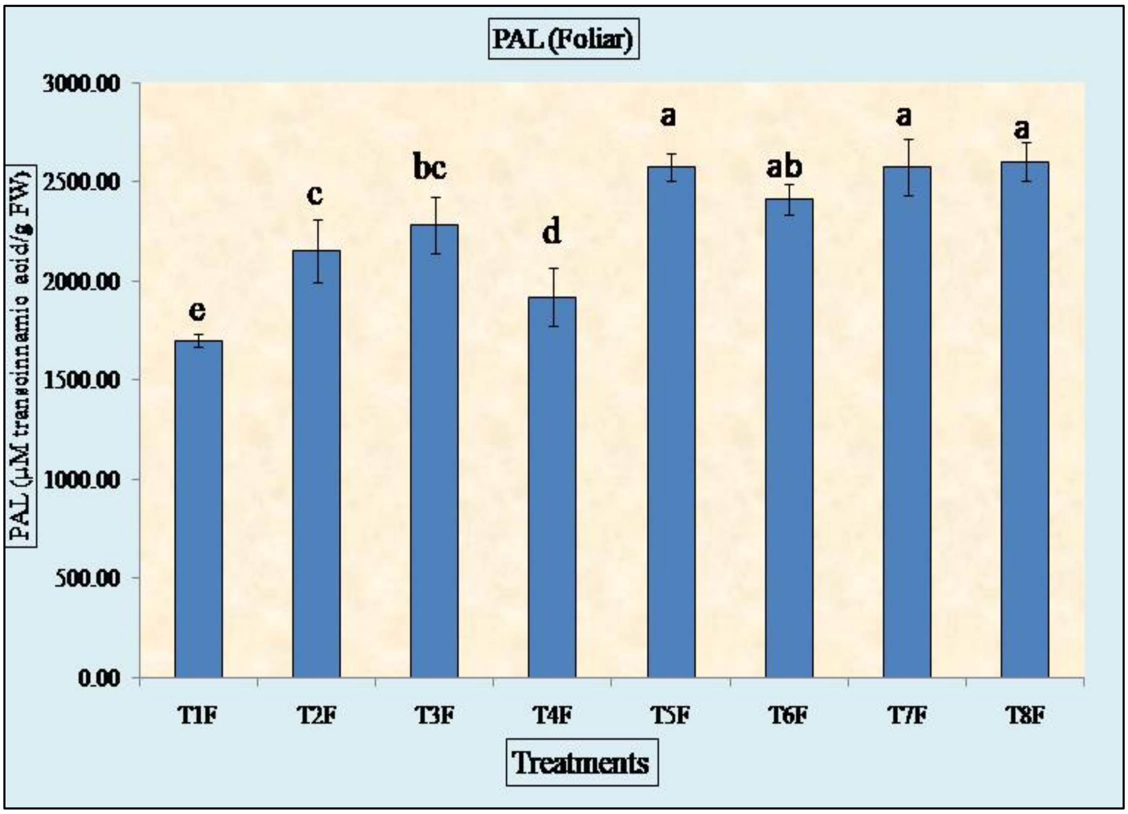


Figure .4.3. The graph showing the value of PAL activity (µM transcinnamic acid g^{-1} FW) in different treatments when the method of application was foliar. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at $p \leq 0.05$

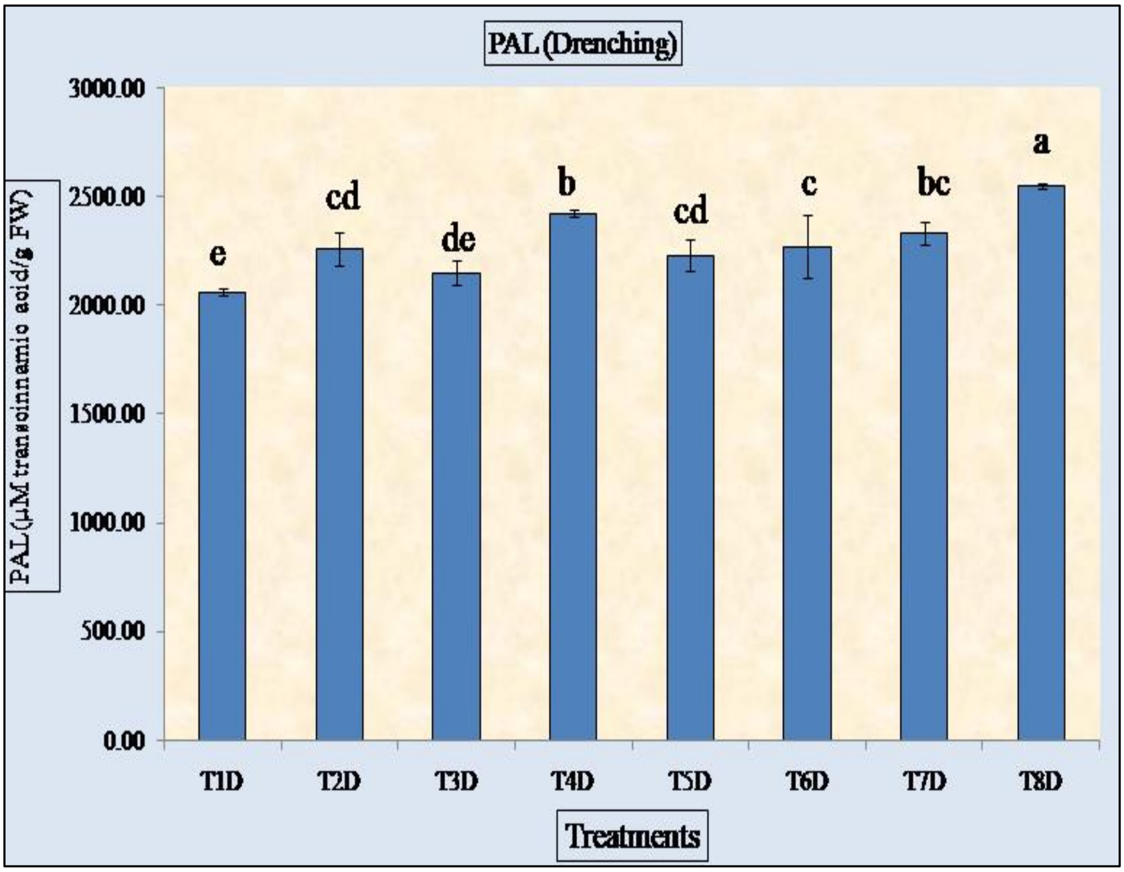


Figure 4.4. The graph showing the value of PAL activity (µM transcinnamic acid g^{-1} FW) (in different treatments when the method of application was soil drenching. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at *p*≤ 0.05

4.3 Peroxidase (PO)

Peroxidase (PO) activity in cucumber was noted 14 days after application of treatments. PO activity was significantly higher in cucumber leaves in all the treatments and in both the methods of applications i.e foliar and drenching. However, the best results were seen in the case of the foliar one. The following amount of PO was observed during the assay.

(A) Foliar Application

In the case of foliar application of biostimulants on cucumber, the maximum value was observed in T8F, which was 3 times higher as compared to the control one (T1F). The best three results were seen in T6F, T5F, and T7F, which were 2.3, 2.1, and 1.8 times higher than the control (T1F) respectively. The lowest value was seen in control followed by T3F and T2F as shown in fig.4.5.

(B) Soil drenching

Overall, the results showed that the PO activity in cucumber after soil drenching. The highest value was observed in T8D which was 2-fold greater than the T1D. In the drenching method of application combined treatments performed better as compared to the treatment alone. Although, the maximum value of PO was found in T8 of the foliar application method when compared with the T8 of drenching, but the results showed that overall average performance in the increase of PO value was observed in the drenching method of application. The value of T8D was leading behind T7D, T6D, and T5D. (Fig no 4.6)

The activity of PO was found to increase nearly two-fold when pea seeds was treated with *Trichoderma asperellum* and *Pseudomonas fluorescens* (OKC) were challenged with powdery mildew (*Erysiphe pisi*) causing pathogen (Patel *et al*., (2017). All the concomitants of biostimulants show the elevation in PO activity as compared to the control. Thus, the present findings also exhibit the increase in PO activity which may be related with their defence role in cucumber.

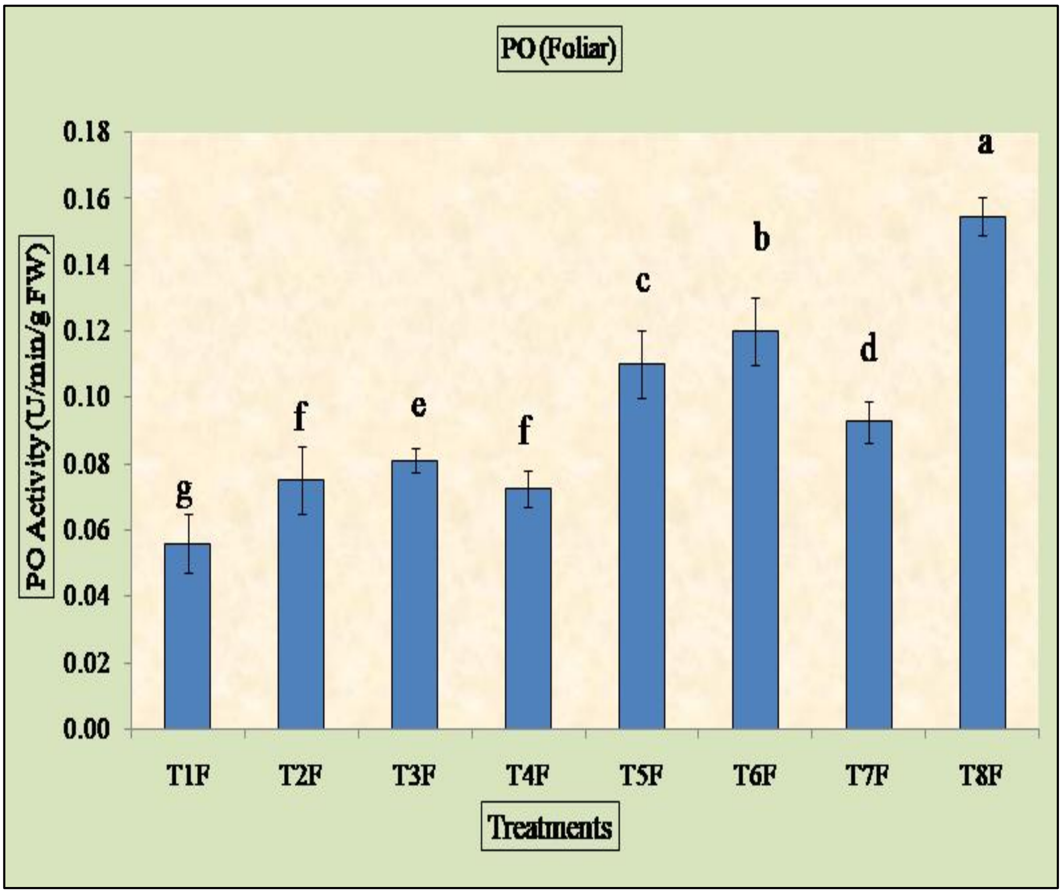


Figure 4.5. The graph showing the value of PO activity in different treatments when the method of application was foliar. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at $p \leq 0.05$

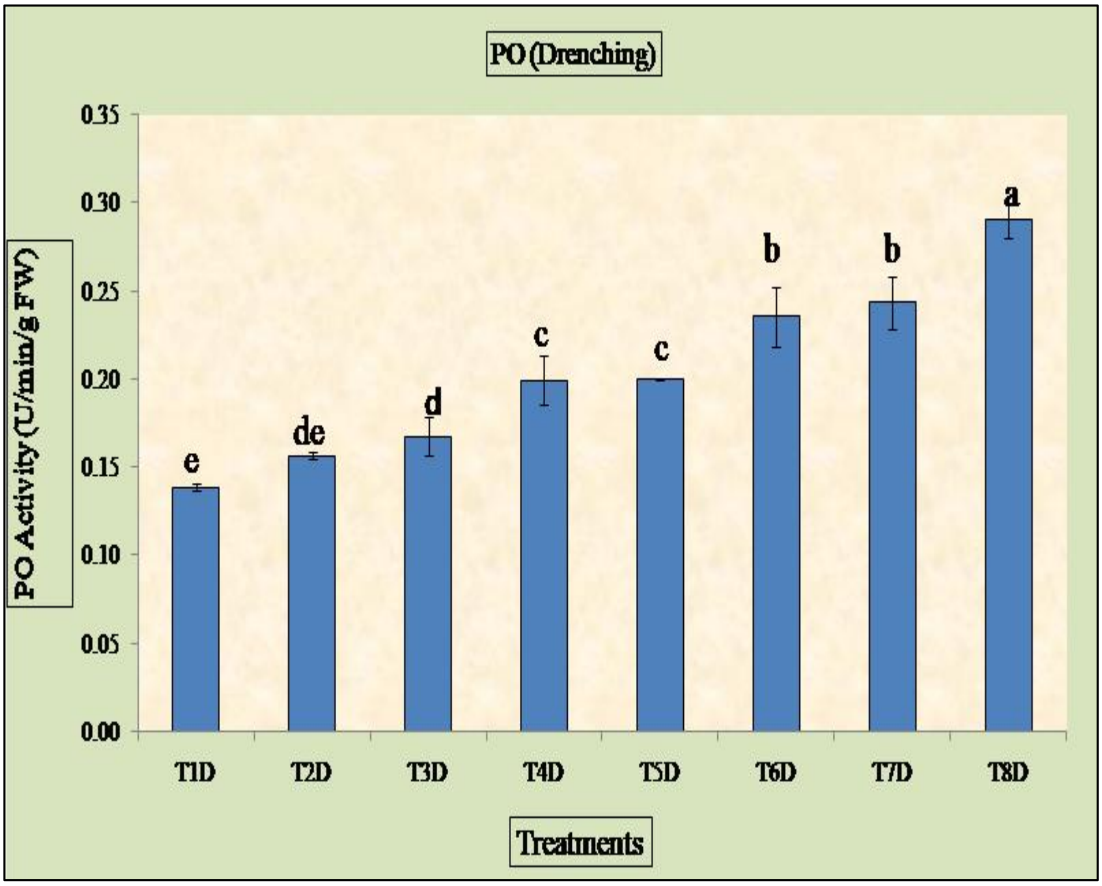


Figure 4.6. The graph showing the value of PO activity in different treatments when the method of application was soil drenching. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at *p*≤ 0.05

4.4 Polyphenol oxidase (PPO)

Polyphenol oxidase (PPO) activity in cucumber was noted 14 days after application of treatments. PPO activity was significantly higher in cucumber leaves in all the treatments and in both the methods of applications i.e foliar and drenching. However, the best results were seen in the case of the foliar one. The following PPO activity observed during the assay.

(A) Foliar Application

The results on PPO activity showed that the PPO activity had increased to 2.1-fold maximum followed by 2-fold, and 1.9-fold in T8F, T6F, and T7F respectively. While PPO activity was relatively less in other treatments, the lowest PPO activity was observed in T1F, which was the control followed by T2F and T3F. (Fig no 4.7)

(B) Soil Drenching

It was observed that the maximum value of PPO in soil drenching method was 2 times higher than the T1D which was the control. T8D showed the best result in all the treatments. The T8D treatment was followed by T7D, T5D, and T6D. The lowest value of PPO activity was seen in T1D. while the remaining treatments had a relatively higher value than the control and lower than T6D. (Fig no 4.8)

In a previous report, the three *Trichoderma* strains (T. *asperellum* 525, T. *harzianum* 610, and T. *pseudokoningii* 866) exhibited excellent growth and disease prevention effects on cucumber. *Trichoderma* 866 was the most successful of the three strains. Polyphenol oxidase (PPO) like stress-resistance enzyme activity significantly increased along with chlorophyll content, nitric nitrogen content, plant height, stem diameter, above and underground fresh weight, and leaf area, in *Trichoderma* inoculated seedlings. Mei *et al*., (2019). The biochemical response at different concentrations of salicylic acid (1.0, 1.5, and 2 mM) was also studied. The response of the plant at 1.5 mM of SA was very fast. It showed higher induction of peroxidase (PO) and polyphenol oxidase (PPO) activities. Similarly, synergistic effects of melatonin and salicylic acid increase the PPO activity in salt-stressed wheat. (Naveen *et al*., 2022). Our experiment finding is matching with these results.

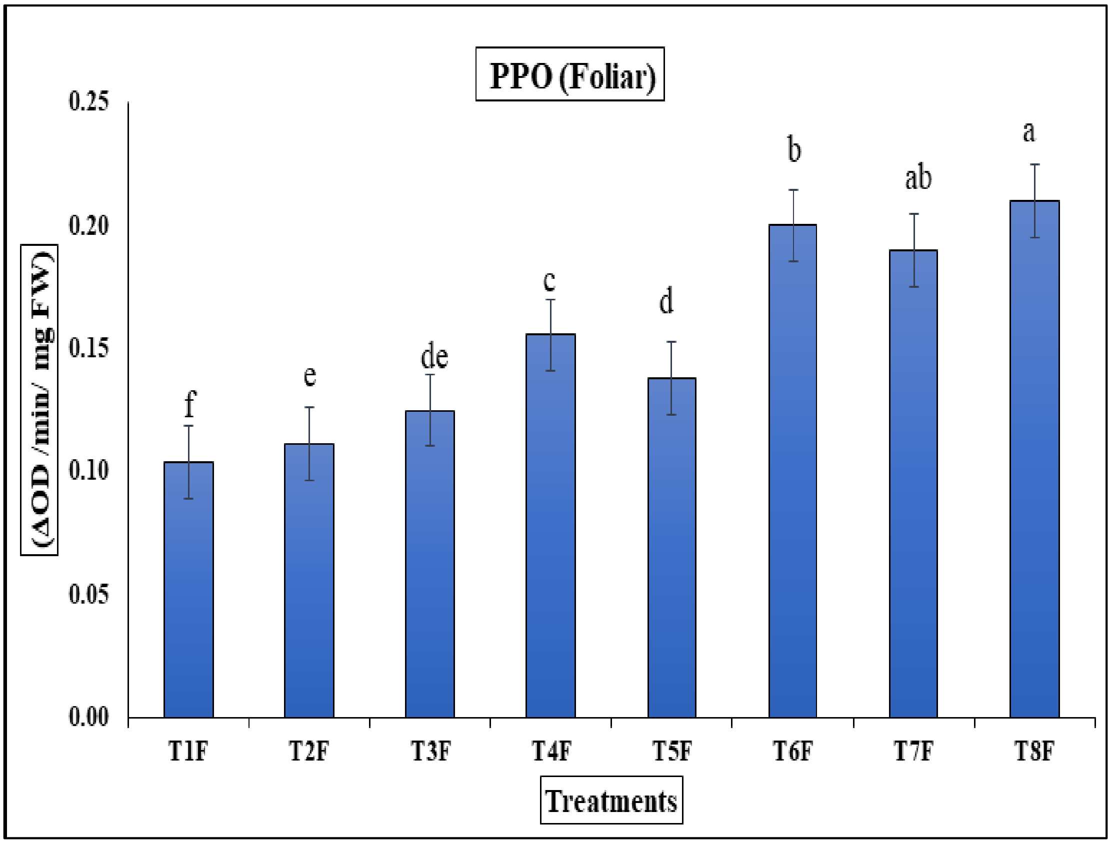


Figure 4.7. The graph showing the value of PPO activity in different treatments when the method of application was foliar. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at *p*≤ 0.05

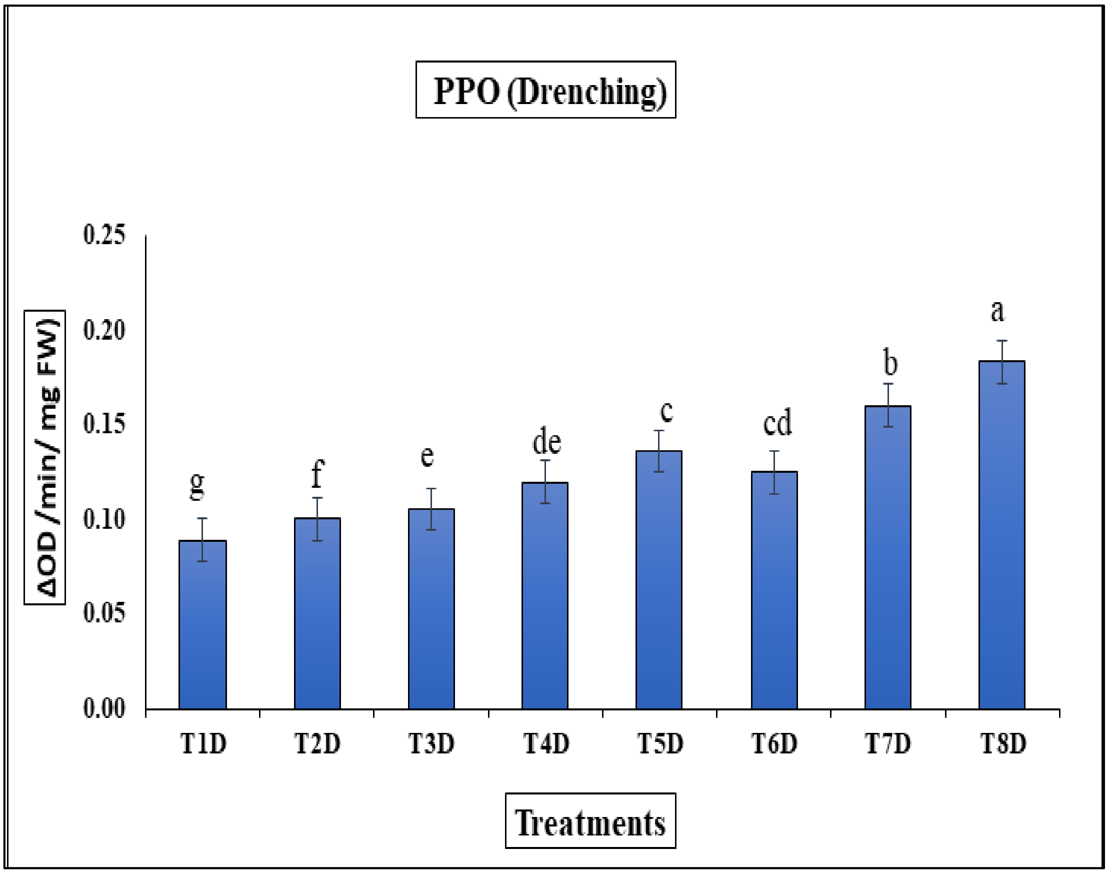


Figure 4.8. The graph showing the value of PPO activity in different treatments when the method of application was drenching. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at $p \leq 0.05$

4.5 Proline content

Proline content in cucumber was noted 14 days after application of treatments. Proline content was significantly higher in cucumber leaves in all the treatments and in both the methods of applications i.e foliar and drenching. However, the best results were seen in the case of the drenching one. The following amount of proline was observed during the assay.

(A) Foliar Application

It had been observed that proline content in T8F was 1.6-fold higher than the control one (T1F). The higher amount of proline found in T8F was 0.0110 µM g^{-1} FW followed by T7F (0.0106 µM g^{-1} FW) and T6F (0.0105 µM g^{-1} FW) while the lowest amount of proline was observed in T1F (0.0065 µM g^{-1} FW) followed by T2F (0.0069 µM g^{-1} FW) and T3F (0.0090 µM g^{-1} FW). (fig.4.9).

(B) Soil drenching

The results showed that there was a 2.3-fold proline increase in T8D followed by 2.1-fold in T7D and 2-fold in T6D as compared to the T1D. The minimum amount of proline was observed in control (0.0063 µM g^{-1} FW) followed by T2D (0.0073 µM g^{-1} FW) and T3D (0.0094 µM g^{-1} FW). (fig. 4.10)

In response to a variety of biotic and abiotic challenges, many plants frequently undergo physiological reactions which results in accumulation of proline. It plays a crucial role in the adaptation of plants under stress. Some evidence suggests that proline act as both metabolites and as a signal molecule. It helps plants during the reproductive phase (i.e from flowering to seed development) (Mattioli *et al.*, 2009). When plants were exposed to salt or heat stress, more proline was released into the rhizosphere. The quantity of this amino acid was greater in exudates collected from plants tolerant to different stress conditions. Furthermore, stress conditions and genotype's tolerance to the particular stress have a considerable quantitative and qualitative impact on the exudation pattern (Vives-Peris *et al.*, (2017).

In the previous study, the foliar applications of salicylic acid (SA) at different concentrations (0.0, 0.25, 0.50, and 1.00 mM) resulted in an increase in proline content. The best result was obtained with 1.00 mM SA treatment in which the greatest proline content was observed (Yildirim *et al.*, 2013). Also, the application of melatonin on tomato seedlings improved proline content in the leaves by 2-fold as compared to the control (Siddiqui *et al*., (2018). When *T. harzianum* was subjected to foliar application on drought stress plants then the concentration of proline was increased to 80.80% as compared to the normal plant (Mona *et al*., 2017). This result can be correlated with the present finding in which combined treatments which has salicylic acid, melatonin, and *Trichoderma* result in the enhancement of proline in the plant.

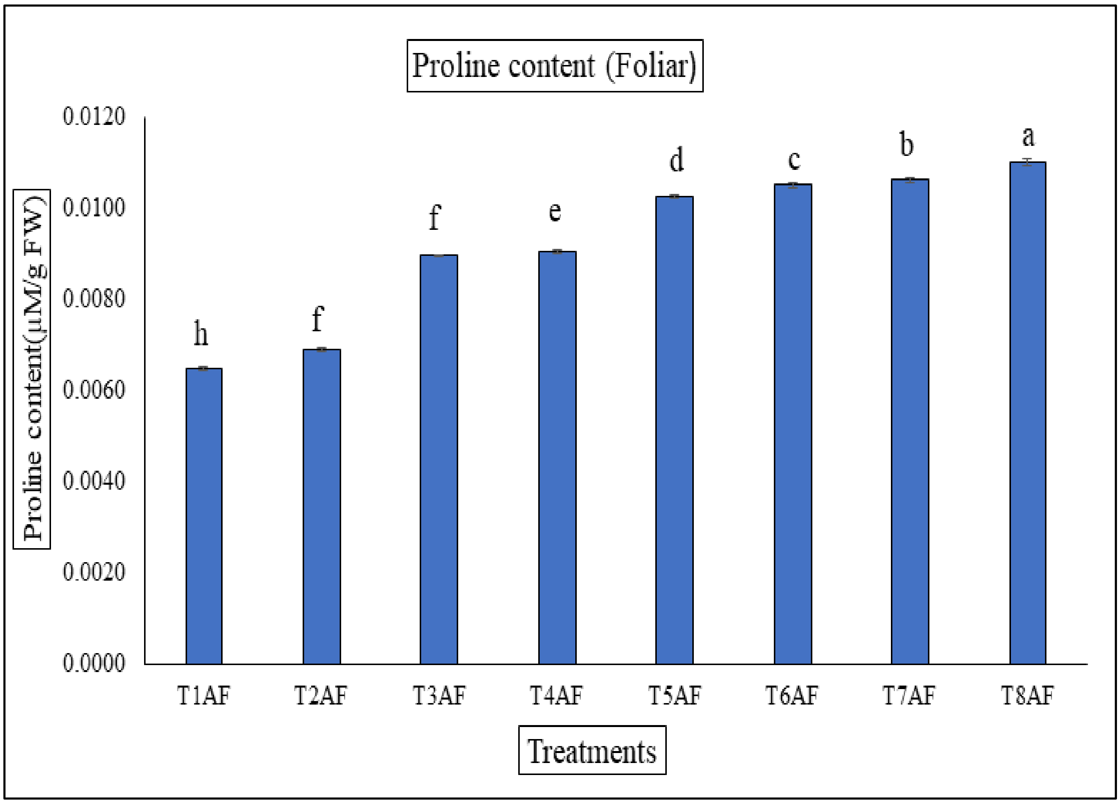


Figure 4.9. The graph shows the value of Proline content in different treatments when the method of application was foliar. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at $p \leq 0.05$

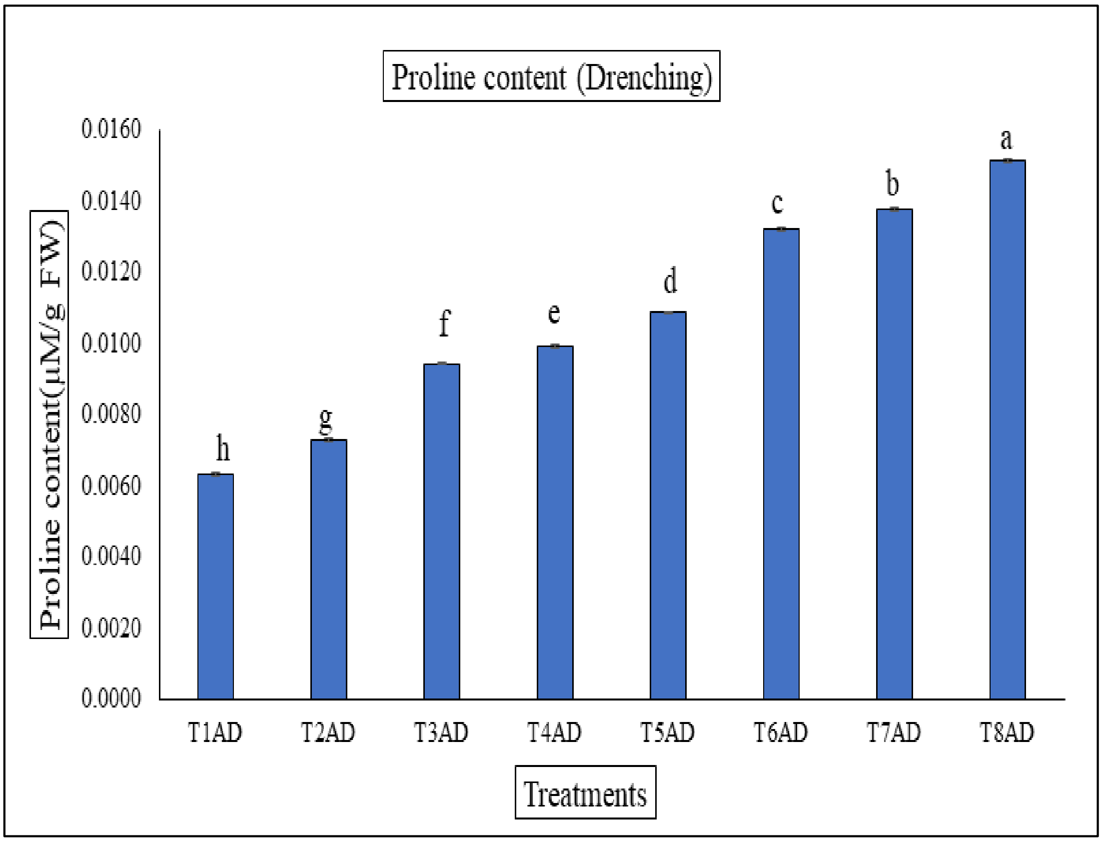


Figure 4.10. The graph shows the value of Proline content in different treatments when the method of application was drenching. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at $p \leq 0.05$

4.6 Catalase Activity

Catalase activity in cucumber was noted 14 days after application of treatments. Catalase activity was significantly higher in cucumber leaves in all the treatments and in both the methods of applications i.e foliar and drenching. However, the best results were seen in the case of the drenching one. The following catalase activity was observed during the assay.

(A) Foliar Application

It was investigated that the highest average value of catalase activity was found in T4F followed by T5F and T8F whereas the lowest average value was observed in T1F followed by T2F and T3F. The results showed that the application of *T. harzianum* alone can better enhance the catalase activity as compared to the combination. However, T8F (salicylic acid + melatonin + *T. harzianum*) also help to alleviate CAT activities significantly (fig.4.11)

(B) Soil drenching

In the case of soil drenching, the catalase activity increased by 3-folds in T4D followed by 2.98-folds in T5D and 2.90-folds in T8D. The lowest value was observed in T1D lagged by T2D and T3D. As compared to the foliar one, catalase activity was found to perform better in the soil drenching method (fig 4.12.)

Biotic stress in plants triggers a Calcium ion influx and increases the cytosolic calcium. The increased level of Ca ion in the cytoplasm directly stimulates the production of H_2O_2. Also, the same calcium ion can also act as a inhibitor of H_2O_2 when combines with calmodulin. H_2O_2 level is down-regulated by Ca^{2+}/Calmodulin which stimulates the catalase activity (Yang and Poovaiah, 2002).   ROS homoeostasis is maintained during biotic stress through the production of catalase and the higher production is an indication of high antioxidant activity. Catalase is involved in scavenging of H_2O_2 and converting it into water and oxygen. Catalase is known to induce the defense related gene associated to Systemic acquired resistance (SAR) (Chen *et al.*, 1993).

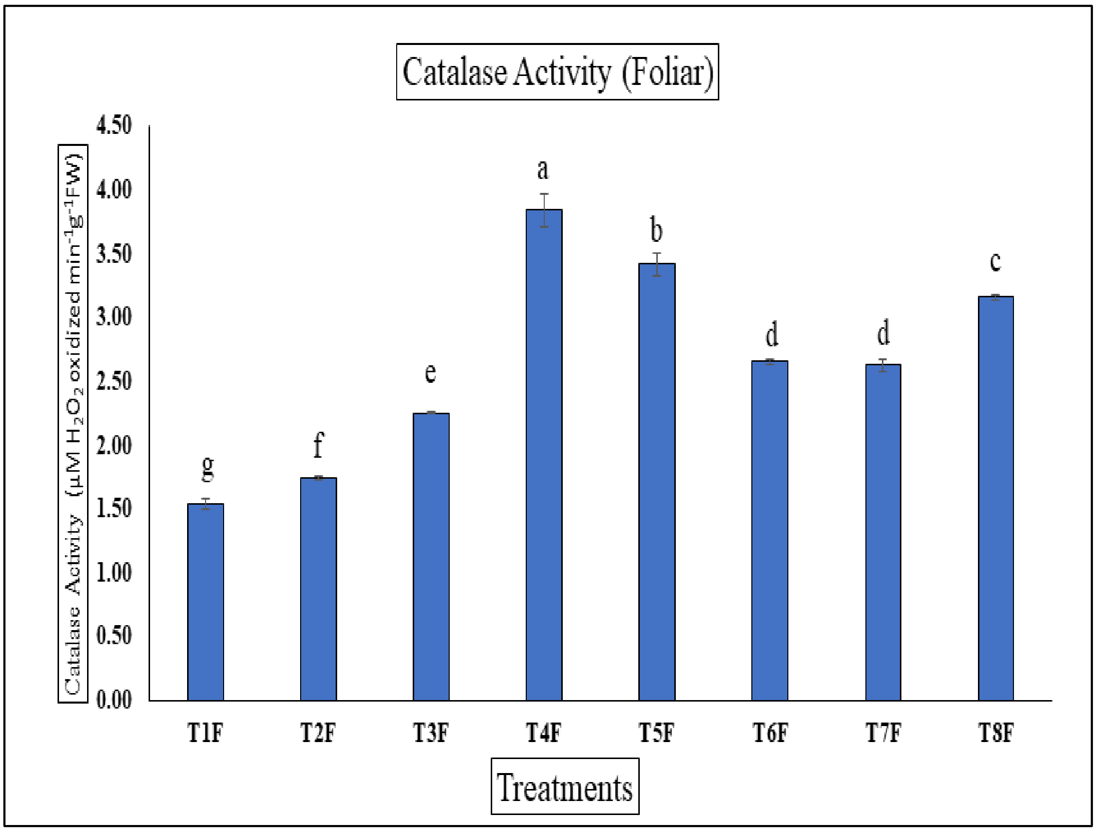


Figure.4.11. The graph shows the catalase activity in different treatments when the method of application was foliar. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at $p \leq 0.05$

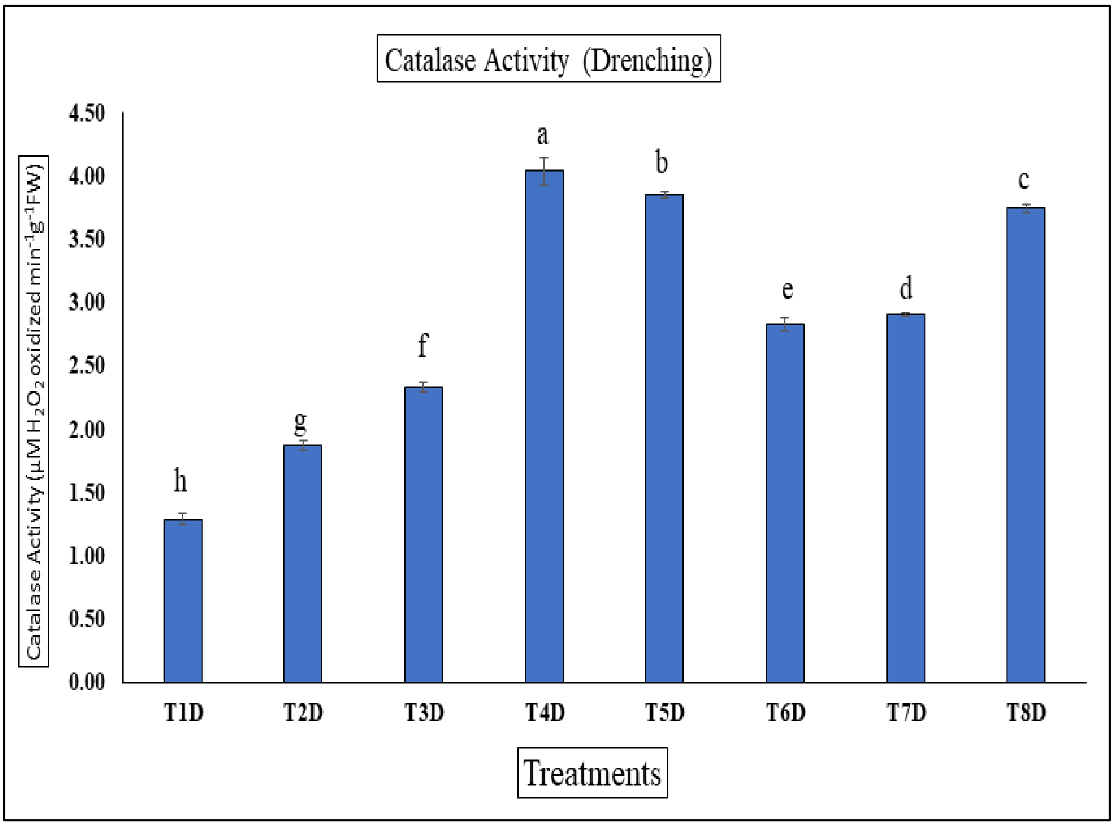


Figure.4.12. The graph shows the catalase activity in different treatments when the method of application was soil drenching. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at $p \leq 0.05$

4.7 Chlorophyll content

Chlorophyll content in cucumber was noted 14 days after the application of treatments. Newly fresh leaves were used to assay the chlorophyll content. Chlorophyll content (Chlorophyll a, Chlorophyll b, and Chlorophyll a + b) was significantly higher in cucumber leaves in all the treatments and in both the methods of applications i.e foliar and drenching. However, the best results were seen in the case of the drenching. The following amount of Chlorophyll a, b and a + b was observed during the assay.

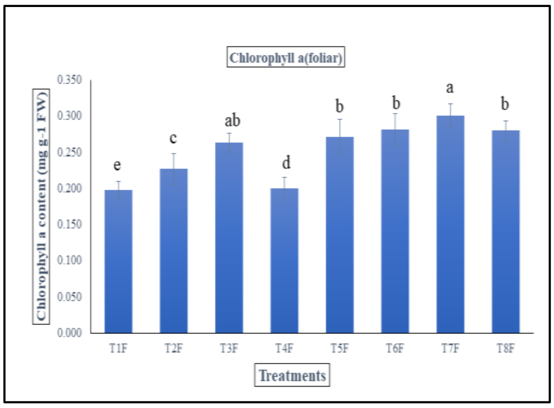
(A) Foliar Application

In case of foliar application, maximum chlorophyll a (0.280 mg g^{-1} FW), chlorophyll b (0.258 mg g^{-1} FW), and chlorophyll a + b (0.525 mg g^{-1} FW) was detected in T8F. The amount of chlorophyll a, b, and a + b detected was 1.4, 2.25, and 1.75- fold higher as compared to the control T1F respectively. The minimum amount of all three chlorophyll was present in T1F i.e chlorophyll a (0.198 mg g^{-1} FW), b (0.114 mg g^{-1} FW), and a + b (0.299 mg g^{-1} FW) (Fig no.4.13)

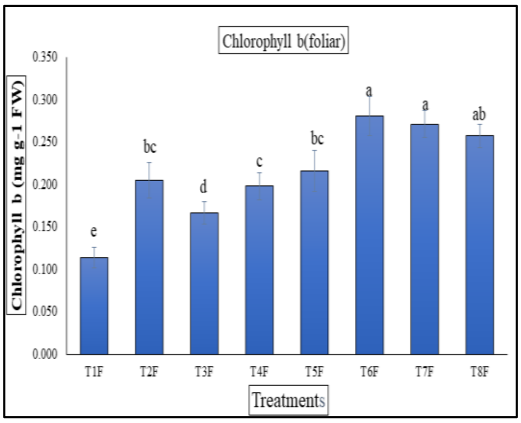
(B) Soil drenching

The results showed that, maximum chlorophyll a (0.345 mg g^{-1} FW), chlorophyll b (0.278 mg g^{-1} FW), and chlorophyll a + b (0.541 mg g^{-1} FW) was detected in T8D followed by T6D and T7D in chlorophyll a, T7D and T6D in chlorophyll b, and T6 and T7D in chlorophyll a + b. The amount of chlorophyll a, b, and a + b detected in T8D was 1.76, 2.78, and 2.4- fold higher as compared to the control T1F respectively. The minimum amount of all three chlorophyll was present in T1F i.e chlorophyll a (0.196 mg g^{-1} FW), b (0.100 mg g^{-1} FW) and a + b (0.224 mg g^{-1} FW). (Fig no.4.14)

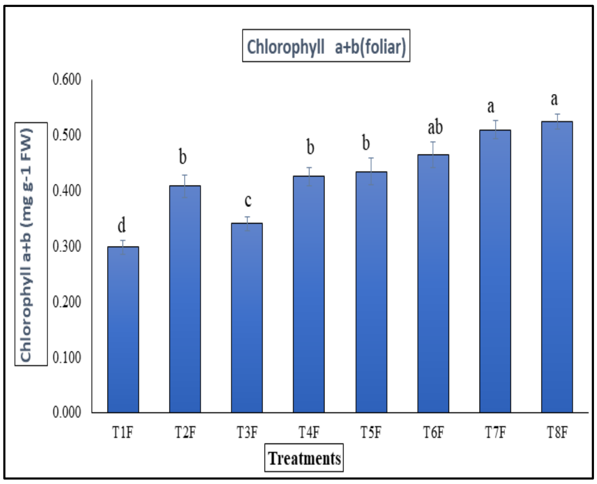
Trichoderma, an endophytic fungus, is well-known and frequently employed as a biocontrol agent for its ability to promote plant growth under biotic and abiotic stress. Several *Trichoderma* spp. strains have been identified, including *T. harzianum, T. asperellum, T. atroviridae, T. virens, and T. viride* strengthening the plant's overall defence by colonizing the rhizospheric layer and triggering systemic resistance, so providing a barrier to pathogen invasion and boosting plant nutrition intake (Patel *et al*., 2019). The three *Trichoderma* strains (*T. asperellum* 525, T. *harzianum* 610, and T. *pseudokoningii* 866) exhibited excellent growth and disease prevention effects on cucumber (Mei *et al*., 2019). Plants treated with *T. asperellum* pool also had an 84 percent higher level of Chlorophyll-a + b (Sousa *et al*., 2020). Foliar applications of SA, on the other hand, resulted in increased shoot fresh weight and dry weight, root fresh weight and dry weight, as well as more plants under salt stress (Yildirim *et al*., 2013). The application of 100 µML^{-1} melatonin followed by inoculation showed an increased level of photosynthetic efficiency and chlorophyll content had also been shown to increase which means that melatonin helps in benefiting fruit yields as well as resistance to downy mildew (Sun *et al*., 2019). In the present study, the level of chlorophyll a, b and c has been found to the maximum in both F (foliar) and D (drenching) in T8 (salicylic acid + melatonin + *Trichoderma).*



(A)

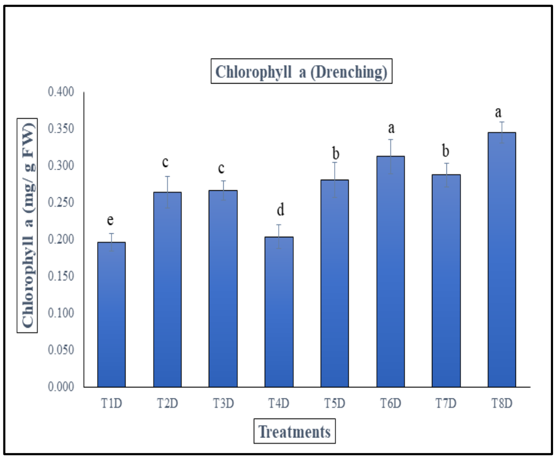


(B)

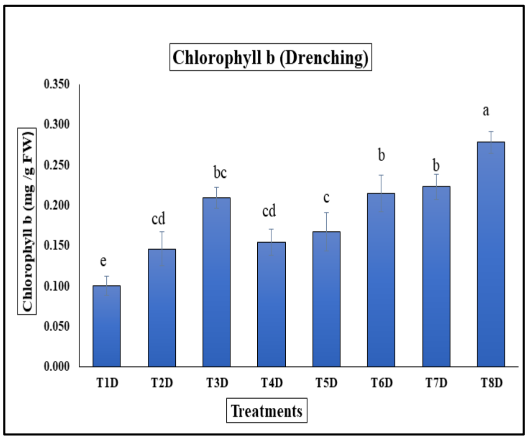


(C)

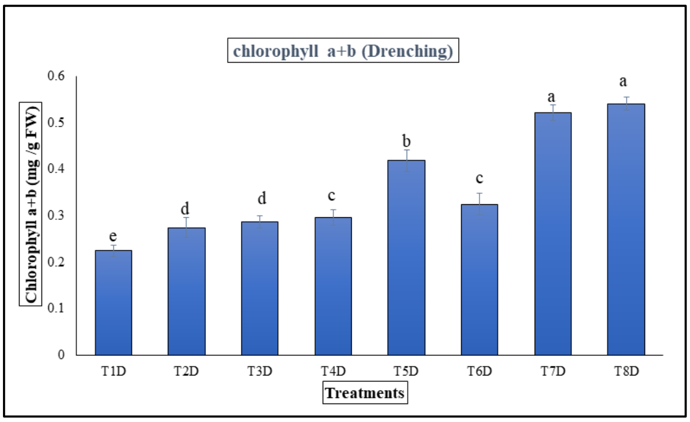
Figure 4.13. Showing (A) Chlorophyll a, (B) Chlorophyll b, (C) Chlorophyll a + b Content in leaves of cucumber after foliar application of treatments. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at $p \leq 0.05$



(A)



(B)



(c)

Figure.4.14 Figure 4.13. Showing (A) Chlorophyll a, (B) Chlorophyll b, (C) Chlorophyll a + b Content in leaves of cucumber after soil drenching method of application of treatments. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at *p*≤ 0.05

4.8 Protein content

Protein content in cucumber was noted after 14 days of application of treatments. Protein content was significantly higher in cucumber leaves in all the treatments and in both the methods of applications i.e foliar and drenching. However, the best results were seen in the case of the foliar one. The following amount of protein content was observed during the assay.

(A) Foliar Application

The results showed that the best result was found in T8F in which the average value of protein content in the leaves was 24.6 µg g^{-1} FW followed by T6F and T5F. The lowest value was observed in T1F which was 20.9 µg g^{-1} FW T8F was 1.7- folds higher than the control. As compared to all the combined treatments T8F (salicylic acid + melatonin + *Trichoderma*) performed better than T6F (salicylic acid + *Trichoderma*) had a better result on the leaves regarding protein content (Fig.no 4.15).

(B) Soil Drenching

It was observed that the value of protein content was maximum in T8D which was 1.17 folds higher than in T1D. In both methods of application, T8 performed well and showed the best results as compared to the other treatment. After T8D, T7D and T5D had better results with average values of 24 µg g^{-1} FW and 23.4 µg g^{-1} FW respectively. The lowest value was 20.8 µg g^{-1} FW followed by 21.2 µg g^{-1} FW and 21.6 µg g^{-1} FW (Fig no. 4.16)

Earlier biochemical response at different concentrations of salicylic acid induced increase in the amount of chlorophyll and protein content in the leaves and the highest value was 1.9-fold higher than the control one (Rashid war *et al.*, 2011). The present study shows similar results in which T8F had higher protein than any other treatments, and had 1.7-fold higher protein than the control one.

The foliar exogenous application of melatonin applied to cucumber seedling which was in an active state resulted in higher protein content in the leaves (Xiang *et al.*, 2010). This result can be correlated with the findings of the present study in which melatonin-treated plant showed increase in protein content inside the leaves.

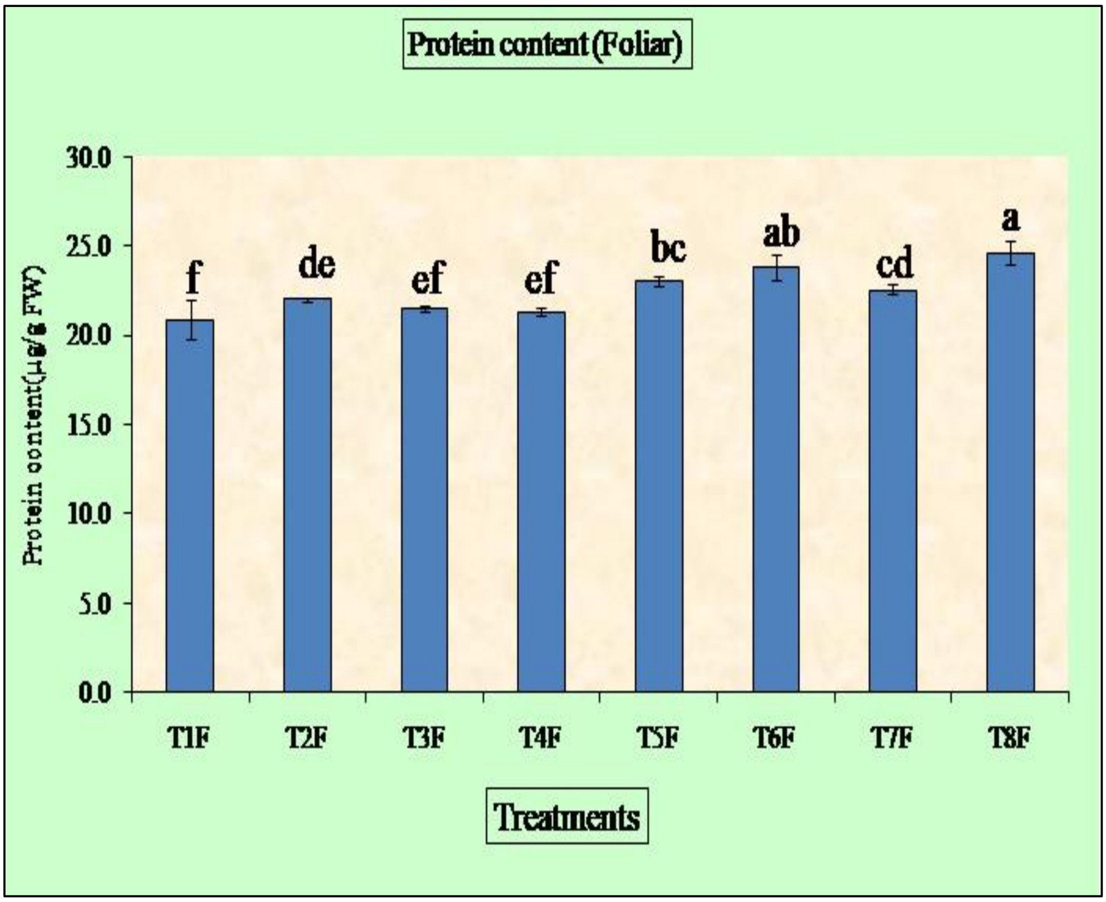


Figure 4.15. showing the protein content in leaves in foliar method of application. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at $p \leq 0.05$

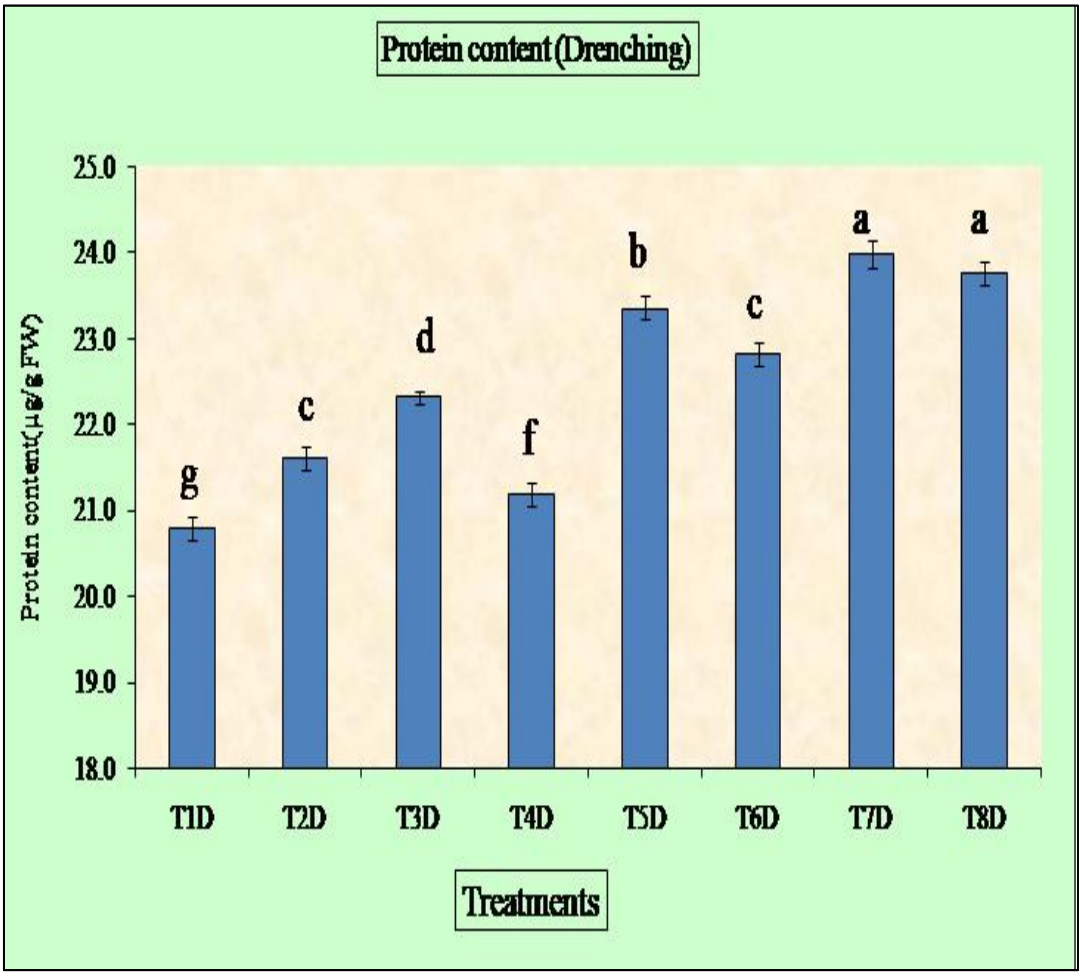


Figure 4.16. showing the protein content in leaves in Soil drenching method of application. Results are presented as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range test at $p \leq 0.05$

Dual culture assay

The two isolates of *Trichoderma* (i.e, *T. harzianum and T. viride*) performed well against all the tested pathogens (*Alternaria solani, Curvularia oryzae, Phomopsis vexans, Colletotrichum capsici, Fusarium oxysporum* f.sp. *lycopersici, and Bipolaris sorokiniana*.). After 4 days of incubation, the isolates of *Trichoderma* inhibited the growth of *Curvularia oryzae* which ranged from 27.74% to 35.77%. The maximum inhibition of 35.77% was observed in the case of *T. viride* whereas 27.74% inhibition was done by *T. harzianum*.

In the case of *Fusarium oxysporum* f.sp. *lycopersici*, the inhibition percentage ranged from 10.78% to 14.71%. *T. harzianum* showed the maximum inhibition which was 14.71% after 4 days of incubation. The minimum inhibition was observed in case of *T. viride* (10.78%).

The inhibition percentage varied from 28.21% to 21.79% as in the case of *Alternaria solani*. The highest inhibition was shown by *T. harzianum* which was 28.21% after 4 days of incubation whereas the lowest inhibition was observed in *T. viride*.

Trichoderma harzianum showed the best result in the case of *Phomopsis vexans*. The inhibition percentage was highest among all the tested pathogens after 4 days of incubation. It was 39.81% inhibition. *T. viride* inhibited 17.48% growth of *Phomopsis vexans*.

In the case of *Colletotrichum capsici*, percentage inhibition ranged from 29.03% to 18.28%. The maximum inhibition was observed after 4 days of incubation in *T. viride*. The inhibition percentage of 18.29% was done by *T. harzianum*.

After *Phomopsis vexans*, both the two strains of *Trichoderma* (i.e, *T. harzianum and T. viride*) performed maximum inhibition in the case of *Bipolaris sorokiniana* after 4 days of incubation. The maximum inhibition percentage was done by T. *harzianum* and the inhibition percentage was 36.36%. *T. viride* inhibited the growth which was 31.31%.

The results showed that among both these strains of *Trichoderma*, the maximum inhibition in the growth of all the tested pathogens was done by *T. harzianum*. It suppressed the highest value of the growth of all the pathogens. However, in the case of *Curvularia oryzae* and *Alternaria solani,* the maximum inhibition percentage was done by *T. viride.* In the in-vitro study of *Trichoderma* strains, *T. harzianum* was found to be performed better against all tested pathogens. Hence, *T. harzianum* was chosen for the in-vivo study.

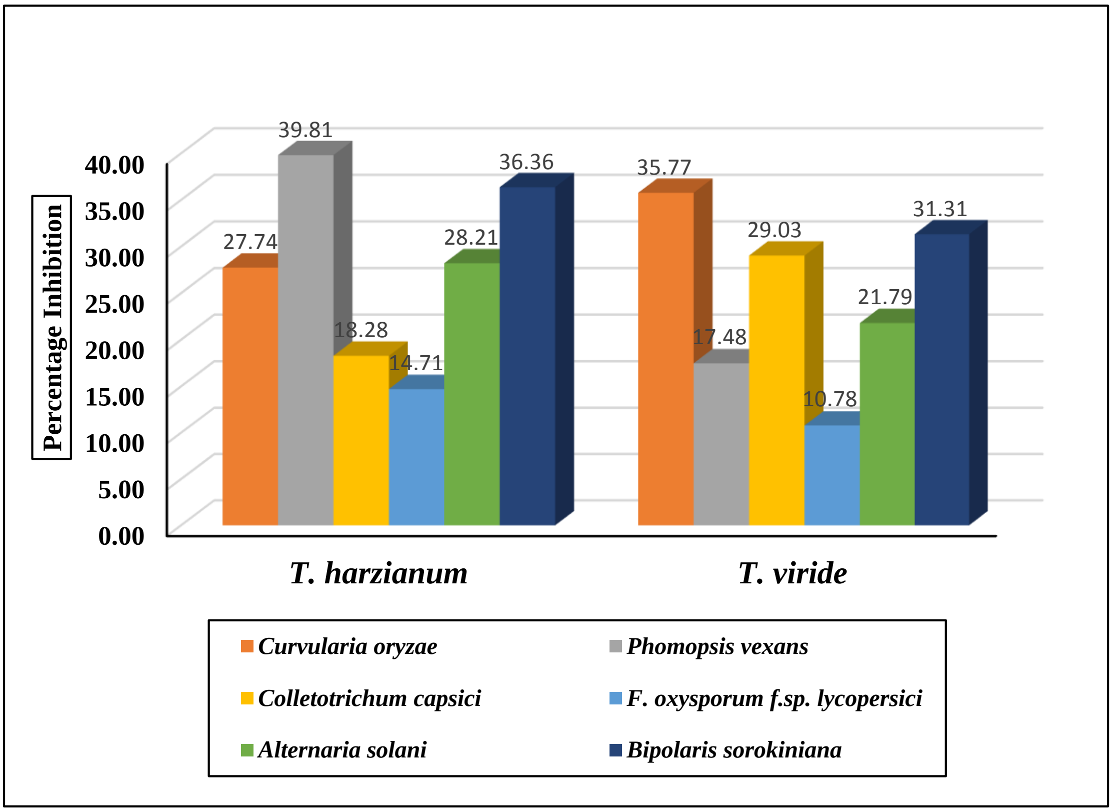


Figure. 4.17. Graph showing percentage inhibition 4 days after incubation by *Trichoderma harzianum* and *Trichoderma viride* against *Alternaria solani, Curvularia oryzae, Phomopsis vexans, Colletotrichum capsici, Fusarium oxysporum* f.sp. *lycopersici, and Bipolaris sorokiniana*.

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Chapter-V

SUMMARY AND CONCLUSION

The present study entitled **"Synergistic effect of bio-stimulants and *Trichoderma* spp. on physio-biochemical parameters of cucumber (*Cucumis sativus* L.)"** was conducted to evaluate the combined effects of salicylic acid, melatonin, and *Trichoderma* spp. in enhancing the defense response against biotic stress along with the promotion of growth and development in cucumber. The different types of biochemical changes were studied to support the objective of the experiment. Total phenol content (TPC), Phenylalanine ammonia-lyase (PAL), Peroxidase (PO), Polyphenol oxidase (PPO) activities, Catalase activity, proline content, chlorophyll (a, b, a + b) content, and protein content were observed. The salient results of the experiments mentioned above are summarized as follows:

- ❖ The results showed that among both the species of *Trichoderma*, the maximum inhibition in the growth of all the tested pathogens was done by *T. harzianum*. It suppressed the highest value of the growth of almost all the pathogens. Thus, *T. harzianum* was taken for further study in objective 2.

- ❖ The application of biostimulants (salicylic acid, melatonin, and *Trichoderma* spp) in different combinations and in two methods of application results in the enhancement of the antioxidants enzymes and molecules such as PAL content, PPO, PO, catalase activities, proline and TPC content which directly take part in plant defense response against biotic and abiotic stress. Among all the combinations, salicylic acid + melatonin + *Trichoderma* was found to be significantly higher in all the biochemical assays and gave most effective results among all the 8 treatments followed by treatment combination of *Trichoderma* + melatonin.

- ❖ The increment in protein content and chlorophyll was almost higher in all treatments as compared to the control but a significant increment was observed in the treatment combination of salicylic acid + melatonin +

Trichoderma. The results of these treatments were also evident in promoting the growth and development of cucumber.

- ❖ In respect to the exogenous application of treatments, foliar application was found to be best as compared to the drenching application of treatments.

On the basis of the present study, it may be concluded that:

The results from this study demonstrated that there were strong synergistic effects between salicylic acid, melatonin along with *Trichoderma harzianum* as compared to the effects alone. *Trichoderma harzianum* strengthens the plant's overall defence response by colonizing the rhizospheric layer and triggering systemic resistance, so providing a barrier to pathogen invasion and boosting plant nutrition intake. *Trichoderma* spp. and their secondary metabolites have shown potential in the management of diseases. Pathogen-induced systemic acquired resistance (SAR) is based on salicylic acid (SA) signalling and leads to the production of pathogenesis-related proteins (PR) and phytoalexins, which may protect against later pathogen attacks. Melatonin is a potent antioxidant that protects the plant against reactive oxygen and nitrogen species (ROS/RNS). Melatonin functions as a protective agent against various stress situations.

In cucumber cultivation practices, excessive use of pesticides, subsequently increase the cost of production and also pollute the environment hence, it is important to reduce its usage and shift to other alternatives such application of these biostimulants that will able to produce good quality marketable fruits and fulfil consumer's demand. Such products will be free from hazardous chemicals and safe to consume.

We have seen that cucumber plants treated with these biostimulants (salicylic acid, melatonin, and *Trichoderma*) showed better results in enhancing cucumber growth and development along with making the plant more tolerant toward biotic and abiotic stress.

The world is getting more concerned about their health and environment day by day. People are now more selective towards the food they eat (i.e vegetables and fruits) due to their health concerns, which leads to creating pressure on growers to fulfil their demands and at the same time practicing sustainable methods of growing crops. After summing up all the above statements, it can be said that biostimulants could be the most efficient alternative to pesticides which will improve cucumber plant health as well as production. Moreover, further investigations will be needed to evaluate, ascertain, and support the details of the mechanisms.

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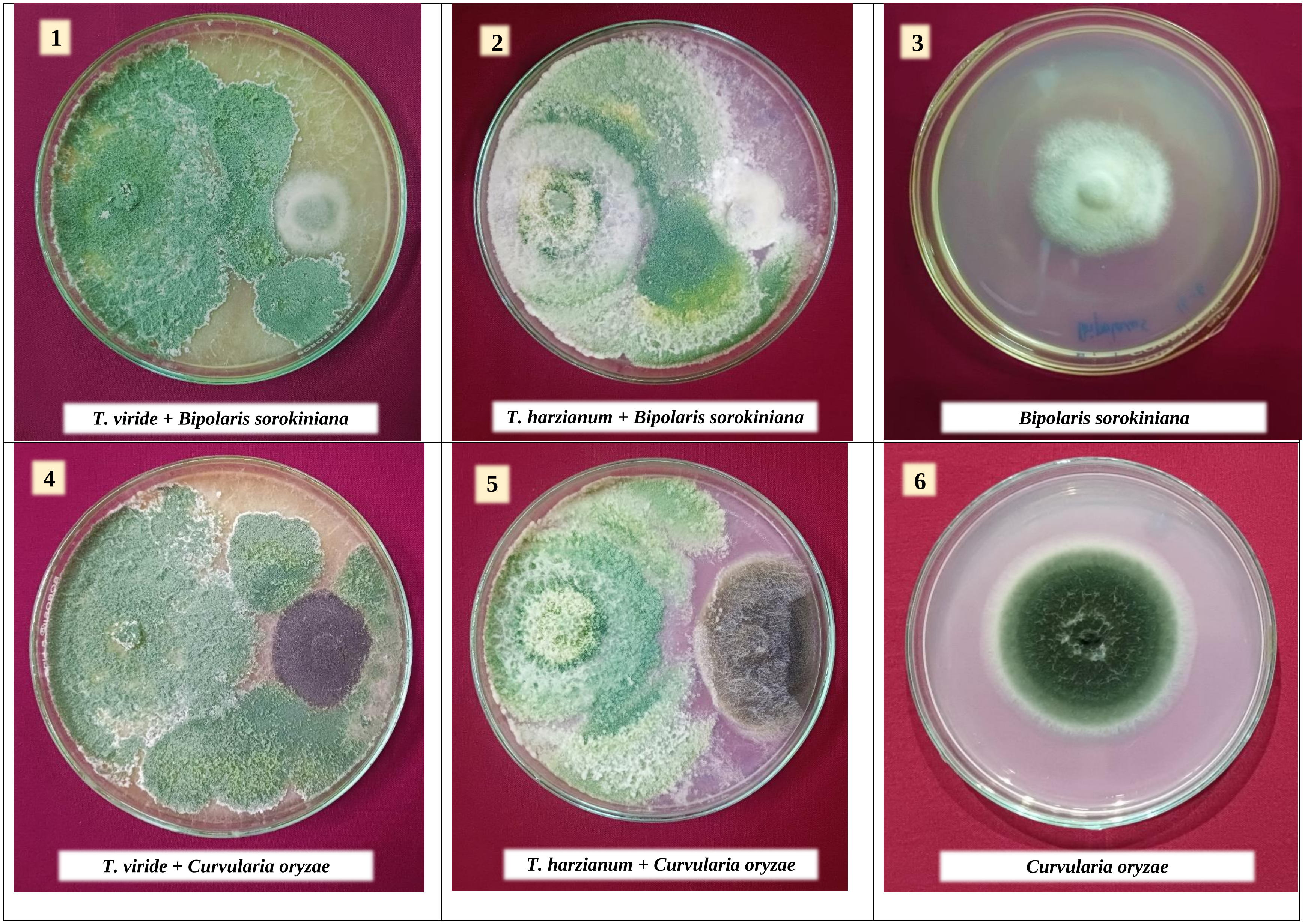
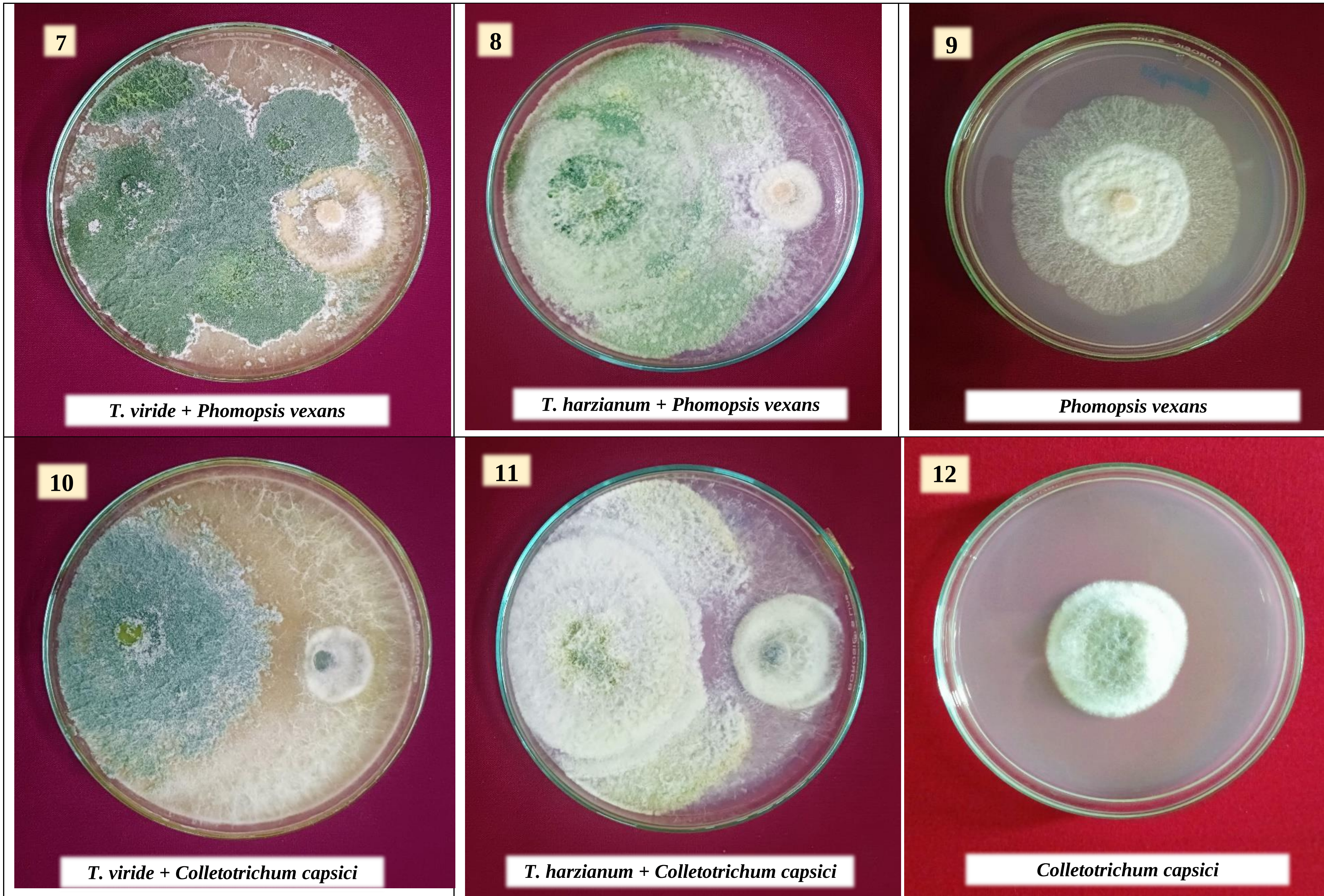
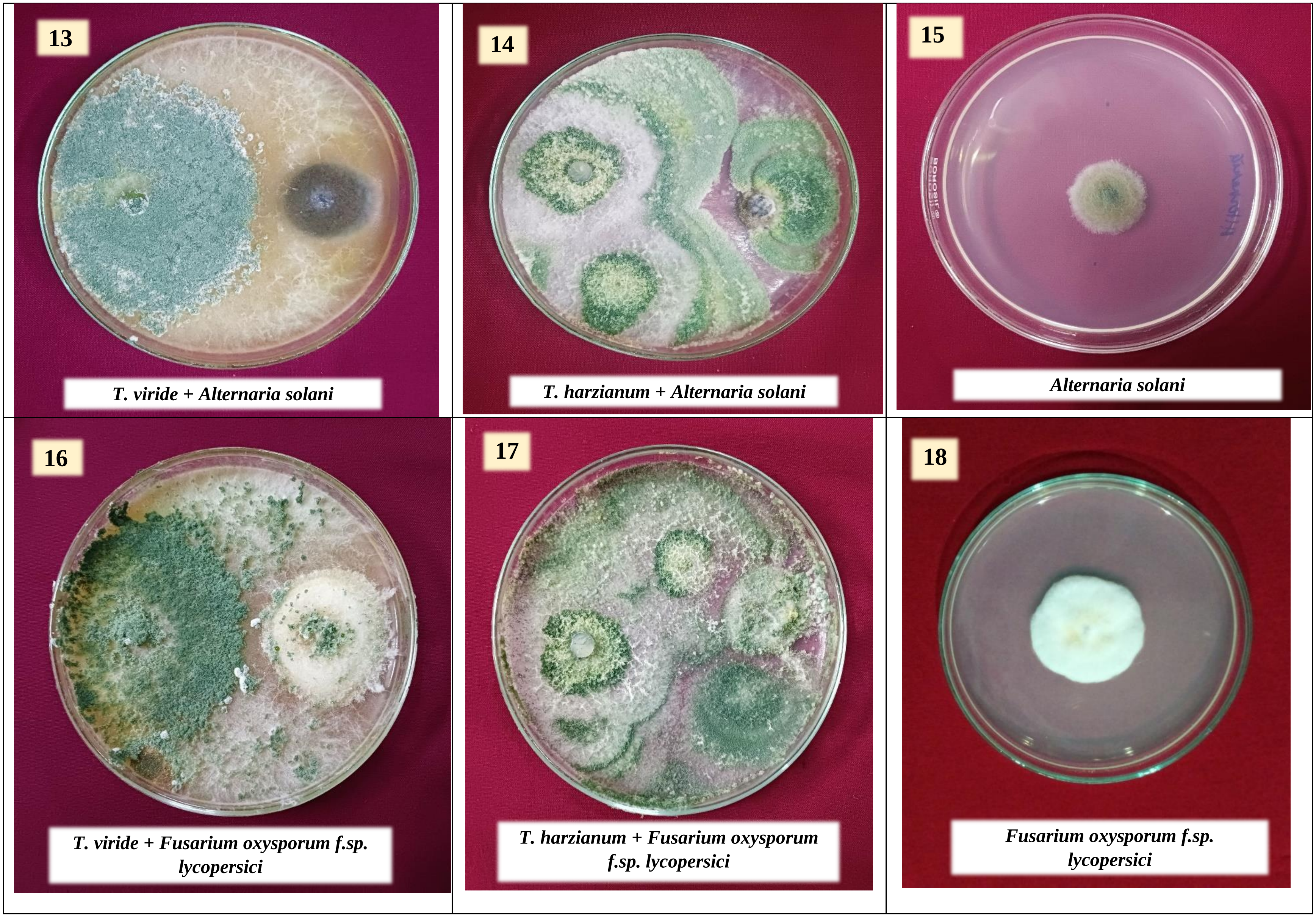


Plates Showing Dual Culture Assay of *Trichoderma harzianum* and *Trichoderma viride* against *Bipolaris sorokiniana* and *Curvularia oryzae* 4 days after incubation.



Plates Showing Dual Culture Assay of *Trichoderma harzianum* and *Trichoderma viride* against *Phomopsis vexan* and *Colletotrichum capsici* 4 days after incubation.



Plates Showing Dual Culture Assay of *Trichoderma harzianum* and *Trichoderma viride* against *Alternaria solani* and *Fusarium oxysporum f.sp lycopersici* 4 days after incubation.