

**Effect of bio-stimulants and *Trichoderma*
spp. on biochemical parameters of
cucumber (*Cucumis sativus* L.) grown
under salt stressed condition**

काशी हिन्दू
विश्वविद्यालय



BANARAS HINDU
UNIVERSITY

THESIS

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF

Master of Science (Agriculture)

in

Plant Pathology

Submitted by

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Dear Sir,

I have great pleasure in forwarding the thesis entitled “**Effect of bio-stimulants and *Trichoderma spp.* on biochemical parameters of cucumber (*Cucumis sativus L.*) grown under salt stressed condition**” submitted by **Mr. Surendra B.K., ID. No. 20412MPP026**, in partial fulfilment of the requirements for 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 university.

I certify that the work has been carried out under my guidance and the data forming the basis of the thesis, to the best of my knowledge are original, genuine and no part of the work has been submitted for any other degree or dissertation.

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Forwarded by

Yours faithfully

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Effect of bio-stimulants and *Trichoderma* spp. on biochemical parameters of cucumber (*Cucumis sativus* L.) grown under salt stressed condition



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ACKNOWLEDGEMENT

*At the outset, I bow my head and offer flowers at the virtuous feet of the founder of Banaras Hindu University, **Late Mahamana Pt. Madan Mohan Malaviya ji**. The auras of Malviya ji spread across the campus has inspired and motivated me to do the best and be the best in every aspect of life in a rightful way.*

I take this golden opportunity to express my heartfelt and deepest sense of gratitude to those who have helped me to complete this manuscript. My debts for many individuals can warmly be acknowledged but never fully recompensed.

*In the ecstasy of delight, I fumble for the words to pen down heartfelt veneration towards my advisor, erudite and revered teacher and elite guide, motive force and path director **Dr. Satyendra Pratap Singh**, Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi for his unimpeachable incessant efforts for planning and execution, valuable guidance through the course of investigation, parental care and painstaking rendered to me which helped me in bringing this surmount task to success. He always encouraged me to take up challenges with confidence. I feel proud to be his student.*

*I am highly thankful to members of my advisory committee, **Dr. Ram Chandra**, Professor, Department of Mycology and Plant Pathology, **Dr. R.S. Meena**, Assistant Professor, Department of Entomology and Agricultural Zoology, for their valuable guidance during the course of study.*

*Words can hardly acknowledge the help made by **Dr. S.S. Vaish**, Professor and Head, Department of Mycology and Plant Pathology for providing necessary facilities and benevolent patronage.*

*I extend my indebtedness to the respected teachers **Prof. Ramesh Chand**, **Prof. Ram Chandra**, **Prof. B.K. Sarma**, **Late Dr. Vineeta Singh**, **Dr. R.K. Singh**, **Dr. Ankita Sarkar**, **Dr. D.D. Bhutia** of Department of Mycology and Plant Pathology, for their discerning comments, valuable suggestions, co-operations and helpful attitude towards me during the course of investigation.*

I am highly indebted to my beloved parents **Mr. Lil Bahadur B.K.**, and **Mrs. Tul Maya B.K.**, who have given me life, taught the concepts of life and their dedicated efforts to educate me to this level, with boundless blessings and affection. I owe an encompassing debt to my beloved sisters **Shova B.K.**, **Rupana B.K.**, **Kalpna B.K.**, and **Samjhana B.K.**, brothers-in-law **Man Bahadur B.K.**, **Thakur Thapa**, **Bikash B.K.**, and **Shyam Raj B.K.**, without their constant support, I could not have completed the study successfully. My words are too feeble to give my inner feelings. Their constant encouragement, moral and emotional support rendered throughout my education for which I will remain indebted to them throughout my life.

I am thankful to **Sri Santosh ji**, **Sri Ram Kumar ji**, **Sri Laxman Prasad ji**, **Sri Kailash ji**, **Sri. Dasharath ji**, **Sri Amit ji**, **Sri Krishna ji**, **Sri Kaushik ji** for whole hearted co-operation and continues inspiration. I owe my sincere thanks to all the non-teaching staff of the Department of Mycology and Plant Pathology, for their keen interest taken in the work, providing the necessary and timely research facilities, inspiration and suggestion throughout the work,

The help rendered by my seniors **Aakansha ma'am**, **Jharjhari ma'am**, **Jeetu ma'am**, **Sumit sir**, **Divya ma'am**, **Rahul sir**, **Abhinandita ma'am** and **Soumya ma'am**, and my batchmates **Suman**, **Samikshya**, **Jiwan**, **Nikita**, **Neha**, **Sushrutha**, **Ganita**, **Rakhi** and **Vandana**, in whose cheerful company I have never felt my work as burden. It is a pleasure to acknowledge the affection and inspiration rendered by my friends **Rashila**, **Avdhesh**, **Kalindi** and **Aashish** for their affection, motivation, inspiration and special upliftment during studies and worries. No words are enough to express my heartiest sense of gratitude to my junior **Kapil** during the study period for his support.

Above all, my humble and whole hearted gratitude to **Pashupatinath Mahadev** and **Baba Vishwanath** for their blessing.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	-	Per cent	rpm	-	Revolution per minutes
&	-	And	Dia.	-	Diameter
<	-	Less than	et al.	-	et alia, and other people
>	-	Greater than	etc.	-	Etcetera
M	-	Molar	g	-	Gram
µl	-	Microliter	i.e.	-	That is
µM	-	Micromolar	Kg	-	Kilogram
mM	-	Millimolar	m	-	Meter
TPC	-	Total phenol content	m ²	-	Square meter
min	-	Minutes	FW	-	Fresh weight
PO	-	peroxidase	ml	-	Millilitre
PPO	-	Polyphenol oxidase	mm	-	Millimetre
/	-	Per	nm	-	Nanometre
@	-	At the rate of	p.s.i	-	Pounds per square inch
°C	-	Degree Celsius	ppm	-	Parts per million
NaCl	-	Sodium Chloride	Conc.	-	Concentrations
SA	-	Salicylic acid	viz.	-	Namely
MT	-	Melatonin	no.	-	Number
TH	-	<i>Trichoderma harzianum</i>	cm	-	Centimetre
TV	-	<i>Trichoderma viride</i>	H ₂ O ₂	-	Hydrogen peroxide

INTRODUCTION

Cucumber (*Cucumis sativus* L., $2n=14$) is a member of the Cucurbitaceae family. It is a warm-season vegetable that grows in tropical and subtropical climates all over the world. It is said to be a native to northern part of India (Pursglove, 1969). Cucumber cultivation has been traced back to 3000 years in India, from where it appears to have migrated eastwards to China and westwards to Asia Minor, North America, and Southern Europe, and then to the rest of Europe (De Candler, 1967). *Cucumis* is a genus of about 30 species in the Cucurbitaceae family, which has 118 genera and 825 species (Jeffrey, 1990). The cultivated species is an herbaceous, trailing, seasonal plant that grows horizontally. It is monoecious in that both female and male flowers are produced on the same plant, but gynoecious lines are also available.

The fruits are nutrient-dense, high in water, and low in calories. In its immature form, the fruit is eaten as a fresh fruit, as sliced cucumber, and pickles. It is abundant in minerals, thiamine, niacin and vitamin C (0.38 g, 0.3 mg, 0.2 mg and 78 mg, respectively per 100 g of edible fruit). Fruits have an edible fraction of about 80 %, with 95 % water, 0.7 % protein, 0.1 % fat, 3.4 % carbohydrate, 0.4 % fiber, and 0.4 % ash (Aykroyd, 1963).

Abiotic stresses have a significant impact on global agricultural output. Salinity, among other abiotic stresses, has become a serious threat to food security, affecting around one-third of the world's irrigated area (Mengel *et al.*, 2001). Soil salinity is a serious environmental issue that has a detrimental effect on the growth and productivity of different plant species. Salinity has a negative impact on agricultural productivity in both irrigated and non-irrigated fields around the world, particularly in arid and semi-arid regions (Ashraf, 2010; Zhang *et al.*, 2010). Salinity has been reported to affect approximately 20% of the world's cultivated areas and half of the world's irrigated areas (Flowers and Yeo, 1995). About a third of the 230 million hectares of irrigable land could not be sown due to soil salinity (Oldeman *et al.*, 1991; Ghassemi *et al.*, 1995). Of

such areas, Africa accounts for 15.57 %, Australia for 5.07 %, Mexico and Central America for 0.57 %, North America for 1.8 %, South America for 20.21 %, North and Central Asia for 26.7 %, South Asia for 24.25 %, and Southeast Asia for 5.82 %. In these locations, a vast number of plant species have been documented. Not just between species, but also within species, these species' salinity tolerances vary dramatically. Paddy is the most vulnerable monocotyledonous cultural plant to salinity, while bread wheat is resilient and barley is the most tolerant species. Dicotyledonous plants are more tolerant of saline circumstances than monocotyledonous plants (Massoud, 1974).

In India, around 60% of the country's total geographical area is cultivable (arable), with crops accounting for nearly 80% (141 million hectares) and rangelands accounting for about 6% (10 million ha) (Mythili and Goedecke, 2016). The remaining arable lands are uncultivated. Approximately 6.727 million ha, or about 2.1 percent of the country's geographical area, is salt-affected, with 2.956 million ha saline and the remaining 3.771 million ha is sodic (Arora *et al.*, 2016; Arora and Sharma, 2017). The Indo Gangetic plains of the country contain approximately 2.347 million ha of salt-affected soils, among which 0.56 million ha are saline and 1.787 million ha are sodic (Arora and Sharma, 2017). Gujarat (2.23 million ha), Uttar Pradesh (1.37 million ha), Maharashtra (0.61 million ha), West Bengal (0.44 million ha), and Rajasthan (0.38 million ha) account for nearly 75 percent of the country's salt-affected soils (Mandal *et al.*, 2018).

Soil salinization has enormous environmental, ecological, agricultural, and social effects, with regard to shrinkage of agricultural lands, low agricultural productivity, uncertain and unstable livelihood security, low economic returns, and poor quality of life. Excess salts content in soil disrupts soil flora and fauna's metabolism, eventually resulting in the extinction of all soil life and transforming nutrient-rich and productive lands into barren and desert lands (Kumar and Sharma 2020). Soils are rendered unusable for agriculture and a variety of other activities (e.g., construction work). The salt deposition harms existing infrastructure, farm machinery, streams, and roadways, among other things. Plants subjected to salt stress face water deficiency and harmful ion toxicity issues (Munns, 2005). The excessive salt concentration in the soil affects the porosity of the soil, lowering the water potential of

the soil and causing physiological drought (Hopkins, 1995). The high salt concentration also has an impact on the plant's physiology, both at the cellular and whole-plant levels (Murphy and Durako 2003). Salinity interrupts the osmotic potential and limits the intake of nutrients such as potassium (K^+), calcium (Ca^{2+}), and phosphorus (P), as well as accumulating high levels of sodium (Na^+) and chlorine (Cl^-) in leaf tissues, causing physiological and biochemical changes in plants (Parida *et al.*, 2005). Salt stress is hazardous to plants, causing metabolic changes such as chloroplast activity loss, photosynthetic rate reduction, and increased photorespiration, all of which result in the creation of reactive oxygen species (ROS) (Parida and Das, 2005). Reduced photosynthesis in saline conditions is said to be one of the most important factors causing plant growth and productivity to decline (Ball *et al.*, 1987).

Changes in the activity of many antioxidant enzymes have been documented in response to salinity stress (Koskeroglu and Tuna, 2008; Venkatesan and Sridevi, 2009; Hernández *et al.*, 2000). The ability to scavenge reactive oxygen species (ROS) and minimise their destructive effects on macromolecules like protein, DNA, lipids, chlorophyll, and other essential macromolecules appears to be an important stress-tolerance factor (Xiong *et al.*, 2002). When plants are stressed by salinity, the equilibrium between reactive oxygen generation and antioxidant enzyme degradation is disrupted, causing oxidative damage (Roy *et al.*, 2005). Antioxidative enzymes such catalase, ascorbate peroxidase, guaiacol peroxidase, glutathione reductase, and superoxide dismutase increase their levels and/or activities in salinity-stressed plants (Chen *et al.*, 2012; Wang and Han, 2009). Plant metabolism and stress tolerance to ROS are both aided by GSH (Szalai *et al.*, 2009). To scavenge ROS, plants have a sophisticated antioxidative defence mechanism that includes both nonenzymatic and enzymatic elements. Non-enzymatic antioxidants include GSH, ascorbate (AsA), carotenoids, and tocopherols. Whereas, Peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT), together with the other enzymes of the ascorbate–glutathione cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) aids in the scavenging of ROS, are enzymatic defense components (Gill and Tuteja, 2010). Szabados and Saviouré (2010) claim that proline is the sole osmolyte

capable of scavenging free radicals, preserving membrane stability and preventing protein denaturation during extreme osmotic stress. Under saline conditions, a rise in Na and Cl, as well as lipid peroxidation, indicates ion toxicity and oxidative damage. However, oxidative damage is regulated by a defense mechanism that includes antioxidants like SOD, POD, and CAT. The findings revealed that salt-tolerant and salt-sensitive okra genotypes exposed to NaCl stress had the highest levels of SOD, POD, and CAT activity in both root and leaf tissues (Abbas *et al.*, 2015). In salt-treated plants, proline concentration was much higher than in non-salt-treated plants (Tripathi *et al.*, 2017). These findings point to a link between stress tolerance and the presence of a well-functioning antioxidant system. Recent studies have demonstrated that phytohormones have the capacity to reduce or eliminate the detrimental consequences of stress factors in plants (Chakrabarti and Mukherjee, 2003).

Biotic stress is defined as harm to crops produced by a variety of living creatures such as fungi, bacteria, viruses, parasitic nematodes, insects, weeds, and other native or grown plants. The severity of these impacts and the subsequent crop loss are determined by a variety of parameters, including causal organisms, ambient circumstances, and crop and causal organism levels (Walters, 2010). Plant diseases are mostly caused by pathogens such as fungi, bacteria, nematodes, and viruses. Fungi and bacteria can produce leaf spots, vascular wilts, and cankers, among other symptoms, and they can affect different plant parts. Nematodes eat plant cells and damage all sections of the plant; they can also let soil-borne diseases get into the root system, causing nutrient deficiency symptoms including stunting and wilting (Moustafa-Farag *et al.* 2019). Viruses cause systemic damage as well as local lesions, causing deformities, stunting, and chlorosis in various plant parts, even when their hosts are not killed. On the other hand, insects and mites should be highlighted. They harm plants by depositing eggs or feeding on them (Schulze-Lefert P, 2010).

During the growing season, the cucumber crop is vulnerable to a variety of fungal, bacterial, and viral diseases, resulting in significant crop loss (Harfoush and Salama, 1992; Reuveni *et al.*, 1993; El-Gamal, 2003). The major fungi that cause significant crop losses economically are powdery mildew [*Sphaerotheca fuliginea* (Schlechtend. Fr.) Pollaci and *Erysiphae cichoracearum* DC]; downy mildew

[*Pseudoperonospora cubensis* (Berk & Curtis)]; *Alternaria* leaf spot [*Alternaria alternata* (Fr.) Keissler]; anthracnose [*Colletotrichum orbiculare* Berk & Mont. Arx (syn. *C. lagenarium* (Pass. Ellis & Halst.))]; Fusarium wilt [(*Fusarium oxysporum* Schlechtend Fr. f.sp. *cucumerinum* (J.H. Owen))]; *Cercospora* leaf spot (*Cercospora citrullina* Cooke); and damping off (*Pythium spp.*), etc.

Pathogens including *Fusarium*, *Colletotrichum*, and *Alternaria* which causes significant losses in cucumber plants, and other important fungal pathogen viz. *Phomopsis*, *Bipolaris*, and *Curvularia* were chosen for the current study.

Fusarium oxysporum [(Schlecht end.: Fr.) f. sp. *cucumerinum* (Owen) Synd. and Hans] is the most common and harmful pathogen on cucumber, causing fusarium wilt which is one of the most damaging diseases of cucumber especially under protected conditions reducing potential yields. Wilting symptoms appear on infected plants which include seedling damping off, plant stunting, yellowing and wilting of older leaves, and brown vascular discoloration. It is common to see vascular discoloration of the roots and stem that extends up to 8-10 nodes (Owen 1955; Takeuchi *et al.* 1978; Vakalounakis 1993). Early infection prevents fruit set, whereas late infection results in small, abnormal fruits. The disease was first reported in Florida in 1955 (Owen 1955), and it has since spread to almost every cucumber-growing region on the planet (Armstrong and Armstrong 1978; Ogura and Ma 1992; Sultana and Ghaffar 2013; Fareed *et al.* 2016). In India, fusarium wilt was first reported in 2012 from Jammu and Kashmir (Pagoch and Raina 2012). Later, it was reported in Lucknow, Uttar Pradesh (Srivastava 2017) and Punjab (Singh 2018). Susceptible plants can be infected through the roots for the rest of their lives, resulting in yield losses of up to 100%. (Chen *et al.* 2011). This disease was estimated to cause 10-50 percent losses in major areas of Pakistan (Fareed *et al.* 2016).

Colletotrichum lagenarium [(Pass) Ellis & Halst] is the causative agent of anthracnose, the other important cucumber disease. According to Sitterly and Keinath (1996), *C. lagenarium* is a member of the family Glomerellaceae in the phylum Ascomycota and that its asexual stage, *C. orbiculare* (also known as *C. lagenarium*), is more common in nature than its sexual stage, *Glomerella lagenarium* (Arx, von 1957).

Anthracnose disease symptoms includes restricted but sunken necrotic lesions on leaves, stems, and flowers, among other things (Agrios, 2005). The disease causes brown circular lesions up to 1 cm in diameter, whereas lesions on the petiole and stem are elliptical, depressed, and round, bordered with a black fungal stroma containing masses of pink spores (Sitterly and Keinath, 1996). The ideal range of temperature for the onset of disease is between 70 and 80⁰ F. Prolonged periods of high relative humidity (RH) in the canopy and a thin layer of water on the surface of the leaves stimulate spore generation, germination, and infection.

Alternaria leaf spot is the other significant economic disease affecting cucumbers. *A. alternata* (formerly as *A. tenuis*) was the type isolate used by Nees to form the genus *Alternaria* in 1817. (Groves and Skolko, 1944; Neergaard, 1945). Nearly all cucumbers in the globe are infected with *A. alternata*, and its spores are spread by wind over greater distances by rain in warm and humid (60–80 %) circumstances (Neeraj and Verma, 2010). For the first time, *Alternaria alternata* f. sp. *cucurbitae*, which causes the cucumber leaf spot disease, was identified in Crete (Greece). The disease reportedly manifested as necrotic flecks encircled by chlorotic halos on middle and higher leaves of plants. The flecks grew larger and merged to form larger lesions that were > 2 cm dia., having pathogenic fructifications on their surface that are brown in color (Vakalounakis and Malathrakis 1982). The ideal temperature for the mycelial growth and the conidia germination is favored by 26 °C. Conidia are usually able to germinate within 2 hours in water and temperature between 10 and 37 degrees Celsius. According to Vakalounakis and Malathrakis (1988), when an infection spreads quickly across a crop, considerable harm is caused within a short period of time.

Previously, plant diseases were managed using chemicals, which resulted in an annual spend of \$15-\$16 billion on fungicides and agrochemicals used for pathogen management (Luca, 2021). However, the usage of chemicals poses a serious threat to the ecosystem due to their toxicity and persistent impacts. Excessive usage of chemical pesticides has resulted in soil pollution and has demonstrated to be detrimental to humans. In recent years, increasing plants' biotic and abiotic stress tolerance has become a major research focus. Bio-stimulants play a critical role in protecting plants from different stress factors (Chakrabarti and Mukherjee, 2003).

Salicylic acid (SA) is named after the first willow (*Salix alba* L.). Hundreds of years ago, American Indians and ancient Romans discovered that the bark and leaves of the willow tree could be used to treat pain and fire (Raskin, 1992). In 1828, Johann Buchner, a researcher in Munich, extracted very minute amounts of salicin from the bark of the willow tree, while Raffaele Piria isolated salicin in the laboratory for the first time in 1838. (Lee *et al.*, 1995; Popova *et al.*, 1997). Salicylic acid is a natural plant hormone that influences a variety of physiological and biochemical activities in plants. It was discovered that this hormone was a critical signal molecule that influenced plant tolerance to stresses in distinct ways (Arfan *et al.*, 2007; Wang *et al.*, 2010). In particular, when plants were exposed to biotic (disease and harmful organisms) and abiotic (heat, cold, light, drought, salinity, etc.) stresses, they tended to produce SA, which play important roles in plant defense mechanisms. Plants produce reactive oxygen species (ROS) under oxidative stress conditions such as salinity, which are hazardous to plant growth due to their deleterious effects on plant subcellular components and metabolism, resulting in oxidative cell damage. It is widely assumed that O_2^- is transformed to hydrogen peroxide (H_2O_2) and then metabolized to water in plants via ascorbate peroxidase and glutathione reductase (Shim *et al.*, 2003). It is worthwhile to maintain membrane integrity for increased stress tolerance. The enhanced activity of antioxidant enzymes also aids in overcoming the negative effects of salt-induced oxidative damage (Andre *et al.*, 2006). SA has recently been shown to induce the expression of 59 proteins in cucumber that have been identified as being involved in various cellular responses and metabolic processes such as antioxidative reactions, cell defense, photosynthesis, carbohydrate metabolism, respiration and energy homeostasis, protein folding and biosynthesis (Nemeth *et al.*, 2002). Yusuf *et al.*, (2008) found that exogenous administration of SA 30 days after sowing in *Brassica juncea* under salt stress improved growth, enzyme activity, and photosynthetic indices. Convincing evidence on the salicylic acid-induced increase in salt resistance of wheat seedlings (Shakirova and Bezrukova 1997), low and high temperature tolerance in tomato and bean have been obtained (Senaratna *et al.*, 2000). Besides abiotic stress, it is also important in the signal transduction pathway that leads to systemic acquired resistance against a wide range of pathogens. Exogenous administration of salicylic acid to sensitive plants at non-toxic amounts could improve disease resistance (Murphy *et al.*, 1998).

Melatonin (N-acetyl-5-methoxytryptamine) is a ubiquitously distributed molecule that is found in all kingdoms, including plant species (Kanwar *et al.*, 2018). Melatonin was discovered in plants for the first time in 1995 and has since been proved to be prevalent in higher plants and plant products (Dubbels *et al.*, 1995). It is a multifunctional biomolecule in plants that can promote photosynthetic activities and growth in response to biotic and abiotic challenges (Ahammed *et al.*, 2019, 2020; Hasan *et al.*, 2019). Exogenous melatonin treatment has been shown to enhance the expression of genes coding for antioxidant enzymes, enhancing SOD, POD, and CAT activities, decreasing oxidative stress (Kostopoulou *et al.*, 2015; Campos *et al.*, 2019), lipid peroxidation, and the concentrations of hydrogen peroxide (H₂O₂; Reiter *et al.*, 2015) and malondialdehyde (MDA; Jiang *et al.*, 2016), and to increase photosynthesis rates, sugar metabolism and growth (Zhao *et al.*, 2015). In addition to acting as an antioxidant, melatonin influences gene expression in several physiological processes. Genes involved in photosynthesis, fatty acid production, and glucose metabolism in soybeans are activated after melatonin application (Wei *et al.*, 2018). Melatonin treatments have been used to improve plant tolerance to salinity in a variety of crops, including *Malus* spp. (Li *et al.*, 2012), cucumber (Zhang *et al.*, 2014; Wang *et al.*, 2016), sour orange (Kostopoulou *et al.*, 2015), maize (Jiang *et al.*, 2016; Chen *et al.*, 2018), rapeseed (Zhao *et al.*, 2018) and tomato (Martínez *et al.*, 2018). Melatonin, a potent antioxidant and inducer of plant stress tolerance, can boost plant resistance to a variety of fungal infections such as gray mold in cherry tomato caused by *Botrytis cinerea* (Li *et al.*, 2019a), anthracnose in banana caused by *Colletotrichum musae* (Li *et al.*, 2019b), tomato fruit decay caused by *Botrytis cinerea* (Liu *et al.*, 2019), powdery mildew in watermelon caused by *Podosphaera xanthii* and crown rot in cucurbits caused by soilborne fungus *Phytophthora capsici* (Mandal *et al.*, 2018), downy mildew in cucumber caused by *Pseudoperonospora cubensis* (Sun *et al.*, 2019), and potato late blight caused by *Phytophthora infestans* (Zhang *et al.*, 2017). In general, melatonin aids plants in resisting fungal infection, reducing lesions, inhibiting pathogen proliferation and reducing disease damage.

The *Trichoderma* (Class: Ascomycetes, Order: Hypocreales, Family: Hypocreaceae) is a young genus in comparison to its teleomorph genus *Hypocrea*.

Trichoderma species, a genus of plant-beneficial fungi, can serve as opportunistic symbionts, inducing plant resistance to biotic and abiotic stressors (Mastouri *et al.*, 2010; Metwally, 2020). Plants and *Trichoderma* strains interact under salt stress, and several mechanisms underpinning these interactions have been discovered (Contreras-Cornejo *et al.*, 2014; Zhao and Zhang, 2015; Yasmeen and Siddiqui, 2018). *Trichoderma virens* Gv29.8 and *Trichoderma atroviride* IMI 206040 boosted *Arabidopsis* seedling growth under salt stress by improving root development, osmolyte production (L-proline and AsA), and Na⁺ elimination (Contreras-Cornejo *et al.*, 2014). *Trichoderma* is the most important fungal bioagent frequently employed in plant disease management. The primary mechanisms of action include competition for nutrients and space, the synthesis of cell wall disintegrating enzymes, antifungal diffusible and volatile compounds, growth hormones, and mycoparasitism (Djonovic *et al.*, 2007). *Trichoderma* strains are used as efficient biocontrol agents for disease management in greenhouse and field crop production, and they are thus regarded as an opportunistic fungal group ((Gupta *et al.*, 2014; Lorito *et al.*, 2010). The mechanisms of *Trichoderma*-induced enhanced tolerance to plant pathogens are still being researched. Pathogens can be suppressed by *Trichoderma* spp. by competing for space and nutrients (Howell 2003; Singh *et al.*, 2014). Pathogen cell walls can be degraded by lytic enzymes secreted by *Trichoderma* spp. They can also cause systemic resistance to pathogens. This suggests that the underlying defensive mechanisms induced by the genus *Trichoderma* are still being researched.

There are inadequate investigations on the influence of bio-stimulants on the physio-biochemical parameters of crop plants under salt stress condition. Considering all these negative effects of salt stress on plants and the significance of bio-stimulants viz. Salicylic acid, Melatonin and *Trichoderma* spp. in increasing plant tolerance to salinity, the current study aims to discover the role of these bio-stimulants in cucumber salt tolerance, with the following objectives:

- To investigate the impact of salt stress on biochemical parameters in cucumber.
- To investigate the role of salicylic acid, melatonin, and *Trichoderma* spp. on biochemical parameters in salt stressed cucumber.

REVIEW OF LITERATURE

Crop plants are vulnerable to a variety of abiotic and biotic stresses throughout their lives, which lower productivity and adversely affect global food security. Abiotic stresses are thought to be responsible for more than half of all crop production losses in the world (Wang *et al.*, 2007; Hamilton and Heckathorn, 2014). Salinity, second only to drought stress, is one of the most common abiotic stresses. Salinity stress affects over 40 million hectares of land, or roughly one-third of all irrigated land on the planet (Norlyn and Epstein, 1984). According to Shrivastava and Kumar (2015), by the year 2050, almost half of the world's arable land would be affected by salinity stress. In view of this massive problem, both conventional breeding and biotechnology approaches are being used to develop salinity-tolerant crop plants. In view of this massive problem, both conventional breeding and biotechnology approaches are being used to develop salinity-tolerant crop plants.

Biotic stress factors include fungi, bacteria, viruses, nematodes, insects, and phytoplasmas etc. The key reason for considerable interest in plant pathogen research is their widespread distribution and high pestiferousness. Many plant diseases are chronic in nature, reducing total plant productivity significantly. Plants become more sensitive to other negative environmental variables, in addition to the significant drop in productivity. Cucumber is frequently infected with a variety of disease. Among those documented to cause significant losses are powdery mildew, downy mildew, anthracnose, fusarium wilt, soil-borne diseases, and viral diseases such as mosaic, bud necrosis, leaf curl, and leaf distortion virus. The degree of plant resistance to various natural phyto-diseases and abiotic stress factors offered by a range of physiological and biochemical markers was discovered to be responsible for changes in plant metabolism and viability in stressful settings (Siebold and von Tiedemann 2012).

2.1. Soil Salinity

Salinity can be defined as the concentration of dissolved mineral salts in soils (soil solution) and waterways. The mineral salts that have been dissolved constitute a

mixed electrolyte of cations and anions. In saline soil solutions, the principal cations are Na^+ , Ca^{2+} , Mg^{2+} and K^+ , while the predominant anions are Cl^- , SO_4^{2-} , HCO_3^- , CO_3^{2-} and NO_3^- . Other constituents that contribute to salinity in hypersaline soils and waterways include B, Sr^{2+} , SiO_2 , M, Ba^{2+} and Al^{3+} .

Salinity is one of the environmental issues that severely limit agricultural production which makes fails to meet the ever-increasing human population's food demands (Botella *et al.*, 2005). Salinity is classified as either primary (natural) or secondary based on the causes of formation. The disintegration of bedrock and salt depot oceans as well as climate conditions, are the causes of primary salinity formation (Munns and Tester, 2008). Furthermore, secondary salinity is caused by an increase in the level of groundwater rich in various salts due to intensive irrigation in agricultural areas up to the surface of the soil, overgrazing, opening of farmlands by destroying a region's natural vegetation, and contamination of soils with chemicals that cause salinity (Pessarakli and Szabolcs, 1999). The majority of salt-affected soils on the planet are saline soils produced by Na_2SO_4 and NaCl (Pessarakli and Szabolcs, 1999).

Salinity problems affect over 950 million hectares of the world's total land area, or about 33% of agricultural fields (Lal and Stewart, 1990; Rowel, 1994). Salt crystals distributed throughout the land surface or in the soil profile are common signs of saline soils. Various attempts have been made to classify salt-affected soils into different groups based on pH of the saturated soil paste (pHs), total soluble salts or electrical conductivity of the saturated soil paste extract (ECe), and exchangeable sodium percentage (ESP) or Sodium adsorption ratio (SAR). Saline soils have an electrical conductivity (EC) of 4 dS m^{-1} and a pH of <8.2 at saturation extract at 25°C . In these soils, those with $\text{EC} > 4 \text{ dS m}^{-1}$ and sodium adsorption rate SAR 15 are categorised as solely saline, whereas those with $\text{EC} > 4 \text{ dS m}^{-1}$ and $\text{SAR} > 15$ are classed as saline-alkaline soils (Mikayilov *et al.*, 1998).

The effects of salinity on plants might differ depending on the type, amount, or concentration of soluble salts and salt compounds readily absorbed by plants. When specific quantities are exceeded, they can be hazardous to plants by interfering with nutrition and metabolism. In addition, when the level of salt in the soil increases, the plant's ability to absorb water becomes more difficult, and plant growth slows or stops

(Kanber *et al.*, 1992; Güngör and Erözel, 1994). A constant quantity of water in the soil that does not inhibit plant growth is required for healthy plant development. On the other hand, plants need less water when there is less water in the root zone. In such instances, salinity is a critical factor that prevents plants from receiving water rapidly from the soil environment. As the salt concentration in the root environment solution medium rises, the amount of energy the plant must expend to obtain this water rises, and as the salinity rises, the amount of water used by plant reduces. Plant yield and quality are affected by difficult use and reduced water use (Yurtseven and Bozkurt, 1997; Yurtseven and Baran, 2000; Kara and Apan, 2000; Yurtseven *et al.*, 2001).

Although removing soluble salts from the soil is vital to protect it from salt damage, if the soil is sodic, the procedures used can cause physical degradation of the soil. The Ca²⁺ ion, on the other hand, prevents the physical degradation caused by sodicity and also defends the plant from the harmful effects of salt (Lauchli, 1990). When compared to potassium, sodium occupies a bigger percentage of the earth's crust (2.8%) than K (2.6%). The Na content of the soil is considerably higher in arid and semi-arid environments. Because sodium is kept very loose on soil complexes, it is easily transported by water and washed away. The Na content of the soil is considerably higher in arid and semi-arid environments. Because sodium is kept very loose on soil complexes, it is easily transported by water and washed away. In the hot zones, the concentration of Na in soil solution is 0.1-1.0 mM, which is similar to or even higher than the K concentration.

2.2. Effect of salinity on growth and development of plants

Salinity is one of the world's most critical environmental issues because increased amounts of NaCl are naturally present in many agricultural fields (Parida and Das 2005). Salt stress can have an impact on a variety of physiological processes, including seed germination and plant development. It causes reduction in agricultural output and changes in plant metabolism, such as lowered water potential, ion imbalance, and toxicity, and severe salt stress can even put a plant's survival in danger. Salinity can have a negative impact on crop performance as a result of nutritional issues. These problems may result from the effect on nutrient availability, competitive absorption, transport, or partitioning within the plant (Silva *et al.*, 2008).

Plants growing in saline soils suffer three major challenges: high salt concentrations in the soil solution, high concentrations of potentially hazardous ions, and nutritional imbalance due to reduced absorption, decreased internal distribution, and transport of mineral (Marschner 1995, Greenway and Munns 1980). Salinity prevents plant growth, affects ionic relationships through ionic and osmotic effects, and causes oxidative stress (Parida and Das 2005, Silva *et al.*, 2008, Molassiotis *et al.*, 2006). Salinity lowers plants' ability to utilize water, resulting in slower development and alterations in plant metabolic processes (Munns 1993, 2003). Plant height is an important indicator of plant growth. When plants are stressed by salinity, their rate of leaf surface expansion slows down, causing them to become stunted. In mungbean plants, salt stress caused changes in plant height and leaf number that were shown to be statistically significant (Raptan *et al.*, 2001). In comparison to the control, increasing the salinity resulted in a reduction in these parameters. In cowpea plants, salt stress resulted in a gradual decline in plant height (Hussein *et al.*, 2007). Salinity also reduced mungbean growth and plant height, according to Rabie (2005). Bierhuizen (1969) discovered a 35% decline in cucumber yield after increasing the chloride concentration of the soil solution by 1 gm. Zhu *et al.* (2020) claimed that NaCl inhibited cucumber development considerably. It was observed that NaCl salinity inhibited cucumber seedling growth by increasing mean emergence time (MET) and decreasing total emergence and dry weight (Esmailpour *et al.*, 2006).

When the level of salinity increased, most of the cucumber growth parameters were decreased such as shoot fresh weight, shoot dry weight, and shoot height (Alrahman *et al.*, 2005). The decrease in cucumber shoot fresh weight was less severe in the progressive shock salinity treatment than in the direct shock treatment, and it was more obvious at 75 and 100 mM (Alrahman *et al.*, 2005). According to Chartzoulakis (1992), cucumber shoot height was reduced by an average of 20, 54, and 80 percent at salinity levels of 2.7, 5.0, and 10.7 dS/m, respectively. Ali *et al.* (2004) showed a significant decrease in leaf area and leaf number under salt stress, which may potentially affect the fresh and dry weights of plants under salinity stress. Cucumber shoot length was decreased by 3% and 6% below control at the lowest concentrations (25 mM and 50 mM, respectively). In contrast, under 100 and 200 mM NaCl concentrations,

however, a significant reduction in shoot length was found (Abdel-Farid *et al.*, 2020). In cucumber, salinity stress had a profound impact on root growth, resulting in a significant reduction in root length. Cucumber seedlings produced under control had the longest roots (7.92 cm), while those grown under 25, 50, and 100 mMNaCl had root lengths of 6.33, 3.37, and 1.43 cm, respectively (Abdel-Farid *et al.*, 2020).

2.3. Effects of Salinity on physiological and biochemical parameters of plants

Salinity has a two-fold effect on plant growth via an osmotic effect on plant water uptake and specific ion toxicities. Osmotic stress was influenced by soil salinity, which reduced water availability and disrupted the cellular ionic equilibrium (Kirst 1989). Plant physiology is affected by high salt levels, both at the cellular and whole plant levels (Murphy and Durako 2003). In maize genotypes, salt stress inhibited plant growth by altering a variety of physiological and biochemical activities, such as photosynthesis, antioxidant capability, and ion homeostasis (Ashraf 2004).

According to Sairam *et al.* (2005), NaCl reduced relative water content, chlorophyll content, and membrane stability index. Salinity stress reduced the rate of photosynthesis and the quantity of chlorophyll in the salt tolerant cultivars, but the sensitive kinds showed a higher loss in cowpea, according to Madan *et al.* (2004). Salinity stress reduced the rate of photosynthesis and chlorophyll content in the salt tolerant varieties, while the sensitive ones showed a higher loss in cowpea (Madan *et al.*, 2004). Photosynthetic pigments were drastically reduced when exposed to salinity condition. Salt stress was hazardous to plants, inducing metabolic alterations such as loss of chloroplast function, reduced photosynthetic rate, and increased photorespiration rate, all of which resulted in an increase in reactive oxygen species (ROS) production (Parida and Das 2005). Photosynthetic pigments were revealed to be degraded by salinity stress (Abdullah and Ahmed 1990, Soussi *et al.*, 1999). The chlorophyll content of several crops' leaves was reduced due to salt stress. Increased salinity resulted in a considerable drop in chlorophyll contents in blackgram cultivars (Ashraf, 1989). Yamani *et al.* (2004) proposed that light was required for salt-induced injury in chloroplasts, and that H₂O₂ and OH⁻ were responsible for the negative effects of salt stress on chloroplast ultrastructure and chlorophyll content. According to Garg and Singla (2004), increasing salinity reduced leaf chlorophyll content (chlorophyll a,

b, and total chlorophyll) considerably in all chickpea cultivars, with the loss being larger in sensitive genotypes. Similar results were found in alfalfa (*Medicago sativa*) by Al Khanjari *et al.* (2003). In green gram, there was a decrease in the total chlorophyll content of the leaves, as well as a significant difference across genotypes and salt levels (Ahmad *et al.*, 2005). With decreasing salinity, the ratio of total chlorophyll to carotenoids fell to 9.37 dS/m. Under the influence of salt stress, there was a decrease in CO₂ assimilation rate, transpiration rate, stomatal conductance, and sub-stomatal CO₂ concentration in sunflower (Noreen and Asharf 2008).

In response to salt stress, leaf relative water content decreased, whereas electrolyte leakage increased (Karlidag *et al.*, 2009). According to Parida and Das (2005), as salinity increases, the relative water content, water potential, and osmotic potential of plants become increasingly negative. Plant membranes undergo alterations in response to environmental stressors, which are frequently associated with increased permeability and loss of integrity (Blokchina *et al.*, 2003). As a result, the ability of a cell membrane to govern the rate of ion transport in and out of cells is utilised to assess tissue damage across a broad spectrum. Wu *et al.* (1998) noted that when plants are exposed to salt stress electrolyte leakage allows cell membrane injury to be measured. At high salinity, increases in membrane permeability were larger at the vegetative stage than at the seedling stage in spinach (Kaya *et al.*, 2001). Salinity reduced membrane permeability and increased electrolyte leakage in maize (Tuna *et al.*, 2007). Xiang *et al.* (2009) discovered that NaCl dosage increased membrane permeability in Lucerne. According to Xiang *et al.* (2009), in Lucerne, membrane permeability increased with NaCl dosage. Siringam *et al.* 2011 found similar results in rice roots.

Plants have evolved various systems to respond to osmotic and ionic stress generated by high salinity in order to overcome salt stress. Osmotic modifications are among the mechanisms involved (Rontein *et al.*, 2002). For osmotic adjustments, Plants utilize or acquire ions like Na⁺ and K⁺, or they produce organic suitable solutes like proline, betaine polyols, and soluble sugars. These organic osmolytes have osmoprotectant properties (Chen and Murata 2000). In response to salt stress, several plant species accumulate proline. Plants have been thought to accumulate some organic solutes as a defense against osmotic stress. Proline content increases more than other

amino acids in plants under water or salt stress, and this result has been utilized as a biochemical marker to select cultivars that can withstand these circumstances (Bates *et al.*, 1973). Proline, asparagine, γ -butyric acid, and other amino acids can play a key role in plant osmotic adjustment under saline conditions (Gilbert *et al.*, 1998).

The most notable parameter in rice grown under salt stress situations is the increase in proline content (Roy *et al.*, 1992). At high salinity, Blackgram cultivars accumulated considerably more proline in the leaves than the control, and this accumulation was significantly larger in salt sensitive varieties than resistant types (Ashraf 1989, Kaur 2009). Chickpea (Soussi *et al.*, 1999) and soybean (Mannan *et al.*, 2009) both showed higher proline levels in response to salt stress. Proline, in addition to its osmolyte function, can preserve enzymes and promote membrane integrity in a variety of situations (Stoeva and Kaymakanova 2008). When compared to control plants, proline concentration increased by more than 19 times in Brassica juncea cultivated under salt stress conditions (Madan *et al.*, 1994). Proline content was similar in all cucumber cultivars' control groups, however salt treatment resulted in a significant (P 0.05) increase in content of proline (Furtana *et al.*, 2010). When compared to the control, proline content in cucumber seedling leaves raised dramatically under NaCl stress; at 2, 4, 6, and 8 days after treatment, proline content was 146 %, 196 %, 299 %, and 356 % of the control plant content, respectively (Fan *et al.*, 2012). Plants suffer from membrane damage as a result of salinity stress, with membrane permeability and lipid peroxidation (MDA concentration) increasing considerably. Malondialdehyde is a lipid peroxidation biomarker that is linked to the peroxidation of polyunsaturated fatty acids in biological membranes, which leads to the release of free radicals (Simaei *et al.*, 2011). The progressive increase in MDA level in mungbean seedlings exposed to salinity revealed an increased rate of lipid peroxidation (Saha *et al.*, 2010). In pumpkin seedlings, sensitive genotypes accumulated more MDA than tolerance genotypes (Sevengor *et al.*, 2011).

Hydrogen peroxide is extremely hazardous in the chloroplasts because it prevents the Calvin cycle enzymes also at low concentrations, lowering photosynthetic carbon dioxide assimilation in wheat (Takeda *et al.*, 1995). Loggini *et al.*, (1999) found an increase in H₂O₂ concentration in wheat seedlings exposed to high salinity.

According to Velikova *et al.* (2000), a rise in H₂O₂ content after stress, emphasising that H₂O₂ can travel a great distance, producing changes in the redox status of surrounding cells and tissues, although it initiates an antioxidative response at low concentrations. In salinity-treated soybean plants, the level of hydrogen peroxide increased, notably at high NaCl salinity concentrations (Simaei *et al.*, 2011). The study of time course of the change in H₂O₂ concentration in cucumber roots demonstrated that H₂O₂ responded quickly to salt stress; H₂O₂ levels spiked within 2 hours of NaCl treatment (Wu *et al.*, 2018). Cucumber leaves from both cultivars (Jinlu4 and Jinyan4) have significantly higher H₂O₂ concentration after being exposed to salt stress (Zhu *et al.*, 2004).

Plants produce reactive oxygen species (ROS) in response to oxidative stress conditions such as salinity, drought, and higher or lower temperature, which are likely to negatively impact growth due to their deleterious effects on the subcellular components and metabolic activity of the plant, resulting in oxidative destruction of the cells. Excess ROS generation under oxidative stress leads to membrane damage, which finally results in cell death. Salt stress, according to Desingh and Kangaraj (2007), enhances the activity of antioxidant enzymes and the production of reactive oxygen species (ROS). These ROS can disrupt cellular metabolism by causing oxidative damage to membranes, proteins, and nucleic acids, as well as protein denaturation, lipid peroxidation and DNA mutation (Imlay 2003). Under saline conditions, ROS production increases (Hasegawa *et al.*, 2000), and ROS-mediated membrane injury has been shown to be a key source of cellular toxicity in rice, tomato, and citrus (Mittova *et al.*, 2004).

Plant antioxidative defense systems include both enzymatic antioxidants like catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR); and non-enzymatic antioxidants like glutathione, ascorbate, and carotenoids (Parida and Das 2005, Silva *et al.*, 2008, Miolassiotis *et al.*, 2006, Sudhakar *et al.*, 2001). ROS react with a wide variety of molecules, resulting in pigment co-oxidation, lipid peroxidation, membrane breakdown, protein degradation, and DNA mutation (Miolassiotis *et al.*, 2006). Malondialdehyde (MDA), a polyunsaturated fatty acid breakdown product, was used

as a ROS biomarker (Azevedo *et al.*, 2006, Juan *et al.*, 2005). Plants' antioxidative enzyme activities, such as CAT, APOX, POD, GR, and SOD, rise in response to salt stress, and there is a link between levels of enzyme and salinity tolerance (Mittova *et al.*, 2002). Potato showed an increase in SOD activity when exposed to osmotic and saline environments. SOD is the initial line of defence against ROS, but it produces the poisonous H₂O₂ as a byproduct. As a result, an effective H₂O₂ scavenging mechanism is necessary to permit quick H₂O₂ removal in plant cells (Daneshmand *et al.*, 2009). Several enzymes control H₂O₂ intracellular levels, but the most significant are CAT, APX, GPOD, and GR (Parida and Das 2005, Sudhakar *et al.*, 2001).

In wheat, antioxidant enzymes protect salt-tolerant plants against oxidative damage. As a result, boosting antioxidant enzyme levels in salt-sensitive plants is regarded to be an effective tool for conferring salt tolerance (Yamane *et al.*, 2004). In the salt-tolerant genotype, the rise in enzyme activity was more marked than in the salt-sensitive genotype. In the salt-tolerant genotype, salt stress seemed to have no effect on catalase (CAT) activity, whereas it was considerably decreased in the salt-sensitive genotype. CAT activity was decreased by both 80 and 120 mM NaCl concentrations on wheat leaves throughout NaCl treatments. The largest statistically significant suppression was seen when 120 mM NaCl was used (Erdal *et al.*, 2011). In the *Oryza sativa* plant, salt stress increased the quantity of H₂O₂ and the function of superoxide dismutase and ascorbic acid peroxidase whereas decreasing catalase activity (Lee *et al.*, 2001). According to Chakrabarti and Mukherji (2003), peroxidase activity in mungbean enhanced under salt stress condition. Antioxidant enzyme activity was maintained or increased, which aided in overcoming salt-induced oxidative damage (Andre *et al.*, 2006). Salt stress dramatically elevated SOD, POD, CAT, and APX activity and gene expression in cucumber seedlings (Tang *et al.*, 2018).

2.4. Effects of salicylic acid on plants under salt stress

Salicylic acid (SA) is an endogenous plant growth regulator which impacts growth and development of plants by triggering a variety of metabolic and physiological responses (Hayat *et al.*, 2010). Furthermore, SA plays an important role in plant defensive responses to local pathogen assault (hypersensitive response) as well as systemic acquired resistance (Alvarez, 2000). As Cameron (2000) mentions, SA is a

plant signalling molecule that is vital in growth, development, and defensive responses under stress situations. SA may modulate plant responses to a variety of stress situations in addition to developing disease resistance (Shirasu *et al.*, 1997).

Shakirova *et al.* (2002) studied the effects of 0.05 mM concentration of SA on wheat seedlings and found that increasing SA dosages boosted cell division rates, enhanced plant growth and development, and hence raised the yields. Researchers also observed that, SA treatments induced an increment in ABA and IAA levels in wheat seedlings subjected to salt stress (2 % NaCl), as well as preventing a drop in cytokine content, all of which protected wheat seedlings from salt stress. An increase in the quantity of proline generated by enhanced ABA synthesis has also been demonstrated to boost tolerance to salt stress.

Tari *et al.* (2002) used salicylic acid to treat salt-stressed tomatoes and found that plants treated with modest doses (0.01 mM) of SA were able to withstand salt stress (100 mM NaCl) for roughly seven days. Furthermore, as SA treatment increased leaf Na⁺ level, there was a decrease in overall sugar content.

El-Tayeb (2005) investigated the effects of 1.0 mM SA treatments on seeded barley seedlings subjected to salt stress (150 mM NaCl) during the seedling phase and found that SA-treated plants had greater seed weight, chlorophyll, glucose, and proline contents. Furthermore, SA-treated plants had decreased relative electrical conductivity values. Peroxidase enzyme activity, a marker of the negative effects of salt stress, was also shown to be reduced in SA-treated plants than those in control plants as a consequence of tests conducted out in plants treated with SA.

Erkılıç (2005) investigated the impact of different salicylic acid concentrations on the buildup of free proline and several physiological parameters in two pepper cultivars. Salt-resistant Demre-8 seedlings were treated with 0.1, 0.5-, and 1.0-mM SA in combination with salt, resulting in shorter root and shoot lengths and larger leaf areas. SA-treated seedlings showed higher leaf tissue fresh and dry weight, as well as LRWC (Leaf Relative Water Content) and free proline levels. SA treatments (0.1, 0.5-, and 1.0-mM SA) given to the salt-sensitive Kahramanmaraş-bitter cultivar enhanced root length, leaf area index (LAI), and fresh leaf tissue.

Tohma (2007) used varied dosages of salicylic acid (SA) (0.0, 0.1, 0.25, 0.5, and 1.0 mM) on Camarosa strawberry types that had been pre-treated with different salt concentrations (0, 2, 4, 6 mS cm⁻¹) and studied the effects of SA applications on physiological responses (membrane permeability, protein, chlorophyll, and proline levels), plant nutritional content (N, P, K, Ca, Mg, Na, Cl, Fe, Cu, Mn, and Zn), and plant development. SA treatment in saline situations was observed to decrease membrane permeability while increasing protein, proline, chlorophyll b, as well as total chlorophyll levels. SA treatments in saline circumstances improved plant development and delayed the onset of salt toxicity.

Kaydan *et al.* (2007) looked at how salicylic acid treatments (0.2 mM, 0.4 mM, and 0.6 mM) affected various physiological properties of wheat cultivated in saline (salinity 8 dS m⁻¹) and salt-free environments. Under salt stress circumstances, researchers found that applying salicylic acid to seedlings enhanced their osmotic potential, shoot and root weights, K⁺/Na⁺ ratio, and photosynthetic pigment (chlorophyll a, b, and carotenoids) composition. It was determined that salicylic acid had a beneficial effect on plant development under saline conditions.

Qing-Mao *et al.* (2007) studied the impact of foliar and root application of SA treatments (50 mg/l SA) on the physicochemical parameters of cucumber response to saline stress in their study. It was revealed that foliar and root application of SA treatments raised sugar content by 11.04 % and proline content by 82.2 % while decreasing electrolyte loss. Furthermore, salicylic acid treatments improved plant characteristics such as plant height, stem diameter, and total weight.

Koçer (2007) investigated the effects of different doses of abscisic acid (ABA) and salicylic acid (SA) applications on salt-stressed maize (*Zea mays* L.) plants. Sugar, fatty acid, and hormone levels were determined using a high-performance liquid chromatography (HPLC) system, whereas chlorophyll a and b, carotenoid, and proline contents were determined using a spectrophotometer. High salt concentrations were found to have a negative impact on morphological development. Treatments with abscisic acid enhanced plant morphology more than treatments with salicylic acid. Endogenous hormone, sugar, and fatty acid contents were considerably affected with both salt and exogenous hormone treatments, and total carotenoid contents were found

to be high in virtually all ABA treatments. Salt treatments raised proline levels, while increasing hormone dosages similarly enhanced proline levels.

Yıldırım *et al.* (2008) studied the effects of foliar salicylic acid applications on cucumber plant mineral content, chlorophyll content, and plant development under salt stress. The experiment was conducted out in pots in a greenhouse setting. Plants were given varied dosages of salicylic acid (0, 0.25, 0.50, and 1 mM SA) and cultivated at two dissimilar salinity levels (0.6 and 1.2 mM NaCl). Plant growth, chlorophyll content, and nutrient flow were all negatively impacted by salinity treatments. Treatments with salicylic acid, on the other hand, had a beneficial impact on plant fresh and dry weights, as well as root fresh and dry weights. Under salt stress, however, reduced stem diameters, leaf number per plant, and leaf relative water content levels were found.

Dolatabadan *et al.* (2009) studied the effects of salicylic acid administrations on wheat seed germination, superoxide dismutase, lipid peroxidation, polyphenol oxidase, catalase, and peroxidase activity under salinity stress environments. Salicylic acid treatments were discovered to have a scavenging impact on reactive oxygen species, which resulted in less membrane damage. Treatments with salicylic acid effectively decreased the harmful effects of salt on embryo development and expedited the restoration of growth and division processes. SA was shown to be quite effective in improving seed germination in arid and semi-arid environments.

Erdal *et al.* (2011) studied the effects of foliar application of SA treatments on salt sensitivity, hydrogen peroxide (H₂O₂) production, and antioxidant enzyme activities such as peroxidase (POX) and catalase (CAT) in plant tissues under salt stress condition. SA treatments considerably raised the fresh and dry weights in either of the root and shoots of salt-stressed wheat plants. Similarly, SA treatments increased the activity of POX and CAT. Increasing antioxidative actions at the same time. In comparison to plants cultivated under saline stress without SA, SA treatments reduced H₂O₂ concentration. According to result, the SA treatments reduced the salt-induced negative effects on wheat seedlings.

Tufail *et al.* (2013) studied the impact of salicylic acid applications on morphological, physiological, as well as biochemical characteristics of two maize

genotypes (Sahiwal-2002 and EV-20) grown in saline and non-saline settings. For 20 days, all pots were watered with Hoagland nutritional solution. After that, 21-day-old plants were salinized using 0 and 120 mM NaCl, as well as three doses of salicylic acid (0, 0.25, and 0.50 mM) administered through rooting media. The administration of 0.50 mM salicylic acid was most effective in reducing Na⁺ and increasing K⁺ and Ca²⁺ concentrations, as well as plant biomass and yield under salt stress. Exogenous administration of varied doses of SA increased the photosynthetic and transpiration rate, stomatal conductance, as well as sub-stomatal CO₂ concentration, chlorophyll b concentrations, and carotenoids in both maize genotypes under salt stress.

Karami Chame *et al.* (2016) investigated the effects of three distinct salinity levels (0, 2, and 4 ds/m) and two levels of the bacteria *Pseudomonas putida* (inoculated but also non-inoculated) with salicylic acid (0 and 1 mM) on bean development in greenhouses. The combination of salt and salicylic acid had a considerable influence on leaf relative water level. Furthermore, the interaction of salt x *Pseudomonas* x salicylic acid had a significant influence on the chlorophyll index, 100-seed weight, and seed protein level. In general, salicylic acid and *Pseudomonas* alleviated the negative effects of salt on plants.

Abbas *et al.* (2016) studied the effects of various salicylic acid doses on growth metrics, chlorophyll & soluble protein content, including antioxidant enzyme functions in cultured mature embryos of several wheat types. In varied combinations, three levels of NaCl (0, 75, 100, and 150 mM) and salicylic acid (0 mM, 0.25, 0.50, and 0.75 mM) were utilised in regeneration medium. The cultures were kept at 26 2°C with a 16-hour photoperiod and a light intensity of 40 moles m⁻² s⁻¹ from a cool white fluorescent tube lamp. According to the findings of this investigation, all NaCl stress levels dramatically inhibited plant development by lowering chlorophyll content and K⁺ levels. The presence of salicylic acid stimulated plant growth in both salt and salt-free circumstances.

2.5. Effects of salicylic acid against plants diseases

Salicylic acid (SA) is a natural plant hormone that has a role in plant immunity as well as response to abiotic stresses beyond the defensive response. Salicylic acid is

an important component of the defensive response to pathogen infection in many plant species. Salicylic acid triggers the oxidative burst which causes cell death in the hypersensitive response and functions as a signal for the progress of systemic acquired resistance (Shirasu *et al.*, 1997).

Amborabé *et al.* (2002) studied the effect of SA against *Eutypa lata*, the causative agent of eutypa dieback, a serious disease of grapevines and other woody plants. SA reduced mycelial development of *Eutypa lata* (Pers. Fr.) Tul. in both a solid and liquid culture media in a concentration-dependent manner, with a threshold value of 0.1 mM. 1 mM SA demonstrated limited fungistatic characteristics in settings that mimicked the plant environment (in particular, a pH approaching the apoplastic value, i.e., 5.5). At several levels, however, changes in the structural structure of the mycelium (wall, mitochondria, vacuole and nucleus) were identified. A fungicidal efficacy was achieved at 2 mM or greater doses, and fungal filaments looked empty after this treatment. When the pH of experiment was adjusted to higher acidic levels (pH 4), the molecule's antifungal effectiveness improved.

Vimala and Suriachandraselvan (2009) investigated the impact of salicylic acid on generating systemic resistance in bhendi towards powdery mildew due to *Erysiphe cichoracearum* DC. They evaluated the use of salicylic acid (1mM) to promote the synthesis of defense-related enzymes and compounds in plants when used as a pre-inoculation, post-inoculation, salicylic acid only, pathogen alone, and water only as control. The results showed that salicylic acid pretreated bhendi plants in which challenge inoculated with *E. cichoracearum* had early and higher phenyl alanine ammonia lyase activities. Plants pretreated with salicylic acid (SA) accumulated more phenolics, which improved resistance to *E. cichoracearum* infection in bhendi.

Hadi *et al.* (2010) investigated the effects of salicylic acid on the initiation and progression of *Rizoctonia solani* in the tuber of Marfona potato cultivar (*Solanum tuberosum* L.) under greenhouse circumstances. They disinfected the soil in pots then contaminated with *R. solani* (21B1 strain) two weeks before transplanting the potato plantlets. Every week, the seedlings were given a dose of 0 (control), 0.1, 0.2, 0.5, 1, 2, and 4 mM salicylic acid. The results revealed that treating the potato tubers using 0.2 mM salicylic acid reduced the infection symptoms (sclerotia) by 73%. Furthermore, an

increment in salicylic acid concentration lowered the severity of infection symptoms even more. When 0.5 mM salicylic acid was used, the least amount of infection was detected. The results also demonstrated that applying 2 mM salicylic acid to fungi-infected plants enhanced the quantity of potato tubers. Based on the findings, applying 0.2 - 0.5 mM salicylic acid reduced tuber losses due to *Rizoctonia* fungus.

Falcioni *et al.* (2014) investigated the possible effect of three dosages of exogenous SA therapy on tomato plants infected with Potato virus X (PVX) under controlled environments and assessed their impacts on: several physiological indicators (gas exchange, stable isotopes, chlorophyll level), secondary metabolism activation, viral accumulation, including induction of pathogenesis-related proteins (PRs) such as β -1, 3-glucanase (PR2), and chitinase expression (PR3). At 7 days after treatment, SA treatment raised the expression of PR2, the function of phenylalanine ammonia lyase (PAL), and the content of antioxidant molecules. PVX detection by ELISA in untreated control leaves of manually infected tomato plants was delayed after SA treatment. Although PVX infection had a minor influence on physiological measures, mild SA treatments resulted in increased photosynthesis, especially in infected plants. The findings show that SA promotes significant changes in the development of resistance in tomato plants, implying that treatment by exogenous SA might be utilised to minimise PVX infections.

Sabbagh and Zinati (2016) explored the role of salicylic acid on induced resistance in cucumbers infected with *Pythium aphanidermatum* (Edson) Fitzp. The expression levels of *Cupi4* and *Lipoxygenase* genes, as well as the activity of many biochemical enzymes, were studied for this proposal. In the greenhouse, a factorial experiment based on randomised complete design comprising four replications was conducted at 25-27° C with 70% humidity and a 14: 8 h light: dark light cycle. The findings of the disease incidence study revealed a significant difference at the 5% level between treated plants with various salicylic acid concentrations (0, 200, and 400 ppm) as well as control plants. The concentration of antioxidant enzymes in treated plants was significantly raised when compared to control, however the activity of Phenylalanine ammonia-lyase was not significantly elevated when compared with untreated plants. The qRT-PCR method of gene expression study revealed that salicylic

acid treatment can raise the expression level of examined genes, with Cupi4 having the greatest expression level. According to the findings of this study, the application of salicylic acid might have a major role in increasing cucumber resistance to damping-off disease.

2.6. Effects of melatonin on plants under salt stress

Liang *et al.* (2015) found melatonin as a strong agent in delaying leaf senescence and cell death in rice. Melatonin treatments dramatically decreased chlorophyll degradation, repressed transcripts of senescence-linked genes, delayed leaf senescence, and improved salinity tolerance. Melatonin is a strong free radical scavenger, according to genome-wide expression profiling using RNA sequencing, and its exogenous administration leads in increased antioxidant protection. Exogenous melatonin can alleviate leaf cell death in *noe1*, a mutant with increased H₂O₂ production. These findings show that melatonin slows leaf senescence and cell death while also improving abiotic stress tolerance either by directly or indirectly mitigating H₂O₂ cellular buildup.

Wang *et al.* (2016) studied the effects of exogenous melatonin treatment on cucumber development and antioxidant system at 200 mM NaCl stress. According to the findings, melatonin-treated plants had dramatically higher growth mass and antioxidant protection. Melatonin substantially alleviated the decline in net photosynthetic activity, maximum quantum efficiency of PSII, including total chlorophyll content during salt stress. Melatonin concentrations of 50–150 M enhanced photosynthetic capability considerably. Furthermore, pretreatment with melatonin decreased oxidative damage during salt stress by directly scavenging H₂O₂ or increasing the activities of antioxidant enzymes (particularly superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase) and antioxidant contents (ascorbic acid and glutathione). As a result, melatonin-treated plants might significantly improve their salt tolerance.

Li *et al.* (2017) investigated the effects of melatonin on photosynthetic activity and redox homeostasis in watermelon under salt stress condition (300 mM NaCl) as well as pretreatment with various dosages of melatonin (50, 150, and 500 M). In the

leaves of watermelon seedlings, NaCl stress hindered photosynthesis while increasing reactive oxygen species buildup and membrane degradation. Melatonin pretreatment on roots, on the other hand, reduced the NaCl-induced reduction in photosynthetic activity and oxidative stress in a dose-dependent way. Melatonin's photosynthetic protection was intimately connected with the suppression of stomatal closure and increased light energy absorption and electron transport within photosystem II. Whereas, reduction in oxidative stress by melatonin was ascribed to better redox equilibrium as well as increased antioxidant enzyme activity. This study revealed that melatonin has an important function in salt stress reduction and may thus be used to reduce salinity problem in watermelon farming.

Zhang *et al.* (2020) evaluated the impact of exogenous melatonin in reducing salt stress in cucumber plant. In comparison to salt exposure alone, exogenous melatonin pretreatment improved cell viability, protected photosynthesis, increased antioxidant enzyme activity, inhibited active oxygen explosion, and reduced malondialdehyde (MDA) concentration and relative conductivity in cucumber seedlings. According to gene expression study, melatonin substantially boosted the expression of enzymatic antioxidants genes, NADPH oxidase genes, MAPK genes (MAPK3, MAPK4, MAPK6), as well as salt overly sensitive (SOS) genes (SOS1, SOS2, SOS3) during salt stress. Finally, exogenous melatonin administration may significantly reduce the damage caused by salt stress on cucumber seedlings, and the effect is linked to hydrogen peroxide (H₂O₂). Furthermore, melatonin-mediated salt tolerance and the MAP kinase signalling pathway may interact.

Brenghi *et al.* (2022) investigated the use of Melatonin (MT) and cobalt (Co) to increase salt tolerance in plants via physiological processes. *Cucumis sativus* L., cv. Barracuda F1) plants were subjected to various doses of salt stress (control = 0.25, 2, 3, and 4 dS m⁻¹) with pre-treatment of MT (100 and 200 M) or Co (15 and 20 mg L⁻¹). The results showed that raising the salt level lowered cucumber plant development and productivity, which might be due to ion toxicity (Na⁺ and Cl⁻ accumulation), oxidative stress such as hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) accumulation as well as a decline in critical nutrient content. Foliar application of 15 mg L⁻¹ Co and 100 μM MT alleviated, at a comparable level, the NaCl-induced reduction of cucumber

growth, fruit number, and production. At the physiological level, these two treatments enhanced the protein content, vital nutrients (N, P, K⁺, and Ca²⁺) quantity, and catalase (CAT) function of stressed plants. The reduction in H₂O₂ and MDA levels was also noted. These findings indicate that Co and MT can enhance salt tolerance in cucumber plants.

2.7. Effects of melatonin against plants diseases

Li *et al.* (2018) investigated the preliminary strategy by which melatonin helps to reduce the impacts of apple replant disease and analysed the regulatory influence of melatonin on plant resistance to this disease. Two-year-old "Fuji" apple trees (*Malus domestica*) grafted over rootstock M.26 were cultivated in "replant" soil during 6 months with or without a 200 µmol/L melatonin treatment. Under replanting conditions, the application of melatonin to the soil significantly enhanced the levels of plant growth, net photosynthesis, and chlorophyll contents. This compound increased K levels in leaves and roots while also increasing the actions of soil enzymes. The makeup of the bacterial and fungal populations in the soil was also changed as a result of this supplementation. These modifications can then increase seedling development, stimulate photosynthesis, and raise K levels, reducing the impacts of apple replant disease.

Mandal *et al.* (2018) conducted research to learn more about the predicted melatonin biosynthetic pathway in the watermelon (*Citrullus lanatus*) and how melatonin, an environmentally friendly immune inducer, can strengthen plant immunity and inhibit pathogen development in areas where fungicide tolerance and absence of genetic resistance are major issues. To assess the function of melatonin in plant defence, they tested the effects of spray-applied melatonin as well as watermelon plants modified with the melatonin biosynthetic genome SNAT (serotonin N-acetyltransferase). In watermelon and other cucurbits, elevated melatonin levels were reported to promote resistance against the foliar disease *Podosphaera xanthii* (powdery mildew) and the soil-borne oomycete pathogen *Phytophthora capsici*. Twenty-seven elevated genes were linked to constitutive defence and the early priming of the melatonin (MT)-induced resistance response in plants.

According to Li *et al.* (2019), exogenous melatonin therapy can considerably decrease the occurrence of anthracnose in mature yellow banana fruit and delayed fruit senescence. Melatonin therapy, on the other hand, had no effect on *Colletotrichum musae* growth *in vitro*. Transcriptomic study of banana peel revealed that 339 genes were up-regulated whereas 241 were down-regulated following melatonin administration, as compared to the control. According to GO keywords and the KEGG pathway, these up-regulated genes were generally classified into signal transmission, cell wall construction, secondary metabolism, volatile chemical production, and stress response, which may be associated to the anti-anthracnose activity of banana fruit generated by melatonin treatment. The metabolomic study revealed an increase in volatile compounds, cell wall elements, and IAA levels in melatonin-treated fruit peel. Auxin, ethylene, as well as mitogen-activated protein kinase (MAPK) sensing pathways were elevated after melatonin treatment, which may be implicated in enhanced fruit resilience via modulating physiological features, disease-resistant proteins, and metabolites.

Zhao *et al.* (2019) studied the melatonin (MEL)-mediated plant tolerance to tobacco mosaic virus (TMV) to investigate local and systemic infection in *Nicotiana glutinosa* and *Solanum lycopersicum*, respectively. Exogenous treatment of 100 μm MEL boosted anti-virus infection efficiency in virus-infected *N. glutinosa* plants to 37.4%. In virus-infected *S. lycopersicum*, the same treatment significantly decreased relative levels of viral RNA and virus titres determined by qRT-PCR and dot-ELISA, respectively while increasing relative expression levels of the PR1 as well as PR5 genes measured by qRT-PCR. MEL treatment resulted in significant accumulations of salicylic acid and nitric oxide, but had no effect on hydrogen peroxide (H_2O_2) generation in virus-infected *S. lycopersicum* plants. A workable model of TMV resistance mediated by MEL is then suggested. Plant resistance to viruses mediated by MEL opens up a new path for controlling plant viral infections.

Sun *et al.* (2019) investigated the impact of melatonin pretreatment on cucumber resistance to *Pseudoperonospora cubensis* that causes downy mildew of cucumber. Following inoculation, 100 mol L⁻¹ melatonin increased the activity of antioxidant enzymes as well as the expression of antioxidant genes, enhancing the

antioxidant capabilities of those plants. This pretreatment successfully lowered the quantity of malondialdehyde as well as relative electrolytic leakage, hence conserving membrane integrity. Melatonin also boosted enzyme activity and the expression of pathogen-related genes. As a result of such pretreatment, the disease index determined for cucumber seedlings was dramatically lowered. These findings illustrate the value of melatonin in increasing fruit yields and improving cucumber resistance against downy mildew disease.

Chen *et al.* (2020) discovered that exogenous melatonin treatment can boost rice resistance to bacterial blight of rice (BB) caused by *Xanthomonas oryzae* pv. *oryzae*. Exogenous melatonin (20 g/ml) treatment of rice plants boosted nitrate reductase, nitric oxide synthase, as well as peroxidase activity, allowing for high intracellular levels of melatonin, nitric oxide, and H₂O₂. When the plants were exposed to melatonin, the expression of NPR1, a crucial regulator in the salicylic acid signalling pathway, increased more than 10-fold. Furthermore, in the treatment of melatonin, three pathogenesis-related proteins, PR1b, PR8a, and PR9, were elevated 20-fold. Melatonin (100 g/ml) treatment of soil-grown rice decreased the prevalence of BB by 86.21 %. As a result, our findings help in the understanding of melatonin-mediated innate immune system in rice against *X. oryzae* pv. *oryzae*.

2.8. Effects of *Trichoderma* spp. on plants under salt stress

Qi and Zhao (2013) investigated the potential application of *Trichoderma* isolate in enhancing cucumber development under salt stress and the processes that are involved. They isolated strain Q1 of *Trichoderma* from the cucumber rhizosphere in a greenhouse and recognized it as *Trichoderma asperellum* on the basis of morphological characteristics and molecular phylogenetic analysis. In pot experiments, strain Q1 greatly enhanced seedling development and reduced the growth suppression caused by salt stress, as evidenced by changes in development phenotype and various biochemical and physiological indicators. These findings suggest that *T. asperellum* Q1 has the ability to boost cucumber development by producing physiological protection against saline stress, and its siderophores displayed signs of reducing the deleterious effects of salinity and available iron shortage.

Contreras-Cornejo *et al.* (2014) studied the ability of *Trichoderma virens* and *T. atroviride* to develop salt tolerance in *Arabidopsis* seedlings. They discovered that salt inhibited plant growth and development in a dose-dependent way by interfering with auxin signalling. Elevated levels of abscissic acid, L-proline, and ascorbic acid, as well as increased Na⁺ removal through root exudates, were seen in *Arabidopsis* seedlings cultivated in salt stress inoculated by *Trichoderma* spp. It has been found that auxin signalling and root architecture play essential roles in salt tolerance in *Arabidopsis*, implying that these fungi may improve plant IAA levels as well as antioxidant and osmoprotective condition during salt stress.

Ahmad *et al.* (2015) investigated the influence of different NaCl doses (0, 100, and 200 mM) on *Brassica juncea's* growth & development, physio-biochemical characteristics, antioxidant enzymes, oil content level, and so on, as well as the protective action of *Trichoderma harzianum* (TH). They found that a higher NaCl dose (200 mM) reduced plant height by 33.7 %, length of root by 29.7%, and dry weight of plant (DW) by 34.5 %. In contrast, adding TH to NaCl-treated mustard seedlings increased shoot, root length, and plant DW by 13.8, 11.8, and 16.7 %, respectively, as contrasted to plants treated with NaCl (200 mM) only. NaCl degenerates the pigment content as well, with a maximum reduction of 52.0 % documented in Chl. 'a'. The treatment of TH to NaCl-treated plants resulted in increased pigment content. Proline levels increased with NaCl stress, reaching a high accumulation of 59.12 % at 200 mM NaCl. The addition of TH resulted in an increase in proline content of 70.37 %. Finally, the result showed that, TH showed to be particularly effective in conferring resistance to NaCl stress on mustard plants via enhanced absorption of critical elements, regulation of osmolytes, and antioxidants.

Zhang *et al.* (2016) studied the influence of the plant-growth-promoting fungus *Trichoderma longibrachiatum* T6 (T6) on growth and the development of wheat plants under salt stress (150 mM NaCl), as well as the function of T6 in generating resistance to salt stress at physiological, biochemical, and molecular scale. They found that the relative moisture content in the roots and leaves, chlorophyll composition, and root activity were all significantly higher, and that the occurrence of proline content in leaves was significantly augmented with plant growth attributes, but the composition of leaf

malondialdehyde was significantly lower in the salt stress condition. With the treatment of the T6 strain under salinity, the antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), as well as catalase (CAT) in wheat seedlings rose by 29, 39, and 19 %, respectively. Their findings showed that the T6 strain considerably reduced the negative impacts, shielding the seedlings from salt stress during their development phase.

Zhang *et al.* (2019) explored the potential mechanism of *Trichoderma harzianum* T-soybean in reducing salt stress injury in cucumber (*Cucumis sativus* L) plants. Their findings show that T-soybean increased cucumber seedling salt tolerance through influencing antioxidant enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (POD), polyphenol oxidase (PPO), catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), and glutathione reductase (GR) via raising proline, soluble carbohydrates & protein, ascorbic acid (AsA), and chlorophyll levels, as well as promoting root activity. Furthermore, T-soybean treatment enhanced the K⁺ content as well as K⁺/Na⁺ ratio while decreasing the Na⁺ concentration and ethylene level.

2.9. Effects of *Trichoderma* spp. against plants diseases

Bokhari and Perveen (2012) investigated the antifungal activity of *Trichoderma harzianum* and *Trichoderma viride* against *Fusarium solani*, the pathogen that causes tomato root rot disease. *In vitro* tests revealed that *T. harzianum* had more antagonist action against *F. solani* as compared to *T. viride*. *T. harzianum* suppressed pathogen growth more effectively in dual culture and volatile metabolites experiments (51.4 and 38.1 %, respectively). Under pot settings, tomato plants inoculated with *T. harzianum* increased plant height as well as dry weight by 117.5 % and 138.9 %, respectively, as compared to the control. The data clearly shows that *T. harzianum* would be a more efficient antagonist against *F. solani* than *T. viride*.

Subash *et al.* (2013) evaluated the antifungal efficacy of *T. harizanium* against *Fusarium oxysporium*, *Alternaria alternate* and *Rhizoctonia solani*, the pathogenic fungus that cause damping disease. *T. harizanium* was mixed with talcum powder and sprayed on damping off infected chilli plants as a foliar spray. *T. harzianum* was shown

to be effective at inhibiting the growth of all of the diseases listed above. *T. harizianum*-treated chilli plants had 60% more leaves, 75% more plant height, and 6% more fruits counts after 49 days of application than control.

Yao *et al.* (2016) conducted the experiment to evaluate effective *Trichoderma* isolates against *P. infestans*, a causal agent of Potato late blight disease, and to investigate the various mechanisms of action involved. *In vitro* dual culture of *P. infestans* and *Trichoderma* isolates revealed that colony of *Trichoderma* isolates greatly inhibited the *P. infestans* colony. When examined under a scanning electron microscope, mycoparasitism appears to assist to the aggressive behaviour of the *Trichoderma* strain HNA14 against *P. infestans*. Among the *Trichoderma* strains tested in the field, the *Trichoderma* isolate HNA14 observed to be most effective against the pathogen and considerably reduced disease severity when compared with the control.

Mei *et al.* (2019) conducted pot culture and greenhouse culture tests to assess the effects of three *Trichoderma* strains, *T. asperellum* 525, *T. harzianum* 610, and *T. pseudokoningii* 886, in reducing cucumber fusarium wilt caused by *Fusarium oxysporum*. With efficacies of more than 78 %, all three *Trichoderma* strains displayed superior control against cucumber fusarium wilt. *Trichoderma* 866 was the most successful of the three strains, with a disease control efficiency of 78.64 % and a 33 % increase in cucumber output. Furthermore, seedlings treated with *Trichoderma* had considerably higher levels of peroxidase (POD), catalase (CAT), polyphenol oxidase (PPO), superoxide dismutase (SOD), and ascorbate oxidase (AAO). Their findings imply that all three *Trichoderma* strains limit *F. oxysporum* infection, boost cucumber metabolism, and increase the activity of stress-resistance enzymes, all of which benefit cucumber plant development.

Sallam *et al.* (2019) investigated the antifungal efficacy of seven *Trichoderma* spp. isolates (T1–T7) *in vitro* against *Fusarium oxysporum* f.sp. *lycopersici* (FOL) causing tomato wilt disease. *Trichoderma* isolate (T7) showed the higher efficacy followed by *Trichoderma* isolate (T3) against the tested pathogen. In greenhouse trials, the treatment of highly antagonistic *Trichoderma* spp. isolates (T3 and T7) resulted in a considerable reduction in disease incidence in comparison to the untreated control. The activation of a defense-related gene (β -1,3-glucanase gene) was examined by real-

time RT-PCR to better understand the efficacy of *Trichoderma* isolates to defend against wilt disease in tomato plants. Tomato plants treated with T3 and FOL showed the highest level of gene expression when compared to control plants (pathogen only).

El-Komy *et al.* (2022) used dual culture and antibiosis experiments against *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (Forc), the causative agent of Fusarium root and stem rot (FRSR) disease in cucumber, to assess the biocontrol capability of 20 native *Trichoderma asperellum* strains. They noticed two *T. asperellum* strains (TAS23 and TAS27) showed the best antagonistic activity against Forc. *in vitro*. In greenhouse experiments, they observed that treating cucumber plants to these antagonistic strains individually was helpful in delaying the onset of FRSR. The findings suggest that TASMix-controlled FRSR is accomplished in cucumber roots by lowering reactive oxygen species buildup, minimizing cellular damage, and enhancing antioxidant enzyme activity.

2.10 Dual culture assay of *Trichoderma* spp. with phytopathogens

Bunbury-Blanchette and Walker (2018) applied dual culture technique to illustrate the biocontrol potential of *Trichoderma* against Fusarium basal rot of onion. They collected 7 different species of *Trichoderma* from several locations of Annapolis valley, Canada and cultured the isolates. From each of *Trichoderma* and *Fusarium* culture 5mm cubes medium were cut and inoculated in 8.5 cm Petri dishes with PDA. In dual culture, all of the *Trichoderma* species tested exhibited antagonistic effect toward *Fusarium oxysporum* thus suggesting that all *Trichoderma* species can be used as potential biocontrol agents against *Fusarium oxysporum*.

Angelini *et al.* (2008) proved the efficacy of tea tree oil (*Melaleuca alternifolia*) against *Trichoderma harzianum* (a fungal contaminant of *Pleurotus* mushroom) through dual culture technique. Different concentration of *M. alternifolia* essential oil was added to the dual culture petriplates where 2mm mycelial disks of *Pleurotus* and *T. harzianum* were placed 30mm apart. The findings suggested that synthetic pesticides now used in mushroom production to prevent and control green mould could be replaced with lower concentrations of *M. alternifolia* essential oil.

Mulemans *et al.* (1987) selected somaclonal variants of Potato showing resistance against *Phytophthora infestans* through dual culture of potato callus and fungus inoculum. About 2500 somaclones were regenerated from potato cultivar Judith, each of which were dual cultured with *P. infestans* to obtain any variant that show antagonistic effect against toxins produced by the fungus.

Zhang and Zhuang (2020) dual cultured large numbers of *Trichoderma* strains against *Rhizoctonia solani* to assess the strength of different *Trichoderma* strains. *T. brevicrassum* (TC967) was observed to perform best against cucumber root rot caused by *R. solani* and it was the first time *T. brevicrassum* was reported as potential biocontrol agent as well as plant growth promoter.

Bastakoti *et al.* (2017) tested biocontrol efficacy of *Trichoderma* against 4 soil borne fungal pathogens (*Sclerotium rolfisii*, *Sclerotinia sclerotiorum*, *Fusarium solani* and *Rhizoctonia solani*) through dual culture technique. 26 soil samples were used to isolate 5 different *Trichoderma* species, which were then tested against the fungi. Three *Trichoderma* isolates completely inhibited the radial growth of *Sclerotium rolfisii*; maximum inhibition of *Rhizoctonia solani* and *Fusarium solani* was found to be 62 percent and 68 percent, respectively; maximum inhibition of *Sclerotinia sclerotiorum* was found to be 23 percent.

Amin *et al.* (2010) evaluated dual culture of six isolates of *Trichoderma* against soil borne pathogens *R. solani*, *S. rolfisii* and *S. sclerotiorum*. *T. viride* (Tv-2) showed superior performance by significantly reducing mycelial growth (71.41 percent over control) in a dual culture of pathogens and *Trichoderma spp.*, followed by *T. viride* (Tv-1) and *T. harzianum* (Th-1), which both demonstrated 65.71 and 60.51 percent inhibition over control respectively.

Tapwal *et al.* (2011) worked on dual culture of *Trichoderma viride* and 5 phyto pathogen that causes serious mortality problem in nursery seedlings of forest trees. The dual culture exhibited significant antagonistic property against phyto pathogens as compared to control. *T. viride* was found to be most effective against *Fusarium oxysporum* and *Rhizoctonia solani*.

Meena *et al.* (2017) evaluated antagonistic effect of *Trichoderma* spp. in respect to volatile and non-volatile compounds produced by it. They followed the dual culture method to determine growth inhibition of pathogen by *Trichoderma*. Growth inhibition was tested for volatile and non-volatile compounds released by *Trichoderma* spp. The nonvolatile compounds produced by *T. harzianum* and *T. viride* were found to have highest inhibition of *A. alternata*, 62.50% and 60.00% respectively while volatile compounds inhibition was 40% and 35% respectively.

Rajendiran *et al.* (2010) tested *Trichoderma* as a biocontrol agent against post-harvest pathogens like *Aspergillus* spp., *Fusarium* sp., *Penicillium* spp. etc. by dual culturing with each of them. It was found to be effective against all these pathogens. Highest radial growth inhibition was observed for *Fusarium* sp. (64%) and *Aspergillus niger* (55%) whereas maximum growth inhibition was observed in *A. niger* (64%), *A. fumigatus* (49%).

Huang *et al.* (2011) investigated *Trichoderma harzianum* (SQR-T37) as a control measure against damping off disease in cucumber seedling. The dual culture showed that SQR-T37 is highly effective against *R. solani*. This is a result of mycoparasitism, in which SQR-T37 coils the *R. solani* Q1 mycelium, excretes hydrolytic enzymes that break down the pathogen's cell wall, penetrates the *R. solani* Q1 cell wall, and causes rupture of its cytoplasm. SQR-T37 reduced the amount of *R. solani* in the soil while defending cucumber seedlings from damping-off illnesses. Additionally, SQR-T37 markedly accelerated the growth of the cucumber seedlings.

MATERIALS AND METHODS

The present study entitled “**Effect of bio-stimulants and *Trichoderma* spp. on biochemical parameters of cucumber (*Cucumis sativus* L.) grown under salt stressed condition**” was conducted in the year of 2022. A detailed account of the materials used and methods adopted during the course of investigation are presented in this chapter.

3.1 Experimental site

The *in vitro* experiment was carried out in the Plant Health Clinic lab and DST-FIST lab of Department of Mycology & Plant Pathology, Institute of Agricultural Sciences (IAS), Banaras Hindu University (BHU), Varanasi, Uttar Pradesh, India. While the *in vivo* experiment was conducted in the vegetable research farm of the same institute situated at 25^o 18’ N latitude and 83^o 3’ E longitude. The clay pot of the size 15×10 cm² were inserted into the soil and cucumber plants were grown on it. Proper precautions were taken in order to prevent the plant from external damage of pests and other environmental factors.

3.2 Materials used

Laminar air flow, Hot air oven, Autoclave, Weighing balance, Compound microscope, Microwave oven, Centrifuge machine, Vortex shaker, UV-VIS Spectrophotometer, BOD (Biological oxygen demand) incubator, pH meter, Micropipette, Eppendorf tube, Conical flask, Beaker, Petri plates, Test tube, measuring cylinder, Spirit lamp, Needle, Forceps, Inoculating needle, Cork borer, Non-absorbent cotton, Cucumber seeds, Zip lock pouch, Tags, Automizer, Pots, etc. were used during experiments.

3.3 *In vitro* assays

3.3.1 Sterilization

All the culture media (PDA) and distilled water were sterilized in autoclave at 121^o C, 15 psi for 20 minutes. Petri plates and other glass wares were sterilized in hot

air oven at 180⁰ C for 20 minutes. The inoculating needles, cork borer, forceps, etc. were first dipped in rectified spirit and then heated on spirit lamp until they become red hot and before using, they were cooled down. UV radiation in laminar air flow was kept on for about 15-20 minutes before using in order to perform the experiments in environment free from any microbes, and prevent any contamination.

3.3.2 Culture media preparation

Potato Dextrose Agar (PDA) was used for isolation, culture maintenance and multiplication of different fungal pathogens as well as *Trichoderma* spp. The composition of medium was as follows:

Table. 3.1 Composition of ingredients to prepare potato dextrose agar (PDA)

Ingredients	Quantity
Peeled potato	200 g
Dextrose	20 g
Agar agar	20 g
Distilled water	1000 ml
pH	6.5 - 7

3.3.3 Isolation of the pathogens

Samples from the different plants showing disease symptoms of respective disease was collected from agricultural research farm of Banaras Hindu University and brought into the laboratory for isolation, identification and preservation purpose. For isolating fungal pathogens from infected leaves and fruit samples, samples were cut into several small sections of 5-10 mm square from the margin of the infected lesion that contain both diseased and healthy-looking tissue. Then surface sterilization was done with sodium hypochlorite (1%) solution by dipping the cut samples for 30 seconds, following this the sections were taken out aseptically, washed three times in sterile water, blotted dry on clean sterile paper towels and was finally placed on the nutrient medium, usually three to five per dish. The pathogens grew from the sections and the colonies of the pathogen were sub cultured aseptically for further study.

For the *in vitro* studies, multiple pathogens were isolated from different plants are shown in the following table.

Table. 3.2 List of pathogens isolated and source of isolation.

Pathogen isolated	Crop	Plant parts used for isolation
<i>Curvularia oryzae</i>	Paddy	Leaf
<i>Bipolaris sorokiniana</i>	Wheat	Leaf
<i>Phomopsis vexans</i>	Brinjal	Fruit
<i>Fusarium oxysporum</i> sp. <i>lycopersici</i>	Tomato	Fruit
<i>Colletotrichum capsici</i>	Chili	Fruit
<i>Alternaria solani</i>	Tomato	Leaf

3.3.4 Maintenance of culture

Pure culture of the fungus was sub cultured on PDA slants and kept in BOD at $27\pm 1^\circ\text{C}$ for 15 days. These cultures were sub cultured routinely once in a month and preserved at 4°C in a refrigerator for future studies.

3.3.5 Bio control agent *Trichoderma* spp.

Two different strains viz. *Trichoderma viride* and *Trichoderma harzianum*, previously isolated and its slants preserved in the refrigerator were collected, sub-cultured in the PDA media and kept at $27\pm 1^\circ\text{C}$ in a BOD. These cultures were sub cultured routinely once in a month and preserved at 4°C in a refrigerator for future studies.

3.3.6 Bioassay of isolated pathogens and *Trichoderma* spp.

3.3.6.1 Dual Culture Assay (Broadbent *et al.*, 1971)

The petri plates poured with PDA media were inoculated with 5 mm mycelial disc of isolates of *Trichoderma* spp. at one edge of 90 mm petri plate and at opposite edge of the plate pathogen tested of 5 mm mycelial disc were inoculated. Both the discs were 20 mm away from the edge and 50 mm apart. The control plates were maintained

by inoculating the 5 mm mycelial disc of tested pathogens and *Trichoderma* spp. on the petri plates separately. All the plates were incubated at $27 \pm 1^{\circ}$ C for 4 days in BOD. Colony diameter of both pathogen and *Trichoderma* spp. was measured up to 4 days. The percent inhibition of each *Trichoderma* isolate against tested pathogens was calculated by the following formula: (Arora & Upadhyay, 1978)

$$I = \frac{(C - T)}{C} \times 100$$

Where,

I = % inhibition of mycelial growth

C = growth of pathogen in control petri plates

T = growth of pathogen in dual petri plates

3.4 In vivo assays

3.4.1 Pot culture experiment

A pot culture experiment was conducted in clay pots which were laid out in a complete randomized design (CRD) consisting of 9 treatments for each of the foliar spray and drenching application maintaining 3 replications for each treatment. The potting mixture contained the loam soil, sand and vermicompost in the ratio of 2:1:1 and these were obtained from the premises of Banaras Hindu University.

3.4.2 Seed sowing

Seeds of cucumber cultivar (Kashi Nootan) was collected from ICAR-Indian Institute of Vegetable Research, Post Bag No. 01; P. O. Jakhini (Shahanshapur), Varanasi - 221 305, Uttar Pradesh, India. Seed sowing was done on 9th March 2022 in each pot at the rate of 4 seeds per pot and were supplied with irrigation water to maintain the level of moisture in the pots. The date of germination starts on 17th March 2022. After two weeks of sowing, crops were thinned out and only 2 healthy seedlings per pot were allowed to grow.

3.4.3 Treatment details

- Number of varieties: 1 (Kashi Nootan)
- Number of treatments: 9

- Number of replications: 3
- Treatment blocks: 2 (Foliar spraying and soil drenching)
- Experimental design: Completely Randomized Design (CRD)

Table. 3.3 An outline of treatment plans

Treatment	Details
Foliar spray application (F)	
T0F	Control
T1F	NaCl (100 mM)
T2F	NaCl (100 mM) + SA (50 mg/L)
T3F	NaCl (100 mM) + MT (50 μ M)
T4F	NaCl (100 mM) + TH (1×10^8 CFU ml ⁻¹)
T5F	NaCl (100 mM) + SA (50 mg/L) + MT (50 μ M)
T6F	NaCl (100 mM) + TH (1×10^8 CFU ml ⁻¹) + SA (50 mg/L)
T7F	NaCl (100 mM) + TH (1×10^8 CFU ml ⁻¹) + MT (50 μ M)
T8F	NaCl (100 mM) + TH (1×10^8 CFU ml ⁻¹) + SA (50 mg/L) + MT (50 μ M)
Soil drenching application (D)	
T0D	Control
T1D	NaCl (100 mM)
T2D	NaCl (100 mM) + SA (50 mg/L)
T3D	NaCl (100 mM) + MT (50 μ M)
T4D	NaCl (100 mM) + TH (1×10^8 CFU ml ⁻¹)
T5D	NaCl (100 mM) + SA (50 mg/L) + MT (50 μ M)
T6D	NaCl (100 mM) + TH (1×10^8 CFU ml ⁻¹) + SA (50 mg/L)
T7D	NaCl (100 mM) + TH (1×10^8 CFU ml ⁻¹) + MT (50 μ M)
T8D	NaCl (100 mM) + TH (1×10^8 CFU ml ⁻¹) + SA (50 mg/L) + MT (50 μ M)

Note: mM = Millimolar, mg = Milligram, μ M = Micromolar, CFU = Colony forming unit, NaCl = Sodium chloride, SA = Salicylic acid, MT = Melatonin, and TH = *Trichoderma harzianum*

3.4.4 Application of salt stress

The salt solution was prepared by dissolving 52.596 g NaCl in 9 litres of water to make concentration of 100 mM. The salt stress was applied for 3 times on 3 days interval after 28 days of sowing. 250 ml of salt solution was applied to the base of plants during each application by soil drenching. Thereafter, plants were irrigated with tap water only when necessary.

3.4.5 Application of salicylic acid, melatonin and *Trichoderma harzianum*

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. Plants in the control group (salt stressed and salt unstressed) received a sterile distilled water spraying.

Similarly, 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. Plants in the control group (salt stressed and salt unstressed) received a sterile distilled water spraying.

In addition, to prepare 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.*, *Trichoderma harzianum* showed higher antifungal efficacy, hence this strain was further mass cultured in the potato dextrose broth (PDB) at $25\pm 2^{\circ}$ 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 by atomizer onto the cucumber plants according to treatments. Plants in the control group (salt stressed and salt unstressed) received a sterile distilled water spraying.

3.4.6 Biochemical observations

3.4.6.1 Sample collection for biochemical analysis

From each treatment, the plant leaf samples were collected randomly including control. The samples were kept into zip lock bags, labelled and immediately placed in cold chamber containing ice packs to prevent degradation of enzymes. Then samples were taken to the laboratory and kept in deep freezer (-20°C) until the biochemical assays were carried out.

The biochemical assays performed in this study are as follows:

3.4.6.2 Total Phenolic content (TPC)

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

Principle:

The Folin-Ciocalteu reagent oxidizes all phenolic compounds found in plant extracts. This reagent is made from a mixture of phosphotungstic acid (H₃PW₁₂O₄₀) and phosphomolybdic acid (H₃PMO₁₂O₄₀), which following the phenol oxidation, is reduced to a mixture of blue oxides of tungsten (W₈O₂₃) and molybdenum (Mo₈O₂₃). 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-Ciocalteu reagent (1N)
 Sodium carbonates (20%)
 Gallic acid

Procedure:

For the estimation, 1 gm of leaf sample was taken in a mortar and pestle and crushed in 10 ml of 50% methanol. The homogenized sample was collected in centrifuge tube and incubated for 1 hour in a cold chamber. The sample was then centrifuged at 13000 rpm for 10 minutes at 4⁰ C. The supernatant separated from the sample was used for further assay. 100 µL of supernatant was pipette out into a test tube and 900 µL distilled water was added to make a volume of 1ml. 500 µL of Folin-Ciocalteau's phenol reagent (1N), and 1.0 ml Na₂CO₃ (20%) were added in the same tube, the whole solution was kept in a vortex for 30 seconds and allowed to incubate for 20 minutes. The mixture is diluted by adding 10 ml distilled water and vortexed for 30 seconds again. Finally, the absorbance was measured at 725 nm in spectrophotometer. Methanol was used in blank in place of the sample. Standard curves were made for each assay using various concentration of gallic acid in 95% ethanol. The absorbance values were converted to mg gallic acid equivalent (GAE) g⁻¹ fresh weight (FW).

3.4.6.3 Phenylalanine ammonia-lyase (PAL) assay

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; 4⁰ C)
β- mercaptoethanol (0.4 mM)
Potassium phosphate buffer (0.2 M; pH-8.7; 4⁰ C)
Trichloroacetic acid (TCA, 1M)
L- phenylalanine (0.1 M)

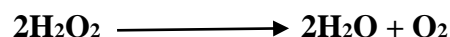
For the estimation, 0.3 gm of leaf sample was taken in a mortar and pestle, crushed in 3ml of 0.1M ice-cold phosphate buffer (K₂HPO₄; pH 7.0) containing 4 µL

β - mercaptoethanol (0.4 mM). All the activities were carried out in chilled condition for this analysis. The homogenized sample was then centrifuged at 16000 rpm for 15 minutes at 4°C. 200 μ L supernatant consisting the enzyme extract was pipette out into a test tube and 500 μ L of 0.2 M phosphate buffer (K_2HPO_4 ; pH 8.7) and 1.3 ml of distilled water was added into it. The reaction was started by adding 1 ml of L-phenylalanine (0.1 M; pH 8.7) and the tube was then incubated at 32° C for 30 minutes. The reaction was then terminated by adding 0.5 ml of 1M trichloroacetic acid (TCA). Reaction mixture without enzyme extract served as blank. The absorbance was recorded at 290 nm. PAL was calculated by formation of Trans-cinnamic acid (TCA) at 290 nm and was expressed in μ M TCA g^{-1} of fresh weight (FW).

3.4.6.4 Peroxidase (PO) assay

Principle:

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



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)

Procedure

The peroxidase assay was performed based on the protocol of Hammerschmidt *et al.*, (1982) with slight modification. 0.1gm sample leaf was homogenized along with 3ml ice-cold 0.1M potassium phosphate buffer (K_2HPO_4 ; pH 7.0; 4⁰ C). The homogenized sample was centrifuged at 16000 rpm at 4°C for 15 minutes. The supernatant obtained was used as enzyme source. The reaction mixture consisted of 50 μ L of enzyme extract, 1.5 ml of 0.05M pyrogallol, and 500 μ L of H_2O_2 (1% v/v). The changes in absorbance were recorded at 420 nm after 30 seconds interval for 3 minutes in a spectrophotometer. The PO enzyme activity was read as a change in the O.D (optical density) and expressed as unit $min^{-1} g^{-1}$ FW.

3.4.6.5 Polyphenol oxidase (PPO) assay

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)

Procedure:

For this assay, 0.1 gm leaf sample was taken in mortar and pestle, crushed in 3ml of ice-cold potassium phosphate buffer (K_2HPO_4 ; 0.1M; pH 6.5). All these activities were carried out by maintaining chilled condition. The homogenized sample was then centrifuged at 16000 rpm at 4°C for 15 minutes. The supernatant obtained was directly used for the enzyme assay. 100 µl of supernatant pipette out into a test tube, 1.5 ml potassium phosphate buffer (K_2HPO_4 ; 0.1 M; pH 6.5) and 0.2 ml of catechol (0.01M) was added. The change in the absorbance was recorded at 495 nm at 30 seconds intervals for 3 minutes. The enzyme activity was expressed as change in the O.D (optical density) $\text{min}^{-1} \text{g}^{-1} \text{FW}$.

3.4.6.6 Protein content estimation

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 (K₂HPO₄; 0.1M; pH 7.0)

Procedure:

For the estimation, 500 mg of leaf samples was taken in a mortar and pestle, crushed in 5 ml of potassium phosphate buffer (K₂HPO₄; 0.1 M; pH 7.0). The homogenized sample was then collected in a centrifuge tube and centrifuged at 16000 rpm at 4⁰ C for 15 minutes. Then, 200 µL of supernatant was pipette out into a test tube and 800 µL of distilled water was added to make up the volume to 1.0 ml. Now, 5.0 ml of alkaline reagent and 0.5 ml of Folin-Ciocalteu's phenol reagent (FCR) was added into the same tube and the mixture was incubated for 30 minutes at room temperature. Finally, the absorbance was recorded at 660 nm. The reaction mix without protein serves as blank. Protein concentration is calculated from standard curve of Bovine Serum Albumin (BSA) and was expressed as µg g⁻¹.

3.4.6.7 Chlorophyll content

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

Reagent used: Acetone (80 %)

Procedure:

1.0 gm of fresh leaf was taken in mortar and pestle, crushed into fine form along with 10 ml of 80% acetone and collected into the centrifuge tube. The samples were then centrifuged at 16000 rpm at 4⁰ C for 15 minutes in dark condition. The supernatant collected on top of tubes was used as enzyme extract for the assay. 80% acetone (v/v) without enzyme extract was used as blank. The absorbance was read at 647 nm and 664 nm on a spectrophotometer. Then the chlorophyll a, chlorophyll b and total chlorophyll was calculated and expressed in mg per gram of fresh weight.

3.4.6.8 Proline content estimation

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

Procedure:

0.5 g of fresh leaves were homogenized in 5 ml of 3 % sulphosalicylic acid and then centrifuged at 16000 rpm for 15 minutes and supernatant was taken. 2 ml of supernatant was taken in a test tube and 2 ml of acid-ninhydrin reagent was added to it. After that 2 ml of glacial acetic acid was added to the tubes. The test tubes were incubated in boiling water bath for 1 hour and the reaction was terminated by keeping tubes in ice-bath immediately. Then 4.0 ml of toluene was added. After vigorous shaking on vortex, the upper organic phase was taken for absorbance. It was recorded at 520 nm by using toluene as blank

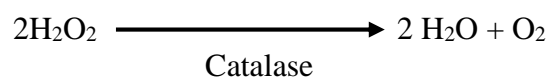
A standard curve was prepared by using graded concentration of proline in 3 % sulphosalicylic acid. The proline content was expressed as $\mu\text{mol g}^{-1}$ FW.

3.4.6.9 Catalase 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 H_2O_2 production.



Catalase assay is based on the absorbance of H₂O₂ 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

0.5 g of leaves samples were crushed along with 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. The homogenized mixture was collected in a centrifuge tube and which was centrifuged at 16000 rpm at 4°C for 15 minutes. The reaction mixture contains 1.5 ml of sodium phosphate buffer (100 mM, pH 7.0), 100 μL of supernatant, 1 ml of 30 mM H₂O₂, and 0.4 ml of distilled water to make the final volume 3 ml. Finally, the reaction is started by addition of 1 ml of 30 mM H₂O₂. The decrease in absorbance was recorded for 1 min at 20 sec intervals at 240 nm. The reaction mixture in which H₂O₂ solution is replaced by phosphate buffer serves as blank.

3.7. Data analysis

The data obtained from the experiments were analyzed statistically by using computer software Statistical Package for the Social Science (SPSS) version 16.0 and subjected to one-way ANOVA (analysis of variance) to find the differences within *in vitro* antagonistic efficacy of *Trichoderma* spp. against fungal pathogens, as well as effect of different treatments on plant defense related compounds to alleviate salt stress. Treatment means were compared by the Duncan's Multiple Range Test (DMRT). The level of significance used in the SPSS analysis of different treatment is $p \leq 0.05$.

RESULTS AND DISCUSSION

4.1 Biochemical analysis

In the present investigation, different biochemical analysis was conducted after 14 days of treatment application with salt and bio-stimulants viz. salicylic acid (SA), melatonin (MT), and *Trichoderma harzianum* (TH) isolate, individually or in association by following two methods of application namely foliar spray and soil drenching, under salt stress condition.

4.1.1 Total Phenolic Content (TPC) Assay

TPC activity was found significantly higher in cucumber in all treatments with salt stress and bio-stimulants as compared to untreated control. Small amounts of TPC were observed in control in all the assays as well.

Foliar application

It becomes noticeable from Figure. 4.1 that highest TPC accumulation under salt stress was observed in T8F (3085.87 $\mu\text{g GAE g}^{-1}$ FW) followed by T5F (2513.50 $\mu\text{g GAE g}^{-1}$ FW) and T6F (2491.50 $\mu\text{g GAE g}^{-1}$ FW). Whereas, control treatment without salt stress viz. T0F exhibited lowest TPC content (1180.67 $\mu\text{g GAE g}^{-1}$ FW) among the treatments which was followed by control treatment with salt stress, T1F (1259.87 $\mu\text{g GAE g}^{-1}$ FW).

Soil drenching

Result showing the effect of different bio-stimulants by soil drenching application on TPC activity in cucumber growing under salt stress condition is shown in Figure 4.2. The highest TPC accumulation was recorded in T8D (2920.50 $\mu\text{g GAE g}^{-1}$ FW) following this T6D (2414.50 $\mu\text{g GAE g}^{-1}$ FW) and T7D (2255.00 $\mu\text{g GAE g}^{-1}$ FW) showed an increased level of TPC. Lowest TPC accumulation was found in T0D (1375.00 $\mu\text{g GAE g}^{-1}$ FW).

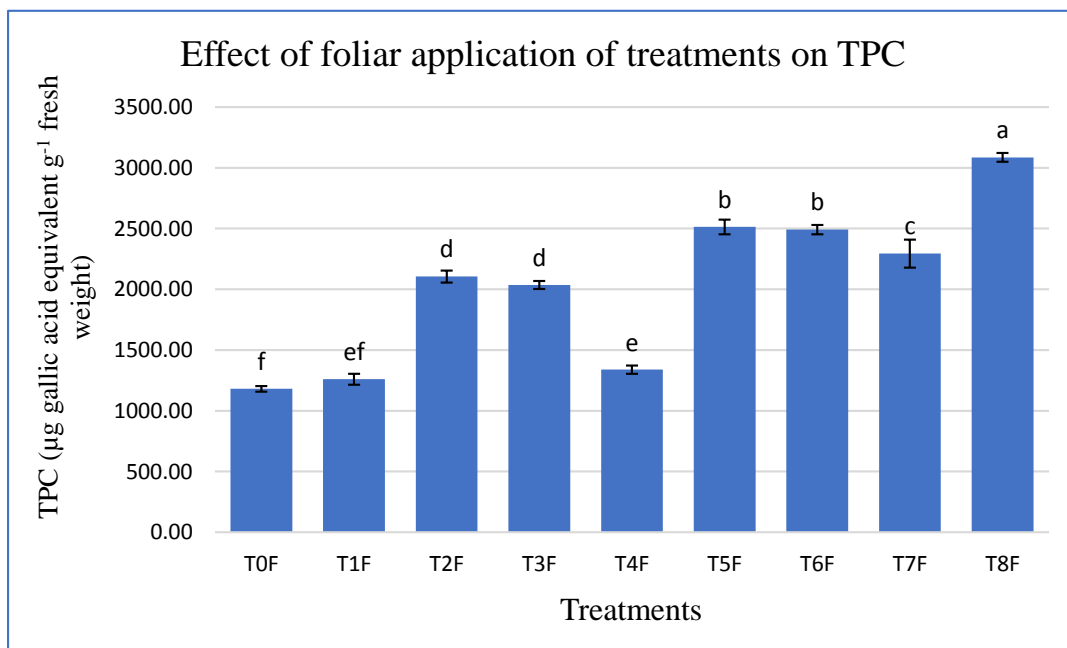


Figure. 4.1 Effect of foliar application of different treatments on TPC of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

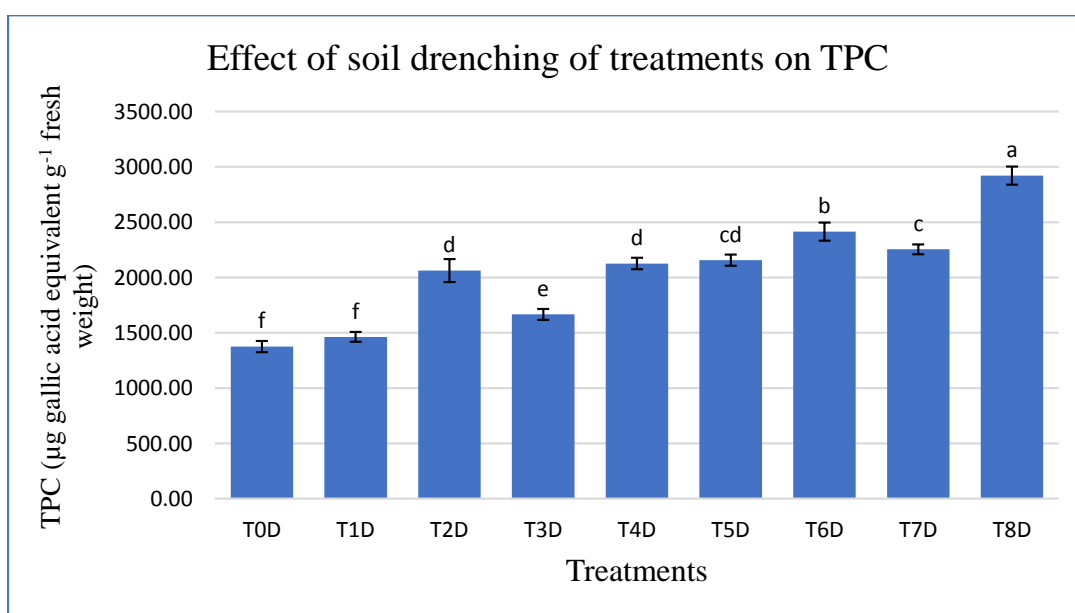


Figure. 4.2 Effect of soil drenching of different treatments on TPC of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Table. 4.1 Comparing the effect of foliar spraying and soil drenching of treatments on TPC of cucumber plants (var. Kashi Nootan) under salt stress condition.

Treatment	Total Phenol Content ($\mu\text{g GAE g}^{-1}\text{ FW}$)	
	Foliar spraying (F)	Soil drenching (D)
T0	1180.67 \pm 22.9 ^{fd}	1375.00 \pm 50.4 ^f
T1	1259.87 \pm 44.9 ^{ef}	1463.00 \pm 44.0 ^f
T2	2104.67 \pm 49.6 ^d	2062.50 \pm 104.5 ^d
T3	2035.00 \pm 33.0 ^d	1666.50 \pm 49.5 ^e
T4	1338.33 \pm 33.6 ^e	2125.93 \pm 51.8 ^d
T5	2513.50 \pm 60.5 ^b	2156.00 \pm 50.4 ^{cd}
T6	2491.50 \pm 38.5 ^b	2414.50 \pm 82.5 ^b
T7	2293.50 \pm 115.5 ^c	2255.00 \pm 44.0 ^c
T8	3085.87 \pm 35.6 ^a	2920.50 \pm 82.5 ^a
CD (0.05)	94.626	113.236
SE (m)	31.603	37.819
SE (d)	44.694	53.484
C.V.	2.692	3.197

Means of 3 replications. Means in column with same superscript is not significantly differed by DMRT, ($P \leq 0.05$)

Although all the bio-stimulants were able to reduce salt stress individually but when these were combined with more bio-stimulants under salt stress, the results on phenol contents showed an upward trend. T8 had the highest phenol content in both foliar spray and soil drenching applications under salt stress. Our findings are consistent with the findings of Grzeszczuk *et al.* (2018), who discovered that foliar application of SA alleviated the negative effects of NaCl and increased total phenol content in *Salvia coccinea*. In a study by Dawood *et al.* (2015), melatonin treatments increased the total phenolic content of faba bean plants. Similarly, Kumar *et al.* (2016) found that in comparison to untreated maize plants, *Trichoderma*-treated plants had significantly higher phenol content (1.67–52 dS m⁻¹ under different salinity conditions).

The formation of lignin begins with phenolic compounds, which are crucial building blocks. Plants respond to biotic and abiotic stressors by accumulating phenolic compounds into their cell walls. Both the hypersensitive response of the cell itself and the reinforcement of the local wall can be achieved through the deposition of phenolic compounds (Datta & Lal, 2012; Nicholson & Hammerschmidt, 1992). Since PAL plays a key role in the plant's arsenal of stress defenses, 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 & Goel, 2019). According to Nguyen *et al.* (2013), gallic acid is thought to produce the enzyme chitinase, which is crucial in the breakdown of the chitin in pathogenic fungi's cell walls and protects plants from disease.

A higher TPC level may also help to more effectively scavenge peroxy, alkyl peroxy, superoxide hydroxyl, nitric oxide, as well as peroxy nitrite in organic and aqueous environments (Jain *et al.*, 2014).

4.1.2 Phenylalanine Ammonia Lyase (PAL) assays

PAL accumulation was observed significantly higher in cucumber in all treatments with salt stress and bio-stimulants as compared to untreated control. Small amounts of PAL were observed in control in all the assays as well.

Foliar application

Results obtained from the Figure. 4.3 shows that maximum PAL activity was observed in T8F (2514.15 $\mu\text{mol transscinnamic acid g}^{-1}$ FW) which is combined treatment of TH (1×10^8 CFU ml^{-1}) + SA (50 mg/L) + MT (50 μM) while minimum PAL activity was found in T0F (1581.92 $\mu\text{mol transscinnamic acid g}^{-1}$ FW) which is control without salt stress. Slightly increased level of PAL was noticed in control with salt stress viz. T1F (1812.44 $\mu\text{mol transscinnamic acid g}^{-1}$ FW).

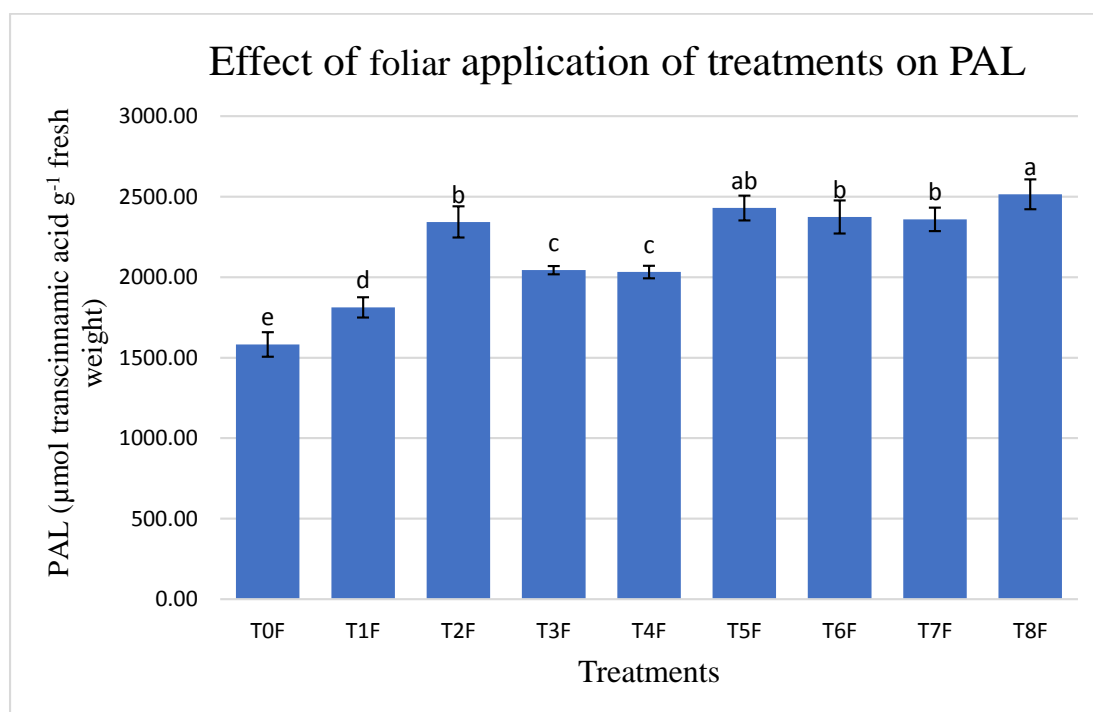


Figure. 4.3 Effect of foliar application of different treatments on PAL activity of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Soil drenching

PAL activity was recorded to be significantly increased in T8D (2600.47 μmol transcinnamic acid g^{-1} FW) and T6D (2451.82 μmol transcinnamic acid g^{-1} FW) treated plants when compared to untreated control TOD (1589.00 μmol transcinnamic acid g^{-1} FW) which shows the lowest activity. An increased PAL activity was also observed in T5D (2353.03 μmol transcinnamic acid g^{-1} FW) and T7D (2335.03 μmol transcinnamic acid g^{-1} FW), however there was no significant difference in between them. Detail of results are presented in the Figure. 4.4.

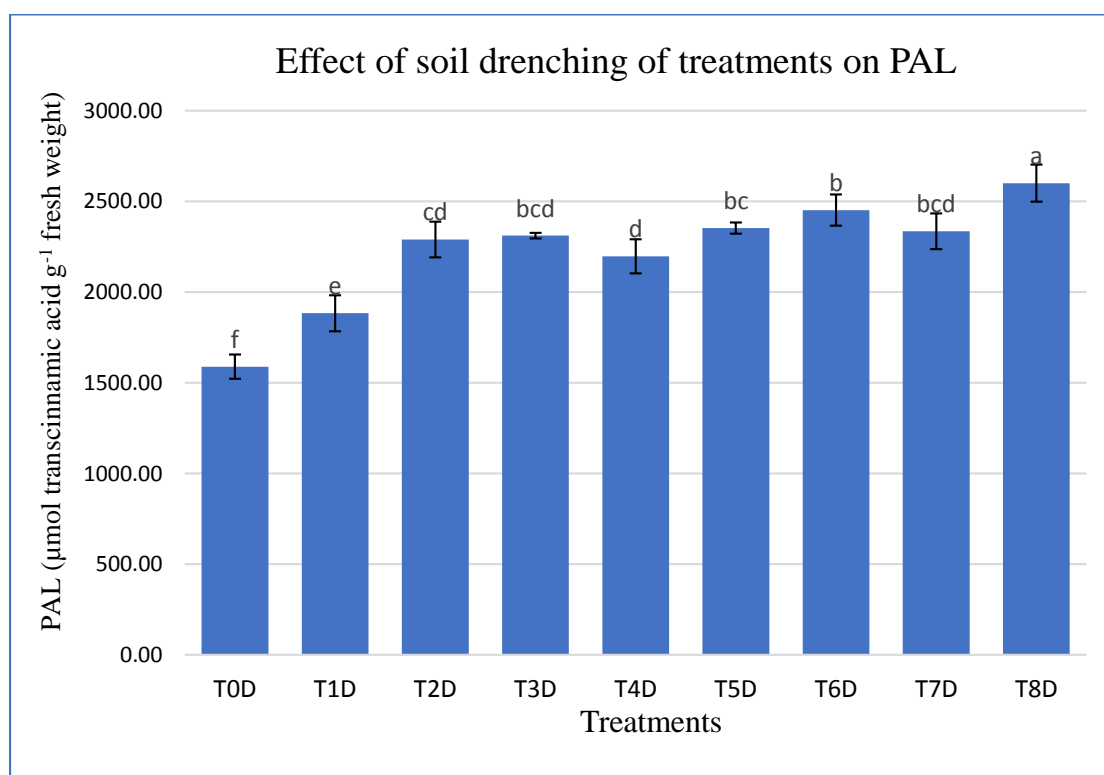


Figure. 4.4 Effect of soil drenching of different treatments on PAL activity of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Table. 4.2 Comparing the effect of foliar spraying and soil drenching of treatments on PAL activity of cucumber plants (var. Kashi Nootan) under salt stress condition.

Treatment	Phenylalanine Ammonia Lyase (PAL) assays ($\mu\text{mol transcinnamic acid g}^{-1}$ fresh weight)	
	Foliar spraying (F)	Soil drenching (D)
T0	1581.92 \pm 75.9 ^e	1588.99 \pm 67.0 ^f
T1	1812.44 \pm 62.8 ^d	1883.23 \pm 98.7 ^e
T2	2342.41 \pm 96.9 ^b	2289.78 \pm 98.8 ^{cd}
T3	2043.72 \pm 25.4 ^c	2311.02 \pm 15.7 ^{bcd}
T4	2031.72 \pm 39.2 ^c	2196.63 \pm 93.9 ^d
T5	2429.20 \pm 76.6 ^{ab}	2353.03 \pm 30.9 ^{bc}
T6	2373.80 \pm 102.8 ^b	2451.82 \pm 86.3 ^b
T7	2358.11 \pm 72.9 ^b	2335.03 \pm 98.3 ^{bcd}
T8	2514.16 \pm 92.8 ^a	2600.47 \pm 102.0 ^a
CD (0.05)	130.985	142.967
SE (m)	43.747	47.748
SE (d)	61.867	67.526
C.V.	3.499	3.720

Means of 3 replications. Means in column with same superscript is not significantly differed by DMRT, ($P \leq 0.05$)

Phenylalanine ammonia lyase (PAL) is an enzyme that links the shikimate and phenylpropanoid pathways which converts L-phenylalanine produced during the shikimate biosynthetic pathway into the trans-cinnamic acid via deamination (Tungmunnithum & Hano, 2020) which is then converted into p-coumaroyl (Yu and Jez, 2008). The Glutamine Synthetase Reaction fixes the ammonia (NH₃) that is released during this process. The PAL enzyme is inhibited by aminooxyphenyl propanoic acid (Heldt and Piechulla, 2011). Lignin biosynthesis as well as other phenolic compounds that build up in response to stress are thought to be preceded by PAL activity (Podile and Laxmi, 1998). The majority of the p-coumaroyl produced is used for the production of lignin (Yu and Jez, 2008).

PAL is the most important enzyme in the phenylpropanoid biosynthesis pathway that produces phytoalexins or phenols, which serve as defence mechanisms in plants by strengthening the cell wall (Nicholson and Hammerschmidt, 1992), acting as antimicrobial agents, and producing signaling molecules like salicylic acid (Wen *et al.* 2005).

In the current study, the plants treated with a combination of all three bio-stimulants (T8) showed the highest level of PAL activity. According to Simaei *et al.* (2012), 100 mM NaCl significantly reduced the activities of PAL, whereas exogenous SA application increased those activities. According to Zhang *et al.* 2019, *Trichoderma harzianum* increased the salt tolerance of cucumber by modifying the activity of phenylalanine ammonia-lyase (PAL).

4.1.3 Peroxidase (PO) assay

In the current study, PO activity was seen following the administration of treatments, and the outcome is shown in Figure 4.5. In an overall trial, it was shown that T8 treated plants had the maximum PO activity, followed by T7 treated plants in both of the foliar spray and the soil drenching applications.

Foliar application

Figure. 4.5 makes it obvious that maximum peroxidase (PO) activity was recorded in T8F (0.21 U min⁻¹ g⁻¹ FW) followed by T7F (0.18 U min⁻¹ g⁻¹ FW) whereas minimum PO activity was noted in untreated control T0F (0.10 U min⁻¹ g⁻¹ FW). Similar PO activities were recorded in T2F and T3F i.e. (0.14 U min⁻¹ g⁻¹ FW).

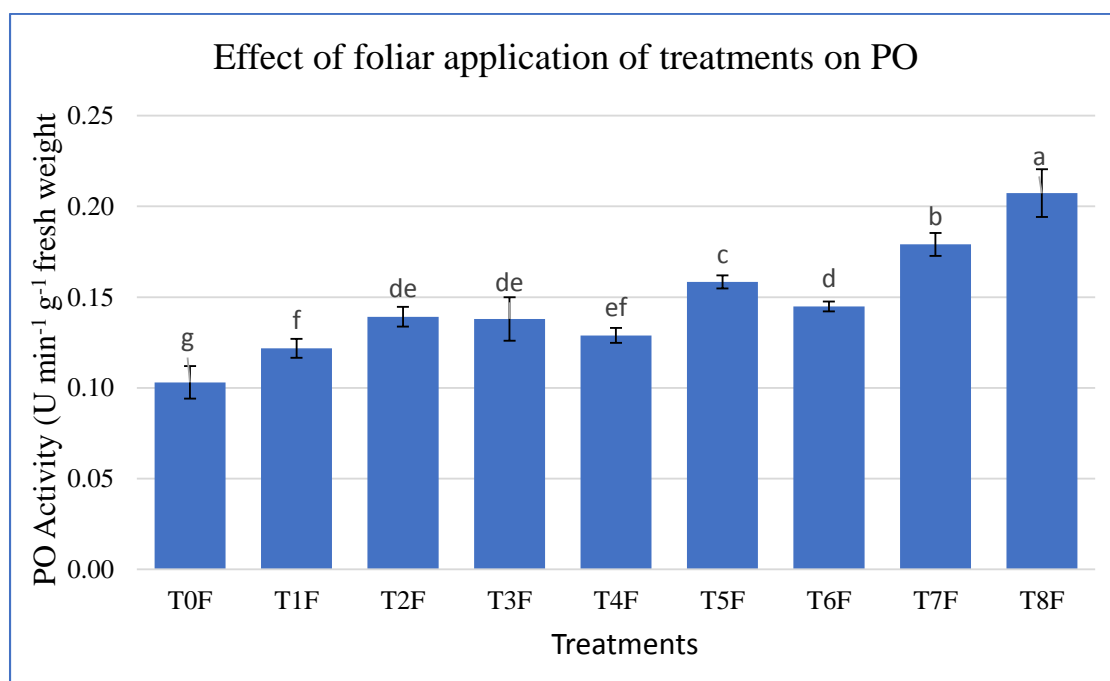


Figure. 4.5 Effect of foliar application of different treatments on PO activity of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Soil drenching

A similar pattern was seen in the soil drenching application, which showed that T8D (0.20 U min⁻¹ g⁻¹ FW) had the highest PO activity, followed by T7D (0.18 U min⁻¹ g⁻¹ FW). The least PO activity of the treatments was shown by T0D (0.11 U min⁻¹ g⁻¹ FW). PO activity was likewise elevated in T5D, T6D, T2D, and T3D, although there was no noticeable difference between them. The Figure 4.6 illustrates the findings in detail.

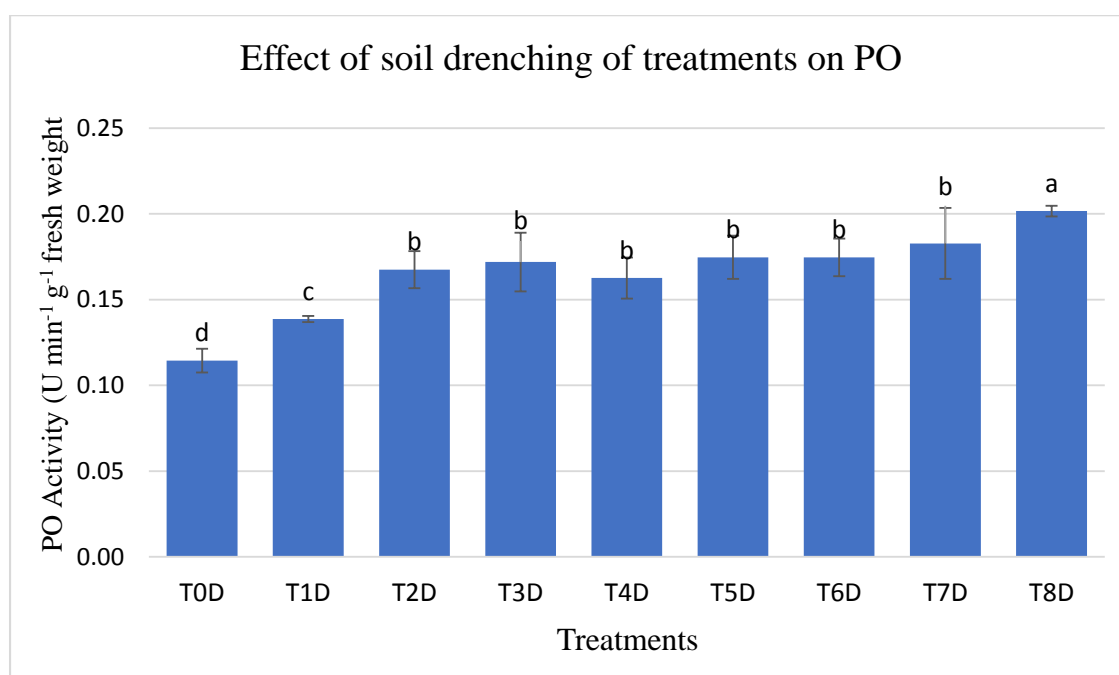


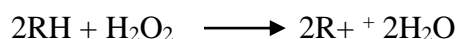
Figure. 4.6 Effect of soil drenching of different treatments on PO activity of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Table. 4.3 Comparing the effect of foliar spraying and soil drenching of treatments on PO activity of cucumber plants (var. Kashi Nootan) under salt stress condition.

Treatment	Peroxidase (PO) assay (U min ⁻¹ g ⁻¹ fresh weight)	
	Foliar spraying (F)	Soil drenching (D)
T0	0.103 ± 0.009 ^g	0.117 ± 0.007 ^d
T1	0.123 ± 0.005 ^f	0.140 ± 0.002 ^c
T2	0.137 ± 0.005 ^{de}	0.170 ± 0.011 ^b
T3	0.140 ± 0.012 ^{de}	0.170 ± 0.017 ^b
T4	0.130 ± 0.004 ^{ef}	0.163 ± 0.012 ^b
T5	0.157 ± 0.004 ^c	0.173 ± 0.013 ^b
T6	0.143 ± 0.003 ^d	0.173 ± 0.011 ^b
T7	0.180 ± 0.006 ^b	0.180 ± 0.021 ^b
T8	0.210 ± 0.013 ^a	0.203 ± 0.003 ^a
CD (0.05)	0.012	0.023
SE (m)	0.004	0.008
SE (d)	0.006	0.011
C.V.	4.897	7.969

Means of 3 replications. Means in column with same superscript is not significantly differed by DMRT, ($P \leq 0.05$)

Peroxidases are enzymes that break down hydrogen peroxide (H₂O₂) while oxidizing a variety of phenolic and non-phenolic substrates (RH) (Kawano, 2003).



Plant peroxidases belong to the Class III peroxidase group and are essential for lignification, ROS metabolism, as well as defence against biotic and abiotic stress (Pandey *et al.*, 2017).

In the current experiment, the plants treated with a combination of all three bio-stimulants (T8) showed the highest level of PO activity. In a wheat crop grown under salt stress, Talaat and Shawky (2022) discovered that the combination treatment of salicylic acid (SA) and melatonin (MT) markedly increased the peroxidase activity as

compared to control. According to Zhang *et al.* (2019), *Trichoderma harzianum* improved cucumber salt tolerance by enhancing peroxidase activity (PO).

4.1.4 Polyphenol oxidase (PPO) assay

Polyphenol oxidase (PPO) activity in cucumber leaves increased over untreated control levels under varying salinity and treatment conditions in both application techniques.

Foliar application

Results from Figure 4.7 reveal that greatest PPO activity was identified in T8F (0.16 $\Delta\text{OD min}^{-1} \text{mg}^{-1}$ of FW), which is a combined treatment of TH (1108 CFU ml⁻¹), SA (50 mg/L), and MT (50 M), followed by T5F and T6F showing the similar PPO activity i.e., (0.13 $\Delta\text{OD min}^{-1} \text{mg}^{-1}$ of FW), whereas minimal PPO activity was discovered in T0F (0.07 $\Delta\text{OD min}^{-1} \text{mg}^{-1}$ of FW), which is a control without salt stress.

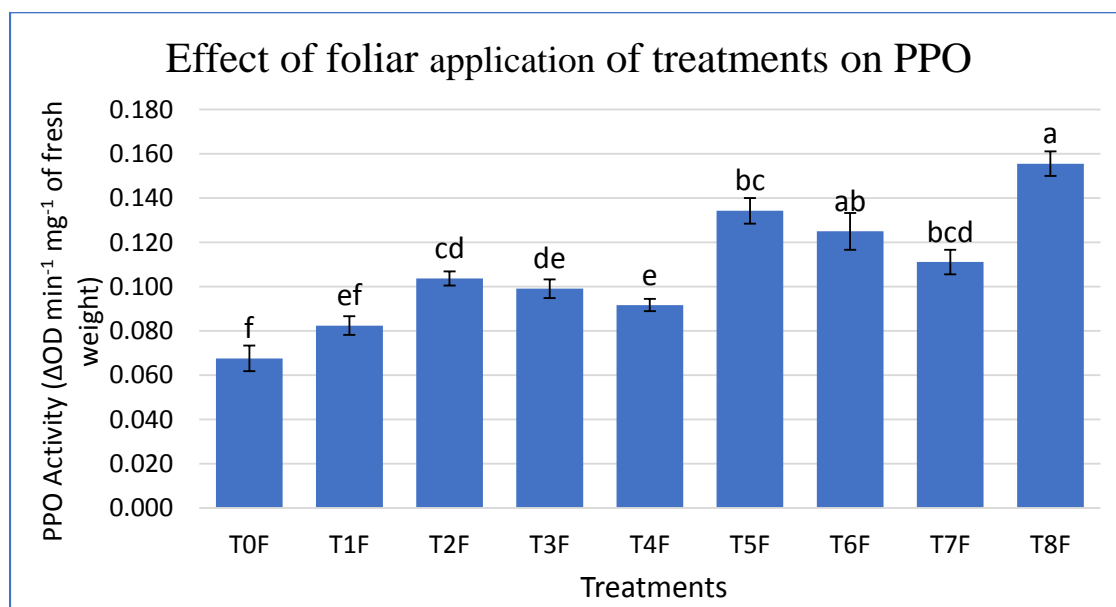


Figure. 4.7 Effect of foliar application of different treatments on PPO activity of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Soil drenching

Comparing treated plants with T8D (0.14 $\Delta\text{OD min}^{-1} \text{mg}^{-1}$ of FW) and T6D (0.13 $\Delta\text{OD min}^{-1} \text{mg}^{-1}$ of FW) to untreated control plants with T0D (0.07 $\Delta\text{OD min}^{-1} \text{mg}^{-1}$ of FW), which exhibits the lowest activity, PPO activity was shown to be considerably higher in the treated plants. Along with T1D (0.09 $\Delta\text{OD min}^{-1} \text{mg}^{-1}$ of FW), which is controlled by salt stress, an elevated PPO activity was also seen in T5D (0.12 $\Delta\text{OD min}^{-1} \text{mg}^{-1}$ of FW) and T7D (0.11 $\Delta\text{OD min}^{-1} \text{mg}^{-1}$ of FW). Figure 4.8 provides a detailed overview of the findings.

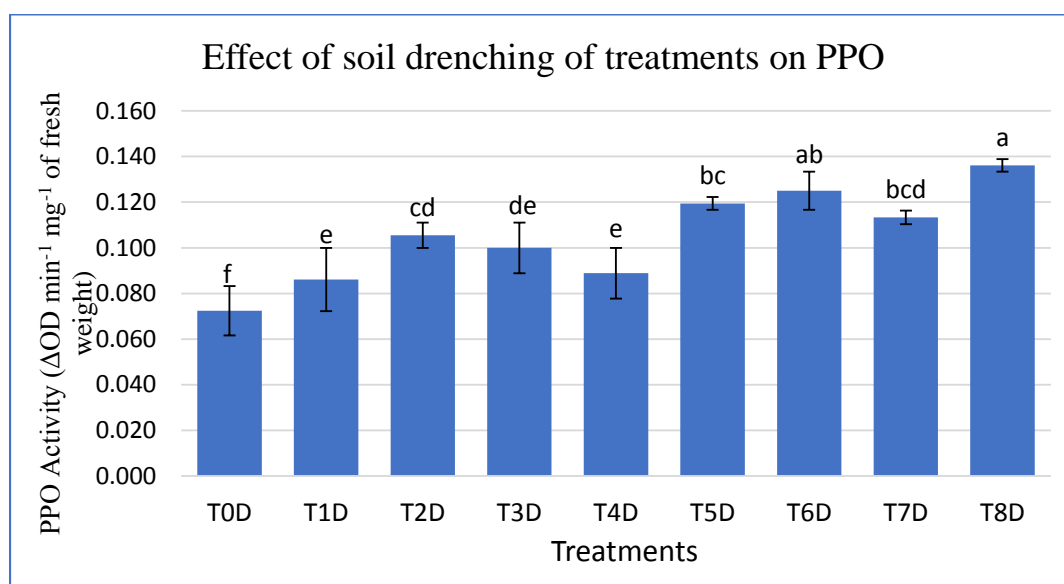


Figure. 4.8 Effect of soil drenching of different treatments on PPO activity of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Table. 4.4 Comparing the effect of foliar spraying and soil drenching of treatments on PPO activity of cucumber plants (var. Kashi Nootan) under salt stress condition.

Treatment	Polyphenol oxidase (PPO) assay ($\Delta\text{OD min}^{-1} \text{mg}^{-1}$ of fresh weight)	
	Foliar spraying (F)	Soil drenching (D)
T0	0.072 \pm 0.006 ^f	0.070 \pm 0.011 ^f
T1	0.086 \pm 0.004 ^{ef}	0.087 \pm 0.014 ^e
T2	0.106 \pm 0.003 ^{cd}	0.107 \pm 0.006 ^{cd}
T3	0.100 \pm 0.004 ^{de}	0.100 \pm 0.011 ^{de}
T4	0.089 \pm 0.003 ^e	0.090 \pm 0.011 ^e
T5	0.119 \pm 0.006 ^{bc}	0.120 \pm 0.003 ^{bc}
T6	0.125 \pm 0.008 ^{ab}	0.127 \pm 0.008 ^{ab}
T7	0.113 \pm 0.006 ^{bcd}	0.113 \pm 0.003 ^{bcd}
T8	0.136 \pm 0.006 ^a	0.137 \pm 0.003 ^a
CD (0.05)	0.015	0.015
SE (m)	0.005	0.005
SE (d)	0.007	0.007
C.V.	8.193	8.154

Means of 3 replications. Means in column with same superscript is not significantly differed by DMRT, ($P \leq 0.05$)

A class of copper-containing enzymes known as polyphenol oxidases (PPOs) catalyses 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.

Recently, Talaat and Shawky (2022) found that the combination treatment of salicylic acid (SA) and melatonin (MT) significantly increased the PPO activity as compared to control in a wheat crop grown under salt stress. In contrast to exogenous SA application, which increased PPO activities, Simaei *et al.* (2012) found that 100 mM NaCl significantly decreased PPO activities. According to Zhang *et al.* (2019), *Trichoderma harzianum* improved PPO activity which improved cucumber's ability to tolerate salt stress.

4.1.5 Estimation of protein content

In this assay, salinity stress lowered protein content of cucumber leaves than the untreated control in both of the foliar spray and soil drenching applications. However, the use of bio-stimulants such as salicylic acid, melatonin, and *Trichoderma harzianum* reduces the effects of salt.

Foliar application

The results shown in Figure 4.9 reveal that the highest amount of total protein content was found in T0F (22.79 mg g⁻¹ of FW), which is an untreated control, and the lowest level of protein content was found in T1F (13.91 mg g⁻¹ of FW), which only received salt stress. Treatments, on the other hand, lessened the impact of salt stress. In combination treatment, T8F (19.85 mg g⁻¹ of FW) had the highest level of total protein content, followed by T5F (18.16 mg g⁻¹ of FW) and T7F (16.94 mg g⁻¹ of FW). In the case of a single treatment, T3F (15.06 mg g⁻¹ of FW) had a higher protein content, but there was no significant difference between T2F and T4F.

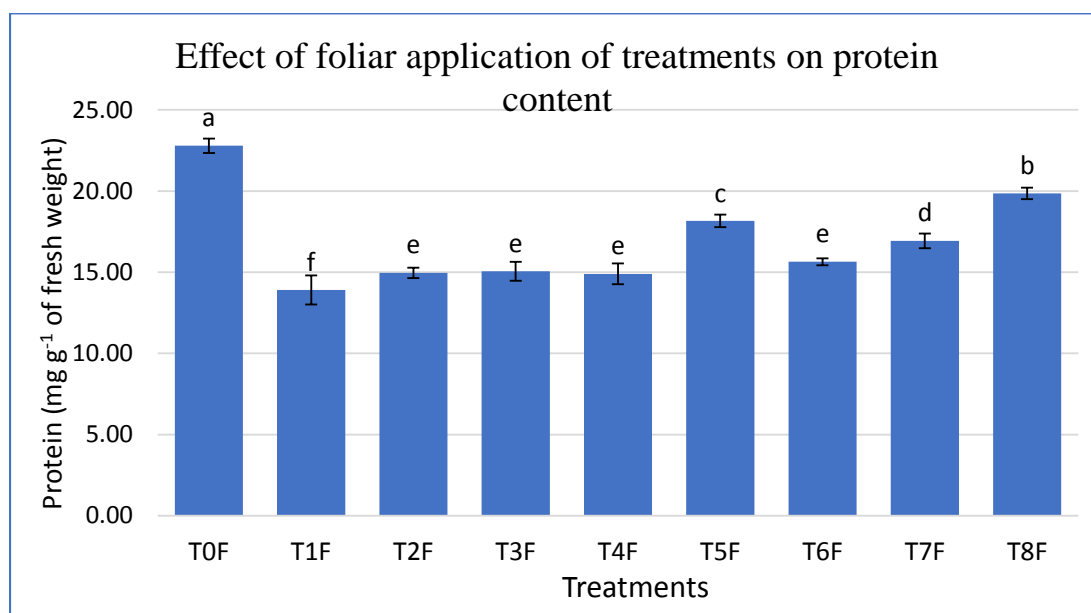


Figure. 4.9 Effect of foliar application of different treatments on Total protein content of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Soil drenching

A similar trend was seen in the soil drenching application, which revealed that T0D (0.46 mg g⁻¹ of FW) had the greatest total protein concentration, followed by T7D (20.03 mg g⁻¹ of FW). T1D (13.22 mg g⁻¹ of FW) had the lowest total protein content among the treatments. Compared to salt stressed alone, total protein level was also higher in T6D, T5D, T4D, and T3D, although there was no significant difference between them. The results are clearly seen in Figure 4.10.

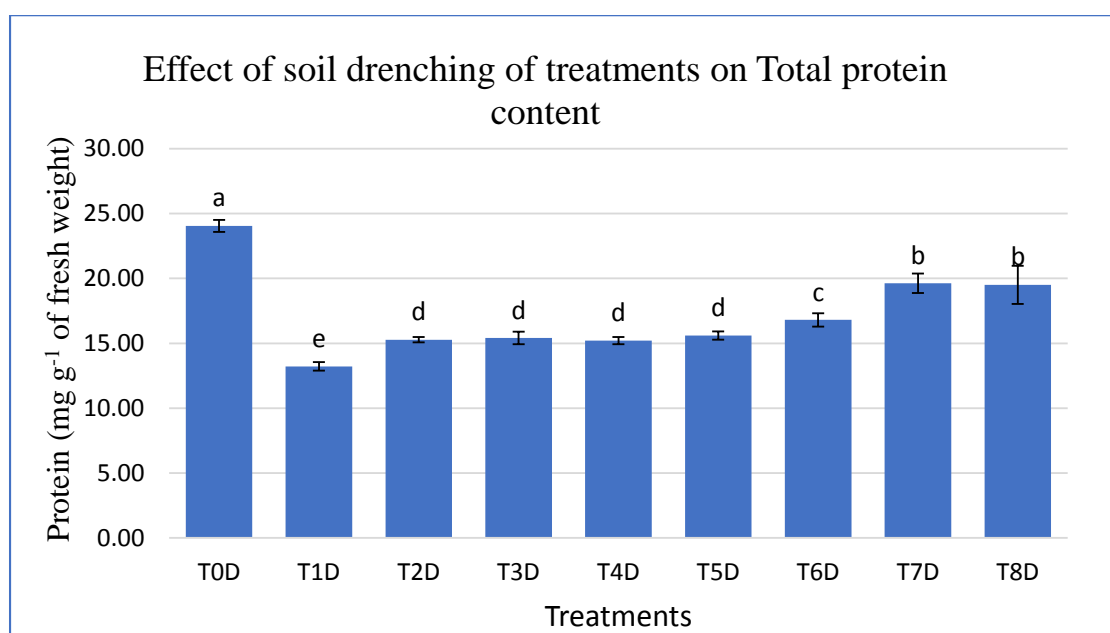


Figure. 4.10 Effect of soil drenching of different treatments on Total protein content of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Table. 4.5 Comparing the effect of foliar spraying and soil drenching of treatments on protein content of cucumber plants (var. Kashi Nootan) under salt stress condition.

Treatment	Protein content estimation (mg g ⁻¹ of fresh weight)	
	Foliar spraying (F)	Soil drenching (D)
T0	22.790 ± 0.45 ^a	24.050 ± 0.46 ^a
T1	13.907 ± 0.89 ^f	13.220 ± 0.32 ^e
T2	14.960 ± 0.32 ^e	15.277 ± 0.21 ^d
T3	15.063 ± 0.58 ^e	15.410 ± 0.48 ^d
T4	14.897 ± 0.64 ^e	15.207 ± 0.27 ^d
T5	18.157 ± 0.39 ^c	15.593 ± 0.32 ^d
T6	15.640 ± 0.21 ^e	16.800 ± 0.51 ^c
T7	16.933 ± 0.45 ^d	19.630 ± 0.75 ^b
T8	19.857 ± 0.36 ^b	19.507 ± 1.47 ^b
CD (0.05)	0.890	1.115
SE (m)	0.297	0.372
SE (d)	0.420	0.526
C.V.	3.044	3.751

Means of 3 replications. Means in column with same superscript is not significantly differed by DMRT, ($P \leq 0.05$)

Cucumber leaves had a lower total protein content due to protein degradation under salt stress, but after treatment with salicylic acid, melatonin, and *Trichoderma harzianum*, the protein content clearly increased due to the stimulation of protein synthesis and inhibition of protein degradation by these bio-stimulants, which preserved cellular stability and physiological activity (Chen *et al.* 2020).

According to Park *et al.* (2021), salinity-damaged mustard had a lower protein content. In comparison to untreated salinity-damaged plants, 1 mM melatonin application increased protein content in salinity stressed plants by 19%. When compared to the control treatment, salinity reduced the amount of total protein in *Vicia faba* (Moussa and Hassan 2016).

Tohma and Esitken (2011) revealed that salt stress treatment significantly reduced protein content in strawberry plants. However, under salt stress conditions, all SA pre-treatments increased the protein content. The highest protein content was determined with a control (24.3 mg g⁻¹) and any SA treatment (14.2–15.9 mg g⁻¹) at a salinity level of 6 mS cm⁻¹.

Regardless of the level of salt stress, Zhang *et al.* (2019) found that root inoculation with *Trichoderma harzianum* led to a significant (P 0.05) increase in soluble protein content.

4.1.6 Estimation of chlorophyll content

Photosynthesis is a crucial process for plants that is impacted by salt stress, hence the amount of chlorophyll in leaves was measured and data from control, salt stressed, and other treated plants were analyzed. In this experiment, both foliar spraying and soil drenching application under salinity stress reduced the chlorophyll content of cucumber leaves compared to the untreated control. However, the effects of salt were diminished by the use of bio-stimulants.

Foliar application

In case of chlorophyll a, figure 4.11 makes it clear that T0F (0.287 mg g⁻¹ of FW), the untreated control, had the highest level of total chlorophyll content, while T1F (0.150 mg g⁻¹ of FW), the treatment to receive salt stress only, had the lowest level. However, this effects of salt in T0F were diminished by bio-stimulants treatment showing enhanced chlorophyll content in T8F (0.283 mg g⁻¹ of FW) which was followed by T7F (0.278 mg g⁻¹ of FW) in combination treatment. Whereas in a single bio-stimulant treatment, T2F (0.230 mg g⁻¹ of FW) exhibited the higher chlorophyll content followed by T4F and T3F.

Similarly in chlorophyll b, figure 4.12 makes it obvious that highest chlorophyll content in all three bio-stimulants combination T8F (0.238 mg g⁻¹ of FW) was recorded followed by T0F (0.223 mg g⁻¹ of FW), the untreated control compared to T1F (0.093 mg g⁻¹ of FW), the treatment to receive salt stress only, had the lowest level.

Higher level total chlorophyll was recorded significantly in T8D (0.521 mg g⁻¹ of FW) followed by T0B (0.511 mg g⁻¹ of FW), the untreated control compared to T1F (0.243 mg g⁻¹ of FW), the treatment to receive salt stress only, had the lowest level. The Figure 4.13 illustrates the findings in detail.

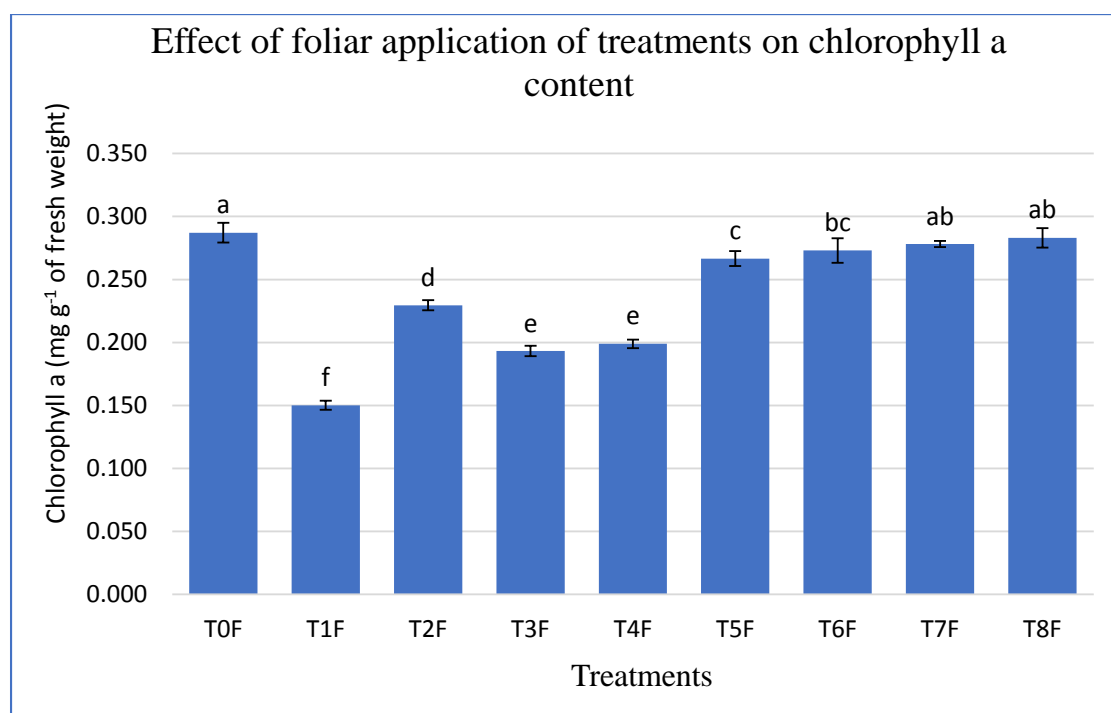


Figure. 4.11 Effect of foliar application of different treatments on Chlorophyll-a content of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

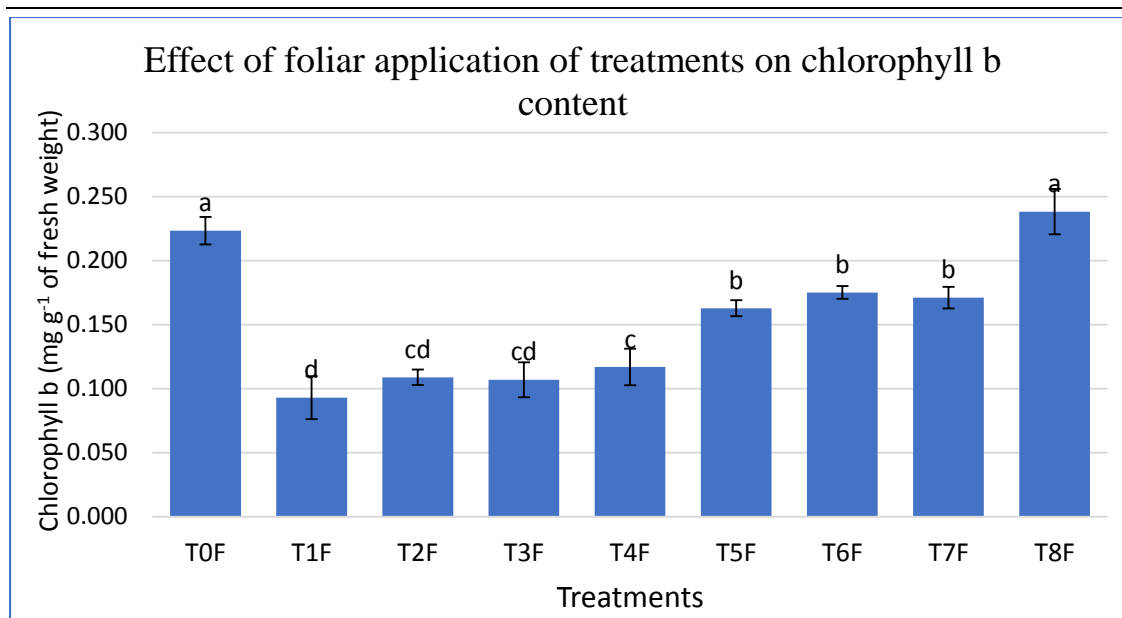


Figure. 4.12 Effect of foliar application of different treatments on Chlorophyll-b content of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

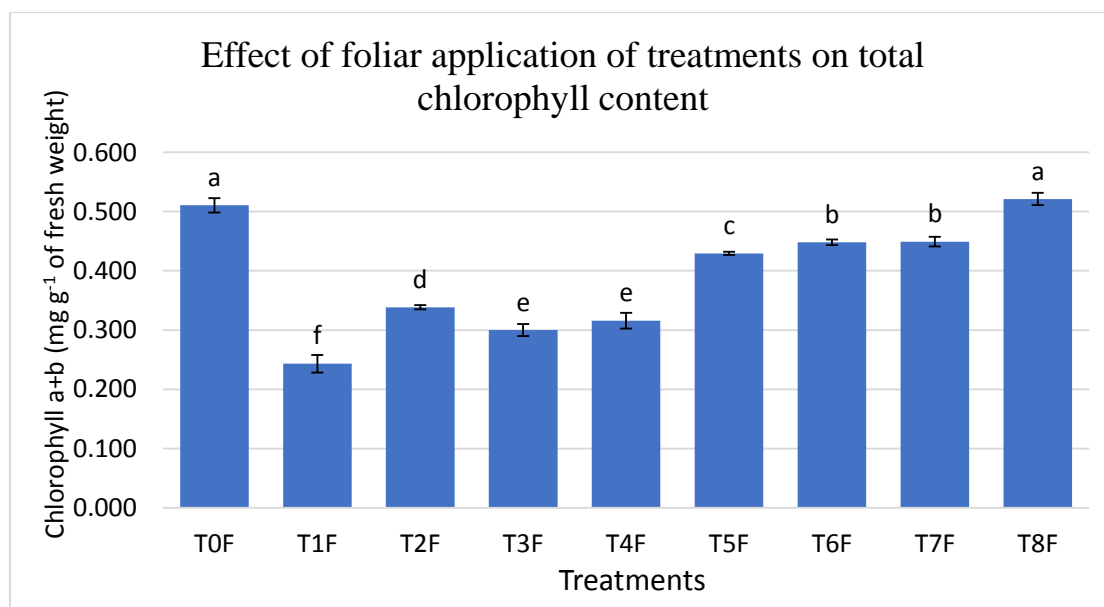


Figure. 4.13 Effect of foliar application of different treatments on Total chlorophyll content of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Table. 4.6 Comparing the effect of foliar spraying of treatments on chlorophyll-a, chlorophyll-b, chlorophyll-a+b content of cucumber plants (var. Kashi Nootan) under salt stress condition.

Treatment	Chlorophyll content estimation (mg g ⁻¹ of fresh weight)		
	Foliar Spraying (F)		
	Chlorophyll a	Chlorophyll b	Chlorophyll (a + b)
T0	0.287 ± 0.008 ^a	0.223 ± 0.011 ^a	0.510 ± 0.012 ^a
T1	0.150 ± 0.004 ^f	0.093 ± 0.017 ^d	0.243 ± 0.015 ^f
T2	0.230 ± 0.004 ^d	0.109 ± 0.006 ^{cd}	0.339 ± 0.004 ^d
T3	0.193 ± 0.004 ^e	0.107 ± 0.014 ^{cd}	0.300 ± 0.010 ^e
T4	0.199 ± 0.003 ^e	0.117 ± 0.014 ^c	0.316 ± 0.013 ^e
T5	0.266 ± 0.006 ^c	0.163 ± 0.006 ^b	0.429 ± 0.003 ^c
T6	0.273 ± 0.010 ^{bc}	0.175 ± 0.005 ^b	0.448 ± 0.005 ^b
T7	0.278 ± 0.002 ^{ab}	0.171 ± 0.008 ^b	0.449 ± 0.008 ^b
T8	0.283 ± 0.008 ^{ab}	0.238 ± 0.018 ^a	0.521 ± 0.010 ^a
CD (0.05)	0.010	0.020	0.017
SE (m)	0.003	0.007	0.006
SE (d)	0.005	0.010	0.008
C.V.	2.443	7.577	2.477

Means of 3 replications. Means in column with same superscript is not significantly differed by DMRT, ($P \leq 0.05$)

Soil drenching

The amount of chlorophyll-a in control and treated plants varied substantially. The content fluctuated between the treatments, with the lowest value of 0.152 mg g⁻¹ fresh weight in salt-stressed untreated (T1D) and the maximum value of 0.307 mg g⁻¹ fresh weight was observed in control (T0D). With the application of salt stress, the chlorophyll-a content drastically reduced as compared to control. As demonstrated in the table. 4.14, the chlorophyll content of leaves under salt stress conditions was improved by the soil drenching application of bio-stimulants showing enhanced content of 0.288 mg g⁻¹ fresh weight (T8D).

A similar trend was seen in leaf chlorophyll-b levels. Figure 4.15 shows that T8F has the highest chlorophyll content (0.256 mg g⁻¹ FW), followed by T0F (0.232 mg g⁻¹ of FW).

The total chlorophyll content of the leaves varied significantly between the treatments, with the lowest value (T1D) of 0.214 mg g⁻¹ fresh weight and the highest (T8D) of 0.544 mg g⁻¹ fresh weight. Salt-treated plants showed a significant decrease in chlorophyll concentration. However, application of bio-stimulants reduced the effects of salinity. The details of the observations are shown in Figure 4.16.

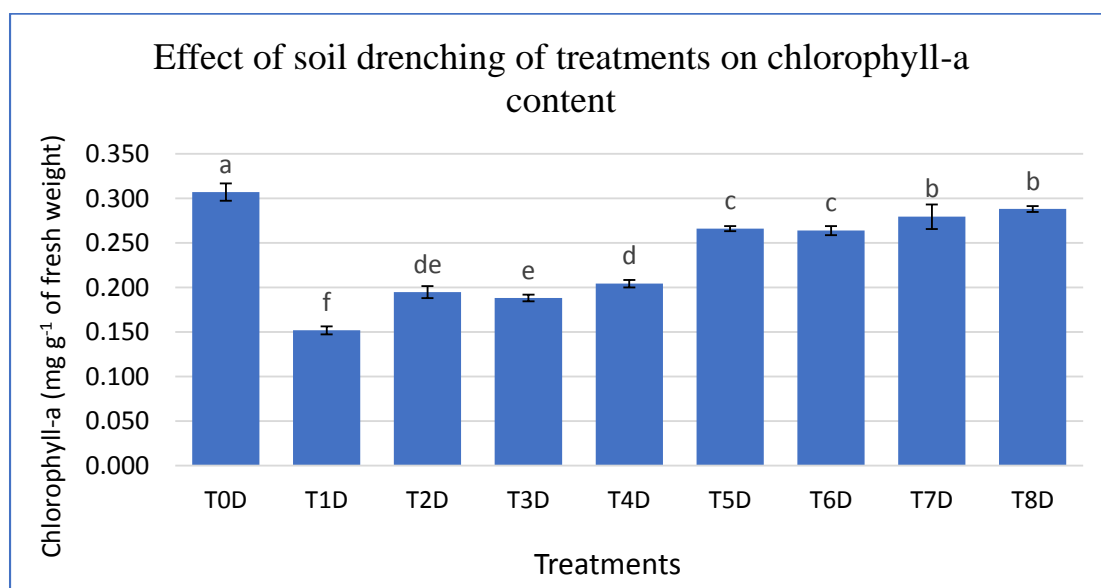


Figure. 4.14 Effect of soil drenching of different treatments on Chlorophyll-a content of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

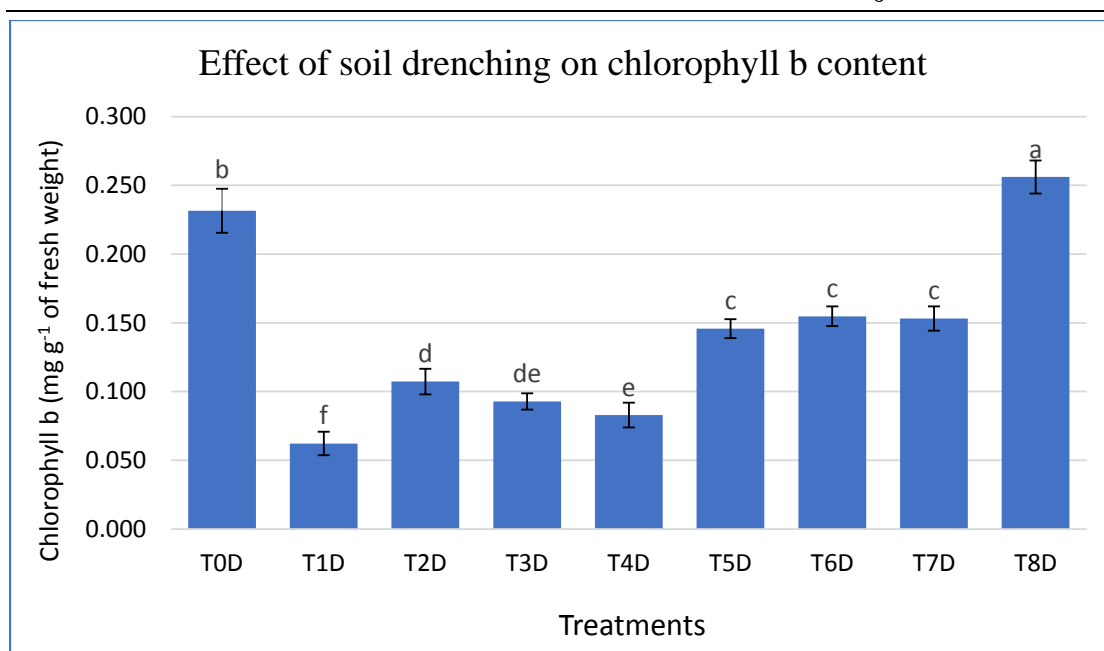


Figure. 4.15 Effect of soil drenching of different treatments on Chlorophyll-b content of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

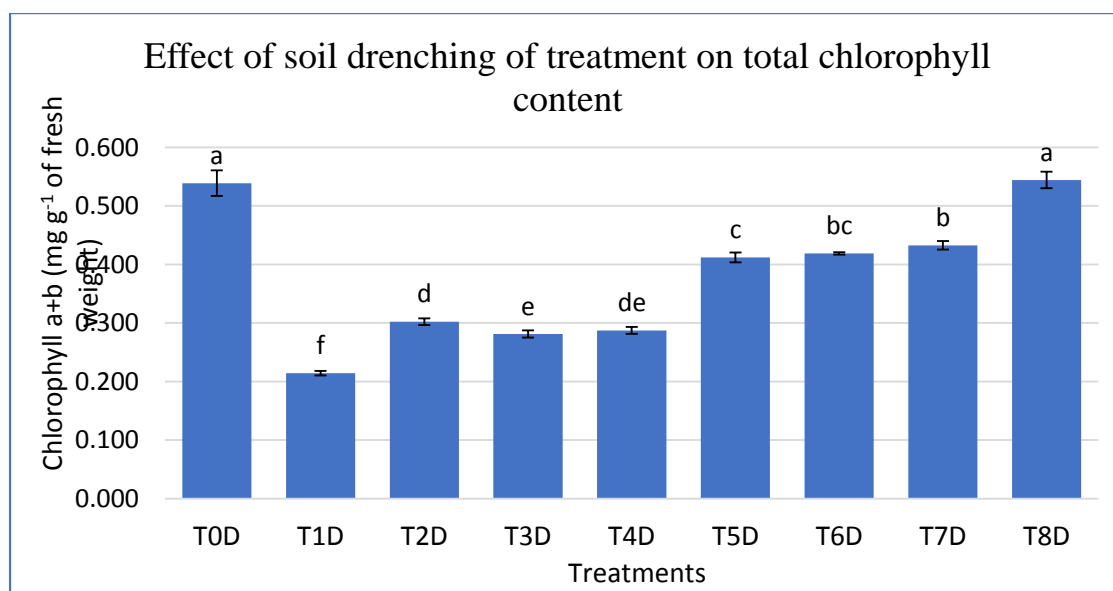


Figure. 4.16 Effect of soil drenching of different treatments on Total chlorophyll content of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Table. 4.7 Comparing the effect of soil drenching application of treatments on chlorophyll-a, chlorophyll-b, chlorophyll-a+b content of cucumber plants (var. Kashi Nootan) under salt stress condition.

Treatment	Chlorophyll content estimation (mg g ⁻¹ of fresh weight)		
	Soil drenching (D)		
	Chlorophyll a	Chlorophyll b	Chlorophyll (a + b)
T0	0.307 ± 0.010 ^a	0.231 ± 0.016 ^b	0.539 ± 0.022 ^a
T1	0.152 ± 0.004 ^f	0.062 ± 0.009 ^f	0.214 ± 0.004 ^f
T2	0.195 ± 0.007 ^{de}	0.107 ± 0.009 ^d	0.302 ± 0.006 ^d
T3	0.188 ± 0.004 ^e	0.093 ± 0.006 ^{de}	0.281 ± 0.006 ^e
T4	0.204 ± 0.004 ^d	0.083 ± 0.009 ^e	0.287 ± 0.006 ^{de}
T5	0.266 ± 0.003 ^c	0.146 ± 0.007 ^c	0.412 ± 0.008 ^c
T6	0.264 ± 0.005 ^c	0.155 ± 0.007 ^c	0.419 ± 0.002 ^{bc}
T7	0.280 ± 0.014 ^b	0.153 ± 0.009 ^c	0.433 ± 0.007 ^b
T8	0.288 ± 0.003 ^b	0.256 ± 0.012 ^a	0.544 ± 0.014 ^a
CD (0.05)	0.012	0.017	0.018
SE (m)	0.004	0.006	0.006
SE (d)	0.006	0.008	0.008
C.V.	2.915	6.758	2.681

Means of 3 replications. Means in column with same superscript is not significantly differed by DMRT, ($P \leq 0.05$)

The findings of this study show that sodium chloride has a detrimental effect on all three chlorophyll (chlorophyll a, chlorophyll b and total chlorophyll) content of cucumber plant leaves. However, it appears from the data that there was an overall rise in chlorophyll content following the application of bio-stimulants as compared to salt stress alone treatment, revealing that bio-stimulants reduced the detrimental effect of salinity stress on the plants.

Stressed plants may have lower chlorophyll concentrations due to decreased chlorophyll production or increased activity of the chlorophyll-degrading enzyme, chlorophyllase (El-tayeb 2005). According to Abdul-Wahid *et al.* (2004), mungbean plants growing under salt stress have lower chlorophyll a and b concentration. Strawberry plants showed negative impacts of salt stress on chlorophyll levels (Karlidag *et al* 2009).

According to Tari *et al.* (2002), SA lessened the reduction in tomato Chl a content caused by salt stress. Liang *et al.* (2015) found melatonin as a strong treatment which dramatically decreased chlorophyll degradation in rice under salt stress condition. Zhang *et al.* (2019) discovered that *Trichoderma harzianum* root inoculation caused a significant ($P < 0.05$) rise in chlorophyll levels regardless of the severity of salt stress.

4.1.7 Estimation of proline content

In the current study, salt stress induced more proline accumulation in the leaves of all the salt-stressed plants than in the salt-unstressed plants.

Foliar application

It was observed that highest proline accumulation was recorded in T8F (0.020 $\mu\text{mol g}^{-1}$ of FW), which is combination treatment of TH (1×10^8 CFU ml^{-1}) + SA (50 mg/L) + MT (50 μM), followed by T5F (0.019 $\mu\text{mol g}^{-1}$ of FW) (Figure 4.17). T6F and T7F showed the similar proline accumulation i.e., (0.017 $\mu\text{mol g}^{-1}$ of FW), whereas lowest proline accumulation was recorded in T0F (0.010 $\mu\text{mol g}^{-1}$ of FW), which is a control without salt stress

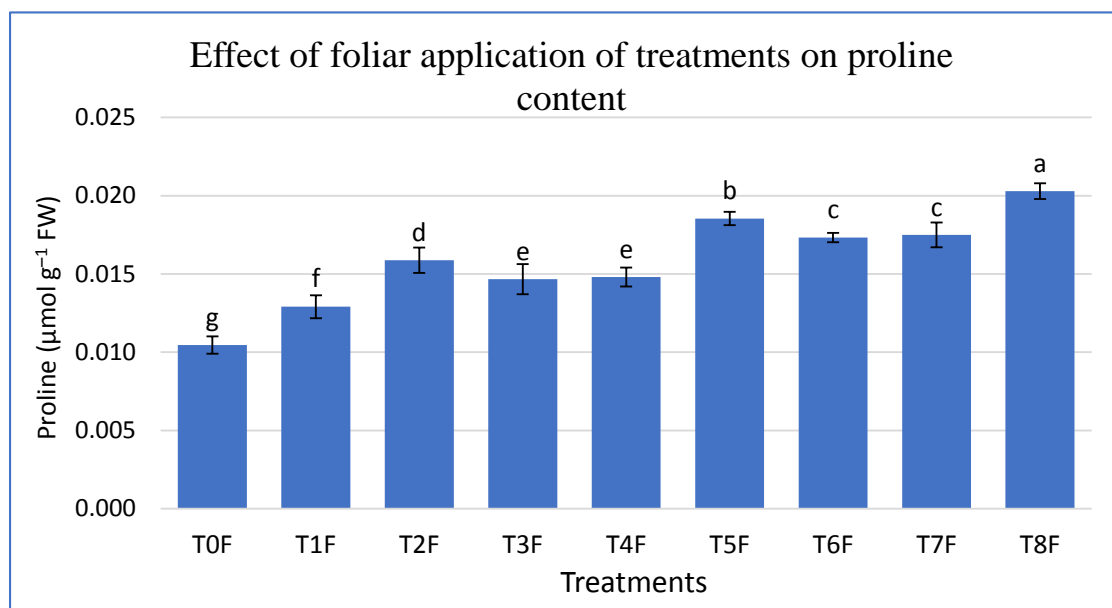


Figure. 4.17 Effect of foliar application of different treatments on Proline content of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Soil drenching

Result showing the effect of different bio-stimulants by soil drenching application on proline content in cucumber growing under salt stress condition is shown in Figure 4.18. It was recorded that T8D and T6D had similar accumulation of highest proline accumulation ($0.021 \mu\text{mol g}^{-1}$ of FW) following by T5D and T7D ($0.018 \mu\text{mol g}^{-1}$ of FW) which had similar accumulation as well. Lowest proline accumulation was found in T0D ($0.011 \mu\text{mol g}^{-1}$ of FW).

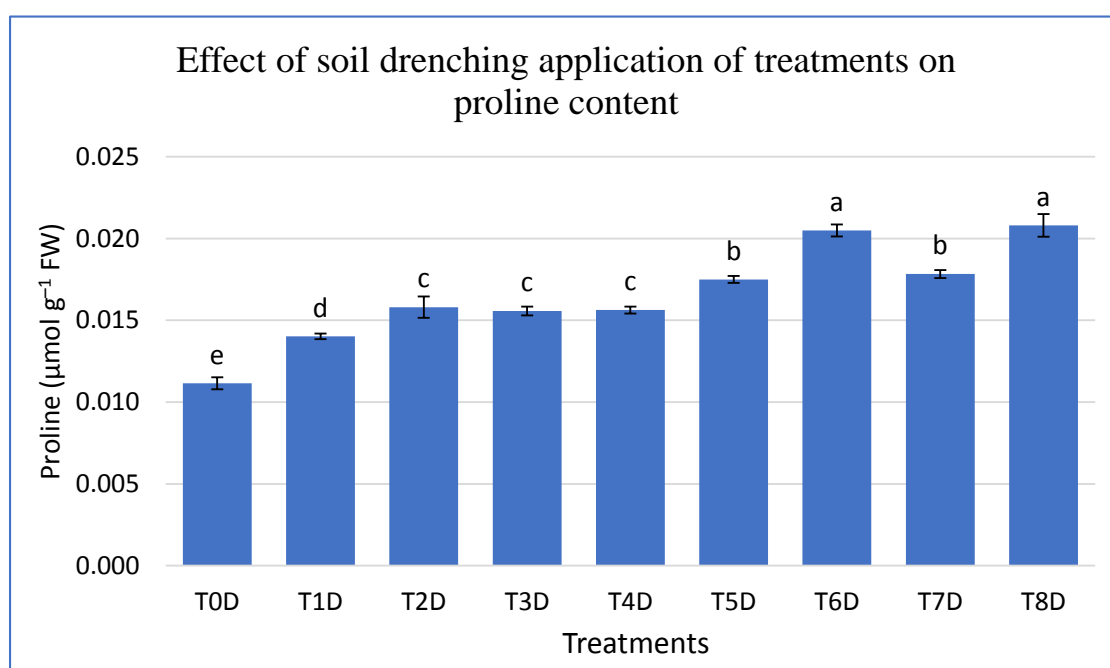


Figure. 4.18 Effect of soil drenching of different treatments on Proline content of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Table. 4.8 Comparing the effect of foliar spraying and soil drenching of treatments on proline content of cucumber plants (var. Kashi Nootan) under salt stress condition.

Treatment	Proline content estimation ($\mu\text{mol g}^{-1}$ of fresh weight)	
	Foliar spraying (F)	Soil drenching (D)
T0	0.011 ± 0.0005^g	0.011 ± 0.0004^e
T1	0.013 ± 0.0007^f	0.014 ± 0.0002^d
T2	0.016 ± 0.0008^d	0.016 ± 0.0007^c
T3	0.015 ± 0.0010^e	0.016 ± 0.0003^c
T4	0.015 ± 0.0006^e	0.016 ± 0.0002^c
T5	0.019 ± 0.0004^b	0.018 ± 0.0002^b
T6	0.017 ± 0.0003^c	0.021 ± 0.0004^a
T7	0.017 ± 0.0008^c	0.018 ± 0.0002^b
T8	0.020 ± 0.0005^a	0.021 ± 0.0007^a
CD (0.05)	0.001	0.001
SE (m)	0.000	0.000
SE (d)	0.001	0.000
C.V.	4.856	2.450

Means of 3 replications. Means in column with same superscript is not significantly differed by DMRT, ($P \leq 0.05$)

Proline is widely thought to be a physiologically appropriate solute required to keep the cell and its surrounds at a favorable osmotic potential (Pollard and Wyn 1979). In addition to its function as an osmolyte, proline can promote membrane stability and protect enzymes in a variety of situations (Stoeva and Kaymakanova 2008).

Govindaraju and Balkrishanan (2002) have also documented an increase in rice leaf proline concentration in saline conditions. Under salt stress, wheat seedlings accumulated a considerable quantity of proline, which was enhanced when salicylic acid was treated exogenously, hence effectively mitigating the negative effects of salinity (Shakirova *et al* 2003). Kaur (2011) showed an increase in proline content caused by SA in mashbean plants grown in saline environments. Ahmad *et al.* (2015) investigated that proline levels increased with NaCl stress, reaching a high accumulation of 59.12 % at 200 mM NaCl. The addition of *Trichoderma harzianum* resulted in an increase in proline content of 70.37 %.

4.1.8 Catalase (CAT) activity estimation

There was an increase in specific activity of catalase in leaves of cucumber cultivar (Kashi Nootan) with salt stress treatment. The foliar spraying and soil drenching applications of bio-stimulants viz. salicylic acid, melatonin, and *Trichoderma harzianum* increased their activity even more.

Foliar application

The results shown in Figure 4.19 reveal that the highest activity of catalase was found in T5F (2.88 $\mu\text{M H}_2\text{O}_2$ oxidized $\text{min}^{-1} \text{g}^{-1} \text{FW}$), which is a combination of salicylic acid and melatonin which was followed by T8F (2.81 $\mu\text{M H}_2\text{O}_2$ oxidized $\text{min}^{-1} \text{g}^{-1} \text{FW}$), and the lowest level of catalase activity was found in T0F (1.54 $\mu\text{M H}_2\text{O}_2$ oxidized $\text{min}^{-1} \text{g}^{-1} \text{FW}$), which is salt-unstressed control. However, there was slightly increased catalase activity in salt stressed untreated plants than salt unstressed control plants.

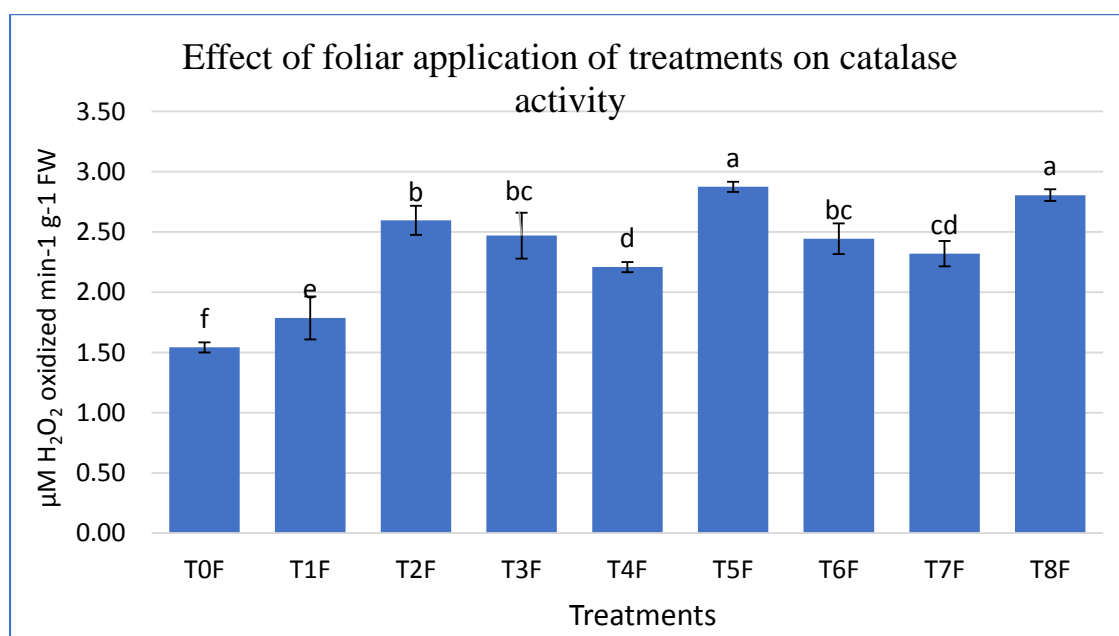


Figure. 4.19 Effect of foliar application of different treatments on Catalase activity of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Catalase activity was recorded to be higher in T6D (2.42 $\mu\text{M H}_2\text{O}_2$ oxidized $\text{min}^{-1} \text{g}^{-1} \text{FW}$) and T8D (2.32 $\mu\text{M H}_2\text{O}_2$ oxidized $\text{min}^{-1} \text{g}^{-1} \text{FW}$) treated plants when compared to untreated control T0D (1.29 $\mu\text{M H}_2\text{O}_2$ oxidized $\text{min}^{-1} \text{g}^{-1} \text{FW}$) which shows the lowest activity. An increased Catalase activity was also observed in T5D (2.24 $\mu\text{M H}_2\text{O}_2$ oxidized $\text{min}^{-1} \text{g}^{-1} \text{FW}$) and T2D (2.17 $\mu\text{M H}_2\text{O}_2$ oxidized $\text{min}^{-1} \text{g}^{-1} \text{FW}$), however there was no significant difference in between them. Detail of results is presented in the Figure. 4.20.

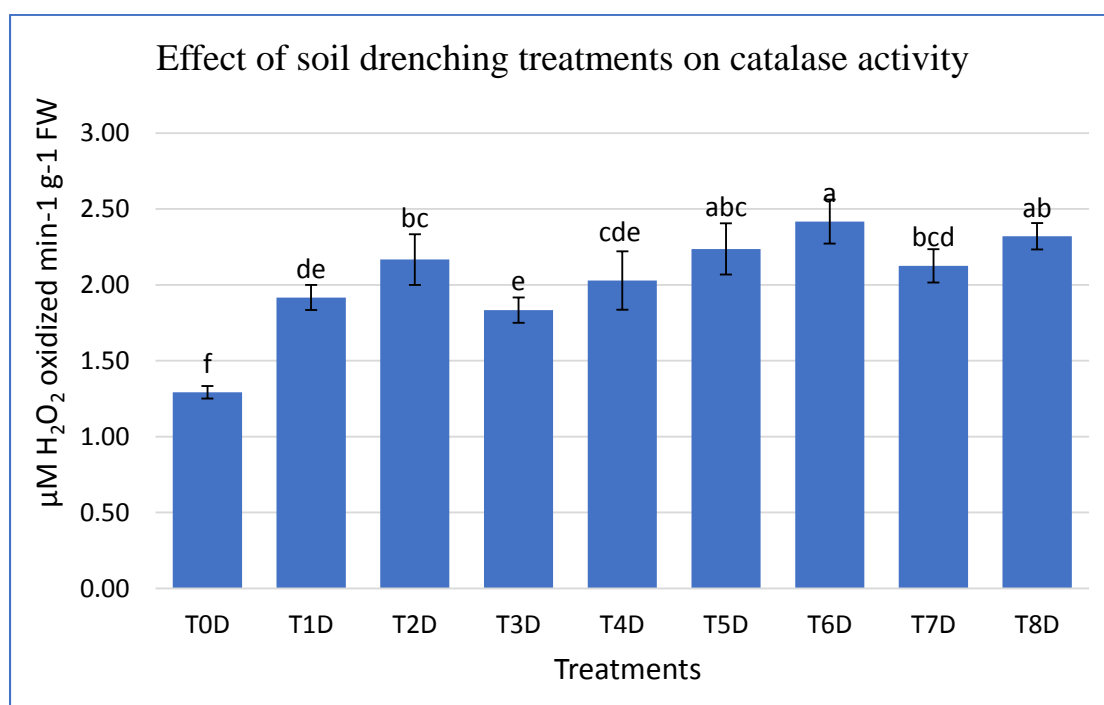


Figure. 4.20 Effect of soil drenching of different treatments on Catalase activity of cucumber plant (var. Kashi Nootan) under salt stressed condition. The results are given as means of three replicates, with vertical bars indicating standard deviation of the means. Bars with different letters indicate significant differences among treatment by Duncan's Multiple Range Test ($P \leq 0.05$).

Table. 4.9 Comparing the effect of foliar spraying and soil drenching of treatments on catalase activity of cucumber plants (var. Kashi Nootan) under salt stress condition.

Treatment	Catalase activity ($\mu\text{M H}_2\text{O}_2$ oxidized $\text{min}^{-1} \text{g}^{-1}$ FW)	
	Foliar spraying (F)	Soil drenching (D)
T0	1.540 ± 0.04^f	1.290 ± 0.04^f
T1	1.787 ± 0.18^e	1.917 ± 0.08^{de}
T2	2.600 ± 0.12^b	2.167 ± 0.17^{bc}
T3	2.470 ± 0.19^{bc}	1.833 ± 0.08^e
T4	2.210 ± 0.04^d	2.030 ± 0.19^{cde}
T5	2.877 ± 0.04^a	2.237 ± 0.17^{abc}
T6	2.443 ± 0.13^{bc}	2.417 ± 0.14^a
T7	2.320 ± 0.10^{cd}	2.123 ± 0.11^{bcd}
T8	2.803 ± 0.05^a	2.320 ± 0.09^{ab}
CD (0.05)	0.198	0.223
SE (m)	0.066	0.075
SE (d)	0.094	0.105
C.V.	4.905	6.342

Means of 3 replications. Means in column with same superscript is not significantly differed by DMRT, ($P \leq 0.05$)

Catalase is a powerful antioxidant enzyme that scavenges excess ROS and prevents oxidative damage (Feng *et al.*, 2013). It defends the cell against H_2O_2 by catalyzing its breakdown into O_2 and H_2O , which is formed in plants as a result of membrane damage (Foyer and Noctor, 2000). CAT is present mostly in peroxisomes and glyoxysomes in plants, where it primarily works to remove H_2O_2 formed during photorespiration (Geetanjali and Neera, 2008), as well as under salinity stress and other abiotic stress conditions (Willekens *et al.*, 1995). Kukreja *et al.* (2006) found that chickpea catalase activity enhanced under mild salt stress but decreased under severe salt stress. Kukreja *et al.* (2006) found that chickpea catalase activity enhanced under mild salt stress but decreased under severe salt stress. Kukreja *et al.* (2006) found that chickpea catalase activity enhanced under mild salt stress but decreased under severe salt stress.

El-Beltagi *et al.* (2017) noted that foliar salicylic acid (200 ppm) application further boosted the activity of these enzymes in stressed cotton plants compared to control plants with increasing salt levels. Manish (2018) also noted a rise in catalase activity following the foliar application of SA under salt stress condition. Wang *et al.* (2016) reported that Melatonin administration decreased the oxidative damage during salt stress by actively scavenging H₂O₂ or increasing the activity of antioxidant enzymes like catalase. Mei *et al.* (2019) reported that inoculation with the 3 *Trichoderma* strains, *T. asperellum* 525, *T. harzianum* 610, and *T. pseudokoningii* significantly enhanced the activities of numerous stress-resistance enzymes, including catalase.

4.2. Bioassay of *Trichoderma* isolates against different fungal pathogens

4.2.1 Evaluation of *Trichoderma harzianum* for antagonism against pathogens

Dual culture of *T. harzianum* with the different fungal pathogens showed the significant antagonistic activity recording the highest mycelial growth inhibition (38%) against *Bipolaris sorokiniana*, which was followed by 36 % against the *Phomopsis vexans* on 4th days of co-incubation at BOD temperature (27⁰ C). Lowest mycelial growth inhibition (14%) was observed against *Fusarium oxysporum* f.sp. *lycopersici*.

4.2.2 Evaluation of *Trichoderma viride* for antagonism against pathogens

The antifungal efficacy of *T. viride* against the different fungal pathogen by using dual culture technique reveals that highest mycelial growth inhibition (MGI) percentage was observed against the *Curvularia oryzae* recording 36% of MGI, which is followed by 32% MGI against the *Bipolaris sorokiniana* on 4th days of co-incubation at 27⁰ C BOD temperature. Whereas, lowest mycelial growth inhibition (MGI) of 10% was observed against *Fusarium oxysporum* f.sp. *lycopersici* under similar incubation conditions.

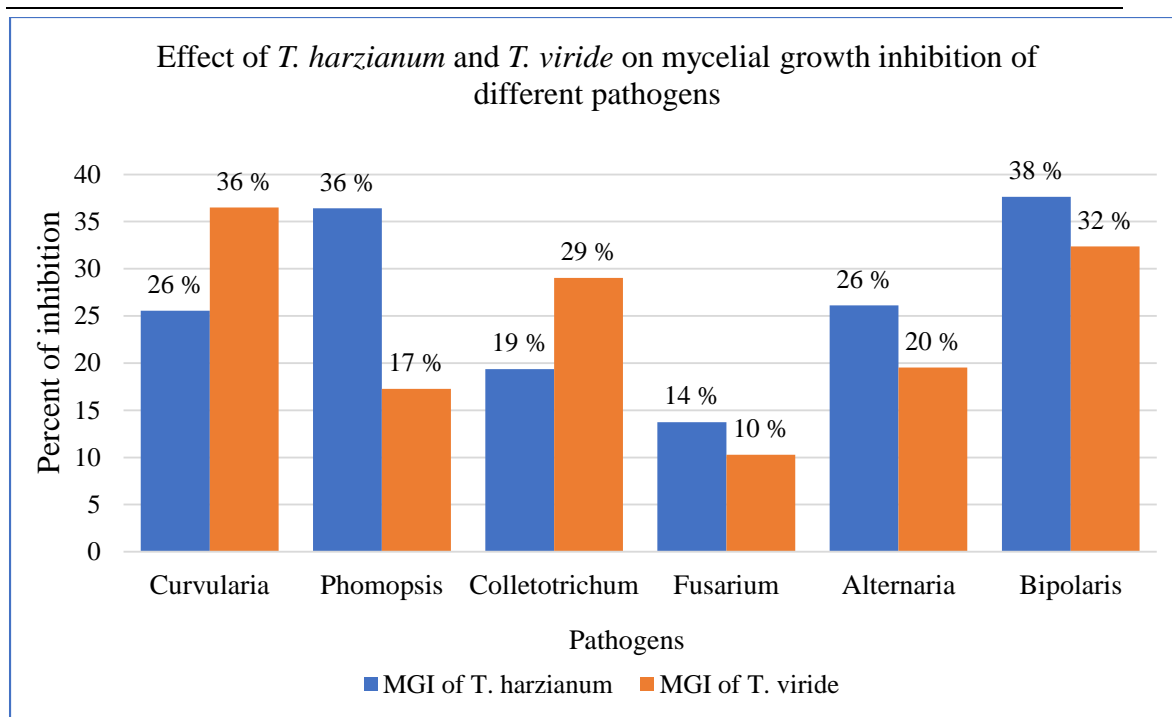


Figure 4.21 Effect of *Trichoderma harzianum* and *T. viride* on the mycelial growth inhibition (MGI) of different fungal pathogens on 4th days of co-incubation period at BOD temperature (27^o C).

Both of the antagonist *Trichoderma harzianum* and *T. viride* Pers. showed the potentials to suppress the radial colony growth of tested pathogens under laboratory condition. In both control and dual cultures, *Trichoderma* isolates grew faster than all other pathogens tested. In addition to producing mycotoxins, *Trichoderma's* intense growth habit offers it a significant advantage over pathogens as they compete for nutrients and space. According to the current *in-vitro* study of biocontrol agents, *Trichoderma* isolates significantly inhibited the radial growth of tested pathogens on the fourth day of incubation. Competition and/or antibiosis were likely the causes of these bio-agents' inhibitory action. According to numerous researchers' results, the antagonistic behavior of *Trichoderma* isolates seen in the current experiments is similar to their findings (Biles and Hill, 1988; Ramachandra, 2000).

Trichoderma's strategy for pathogen control may involve targeting and binding harmful organisms via sugar linkage then secreting extracellular protease and lipase (Cal *et al.*, 2004). *Trichoderma* sp. grows over pathogenic fungal hyphae, coils around them, and destroys the cell walls. This parasitic action limits the growth and functions of pathogenic fungi. Furthermore, or in combination with mycoparasitism, certain *Trichoderma* species produce antibiotics (Harman, 1996).

SUMMARY AND CONCLUSION

Cucumber (*Cucumis sativus* L., 2n=14) is a member of the Cucurbitaceae family. It is a warm-season vegetable that grows in tropical and subtropical climates all over the world. Cucumber has a significant economic impact because global cucumber production in 2019 was over 87.8 million tonnes, whereas India produced 1.9 lakh tonnes, making this crop important for commercial farming (FAOSTAT Statistical Database 2021). Agriculture is seriously threatened by abiotic factors such as chemical toxicity, drought, salinity, and extremely high or low temperatures. Salinity has a negative impact on growth, development and survival of plant. Around one-third of the world's arable land is thought to be impacted by salinity and has a detrimental effect on soil fertility. Similarly, biotic stress produced by variety of living creatures such as fungi, bacteria, viruses, parasitic nematodes, insects, weeds, etc. that caused harm to crops. Plants must have mechanisms for stress avoidance and tolerance in order to compensate for yield losses brought on by biotic and abiotic stresses. Salicylic acid (SA), melatonin (MT), and *Trichoderma* spp. are important bio-stimulants that play an essential role in the response to these biotic and abiotic stresses.

In view of this, the present study entitled “**Effect of bio-stimulants and *Trichoderma* spp. on biochemical parameters of cucumber (*Cucumis sativus* L.) grown under salt stressed condition**” was conducted with the objective to assess the effect of foliar spray and soil drenching application of SA, MT and TH on cucumber under induced salt stress condition. The *in vitro* experiment was carried out in the Plant Health Clinic lab and DST-FIST lab of Department of Mycology & Plant Pathology, Institute of Agricultural Sciences (IAS), Banaras Hindu University (BHU), Varanasi, Uttar Pradesh, India. Whereas the *in vivo* experiment was conducted in the vegetable research farm of the same institute. Cucumber cultivar Kashi Nootan was used for the study. The crop was induced with salt stress through soil application of 100 mM NaCl solution at 3 days interval after 28 DAS. SA, MT, and TH was applied as foliar spray and soil drenching at the concentration of 50 mg/L, 50 µM, and 1×10⁸ CFU ml⁻¹

respectively. The experiment was carried out in a complete randomized design (CRD) with 9 treatments and 3 replications. At 14 DAT, leaf samples were collected, and observations were made to investigate the physiological and biochemical parameters. The results of the experiments are summarized below.

- ❖ When compared to bio-stimulants untreated plants that were either challenged or not challenged with salinity stress, total phenol content (TPC) buildup in plants treated with bio-stimulants was significantly higher. In both of the soil drenching and foliar spray assays, minute amounts of phenols were also found in salinity unchallenged controls (T0F & T0D). Plants treated with all three combinations of bio-stimulants showed 2.61- and 2.12-times higher TPC activity in foliar spray (T8F) and soil drenching (T8D) treatments respectively as compared to salt-unchallenged control (T0F & T0D).
- ❖ All bio-stimulant treated plants either individual or in combination application had significantly higher phenylalanine ammonia lyase (PAL) levels, which were followed by a fall in activity in the salinity-unchallenged control plants (T0F & T0D). Foliar spray and soil drenching assays of all three bio-stimulants treatment exhibited 1.59- and 1.64-times greater PAL activity respectively as compared to salt-unchallenged control.
- ❖ The three-bio-stimulants consortium among both of the foliar spray (T8F) and soil drenching (T8D) assays exhibited 2.01- and 1.75-times higher peroxidase (PO) activity respectively in comparison to their respective salinity-challenged and -unchallenged controls and when compared to all other treatments, its level was significantly higher.
- ❖ A similar trend in polyphenol oxidase (PPO) activity was observed in all bio-stimulant treated plants in both of the application methods when compared to their salinity challenged and unchallenged control counterparts. However, the highest activity of PPO was reported in foliar spray rather than soil drenching assay. The three-bio stimulant mixture recorded 2.30- and 1.9-fold greater PPO activity in foliar spray and soil drenching assays respectively when compared with salinity unchallenged control (T0F & T0D).

- ❖ In the presence of salinity, the protein content of cucumber leaves significantly decreased. The lowest level of protein was recorded in salt stressed control plants (T1F & T1D), whereas highest level was observed in salt unstressed control (T0F & T0D) plants. However, the protein content in salt-stressed plants was boosted by foliar spraying and soil drenching with bio-stimulants. The three-bio stimulant combination recorded 1.43- and 1.47-fold higher protein content in foliar spraying and soil drenching assays respectively when compared with salt stressed control (T1F & T1D).
- ❖ A similar pattern was observed in chlorophyll content (chlorophyll-a, chlorophyll-b and chlorophyll-total). The chlorophyll content of cucumber leaves significantly decreased under salt stressed condition. The lowest level of chlorophyll (a, b, and total) was recorded in salt stressed control plants (T1F & T1D). It was recorded that bio-stimulant treated plants in both of the foliar spraying and soil drenching application helped to reduce the impact of salt stress by increasing the chlorophyll content in bio-stimulants treated plants under salt stress as compared to salt stressed control plants (T1F & T1D). The three-bio stimulant combination (T8F & T8D) recorded increased chlorophyll (a, b and total) content in both of the foliar spraying and soil drenching assays as compared to salt stressed control plants.
- ❖ The three-bio-stimulants consortium among both of the foliar spray (T8F) and soil drenching (T8D) assays exhibited 1.94- and 1.86-times higher proline content respectively in comparison to their respective salinity unchallenged controls and when compared to all other treatments, its level was significantly higher.
- ❖ Catalase activities were significantly enhanced by application of bio-stimulants compared to the salt unchallenged control. In foliar spraying application, T5F and T8F showed the higher activity compared to others but there was not significant difference between them. Whereas in soil drenching, combination of *T. harzianum* and salicylic acid (T6D) showed the higher catalase activity.
- ❖ In dual culture assay, *T. harzianum* showed the higher antagonistic activity against the different tested pathogens as compared to the *T. viride*.

Based on the findings of this study, it can be concluded that:

Salt stress affected the physio-biochemical parameters of cucumber plant. The ameliorating effect was observed after foliar spraying and soil drenching application of bio-stimulants based on the enhanced accumulation of protein and chlorophyll, further increase in antioxidative activities. There were strong synergistic effects between all three bio-stimulants viz. salicylic acid, melatonin and *Trichoderma harzianum* and their combined application showed significant results as compared to their dual combination and individual application.

Hence, this study suggested that combined application of salicylic acid, melatonin, and *Trichoderma harzianum* at the concentration of 50 mg/L, 50 µM, and 1×10^8 CFU ml⁻¹ respectively were efficient in alleviating the salinity effect in the cucumber genotype (Kashi Nootan) at pot condition, and that this may be further utilized in natural field conditions.

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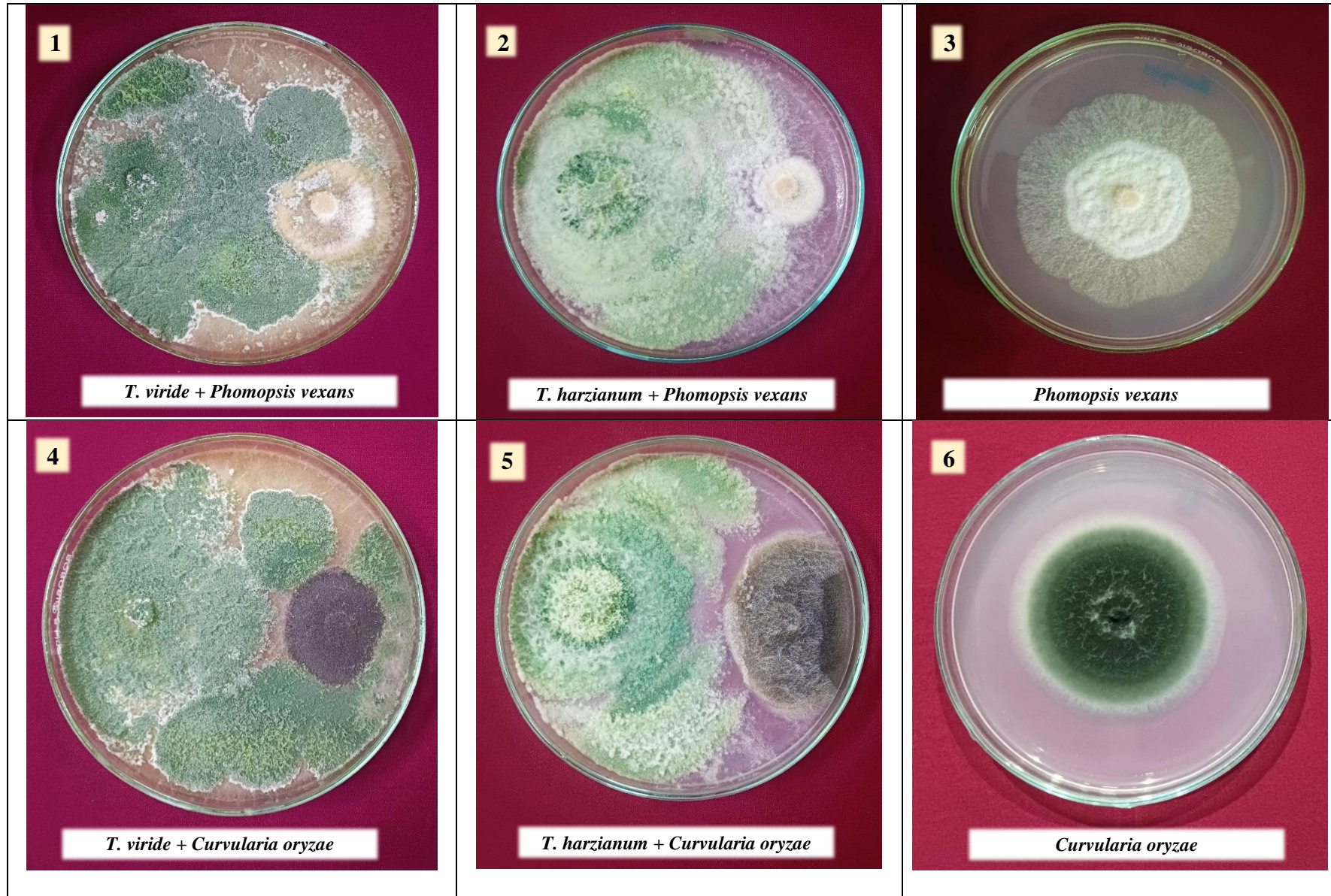
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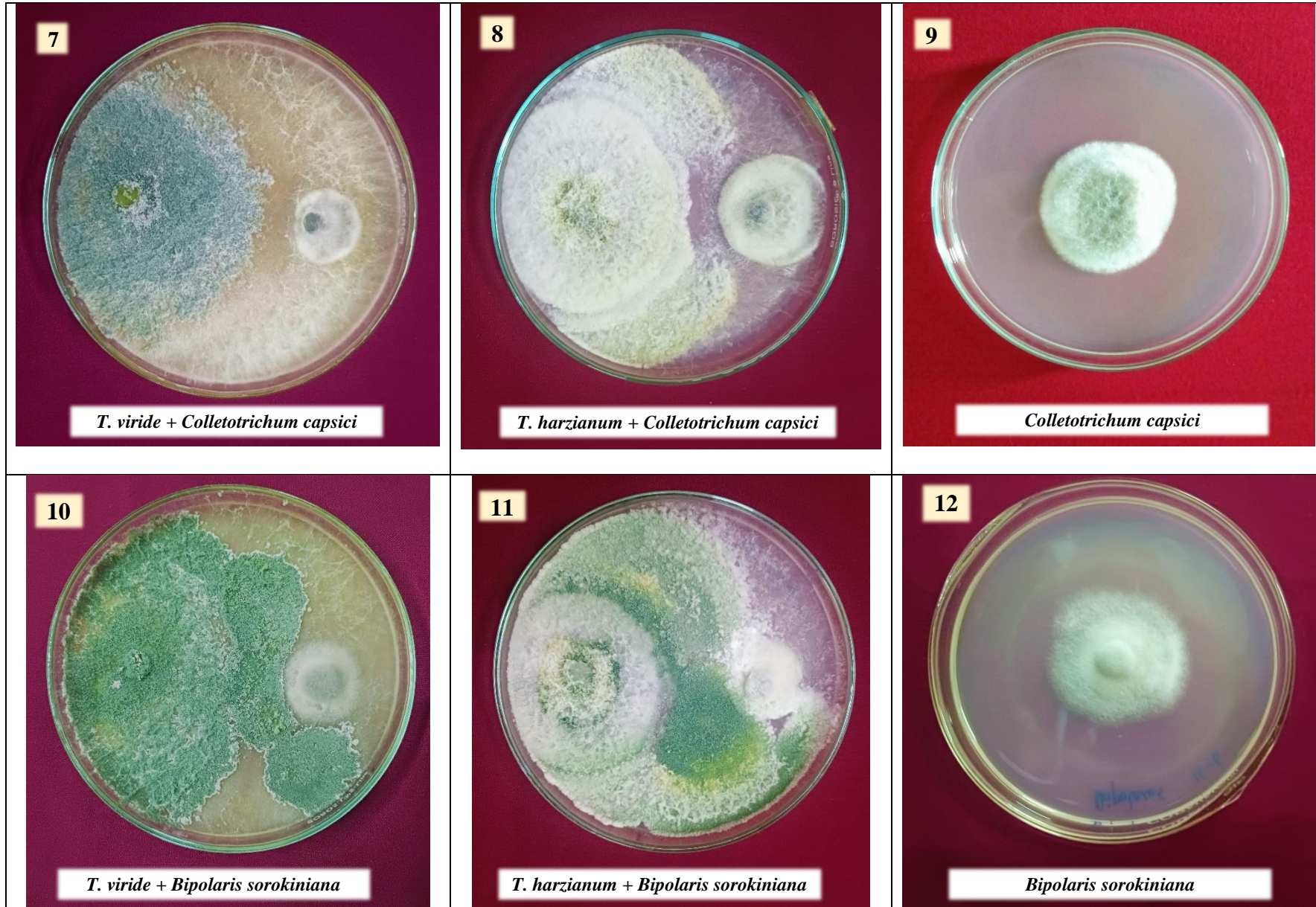
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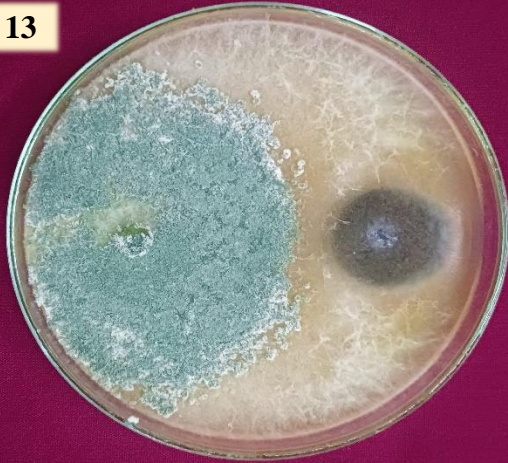


Dual culture assay of *Trichoderma viride* and *Trichoderma harzianum* against *Phomopsis vexans* and *Curvularia oryzae* on 4th days after incubation at 27^o C temperature.



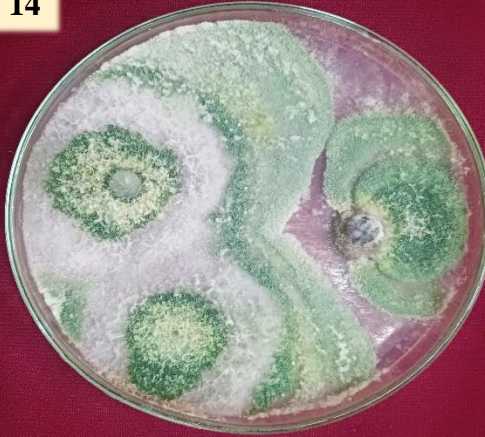
Dual culture assay of *Trichoderma viride* and *Trichoderma harzianum* against *Colletotrichum capsici* and *Bipolaris sorokiniana* on 4th days after incubation at 27^o C temperature.

13



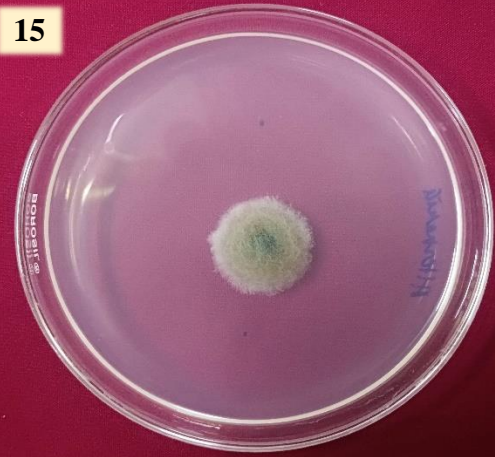
T. viride + *Alternaria solani*

14



T. harzianum + *Alternaria solani*

15



Alternaria solani

16



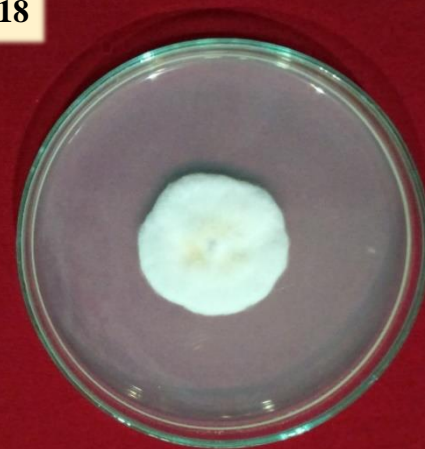
T. viride + *Fusarium oxysporum* f.sp.
lycopersici

17



T. harzianum + *Fusarium oxysporum*
f.sp. *lycopersici*

18



Fusarium oxysporum f.sp.
lycopersici

Dual culture assay of *Trichoderma viride* and *Trichoderma harzianum* against *Alternaria solani* and *Fusarium oxysporum* f.sp. *lycopersici* on 4th days after incubation at 27^o C temperature.