IDENTIFYING OXIDATIVE STRESS MARKERS TO CONTROL FUSARIUM WILT AND DAMPING OFF IN TOMATO THROUGH INTERACTION OF TRICHODERMA SP. AND JASMONIC ACID

Thesis Submitted for the Award of the Degree of

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in

Botany

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DECLARATION

I, hereby declared that the presented work in the thesis entitled "Identifying Oxidative Stress Markers to Control Fusarium Wilt and damping off in tomato through interaction of *Trichoderma* sp. and Jasmonic Acid" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of Dr. Sarvjeet Kukreja, working as Associate Professor of Agronomy Department, School of Agriculture of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of another investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Identifying Oxidative Stress Markers to Control Fusarium Wilt and damping off in tomato through interaction of *Trichoderma* sp. and Jasmonic Acid" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Botany/ Bioengineering and Biosciences, is a research work carried out by Monika Sood, 11816033, is bonafide record of her original work carried out under our supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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Abstract

To meet the needs of a projected global population of 9.1 billion by 2050, agricultural food production must increase by at least 70%. This challenge is further worsened by biotic and abiotic stresses driven by several factors. Biotic challenges include pathogenic fungi, bacteria, nematodes, herbivores etc., and among these, fungal pathogens cause significant yield losses to several economically important crops. Current agricultural practices rely heavily on chemical fungicides, particularly for vegetable and ornamental crops, which pose numerous health and environmental risks, produce resistant varieties of pathogens and cause harm to beneficial organisms in the soil. Biopesticides offer a sustainable alternative over these chemicals, in enhancing disease resistance in affected plants. The present study aimed to examine the seed priming effects of *Trichoderma virens* and Jasmonic acid (JA) on tomato plants infected with *Fusarium oxysporum lycopersici* (Fol) and *Rhizoctonia solani*, which cause Fusarium wilt and damping-off, respectively. The experiment was conducted at Lovely Professional University by utilizing the Punjab Ratta tomato cultivar, and followed a Random Block Design with twelve treatments, each replicated three times. Results indicate that seed priming with these ameliorative agents significantly improved growth parameters, reduced disease incidence, and enhanced physiochemical and molecular responses. Treated plants exhibited better photosynthesis, higher pigment levels, improved stomatal behaviour, and increased accumulation of protective compounds like osmolytes, secondary metabolites and antioxidants. The enhanced activity of the antioxidative enzymes and reduction in the oxidative stress markers indicate improved defence system in the presence of biostimulants. The combined pre-treatment of *T. virens* and JA effectively reduced pathogen-induced damage and promoted plant growth. Thus, combined application of *T. virens* and JA offers a cost-effective, environmentally friendly strategy to improve tomato crop yield and resilience against the studied soil-borne pathogens. This approach can be recommended for commercial-scale tomato production, providing a sustainable alternative to chemical fungicides.

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"Every great accomplishment starts with the decision to begin and the commitment to see it through to the end."

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Monika Sood

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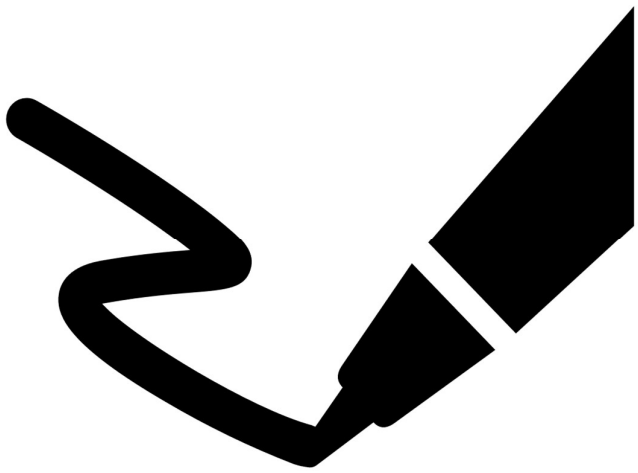


Chapter 1 Introduction

In recent years an upsurge in global temperature has initiated significant alterations in several agricultural approaches. During their entire life cycle, crop plants have been exposed to numerous types of abiotic (temperature extreme, salt and drought stress, heavy metals induced toxicity etc.) and biotic (viz. virus, bacteria, nematodes, fungi, herbivores etc.) challenges. Furthermore, an increase in the levels of stresses has forced an immediate adverse outcome on overall output through an increased vulnerability of crops to pathogens and pests (Mahalingam, 2015; Ramegowda and Kumar, 2015; Umar et al., 2021). In addition, the problems of climatical alterations have been documented to trigger terrible pandemics around the globe. Consequently, the climate change influences have worsened the magnitude and consequences of biotic stresses inflicted by plant pathogens and ultimately raised the hazard and expenses of controlling plant diseases (Raza et al., 2019).

These climatic conditions have harshly changed the physiological characteristics and the disease-resistance system of host plants (Velásquez et al., 2018). In addition, severe changes in climatic conditions have modulated the host-pathogen communications, virulence strategies of pathogens and epidemiology of mycological ailments (Abdullah et al., 2017; Pontes et al., 2020). Global productivity of crop plants has been limited by several plant pathogens which ultimately has caused a severe threat to worldwide food security. Moreover, the population of phytopathogens is exceptionally adaptable regarding space, time and genetic constitution. Consequently, advancing new pathogenic races over existing host defensive mechanisms has raised the issues of agroeconomic production very drastically (Fones et al., 2020). In this regard, it is crucial to identify the problem of crop loss due to various kinds of plant pathogens and propose novel and practical approaches to combat these destructive pathogens.

1.1 A brief introduction to the experimental host plant

Tomato (*Solanum lycopersicum* L.) is a rabi crop that belongs to the Solanaceae family. This important horticultural vegetable crop is widely grown and popularly known as “poor man’s orange” in India and “Love of Apple” in England (Gatahi, 2020). Several scientific reports about cultivated tomatoes suggest that they originated from a wild cultivar of the Peru-Ecuador- Bolivia area of the Andes (Rick and Fobes, 1975). Fruits of tomato can be eaten either fresh or cooked. Tomato is

mainly consumed in sauce, soups, ketchup, stews, and canned tomato. Food products of this herbaceous crop are rich sources of several minerals (Ca, Fe, P, K, Mg etc.), Vitamins (A and C), fibres and rich quality plant proteins (Chaudhary et al., 2018). A vital carotenoid, lycopene in tomatoes, has been proven to hold a range of pharmacological and dietary properties with favourable health advantages as a biological antioxidant (Arballo et al., 2021). These redox-homeostasis characteristics of lycopene have been proven to be implicated in carcinogenesis and atherogenesis by shielding LDL, DNA and lipids in the cell (Kelkel et al., 2011; Bin-Jumah et al., 2022). In addition to all its valuable perspectives, this crop attracts great interest from scientists and researchers because it represents a model plant system to study resistance mechanisms in plants against pathogens at the molecular level (Abdelkhalek et al., 2022; Lin et al., 2014).

1.2 Origin of the Problem

Tomato is cultivated all over the world because of its wide range of adaptability as well as versatility. Climatic regions which support this crop chiefly include tropical, subtropical, and slightly cold zones and cover an area of approximately 5,051,983 hectares 9,05,000 hectares with 186.821 million metric tons of annual production and usual yield of 37.1 metric tonnes/hectare (MT/H) in 2020. Worldwide (total fresh) tomato production exceeded 187 million Tonnes in 2020 - Tomato News, 2022. Despite all these, the growth and productivity of this essential horticultural crop are limited due to various biotic and abiotic stresses. Above all, diseases are the most common limiting factors in tomato production. It is susceptible to being infected by different kinds of pathogens viz. viral, bacterial, nematodes, fungal etc. In contrast to others, fungal pathogens are responsible to a greater extent for lowering the quantity as well as the market value of this commercial crop (Singh et al., 2017).

Fusarium oxysporum f.sp. *lycopersici* (*Fol*) is a cosmopolitan, soil-borne, hemi biotrophic, fungal pathogen. Depending upon the structure of conidiogenous hyphae, different spp. of *Fusarium* has been classified under Hyphomycetidae and the subclass of Deuteromycetes (Ashwathi et al., 2017). In general, most wilt-inducing *Fusarium* belongs to sp. *F. oxysporum*. Furthermore, different kinds of host plants have been attacked and diseased by various special forms (*formae speciales*) of *F. oxysporum* (Edel-Hermann and Lecomte, 2019). Infection with this pathogen leads to water loss and wilting of tomato plants by directly clogging their xylem vessels.

Being a soil-borne pathogen, *Fol* persists in the soil as resting propagules known as chlamydospores. After successful invasion into the cortical cells, fungi migrate intercellularly towards the vascular tissue and enter the xylem. Inside these vessels, pathogens initiate the production of microconidia, which further spread the infection in upward vessels through migration with sap streams. The distinctive wilt indications appear because of obstruction prompted by the congregation of pathogen hyphae and a blend of host-pathogen interfaces such as secretion of toxins (fusaric acid, dehydrofusaric acid, lycomarasmin etc.) and development of gums, gels and tyloses (Perincherry et al., 2019).

Rhizoctonia solani Kuhn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is a cosmopolitan, soil-borne, necrotrophic pathogenic fungus responsible for inducing infection and lowering the productivity of many commercially significant crops (Gondal et al., 2019). *R. solani* is an imperfect stage of the fungus *Thanatephorus cucumeris,* which belongs to the class Basidiomycetes and order Homenomycetales (Senapati et al., 2022). Moreover, members of this species have been distributed in somatically incompatible 14 anastomosis groups (AGs) based on characteristics like morphology, pathogenicity, molecular and biochemical markers and aggressiveness. This fungus is responsible for causing damping off disease in seedlings and very young plants. Subsequently, it also spreads infection in the roots or near collar regions, from where tiny red or brown colour spots of dead tissues seem to emerge. With the further expansion of disease, the root system of the diseased plant also becomes brown, and chlorosis is observed in the upper parts. In addition, cellulose and lignin decomposing enzymes of the pathogen further assist in the distant transfer of the pathogen (Clocchiatti et al., 2021). This pathogen persists in the soil inside contaminated plant material through mycelia or thick-walled sclerotia during unfavourable settings for numerous years (Gondal et al., 2019).

Based on the above facts and substantial losses observed in the tomato crop by the mentioned pathogens it becomes imperative to find suitable ecofriendly methods to manage the pathogen mediated economic loss. Therefore, in this research work, we have studied the effects of two diseases, i.e., fusarium wilt and damping off, on infected tomato plants in terms of growth characteristics and biochemical and molecular alterations.

1.3 Limitations of chemical fungicides for disease management

Even though presently a broad range of chemical pesticides are accessible to handle plant diseases, the incessant and injudicious application of these fungicides not only alters the nutritional innards of tomatoes but also affects the consistency, efficiency and overall health of soil (Meena et al., 2020). Moreover, these fungicides cannot be considered long-term remedies against pathogenic fungi because of health and environmental concerns. Furthermore, regular application of these chemicals not only mutates and establishes tolerance in target organisms, but their continuous application introduces several adverse effects in numerous beneficial organisms in the rhizosphere. Nowadays, restrictions on the use of these chemicals have been increasing progressively. In recent times, agriculturists and business-related sectors have shown intense concern and determination concerning the generation of sustainable and cost-effectual approaches for disease control (Panth et al., 2020). Biological control procedures are intended as substantial remedies for control since fungicides negatively affect other beneficial organisms, too (Köhl et al., 2019).

1.4 Biological control for management of phytopathogens induced disease

In recent years, the essential need for safe and chemical free food has increased the demand and market for biopesticides. Moreover, in sustainable agriculture, biopesticides serve as an eco-friendly approach by minimising the application of fertilizers and other harmful chemicals (Fenibo et al., 2021). The efficiency of specific biochemical and bio-control factors in eliciting induced resistance in infected plants against several biotic challenges has been successfully reported. Pre-treatment of a susceptible host with either a biocontrol agent (biotic inducer) or a chemical compound individually or together ensures an enhanced tolerance towards a wide variety of pathogens not only at the application site but also at distant locations. Subsequently, the expression of many defence-linked genes has been upregulated following the treatment with biotic and abiotic resistance inducers (Lahlali et al., 2022).

Trichoderma spp. are free-living, fast colonizers, intrusive, filamentous, adaptable, avirulent, plant symbiotic fungi which assist the plant in having better growth and metabolism in pathogen-inoculated soil by inhibiting the growth of pathogens through several mechanisms of antagonisms (Sood et al., 2020). There are several reports which confirmed the point that microorganisms growing in their

vicinity cause the inhibition of the growth of pathogenic spp. by upsetting their metabolisms. Around 90% of fungi used as biocontrol mediators against several biotic stresses in plants belong to the genus *Trichoderma* (Hermosa et al., 2012). Around the world, more than 60% of successful bio-fungicides are derived from *Trichoderma*-founded innovations (Abbey et al., 2018). In our country too, about 250 *Trichoderma*-isolated products are applied as bio fungicides. However, our farmers still rely on artificial chemical fungicides to a more considerable extent than biological control (Dinesh and Prateeksha, 2015; Mishra et al., 2020).

During exposure to stressful stimuli, plants respond to them by operating a brilliant communication network of chemical messengers known as the hormonal signal transduction pathway. A unique characteristic of this communication system is that it can communicate over long distances through signal detecting, transmitting and responding to cells and even organelles of different tissues and organs (Jung et al., 2018). It has been commonly acknowledged that pathogen-induced attacks on the host plant led to the generation of specific Microbe/Pathogen Associated Molecular Patterns (MAPS/PAMS). Subsequently, these are identified by cell membrane-situated PRRs. Following pathogen attack and successful recognition by specific PRR, JA biosynthesis is initiated through the oxylipin biosynthesis pathway (Gfeller et al., 2010; Zhang et al., 2017). Under the conditions of pathogen contamination, JA and its derivatives can be instantly coming into effect (Fragoso et al., 2014), which consequently induces nearly all chief secondary metabolites and protein expression implicated in defence reaction, comprising of phenylpropane, alkaloids, anti-nutritional proteins, terpenoids, amino acid derivatives, and some pathogen-related proteins (Wang et al., 2021).

1.5 Oxidative stress markers

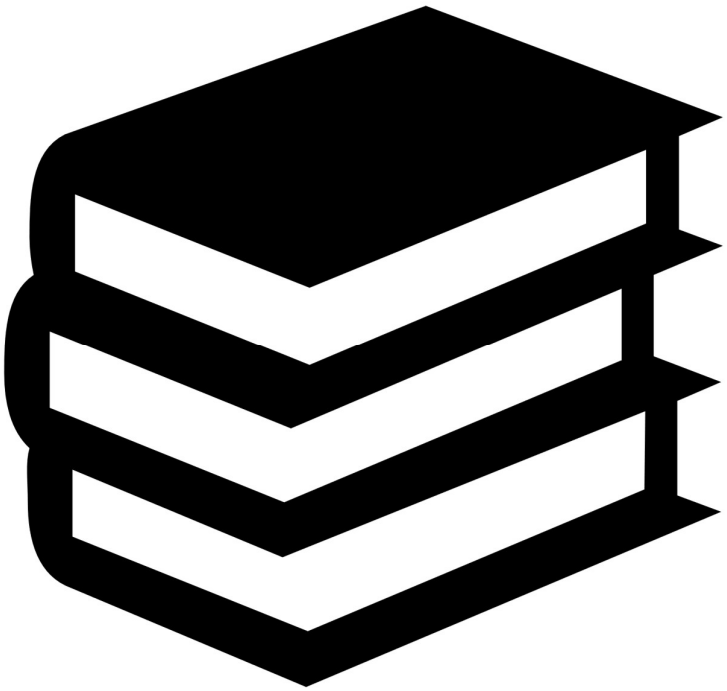
Following exposure to a virulent pathogen, a susceptible host plant develops several resistance mechanisms to either exclude or overcome partially or entirely the damage convinced by the pathogen attack (Sood et al., 2021). Resistance approaches in diseased plants limit pathogens' growth, multiplication and spread or confine the pathogen to necrotic lesions by hypersensitive responses. Subsequently, the generation of ROS like OH^{-}, O^{2-} and H_2O_2 because of pathogenic attack serves as a primary indication of early ameliorating response against these devastating diseases (Juan et al., 2021). These ROS, once generated through the various biochemical pathways, are extremely toxic and induce impairment to lipids, proteins,

carbohydrates and nucleic acid at the cellular level. Furthermore, these ROS also act as critical regulators of various biological and physiological processes associated with mitigating several types of biotic or abiotic stresses in plants by indirectly functioning as a second messenger in cellular signalling (Sachdev et al., 2021).

In addition to this, intermediates associated with ROS initiate defence-related responses in plants through a surfeit of mechanistic activities, e.g., strengthening of plant cell wall through the cross-linking of lignin and proteins, advancement in systemic acquired resistance (SAR), an acquaintance of hypersensitive response (HR) against the targeted organisms. ROS enhance the expression of numerous defence-associated genes by acting as second emissaries. Their gene products mainly include peroxidase (POD) and polyphenol oxidase, whose catalysis leads to lignin formation in plants. Similarly, phenyl ammonia lyase produced cause the synthesis of phytoalexins and phenolic compounds (Nita and Grzybowski, 2016). Furthermore, due to the antifungal activity of some PR-proteins such as chitinase and glucanase, oligomers such as chitin and glucan are produced because of fungal cell wall degradation, which further provokes the numerous defence systems by acting as powerful elicitors. In general, plants own very competent enzymatic and non-enzymatic, alkaloids, phenolic compounds as well as non-proteinaceous amino acids] antioxidative defensive systems which act in collaboration to regulate the problems of unrestrained oxidation and ultimately shield the plant cells from oxidants injury by scavenging these toxic ROS (Sharma et al., 2022).

Even though till now several reports have documented the initiation of disease resistance mechanisms in plants through the pre-treatments with bioagent fungi or chemical inducers on their own, their combined efficiency in amelioration of fungal-induced biotic stress in plants has been explored to a much lesser extent. Thus, the present study aimed to investigate the impact of pre-treatment of bioagents, i.e., *T. virens* and jasmonic acid, as an individual or integrated remedy on the damping off and fusarium wilt of tomato plants. Therefore, the chief objective behind this research work focussed upon the alteration in growth and physiological activities comprising of enzymatic/non-enzymatic defence systems and expressions of defence-related genes of treated as well as untreated diseased tomato leaves.

Chapter 2
Review of Literature



Chapter 2 Review of Literature

Plants persistently face a wide range of stresses which limits their productivity. Stress can be well defined as a stimulus that is outside the acceptable range of homeostatic limit in a living being (Gull et al., 2019). These environmental stresses to which plants have been exposed can broadly categorize as 1) Abiotic and 2) Biotic stress. Exposure of plants to these circumstances induces a disturbance in plant metabolism, leading to a decline in growth and, eventually, productivity (Gull et al., 2019). Abiotic stresses involve salinity, extremes in temperature, radiation, flood, drought, heavy metals toxicity, etc. (Yadav et al., 2020). Conversely, biotic stress includes pathogenic attacks by fungi, bacteria, nematodes, and herbivores (Iqbal et al., 2021). These pathogens cause several plant diseases responsible for significant crop yield loss globally. Being a sessile organism, the plant must face these environmental signals. To complete their life cycle, plants display stress tolerance or avoidance through mechanisms like acclimation and adaptation that ultimately initiate cellular or organismal homeostasis (Lamalakshmi Devi et al., 2017).

Disease-instigating pathogens are responsible for roughly a 15% decline in global food production. It has been estimated that around 800 million persons worldwide do not have access to enough food (Strange and Scott, 2005). Because of the population explosion, the adequate amount of food needed to feed people sufficiently are increasing. Food assurance occurs when people forever have a physical and economic approach to sound, safe and healthful food that greets their eating supplies for an active and healthy life. In several developing countries, the statistics of hungry, undernourished people who don't have diet security are increasing alarmingly. Therefore, in the changing world, there is a critical requirement to refer the serious risks to food security triggered by plant diseases on a privileged basis to allow constant access to enough nutritious food (Strange and Scott, 2005). The food scarcity caused pathogens is an essential factor to consider carefully. It is estimated that almost 20 to 40% of agricultural yield is lost globally due to multiple types of biotic challenges (van Dam and Bouwmeester, 2016; Junaid and Gokce, 2024). Further, global warming and climate change increase the reproductive capacity and geographic extension of these phytopathogens which eventually causes the production of excessively virulent and aggressive pathogens which negatively interact with a wide range of host plant species; and consequently, the yield destruction will be the uppermost consequence of this

phenomenon (Das et al., 2016; Lahlali et al., 2024). Hence, we can say that in future, the probability of plants confronting exposure to biotic/ abiotic stress will be of elevated magnitude with repeating stress incidences.

1. Host-Pathogen Interaction

A virulent pathogen is a living organism that exhibits the potential to induce damage in susceptible hosts during plant-pathogen interaction. Further, the virtue with which a pathogen ruins the metabolism of its host plant is termed its pathogenicity. After successful penetration and establishment in diseased plants or tissue, it spreads the infection into the healthy area of plants by suppressing the immunity of the host plant. Based on their disease initiation mechanisms, pathogens can be broadly categorized into opportunistic, facultative, and obligate domains. Obligate pathogens communicate an infection inside a healthy, susceptible host species (Pandit et al., 2022; van Baarlen et al., 2007). In addition to contaminating within a confined host series, the facultative ones can survive exterior to the host in a non-living environment. Contrary to both, opportunistic pathogens express small virulence for many host species. When host species become immunologically vulnerable, opportunistic pathogens hit the host plant vigorously (Cornelis and Dingemans, 2013). Further, based on the metabolic relationship with their host plants, pathogens have been grouped into biotrophs and necrotrophs (Priyashantha et al., 2023; Wang et al., 2014). The first requires a living host for their growth as well as reproduction. On the other hand, by forming destructive enzymes and toxic substances, necrotrophic ones cause metabolic injury and death of the infected plants. In addition to studying the host-pathogen interface during a particular disease, a third component that should also be considered is the 'environment.' These three factors together establish a 'disease triangle', as for the occurrence of a successful infection, all the environmental conditions must be conducive to the growth and proliferation of the pathogen (Dixon et al., 2003; Sood et al., 2021). Subsequently, a chain of relatively linked events in a series that gives a precise idea about the induction and establishment of disease because of pathogenic activities is mentioned to as the 'disease cycle', which demonstrates a clear idea about the development and establishment of an ailment as an affair of pathogen action (van Baarlen et al., 2007).

2. Consequences of pathogen-induced damage in plants

Owing to pathogen infections, annual yields of several economically important food crops have been reported to be diminished by the regular occurrences of different plant diseases. Considering that food crops affected by pathogen-induced destruction can feed millions of people, the socio-economic influences of these phytopathogens on plants should not be misjudged (de Vos et al., 2005). Therefore, for better control of plant illnesses, an organised and wide-ranging interpretation of the destructive effects of pathogens on plants is obvious. After the invasion and successful establishment into their respective host, metabolisms of both the pathogen and the host plant become tightly interconnected. This mainly causes severe nutrient loss from the plant side for the better growth and proliferation of pathogens (de Vos et al., 2005; Sood et al., 2021). Upon challenges imposed by the pathogen or its derived elicitors, several critical metabolic pathways in the diseased plants are down regulated, while others essential in plants' defence are upregulated (Less et al., 2011; Riseh et al., 2024). For example, metabolic pathways associated with energy production like pentose phosphate pathway, glycolysis, Kerb's cycle, Electron transport Chain of mitochondria, biosynthesis of Adenosine triphosphates (ATPs), synthesis of energy generating amino acids like lysine and methionine and photorespiration linked amino acids such as arginine, serine, glutamic acid, and glycine were reported to be upregulated. On the other hand, assimilatory metabolic processes like photosynthesis, lipid and starch metabolism, and biosynthesis of specific amino acids such as valine, leucine and isoleucine are observed to be down-regulated because of pathogen-induced damages in plants (Less et al., 2011).

The severity of the disease symptoms the infected plant expresses depends on how much its physiology has been altered because of the pathogen attack. For example, interference in photosynthesis by a virulent pathogen was depicted as chlorosis (yellowing) and necrosis (browning as death) of leaves and stems in infected host plants. Even a slight reduction in the magnitude of photosynthesis not only impairs the plant but also increases its susceptibility to many pests and pathogens (Nazarov et al., 2020). In addition, pathogenic invasion also leads to disturbances in the availability and translocation of minerals and water via the vascular system of the host plants. An adequate nutrient supply ensures optimal plant growth and is also needed for better resistance and tolerance against pathogens. Several mineral nutrients

are used to build strong mechanical barriers and biosynthesis of defensive compounds like antioxidants, phytoalexins, flavonoids, etc., providing protection against pathogens (Mitter et al., 2021). The presence of pathogens affects the water and mineral uptake by affecting transpiration from aerial plant tissues or influencing their absorption from the affected roots. Subsequently, slow translocation of these ultimately causes wilting, chlorosis, and probably necrosis in diseased plants (Fig. 2.1).

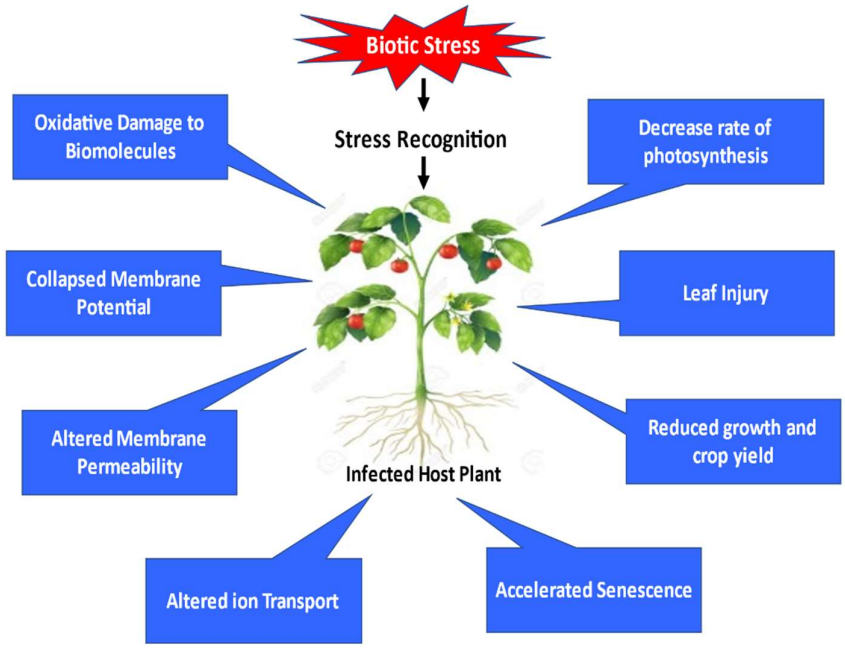


Fig. 2.1 Depicts the discrete effects of biotic stress on infected plants

Furthermore, change in the permeability of cellular and organellar membranes is another important outcome of pathogen-induced damage in plants. This detectable response causes the loss of several physiologically essential electrolytes, especially Ca^{2+} and K^{+} from the infected tissues. In addition, most invading fungi release toxic substances that directly affect the permeability of diseased plant cells (Tsuge et al., 2013). For instance, in oat leaves, a cyclic peptide termed victorin is produced by *Helminthosporium victoriae,* which alters the membrane permeability by binding to its proteins and subsequently leads to the development of chlorosis and necrotic stripes on leaves (Kessler, 2020). The general retort of plants to most types of stresses includes an increase in respiration rate. In pathogen-induced injured host tissue, an

increase in respiration is often observed. Aftereffects of the intensified oxygen uptake and increased activity of respiratory enzymes induced an insignificant elevation in temperature, deposition of metabolites across infection points, and even an enhancement in the host tissue dry weight (Hasanuzzaman et al., 2020).

Exposure of plants to unique kinds of challenges leads to the assembly of ROS. An inconsistency in ROS production and subsequent disposal through enzymatic and non-enzymatic responses results in oxidative stress in diseased plants. Besides acting as a stress indicator, ROS are served as a signalling molecule during pathogen defence reactions such as HR, SAR, stress hormones synthesis and accumulation, PCD and acclimation (Kaur et al., 2022). Elevation in the levels of oxidising molecules at the time of oxidative stress disturbs the functioning of biological systems by extracting electrons from essential organic molecules and ultimately leads to cell damage and death by ROS toxicity.

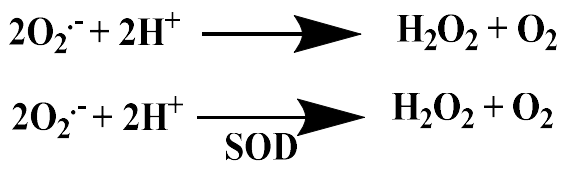
After successful pathogen recognition, several enzymes, particularly plasma membrane-located oxidase and cell wall peroxidase, have been reported to be participating in the apoplastic production of ROS. The NADPH oxidase, also termed respiratory burst oxidase (RBO), is a multiunit complex that allocates electrons to molecular oxygen and generates superoxide radicals in infected plants (Arthikala et al., 2017). In addition, peroxidase catalyses the oxidation and reduction of the substrate by using hydrogen peroxide. Following pathogen interaction, the expression of these enzymes is reported to be upregulated. As reported in the case of French beans in the vicinity of pathogenic bacteria, both H_2O_2 and cationic peroxidase coexisted together (Bestwick et al., 1998). Although consequent to pathogen recognition, the apoplast is the primary site for ROS generation; other cellular compartments may also be associated with forming defence-related ROS (Dumanović et al., 2021).

The most common types of ROS mainly comprise $^1O_2$, $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ (Fig. 2.2). Molecular O_2 in the ground state remains inactive and paramagnetic due to occurrence of two parallel spined unpaired electrons. Its activation mainly occurs through two mechanisms: 1) absorption of an efficient amount of energy to reverse the spin of one of the paired electrons (Khorobrykh et al., 2020). This leads to the production of $^1O_2$. 2) step-by-step monovalent reduction of molecular oxygen causes

the formation of $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$. Moreover, in the presence of light, $^1O_2$ can be formed through ^3Chl synthesis in the PS II reaction centre. This triplet Chl produced can further react with $^3O_2$ to produce a highly damaging ROS, i.e., $^1O_2$.

In addition, the limited availability of CO_2 due to the closure of stomata at the time of different kinds of stresses initiates the formation of $^1O_2$. This ROS causes the oxidation of unsaturated fatty acids, proteins and DNA. Furthermore, it alters the nucleic acid composition by reacting selectively with deoxyguanosine (Kasai, 1997; Ma and Bai, 2021). Consequently, by promoting light-inducing damage to photosystem II, it triggers cell death in stressed plants (Krieger-Liszkay et al., 2008; Roeberet al., 2021). β-carotene and α-tocopherol are the agents which the plants can employ for the successful quenching of $^1O_2$ (Krieger-Liszkay, 2005; Kumar et al., 2020).

Due to spin constraints, O_2 cannot allow four electrons concurrently to produce a water molecule. However, during reduction, it takes one electron at each step and can give rise to stable intermediates at the respective stage (Halliwell and Gutteridge, 1984; Juan et al., 2021). After this, $O_2^{\bullet-}$ is the chief ROS developed, which begins a system of responses to create "secondary" ROS, either straightforwardly or usually through enzyme- or metal-catalysed progressions (Hong et al., 2024; Valko et al., 2005). Owing to both oxidising and reducing features, $O_2^{\bullet-}$ has been reported to oxidize enzymes encompassing the [4 Fe-S] groups and was competent to reduce cytochrome C. By accepting one electron and two protons, $O_2^{\bullet-}$ can be readily converted to H_2O_2 either nonenzymatically or through SOD catalysed reactions (Imlay, 2003; Mandal et al., 2023).



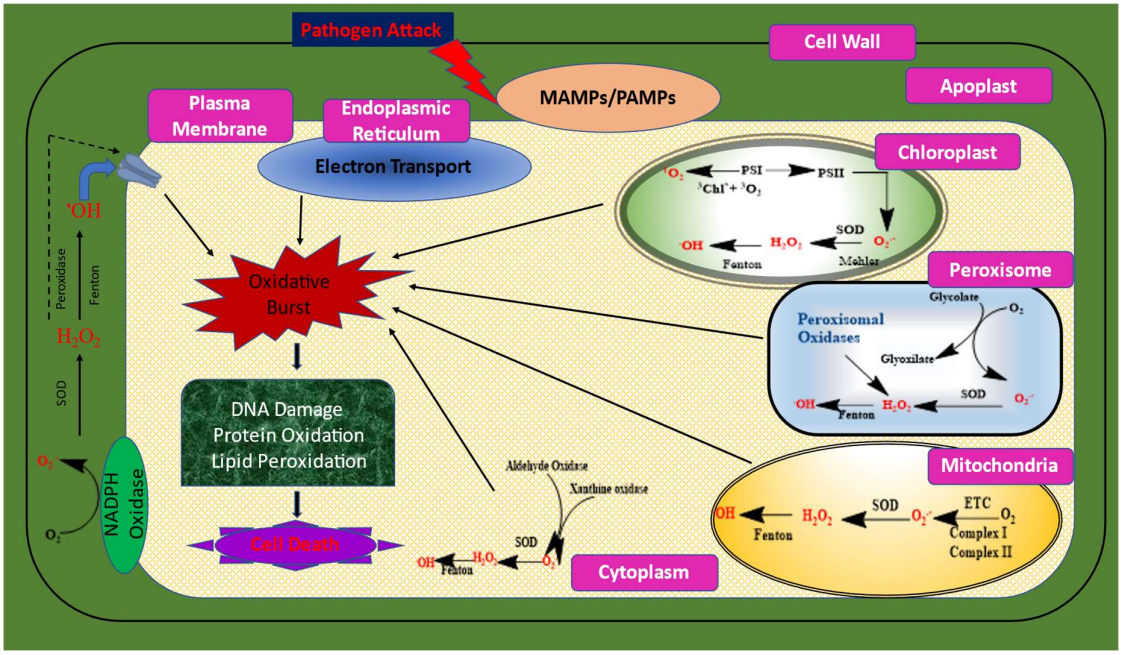


Fig. 2.2 Represent Pathogen Induced Production of ROS

Production of ROS in multiple organelles leads to oxidative damage in the respective cell following pathogenic attack on the host plant. By ETC in chloroplast at PSI and PSII, ROS like $^1O_2$, O_2•−, H_2O_2, and $^{\bullet}OH$ are produced through Chl molecules. SOD catalyses the dismutation of O_2•− via Fenton reaction. At peroxisome photorespiratory enzymes induce ETC dependent generation of O_2•− and H_2O_2. Similarly, ETC at mitochondria generate O_2•− which on dismutation through SOD forms H_2O_2. Likewise, production of ROS has also been reported in ER, Cell wall and apoplast. ROS at low or moderate concentrations mediate cellular signaling but at higher concentration cause oxidative burst and ultimately apoptosis in the infected plant cell.*

In addition to this, H_2O_2 is produced in cells both under ordinary as well as varied series of adverse circumstances. Chiefly, ETC operated in chloroplast, ER, mitochondria, and cell membrane; along with fatty acid oxidation as well as photorespiration are key foundations of H_2O_2 production in plant cells (Fig. 2.2). Reactions associated with photooxidation, NADPH oxidase and xanthine oxidase (XOD) also play a pivotal role in H_2O_2 generation in plants. Furthermore, it also acts as a substrate during biosynthetic processes such as lignification and suberization (Mittler and Zilinskas, 1992; ul Islam et al., 2023; Sharma et al., 2021). Due to a lack

of unpaired electrons, it can easily penetrate the biological membranes and accordingly trigger oxidative destruction far away from the place of its formation. Since its diffusion ability through aquaporins in the membranes and its stability in comparison to other ROS, it has been accorded special consideration as a signalling molecule participating in the control of biological progressions and stimulating easiness against numerous strains, especially during plant-pathogen interfaces even at a small concentration (Aranda-Rivera et al., 2022; Bienert et al., 2007; Neill et al., 2002; Yan et al., 2007; Torres et al., 2002). In opposite to this, at high concentrations, it can oxidize the methionine/ cysteine residues, which subsequently leads to the inactivation of enzymes like C_3 cycle enzymes, by their thiol groups oxidation (Halliwell and Gutteridge, 2015). Once the concentration of hydrogen peroxide reaches 10 μM under stress, various enzymes participating in the C_3 cycle show 50% reduction in their activity (Kaiser, 1979; Leegood and Walker, 1982). Furthermore, its accumulation also oxidizes phosphatases, protein kinases, and transcription factors comprising thiolate residues. Above all, it coordinates PCD at very elevated concentrations in stressed plants (Dat et al., 2000; Yang et al., 2019).

Among all ROS, $^{\bullet}OH$ is the most reactive species. Due to an unpaired electron, it reacts with practically all biological molecules and subsequently causes cellular damage in the form of lipid peroxidation, proteolysis, and membrane devastation (Foyer, 1997; Meitha et al., 2020). As cells lack an effective enzymatic system to eliminate $^{\bullet}OH$, its surplus accumulation can ultimately cause cell death (Kesawat et al., 2023; Pinto et al., 2003). Due to the short life span and sharply positive redox potential (~ +2 V), the site of the formation lies nearby to its place of reaction (Elstner, 1982). Furthermore, the synthesis of $^{\bullet}OH$ at the catalytic site of a large subunit of RUBISCO through the Fenton reaction causes its disintegration in chloroplast lysates (Ishida et al., 1997; Luo et al., 2002).

3. Host Plant- *Solanum lycopersicum*

Tomato belongs to the family Solanaceae, which includes more than 3000 spp. Solanaceae comprises several economically important crop plants with diverse uses, e.g., as food (potato, eggplants, peppers, tomatoes etc.), medicinal importance (henbane, datura, deadly nightshade) as well as decorative uses (Petunias). Linnaeus, in 1753 named this genus as *Solanum lycopersicon,* but after about 15 years, Philip

Miller substituted this name with *L. esculentum* (Gerszberg et al., 2015). Presently, most taxonomists and plant breeders have agreed with the restoration of these into *Solanum* (Caicedo and Schaal, 2004; Fridman et al., 2004; Mueller et al., 2005; Schauer et al., 2006; Tomato Genome Consortium, 2012). 13 closely correlated taxa have been included into the section Lycopersicon of Solanum. Cultivated tomato, i.e., *S. lycopersicum* has existed as domesticated, while 12 others have been considered wild spp. (Peralta et al., 2005, 2008; Spooner et al., 2005). Due to the exhibition of bright yellow flowers and the presence of pinnate/ pinnatifid non-spiny leaves, tomatoes and their close relatives have been differentiated from other groups of Solanum species. Tomatoes have been considered a new world crop and originated in the Andean region, comprising parts of Peru, Columbia, Ecuador, Chile and Bolivia (Rick, 1973; Taylor, 1986). The most probable ancestor of this plant is the wild cherry tomato, previously *L. esculentum* var. *cerasiforme* (Dun.) Gray. Overall, tomatoes are among the most eaten vegetables. They can be consumed fresh or processed into various eatable forms such as juice, puree, powder, flakes, soup, sauces etc.

Generally, tomato plants are branched and slightly trailing when fruiting. Leaves are hairy, odorous and pinnately compound. Flowers are five-petaled, yellow in colour, pendant and clustered. Fruits are berry and usually red, scarlet or yellow and differ in outline ranging from pear-shaped, spherical, elongate or oval. Every fruit comprises at least two cells of tiny seeds bordered by jelly-like pulp. It is a perennial diploid (2n=24) dicotyledonous plant. Tomato plants flourish well in the temp. between 10-30 $^{\circ}$C (optimum temp. 21-24 $^{\circ}$C). The plant requires low to medium rainfall and can't withstand frosting conditions. In different cellular, physiological, biochemical, molecular and genetic investigations, tomato is commonly used as a 'model crop' because of their ease of cultivation, manipulation and short life span. Furthermore, we can conclude that the tomato is a brilliant device to enhance knowledge about horticultural crops.

Despite its shorter life cycle, the yield of this critical vegetable crop has been limited due to various factors. Among these, yield destruction caused by soil-borne pathogens is the worst. Soil-borne infection complexes include *Fusarium* wilt, verticillium wilt, black dot root rot, corky root rot, root-knot nematodes, *Rhizoctonia* root rot, *Sclerotinia* white mould, and *Pythium* root rot. Moreover, soil-borne pathogens are considered difficult to handle because of a wide range of hosts and the

fact that they persist in the soil longer by developing different kinds of perennating structures. Because of the susceptibility of cultivated tomato to multiple pathogens, it has been considered a prototype to study plant-pathogen interfaces and thereby assisting in the establishment of effective control against various diseases to enhance universal production (Arie et al., 2007; Piquerezt et al., 2014; Takahashi et al., 2005).

4. Fungal Pathogen-I

***Fol* [W.C. Snyder and H.N. Hansen]** is a cosmopolitan, soil-borne, hemi biotrophic, fungal pathogen. Depending upon the structure of conidiogenous hyphae, different spp. of *Fusarium* has been classified under Hyphomycetidae and the subclass of Deuteromycetes (Ashwathi et al., 2017). In general, most wilt-inducing *Fusarium* belong to sp. *F. oxysporum*. Furthermore, different kinds of host plants have been attacked and diseased by various special forms (*formae speciales*) of *F. oxysporum* (Edel-Hermann and Lecomte, 2019). *Fol* leads to water loss and wilting of tomato plants by directly clogging the xylem vessels. Saccard, in 1886 reported this pathogen for the first time from Italy. In our country, Butler described this pathogen in 1918 (Reis et al., 2005).

Morphologically, diseased plants are identified by symptoms of wilt like epinasty of leaves, vein clearing, wilting and defoliation, which finally leads to the death of the host plant (Joshi, 2018). *Fol* persists in the soil as inactive propagules known as chlamydospores. Their germination is hastened in the soil in the presence of host plant roots. Infectious hyphal threads attach and then penetrate the root cells. After successfully invading the cortical cells, fungi migrate intercellularly towards the vascular system and enter the xylem vessels. Inside these vessels, pathogens initiate the production of microconidia, which further spreads the infection in upward vessels through migration with sap streams. The distinctive wilt indications appear because of obstruction prompted by the congregation of pathogen hyphae along with a blend of host-pathogen interfaces such as secretion of toxins (fusaric acid, dehydrofusaric acid, lycomarasmin etc.) and development of gums, gels and tyloses (Perincherry et al., 2019) (Fig. 2.3).

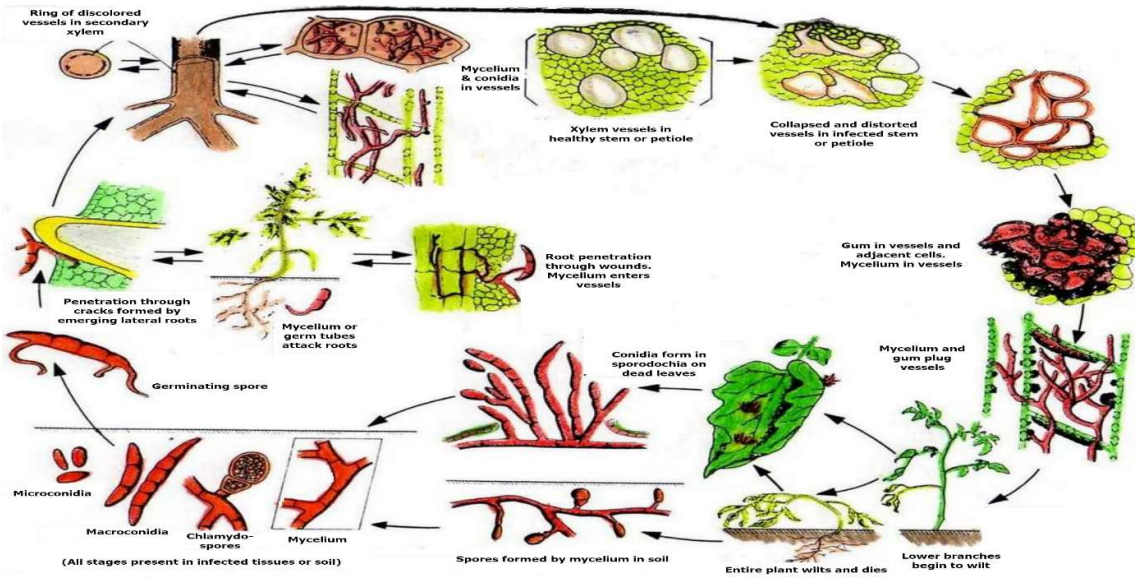


Fig. 2.3 Shows *Fol*-induced development of vascular infection in diseased tomato plants

It is a complex process and occurs in sequential steps as mentioned ahead 1) After successful root recognition through biochemical cues, infectious hyphal threads attach and penetrate the root cells. 2) After successfully invading the cortical cells, fungi migrate intercellularly towards the vascular system and enter the xylem vessels. 3) Inside these vessels, pathogens initiate the production of microconidia, which further spread the infection in upward vessels through migration with sap streams. 4) The specific wilt indications appear because of obstruction prompted by the congregation of pathogen hyphae along with a blend of host-pathogen interfaces like the secretion of toxins and development of gums, tyloses and gels (Perincherry et al., 2019). (Picture Source Plant Pathology 5th Edition; Agrios, 2004)

In pathogen-contaminated moist soil, the germ tube of *Fol* spores directly penetrates the root tips of healthy plants (Arie, 2019). Through the root cortex mycelium of fungus advances towards the xylem vessels and eventually reaches the plant's crown by travelling through the xylem vessel of the stem. In vessels, infection is further accelerated through the production of microconidia on mycelial phialides. Due to vessel clogging, water scarcity comes into existence and excessive transpiration via leaves leads to wilting due to the closure of stomatal pores and ultimately, the death of leaves and finally of the whole plant occurs (Srinivas et al., 2019) (Fig. 2.3). From the above inferences, we can conclude that this fungus causes pathological wilting in the tomato plants through a combination of various factors like presence of mycelial growth in and around the xylem vessels, synthesis and accumulation of mycotoxins, deactivation of host defensive system and above all

production of gums and tyloses. Several researchers have confirmed that *Fol*, responsible for the vascular wilt of tomatoes, lowered the crop produce to the utmost extent (Nirmaladevi et al., 2016; Asha et al., 2011). Singh et al. stated approximately 60-70 % of yield loss in wilted tomatoes with yellowed leaves (Singh et al., 2015).

In addition to crop destruction, certain *Fusarium* spp. efficiently produce mycotoxins in agricultural commodities and foodstuffs (Mudili et al., 2014; Nayaka et al., 2008, 2009). Further, toxins produced by this species are the amplest natural contaminates of cereal and grain comprising diets (Divakara et al., 2014; Venkataramana et al., 2014; Kumar, 2016; Kalagatur et al., 2015) and are supposed to cause several illnesses among humans and others (Kalagatur et al., 2017, 2018; Nayaka et al., 2010; Venkataramana et al., 2014). Several PCR-based findings demonstrate the boost in expression of fumonisin biosynthetic genes in different strains of *Fol* (Nirmaladevi et al., 2012). During pathogenesis, polygalacturonase and pectate lyase are the key enzymes that assist in the host plant's cell wall destruction (Pareek and Rajam, 2017; Guo et al., 2016). As chitin is an important constituent of the fungal cell wall, the *chs V* gene encodes the chitin synthase enzyme - which participates in membrane linked chitin production and further enhances the resistance of pathogens against plant based secondary metabolites (de Coninck et al., 2015).

5. Fungal Pathogen-II

***Rhizoctonia solani* Kuhn (teleomorph = *Thanatephorus cucumeris* (Frank) Donk)** The name of this fungus is derived from ancient Greek words i.e., 'rhiza means root' and '*ktonos* means murder.' This is a cosmopolitan, soil borne necrotrophic pathogenic fungus that is responsible for inducing infection and lowering the produce of lots of horticulturally essential crops (Gondal et al., 2019). For the first time de Candolle in 1815 has studied this genus in alfalfa and saffron. In accordance with his studies, *Rhizoctonia* exhibits two peculiar characteristics. The first one is that it produces a unique kind of sclerotium which is different from *Sclerotia* and the second is that the fungal mycelium of this pathogen makes association with the roots of host plants. In fact, *R. solani* is an imperfect stage of the fungus *Thanatephorus cucumeris* which belongs to the class Basidiomycetes and order Homenomycetales (Khosravi et al., 2011). Moreover, members of this species have been distributed in 14 anastomosis groups (AGs) based on some characteristics like morphology, pathogenicity, molecular and biochemical markers and aggressiveness. However,

these groups are somatically incompatible. In 1858 Kuhn, for the first time, observed and studied *R. solani* fungus on potato tubers. *R. solani* chiefly causes infections in the seeds below the soil line, but it can also attack the roots, leaves, pods, and stems of infected host plants. Consequently, this fungus is responsible for causing damping off disease in seedlings and very young plants. In addition, it also spreads infection in the roots or near collar regions from where tiny red or brown spots of dead tissues seem to emerge. With the further spread of infection, the root system of the diseased plant also becomes brown, and chlorosis is observed in the upper parts. In addition, cellulose and lignin decomposing enzymes of the pathogen further assist in the distant dispersal of the pathogen (Sturrock et al., 2015).

Fungal pathogens persist in the soil inside contaminated plant material in the form of mycelia or thick-walled sclerotia, even during unfavourable settings for numerous years (Gondal et al., 2019). Pathogens are attracted to their host crops through chemical stimuli released by the nearby plant roots. After successful penetration into the host cells, they initiate appressorium production, which helps them to derive nutrients from plant cells. In addition, pathogens release Cell Wall Degrading Enzymes (CWDEs) within the host cells, which further support in colonization and growth of pathogens inside the plant's dead tissues. Consequently, when new host plants are available, a new disease cycle is repeated (Xue et al., 2018) (Fig. 2.4).

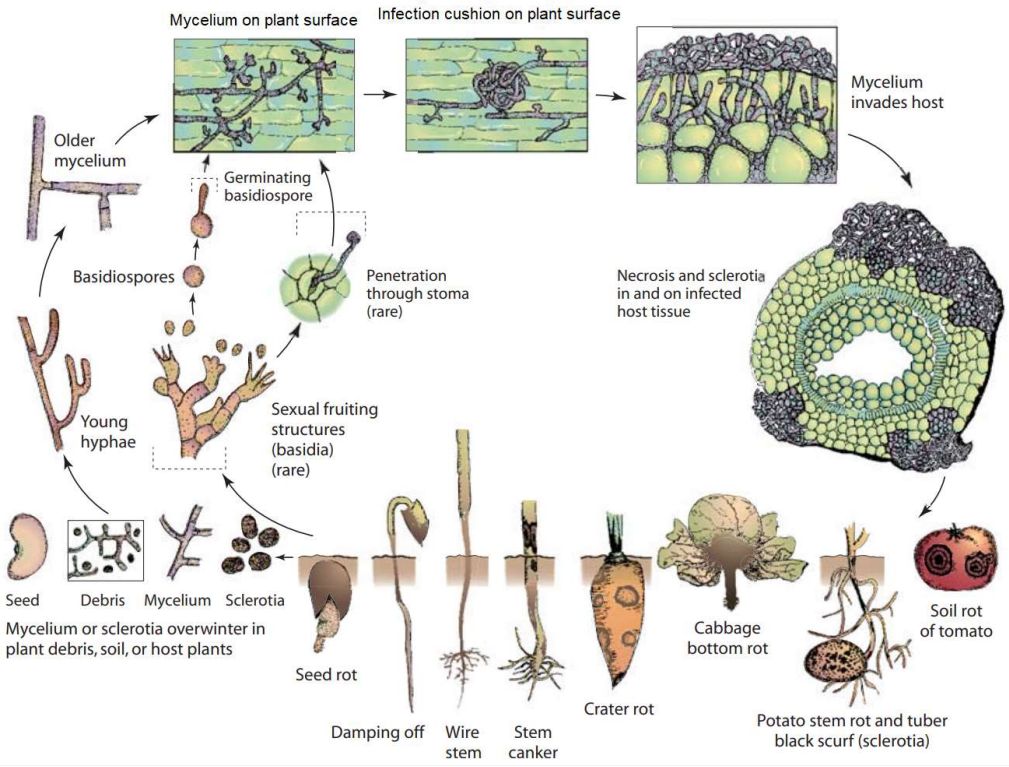


Fig. 2.4 Represents the disease cycle of *Rhizoctonia solani*

The pathogens perennate in the soil in the form of mycelia from plant debris or soil, or in the form of thick-walled sclerotia and are attracted towards the roots of host plants through chemical signals released by these plants in their vicinity. Following successful invasion and establishment into the host cell, pathogens derive nutrients from the plant cells through appressorium formation. Moreover, the synthesis of CDWE further assists in the establishment and development of mycelium into the host plant (Picture Source Plant Pathology 5th Edition; Agrios, 2004)

6. Biocontrol agents (BCA) and chemical inducers as promising ameliorating approaches in the mitigation of pathogen-generated damages in plants

As previously mentioned, plant diseases result from multifaced interactions among pathogens, host plants and the environment. The long history of agriculture signifies the development of various practices to manipulate this interaction to construct an approach which encourages the growth and advance of the host plants. Still, it simultaneously induces adverse effects on pathogens' transmission, reproduction and existence (Bisht et al., 2020). Depending upon several factors like the crop, nature of the pathogen, technology accessibility, geographic locations and many more; these approaches can be broadly classified into five categories, i.e.,

agronomic, regulative, genetic, physical and chemical. Furthermore, these technologies can be employed either individually or in combination (integrated disease management, IDM) to overcome destructive pathogens, intensify host immunity, or alter the abiotic/biotic environment where this interaction occurs (He et al., 2021). The ideology of disease-free agriculture emphasizes the applications of chemical fungicides to extreme limits, especially in the case of vegetable and ornamental crop production. Besides their high financial burden and the threat of lowering efficacy, improper and wide-scale application of these fungicides leads to several environmental risks due to their ill effects on water quality, soil, humans, and animals' health (He et al., 2021). Owing to the literature mentioned above, we can conclude that the use of biological control agent seems to be a promising alternative for disease management.

In general, plant disease management through biological control is based on the fact that these microorganisms diminish the plant pathogens by enhancing plant immunity and ensuring more remarkable survival by facilitating the environment and healthy cropping system (Poveda et al., 2020). Compared to chemical fungicides, BCA offers better advantages because they generally attack a specific type of pathogen and thereby induce fewer adverse effects on the ecosystem. Secondly, compared to chemical fungicides, these BCA can maintain themselves longer in the plant rhizosphere. In general, these BCA inhibit the constitutive expression of the disease-suppressive genes of the host and simultaneously help it to distribute more energy and assets towards the betterment of agronomic traits (Wang et al., 2018). Despite the recognized documentation of these BCA as a significant component of IDM, its commercial importance in the crop protection market is less than five per cent (Kiran Kumar et al., 2018). The top reason behind this is highly linked to the lack of technology transfer, as its economic significance is not recognized yet, especially in developing countries.

6.1 *'Trichoderma'* A multitalented Bio Control Agent

Trichoderma (teleomorph *Hypocrea)* belong to fungi imperfecti, and are filamentous, free-living, opportunistic, rapid colonizing, avirulent plant symbiotic fungi which assist the host plant in better growth and metabolism in pathogen-contaminated rhizosphere by suppressing the proliferation of pathogenic organisms through several antagonistic mechanisms (Lorito et al., 2010; Sood et al., 2020). *Trichoderma* spp. exhibited negative behaviour against pathogenic microorganisms, especially fungi - either through direct (inhibiting its growth) or indirect mechanisms (inducing disease resistance and improving plant growth) (Fig. 2.5).

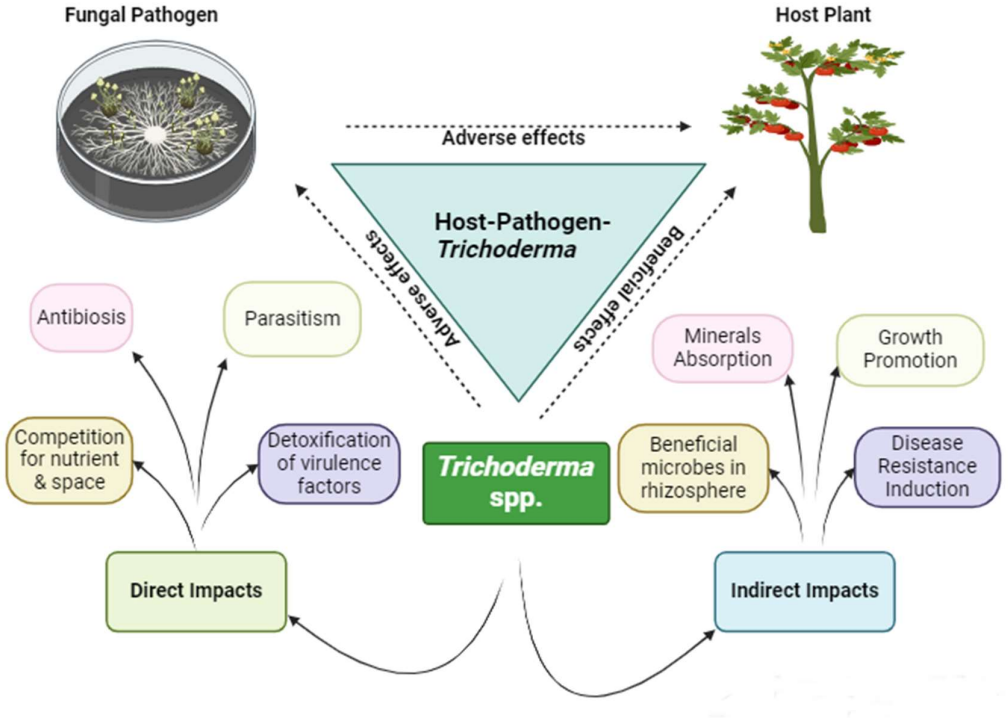


Fig. 2.5 Depicts the plant-pathogens-*Trichoderma* interaction triangle.

This interaction is a complex network of various processes. Trichoderma spp. exhibit a symbiotic relationship with plants and imparts multiple disease resistance mechanisms, better absorption of minerals and nutrients, improved development, and enhanced crop yield etc. In the pathogen-contaminated rhizosphere, Trichoderma spp. adversely affects the pathogenic organisms through some peculiar biocontrol mechanisms like antibiosis, mycoparasitism, and competition and ultimately inhibits their proliferation near the host plant.

6.1.1 Indirect Mechanisms

6.1.1.1 Antibiosis

Antibiosis is a phenomenon by which low-molecular weight composites interact and decrease the development of other harmful microorganisms. Generally, it deals with the synthesis of approximately 180 kinds of secondary metabolites belonging to different classes of chemical compounds associated with this genus (Hu et al., 2016; Sood et al., 2020.). These antibiotics assist the different spp. of *Trichoderma* to inhibit/reduce the growth of a wide range of soil-borne pathogens like *Fusarium, Rhizoctonia, Phytophthora, Pythium, Sclerotinia, Verticillium* etc. (Abdel-Mageed Zaher et al., 2013; Ragab et al., 2015; Yao et al., 2023). Numerous volatile and non-volatile antibiotics generated by different strains of *Trichoderma* were described to be antagonistic against a wide selection of pathogenic fungi (Nagamani et al., 2017; Tyśkiewicz et al., 2022) (Table 2.1). However, it has been monitored that the combined cooperative activity of antibiotics and lytic enzymes express antagonistic activity to a greater extent than their individual performance (Monte, 2001; Guzmán-Guzmán et al., 2023). For instance, lytic enzymes catalyse the disintegration of the cell wall of *Botrytis cineria* and *F. oxysporum,* which leads to enhanced diffusion of antibiotics into the targeted mycelium (Howell, 2003).

Table 2.1 List of useful compounds produced by *Trichoderma* spp. engaged in plant-*Trichoderma*-pathogen interaction.

S. No.	Category	Function performed	Reference
1	**Phytohormones**	Better growth, improved metabolism, enhancement in tolerance against different kinds of biotic and abiotic stress through upregulation of expression of defensive genes.	Contreras-Cornejo et al., 2009; Guzmán-Guzmán et al., 2019; Pieterse et al., 2012; Ruocco et al., 2015; Seyfferth and Tsuda, 2014; Wang et al.,

			2002; Yoshioka et al., 2012
1.	**Enzymes**		
ACC synthase ACC oxidase		Raise biosynthesis of ethylene	Polko and Kieber, 2019
Antioxidative enzymes		Improve antioxidative defense	Mastouri et al., 2012; Zhang et al., 2016
ETR1 and CTR1		Ethylene signaling	Shoresh et al., 2005
Exopeptidases, Endopeptidases		Cause the cleaving of peptide bonds	Flores et al., 1997; Goldman and Goldman, 1998
Glucan and Chitin synthases		Protect and repair *Trichoderma* cell wall during interaction with pathogen	Ribeiro et al., 2019
Glucanase, Glucosidases, Xylanase, Chitinases.		Catalyse the breakdown of their substrate, i.e., complex polysaccharides like cellulose, chitin, xylans etc., to their respective monomers	Harmanet et al., 1993; Kim et al., 2002; Li et al., 2011; Lorito et al., 1993; Okada et al., 1998; Peterbauer et al., 1996; Sandgren et al., 2001; Strakowska et al., 2014; Vázquez-

			Garcidueñas et al., 1998; Viterbo et al., 2002; Wong and Saddler, 1992
Glucose oxidase		Produce ROS	Contreras-Cornejo et al., 2014; Djonović et al., 2006; Gupta et al., 2014
Lipase		Lipase hydrolyses ester bonds of triacylglycerols.	Bhale and Rajkonda, 2012
LOX1 (Lipoxygenase 1) PAL1 (phenylalanine ammonia lyase),		Participate in jasmonic acid (JA) and SA biosynthetic pathways.	Sharma et al., 2020
MAPK		Convey signals from receptors to initiate signaling cascade and defense responses.	Jagodzik et al., 2018; Shoresh et al., 2005
PAL and CHS		Produce phytoalexins	Ahluwalia et al., 2015
δ-cadinene synthase		Serve as a precursor for synthesis of phytoalexin	Djonović et al., 2006; Yoshikuni et al., 2006
2.	**Phytohormones**	Better growth, improved metabolism, enhancement in	(Contreras-Cornejo et al., 2009; Guzmán-

		tolerance against different kinds of biotic and abiotic stress through upregulation of expression of defensive genes.	Guzmán et al., 2019; Pieterse et al., 2012; Ruocco et al., 2015; Seyfferth and Tsuda, 2014; Wang et al., 2002; Yoshioka et al., 2012)
3.	**Secondary metabolites**		
Butenolides and Hydroxy-Lactones		Antifungal	Vinale et al., 2016; Zou et al., 2019
Diketopiperazines		Antifungal	Howell, 2019
Hydrophobins		Assist in the plant growth and defense induction	(Guzman-Guzman et al., 2017; Y. Huang et al., 2015
Isocyanic compounds such as dermadin and trichoviridin		Antifungal	Pyke and Dietz, 1966
Koninginins, Trichodermamides, Viridins,		Antimicrobial	Brian and Mcgowan, 1945; Garo et al., 2003; Ghisalberti and Rowland, 1993
Lactones		Participated in IAA and Et induced signaling and	Vinale et al.,

	improvement of plant growth and root development.	2012
N-heterocyclic compounds	Antifungal	Dickinson et al., 1989; Vinale et al., 2006
Peptaibol like Alamethicin and Trichokonin VI	It is a non-ribosomal short peptide with antimicrobial nature, rich in 2-amino-IBA acid, implicated in plant protection mechanisms	Rippa et al., 2010; Shi et al., 2016
Polyketides	Exhibit antimicrobial activities by stimulating SA-mediated signaling pathways	Mukherjee et al., 2012; Pang et al., 2018
Pyrones	Antimicrobial	Cardoza et al., 2005
Terpenes	Antimicrobial	Fang et al., 2019; Liang et al., 2016, 2019
Volatile Organic Compounds	Smoothen the rhizospheric plant-microbial interactions	Cruz-Magalhães et al., 2019; Liang et al., 2019; Schenkel et al., 2015
4.	**Soil modifiers**	
Gluconic, citric and fumaric acids	Assist in the solubilization and absorption of phosphates and	Vinale et al., 2008; Zhao et al.,

	micronutrients.	2014
Siderophore	By chelating with insoluble Fe^{3+} siderophores convert them to soluble Fe^{2+}	Srivastava et al., 2018

6.1.1.2 Competition

The struggle for micro-macro nutrients like C, N and Fe is crucial during beneficial and detrimental fungi interactions and is linked with the biocontrol systems (Marra et al., 2008; Chauhan et al., 2023). It has been documented that *Trichoderma* spp. fight for nutrients and biological niches against pathogens in the rhizosphere of plants. Limited supply and competition for available nutrients induce the natural control of fungal populations. Though rhizosphere, as well as root exudates, are valuable resources of various nutrients like carbohydrates, vitamins, amino and organic acids, iron etc. but the struggle for carbon between fungal pathogens like *R. solani, F. oxysporum* etc. and different strains of *Trichoderma* was believed to be most striking (Alabouvette et al., 2009; Guzmán-Guzmán et al., 2023). Compared to other soil microbes, skilled mobilization and utilization of immobile nutrients offer superiority to *Trichoderma* species. Furthermore, different strains of *Trichoderma* produce and secrete organic acids such as fumaric, citric, gluconic etc., which decrease the pH of the soil and ease the solubilization of mineral cations like phosphates, Mn, Fe and Mg (Vinale et al., 2008; Tyśkiewicz et al., 2022). Under neutral pH and aerobic conditions, Iron primarily exist as Fe^{3+} and forms an unsolvable ferric oxide which eventually makes it unavailable for absorption by plant roots. *Trichoderma* spp. form Fe-chelating complex named siderophore. The siderophore first combines to the Fe^{3+} and then transforms it to a highly absorbable form, i.e., Fe^{2+}. In this way, *Trichoderma* depletes the Iron supplies of the soil and, by this means, reduces the growth of target fungi (Srivastava et al., 2018).

6.1.1.3 Mycoparasitism

Mycoparasitism, first reported by Weindling (Weindling, 1932), is amongst the most significant antagonistic mechanisms articulated by approximately seventy-five different spp. of genus *Trichoderma.* Their mycoparasitic activities have been

registered against many pathogenic fungi (Harwoko et al., 2021). Several serial events bring about this complex process. Firstly, cell wall carbohydrates in *Trichoderma* bind with lectins of pathogenic fungi. The hyphal whirling and formation of appressoria follow this initial recognition step. After that, a wide variety of fungitoxic CWDE like, glucanases, chitinases as well as proteases that have been synthesised and secreted by *Trichoderma* strains ease the direct entrance of their hyphae into pathogen's lumen. The CWDE and numerous fungitoxic peptaibol antibiotics produced by *Trichoderma* spp. collectively cause dissolution as well as parasitism of pathogenic fungi. Numerous genes, proteins and secondary metabolites have been reported to be associated with this activity. Besides causing cell wall degradation of target fungi, *Trichoderma* attack leads to the inactivation of its essential enzymes required for the successful penetration and colonization into the host tissues (Bargaz et al., 2018; Harman et al., 2004; Manzar et al., 2022).

6.1.2 Direct Mechanisms

Trichoderma spp. interact with the host in the rhizosphere through the distinctive structure known as MAMPs. These MAMPs engaged in the initiation of signal transduction pathways related to antimicrobial defensive compounds and enzymes. After pathogen infection and *Trichoderma* colonization, plants display a protective attitude by synthesising and accumulating antimicrobial molecules like PAL, PPO, and POX, lipoxygenase, PR Proteins, terpenoids, antioxidants, phytoalexins etc. (Dutta et al., 2023; Howell et al., 2000). Remarkably, *Trichoderma* strains are resistant to these compounds mainly due to the presence of the ABC (ATP-binding cassette) transport system. Furthermore, Mitogen-Activated Protein Kinase (MAPK) participate in the signalling process associated with plant defence by conveying signals from receptors to stimulate a cascade of plant cellular reactions (Jagodzik et al., 2018). For instance, applying *T. harzianum* in lemons intensifies the levels of SA and JA and ultimately enhances resistance against *F. oxysporum* (Martinez-Medina et al., 2010). Similarly, in the root system of *A. thaliana* colonized by two spp. of *Trichoderma* i.e., *T. atroviride* and *T. virens*, it enhanced the concentration of a phytoalexin named camalexin (Contreras-Cornejo et al., 2011).

6.2 Jasmonic acid as a signalling biomolecule under pathogen stress

A phytohormone in plants can be defined as a naturally persisting organic compound required in minimal amounts, i.e., less than 1mM or even at 1µM concentration. These biological compounds regulate various physiological, morphological and biochemical functions in plants and are effective under different biotic and abiotic stressful circumstances (Egamberdieva et al., 2017; Zheng et al., 2023). During exposure to stressful stimuli, the plant responds to them by operating a brilliant communication network of chemical messengers known as the hormonal signal transduction pathway. A unique characteristic of this communication system is that it can communicate over long distances through signal detecting, transmitting and responding to cells and even organelles of different tissues and organs (Klumpp and Krieglstein, 2002; Zhang et al., 2020). Among numerous phytohormones present in plant spp. ABA, JA, SA and ethylene in adequate proportion play a significant role as stress signals. Where ABA chiefly contributes to mitigating abiotic stress, SA, JA, and ET play crucial roles in response to biotic stress signalling (Cramer et al., 2011; Liu et al., 2008; Sood, 2023). In general, SA executes a defensive response against pathogenicity of biotrophic pathogens; and necrotrophic induced severe infection has been reported to be alleviated by JA/ET mediated defence signals (Glazebrook, 2005; Hönig et al., 2023). JA is a fatty acid with 12-C unsaturation and a cyclopentane ring with a keto group. In addition, its methyl ester, i.e., methyl jasmonates comes into existence by transferring a methyl group from SAM to the carboxyl group of JA by the action of JMT. JA, as well as MeJA, are biologically active compounds and, along with their derivatives, are known as jasmonates (Lalotra et al., 2020).

Like other oxylipins, JA is created by the octadecanoid path. The family of these defence-related compounds has been derived from oxidation of polyunsaturated fatty acids. Biosynthesis of JA is initiated from 18 carbon with three unsaturated fatty acids named linolenic acid (LA) (Lalotra et al., 2020). Several investigations have reported that activation of the phospholipase enzyme, which releases LA from membranes, is responsible for enhancing JA concentration in wounded tissues (Canonne et al., 2011; Ruan et al., 2019). Moreover, plants enzyme-like acyl hydroxylase can assist in releasing fatty acids from lipids.

JA, with IUPAC name, 3-oxo-2-(pent-2′-enyl) cyclopentane acetic acid is a ubiquitous phytohormone in the plant kingdom (Lalotra et al., 2020). At the very beginning of JA biosynthesis, LA is converted to 13-hydroperoxylinolenic acid (13-HPLA) by the action of the lipoxygenase (LOX) enzyme (Wasternack and Strnad, 2018). 13-HPLA serves as a substrate for two enzymes which are Allen Oxide Synthase (Hydroperoxide dehydratase/hydroperoxide dehydrase) and Allen oxide Cyclase which convert it to an intermediate named 12-oxo-PDA (Rustgi et al., 2019). Subsequently, upon reduction and 3 cycles of β-oxidation, 12-oxo-PDA gives rise to JA. (+)-7-iso-JA so formed has been epimerized to a more stable trans configuration, i.e. (-)- JA (Liu and Park, 2021).

A significant factor that ascertains the better vegetative and reproductive growth of plants is 'defence'; a well-defended plant can survive under various biotic stresses. The defensive strategies of plants are based on the rapidity to recognise a specific pathogen and mounting a signalling network to initiate the synthesis and accumulation of defence molecules. JA and its derivatives play a crucial role in plants' immune systems to defend them against pathogens (Pieterse et al., 2012; Sood, 2023) Multiple reports signify the importance of JA in inducing disease resistance against several biotrophic and necrotrophic pathogens (Table 2.2). Constitutive expression of the AOS gene participating in JA biosynthesis exhibited enhanced resistance to fungal pathogens by upregulating the expression of anti-pathogen-related genes in transgenic rice (Mei et al., 2006). Similarly, two AOS mutants *cpm2* and *hebiba* deficient in JA biosynthesis, are observed to be susceptible to infection by even an avirulent strain of *Magnaporthe oryzae* (Riemann et al., 2013).

Table 2.2 Various reports showing the defensive role of JAs application against distinct kinds of biotic stresses.

Plant Name	**Biotic Stress**	**JA (Dose and Mode of Application)**	**Post- JA treatment effects**	**References**
Arabidopsis	*Botrytis cinerea* and *Alt*	2 mM SA, 100 μM	WRKY75 transcriptionally upregulates the genes	Chen et al.,

thaliana	*ernaria brassicicola*	JA and 2 mM ACC solutions were sprayed on to plants.	associated with the JA-mediated signalling pathways and thereby clearly synchronizes the defence responses of *A. thaliana* against necrotrophic pathogens.	2021
Citrus reticulata, *C. limon*, and *C. limetta*	*Penicillium digitatum* and *P. italicum*	Preceding inoculation with pathogens, citrus fruits were immersed in SA and JA solutions for 15 minutes.	Applications of SA and JA suppress post-harvest infections and reduce disease severity in citrus fruits. In addition, it enhances disease resistance against green mould and blue mould pathogens by upregulating the catalytic activities of POD and PPO.	Moosa et al., 2019
Helianthus annuus, Arabidopsis thaliana, Zea mays	Wounding, insect attack, infection with *Pseudomonas syringae*	200 µM MeJA/ 30 µM ACC	Induces the expression of HAHB4 transcription factor which upregulates the transcriptional level of several defense related products such as green leaf volatiles, trypsin protease inhibitors (TPI), lipoxygenase, hydroperoxide lyase.	Manavella et al., 2008
Oryza sativa	Rice stripe virus (RSV)	50 µM Me-JA+1 µM	Enhances resistance against RSV	Hu et al., 2020)

		Epibrassinolide (BL) sprayed on rice seedlings		
Phoenix dactylifera	*Fusarium oxysporum* f. sp. *albedinis*	Roots were injected with 50 µM JA	Improved resistance against Bayoud disease by enhancing the activity of defensive enzymes, i.e., polyphenol oxidase and peroxidase.	Jaiti et al., 2009
Prunus avium	*Penicillium expansum*	10 µmol/L MeJA	Controlling blue mould decay enhances the activity of chitinase, glucanase, catalase, calmodulin, glutathione, NPR1-like, Thaumatin like- proteins and reduction in the action of polygalacturonase and pectin-methylesterase enzymes.	Wang et al., 2015
Prunus persica	*Penicillium expansum, Botrytis cinerea,* and *Rhizopus stolonifer*	1 µmol L^{-1} Me-JA vapour	Reduction in the disease incidence as well as lesion for pathogens by upregulating the activity of defensive enzymes like glucanase, chitinase, PAL, POD, PPO and level of total phenolic contents.	Jin et al., 2009

			Also increases the action of APX, SOD, CAT and the concentration of H_2O_2.	
Solanum lycopersicum	*Botrytis cinerea*	10 mM, Fruits immersed in solution	Inhibit lesion diameter of grey mould rot by alleviating oxidative damage due to upregulation of SOD and APX gene expression, enhancement in the content of ascorbate and glutathione, inhibition of protein carbonylation and negatively affecting plasma membrane integrity of pathogens.	Zhu and Tian, 2012
Solanum lycopersicum	*Meloidogyne incognita*	Seeds were immersed in 100 nM JA	Under nematode stress, expression of *SOD, POD, CAT*, and *GPOX* was upregulated. An upsurge in the concentrations of several important amino was reported in JA pre-treated tomato seedlings.	Bali et al., 2020
Triticum aestivum	*Tilletia laevis*	100 µM Me-JA applied to seedlings	Improved resistance against pathogens by upregulation of gene expression of Chitinases,	Lu et al., 2006

			PR-proteins and lipase.	
Triticum aestivum	*Fusarium pseudograminearum*	300 µM Me-JA by spraying plants	Induces the expression of PR1.1, PR2, PR3, PR4, PR5, PR10 genes.	Desmond et al., 2006
Triticum aestivum	*Fusarium culmorum*	200 µM MeJA applied in soil around the seedling	Enhancement in the catalysis of defense related enzymes like SOD, CAT, POX, PPO, LOX and PAL. An increase in the total phenolics and callose contents was also observed. In addition, a decrease in wheat seedlings' H_2O_2 and lipid peroxidation levels was also observed.	Motallebi et al., 2017

Previous investigations revealed that exogenous exposure to JA or its derivatives induces the expression of defence-linked genes, thereby improving resistance against several necrotrophic pathogens (Ameye et al., 2015; Macioszek et al., 2023; Wang et al., 2012). As examined in the case of *Fusarium graminearum*-infected wheat, significant alterations in defensive enzymes and secondary metabolites have been monitored, which later enhance disease resistance by directly inhibiting pathogen invasion (Moosa et al., 2019; Zhao and Li, 2021). Jasmonates are pivotal in regulating the biosynthesis of diverse compounds of specific metabolic pathways. As mentioned previously, JA assists in producing distinct types of secondary metabolites like indole alkaloids, terpenes, phenylpropanoids, nicotine, flavonoids etc. (Wasternack and Hause, 2013).

6.2.1 JA induced signalling and gene regulation mechanisms

Due to pathogenic attacks, multiple invader-derived or damage-associated plant-originated signals, which may be either chemical or physical, have been reported in local responses. These cues are later recognized by PRR situated on the cell membrane. Consequently, this recognition event initiated the *de novo* formation of JA and JA-Ile (JA-Isoleucine). The cytoplasmic enzyme JASMONATE RESISTANT 1 (JAR1) (Staswick and Tiryaki, 2004) is known to be responsible for the conjugation of JA with Isoleucine to convert it into the biologically active form, i.e., JA-Ile. In addition, other active metabolites of JA, including Me-JA (an airborne signalling molecule) and cis-jasmone, which function as inter-plant signalling compounds, also come into existence.

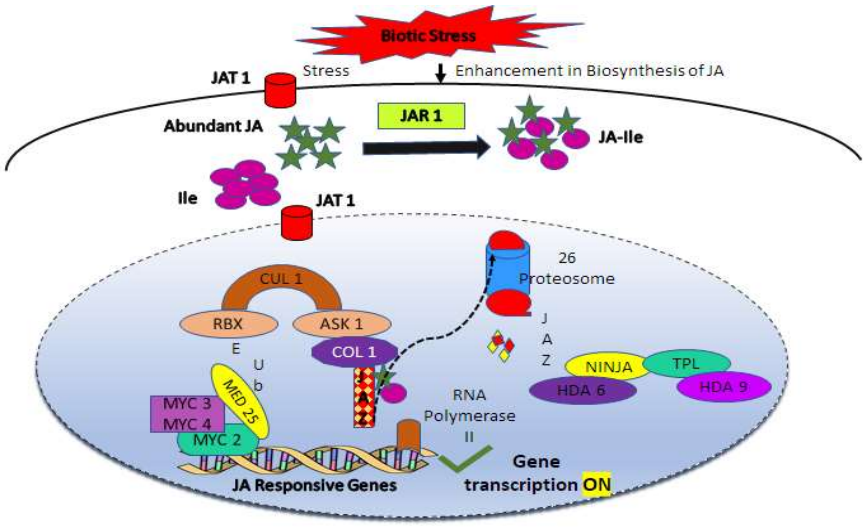


Fig. 2.6 Represents JA induced signalling and gene regulation mechanisms.

A massive increase in JA-Ile concentration has been reported under stressful circumstances. On entering the nucleus, the SCF complex recognises the JA-Ile complex. After that, the JA-Ile complex is sensed by the COI1-JAZ coreceptor complex, which elicits rapid interaction of JAZ with COI1. This interaction causes the degradation of JAZ repressor proteins in the 26S proteasome. After degradation, transcription factors linked with JA-responsive genes are activated. Furthermore, a mediator termed MED 25 modulates the gene transcription by assisting in binding TFs (MYC 2) with RNA II polymerase.

Several reports emphasize that in JA signalling, (+)-7-iso-JA-L-Ile is the natural and direct ligand in the plant system (Yan et al., 2016). Coronatine insensitive1 (COl1) is a potent receptor for this ligand (Xie et al., 1998). COI1 locus translates an F-box protein that assembles with its other companions, SKP1, Cullin and Rbx proteins, creating an E3 ubiquitin ligase during the signal transduction pathway (Xie et al., 1998). The Skp1/Cullin/F-box (SCF) complex is a proteinaceous ubiquitin-proteasome complex. A protein family known as jasmonate zim-domain (JAZ) serves as a critical down regulator of the JA signalling pathway and tasks as a substrate for this SCFCOI1 E3 ubiquitin ligase complex (Sood, 2023).

7. Seed Priming as a novel method to induce disease resistance in stressed plants

Seed priming is an evolving technique for sustainable agricultural practices, designed to increase seed value, seedling strength, and productivity and, most importantly, induce resistance against several kinds of biotic and abiotic stresses by minimising the uses of several harmful chemical compounds (Sood et al., 2021). The concept of seed priming was quite ancient and historically dates to CE 60. Several reports confirmed that water imbibing seeds before sowing causes faster seed germination. Similarly, premature germination of tomato seeds was observed to be triggered by two days of water soaking at 23.6 °C (Ells James E., 1963). In addition to better growth and efficiency of seedlings, seed priming also leads to significant changes in treated seeds, including upregulation in enzyme actions associated with the detoxification of ROS and hydrolytic activities. Moreover, modification in internal levels of hormones and variable gene expression contributes to increased plant growth and better resistance (Lutts et al., 2016). However, this technique involves seed hydration to the extent needed for the activation of various metabolic processes but is not suitable for radicle emergence from the treated seeds.

8 Antioxidative defence system in plants

In plants, oxidative damage is a strategy in which the generation of ROS through various metabolic processes causes unfavourable changes in critical cellular components like proteins, DNA, lipids etc (Hasanuzzaman et al., 2020). Broadly, ROS can be categorised into radical (including O_2 •−, OH• and RO•) and non-radical (comprising H_2O_2, O_2, and O_3 and HClO) species. These species usually are produced as typical by-products in plant metabolic processes like the ETC in chloroplast and mitochondria (Choudhury et al., 2017; del Río, 2015; Guo et al.,

2023; Halliwell, 2006). Overproduction of these ROS swing the equilibrium of oxidant/antioxidant in favour of antioxidants. On the other hand, the presence of unpaired free electrons in their construct makes these ROS highly reactive to induce damage in several biologically important cellular macromolecules.

As too much oxidation and reduction of cellular compounds are highly disadvantageous, maintaining redox homeostasis is essential for plants' survival and growth (Foyer and Shigeoka, 2011). Interestingly, plants are exceptionally rich in complexes with antioxidative properties (Pulido et al., 2009; Vivancos, Dong, 2010; Vivancos, Wolff, 2010; Wachter et al., 2005). Antioxidants refer to molecules that can quench or inhibit free radical reactions and postpone or avoid cellular damage (Dumont and Rivoal, 2019; Nimse and Pal, 2015). To manage ROS induced toxicity, plants have evolved an effective antioxidative defence system mainly comprised of lipid-solvable membrane-associated antioxidants like β-carotene and α-tocopherol and water-soluble ones like ascorbate (AsA) and glutathione (GSH) (Khan et al., 2012; Zandi and Schnug, 2022). AsA-GSH cycle constitutes a primary H_2O_2 cleansing system involving the catalysed oxidation and reduction of AsA and GSH. Enzymes which have participated in these essential detoxification mechanisms comprise APX, MDHAR, DHAR and GR (Rajput et al., 2021).

8.1 Antioxidative molecules

8.1.1 AsA

AsA functions as the first line of defence by shielding plant cells from damaging factors that lead to ROS production. In addition, AsA shows a fundamental task in regulating the consequence of the plant-pathogen interface by directly interacting with critical elements associated with plant defence mechanisms. As mentioned previously in harmonisation with GSH and vital enzymatic antioxidants, AsA can provide an important redox environment, essentially required for various defence-related pathways like controlling the expression of defensive genes through the stimulation of the NPR1, cell wall strengthening, regulatory transcription factors, alteration of defence inducing hormone-signalling systems (Boubakri, 2018). The AsA-GSH cycle was operational in different cellular organelles, including chloroplast, mitochondria, peroxisome, and cytosol, as well as in the apoplastic region (Hasanuzzaman et al., 2019; Noctor and Foyer, 1998). In these compartments, Ascorbic acid could be retrieved either in reduced or in two oxidized models.

Owing to pathogen exposure, an upsurge in ROS accumulation leads to an increase in the amount of DHA mainly due to the oxidation of AsA. For instance, in tomato fruits, an approximately 40% rise in AsA levels has been detected after infection with cucumber mosaic virus (Tsuda et al., 2005). Similarly, due to the infection with TuMP in the resistant cultivar of *Brassica rapa*, enhancement in the magnitude of AsA has been monitored (Fujiwara et al., 2013). On the other hand, in susceptible cultivars, a remarkable decrease in the AsA levels has been detected (Fujiwara et al., 2013).

8.1.2 Glutathione (GSH)

Glutathione is a universally dispersed sulphur comprising antioxidative molecule that performs crucial tasks in regulating plant development, growth and stress tolerance (Hasanuzzaman et al., 2017; Rodrigues de Queiroz et al., 2023). It is a small-molecular-weight thiol, which swiftly gathers in the cells under stressful circumstances. The vibrant connection between reduced GSH and ROS has been well established and its dynamical participation in cell signalling and physiological processes comprising protein synthesis, transport of amino acid, DNA repair and regulation of cell division and senescence has been documented in several reports (Aquilano et al., 2014; Hasanuzzaman et al., 2019). GSH play a crucial role in the recovery of the reduced form of ascorbate in dehydroascorbate reductase (DHAR) catalysed reaction, well known as the AsA-GSH cycle. The equilibrium between the GSH/GSSG content is a fundamental component in sustaining the cellular redox status. To counter the repressing effects of ROS mediated oxidative stresses in plant cells, it is essential to maintain appropriate levels of reduced GSH. It is reported as a capable scavenger of $^1O_2$, H_2O_2 and OH^-.

8.1.3 Tocopherol

Tocopherols are lipid-soluble antioxidants essential for membrane integrity synthesized in the plastid envelope from homogentisic acid and isopentenyl diphosphate. These tocopherols shield cellular membranes from oxidative stress by neutralizing singlet oxygen and dropping superoxide radicals (Shah et al., 2021). They prevent lipid peroxidation by scavenging the fatty acyl peroxy radicals. In addition to this, α-Tocopherols may influence intracellular signal transduction in plant cells. They either interact directly with the significant participants of the signalling cascade or may affect the signalling process indirectly by regulating the levels of ROS and

phytohormones like Jasmonic acid, which thereby later control the plant reactions to development and stress tolerance (Munné-Bosch et al., 2007). α-Tocopherols deactivate $^1O_2$ to $^3O_2$ through the mechanism of charge transfer. Tocopherol loses one electron to electron-deficient $^1O_2$ and subsequently forms a charge transfer complex, which undertakes intersystem crossing and splits into α-Tocopherols and $^3O_2$. Interestingly, it has been reported that before complete degradation, one tocopherol molecule is responsible for the deactivation of approximately 120 singlet oxygen molecules by resonance energy transfer (Kurutas, 2016).

8.2 Enzymatic Antioxidants

Table 2.3 List of antioxidative enzymes in plants

Antioxidative Enzyme	Isoform	Cellular Location	Catalytic Reaction
APX	cAPX, Chl APX, mit APX and mAPX.	Cytoplasm, chloroplast, Mitochondria, Peroxisome and glyoxisome.	$AsA+H_2O_2 \rightarrow DHA+2H_2O$
CAT	CAT-I, CAT-II and CAT III	Peroxisome, Mitochondria and cytoplasm	$H_2O_2 \rightarrow H_2O + 1/2\ O_2$
DHAR	Different in different plants	Chloroplast, cytosol, mitochondria, peroxisome	$DHA+2GSH \rightarrow AsA+GSSG$
GPX	-	Chloroplast, Cytosol, Mitochondria and endoplasmic reticulum	$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$
GPOX	GPX1-	Chloroplast,	$2H_2O_2 + Guaiacol \rightarrow 2H_2O$

	GPX7	cytosol, mitochondria, endoplasmic reticulum.	+ oxidized Guaiacol
GR	Different in different plants	Chloroplast, cytosol, mitochondria, peroxisome	GSSG+NAD(P)H→ 2GSH + $NAD(P)^+$
GST	-	Chiefly cytoplasm	GSH+X→GSH-X conjugate
MDHAR	Different in different plants	Chloroplast, cytosol, mitochondria, peroxisome	MDHA+NAD(P)H → AsA+ $NAD(P)^+$
PPO	-	Chloroplast	*o*-diphenols +O_2 → *O*-quinone +H_2O → complex brown polymers
SOD	Cu/Zn-SOD, Fe-SOD and Mn-SOD	Mitochondria, chloroplasts, nuclei, cytoplasm, peroxisomes, and apoplasts	$2O_2\bullet^- + 2H^+ \rightarrow O_2 + H_2O_2$

8.2.1 Superoxide Dismutase (SOD)

SODs are omnipresent metal containing enzymes and serve as the primary defence against ROS. It causes the dismutation of superoxide ($O_2^{\cdot -}$) radicals generated in various metabolic processes (Wang et al., 2018). Based on the occurrence of an active site metal co-factor, SOD can be classified into three isoforms i.e., Cu/Zn-SOD, Fe-SOD and Mn-SOD (Fukai and Ushio-Fukai, 2011). By eliminating $O_2\bullet^-$

radicals, SODs reduce the promise of OH• development through metal catalysed Haber-Weiss-type reaction since, in comparison to spontaneous dismutation, the rate of this reaction is about 10,000 times faster (Gill and Tuteja, 2010; Yadav et al., 2019) (Table 2.3).

8.2.2 Catalase (CAT)

CAT is a heme-encompassing antioxidative enzyme present in all aerophilic organisms. This enzyme exhibits a dumbbell-shaped homo tetrameric structure with the molecular weight of each monomer ranging between 2,20,000-3,50,000 kD. The presence of the heme group constitutes a crucial component of its activity. Being an enzymatic antioxidant, catalase prevents cellular oxidative damage by facilitating the decomposition of H_2O_2 into oxygen and H_2O with extraordinary efficiency (Ransy, 2020). The occurrence of this enzyme was reported in peroxisome, but several investigations have also supported its existence in mitochondria and cytoplasm. Various physiochemical processes associated with the generation of H_2O_2 in plants include mitochondrial ETC, fatty acids' β-oxidation, and, most significantly, photorespiratory oxidation under stressed or normal conditions. Through Fenton reaction exhibits great reactivity towards Fe and other transition elements which thereby leads to the lysis of H_2O_2 to ·OH, radicals. Furthermore, reacting directly with -SH groups of enzymes like sedoheptulose biphosphatase, fructose biphosphatase and glyceraldehyde 3-phosphatase, H_2O_2 inactivates these enzymes. Similarly, by dissociating metal ions from metalloproteins, H_2O_2 obstructs their biological activities. Depending upon the size of the subunit, quaternary structures, sequence and prosthetic groups, catalase can be organized into three groups (I-monofunctional Catalases, II- Catalase peroxidase and III- Nonheme catalase) (Loewen et al., 2000; Hansberg, 2023) (Table 2.3). Among all the antioxidative enzymes, catalase exhibits the highest turnover rate. Several reports have estimated that a single catalase molecule efficiently converts 6 million molecules of hydrogen peroxide into water and O_2 per minute.

8.2.3 Ascorbate Peroxidase (APX)

APX is another H_2O_2-scavenging enzyme that protects the chloroplast and other cellular constituents from the damaging effects of H_2O_2 and •OH radicals (Caverzan et al., 2012; Gomes et al., 2022). APX belongs to class I heme-peroxidases; therefore, iron is essentially required for the catalytic activity of this oxidoreductase enzyme. Depending on the composition of amino acids, APX has been classified into

five isoforms including cytosolic (cAPX 1and2), chloroplastic (chlAPX: stromal and thylakoidal APX), mitochondrial (mitAPX) and peroxisomal/glyoxisomal (mAPX) (Caverzan et al., 2012; Jardim-Messeder et al., 2022). APX differs from other peroxidases since it utilizes ascorbate (AsA) as its definite electron donor to reduce hydrogen peroxide to H_2O with the consequent production of monodehydroascorbate (MDHA), a monovalent oxidant of ascorbate. Later this MDHA mechanically breaks down into AsA and dehydroascorbate (DHA) (Shigeoka, 2002; Li, 2023) (Table 2.3). The major H_2O_2 detoxification mechanism in plant chloroplast involves the participation of ascorbate peroxidase-ascorbate and glutathione, identified as the AsA-GSH pathway or the Foyer-Halliwell-Asada pathway. It is well acknowledged as the "heart of redox homeostasis" and plays the role of a combined ROS scavenger.

8.2.4 Guaiacol peroxidase (GPOX)

GPOX belongs to heme-comprising peroxidase proteins that catalyse the redox reaction of aromatic e^- donors like pyrogallol and guaiacol at the expenditure of H_2O_2 (Sharma et al., 2012) (Table 2.3). These enzymes are structurally a combination of 4 restricted disulphide bridges and possess two interconnected Ca^{2+} ions. GPOX plays a crucial role in multiple metabolically important processes in plants, comprising cell wall lignification, creation of free radical intermediates through the oxidation of hydroxy-cinnamyl, decomposition of IAA, ethylene biosynthesis, regulation of extension of the plant cell wall, wound repair and crosslinking of polysaccharides. Different isoforms of GPOX are known to be distributed in various plant tissues and compartments like cytoplasm, cell wall and vacuole (Zandi and Schnug, 2022).

8.2.5 Monodehydroascorbate reductase (MDHAR)

To protect themselves against the damaging effects of ROS, plants possess an efficient system of reductase enzymes that can keep the AsA pools in a reduced state. Therefore, MDHAR, an ascorbate reductase, displays a significant defensive role in oxidative stress tolerance (Polle, 2001; Mishra et al., 2023). Owing to the exposure of plants to different kinds of stresses causes fast oxidation of AsA to MDHA in the chloroplast. For the reason of endurance of plants, that MDHA must be reduced back to revive AsA. MDHAR recovers MDHA into AsA by bringing about their reduction. In the thylakoid membrane, MDHA is reduced to Ascorbic acid by photo-reduced ferredoxin at an elevated rate, constituting the major pathway for AsA regeneration (Table 2.3). On the other hand, away from the thylakoids, MDHA reduction is carried

out by two enzymes i.e., DHAR and MDHAR of the AsA-GSH route (Asada, 1999; Hasanuzzaman et al. 2019). MDHAR uses NAD(P)H as an e^- donor for direct reduction of MDHA. Otherwise, two MDHA molecules can react non-enzymatically to produce AsA and DHA. In higher plants, MDHAR comprises a multigene family with multiple subcellular isoforms. The activity of this enzyme has been detected in several cellular compartments like chloroplast, peroxisome, cytoplasm and mitochondria.

8.2.6 Dehydroascorbate reductase (DHAR)

The expression of DHAR has been upregulated by several types of stress in plants which imparts tolerance mechanisms to affected plant spp. Ascorbic acid is oxidised to DHA in plants through sequential electron transmissions with MDHA as an intermediate free radical (Pandey et al., 2014). Thus, by using GSH as an e^- donor, DHA so formed, is transformed to AsA by DHAR catalysed reduction process (Table 2.3). In addition to oxidative stress tolerance, DHAR plays a crucial task in plant growth and development. As reported in the case of *O. sativa*, a lack of DHAR leads to a sharp decrease in AsA content and is ultimately responsible for the slower pace of leaf expansion (le Martret et al., 2011; Ye et al., 2000).

8.2.7 Glutathione Reductase (GR) (NADPH: oxidized glutathione oxidoreductase)

GR is a flavoprotein redox enzyme ubiquitously distributed in both pro- and eukaryotes. This enzyme is required to maintain a cellular redox state by sustaining a balance between reduced Glutathione and AsA pool by regenerating the reduced form of GSH (Noctor and Foyer, 1998; Foyer and Kunert, 2024). The GR and GSH perform a fundamental role in asserting the tolerance of plants under multiple kinds of stresses. GR maintains the GSH pool in the affected plants by catalysing the NADPH-reliant reduction of the disulphide bond of GSSG (Trivedi et al., 2013b; Rai et al., 2023) (Table 2.3). Although this enzyme is chiefly located in chloroplast, its existence in other organelles like cytosol, peroxisome and mitochondria has also been reported. Numerous isoforms of GR have been registered in many plants (Contour-Ansel et al., 2006; Edwards et al., 1990; Lascano et al., 2001; Rao and Reddy, 2008; Trivedi et al., 2013a; Tanwir et al., 2021).

8. 2. 8 Glutathione peroxidase (GPX)

Plant GPX is an important antioxidative enzyme that maintains H_2O_2 homeostasis and is an efficient scavenger of ROS. This enzyme corresponds to the non-heme thiol peroxidase family that utilizes either glutathione (GSH) or thioredoxin (Trx) as reducing substrates (Bela et al., 2015) (Table 2.3). Compared to their animal counterparts, plant GPX is inclined towards Trx rather than GSH as the reducing substrate. Furthermore, in their active sites, plant GPX comprises cysteine residue but in the case of mammalian GPX, selenocysteine (SeCy) rather than Cys residue is required for catalytic activity (Islam et al., 2015; Koh et al., 2007). Depending upon in silico analysis, GPXs were expected in the chloroplast, mitochondria, cytosol, and ER localizations synthesizes H_2O_2. Conclusively, this hydrogen peroxide gets converted into H_2O and O_2 by the catalytic activity of catalase and peroxidase. Moreover, their crucial role in plants' antioxidative defence system, GPX also regulates other critical metabolic processes like root-shoot development, stomatal functioning, regeneration, immune response, photosynthesis and hormonal signalling.

8.2.9 Glutathione-S-Transferase

GSTs activities lead to labelling various xenobiotics for vacuolar sequestration or transfer to apoplast by catalysing their conjugation with electrophilic centres and nucleophilic tripeptide GSH (Martinoia et al., 1993) (Table 2.3). Further, lipid peroxides like 4-hydroxyalkenals or 13-hydroperoxylinoleic acid are detoxified by GST-induced conjugation of these molecules to GSH. Interestingly, it has been revealed in several studies that multiple GSTs contribute to antioxidative defence mechanisms by exhibiting the activities of glutathione peroxidase by acting on toxic base propenal like thymidine hydroperoxide (Dixon and Edwards, 2009). In plants, GSTs are cytosolic and correspond to about 2% of soluble protein. In addition, several GSTs are reported to take part in auxin transport and auxin binding as non-substrate ligands. It has also been observed that GSTs perform a significant part during the regular metabolism of secondary metabolites like anthocyanins (Marsh et al., 2008; Gao et al., 2020).

8.2.10 Polyphenol oxidases (PPOs)

PPOs are omnipresent Cu-containing enzymes which utilize molecular Oxygen for catalytic hydroxylation and dehydrogenation of *o*-diphenolic compounds like catechol and caffeic acid to their respective quinones, which leads to the

alkylation of nucleophilic groups and self-polymerization to produce dark-coloured melanin polymers (Dirks-Hofmeister et al., 2013) (Table 2.3). In plants, PPOs are primarily sequestered in the chloroplast as latent enzymes, whereas their substrates are reported in other cytosolic compartments. Pathogen-induced cell disruption would facilitate the union of PPOs with their substrates. Critical characteristics of PPO comprise two conserved Cu-binding domains and chloroplast and thylakoid transit peptides at the N-terminal (Joy IV et al., 1995; Taranto et al. 2017).

9. Defensive Secondary Metabolites in Plants

Several physiological processes related to the fundamental growth and development of plants constitute their primary metabolism (Zaynab et al., 2018). However, secondary metabolism is not necessary for basic processes, but it is extremely important for plant defence under adverse conditions (Edreva et al., 2008; Divekar et al., 2022). The method(s) of defence response necessitates a certain change in the expression levels of metabolic gene organization, which affects protein synthesis to control related primary and secondary metabolite pathways. Primary molecules create and accumulate a wide range of secondary metabolites in plant cells. Furthermore, when plant cells are stimulated with abiotic/biotic stress mediators and signalling molecules, their biosynthesis and accumulation may be upregulated (Al-Khayri et al., 2023; Narayani and Srivastava, 2017; Ramakrishna and Ravishankar, 2011; Rejeb et al., 2014). There is additional evidence from numerous studies that under stressful circumstances, carbon supplies have been diverted toward creating secondary metabolites rather than biomass (Bryant et al., 1983; Isah, 2019).

SMs can be classified as phenolic, terpenoids, alkaloids, and sulfur-containing chemicals according to their biosynthesis routes (Hänsch and Mendel, 2009; Shiade et al., 2024). Shikimic acid routes are used to create aromatic phenolic metabolites. Generally, these can be grouped as insoluble, cell wall-binding substances such as condensed tannins, lignin, and hydroxamic acid. Conversely, the soluble phenolic group comprises phenolic acids and flavonoids (Dehghanian et al., 2022; Jimenez-Garcia et al., 2013). These compounds lessen the impact of pathogen-tempted damage to crop plants by employing different mechanisms. Owing to pathogen infection, these substances break down into phenolate ions and phenolic hydroxyl groups, which then join with pathogenic peptides and proteins to produce ionic and hydroxyl linkages that eventually cause their denaturation. Furthermore, they intervene with pathogen signalling molecules and trigger changes in their reproductive systems and

physiological processes via enzyme inhibition, alkylation of DNA, and so on. They also function as allelochemicals and display diverse antifungal, antipredator, and antibiotic molecular functions; for example, phenolic acids like vanillic, hydroxybenzoic, benzoic, and caffeic, exhibit antimicrobial properties. Like this, substances like synaptic, Caffeic, p-coumaric, chlorogenic, and ferulic acids shield plants from antioxidative stress by removing ROS produced because of membrane lipid peroxidation (Khalid et al., 2019).

Terpenoids are lipophilic SMs that are produced via mevalonate pathways. These are biosynthesized and categorized based on the number of C_5 Isoprene units (Morrissey and Guerinot, 2009; Mosquera et al., 2021). Carotenoids are chemically tetraterpenes which serve as accessory pigments during photosynthesis, and by photoinhibition, guard the photosynthetic apparatus from photooxidation (Sahebi et al., 2017). In the plant kingdom, terpenes perform a crucial protective role as a feeding deterrent against herbivores and mammals, such as pyrethroids in the leaves of *Chrysanthemum* exhibit insecticidal action (Haouas et al., 2008). Numerous volatile terpenes generated essential oils, such as limonoids from citrus fruits and azadirachtin from neem, act as potent insect repellents and feeding deterrents. Cardenolides also have a bitter taste and are particularly poisonous to higher animals (Okumu et al., 2007).

Familiar amino acids like tryptophan, tyrosine and lysine serve as precursor molecules for the biosynthesis of Nitrogen bearing SMs mainly comprising alkaloids and cyanogenic glycosides (Ziegler and Facchini, 2008). Consequent to herbivory, an elevated accumulation of antiherbivore alkaloid compounds has been described in several investigations, for instance nicotine uprise in *Nicotiana attenuata* (Pandey et al., 2008). Whenever a plant comprised of cyanogenic glycosides and glucosinolates as nitrogenous SMs is crushed, these metabolites are broken down. By means of enzymatic hydrolysis, cyanogenic glycosides release toxic gas named hydrogen cyanide (HCN) (Nyirenda, 2021). Being a fast-acting toxin, HCN shows a unique ability to bind metalloproteins like cytochrome oxidase, an enzyme essentially required in mitochondrial respiration. Furthermore, numerous alkaloids, for instance, strychnine, coniine and atropine are testified to be toxic, while others, like caffeine, cocaine and nicotine, act as sedatives in human beings.

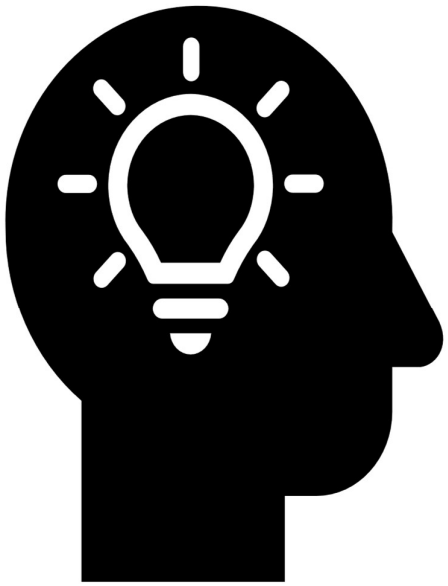
10. Protective Osmolytes in plants

To adapt to stressful circumstances, plants produce and accumulate compatible solutes designated as osmolytes. Various low molecular weight substances or metabolites, including sugars, polyamines, secondary metabolites, amino acids, and polyols, are called osmolytes (Ghosh et al., 2021). These serve as significant cytoprotectants by maintaining cellular homeostasis through various mechanisms like facilitating driving gradient for water absorption, regulating cell turgor through fine osmotic tuning, and establishing cellular redox status by eliminating excess ROS, and providing protection to cellular machinery from oxidative injury and osmotic stress. Numerous stress signalling pathways such as hormones, mitogen activated protein (MAP) kinase, and calcium-signaling cause excessive formation of osmolytes. In response to host plants' osmotic and oxidative stress-induced damages, these accumulate numerous small molecular weight metabolites, specifically, sugars, amino acids, polyamine, secondary metabolites, and polyols (Ghosh et al., 2021; Roychoudhury and Banerjee, 2016).

In many plant species, sugars act as key osmolytes in osmotic adjustment. Their contribution towards free radical scavenging, Osmo-protection, carbon-storing and maintenance of the structure of essential proteins like RUBISCO has been widely acknowledged. In addition to compatible osmolytes, sugars act as crucial substrates for the growth and regulation of gene expression under stressed conditions. Among the several amino acids in plants, proline, glycine betaine (GB) and Gamma-Aminobutyric Acid (GABA) function as stress-responsive amino acids and play a key role in oxidative and osmotic adjustment in plants (Dabravolski et al., 2023; Giri, 2011; Huang et al., 2000; Suprasanna et al., 2014). Proline is a proteinogenic amino acid; it is a secondary amine and does not possess an amino group. Proline serves as compatible solutes under osmotic stress conditions. In addition to this, because of its chaperone-like nature, it also performs as a brilliant antioxidant as well as a potent scavenger of ROS (Alia et al., 2001; Matysik et al., 2002; Smirnoff and Cumbes, 1989; Spormann et al., 2023). Proline accumulation in the plant cell is executed either by new synthesis or reduction in degradation processes or can be both. GABA is a non-protein amino acid that substantially regulates various metabolic processes in abiotically stressed plants because of its significance in plant antioxidant defence, osmoregulation and being a signalling molecule (Michaeli and Fromm, 2015; Nayyar

et al., 2014). GB is a nitrogenous substance, more specifically a quaternary amine, which possesses zwitterions. The chemical structure of glycine betaine exhibits positively charged functional groups like phosphonium cation or quaternary amine, and on the other side, it displays negatively charged functional groups such as the carboxylate group. In stressed plants, GB alleviates different kinds of abiotic stresses, mainly by reducing the levels of ROS accumulation, protecting membrane integrity and photosynthetic machinery, and inducing the expressions of genes responsible for stress tolerance in plants (Chen and Murata, 2008, 2011; Zulfiqar et al., 2022).

Chapter 3
Hypothesis



Chapter 3 Hypothesis

Presently, the ideology of disease-free agriculture emphasises the applications of chemical fungicides to extreme limits, especially in the case of vegetable and ornamental crop production. Besides their high financial burden and the threat of lowering efficacy, improper and wide-scale application of these fungicides leads to several environmental risks due to their ill effects on water quality, soil, and human and animal health. Moreover, these fungicides cannot be considered long-term remedies against pathogenic fungi because of health and environmental concerns. Subsequently, the essential need for safe and chemicals free food has increased the demand and market for biopesticides. Moreover, in the pursuit of sustainable agriculture, biopesticides serve as an eco-friendly approach by minimising the application of harmful chemicals. In this direction, the efficiency of specific biochemical and bio-control factors in eliciting induced resistance in infected plants against several biotic challenges has been successfully reported. Furthermore, pre-treatment of a susceptible host with either a biocontrol agent or a chemical inducer, individually or together, initiates a defensive response against the devastating pathogens comprising several interlinked biomolecules with ameliorative nature. Synthesis and accumulation of these molecules inside diseased plants lead to induced systemic resistance (ISR). Further, seed priming with these ameliorative agents is an evolving technique for sustainable agricultural practices, designed mainly to increase seed value and, most importantly, to induce resistance against several kinds of stresses, either biotic or abiotic, in plants by minimising the use of several harmful chemical compounds.

In the present investigation, tomato is taken as a 'model crop' for studying resistance mechanisms against pathogens. Cultivated tomatoes have low genetic diversity and are susceptible to over 200 diseases. Among these pathogens, especially soil-borne like *Fol* and *R. solani*, are difficult to manage due to their persistence in soil for longer duration. Thus, due to susceptibility of cultivated tomato to multiple pathogens, it has been considered a prototype to study plant-pathogen interactions and thereby assisting in the establishment of effective control against various diseases to enhance universal production. Among these, yield destruction caused by two soil-borne fungal pathogens of tomato viz. *Fol* and *R. solani* are most devastating pathogens and are accountable for a significant yield loss in the greenhouse and open

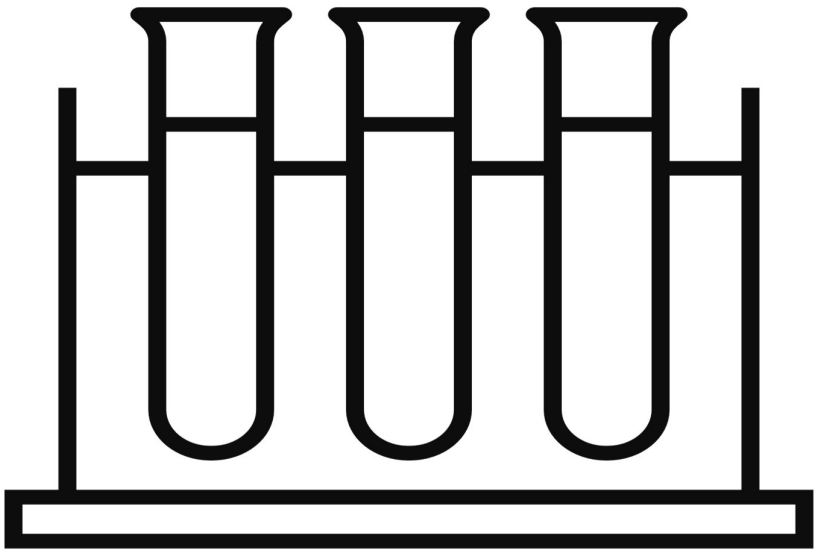
field conditions. Applying chemical elicitors like jasmonic acid upregulates various defence-related pathways in pathogenically stressed plants. In addition, bioagents such as *Trichoderma virens* limit the growth of fungal pathogens through multiple strategies such as mycoparasitism, competition and antagonism and, on the other hand, symbiotically enhance the plant growth in the pathogenically contaminated rhizosphere. Furthermore, the ameliorative potential of *T. virens* in coordination with JA in fusarium wilt and damping-off stressed tomato plants had not been studied until now. Hence, the main objective of the current investigation is to work out the morphological, biochemical/ physiological and molecular aspects of defence response against *Fol* and *R. solani* in the existence of either chemical inducer or biological control agent individually or together in biotically stressed tomato plants.

Chapter 4
Research Objectives

Chapter 4 Research objectives

- Isolation, identification, and purification of *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani*
- Pathogenicity test of fusarium wilt and damping off diseases on tomato plants
- To monitor the effects of *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani* induced biotic stress in terms of morphological, biochemical, and molecular aspects of tomato plants
- To monitor the ameliorative efficacy of *Trichoderma virens* and jasmonic acid in controlling fusarium wilt and damping off diseases in tomato plants

Chapter 5
Methods and Materials



1. Isolation, Purification and Identification of Pathogens

1.1 *Fusarium oxysporum lycopersici*

The stem of the infected plant was washed, chopped into pieces and then kept in 1% sodium hypochlorite (NaOCl) for 3 min., and they were then rinsed again with DW thoroughly. These pieces were dried between the folds of sterilized blotting paper. The single piece was then kept in a sterilized Petri plate (90 mm) comprising Potato Dextrose Agar (PDA) medium poured previously under aseptic conditions. To inhibit bacterial contamination, the antibiotic chloramphenicol was supplemented into this medium. Lastly, these plates were incubated at 28 ± 2ºC in a BOD incubator for seven days. The purification of the pathogen was carried out regularly by sub-culturing. The culture of the pathogen was sustained on PDA slants in culture tubes and preserved at 4ºC for further use. The cultural and morphological features of the fungi such as the colour of the colony, the pattern of growth and the asexual spores' formation were examined and confirmed with the help of standard text for establishing their identity. Furthermore, the pathogenic mycelium was identified by the ITCC, IARI with ITCC No. 8111.

1.2 *Rhizoctonia solani*

The pathogen was procured from ITCC, IARI, New Delhi, with ITCC No. 1142. By using the single hyphal tip method, the pathogen was purified. The fungus was cultured in the middle of an agar Petri plate. Hyphal strands were examined in an inverted Petri plate under a low-power microscope, and isolated hyphal tips were marked with a permanent marker. Finally, these tips were aseptically transferred in PDA slants and incubated usually at 28 ± 2ºC for 7 days. The cultural and morphological features of the fungus were examined and confirmed with the help of standard text to establish their identity.

2. Plant material and its growth

Tomato seeds, cultivar *Punjab Ratta* were obtained from the Department of Vegetable Science, Punjab Agriculture University, Ludhiana, Punjab. The seeds were sterilized with 0.01% mercuric chloride and grown in seedling trays containing sterilized soil and vermicompost in the ratio of 3:1 in the plant growth chamber at a temperature between 25-27 ºC, 16 hours (h) light and eight h dark cycle and relative humidity of

70 % for thirty days. The thirty days old tomato seedlings were then used to determine sensitivity against pathogens and further experimentations.

3. Pathogenicity Test

The pathogenicity test was conducted to confirm the principles of Koch for pathogenicity validation of the concerned pathogen.

3.1 *Fusarium oxysporum lycopersici*

3.1.1 *Preparation of inoculum*

Two mycelial plugs (0.5 cm in diameter) from the 7-day-old culture of *Fol* were inoculated in 250 mL PDB (Potato Dextrose Broth) containing an Erlenmeyer flask. This flask was incubated at 28 ± 2° C at 100 rpm for 10 days. The mycelial mat was filtered through a double layer of sterilized cheesecloth to obtain conidial stock. The density of conidial stock was counted using a hemocytometer, and the desired density i.e., 1×10^6 spores/mL, was acquired by diluting the stock solution.

3.1.2 *Pathogenicity Test*

Thirty days old healthy tomato seedlings were infected with the pathogen inoculum with the standard root dip method. Healthy tomato seedlings were relocated, shaken to eliminate any stuck soil particles without disturbing the root integrity, and cleaned gently under running tap water. The root apex was trimmed (~ 1cm) with the help of a sterile scissor, and then these roots were immersed in the formulated spore suspension (1×10^6 spores/mL) of *Fol* for (Nirmaladevi et al., 2016). These inoculated seedlings were transplanted to sterilized soil (2 Kg) containing plastic pots (diameter 20 cm and surface sterilized with 0.1% $HgCl_2$). In each pot, three seedlings were transplanted. The plants were kept in a greenhouse at 16 h light and 8 h dark conditions, with temperatures ranging from 25-28 ºC. The vascular wilt symptoms were observed 10-15 days after post-pathogen inoculation.

3.2 *Rhizoctonia solani*

3.2.1 *Preparation of inoculum*

Two mycelial plugs (0.5 cm in diameter) from a 7-day-old culture of *R. solani* were transferred to a 250 mL PDB containing an Erlenmeyer flask. This flask was incubated at 28 ± 2° C at 100 rpm for ten days. The mycelial mat was filtered out with

a double layer of sterilized cheesecloth. A known amount of mycelial mat was blended in sterilized distilled water (2 % W/V) in a blender for 30-60 sec.

3.2.2 *Pathogenicity test*

To evaluate the pathogenicity of *R. solani* suspension, 10 mL was inoculated into pots with the exact specification and conditions as mentioned in the case of Fol inoculum. These pots were allowed to incubate for ten days before the transplantation of tomato seedlings into them. The damping-off symptoms were monitored after 10-15 days post-pathogen infection in tomato seedlings (Manganiello et al., 2018).

4. Evaluation of antagonistic activities of *T. virens* and *T. viride* against *Fol* and *R. solani*

4.1 *Dual culture assay*

In dual plate assay, *T. virens* (ITCC No. 4177) and *T. viride* (ITCC 8315) were screened for assessment of antagonistic activity against test pathogens, i.e., *Fol* (ITCC no. 8111) and *R. solani* (ITCC No. 1142) by inserting a mycelial plug in the Petri dishes (Morton and Stroube, 1955). Similarly, a mycelial disc (5 mm) of a 5-day-old test pathogen culture was kept 6 cm distant on the opposed side of the Petri petriplate, at right angles to the *Trichoderma* fungal disc. The same disc of test fungus was placed in another PDA containing a Petri plate, which served as untreated control. After this, these plates were incubated in a BOD incubator at 28 ± 2ºC. The growth of pathogens mycelium towards colonies of *Trichoderma* spp. was monitored, and inhibition percentage was measured after seven days of incubation. The experiment was carried out in triplicate and repeated twice.

Calculations

The following formula has been applied to calculate the per cent inhibition in the radial colony growth (Garrett, 1956).

$$\text{Inhibition } \% = \frac{\text{Control} - \text{Treated}}{\text{Control}} \times 100$$

Control- Radial growth (cm) in the control set, Treated- Radial growth in the treated set

4.2 Screening of *Trichoderma* spp. for the synthesis of extracellular enzyme, i.e., Chitinase assay

The Chitinase Detection Medium (Agrawal and Kotasthane, 2012) comprised of a basal medium containing 4.5 g of chitin, 0.30 g of $MgSO_4.7H_2O$, 2.00 g of KH_2PO_4, 3.00 g of $(NH_4)_2SO_4$, 1.00 g of Citric acid monohydrate, 0.15 g of Bromo cresol purple 15.00 g of Agar, and 0.20 ml of Tween-80 per litre. The pH of this medium was maintained at 4.7 and then autoclaved at 121ºC for 15 min. After cooling, the medium was transferred into petri plates and permitted to solidify. The actively growing *T. virens* and *T. viride* culture plugs of the isolates to be analyzed for chitinase activity were inserted into the medium containing Petri plates and perceived for the coloured zone development. Chitinase activity was characterized by the construction of a purple-coloured zone. The Colour intensity and diameter of the coloured area were considered as the benchmarks to verify the chitinase activity.

Preparation of Colloidal chitin

In the chitinase assay, the detection medium is supplemented with chitin as the sole carbon precursor. It was prepared corresponding to the method of (Roberts and Selitrennikoff, 1988) with a slight modification. For this, 5 g chitin was acid hydrolysed in 40 ml conc. Hydrochloric acid by continuous stirring in a magnetic stirrer for 24 hours at 4 ºC. This step is followed by adding 200 mL of chilled ethanol (95%) and then kept at 26 ºC for 24 hours. Subsequently, it was centrifuged for 20 min at 3000 rpm and 4 ºC. After that, the pellet so obtained was eroded with sterile DW by centrifugation at 3000 rpm and 4 °C for 5 min until the complete removal of alcohol smell. The procured chitin had a pasty and soft consistency with wetness up to 90-95 %. It was preserved at 4 °C till further use.

5. Seed Treatment and Germination

Tomato seeds were surface decontaminated with 0.01% mercuric chloride ($HgCl_2$), followed by frequent cleaning with double-DW. Afterwards, adequate concentrations of *Trichoderma virens* and Jasmonic acid were standardised for seed treatment.

5.1 Preparation of spore suspension of *Trichoderma virens*

T. virens with ITCC no. 4177 was raised on PDA for 7 days at 28 ± 2º C in a BOD incubator. Two mycelial plugs from a 7-d old culture of *T. virens* were transported to 250 ml PDB containing Erlenmeyer flask. This flask was incubated at 28 ± 2º C at 100 rpm for 10 days. The mycelial mat was passed through a double layer of sterilized cheesecloth to get the spore suspension. This suspension was further diluted, and the spores were counted through a hemocytometer under the microscope. To this spore

suspension, 1% carboxy methyl cellulose (CMC) was added, serving as an adherent during seed treatment. The stock solution was further diluted to 1×10^6, 1×10^7, and 1×10^8 spores/mL to get the optimum concentration for seed treatment. Sterilized tomato seeds were applied with different concentrations of *T. virens* for 4 hrs (50 seeds in 20 ml) and allowed to germinate in Petri plates at 25 ± 2 ºC and 80 % humidity in a seed germinator.

5.2 Jasmonic acid

A JA stock solution of concentration 10 mM was prepared. This stock was further diluted to attain 0.1, 1.0 and 10 µM concentrations. Sterilized tomato seeds were treated with different concentrations of JA (50 seeds in 20 ml) for 4 hrs and allowed to germinate in Petri plates at 25 ± 2 ºC and 80 % humidity in a seed germinator. Each treatment was maintained with three replicates.

5.3 *In vitro* seedling germination and vigour index under pathogens infection

Sterilized tomato seeds of uniform size were selected and imbibed with 0.1, 1, 10 µM of JA and 1×10^6, 1×10^7, and 1×10^8 spores/mL of *T. virens* for four hours to get the optimum concentration for seed treatment. Then, these imbibed seeds were infected with the desired pathogens inoculum, as described previously, by soaking them in their respective inoculum for 30 minutes. After that, the seeds were washed with distilled water (DW) and then 20 seeds were transferred to each Petri plate for germination. Petri plates were lined with three layers of filter paper made wet with 7 mL of DW. After 10 days, seedlings' growth was measured in terms of root length, shoot length and percentage germination.

In vitro seedling growth promotion activity of JA and *T. virens* was determined according to the value of the seedling vigour index. For this, seed germination percentage and root-shoot length were monitored after 10 days of germination. The seedling vigour index was measured through the formula as defined by (Abdul-Baki and Anderson, 1973).

$$\text{Vigour Index} = (\text{Mean Root Length} + \text{Mean Shoot Length}) \times \text{Germination (\%)}$$

$$\text{Seed germination \%age} = \frac{\text{Total no.of seeds} - \text{no.of seeds germinated}}{\text{Total no.of seeds}} \times 100$$

5.4 Seed Priming and germination

After ascertaining the effective concentration of *T. virens* and JA for seed treatment, tomato seeds were sterilized with 0.01% $HgCl_2$, pursued by frequent cleaning with double-DW and then dried in laminar air flow on sterilized blotting paper (Jain et al., 2012). Thereafter, these dried and surface sterilized seeds were treated by immersing them for 4 hours in 1×10^7 spores/mL in 1% CMC of *T. virens* and 1µM JA. Seed treatment in combination (*T. virens* + JA) comprised an equal volume of individual preparation of *T. virens* and JA. The control ones were treated with distilled water alone. Furthermore, all these seeds were kept in the seed germinator at 80 % relative humidity and 25–27 ºC and kept for 24 h (Jensen et al., 2004). These treated seeds were then grown for thirty days in a plant growth chamber in seedling trays. After this, they were exposed to a selected concentration of pathogens in the field.

6 Experimental design and treatments

The experiment about this study was conducted under open field conditions at the experimental farm at Lovely Professional University, with latitude 31°24.500' NS and longitude 75°69.507' EW (Fig 5.1). The design of the experiment was Random Block Design (RBD), and a total of twelve treatments with three replications each were distributed in a 20×20 m experimental field separated by a 60×90 cm distance from each other. (Fig. 5.2) The following twelve treatments were examined (Table 5.1):

Fig. 5.1 Pictorial presentation of experimental field [A] Field Preparation, [B] Transplantation of tomato seedlings, [C] overview of experimental field and [D] Sample collection for further analysis

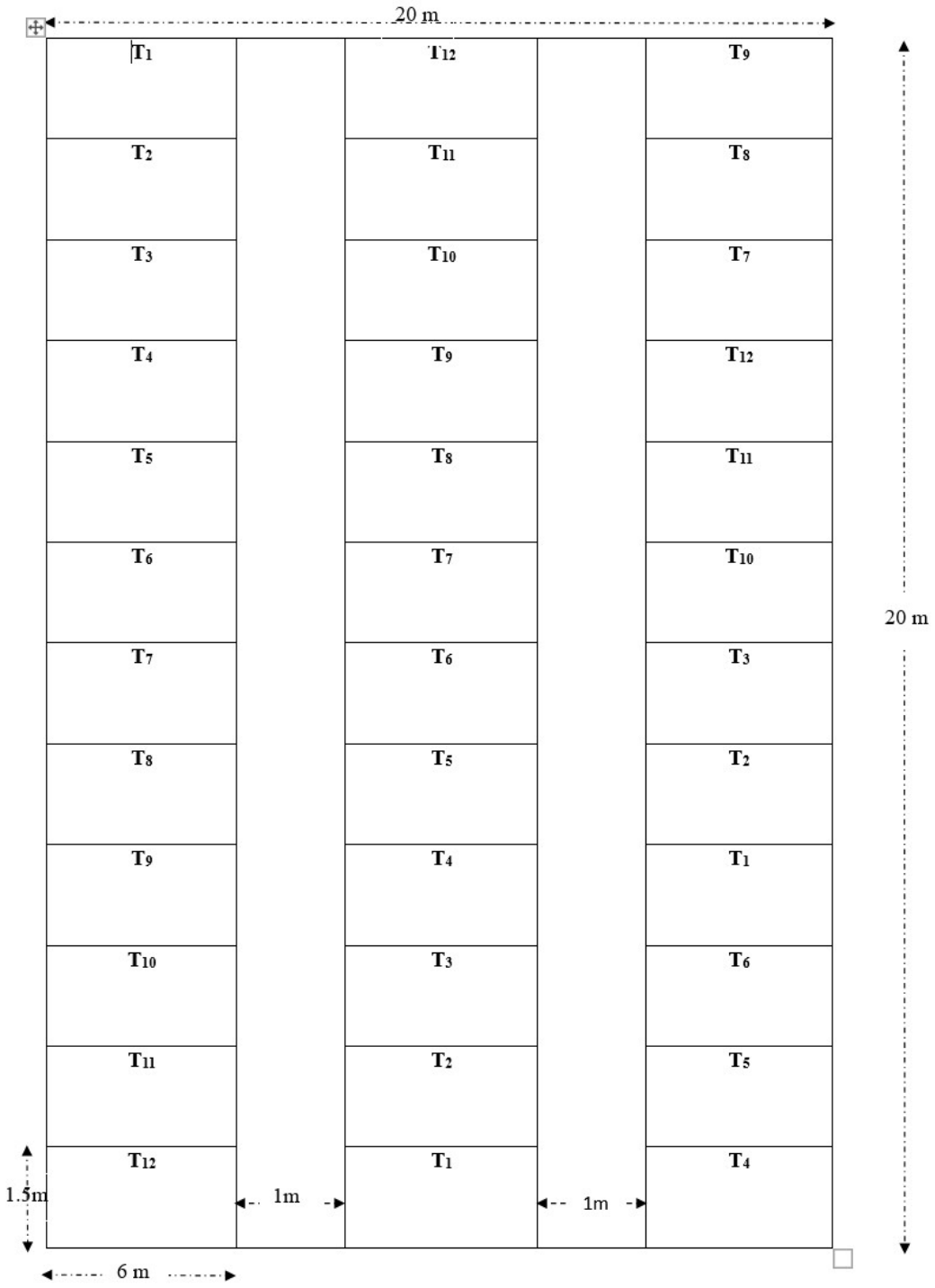


Fig. 5.2 Overview of experimental field layout

Table 5.1 Details of treatments

Treatment	*Fol*	*R. solani*	*T. virens*	JA
T_1 (CN)	-	-	-	-
T_2 (Tv)	-	-	+	-
T_3 (JA)	-	-	-	+
T_4 (Tv+JA)	-	-	+	+
T_5 (Fol)	+	-	-	-
T_6 (Fol+JA)	+	-	-	+
T_7 (Fol+Tv)	+	-	+	-
T_8 (Fol+Tv+JA)	+	-	+	+
T_9 (Rs)	-	+	-	-
T_{10} (Rs+JA)	-	+	-	+
T_{11} (Rs+Tv)	-	+	+	-
T_{12} (Rs+Tv+JA)	-	+	+	+

+ (Present), - (Absent)

A Determination of Morphological Aspects

7.1.1 *Shoot length*

In sixty days old tomato plants, shoot length was determined in cm from the base to the tip of the shoot.

7.1.2 *Root length*

It was determined (cm) from the stem's base to the root's tip and recorded.

7.1.3 *Fresh weight and number of leaves*

These were taken immediately at the time of harvest of plant samples.

7.1.4 *Dry weight*

After the estimation of fresh weight, the samples were placed in a pre-heated hot-air oven, dried at 60 ºC for 6 hours, and obtained dry weight was recorded in g/plant.

7.2 Disease Incidence

After thirty days of pathogen inoculation, the percentage disease incidence (PDI) was determined by using equation:

$$\text{PDI} = \frac{\text{No. of plants}}{\text{Total number of observed plants}} \times 100$$

8. To study stomatal opening/closing through Scanning Electron Microscopy (SEM)

Opening and closing of stomata in tomato leaves under different treatments at 60 days old seedlings were determined by Field Emission scanning electron microscope JEOL.

8.1 Principle

The SEM is based upon the principle that whenever the accelerated primary electrons hit the sample tissue, it produces secondary electrons, which are gathered by a +vely charged electron detector that sequentially gives a 3-D image of the sample.

8.2 Procedure

Leaf tissues from sixty days old tomato plants were dehydrated in ethanol series and then air dried. Then with the help of double-sided carbon adhesive tape, these samples were fixed on metallic supports known as stubs. After fixation, these samples were metalized with gold in JEOL Smart Coater. The observation and electron micrographs were obtained with a JEOL device at Central Instrumentation Facility at LPU.

B Biochemical Aspects

9.1 Chlorophyll Content

Chl. content was estimated as per the method of Lichtenthaler, 1987.

Principle

The basic structure of chlorophyll molecule possesses a phytol chain linked to a porphyrin ring system. In general, chlorophylls exhibit lipid-like properties because of the presence of a phytol side chain. This phytol chain is esterified to the carboxyl group of the ring. As chlorophyll molecules are fat-soluble compounds, they can be disengaged from plant tissue with the help of either pure or aqueous solution of organic solvents like acetone, methanol, or ethanol.

Reagent

Acetone – 80 %

Procedure

250 mg of leaf tissue from the treated and control plant was homogenized with 80% acetone and then subjected to centrifugation at 13,000 rpm for 20 minutes at 4ºC. After that, the supernatant from this plant extract was procured to determine chlorophyll content. The Ab. of the supernatant was pick up at 645 and 663 nm operating a UV-vis. spectrophotometer.

$$\text{Chl a} = [((A_{663} \times 12.7) - (A_{645} \times 2.69)) \times \frac{v}{w}]$$

$$\text{Chl. b} = [((A_{645} \times 22.9) - (A_{663} \times 4.68)) \times \frac{v}{w}]$$

$$\text{Total Chl.} = [((A_{645} \times 20.2) + (A_{663} \times 8.02)) \times \frac{v}{w}]$$

Where v = volume of plant extract in ml, W = fresh weight in grams

9.2 Total carotenoid content (TCC)

TCC was assessed by following the protocol of Maclachlan and Zalik, 1963.

Principle

The carotenoids constitute a chemically diverse group of yellow-red coloured polyenes possessing 3 to 13 double bonds in conjugation. Further, a hydroxylated C6 ring structure at one or both ends has also been reported in a few cases. These peculiar characteristics allow the active delocalization of electrons along the total stretch of the chain. They are responsible for light-harvesting capacity during photosynthesis, pigmentation, and various chemical reactivities. Approximately 700 compounds have been reported under this important plant pigment family, broadly categorized into carotenes and oxygenated derivatives known as xanthophylls. Due to the lipid-like properties of these molecules, they can be easily extracted in an aqueous solution of 80% acetone.

Procedure

250 mg leaf tissues from different plant treatments were homogenized with 80 % acetone and centrifuged at 13,000 rpm for 20 minutes at 4ºC. The OD of the supernatant was read out at 480 and 510 nm utilizing a UV-visible spectrophotometer.

Calculations

$$\text{Total Carotenoid contents} = \frac{7.6\ (\text{O.D.}\,480) - 1.49\ (\text{O.D.}\,510) \times \text{v}}{\text{d} \times 1000 \times \text{w}}$$

Where, v = Volume of plant extract, w = fresh weight and d = path length of cuvette (1cm)

9.3 Total Anthocyanin Content (TAC)

TAC was estimated according to Mancinelli, 1984.

Principle

Anthocyanin comprises an important subclass of flavonoids in plants. These are water-soluble vacuolar pigments accountable for violet, purple, blue, red and scarlet colours of leaves, stems, fruits and flowers. In addition to providing vibrant colours, anthocyanins are also responsible for incorporating adaptation to different types of stressful conditions. They are necessary secondary metabolites in the plant system and

are usually separated using different solvents, particularly acidified methanol solution. It has been suggested that "slight" acidification of solvents avoids the degradation of these compounds.

Procedure

0.5 g leaf tissue with different treatments was homogenized with 1.5 mL of extraction mixture (methanol: water: HCl, 79:20:1). This crumpled material was then centrifuged for about 20 minutes at 13,000 rpm and 4 ºC. Then Ab. was read at 530 and 657 nm through a spectrophotometer.

Calculations

$$\text{TAC} = \text{O.D.}\,530 - 0.25 \times \text{O.D.}\,657$$

9.4 Total flavonoid content (TFC)

TFC was quantified in accordance with the method of Kim et al., 1999.

Principle

Flavonoids are a complex group which comprises flavanones, flavanols, flavones, chalcones, catechins, and anthocyanins. The primary chemical structure of flavonoids is the $C_6C_3C_6$ assembly of flavanone synthesized through the catalytic activity of flavanone synthase enzyme from the condensation reaction of 4-coumaroyl-CoA and malonyl-CoA. For the determination of total flavonoid content, $AlCl_3$ spectrophotometric technique was utilized. It is based upon the principle that $AlCl_3$ makes acid-stable complexes with the C-4 keto group and the hydroxyl group of flavones and flavanols at either the C-3 or C-5 position. Moreover, it also creates labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids.

Procedure

Extract preparation

The plant extract was made by crushing the 1 g leaf sample in 3 mL absolute methanol in a pestle and mortar, trailed by centrifugation at 13,000 rpm for 20 minutes and 4 ºC.

Reagents

NaOH 1mol\L

$NaNO_2$ 5%

$AlCl_3$ 5%

Absolute alcohol

Procedure

1 mL of this supernatant was mixed with 4 mL double DW, 0.3 mL 5% sodium nitrite and 0.3 ml 5% aluminium chloride, followed by incubation for five minutes. After 5 minutes, 2 mL of 1mol\L NaOH and 2.4 mL of DW were added, and absorbance was taken at 510 nm by operating a spectrophotometer.

Calculations

Flavonoid contents were estimated in term of rutin equivalents (mg rutin/ g FW) from standard curve of rutin.

$$C = \frac{c \times v}{m}$$

where 'C' is the total flavonoid content in mg g^{-1} plant extract c, which indicates the quantity of rutin quantified by the calibration curve (mg/mL), 'v' indicates the volume of plant extract in mL and 'm' is the weight of crude plant extract in grams.
Concentrations of extracts were calculated using the following regression equation

$$Y = mx + c$$

Where Y= extract absorbance, m= slope of the calibration curve, x= extract concentration, and c= signified the intercept.

9.5 Gaseous Exchange Parameters by Infra-Red Gas Analyzer (IRGA)

The gaseous exchange of plant leaves was monitored with the help of IRGA (Li-COR 6400). Various parameters were quantified by regulating the air temperature at 25 °C,

air relative humidity at 80–90%, CO_2 concentration and photosynthetic photon flux density (PPFD) at 400 μ mol mol^{-1} and 1000 μ mol $m^{-2}s^{-1}$, respectively.

Principle

Hetero atomic gas molecules like CO_2, NH_3, N_2O, and H_2O absorb infrared (IR) radiation, particularly IR wavebands. An infrared gas analyser measures the decrease in the transmission of IR wavebands due to the existence of gas in between the source of these radiation and a detector. This decline in the transmission is an act of gas concentration. Therefore, the chief role of IRGA is to measure the intensity of CO_2 concentration.

Procedure

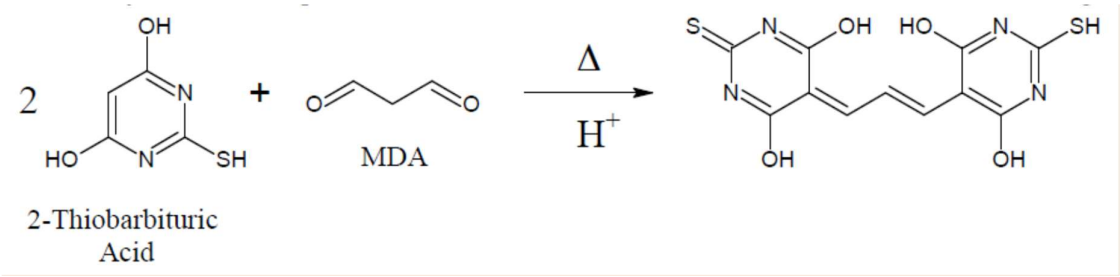
Gaseous exchange parameters comprise PR, TR, SC, and IC in tomato leaves under different treatments detected by an Infrared Gas analyser by maintaining optimum conditions for analysis.

10.1 Malondialdehyde (MDA) content

The MDA content estimation has long been done as a marker of lipid peroxidation in studies associated with oxidative stress and redox signalling as plant reactions to different stresses. Generally, synthesis of MDA content can either be stimulated nonenzymatically through ROS or enzymatically via lipoxygenase activity. However, under both conditions, determining primary lipid hydrogen peroxide compounds is problematic because of their reactivity and instability. Therefore, estimation of lipid peroxidation is generally quantified by calculating the concentration of secondary oxidation products (mostly aldehydes) derived from these primary hydroperoxides.

Principle

In this test, one molecule of MDA reacts with two molecules of TBA, producing peach orange-coloured chromogen exhibiting an absorption maximum at 532 nm.



Procedure

MDA content of tomato leaves with different treatments was quantified according to the Heath and Packer, 1968 method. For this, 1g of plant tissue was homogenized by adding 5 mL of TCA and then centrifuged at 5,000 rpm for 10 minutes. After that, to 1mL supernatant, 6 mL of 20 % (w/v) TCA comprising 0.5 % (w/v) TBA was added. The reaction mixture was warmed to 95ºC for 30 min. and immediately cooled on ice. The absorbance of this was then taken at 532 nm. The nonspecific Ab. was rectified by deducting the Ab. taken at 600 nm. Finally, the MDA content was quantified applying an extinction coefficient, i.e., 155 mM cm^{-1}.

$$\text{MDA} = \frac{\text{Absorbance} \times \text{Total Volume} \times 1000}{\text{Ext. coefficient} \times \text{sample vol.} \times \text{sample weight}}$$

10.2 Hydrogen Peroxide (H_2O_2) content

Principle

The quantitative measurement of H_2O_2 content in stressed plants is vital because of the involvement of H_2O_2 in oxidative cellular destruction and its importance in cell signalling (Neill et al., 2002). In this investigation, H_2O_2 content was quantified by following the method of Velikova et al., 2000. This method is grounded upon the principle that in acidic mediums, potassium iodide (KI) is oxidised by H_2O_2 as per the following equation:

$$2I^- + 2H^+ + H_2O_2 \longrightarrow I_2 + 2H_2O$$

$$I_2 + I^- \longrightarrow I_3^-$$

On adding hydrogen peroxide to a colourless solution of KI, the I^- (iodine ions) are oxidised slowly to I_2 (iodine). Further, I_2 react with I^- to form triiodide I_3^- and develop a reaction mixture with yellow colour. In this manner, H_2O_2 could be estimated spectrophotometrically by determining I_3^- absorption at 390 nm.

Reagents

Potassium phosphate buffer (PPB) – 10 mM

Trichloroacetic acid (TCA)– 1M

Potassium iodide (KI)– 1M

Procedure

500 mg of plant tissue was homogenized with 2 mL of TCA and then subjected to centrifugation at 12,000 rpm for 15 minutes. After centrifugation, to 0.5 mL of supernatant equal volume of PPB (10 mM) and 1 mL of KI (1 M) were added. O.D. was taken at 390 nm.

Calculations

H_2O_2 content was quantified against the standard curve.

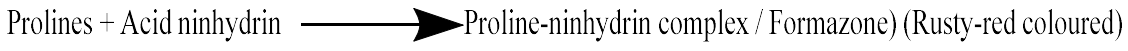
11 Estimation of Osmolytes

11.1 Free Proline content

Proline content was evaluated corresponding to the Bates et al., 1973 method.

Principle

Proline is extracted in Sulphosalicylic acid. This proline was made to react with acidic ninhydrin to form a rusty red colour chromophore having absorption maxima at 520 nm. Proline-ninhydrin complex is hydrophobic and dissolves in an organic solvent, i.e., toluene. As toluene is immiscible in water, two layers or phases formed when we added toluene to a water-based solution, vortexed and allowed it to settle.



Reagents

Sulphosalicylic acid (SSA)- 3%

Acid Ninhydrin- Created by heating 1.25 g ninhydrin in 30 ml GAA and 20 ml 6 M orthophosphoric acid, with stirring, until dissolved

Glacial acetic acid (GAA)

Toluene

Proline

Procedure

For proline estimation, 250 mg of plant sample was homogenized with 3% SSA followed by centrifugation at 10,000 rpm for 10 minutes. 1 mL of obtained supernatant was reacted with 1 mL of freshly prepared ninhydrin and 1 mL of GAA in and then placed for 1 h in a water bath at 100°C. The reaction ceased in an ice bath and was then brought to room temperature. Subsequently, extracted with 2 mL toluene and mixed strongly with a vortex. The chromophore comprising toluene was extracted from the aqueous phase, and Ab. was taken at 520 nm taking toluene as blank. The proline intensity was quantified from the standard curve.

11.2 Glycine-Betaine (GB) Content

GB content was quantified by following Grieve and Grattan, 1983 method.

Principle

Glycinebetaine (GB) is a nitrogenous compound and a quaternary amine which contains zwitterions. It is a chemical having a +vely charged cationic group, such as a quaternary ammonium/ phosphonium cation, with no H atom. Conversely, a functional group containing a negatively charged carboxylate group may not be present in front to the cationic position. It is also recognized as a methyl donor. This method uses periodide to precipitate quaternary ammonium compounds to determine glycine betaine content in a given plant sample.

Reagents

HCl – 2 M

Potassium triiodide (Lugol's Reagent)

1-2 dichloromethane

Procedure

1 g of dried leaf sample was crumpled in 10 mL of distilled water and then filtered through Whatman filter paper (No. 1). 1 mL of this filtrate was mixed with a mL of 2 M HCl. To this mixture, 0.2 mL of Lugol's reagent was added. The constituents were correctly mixed, followed by cooling for 90 min. by using an ice bath with constant shaking. After this, 2 mL of ice cooled DW and 20 mL of C_2H_4Cl were supplemented. The 2 layers formed in the mixture were appropriately combined by giving it a nonstop flow of air. The upper one was thrown away, and the absorbance of the lower one was recorded at 365 nm.

Calculations

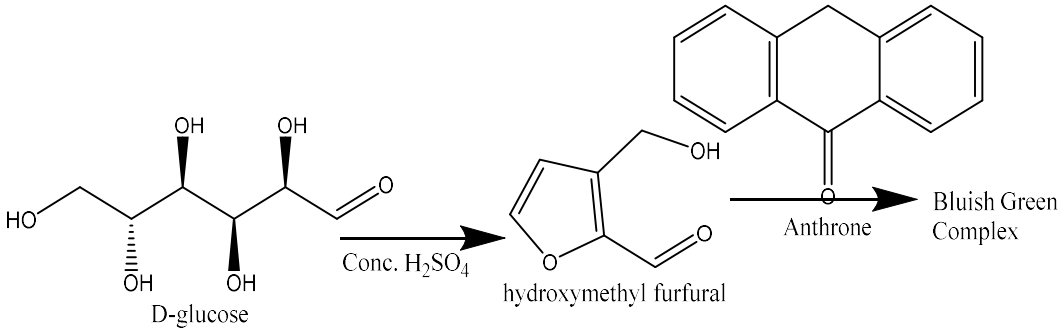
GB content in the given sample was quantified against the value of the standard curve.

11.3 Total soluble sugar (TSS) content

TSS content was spectrophotometrically detected corresponding to the method explained by Scott and Melvin, 1953.

Principle

First, the concentrated acid in the anthrone reagent causes the hydrolysis of carbohydrates (present as poly- or monosaccharides/ free or bound) into their constituent monosaccharides. Correspondingly, the presence of acid activates the dehydration of the monosaccharides to create furfural (in the case of pentose) or hydroxy furfural (from hexose). These furfurals or hydroxy furfurals so formed condense with two naphthol molecules (from anthrone reagent) to produce a blue-greenish colour complex. The concentration of the complex is then quantified by measuring the absorbance at 630 nm.



Reagents

HCl – 2.5 N

Na_2CO_3

Anthrone reagent (0.2 % in conc. H_2SO_4)

Procedure

To 25 mg plant tissue, 1.25 mL of HCl was mixed and allowed to cool down to room temperature. After that, solid Na_2CO_3 was added to it for neutralization, and then with the addition of DW, volume was prepared to 25 mL. To 1 mL of this, 4 mL of freshly prepared anthrone reagent was mixed. This mixture was boiled in a water bath for 8 minutes and then cool down to room temperature. O.D. was logged at 620 nm.

Calculations

The total sugar content in a sample was quantified from a standard curve of glucose solution.

12 Antioxidative Défense System

12.1 Antioxidative Enzymes

Preparation of enzyme extract

1 g plant sample with different treatments was homogenized in a pre-chilled pestle and mortar utilizing 3mL of potassium phosphate buffer (PPB) (100 mM, pH=7). The

crushed sample was centrifuged for about 20 minutes at 13,000 rpm and 4 °C. The supernatant thus procured was utilized for different biochemical analyses.

12.1.1 Protein Content

Protein content estimation was done by ensuing the method of Lowry et al., 1951.

Principle

Determination of protein concentration by the Lowry method is dependent upon the reaction of peptide nitrogen [s] with the Cu [II] ions in an alkaline condition and the succeeding reduction of the Folin- Ciocalteu phosphomolybdic phosphotungstic acid to hetero-poly molybdenum blue via the aromatic acid's oxidation because of Cu-catalyzed reaction.

Reagents

A. 2% Na_2CO_3 in 0.1 N NaOH

B. 1% $C_4H_4O_6KNa \cdot 4H_2O$ (Potassium sodium tartrate) in H_2O

C. 0.5% $CuSO_4.5\ H_2O$ in H_2O

D. Reagent I: 48 ml of A, 1 ml of B, 1 ml C

E. Reagent II- 1-part Folin-Phenol [2 N]: 1 part water

BSA Standard - 1 mg/ ml

Procedure

100 µL of plant extract and a series of the standard were taken into test tubes, and in each tube, the volume was made to 1 mL using DW. A test tube containing 1 mL DW is considered a blank. To each tube, 5 mL of reagent I was added, thoroughly mixed and let to stand for 10 min. After that, 0.5 mL of Reagent II was supplemented and incubated under room conditions for 30 minutes under dark conditions. A blue colour complex was formed. O.D. was taken at 660 nm, and the standard was designed. The protein content present in each sample was quantified from the standard graph.

12.1.2 Guaiacol Peroxidase (GPOX) Activity

Guaiacol peroxidase was quantified by following the method given by Pütter, 1974

Principle

Guaiacol peroxidases are heme-bearing enzymes which catalyze the oxidation of aromatic electron donors like guaiacol at the cost of H_2O_2. In the presence of peroxidase, H_2O_2 is quickly converted to water and oxygen, and this oxygen, thus produced, reacts with guaiacol to generate a brown colour product termed oxidized guaiacol.

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

$$O_2 + \text{Guaiacol} \longrightarrow \text{Oxidised Guaiacol}$$

Reagents

Phosphate buffer – 0.1 M, pH 7.0

Guaiacol solution – 20 mM

H_2O_2 solution – 12.3 mM

Procedure

The total 3 mL of reaction mixture consisted of 0.1 M phosphate buffer, 20 mM guaiacol solution and 12.3 mM H_2O_2 and 30 µL enzyme extract in the test cuvette. The rate of production of oxidised guaiacol was monitored spectrophotometrically at 436 nm.

Calculations

Enzymatic activity is described as the extent of the enzyme catalyzing the development of 1µM of Guaiacol Dehydrogenation Products (GDHP) $min^{-1}g^{-1}$ FW and assessed by using 25.5 mM^{-1} cm^{-1} extinction coefficient. Specific activity was calculated by using the following equation:

$$\text{Specific Activity (U mg}^{-1}\text{ protein)} = \frac{\text{Unit Activity (U min}^{-1}\text{ g}^{-1}\text{ FW)}}{\text{Protein Content (mg g}^{-1}\text{ FW)}}$$

12.1.3 Catalase

CAT activity was assessed by following the method depicted by Aebi, 1974.

Principle

CAT cause the decomposition of H_2O_2 into water and O_2. The decrease in the concentration of H_2O_2 was monitored after incubating the given sample with a known amount of H_2O_2 spectrophotometrically at 240 nm.

$$2H_2O_2 \xrightarrow[240\text{ nm}]{\text{Catalase}} 2H_2O + O_2$$

Reagents

Phosphate buffer – 0.1 M, pH 7.0

H_2O_2 solution – 150 mM

Procedure

The breakdown of H_2O_2 was logged as the rate of decline in absorbance of the 2 mL reaction mixture comprising 0.1 M phosphate buffer, 150 mM H_2O_2 and 30 µL enzyme extract in a cuvette at 240 nm spectrophotometrically.

Calculations

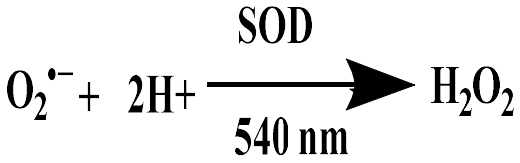
1 UA is quantified as the enzyme content needed to oxidize1 µM hydrogen peroxide $\text{min}^{-1}\text{g}^{-1}$ FW by using 36 μM^{-1} cm^{-1} extinction coefficient. Afterwards, specific activity was calculated.

12.1.4 Superoxide Dismutase (SOD)

The enzyme activity of SOD was measured in accordance with the method of Kono, 1978.

Principle

This method is founded upon the theory that superoxide radicals ($O_2^{\bullet-}$) interact with nitro blue tetrazolium (NBT) causing its reduction from yellow tetrazolium to blue coloured formazan. The presence of SOD in the reaction mixture competes with NBT for $O_2^{\bullet-}$. Therefore, a decrease in the formation of the coloured complex directly corresponds to the amount of SOD present in the reaction mixture.



Reagents

Sodium carbonate buffer – 50 mM, pH 10.0

Nitroblue tetrazolium (NBT) – 96 µM

Triton X-100 – 0.6%

Hydroxylamine Hydrochloride (NH_2OH. HCl) – 20 mM, pH 6.0

Procedure

A reaction mixture comprising 1350 µL of 50 mM Na_2CO_3 buffer, 96 µM NBT, and Triton X-100 (0.6 %) was taken in a test cuvette. The addition of 20 mM NH_2OH commenced the reaction. After the incubation of 2 minutes, 50 µL of plant extract was added to this reaction mixture in the cuvette and the sample's Ab. was noted at 540 nm.

Calculations

1 UA is described as, under the assay conditions, the enzyme content needed to decrease chromogen production's absorbance by 50% at 540 nm in one minute.

12.1.5 APX

The APX activity was quantified by ensuing Nakano and Asada, 1981 method.

Principle

The principle behind this method is that APX catalyzes the reduction of hydrogen peroxide by using ascorbic acid (AsA) as a substrate which leads to its oxidation to

$$AA + H_2O_2 \xrightarrow[290\ nm]{APX} DHA + 2H_2O_2$$

monodehydroascorbic acid (MDHA). Later this MDHA is mechanically unevenly broken to AsA and dehydroascorbate (DHA). The decrease in Ab. at 290 nm relates to the oxidation of AsA.

Reagents

Phosphate buffer – 100 mM, pH 7.0

Ascorbate – 5 mM

Hydrogen peroxide – 0.5 mM

Procedure

In total, 2 mL of the mixture, 100 mM K-buffer, ascorbate (5 mM) and hydrogen peroxide (0.5 mM) were taken in a test cuvette, to which 50 µL enzyme extract was included, and the decrease in O.D. was recorded at 290 nm for 1 minute against a suitable blank.

Calculations

1 UA was determined as the enzyme content essential to oxidize ascorbate (1µM) min^{-1} g^{-1} FW by using extinction coefficient = 2.8 mM^{-1} cm^{-1}. Further specific activity was quantified by dividing the unit activity with protein content (mg g^{-1} FW)

12.1.6 GR Activity

GR activity was quantified according to Carlberg and Mannervik, 1975, method.

Principle

GR preserves the GSH pool in the affected plant by catalysing the NADPH-reliant reduction of GSSG disulphide bond.

$$GSSG + NAD(P)H \xrightarrow[340\ nm]{GR} 2GSH + NAD(P)^{+}$$

Reagents

Potassium phosphate buffer – pH 7.6

Ethylenediaminetetraacetic acid (EDTA) – 3.0 mM

Nicotinamide adenine dinucleotide phosphate (NADPH) – 0.1 mM

Glutathione disulfide (GSSG) – 1mM

Procedure

In total, 2 mL of assay mixture consisting of K-phosphate buffer (50 mM, pH 7.6), EDTA (3 mM), NADPH, oxidized glutathione (1 mM) and 200 µL of enzyme extract in a cuvette. The change in Ab. was monitored for 1 minute at 340 nm.

Calculations

Enzyme activity corresponds to the enzyme needed to oxidise 1 nmol of NADPH in one minute, applying extinction coefficient = 6.2 mM^{-1} cm^{-1}. After that specific activity was calculated.

12.1.7 Dehydroascorbate Reductase (DHAR)

DHAR activity was estimated by following Dalton et al., 1986 method.

Principle

In plants, ascorbic acid is oxidised to DHA employing sequential electron transmissions with MDHA as an intermediate free radical. Thus, by using GSH as an electron contributor, DHA so formed is transformed to AsA by DHAR catalyzed reduction process.

$$DHA + 2\ GSH \xrightarrow[265\ nm]{DHAR} AsA + GSSG$$

Reagents

Phosphate buffer – 50 mM (pH 7)

EDTA – 0.1 mM

Glutathione (GSH) – 1.5 mM

Dehydroascorbate – 0.2 mM

Procedure

In total, 2 mL of assay mixture consisting of K-phosphate buffer (50 mM, pH 7), EDTA (0.1 mM), 0.2 mM dehydroascorbate, reduced GSH (1.5 mM), and 50 µL of tissue extract was taken in a test cuvette. The change in O.D. was recorded for 1 minute at 265 nm.

Calculations

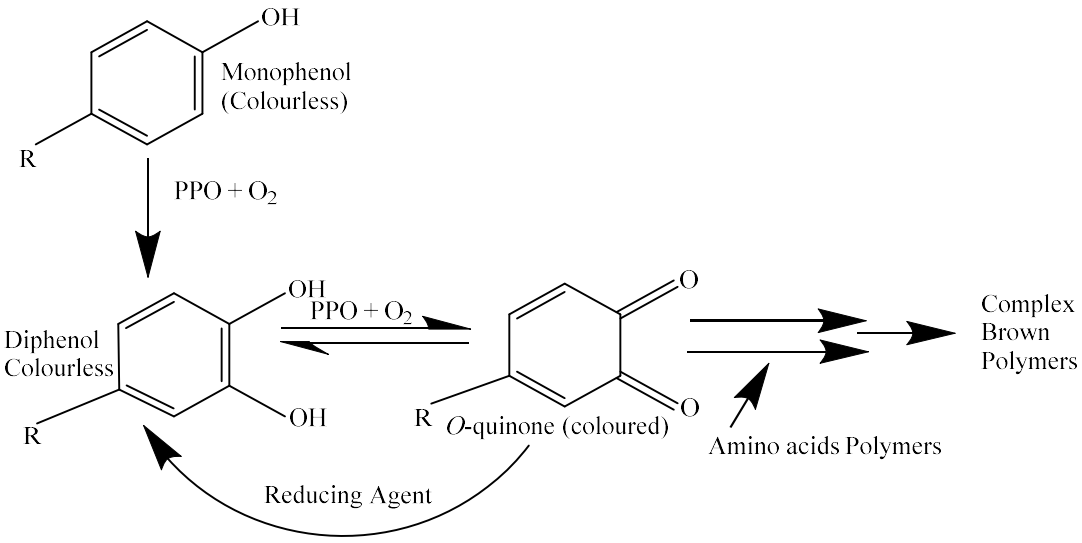
The enzyme activity corresponds to the enzyme content needed to produce 1 µ mol of ascorbate in one minute by employing ε=14 mM^{-1} cm^{-1}.

12.1.8 PPO

The activity of PPO was quantified by following Kumar and PA, 1983 method.

Principle

PPOs are omnipresent Cu-containing enzymes which utilize molecular oxygen for catalytic hydroxylation and dehydrogenation of *o*-diphenolic compounds like catechol and caffeic acid to their respective quinones; which leads to alkylation of nucleophilic groups and self-polymerization to produce dark-coloured melanin polymers.



Reagents

Potassium phosphate buffer - 0.1 M

Catechol - 0.1 M

Sulphuric acid - 2.5 N

Procedure

In total, two mL of the reaction mixture, 0.1 M K-Phosphate buffer, 0.1 M catechol and 100 µL of extract were taken, and then was kept at 25°C for 2 minutes. The reaction was completed by adding 2.5 N sulphuric acid to this, and the change in Ab. was recorded at 495 nm.

Calculations

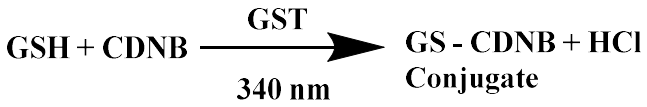
1 UA was expressed as the enzyme essential for the oxidation of 1µM of catechol by using ε=2.9 mM^{-1} cm^{-1}.

12.1.9 GST

GST activity was quantified according to Habig et al., 1974 method.

Principle

The principle behind this method is that GST activates the conjugation of L-GSH to 1-chloro-2,4-dinitrobenzene (CDNB). This conjugate absorbs λ = 340 nm. The rate of enhancement in Ab. directly corresponds to the activity of the GST enzyme.



Reagents

Potassium-phosphate buffer

GSH- 20 mM

1-chloro-2,4-dinitrobenzene (CDNB) – 20 mM

Procedure

The activity of the GST enzyme was determined in 2 mL of the reaction mixture comprising 0.2 M K-phosphate buffer, GSH (20 mM), CDNB (20 mM) and 50 µL enzyme extract. The change in Ab. at 340 nm was logged spectrophotometrically.

Calculations

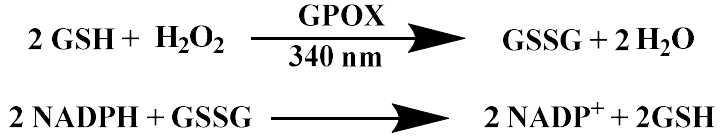
1 UA of GST is labelled as the amount of enzyme that activates the formation of 1 µM of the centrifuged product of CDNB/min/g tissue at 25°C. This was calculated by using ε= 9.6 mM^{-1} cm^{-1}.

12.1.10 Glutathione peroxidase

The activity of the GPOX enzyme was determined corresponding Flohé and Günzler, 1984 method.

Principle

The method is established on the principle that by using GSH as a reducing substrate, GPOX catalyzes hydrogen peroxide reduction and converts GSH to oxidised form GSSG. Later in the presence of NADPH, this GSSG is reduced to GSH form again.



Reagents

Potassium phosphate buffer - 50 mM, pH = 7

Ethylenediamine tetra acetic acid (EDTA) - 0.5 mM

GSH - 1 mM

Sodium azide - 0.15 mM

NADPH - 0.15 mM

H_2O_2 - 0.15 mM

Procedure

In a cuvette, a total of 2 mL of the reaction mixture comprising 50 mM K-phosphate buffer, EDTA (0.5 mM), GSH (1 mM), 0.15 mM NADPH, 0.15 mM H_2O_2 and 30 µL enzyme was taken. The rate of change of Ab. was recorded after 1 minute at 340 nm.

Calculations

1 UA corresponds to the oxidation of 1 µM NADPH/min/g tissue at 25 ºC and is estimated by applying the ε= 6.22 mM^{-1} cm^{-1}.

12.2 Antioxidant

Preparation of plant extract

In a pre-chilled pestle and mortar, 1 g of fresh leaves with different treatments were homogenized with 3 mL Tris buffer (50 mM, pH=10). Then this crushed material was centrifuged in a cooling centrifuge at 13,000 rpm, 4°C for 20 min. This collected supernatant was then utilized for further analysis.

12.2.1

AsA content was quantified by **Ascorbic acid (AsA) Content** pursuing Roe and Kuether, 1943 method.

Principle

In this method, Ascorbic acid content was quantified through a coupling reaction using 2,4 dinitrophenyl hydrazine (DNPH) as a reaction dye. The presence of Cu^{2+} ions cause the oxidation of AsA to dehydro AsA. After that, DNPH reacts with the ketone group of dehydroascorbic acid under strong acidic conditions to form a yellowish orange coloured complex known as osazone. The intensity of formation of

osazone is directly related to the ascorbic acid content in the analysed sample and can be detected spectrophotometrically at 520 nm wavelength.

Reagents

Trichloroacetic acid (TCA) - 50%

2,4 dinitrophenyl hydrazine (DNPH) reagent - 2 g 2,4-DNPH; 250 mg Thiourea; 30 mg $CuSO_4.5H_2O$ in 9N H_2SO_4 (100 mL)

H_2SO_4 - 65%

Standard ascorbic acid - 1 mg 100 mL^{-1}

Procedure

4 mL of double DW, 0.5 mL of 50 % TCA and 0.5 mL of plant extract and 100 mg charcoal were added together. The reaction mixture was then passed through Whatman filter paper 1. 0.4 mL of DNPH was added to this filtrate, and the mixture was incubated at 37º C for three hours, followed by chilling in an ice bath. After that, to this solution, 1.6 mL of 65 % H_2SO_4 was added and kept for 30 min. at RT. Ab. was recorded at 520 nm, and AsA (1mg/100 ml) was consumed as a standard.

Calculation

$$\text{AsA (mg/g tissue)} = \frac{\text{Ab of test} \times \text{conc. standard} \times \text{total vol.}}{\text{Ab of standard} \times \text{vol. of sample taken}}$$

12.2.2 Tocopherol Content

Tocopherol content was determined by following the method of Martinek, 1964.

Principle

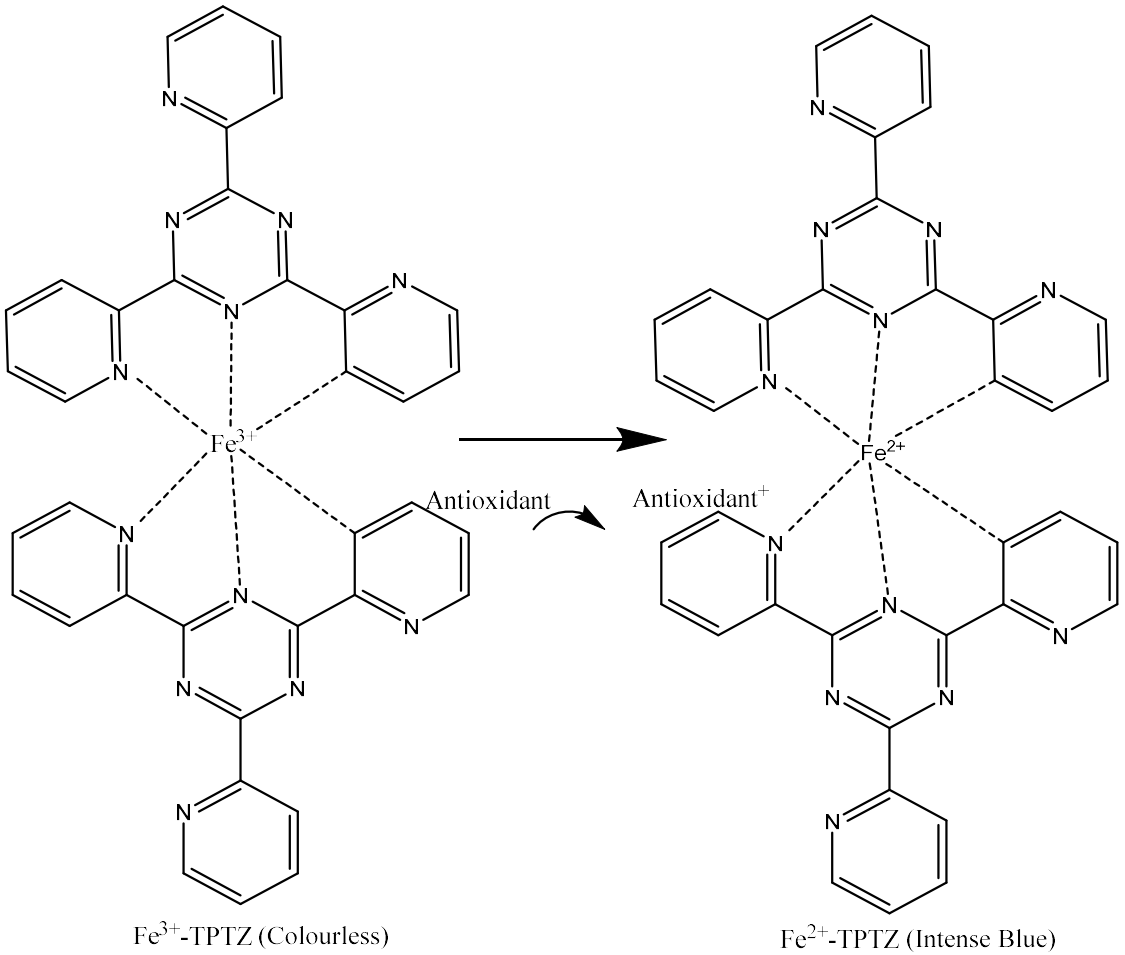
In the presence of tocopherol, ferric ions are reduced to ferrous ions. These reduced ions bind to 2,4,6-tripyridyl-S-triazine (TPTZ). The strength of the colour produced is relative to the concentration of vitamin E in the observed sample and quantified against blank.

Reagents

2,4,6-tripyridyl-S-triazine (TPTZ) - 0.12 % in n-propanol

Ferric chloride - 0.12 % $FeCl_3.6\ H_2O$ in absolute ethanol

Standard tocopherol - 1 mg $100ml^{-1}$



Fe^{3+}-TPTZ (Colourless) Fe^{2+}-TPTZ (Intense Blue)

Procedure

To 1/2 mL of plant extract, 1/2 mL of C_2H_5OH and the same quantity of double distilled water were added, followed by shaking to get divided residues of proteins. After that, 1/2 mL of xylene was inserted, and centrifuge tubes were stirred for 30 sec. vigorously. Then centrifugation was performed for 10 minutes at 3000 rpm. After centrifugation, 1/2 mL of xylene from the top layer was combined with 1/2 mL of

TPTZ reagent and Ab. was read at 600 nm. For tocopherol content estimation, 1 mg/100 mL^{-1} tocopherol was taken as standard.

Calculations

$$\text{Tocopherol (mg/g tissue)} = \frac{\text{Ab of test} \times \text{conc. standard} \times \text{total vol.}}{\text{Ab of standard} \times \text{vol. of sample taken}}$$

12.2.3 Glutathione (GSH) Content

The glutathione content was estimated according to the method given by Sedlak and Lindsay, 1968.

Principle

This method is dependent upon the practice of Ellman's reagent. As per the principle, the presence of -SH groups lead to the reduction of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), which thereby results in the generation of 2- nitro- 5- mercaptobenzoic acid (NMB). The product so formed is brilliant yellow in the shade and supportive in detecting the sulfhydryl group.

Reagents

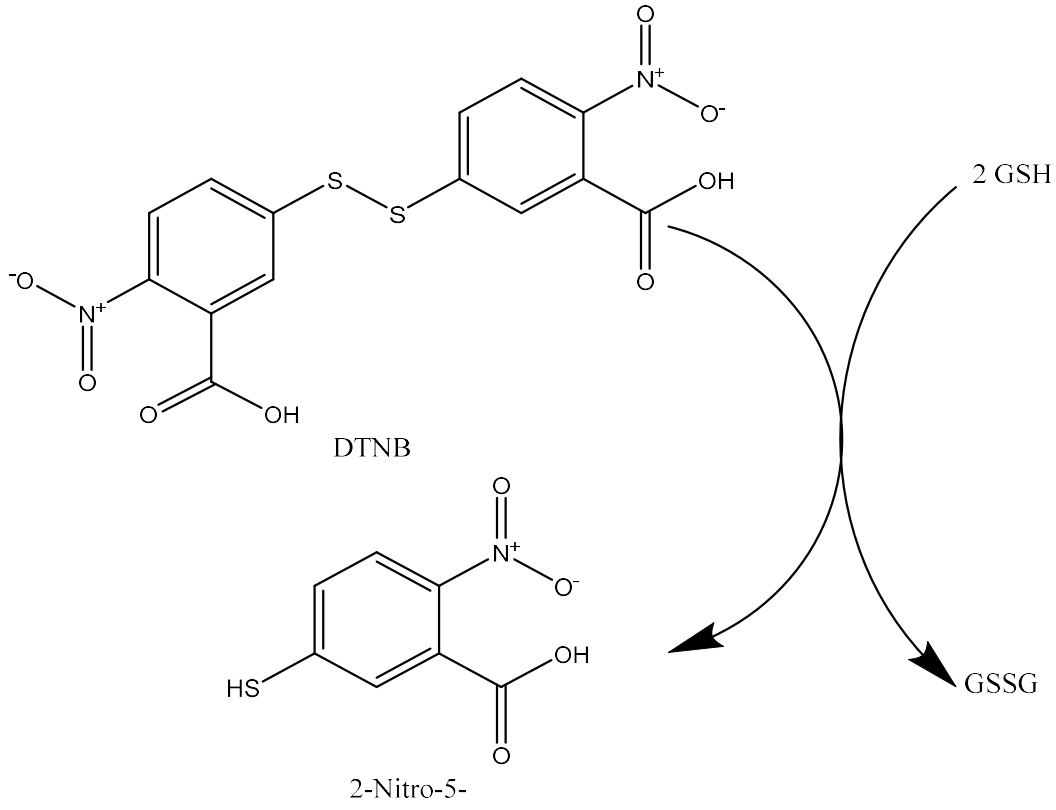
Tris buffer – 0.2M, pH 8.2

5,5'-dithio-bis-(2-nitrobenzoic acid) DTNB – 0.01M

Absolute methanol

Procedure

For GSH content determination, 100 µL of extract was integrated with 1 mL Tris buffer. To this, 50 µL of DTNB and 4 mL of absolute CH_3OH were inserted, and the reaction mixture was allowed to incubate at room temperature for 15 minutes. This step is pursued by centrifugation at 3000 rpm for 15 min. The O.D. of the supernatant was taken at 412 nm and 1mg/100 mL glutathione conc. was utilized as the standard for quantification.



Calculations

$$\text{GSH (mg/ g tissue)} = \frac{\text{Ab of test} \times \text{conc. standard} \times \text{total vol.}}{\text{Ab of standard} \times \text{vol. of sample taken}}$$

12.2.4 Total Phenolic Content (TPC)

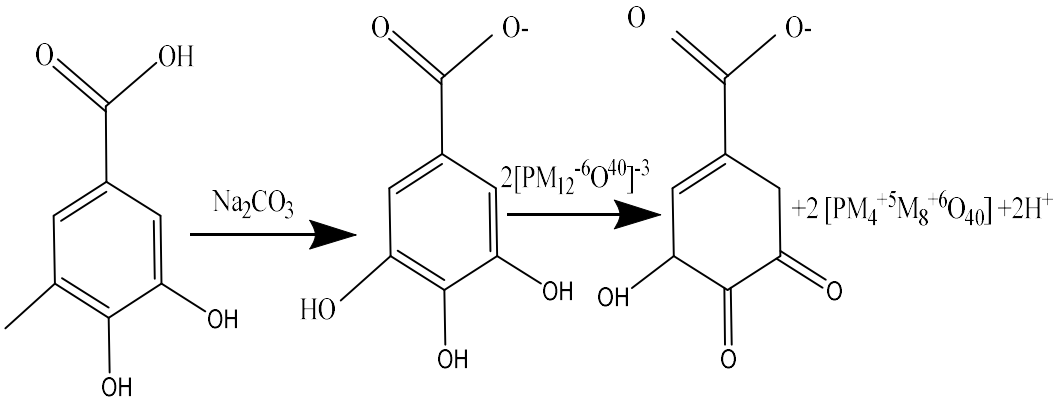
TPC was estimated by following Singleton and Rossi, 1965 method.

Principle

In this method, the Folin-Ciocalteu reagent was used to quantitate the total phenolic content in the given sample. This reagent is developed from a blend of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$), which after oxidation of phenolic compounds reduced to the blue oxides of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}), respectively. With the use of sodium carbonate, this reaction is carried out under alkaline conditions. This atmosphere eases the release of

electrons from phenols. The resulting blue colouration has absorbance maxima at 760 nm.

M=Mo or W



Reagents

Folin-Ciocalteu (FC) reagent

Sodium carbonate –7.5 %

Ethanol – 60%

Procedure

To determine total phenolic contents in given plant samples, 1 g of leaf tissue was homogenized in 5 mL of C_2H_5OH (60 %) and incubated (60 °C) for 30 minutes. From this, 0.25 mL was added to 1.25 mL of FC and after that 1 mL of 7.5 % sodium carbonate was added to this reaction mixture. The mixture was let to incubate for 2 h at RT. Intense blue colour had developed. Ab. was recorded at 765 nm spectrophotometrically.

Calculations

A graph of concentration vs absorbance was plotted with Gallic acid and the total phenolic content was quantified as mg GAE / g FW from the graph.

13. Radical Scavenging Activities

Extract preparation

Plant samples (20 mg) with different treatments were cleaned thoroughly and dried out in an oven. After that, these samples were extracted with continuous shaking in 80 % methanol for 24 hours. Obtained extracts were then filtered with Whatman filter paper (No. 1). supernatant was then utilized for the estimation of radical scavenging activity.

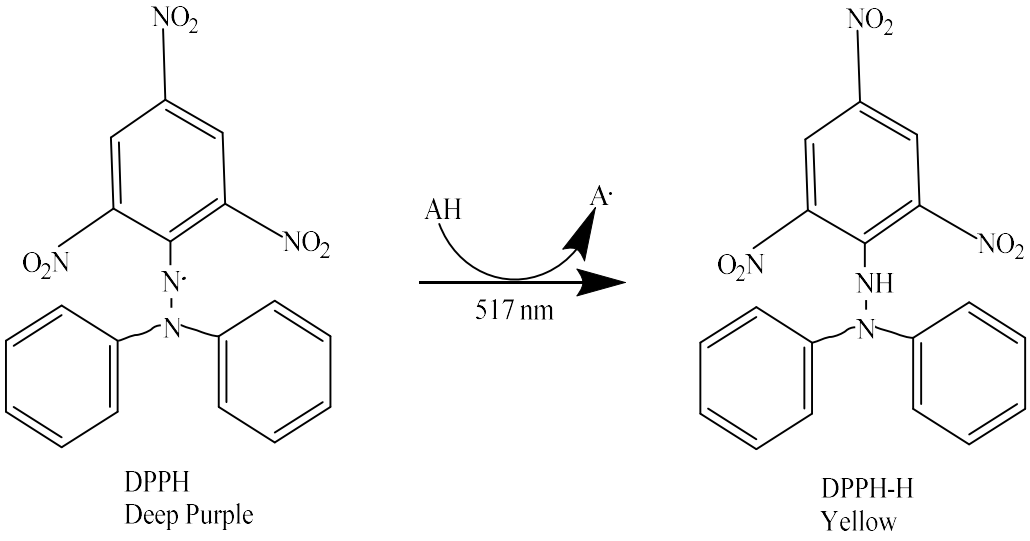
The following activities were performed in tomato leaves subjected to different treatments

13.1 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The assay was accomplished by following the method given by Blois, 1958.

Principle

DPPH is a stable, free, π-radical characterized by its low reactivity mainly due to effective screening of the hydrazyl structure in the compound. This method is based upon reducing DPPH in the methanol solution to DPPH-H in the presence of the H-donating antioxidant group. After accepting hydrogen from a corresponding donor, colour transformation from purple to yellow occurs due to a decline in Ab. This decrease is directly relative to the radical scavenging activity.



Reagents

1,1-Diphenyl-2-picrylhydrazyl – 0.1mM

Methanol – 80%

Procedure

In total 3 mL of the reaction mixture, 100 µL of plant extract was mixed with 0.1 mM DPPH solution in methanol. The reaction mixture was incubated in the dark for twenty minutes, and absorbance was taken at 517 nm.

Calculations

The % age inhibition of DPPH was quantified corresponding to the equation given below

$$\%\ \text{age inhibition} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100$$

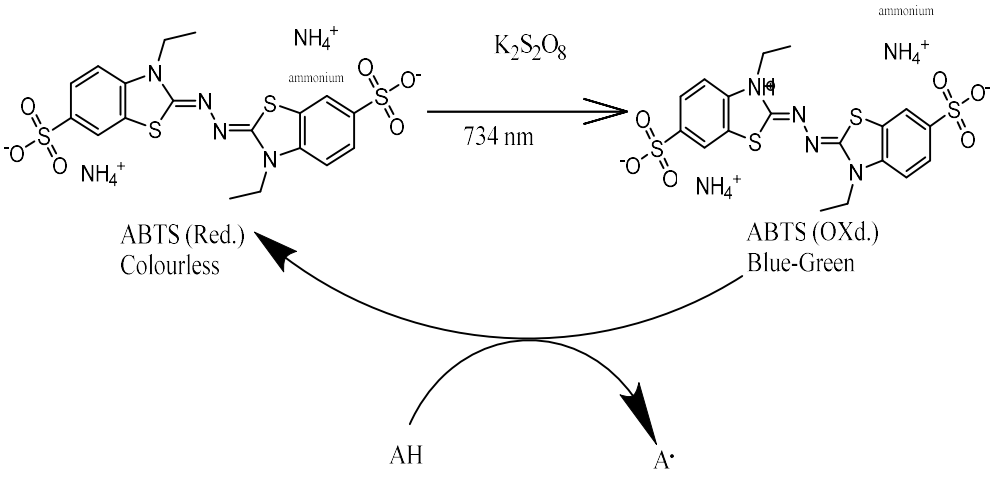
Where Ac = absorbance of the control and As = absorbance of the sample

13.2 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) Radical Scavenging Activity

This assay was executed by following Re et al., 1999 method.

Principle

First, ABTS is oxidised to its stable radical cation as $ABTS^{\cdot+}$ through an oxidising agent known as potassium persulphate. $ABTS^{\cdot+}$ is a blue-green chromophore and exhibits maximum absorption at 734 nm. The level of discolouration of blue-green colour is measured as a rapid decrease in absorbance at 734 nm, based upon the duration of the reaction, innate antioxidant activity, and the concentration of the given sample.



Reagents

ABTS-7 mM

Potassium persulphate-2.45 mM

Ethanol

Procedure

$ABTS^{\cdot+}$ radicals were formed by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulphate in the ratio of 1: 0.5, left in the dark at room temperature for 16 h before use. $ABTS^{\cdot+}$ solution was then diluted with C_2H_5OH to get a working solution with an Ab. of 0.700 at 734 nm. This solution was then added to 20 µl plant extract and the change in Ab. at 734 nm was recorded.

15. Real Time Quantitative Reverse Transcription-PCR (qRT-PCR) Gene Expression Analysis

15.1 Isolation of RNA

The relative gene expression of two antioxidative enzymes i.e., *SOD* and *Catalase* under different treatments was carried out quantitatively in sixty days old tomato leaf tissue. The RNA was extracted by applying the TRIzol method. For RNA isolation, 100 mg fresh leaf samples from treated and control plants were homogenized in a pre-chilled mortar pestle in liquid N_2 and TRIzol reagent. After incubation at 15-30 ºC for 5 minutes, 0.2 mL of chloroform was added to this homogenate and again allowed to incubate for 2-3 minutes at 15-30 ºC. Later these were centrifugated at 12000 rpm for 15 minutes at 4 ºC. Afterwords, this mixture partitioned into a lower-red phenol-chloroform phase, an interphase and a colourless upper RNA containing an aqueous phase. The upper one was transmitted to a fresh tube. RNA was precipitated by adding 0.5 mL of isopropanol and allowed to incubate at RT for 10 minutes and thereafter centrifuged at 10,000 rpm for 10 minutes at 4 ºC. These precipitates form gel-like residue on the sides and bottoms of the tube. The supernatant was discarded, and the pellet thus obtained was cleansed with 1 mL of 75% C_2H_5OH and then dissolved in 50 µL of RNAase-free DEPC-treated water. Furthermore, the extracted RNA was assessed for quantification, integrity and purity using a nanodrop spectrophotometer at an absorption ratio of 230/260/280 nm.

15.2 cDNA Synthesis

A commercial kit by Thermo Scientific was used to synthesize first-strand cDNA from 1.5 µg extracted RNA template by following the manufacturer's instructions. This cDNA was used as a template for further RT-PCR experimentation.

15.3 Real-Time Quantitative PCR analysis

Two stress-associated genes, i.e., *catalase-1* and *Cu-Zn SOD* and one housekeeping gene, i.e., *Actin* were selected, and their respective nucleotide sequence were retrieved from the NCBI database. Nucleotide sequence of gene-specific primers is given in Table 5.2. The RT-PCR reactions were carried out in a 20 µL final reaction mixture containing cDNA (80 ng), each gene-specific primer (400 nM) and 10 µl of 2× SYBR green qPCR master mix. qRT-PCR was duplicated using the Applied Biosystems QuantStudio 5 Real-time PCR system. The RT-PCR programme was as follows: Initial denaturation at 94 ºC for 5 min. then 35 cycles of denaturation at 94 ºC for 30 sec., annealing at 61 (*cat-1*) and 63 ºC (*Actin* and *Cu-Zn SOD*) for 30 sec., extension for 72 ºC for 1 min. and final extension at 72 ºC for 5 min. and then 4 ºC hold.

Table 5.2 Sequence of primers used for qRT-PCR analysis.

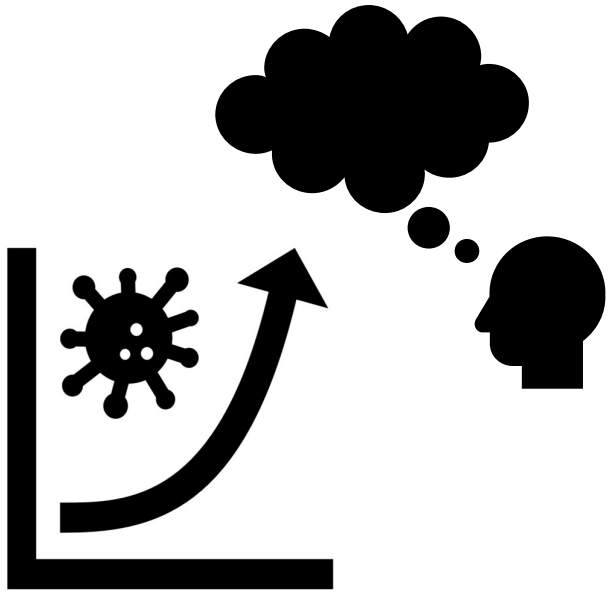
Gene	Accession Number	Primer Sequence (5'-3')	Tm (ºC)	GC %	Annealing Temp. (ºC)
Cat-I	NM_001247898.1	F TGGAAGCCAACTTGTGGTGT	60.03	50.00	61
		R AAGCGACCTTCTGACCACAG	59.97	55.00	
Cu-Zn SOD	NM_001311084.1	F GGTGTTAGTGGCACCATCCT	59.67	55.00	63
		R AGCACCATGCTCCTTACCAG	59.75	55.00	
Actin	NM_001330119.1	F TCCACATGCCATTCTCCGTC	60.11	55.00	63
		R GGTGGAGCGACCACCTTAAT	59.75	55.00	

The mRNA levels were normalized with *Actin* (as endogenous control) as the reference gene. The fold change in gene expression was examined by adopting the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). To determine ΔCT values, CT value of the housekeeping gene was subtracted from the CT values of our target genes.

Statistical Analysis

The data are expressed as the mean of three independent experiments ± standard error. The one-way variance analysis (ANOVA) was performed to test the significance of the observed differences using IBM SPSS Statistics version 24. The differences between the parameters were evaluated by means of the *Tukey post hoc test* and p values ≤ 0.05 were considered as statistically significant.

Chapter 6
Results & Discussion



Results

The present investigation was carried out to check out the seed-priming induced ameliorative potential of *Trichoderma virens* (Tv-Biocontrol agent) and Jasmonic acid (JA-Chemical inducer) individually and in combination against two soil-borne pathogens of tomato viz., *Fusarium oxisporum* f. sp. *lycopersici* (*Fol*) and *Rhizoctonia solani* (Rs). The data were collected at thirty- and sixty-days post-pathogen inoculation. The observations were recorded for morphological, biochemical and molecular aspects.

1. Samplings of Wilt-infected Tomato plants

Samples of Fusarium wilt-infected tomato plants were collected from tomato-growing agricultural fields at Lovely Professional University, Phagwara, Punjab, India. The diseased plants were recognized by monitoring symptoms like wilting, chlorosis, stunting, slight vein clearing, marginal necrosis, epinasty and defoliation of leaves and drooping of petioles (Fig. 6.1 A & B).

Fig. 6.1 Disease symptoms exhibited by tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici* [A] yellowing, browning, and wilting of tomato plants [B] Browning and discoloration of infected stem.

2. Isolation and Identification of Pathogens

Morphological and Cultural Identification

Fol

To isolate the pathogen surface sterilized pieces of the infected stem were placed in PDA-containing Petri plates and allowed to incubate in a BOD incubator.

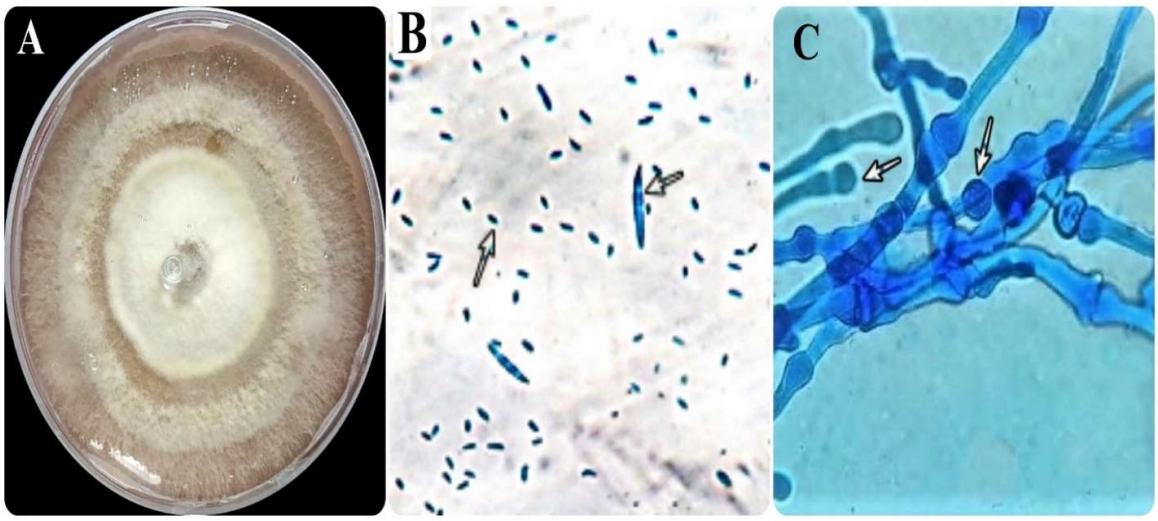


Fig. 6.2 Depicting the Cultural and microscopic observations of *Fusarium oxysporum* f. sp. *lycopersici* [A] Pure culture, [B] Micro and Macro Conidia [C] Chlamydospores.

The fungal mycelium established on these plates was colourless at first, subsequently with time turning cream or pale-yellow coloured, and it also has been observed to produce a pale pink colouration with a purplish tinge (Fig. 6.2 A). This fungus grows three kinds of asexual spores, microconidia, macroconidia and chlamydospores. Microconidia are single-celled, ovoid-ellipsoidal in shape and colourless. Macroconidia are larger and represent typical '*Fusarium*' spores with tapering and curvy ends at both sides. Macroconidia are generally 2-4 septate (Fig. 6.2 B). Chlamydospores are rounded, thick-walled, one or two-celled spores which are either produced terminally or intercalary on aged mycelium or in macroconidia (Fig. 6.2 C). Based on these morphological and cultural characteristics, the isolated pathogen was recognized as *Fol* by following the standard manual of Synder and Hansen (1941). Further Pathogen was identified from IARI with ITCC No. 8111.

Rhizoctonia solani

The pathogenic fungus was procured from the Division of Plant Pathology, Indian Agriculture Research Institute, New Delhi, with ITCC No. 1142. The fungal mycelium does not produce spores but is identified only by mycelial characteristics.

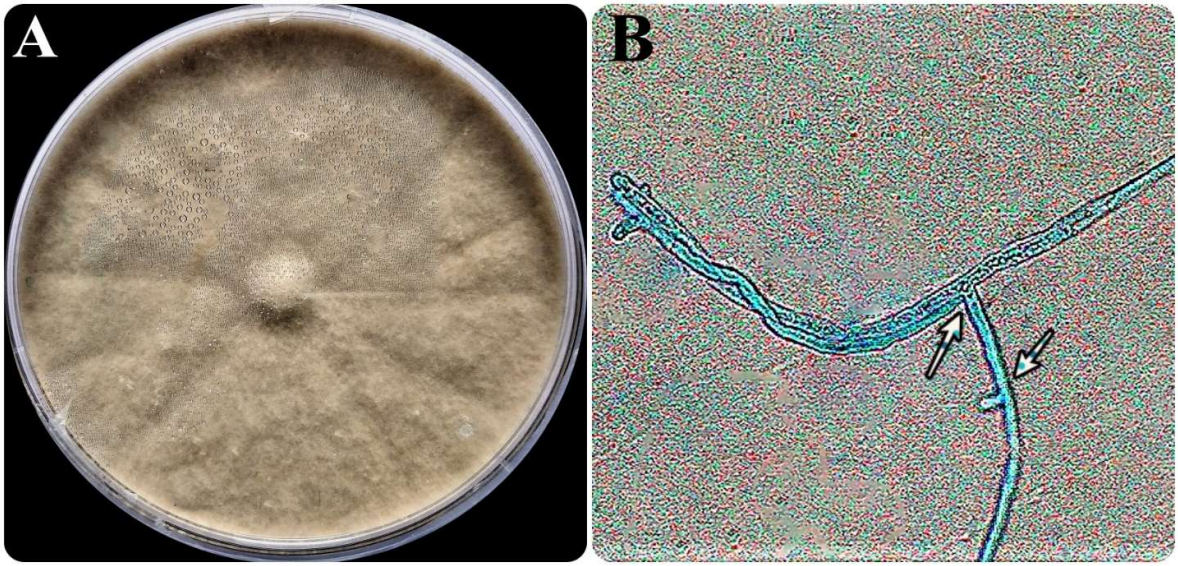


Fig. 6.3 Depicting the Cultural and microscopic observations of *Rhizoctonia solani* [A] Pure Culture, [B] Right-angle branched fungal hypha.

The fungal culture was initially greyish white, turning brown and black afterwards (Fig. 6.3 A). The hyphae produce right-angled branches, and these branches exhibit constriction at the point of origin (Fig. 6.3 B). Furthermore, near the originating point of the branch, a septum has always been reported. In approximately sixty days old culture, the fungus formed microsclerotia of 0.8-1.0 mm in diameter. Based on these cultural characteristics, the fungal species has been identified as *Rhizoctonia solani* following Sneh et al. (Sneh et al., 1991).

3. Pathogenicity Test

Fusarium oxysporum* f. sp. *lycopersici

The Pathogenicity test was performed under Koch's postulates. Thirty days old tomato seedlings were inoculated with *Fol* suspension by the standard root dip method. Typical wilt symptoms like chlorosis of lower tomato leaves occurred within 4-5 days of infection. With the progression of the disease, the leaves were wilted and dried. Further, wilting and drooping of stem tips were also monitored after 10-15 days of pathogen inoculation (Fig. 6.4 A & B).

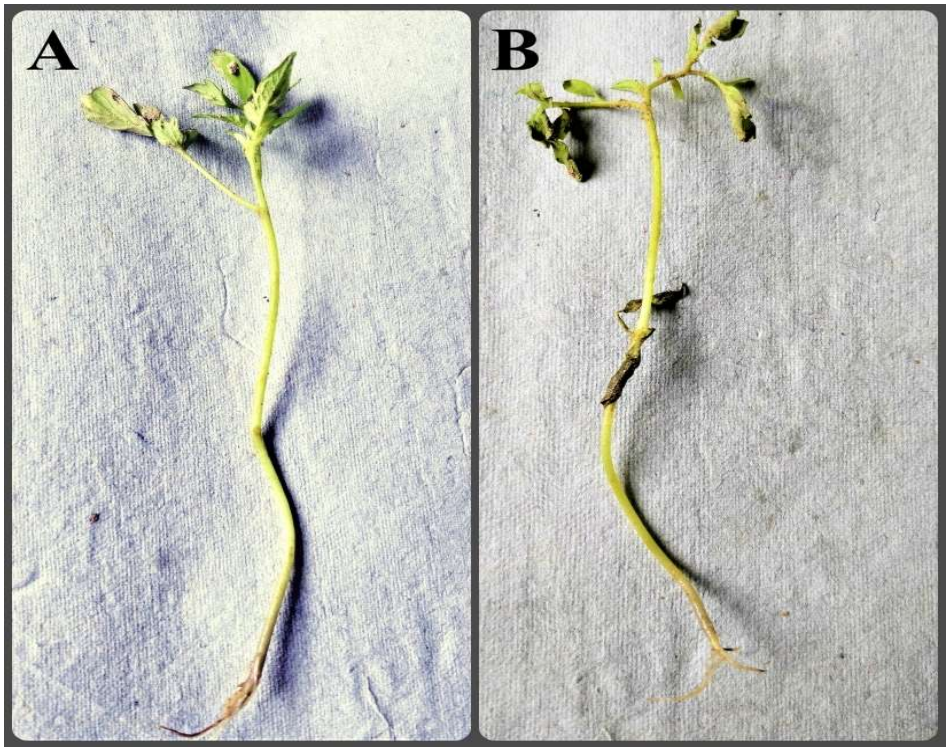


Fig. 6.4 *Fusarium oxysporum* f. sp. *lycopersici* inoculated plants showed symptoms of yellowing and wilting of leaves and stunting [A and B]

In contrast, control plants were healthy and completely free from disease. When the pathogen was re-isolated from the experimental diseased plants exhibited similar kinds of cultural growth and conidial morphology and could reproduce the disease on inoculation again. These results validated the isolated pathogen as *Fol.*

Rhizoctonia solani

To validate the Koch postulates, a pathogenicity test was carried out in tomato seedlings. Thirty days old tomato seedlings were transplanted in *R. solani* pre-inoculated soil under open field conditions. Initially, water-soaked lesions are formed which later transformed into brown colour and enlarged in size to become damaged portions (Fig. 6.5 A). The older leaves on these plants turn yellow. The stem on the damaged region turned dry with typical cracks on them (Fig. 6.5 B). When the pathogen was re-isolated from the experimental diseased plants exhibited similar kinds of cultural growth characteristics as explained in the case of the original pathogen as mentioned previously. Hence these findings validated this pathogen as *Rhizoctonia solani*

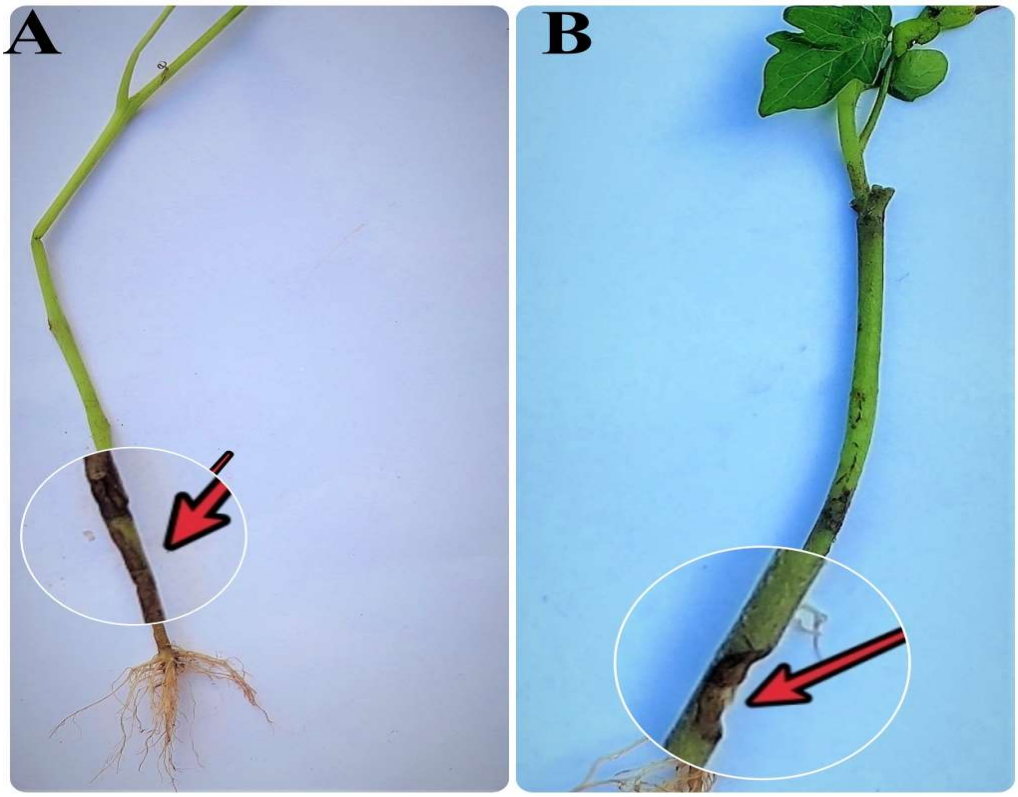


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Fig. 6.5 *R. solani* inoculated plants showed symptoms of [A] Brown colored, water-soaked lesion near collar region, [B] dry sunken stem canker.

4. **Evaluation of antagonistic activities of *T. virens* and *T. viride* against *F. oxysporum* f.sp. *lycopersici* and *R. solani* by dual culture assay**

Two *Trichoderma* spp. i.e., *T. virens* (Fig. 6.6 A) and *T. viride* (Fig. 6.6 B) were screened for their antagonistic activity against *Fol* and *R. solani*. Efficacy of two spp. of *Trichoderma* i.e., *T. viride* and *T. virens* against pathogens were determined by performing dual culture (Fig. 6.7 & 6.8) assay.

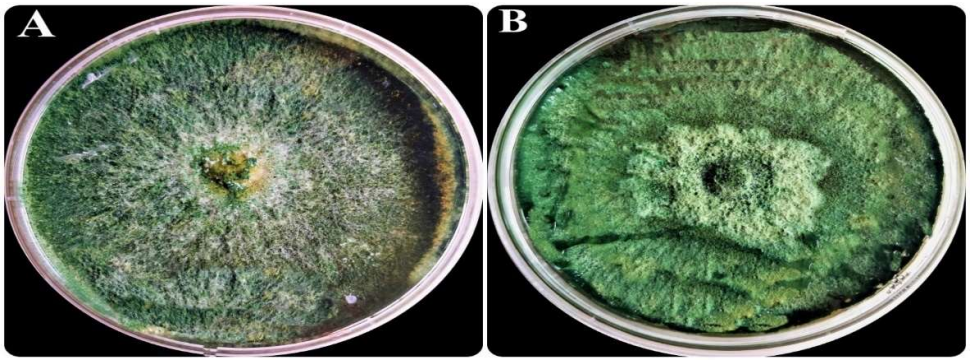


Fig. 6.6 Pure culture of *Trichoderma* spp. [A] *T. virens* [B] *T. viride*

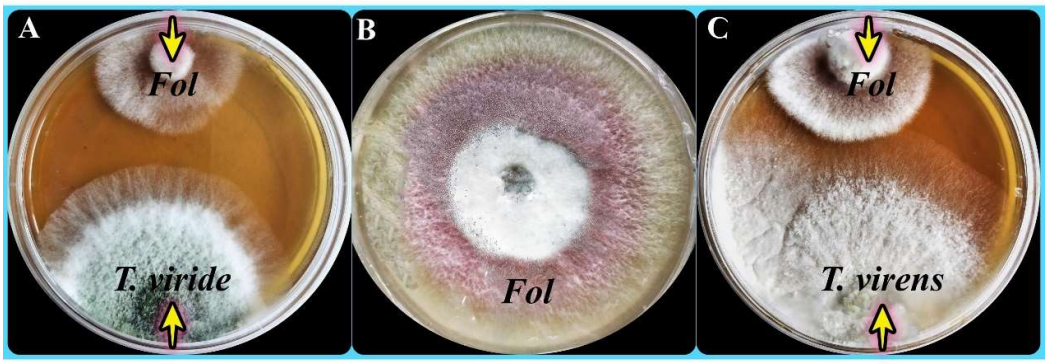


Fig. 6.7 Antagonistic activity of *Trichoderma* spp. against *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) in dual culture assay. [A] *T. viride* + *Fol*, [B] Control (*Fol*) and [C] *T. virens* + *Fol*.

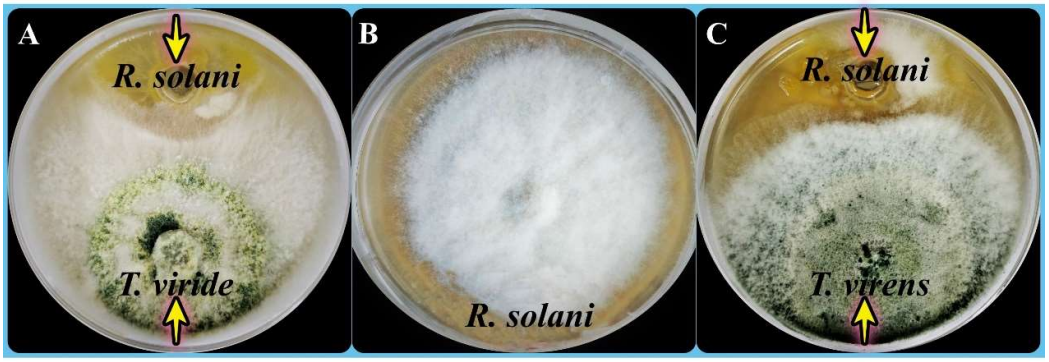


Fig. 6.8 Antagonistic activity of *Trichoderma* spp. *Rhizoctonia solani* (*R. solani*) in dual culture assay. [A] *T. viride* + *R. solani*, [B] Control (*R. solani*) and [C] *T. virens* + *R. solani*.

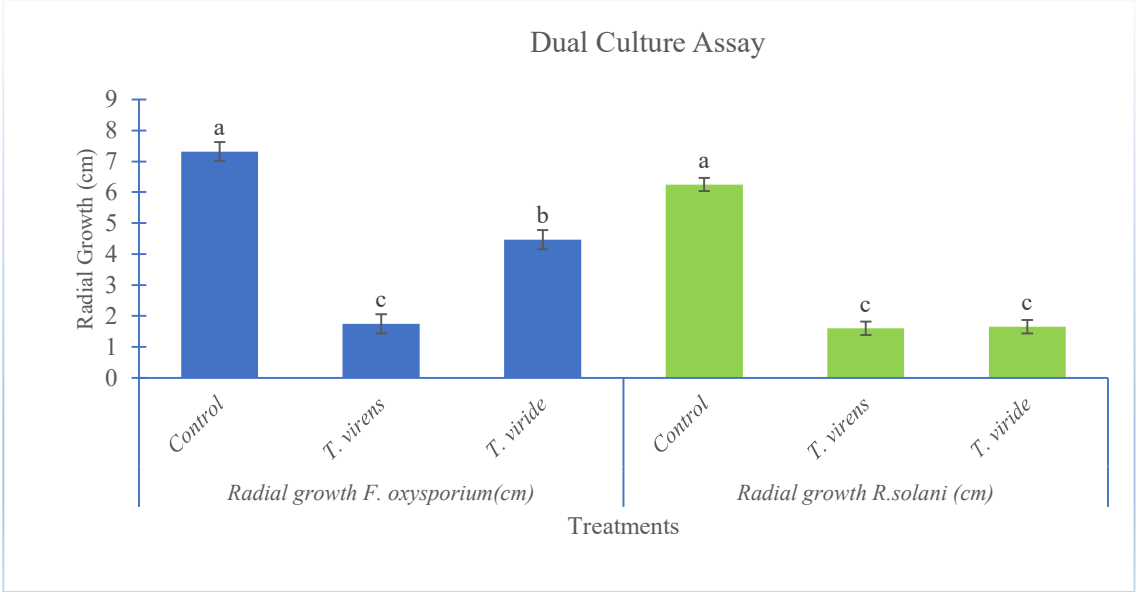


Fig. 6.9 Screening of *Trichoderma* spp. against pathogens in dual culture technique

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

As compared to the control, *T. virens* and *T. viride* cause a significant mycelial growth reduction to the magnitude of 76.2, 39 % in the case of *Fol* and 78.9, 73.6 % in the case of *Rhizoctonia solani*, respectively, but statistically non-significant differences were observed between *T. virens* and *T. viride* (Fig. 6.9)

5. Screening of *Trichoderma* spp. for the synthesis of chitinase, an extracellular enzyme assay

The chitinolytic activity was assessed by determining the release of reducing saccharides from colloidal chitin. Two *Trichoderma* spp. i.e., *T. viride* and *T. virens* were grown on basal chitinase detection medium with colloidal chitin as a sole source of carbon. Out of two observed spp. of *Trichoderma, T. virens* exhibited approximately 16 percent more chitinase activity than *T. viride* (Fig. 6.10 & 6.11).

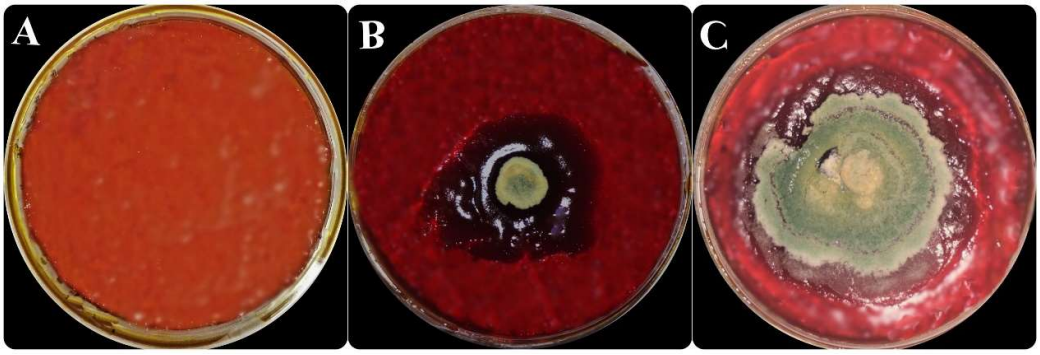


Fig. 6.10 Comparison of chitinase activity of two *Trichoderma* spp. with control. [A] Control (without *Trichoderma*) [B] *T. virens* [C] *T. viride*.

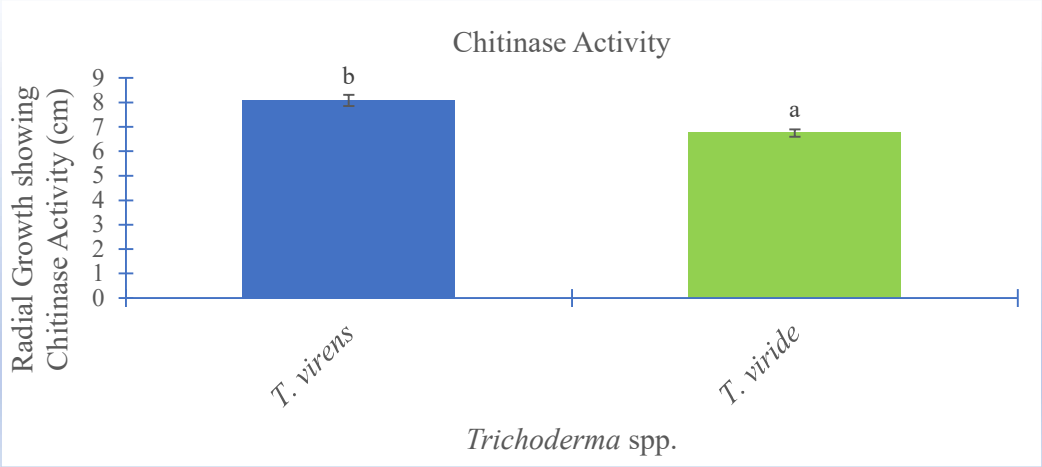


Fig. 6.11 Screening of *Trichoderma* spp. for chitinase activity on medium supplemented with colloidal chitin.

Bars represent the Standard Error of Mean [n=6]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

6. Effect of different concentrations of JA and *T. virens* on in vitro seed germination and seedling vigour of tomato seeds under pathogen infection

In comparison to non-primed tomato seeds, pre-treatment with *T. virens* and JA had significant improvement in mean root-shoot length, percentage germination and vigour index. Under *Fol*-challenged tomato seedlings, the greatest improvement in vigour index was achieved through priming with 1µM JA and 1×10^7 spores/mL *T. virens*. A similar trend of improvement has been repeated by *R. solani*-infected tomato seedlings with the same concentrations of *T. virens* and JA (Table 6.1).

Table 6.1 Effect of different concentrations of JA and *T. virens* on *in vitro* seed germination and seedling vigor of tomato seeds under pathogen infection.

Values shown are means followed by ± Standard Error of Mean (n = 15). Different lower-case letters in the table indicate significant differences between treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Pathogen	Priming agent	Mean Root Length (cm)	Mean Shoot Length (cm)	Percentage Germination	Vigour Index
Fusarium oxysporum lycopersici	**Jasmonic Acid (µM)**				
	0	2.12^{a} ± 0.15	4.17^{a} ± 0.10	63.33^{a} ± 4.41	398.35
	0.1	2.34^{a} ± 0.07	5.10^{b} ± 0.09	76.67^{ab} ± 4.41	570.43
	1	3.01^{b}± 0.13	6.03^{c} ± 0.12	90.00^{b} ± 2.89	813.6
	10	2.91^{b}± 0.14	5.49^{b} ± 0.14	85.00^{b} ± 2.89	714
	***T. virens* (Spores/ mL)**				
	0	2.12^{a} ± 0.15	4.17^{a} ± 0.10	63.33^{a} ± 4.41	398.35
	10^{6}	2.55^{a} ± 0.06	5.22^{b} ± 0.11	81.67^{b} ± 1.67	634.58
	10^{7}	3.30^{b} ± 0.11	6.19^{c} ±0.12	90.00^{b} ± 2.89	854.1
	10^{8}	3.14^{b} ±	5.90^{c} ±	85.00^{b} ± 2.89	768.4

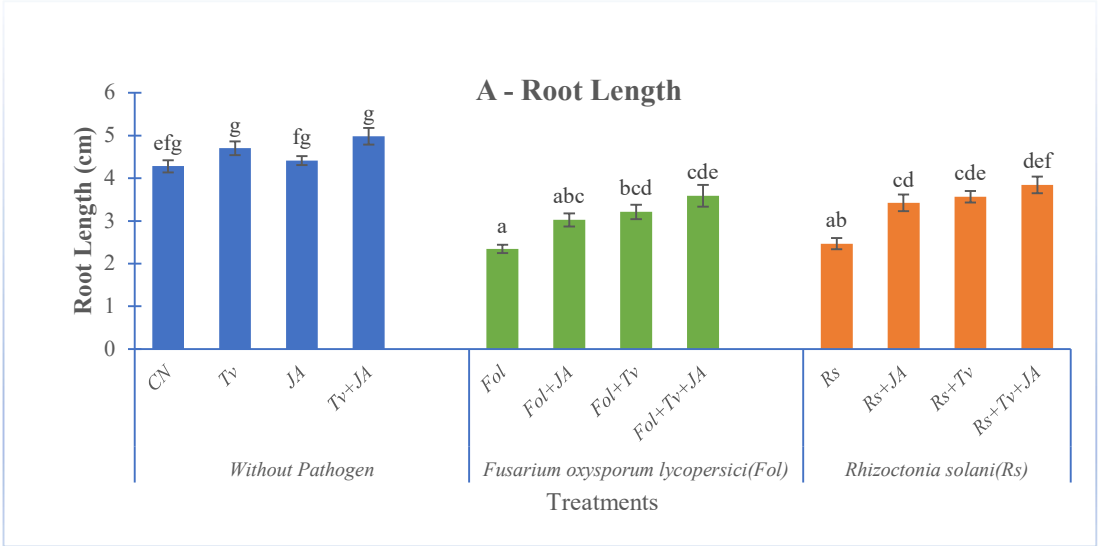
		0.14	0.11		
Rhizoctonia solani	**Jasmonic Acid (µM)**				
	0	$1.89^{a} \pm 0.13$	$3.99^{a} \pm 0.10$	$61.67^{a} \pm 6.67$	362.62
	0.1	$2.21^{a} \pm 0.09$	$5.01^{b} \pm 0.09$	$80.00^{ab} \pm 2.89$	577.6
	1	$2.95^{b} \pm 0.13$	$5.79^{c} \pm 0.15$	$85.00^{b} \pm 2.89$	742.9
	10	$2.71^{b} \pm 0.15$	$5.52^{c} \pm 0.14$	$85.00^{b} \pm 2.89$	700.0
	***T. virens* (Spores/ mL)**				
	0	$1.89^{a} \pm 0.13$	$3.99^{a} \pm 0.10$	$61.67^{a} \pm 6.67$	362.62
	10^{6}	$2.45^{b} \pm 0.10$	$5.19^{b} \pm 0.09$	$83.33^{b} \pm 4.41$	636.64
	10^{7}	$3.04^{c} \pm 0.12$	$5.95^{c} \pm 0.14$	$88.33^{b} \pm 1.67$	794.09
	10^{8}	$3.07^{c} \pm 0.12$	$5.76^{c} \pm 0.10$	$85.00^{b} \pm 2.89$	750.55

7. Morphological Aspects

After thirty days of pathogen inoculation, morphological parameters viz., shoot-root length, fresh-dry weight and the number of leaves were recorded in sixty days old tomato plants under different treatments.

Root-Shoot Length

Significant variations were observed among root and shoot length in sixty days old treated/untreated tomato plants. When compared with the control, a remarkable decrease in root length was detected in tomato plants infected with *Fol* (45.2%) and *R. solani* (42.3%). Pre-treatment of tomato seeds with *T. virens* and JA - either individually or together, brought significant improvement in root length in infected plants as observed in the case of *Fol*-treated plants. Enhancements of 29, 37 and 53% were observed in the case of treatments with Fol+JA, Fol+Tv and Fol+Tv+JA, respectively. A similar trend of significant increase has also been perceived in the case of *R. solani* inoculated tomato plants with the treatments Rs+JA (38.7%), Rs+Tv (44.6%) and Rs+Tv+JA (55.9 %), respectively (Fig. 6.12 A). In continuation to root length observations, shoot length was also observed to decrease in *Fol* (21.6 %) and *R. solani* (24%) challenged tomato plants. However, the most significant improvement in shoot length was also observed in the treatments Fol+Tv+JA (17.6%) and RS+TV+JA (16.7%) (Fig. 6.12 B).



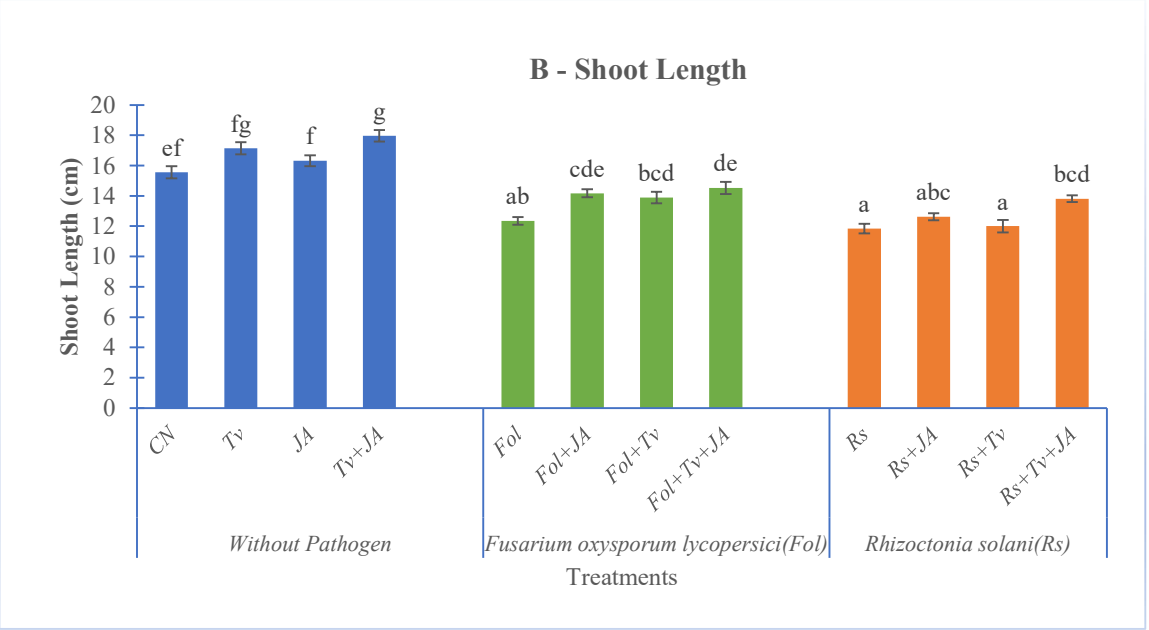
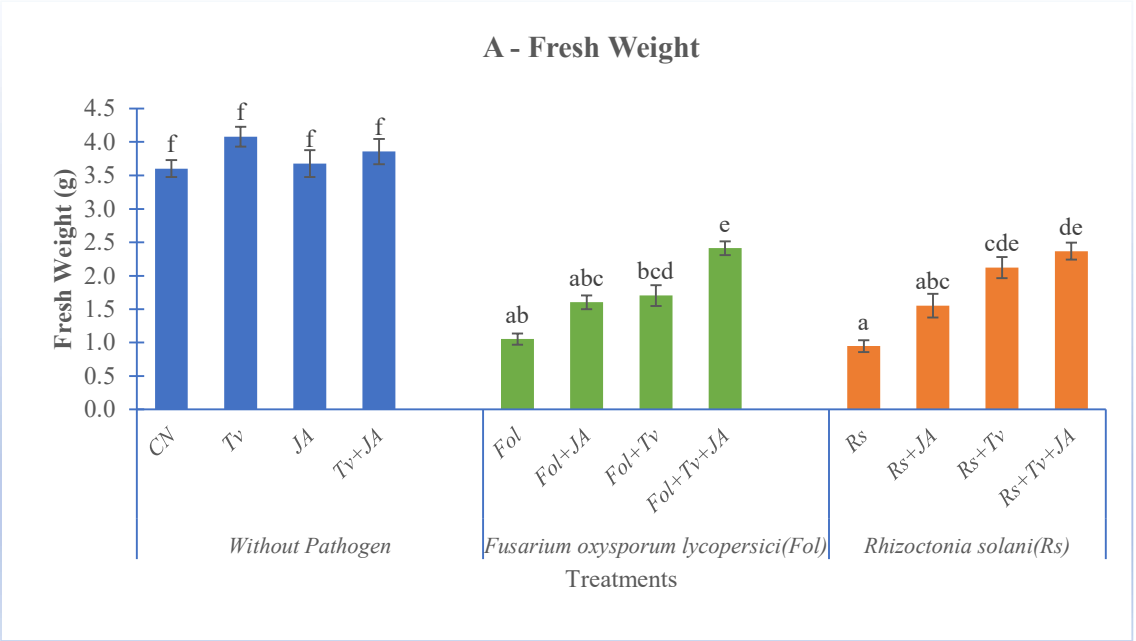


Fig. 6.12 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on [A] Root length and [B] Shoot length in sixty days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=9]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Fresh-Dry Weight

Like root-shoot length, tomato plants' fresh and dry weights were also affected adversely due to pathogen-induced damage to them. A significant decrease in fresh (70.8 and 73.7 %) and dry weight (73.6 and 76.5%) was monitored in the case of tomato plants challenged by *Fol* and *R. solani*, respectively. In comparison to pathogens-stressed plants, maximum fresh weight improvement of 2.3 and 2.5-fold was observed in the treatments with Fol+Tv+JA and Rs+Tv+JA, respectively (Fig. 6.13 A). A similar pattern of a significant reduction in dry weight was also observed in pathogen infected tomato plants. However, after the application of bio stimulators, a similar trend of enhancement in dry weight has also been repeated in *T. virens* and JA-primed tomato plants under different treatments Fol+Tv+JA (123%), Rs+JA (83.9%), Rs+Tv (139.7%) and Rs+Tv+JA (137.6%) (Fig. 6.13 B).



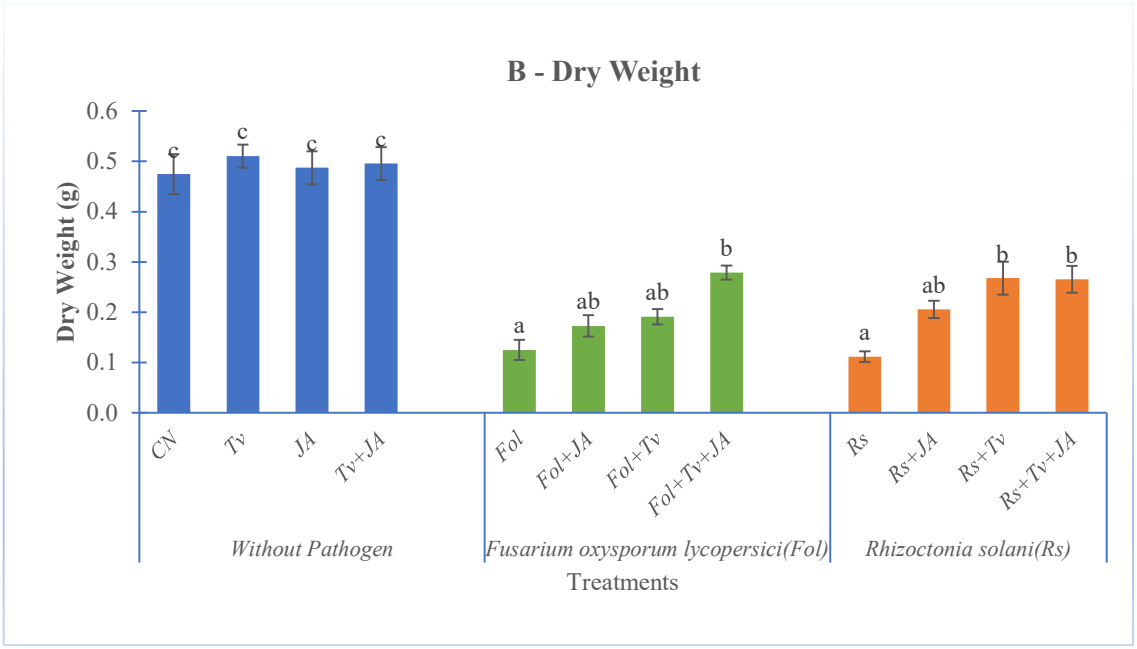


Fig. 6.13 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on [A] Fresh and [B] Dry weight in sixty days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=9]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Number of Leaves

As observed in the previous growth parameters, a significant increase in the number of leaves was observed in JA+ Tv seed-primed tomato plants under pathogen inoculation. In pathogen-challenged plants, a percentage reduction of approximately 43 and 46% was observed in the case of *Fol* and *R. solani* infected plants, respectively. However, due to seed priming, a slight improvement of 25% was observed in the treatments with Fol+Tv and Fol+Tv+JA while a remarkable improvement of 37% was achieved in the treatment with Rs+Tv+ JA in the case of *R. solani* diseased tomato plants (Fig. 6.14).

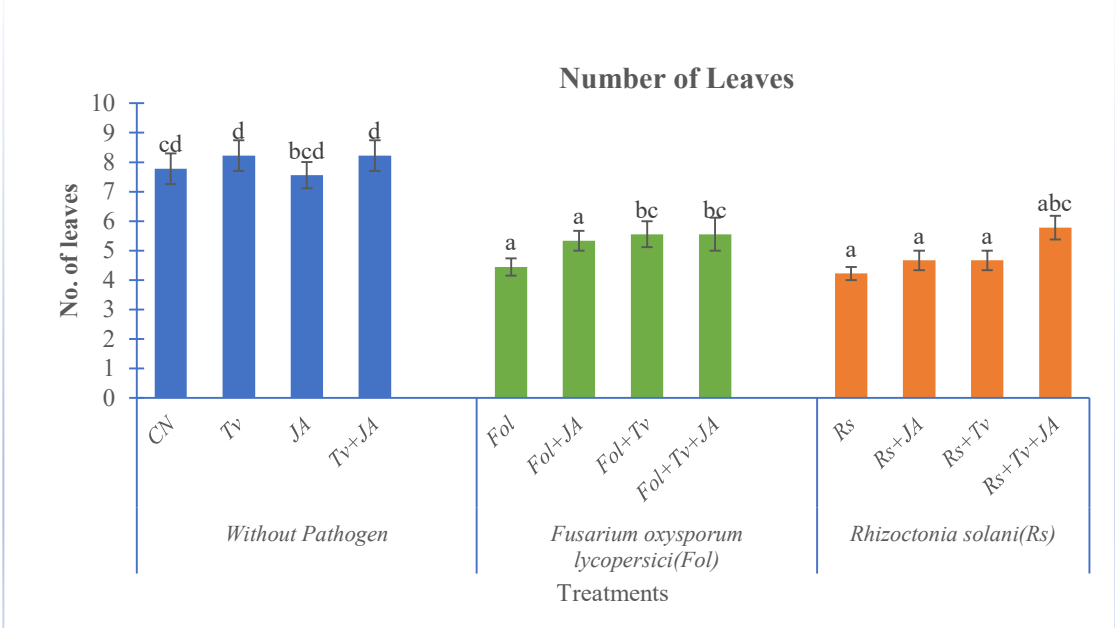
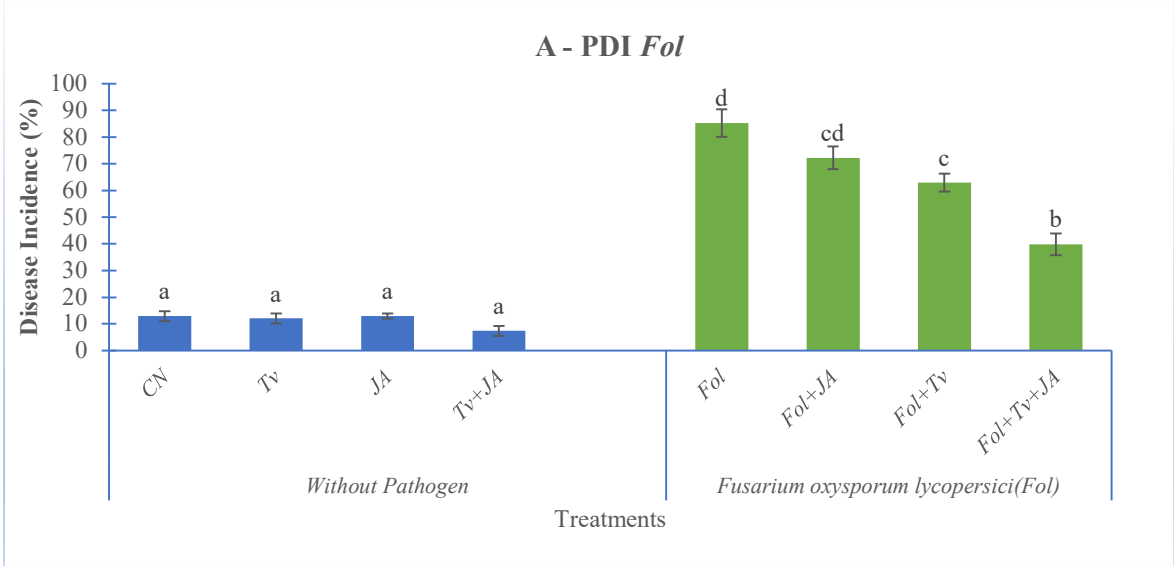


Fig. 6.14 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on number of leaves in sixty days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=9]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

8. Disease Incidence

The extent of disease suppression in tomato plants through pre-treatment with *T. virens* and JA was revealed by the reduction of wilt and damping-off incidence in tomato plants infected with *Fol* and *R. solani,* respectively. Our results showed that the highest PDI of 85 % was detected in pathogens challenged plants. It has been further observed that priming the tomato seeds with *T. virens* and JA, alone or in combination, significantly reduces the PDI when compared to pathogen-inoculated plants. However, in the case of *Fol*, the lowest PDI (40%) was observed in the case of treatment with Fol+Tv+JA (Fig. 6.15A). A similar trend of reduction has been followed by treatment with Rs+Tv+JA with the PDI falling to 42 % (Fig. 6.15 B).



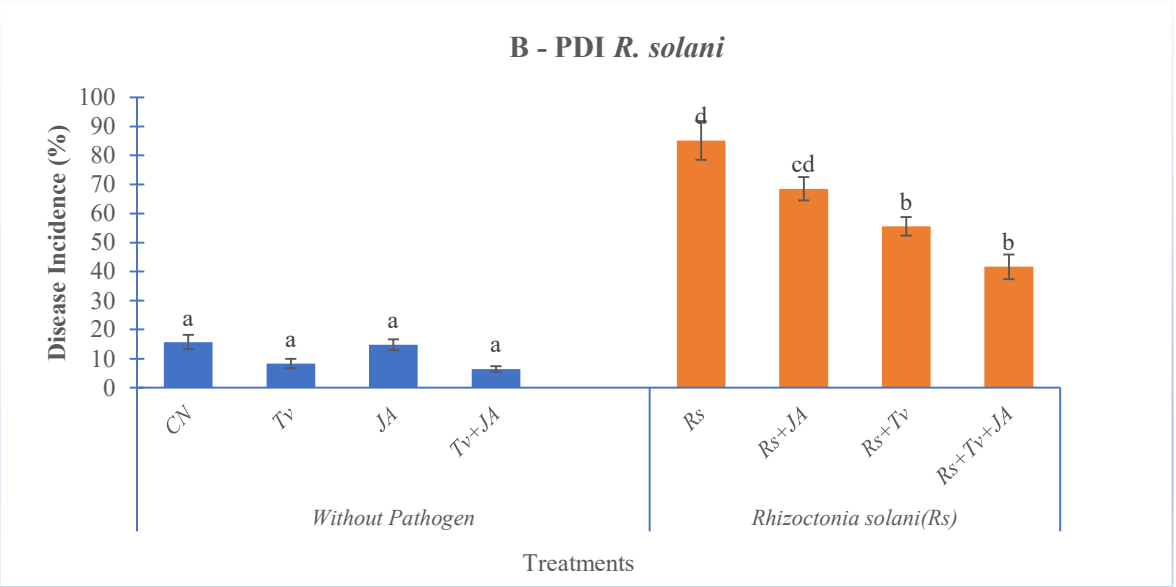


Fig. 6.15 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Percentage Disease Incidence (PDI) of [A] *F. oxysporum lycopersici* and [B] *R. solani* and in sixty days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=36]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

9. Stomatal opening and closing through the Scanning Electron Microscopy

In the present investigation lower surface of tomato leaves under different treatments was scanned with SEM. Under control conditions, the leaf cells have normal stomata with characteristics of guard cells. Pathogen-induced damage in tomato leaves leads to stomatal closure as depicted in (Fig 6.16 A-E). Furthermore, fusarium wilt and damping off diseases also resulted in stomata being remarkably shorter and narrower in addition to this exhibiting smaller aperture size. However, priming of tomato seeds with combined treatment of *T. virens* and JA in reduced the pathogen-induced damage to tomato leaves depicted in terms of partially recovered stomatal response and comparatively opened stomatal aperture.

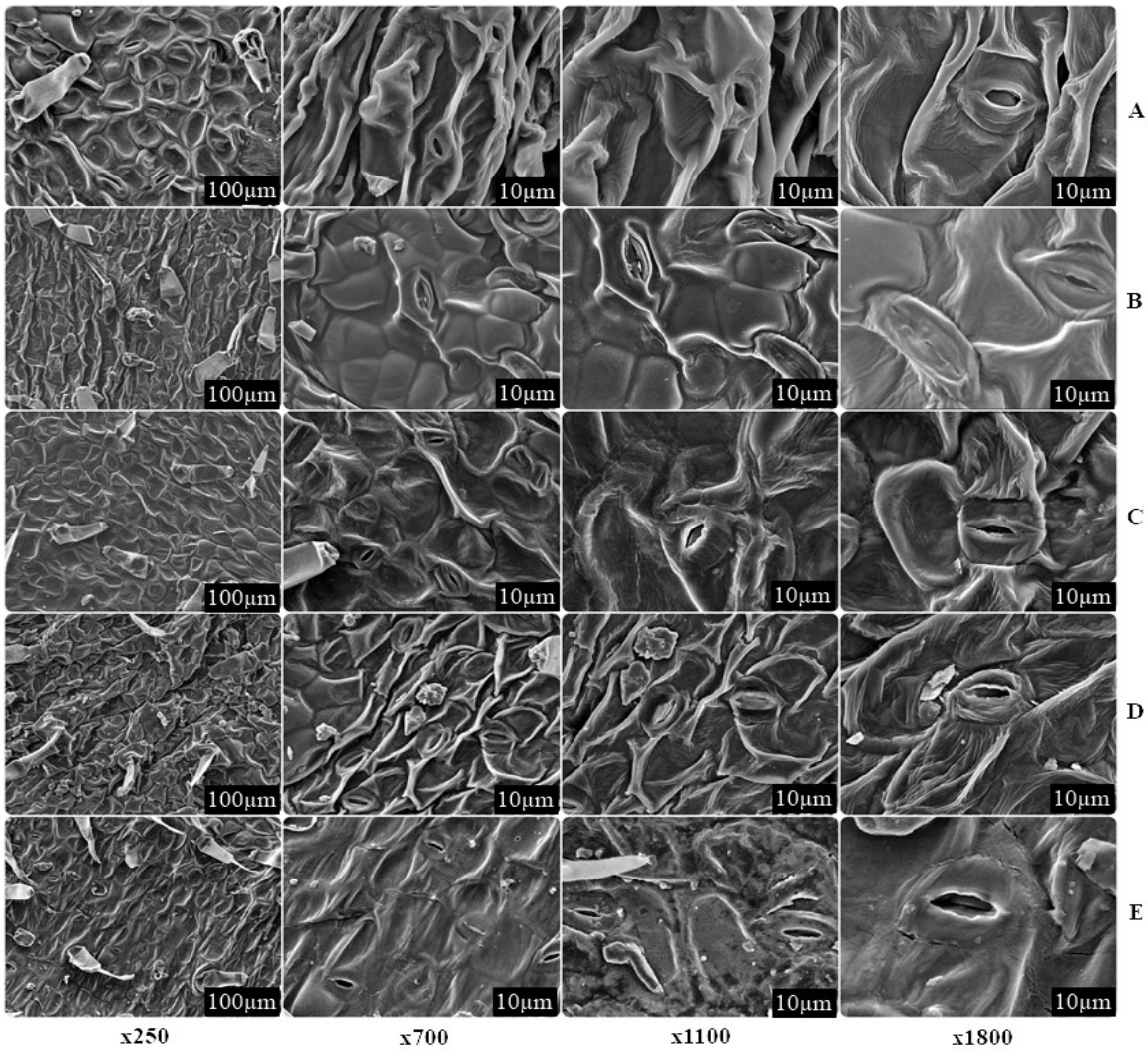


Fig. 6.16 Effect of *T. virens* (Tv) and Jasmonic acid (JA) on stomatal morphology in sixty days old tomato leaves stressed with *F. oxysporum lycopersici* (Fol) and *R. solani* (Rs) observed with the help of Scanning Electron Microscopy.

A, B, C, D and E respectively represent the treatments viz., Control, Fol, Fol+Tv+JA, Rs and Rs+Tv+JA. The samples were observed and photographed with SEM at 15.0 kV. The epidermis along with stomata in leaves was observed at different magnifications (× 250 - ×1800)

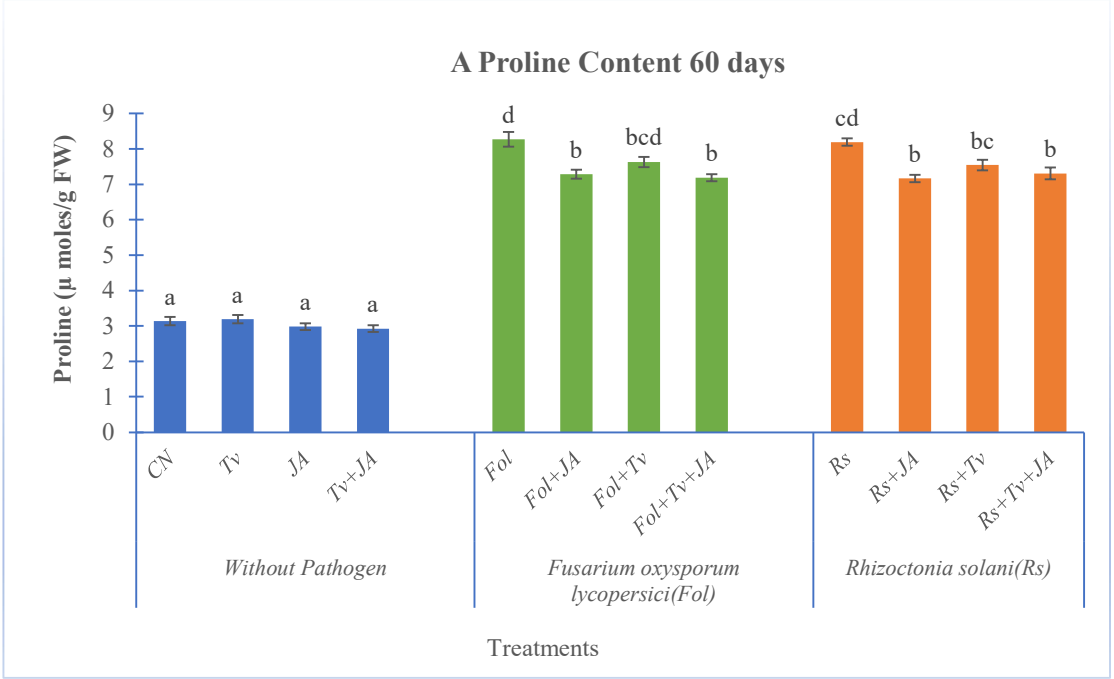
Biochemical Aspects

Osmolytes

Compatible osmolytes play a tremendous role in plant defence by maintaining cellular turgor by decreasing the water potential and acting as signalling molecules to initiate a cascade of defensive responses in diseased plants.

10. Free Proline Content

In sixty days old tomato plants when compared with uninoculated plants, a significant increase in the levels of free proline has been observed in the case of both wilting and damping off stressed tomato plants. As compared to control, maximum enhancement in proline content was depicted in the *Fol* i.e., 2.7- fold followed by *R. solani* treated plants (2.6- fold). However, pre-treatment of tomato seeds with JA and *T. virens,* either individually or in combination, significantly decreases the accumulation of free proline content in the case of both pathogens (Fig. 6.17 A). Our results also revealed that, after sixty days of pathogen inoculations in comparison to control plants a 2.3- and 2.6-fold increase in free proline content has been measured in *Fol* and *R. solani* diseased tomato plants, respectively. Additionally, pre-treatment of tomato seeds with *T. virens* or/and JA further increase the free proline content in tomato plants in the case of both studied pathogens except in the treatment Rs+Tv (Fig. 6.17 B).



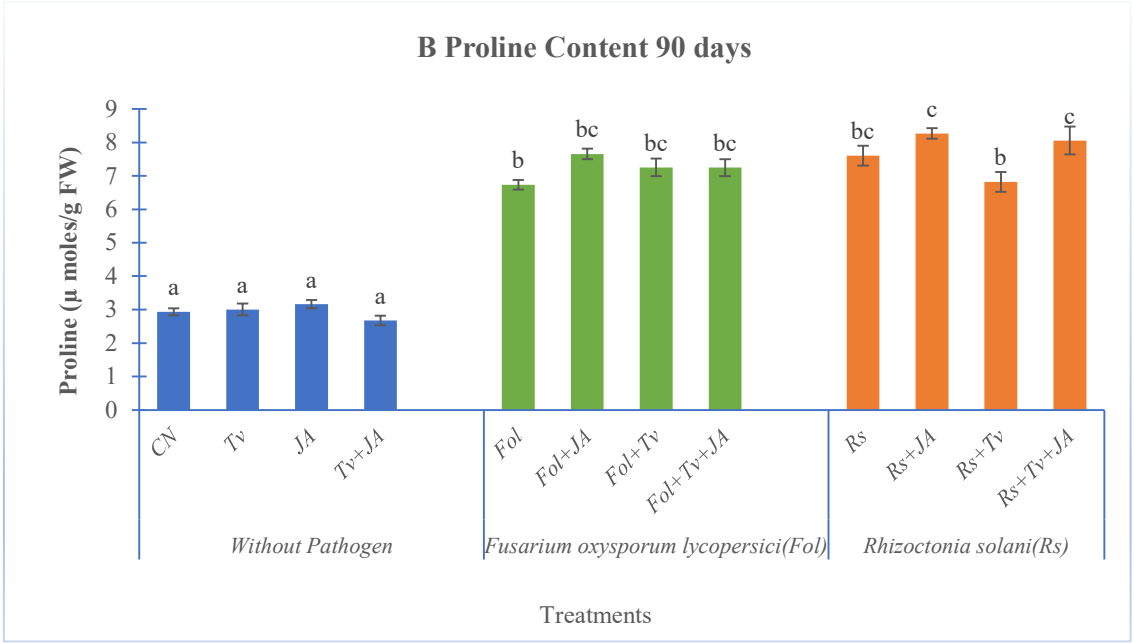
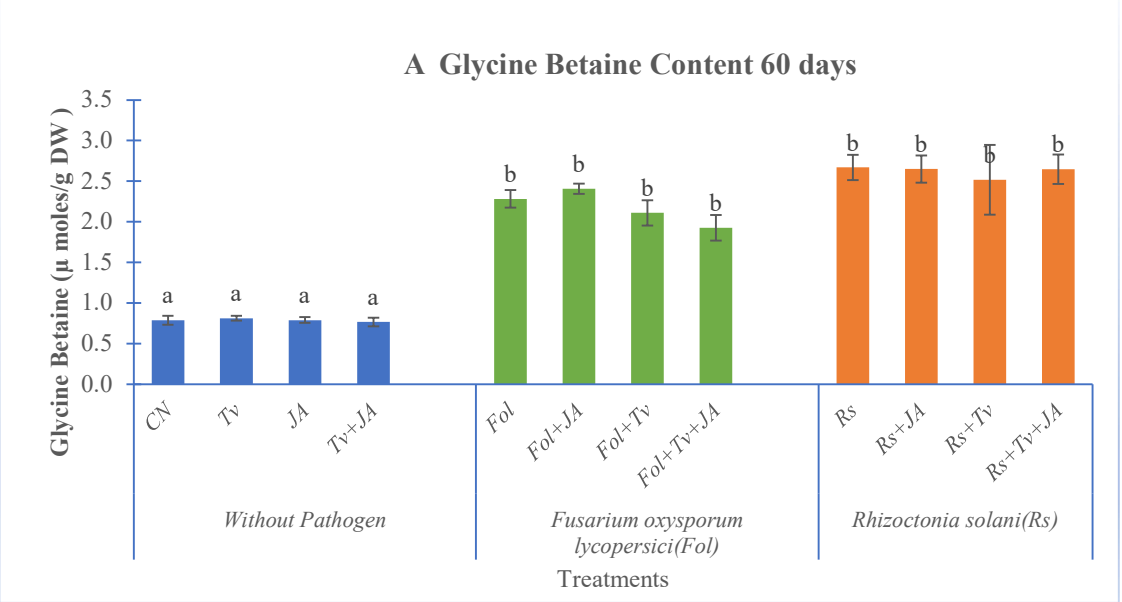


Fig. 6.17 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Free Proline content in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress. Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

11. Glycine Betaine Content

After thirty days of pathogens inoculations, a marked increase of approximately 3- and 3.4- fold as compared to control in glycine betaine content has been observed in *Fol* and *R. solani* stressed tomato plants, respectively. Further, pre-treatment of tomato seeds with *T. virens* and JA non-significantly altered the accumulation of glycine betaine in diseased plants (Fig. 6.18 A). Likewise, compared with control plants, a similar trend of increase in glycine betaine content has also been followed in ninety days old tomato plants challenged with fusarium wilt (3.2- fold) and damping off (3.8-fold) diseases. In the case of *Fol*-stressed plants, a significant reduction in glycine betaine content has been observed in the treatments Fol+Tv and Fol+Tv+JA though they are non-significant to each other. On the other hand, in the case of *R. solani* stressed plants highest content of Glycine betaine was quantified in the treatment Rs+Tv+JA (Fig. 6.18 B).



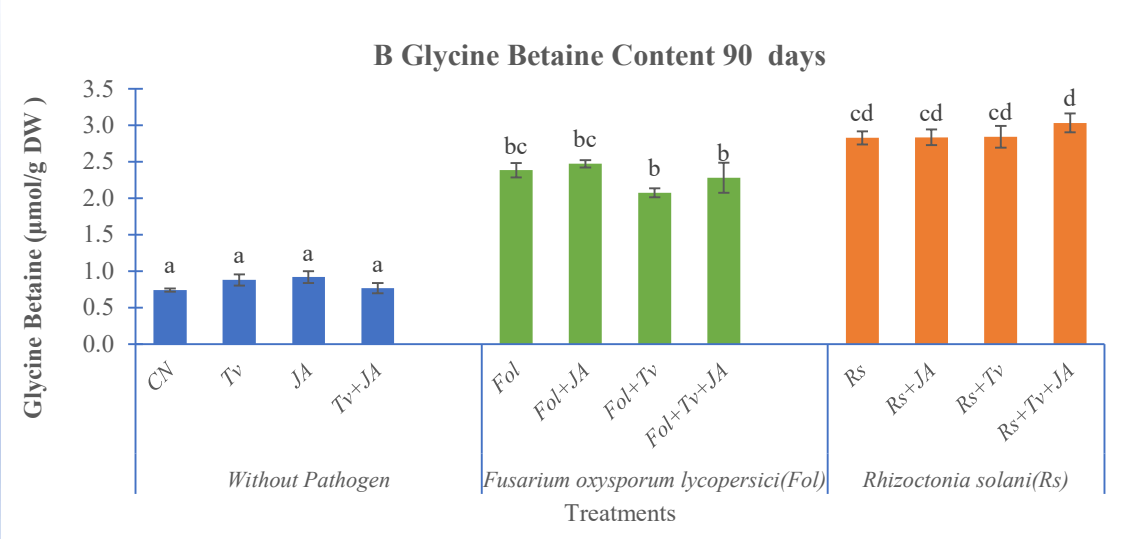
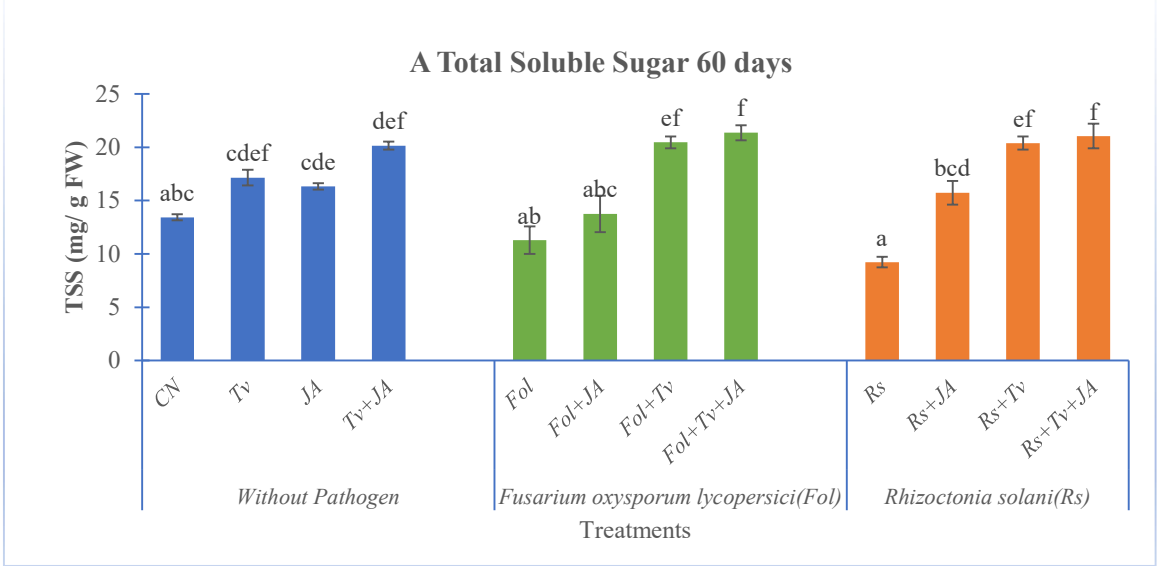


Fig. 6.18 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Glycine Betaine content in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress. Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

12. Total soluble sugar (TSS)

Our findings revealed that compared to the control in sixty days old tomato plants, the levels of total soluble sugar significantly reduced in the leaves of tomato plants infected with *Fol* (16 %) and *R. solani* (31 %). Compared to Fol inoculated plants, the maximum soluble sugar content was quantified in the tomato leaves in which seeds were primed with *T. virens* and JA together. Similarly, in the case of *R. solani* infected plants, the maximum total soluble sugar content was measured in the treatment Rs+Tv+JA (2.3- fold) when compared with the treatment Rs and its TSS content was statistically like the treatment Fol+Tv+JA (Fig. 6.19 A). In addition, a significant reduction in the magnitude of total soluble sugar content has been observed in *Fol* (52 %) and *R. solani* (50 %) challenged ninety days old plants compared to uninfected control plants. In comparison to *Fol,* a significant enhancement in total sugar content has been observed in the JA (2.3-), Tv (2.7-) and JA+Tv (3.3-fold) primed, 90 days old tomato plants. Correspondingly in the case of *R. solani*-infected tomato plants, a similar trend of increase in total sugar content has also been observed in the *T. virens* and JA-primed tomato plants (Fig. 6.19 B). Furthermore, after sixty days of pathogen inoculation, priming tomato seeds with *T.*

virens alone or combined with Jasmonic acid induced a statistically similar influence on TSS content in *R. solani*-infected tomato plants.



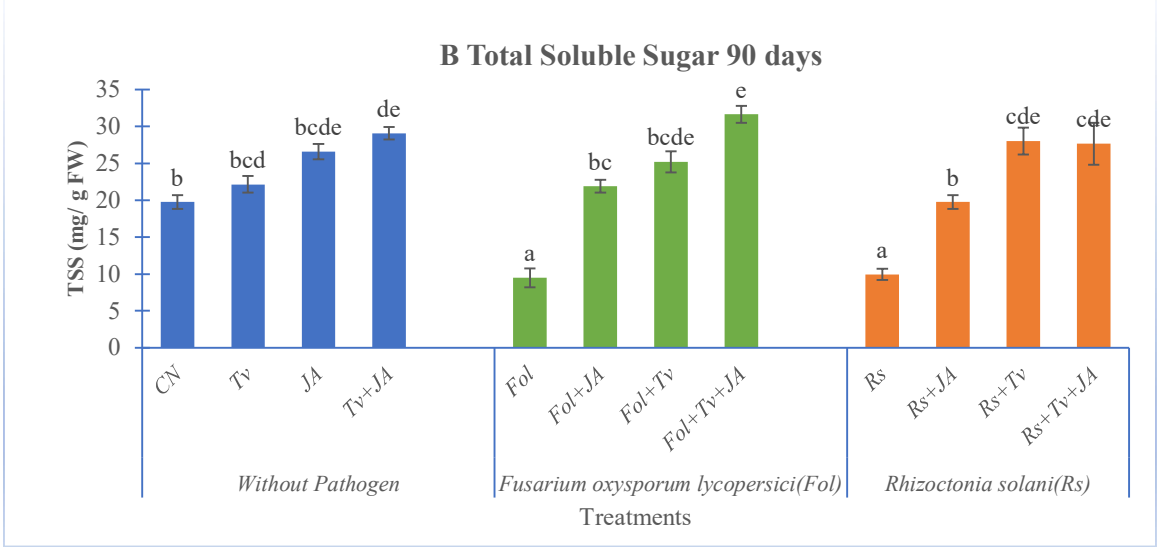
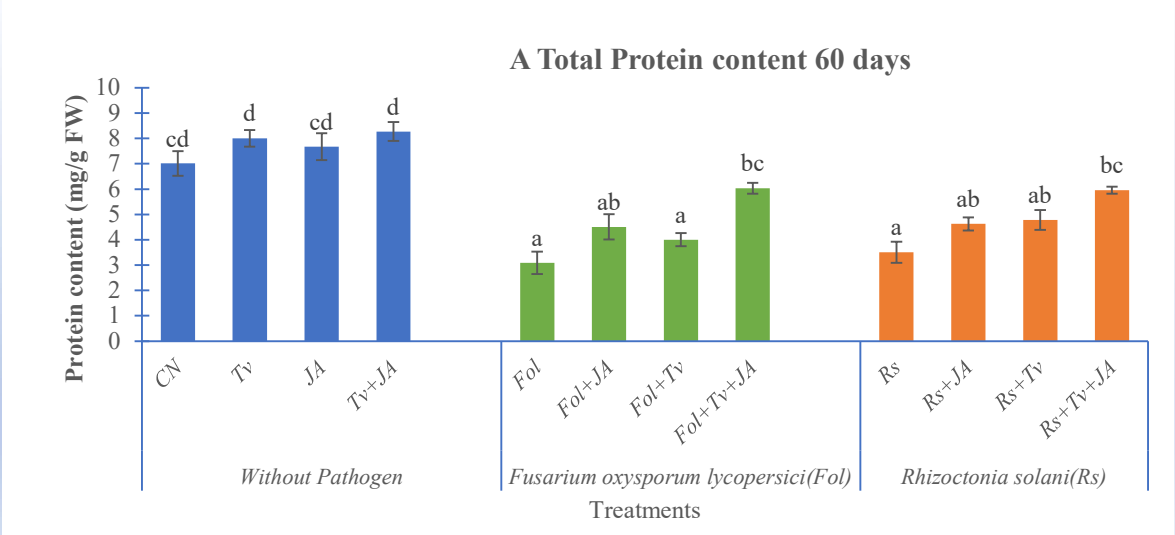


Fig. 6.19 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Total Sugar content in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress. Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

13. Total Protein Content

In sixty days, old tomato plants, total protein contents were observed to be decreased significantly under *Fol* (56 %) and *R. solani* (50 %) inoculations as compared to control plants. In the case of *Fol*-challenged plants, the most significant increase (95.2 %) has been quantified in the treatment Fol+JA+Tv compared to the non-primed tomato plants. Similar findings have also been monitored in the case of *R. solani*-infected plants as 69.8 % improvement has been reported in the case of treatment Rs+JA+Tv (Fig. 6.20 A). Likewise, after sixty days of pathogen inoculation in ninety days old tomato plants compared to the control, a significant reduction in total protein content has been reported in *Fol* (57.6 %) and *R. solani* (42.3 %) diseased plants. Further pre-treatment of tomato seeds with *T. virens* and JA alone or in combination brought a marked increase in total protein content in both *Fol* and *R. solani* inoculated tomato plants. Maximum enhancement in total protein content, i.e., 107.8 % has been measured in the treatment Fol+Tv+JA under *Fol* infection. A similar trend has also been repeated by the treatment Rs+Tv+JA with a significant increase of 52.35 % in *R. solani* diseased plants (Fig. 6.20 B).



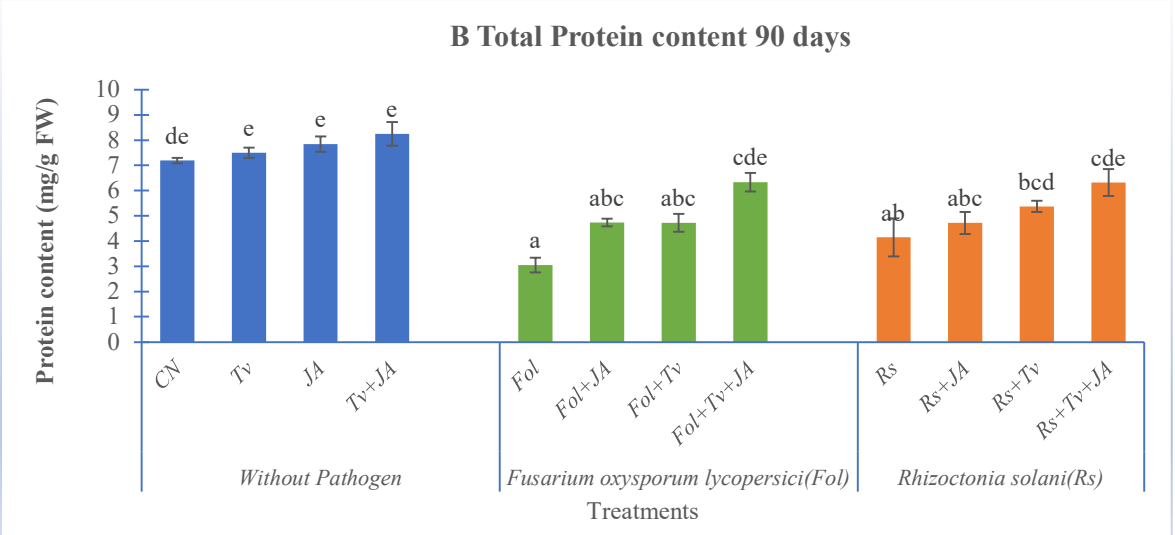


Fig. 6.20 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Total Protein content in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Pigments Molecules

14. Chlorophyll Content

In comparison to control plants, a significant reduction in the content of chlorophyll a, b and the total have been monitored in *Fol* and *R. solani*-stressed tomato plants. In diseased plants an approximate decrease of 64 and 68 % in chl. a content has been recorded in *Fol* and *R. solani* challenged tomato leaves, respectively, compared with control plants. Similarly, in comparison to the control, a similar trend of decrease has also been repeated by Chl. b and total chl. contents in diseased plants. Moreover, pre-treatment of tomato seeds with *T. virens* and JA, either individually or in combination, increases the values of chlorophyll content in both infected as well as non-infected plants. In the case of *Fol*-challenged tomato plants most significant increase in the level of chl. a, and total chl. content has been observed in the treatment Fol+Tv+JA. Similar findings under different treatments have also been depicted in *R. solani*-challenged tomato plants in the context of Chl. b and total chlorophyll content (Table 6.2).

Table 6.2 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Chl. a, Chl. b and total Chl. in 60 days old tomato plants with and without pathogenic stress.

Values shown are means followed by ± Standard Error of Mean (n = 3). Different lower-case letters in the table indicate significant differences between treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Chlorophyll Content 60 days				
Treatments	**Chlorophyll a (mg/g FW)**	**Chlorophyll b (mg/g FW)**	**Total Chlorophyll (mg/g FW)**	**Chl a/Chl b**
CN	$1.91^{f} \pm 0.06$	$0.89^{f} \pm 0.03$	$2.8^{g} \pm 0.07$	2.16
Tv	$2.13^{g} \pm 0.02$	$1.06^{g} \pm 0.02$	$3.19^{h} \pm 0.03$	2.02
JA	$1.61^{e} \pm 0.04$	$0.77^{e} \pm 0.01$	$2.39^{f} \pm 0.03$	2.08
Tv+JA	$1.93^{f} \pm 0.02$	$0.88^{ef} \pm 0.03$	$2.8^{g} \pm 0.03$	2.19
Fol	$0.69^{ab} \pm 0.02$	$0.34^{a} \pm 0$	$1.03^{ab} \pm 0.02$	2.05
Fol+JA	$1.05^{c} \pm 0.02$	$0.57^{cd} \pm 0.01$	$1.62^{cd} \pm 0.02$	1.83
Fol+Tv	$1.15^{c} \pm 0.04$	$0.62^{d} \pm 0.04$	$1.77^{d} \pm 0.08$	1.86
Fol+Tv+JA	$1.35^{d} \pm 0.02$	$0.64^{d} \pm 0.02$	$2^{e} \pm 0.01$	2.1
Rs	$0.61^{a} \pm 0.02$	$0.3^{a} \pm 0.02$	$0.91^{a} \pm 0.05$	2.02
Rs+JA	$0.82^{b} \pm 0.02$	$0.41^{ab} \pm 0$	$1.23^{b} \pm 0.02$	2.03
Rs+Tv	$1.01^{c} \pm 0.02$	$0.5b^{c} \pm 0.02$	$1.51^{c} \pm 0.01$	2.01
Rs+Tv+JA	$1.07^{c} \pm 0.03$	$0.56^{cd} \pm 0$	$1.62^{cd} \pm 0.03$	1.92

Owing to pathogens infection, a similar pattern of decrease in chlorophyll contents has also been repeated in ninety days old tomato plants. In comparison to the control, a percentage reduction of 71 and 72 % in Chlorophyll a content has been measured in tomato plants challenged with the pathogenicity of *Fol* and *R. solani,* respectively. In the case of *Fol* infected plants, a maximum per cent improvement of 123.9 and 69.2 % in the case of Chl. a and total chl. content has been depicted by the treatment Fol+Tv+JA. Similarly in the case of *R. solani* inoculation, the maximum improvement in chl. a (111.6 %) content has been recorded in the treatment Rs+Tv+JA. On the other hand, priming agents non-significantly altered the content of Chl. b in *R. solani* diseased plants (Table 6.3).

Table 6.3 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Chl. a, Chl. b and total Chl. in 90 days old tomato plants with and without pathogenic stress.

Values shown are means followed by ± Standard Error of Mean (n = 3). Different lower-case letters in the table indicate significant differences between treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Chlorophyll Content 90 days				
Treatments	**Chlorophyll a**	**Chlorophyll b**	**Total Chlorophyll**	**Chl a/Chl b**
CN	$1.95^{e} \pm 0.04$	$0.97^{ab} \pm 0.02$	$2.93^{c} \pm 0.05$	2.01
Tv	$2.17^{e} \pm 0.03$	$1.02^{ab} \pm 0.01$	$3.19^{c} \pm 0.04$	2.12
JA	$1.95^{e} \pm 0.15$	$1.33^{b} \pm 0.39$	$3.28^{c} \pm 0.47$	1.46
Tv+JA	$2.28^{e} \pm 0.07$	$0.93^{ab} \pm 0.05$	$3.21^{c} \pm 0.11$	2.45
Fol	$0.57^{ab} \pm 0.04$	$0.46^{a} \pm 0.09$	$1.02^{ab} \pm 0.08$	1.25
Fol+JA	$1.11^{cd} \pm 0.07$	$0.56^{a} \pm 0.15$	$1.67^{ab} \pm 0.12$	1.99
Fol+Tv	$1.21^{cd} \pm 0.08$	$0.45^{a} \pm 0.06$	$1.66^{ab} \pm 0.13$	2.72
Fol+Tv+JA	$1.27^{d} \pm 0.06$	$0.46^{a} \pm 0.05$	$1.73^{b} \pm 0.09$	2.76
Rs	$0.54^{a} \pm 0.05$	$0.35^{a} \pm 0.07$	$0.9^{a} \pm 0.07$	1.54
Rs+JA	$0.64^{ab} \pm 0.05$	$0.39^{a} \pm 0.06$	$1.03^{ab} \pm 0.03$	1.65
Rs+Tv	$0.91b^{c} \pm 0.06$	$0.42^{a} \pm 0.06$	$1.33^{ab} \pm 0.12$	2.14
Rs+Tv+JA	$1.15^{cd} \pm 0.03$	$0.39^{a} \pm 0.05$	$1.55^{ab} \pm 0.06$	2.93

15. Total Carotenoids Content

As with the chlorophyll content, a similar trend of decrease in the total carotenoid content has been exhibited by the pathogens-inoculated tomato plants when compared with their control counterparts. In sixty days old plants concerning control plants, a percentage decline of 17 and 39 per cent have been reported in *Fol* and *R. solani*-challenged plants, respectively. Furthermore, seed priming with Tv+JA improved carotenoid content to most significant extent (22 %) in *Fol* stressed plants. Similarly, priming of tomato seeds with *T. virens* alone and in combination with JA most significantly improved the carotenoid content in *R. solani*-infected tomato plants (Fig. 6.21 A). Furthermore, pre-treatment of tomato seeds with *T. virens* not only enhanced

the total carotenoid content in diseased plants, but a remarkable increase has also been exhibited in the non-inoculated plants at both the observed growth stages. Likewise, in ninety days old plants, a significant decrease in carotenoid content of magnitude 21 and 41 % had been reported in *Fol* and *R. solani* treated plants compared to control plants. However, priming of tomato seeds with Tv and Tv+JA brought the most significant improvement in total carotenoid content in both *Fol* and *R. solani*-challenged tomato plants after 60 days of pathogen inoculation (Fig. 6.21 B).

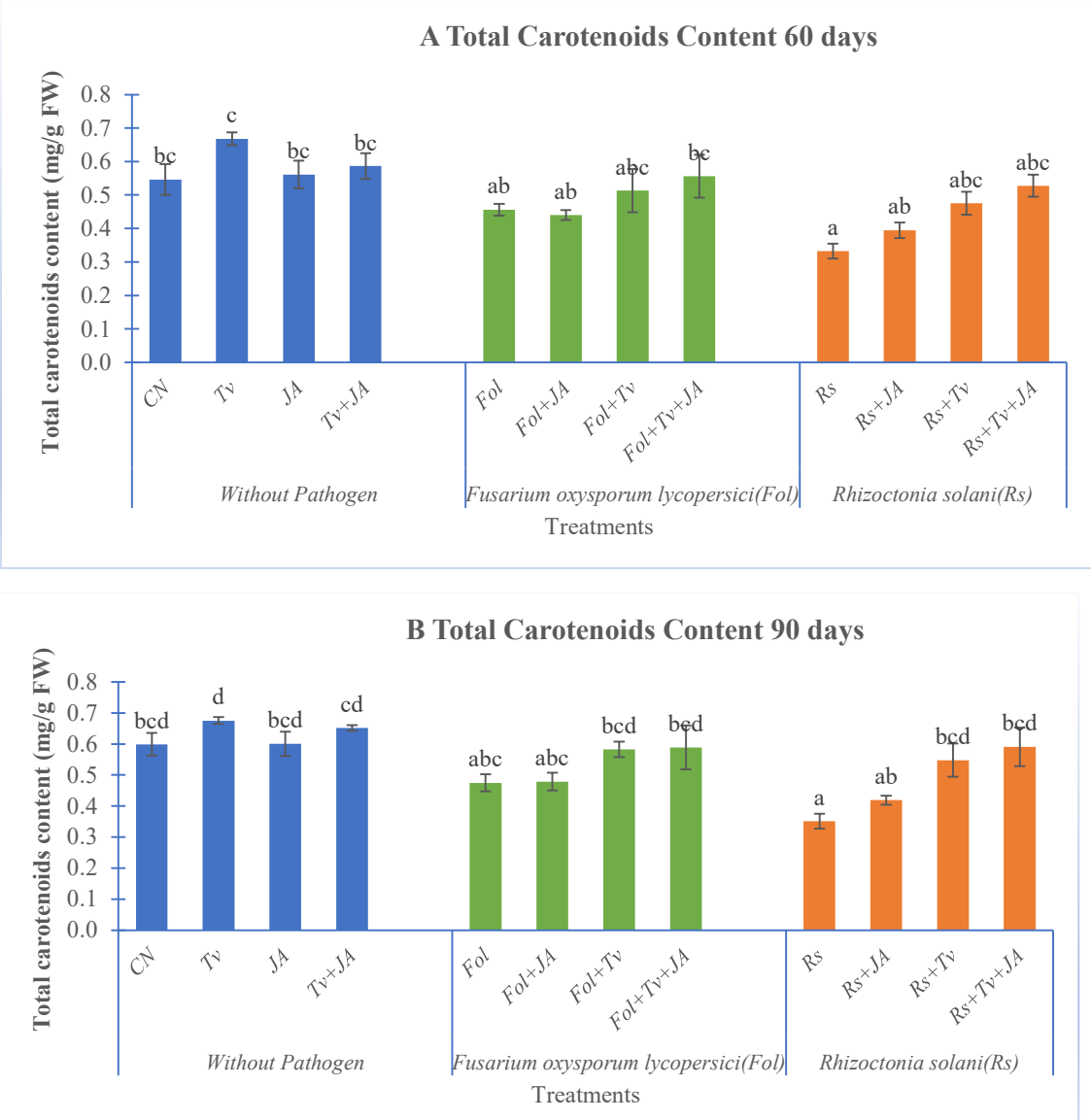
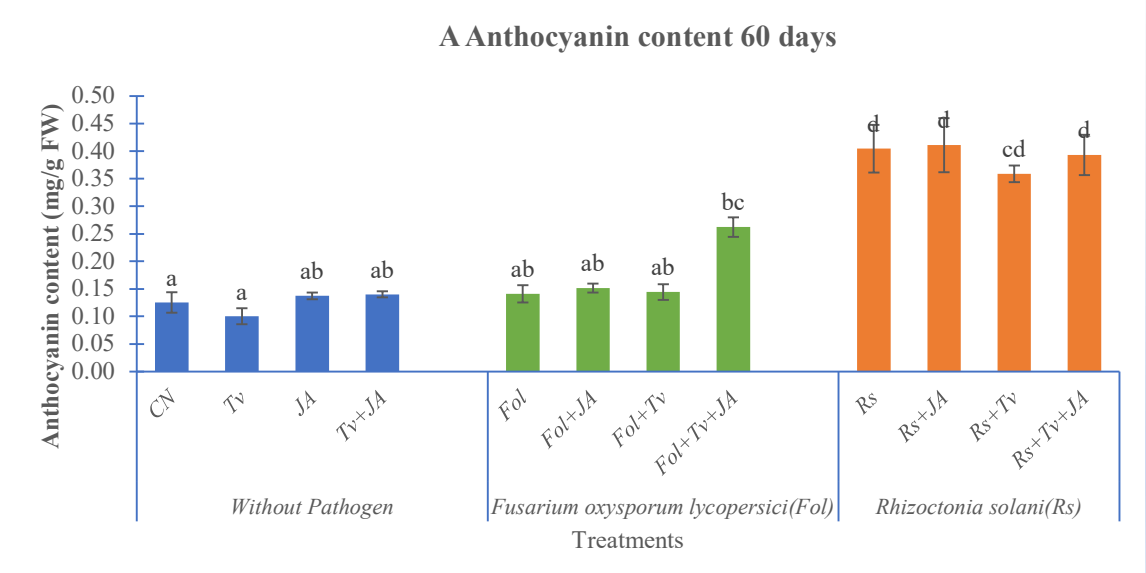


Fig. 6.21 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Total carotenoids content in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

16. Total Anthocyanin Content

Our investigation revealed that compared to the control, due to pathogen stress, the build-up of total anthocyanin contents has increased significantly in sixty days old plants. Though pre-treatment of tomato seeds with JA and Tv+JA also caused a significant increase in anthocyanin content in non-inoculated plants too. The magnitude of accumulated content was more abundant, i.e., 3.2-fold in the case of *R. solani* infected plants and less (12.43 %) in *Fol* inoculated ones compared with the control value. In the case of *Fol*-challenged plants a significant increase, i.e., 86 % has been observed in the treatment Fol+Tv+JA, while in the case of *R. solani* infected plants, no substantial change in anthocyanin content has been monitored except a decrease of 11.3 % has been reported in the treatment Rs+Tv (Fig. 6.22 A). A similar trend of enhancement in anthocyanin content has been observed in ninety days old tomato plants under *R. solani* inoculation. Pre-treatment of tomato seeds with *T. virens* and JA, either individually or in combination, does not have any significant influence on anthocyanin content accumulation in the case of *R. solani* infected plants, while 45.5 % enhancement has been recorded in the treatment Fol+Tv+JA in *Fol* infected plants (Fig. 6.22 B).



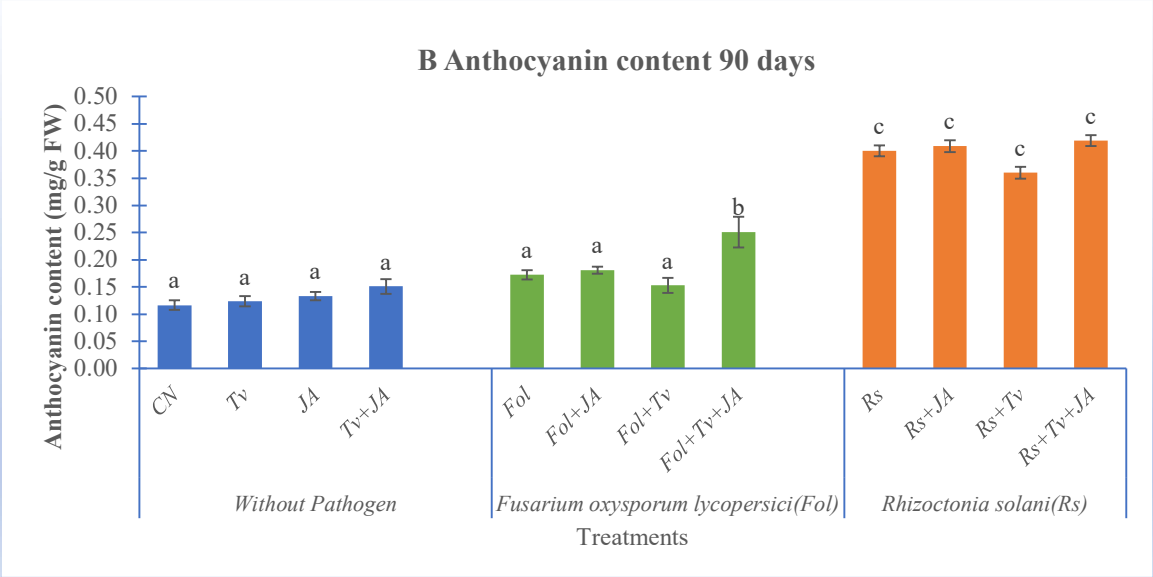


Fig. 6.22 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Anthocyanin content in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

17. Gaseous Exchange

In addition to various influences of pathogen-induced stress on plant pigments, these also affect the photosynthetic activity of plants concerning photosynthetic rate, transpiration rate, stomatal conductance and intercellular carbon contents.

Photosynthetic Rate (PR)

Regarding the photosynthetic rate in sixty-day-old plants, approximately 66.7 and 64.5 % inhibition has been observed in Fol and R. solani-treated plants, respectively, compared with the control. However, pre-treatment with *T. virens* alone and JA leads to a substantial increase in photosynthetic rate. In the case of *Fol* inoculated plants, priming of tomato seeds with each ameliorating agent treatment exhibit significant improvement in photosynthetic rate. A similar trend of the significant increase in photosynthetic rate has also been repeated in the case of *R. solani* diseased plants. A marked rise of approximately 96.7 % has been achieved in the treatments Rs+Tv+JA (Fig. 6.23 A). A similar trend of decrease in PR in diseased plants has been repeated in ninety days old tomato plants. In the case of *Fol*-infected plants, pre-treatment of tomato seeds with bio stimulants alone or in combination significantly improves the

photosynthetic rate. Similarly, in the case of *R. solani* diseased plants, a significant increase in the photosynthetic rate has been observed in the treatment Rs+Tv+JA (Fig. 6.23 B).

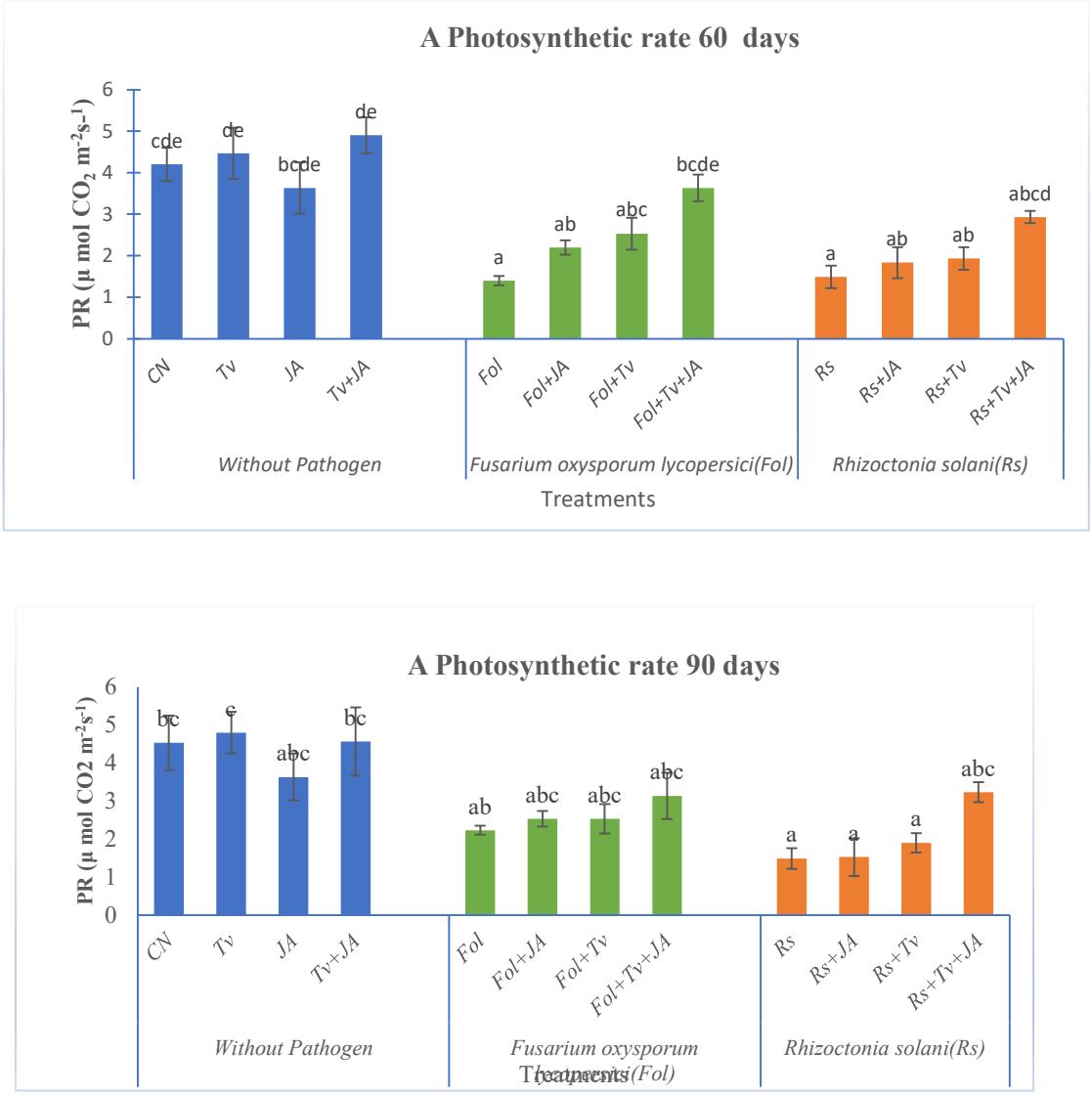
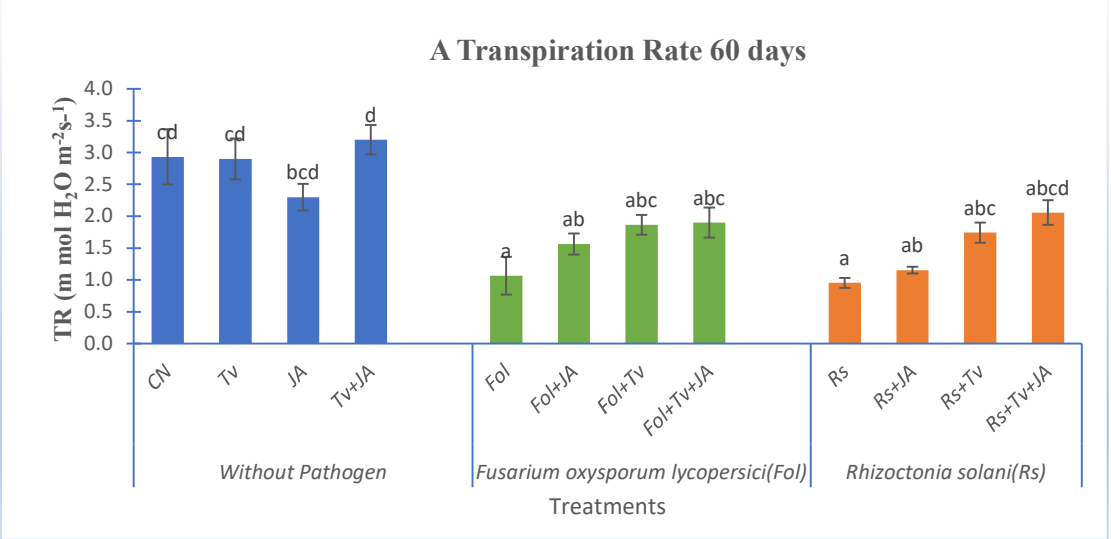


Fig. 6.23 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Photosynthesis rate in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Transpiration rate (TR)

As depicted in the case of photosynthetic rate, the transpiration rate is also observed to be affected because of pathogen-stimulated damage to tomato leaves. In sixty days, old tomato plants, compared to control, a percentage reduction in transpiration rate of 63.6 and 67.50 was quantified in *Fol* and *R. solani*-infected tomato leaves, respectively. But pre-treatment of host plants seeds with *T. virens* and JA alone or in combination leads to a significant enhancement in TR in the case of *Fol* stressed plants. Likewise, in the case of *R. solani* stressed plants, marked improvement in TR has been observed in the primed tomato plants (Fig. 6.24 A). Similarly, in 90 days old tomato plants, a 60.5 and 67.5 % reduction in TR has been observed in *Fol* and *R. solani*-challenged plants compared to the control one. In *Fol* infected plants, priming of tomato seeds with bio stimulants caused a significant enhancement in TR to a statistically equal extent. Likewise, compared to *R. solani* inoculated 90 days old plants a considerable improvement of 81 % in TR has been observed in the treatment Rs+Tv+Ja (Fig. 6.24 B).



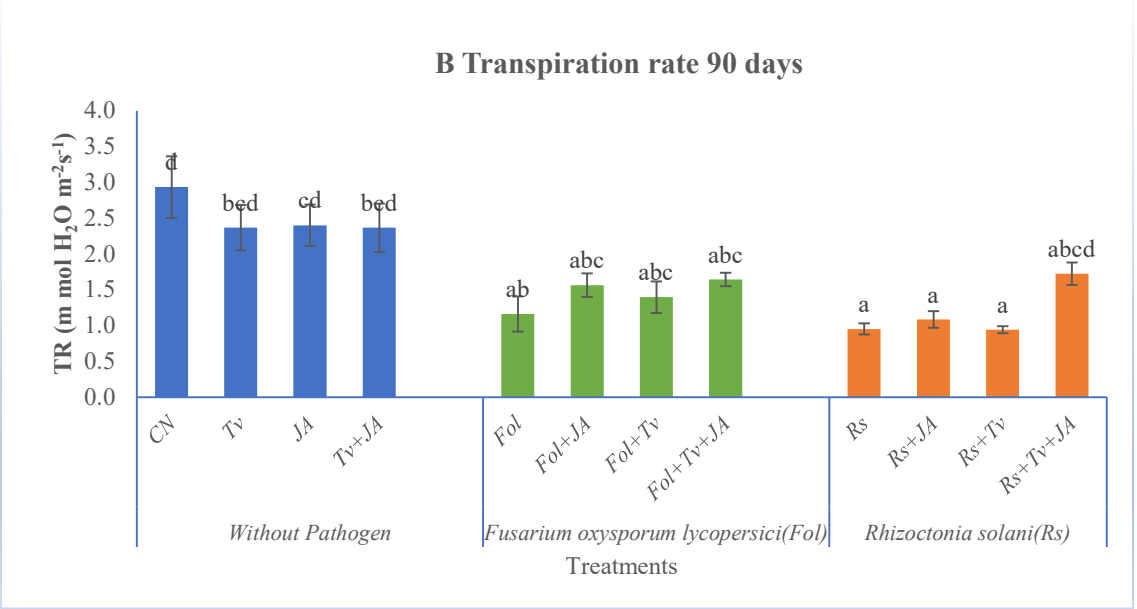


Fig. 6.24 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Transpiration rate in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Intercellular Carbon (IC)

As with the rates of transpiration and photosynthesis, intercellular carbon is also observed to decrease in the pathogenically challenged tomato plants. After thirty days of pathogen inoculation, the lowest and highest intercellular carbon was monitored in the pathogenically infected and non-infected plants with treatment Tv+JA, respectively in the case of sixty days old tomato plants. Further, in the case of *Fol* diseased plants, maximum improvement (30.8 %) in intercellular carbon was depicted in the Tv+JA-seed primed tomato plants. Similar trend of enhancement in IC has also been followed by the treatment Rs+Tv+JA in the case of *R. solani* infected plants (Fig. 6.25 A). As observed in the case of sixty days old tomato plants, a similar trend of decrease in the intercellular carbon has also been observed in the *Fol* (33.8 %) and *R. solani* (36 %) diseased plants when compared with the control counterpart. However, in *Fol*-challenged plants, a significant increase in intercellular carbon has been monitored in the treatment Fol +JA (34.8 %) followed by Fol+Tv+JA (32.7 %).

Similarly, in the case of *R. solani* stressed plants, the maximum increase in intercellular carbon has been monitored in the treatment Rs+Tv+JA (Fig. 6.25 B).

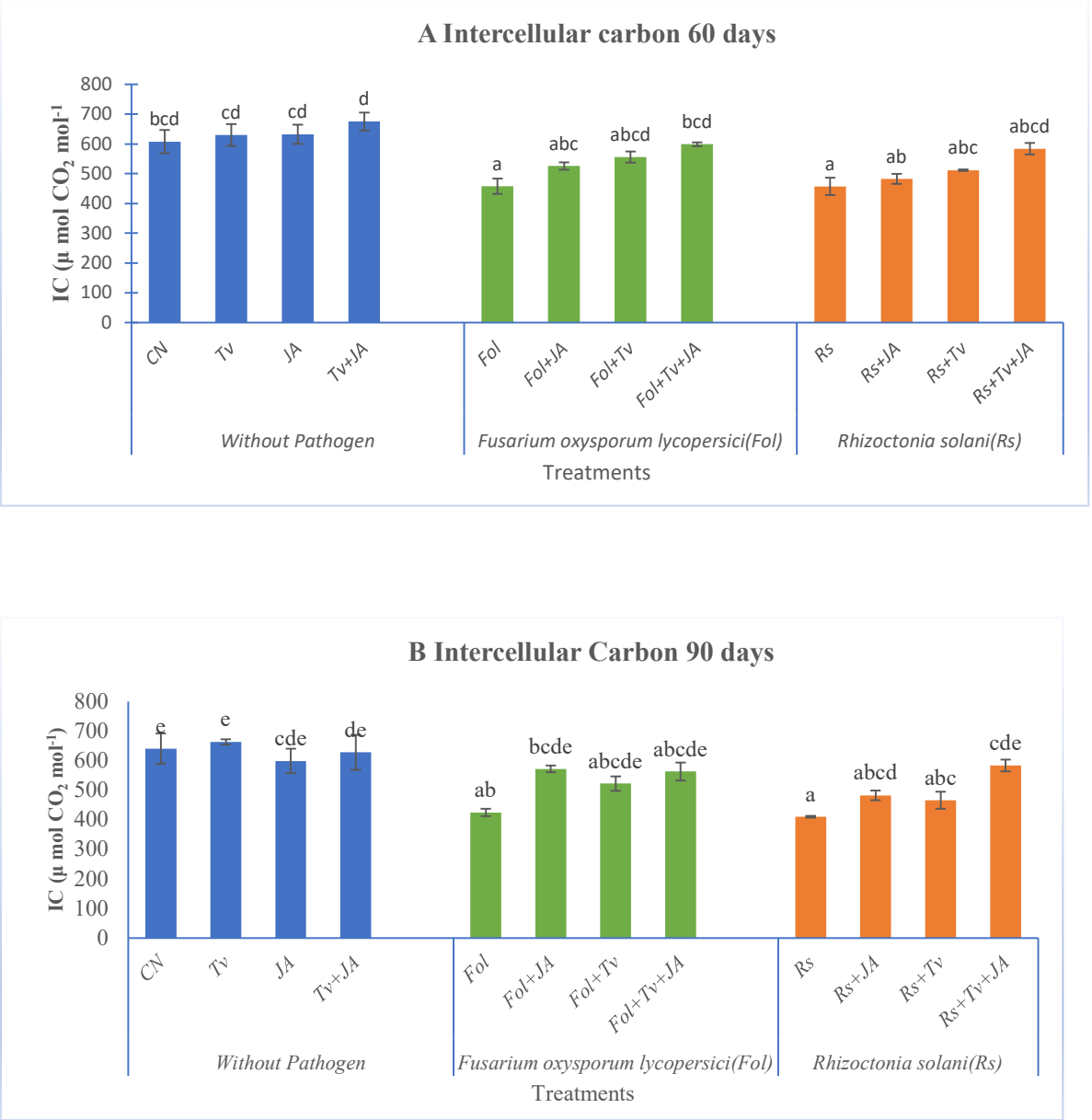
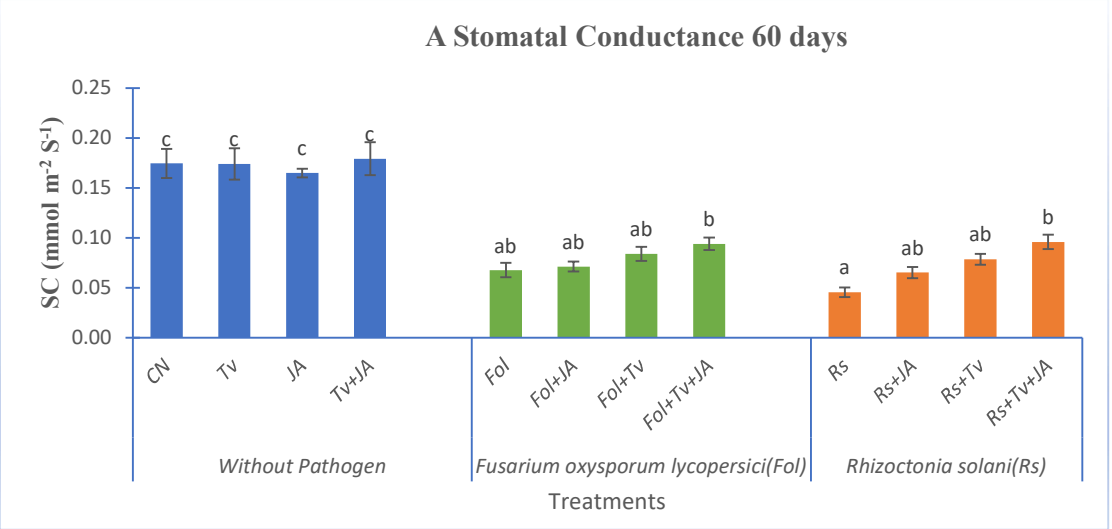


Fig. 6.25 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Intercellular carbon in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Stomatal Conductance (SC)

Stomatal conductance measures the level of stomatal opening and can be utilized as a marker of the water status of plants. In other words, this term is generally referred to as the rate of entering CO_2/ exiting of water vapours through stomata. Compared to control, owing to the exposure of these two soil-borne pathogens, a significant reduction in stomatal conductance has been observed in *Fol* (61.3 %) and *R. solani* (73.9 %) infected sixty days old diseased plants. Our findings also demonstrated that the pre-treatment of tomato seeds with *T. virens*+JA caused the most significant improvement in the stomatal conductance in the case of both *Fol* and *R. solani*-challenged tomato plants (Fig. 6.26 A). Furthermore, in 90 days old tomato plants, a similar trend of decrease in stomatal conductance has also been followed by pathogenically challenged tomato plants compared to control plants. Similarly, seed priming with both the ameliorating agents together led to the most significant enhancement in stomatal conductance in the plants infected with their respective pathogens (Fig. 6.26 B).



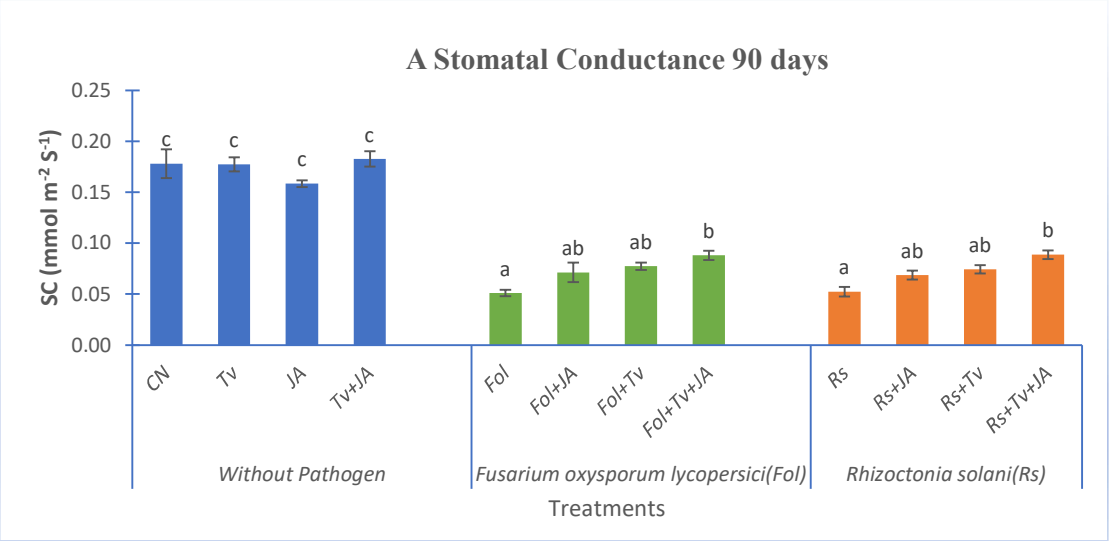


Fig. 6.26 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Stomatal Conductance in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Oxidative Stress Markers

18. Lipid peroxidation

Our results indicate that pathogens-induced cellular damage in tomato leaves led to lipid peroxidation during pathogenic stress. The extent of lipid peroxidation was determined in terms of MDA content. In sixty days old tomato plants in comparison to the control, MDA contents were reported to be the highest in the case of *R. solani* (2.5- fold) followed by *Fol* (2.3-fold), respectively. However, pre-treatment of tomato seeds with *T. virens* and JA lowers the MDA content accumulation in the case of both pathogens. Further, the tomato seeds priming with the combination of *T. virens* + JA resulted in a significant reduction to the magnitude of 25.3 and 28.1 % in the case of *Fol* and *R. solani* diseased plants, respectively in comparison to the individual pathogens treatments alone (Fig. 6.27 A). In addition, due to pathogen inoculation, MDA content was also reported to be increased in ninety days old tomato plants. As a significant increase of 3.5- and 3.3- fold of control has been quantified in *Fol* and *R. solani* diseased ninety days old tomato plants, respectively. Moreover, pre-treatment of tomato seeds with *T. virens* and JA, either individually or combined led to a further

reduction in MDA contents in plants affected by respective pathogens. In the case of *R. solani* infected plants, the maximum decrease in the lipid peroxidation was reported in the treatments where seeds were primed with both the ameliorating agents in the treatment Rs+Tv+JA. On the other hand, priming of tomato seeds with either *T. virens a*lone or in combination with JA have statistically equal influence on the extent of lipid peroxidation in *Fol*-diseased plants (Fig. 6.27 B).

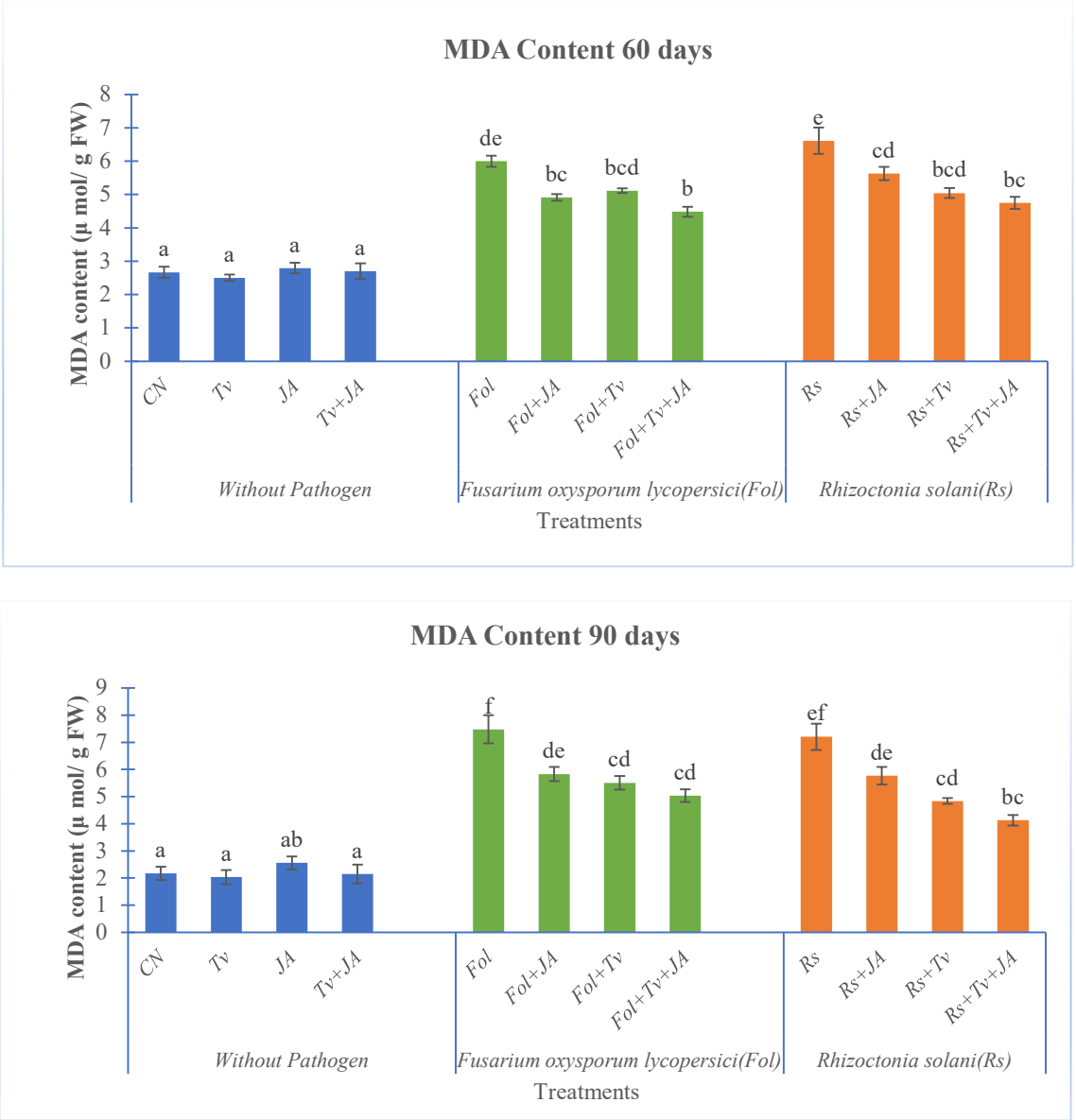
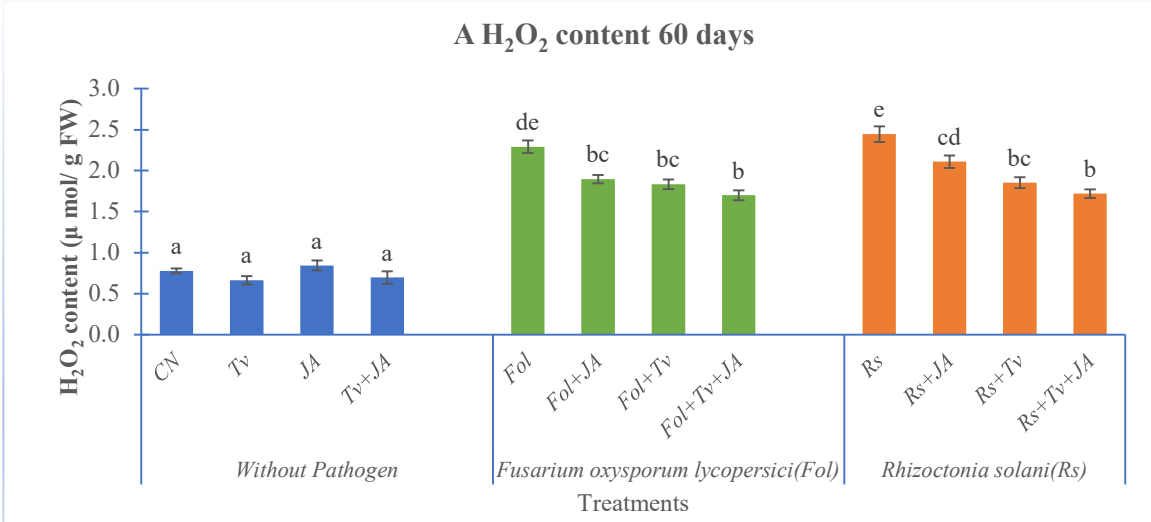


Fig. 6.27 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on MDA content in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

19. Hydrogen Peroxide Content

Inoculation of tomato plants with pathogens leads to the production of the oxidative burst in the leaves of infected plants and is detected in the form of H_2O_2 content. In the sixty days old tomato plants, in comparison to the control H_2O_2 contents were reported to be the highest in the case of *R. solani* (3.2- fold) followed by *Fol* (3- fold), respectively. Moreover, the maximum reduction in H_2O_2 contents was observed in *Fol* (25.9 %) and *R. solani* (29.7 %) diseased tomato plants where tomato seeds were previously primed with *T. virens* and JA together under the treatments Fol+Tv+JA and Rs+Tv+JA, respectively (Fig. 6.28 A). Likewise, as compared to the control a similar trend of enhancement in H_2O_2 contents has also been repeated in ninety days old tomato plants challenged with *Fol* (5- fold) and *R. solani* (6- fold). Though pre-treatment of tomato seeds with *T. virens* and JA alone or in combination did not bring any significant decrease in the level of H_2O_2 accumulation in *Fol* stressed plants, a marked decrease has been observed in *R. solani* stressed plants under the treatments Rs+JA, Rs+Tv and Rs+Tv+JA (Fig. 6.28 B).



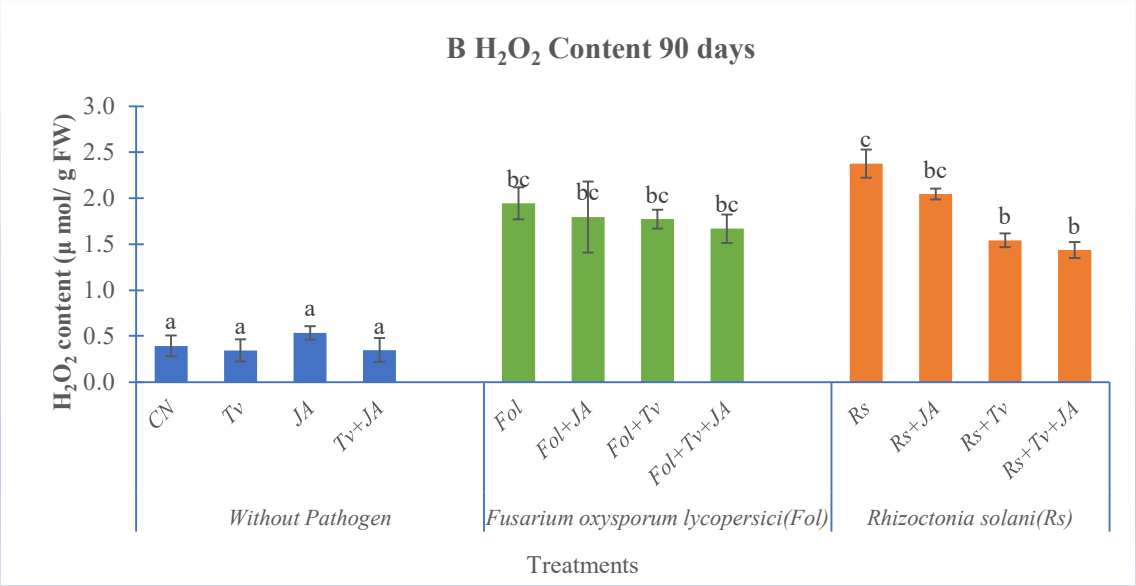


Fig. 6.28 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on H_2O_2 content in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Antioxidative Defence System

Non-Enzymatic Antioxidants

The effect of pathogens induced stress in tomato plants pre-treated with *T. virens* and JA, in terms of non-enzymatic antioxidants was observed by monitoring the values of Ascorbic acid, Glutathione and Tocopherol content under different treatments after 30 and 60 days of pathogens inoculation.

20. Ascorbic Acid (AsA)

In the case of sixty days old plants, maximum contents of ascorbic acid were reported in plants with treatment RS+Tv+JA (0.46 mg/ g FW). In comparison to the control, a significant rise of 82.5 and 104.3 % in ascorbic acid content has been reported in the case of *Fol* and *R. solani* inoculated tomato plants, respectively. Further, priming of tomato seeds with *T. virens* and JA either alone or in combination lead to a significant increase in the AsA content in diseased plants. A similar trend of AsA content increase has also been followed in 90- days old tomato plants and maximum content has been observed in the treatment Fol+Tv+JA (0.52 mg/ g FW) (Table 6.4 & 6.5).

21. Glutathione (GSH)

Pathogens induced oxidative damage in tomato plants also led to the enhancement in GSH content in diseased plants after both 30 and 60 days of pathogens inoculation. Moreover, pre-treatment of tomato seeds with *T. virens* and JA alone or in combination further raises the GSH content in both inoculated as well as non-inoculated tomato plants. In comparison to the control, maximum GSH content was observed in the treatments Fol +Tv +JA (100 %) and Rs+Tv+JA (99.4 %) in sixty days old tomato plants, though w.r.t GSH content, these treatments were non-significant to each other. Similarly, in the case of ninety days old plants, compared to the control a significant enhancement in GSH content has been observed in the treatments Fol+JA, Fol+Tv and Fol+JA+Tv in the case of *Fol* diseased plants. Similar findings have also been observed in *R. solani* stressed tomato plants in the case of treatments Rs+JA, Rs+Tv and Rs +JA+Tv (Table 6.4 & 6.5).

Table 6.4 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on AsA, GSH and Tocopherol contents in sixty days old tomato plants with and without pathogenic stress.

Values shown are means followed by ± Standard Error of Mean (n = 3). Different lower-case letters in the table indicate significant differences between treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Treatments	Ascorbic acid (mg/ g FW)	Glutathione (mg/ g FW)	Tocopherol (µg/ g FW)
CN	$0.205^{a} \pm 0.003$	$0.215^{a} \pm 0.004$	$18.45^{a} \pm 2.54$
Tv	$0.21^{a} \pm 0.0018$	$0.23^{ab} \pm 0.0062$	$19.4^{ab} \pm 2.8$
JA	$0.26^{a} \pm 0.017$	$0.25^{ab} \pm 0.0062$	$20.12^{ab} \pm 1.91$
Tv+JA	$0.23^{a} \pm 0.013$	$0.266^{b} \pm 0.0051$	$22.32^{abc} \pm 1.2$
Fol	$0.37^{b} \pm 0.015$	$0.377^{c} \pm 0.0090$	$29.7^{bcd} \pm 1.6$
Fol+JA	$0.44^{bc} \pm 0.029$	$0.418^{cd} \pm 0.0094$	$35.13^{de} \pm 3.13$

Fol+Tv	$0.408^{bc} \pm 0.016$	$0.39^{cd} \pm 0.0112$	$31.13^{cde} \pm 1.9$
Fol+Tv+JA	$0.425^{bc} \pm 0.013$	$0.429^{d} \pm 0.0129$	$40.3^{e} \pm 1.8$
Rs	$0.42^{bc} \pm 0.018$	$0.379^{c} \pm 0.0065$	$34.62^{de} \pm 2.6$
Rs+JA	$0.45^{bc} \pm 0.01$	$0.425^{d} \pm 0.0126$	$34.5^{de} \pm 1.4$
Rs+Tv	$0.411^{bc} \pm 0.014$	$0.379^{c} \pm 0.0068$	$31.26^{cde} \pm 1.44$
Rs+Tv+JA	$0.462^{c} \pm 0.016$	$0.428^{d} \pm 0.0103$	$40.25^{e} \pm 1.8$

22. Tocopherol Content

In addition to AsA and GSH content, tocopherol content has also been monitored to increase in pathogen-challenged tomato plants post 30 and 60 days of pathogen infection. Furthermore, seed priming with *T. virens* and JA alone or together raises the content of tocopherol in diseased as well as non-infected plants. In comparison to the control, maximum tocopherol content (40.3 µg/ g FW) was monitored in the treatment Fol+ Tv+JA followed by Rs+Tv+JA (40.3 µ g/ g FW) in sixty days old plants, and both of these values are non-significant to each other. A similar trend of tocopherol enhancement has also been followed in 90 days old tomato plants under different treatments. In the case of *F. oxysporum lycopersisci*, priming of tomato seeds with JA and *T. virens* in combination caused a significant increase of 47. 7 % in tocopherol content, while a percentage increase of 39 % has been observed in the treatment Rs+Tv+JA compared to the treatment Rs alone (Table 6.4 & 6.5).

Table 6.5 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on AsA, GSH and Tocopherol contents in ninety days old tomato plants with and without pathogenic stress.

Values shown are means followed by ± Standard Error of Mean (n = 3). Different lower-case letters in the table indicate significant differences between treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Treatments	Ascorbic acid (mg/ g FW)	Glutathione (mg/ g FW)	Tocopherol (µg/ g FW)
CN	$0.19^{a} \pm 0.01$	$0.2^{a} \pm 0.01$	$17.42^{a} \pm 2.13$
Tv	$0.23^{ab} \pm 0$	$0.21^{ab} \pm 0$	$19.69^{ab} \pm 0.86$
JA	$0.26^{abc} \pm 0.01$	$0.25^{ab} \pm 0.01$	$21.03^{ab} \pm 1.59$
Tv+JA	$0.28^{bc} \pm 0.02$	$0.27^{b} \pm 0.01$	$22.45^{abc} \pm 1.64$
Fol	$0.33^{cd} \pm 0.02$	$0.35^{c} \pm 0.01$	$28.77^{bcd} \pm 1.4$
Fol+JA	$0.46^{fg} \pm 0.03$	$0.43^{de} \pm 0.01$	$36.98^{de} \pm 2.85$
Fol+Tv	$0.41^{def} \pm 0$	$0.4^{cde} \pm 0.01$	$32.77^{d} \pm 3.35$
Fol+Tv+JA	$0.51^{g} \pm 0.01$	$0.44^{e} \pm 0.01$	$42.48^{e} \pm 1.32$
Rs	$0.38^{de} \pm 0.02$	$0.35^{c} \pm 0.01$	$31.18^{cd} \pm 2.14$
Rs+JA	$0.49^{g} \pm 0.01$	$0.44^{e} \pm 0.02$	$37.2^{de} \pm 1.27$
Rs+Tv	$0.44^{efg} \pm 0.02$	$0.38^{cd} \pm 0.01$	$34.74^{de} \pm 1.58$
Rs+Tv+JA	$0.49^{g} \pm 0.01$	$0.45^{e} \pm 0.01$	$43.34^{e} \pm 0.98$

Enzymatic Antioxidants

23. Superoxide Dismutase (SOD)

Infection of tomato plants with both pathogens leads to an increase in the activity of the SOD enzyme. In comparison to ninety days old plants, SOD activity was observed to be more in sixty days old plants under different applied treatments (Fig 6.29 A). Further, combined seed priming with *T. virens* and JA raised the activity of the SOD enzyme in the case of both *Fol* and *R. solani*-infected plants to the maximum level. As in the case of *Fol* inoculated plants a significant increase of 56 and 48 % has been reported in the treatment Fol+Tv+JA in 60- and 90-days old tomato plants, respectively. Similarly, in comparison to non-primed seedlings, in the case of *R.*

solani diseased plants highest activity of SOD in sixty days (57 %) and ninety days old (62 %) plants have been monitored under the treatment Rs+Tv+JA (Fig..6.29 A & B).

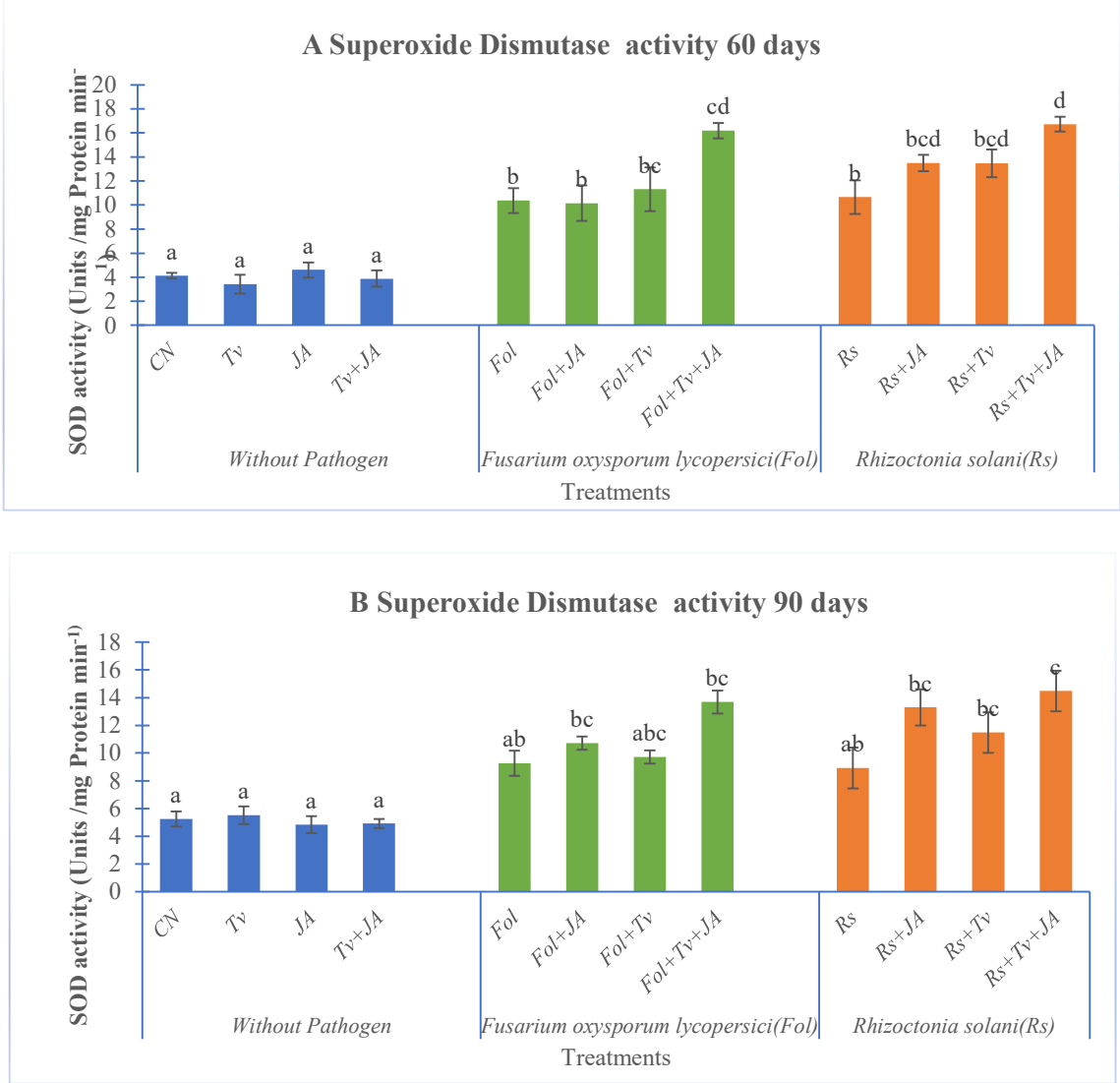
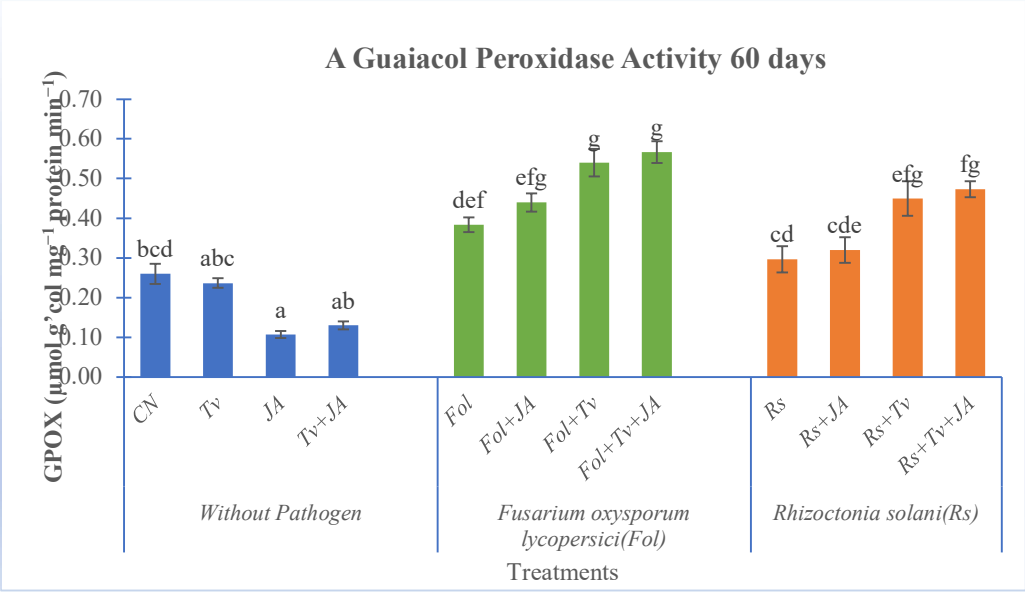


Fig. 6.29 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on the enzymatic activity of SOD enzyme in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

24. Guaiacol peroxidase (GPOX)

Pathogens inoculation caused a marked increase in the activity of GPOX enzyme at both stages of growth in contrast to the control plants. The activity of GPOX enzyme was observed to be slightly higher in sixty rather than ninety days old plants. In the case of sixty days old plants, the most increased activity was reported in the treatment Fol+Tv+JA followed by Fol+Tv though they are non-significantly different from each other. Though in non-inoculated plants, seed priming with biostimulants decreased this enzyme's activity. Furthermore, in the case of *R. solani* diseased plants, maximum GPOX activity (0.47 µ mol mg^{-1} protein min^{-1}) has been observed in the treatment Rs+Tv+JA (Fig. 6.30 A). In comparison to the control, a percentage increase in GPOX activity of magnitude 118 and 82 % has been reported in the treatment Fol+Tv+JA and Rs+Tv+JA, respectively. Likewise, in ninety days old plants, a significant rise of 2.9- fold of control in GPOX activity has been observed in the treatments Fol+Tv+JA and Rs+Tv+JA. Though priming tomato seeds with ameliorative agents non-significantly altered GPOX activity in ninety days old *Fol* challenged tomato plants, in ninety days old plants, a marked increase in GPOX activity has been monitored under the treatments Rs+JA and Rs+Tv+JA in *R. solani* infected tomato plants (Fig. 6.30 B).



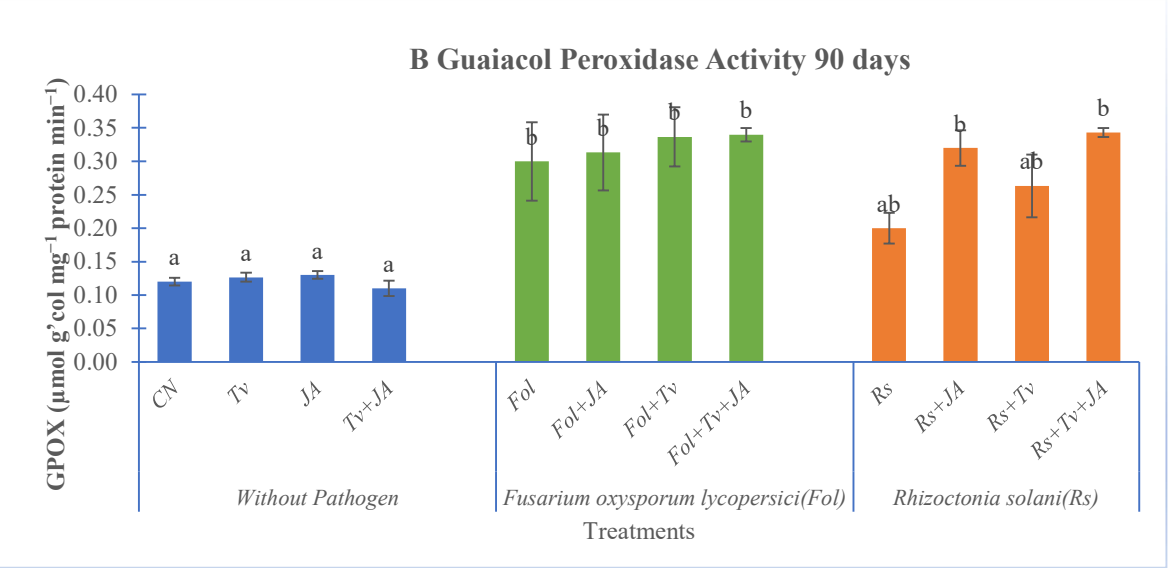


Fig. 6.30 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on the enzymatic activity of GPOX enzyme in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

25. Ascorbate peroxidase (APX)

It has been observed that the activity of APX has been increased in the pathogenically stressed tomato plants. Among these, a maximum elevation in APX activity was observed in the plants seed-primed with *T. virens* and JA in combination in sixty days old plants challenged with the respective pathogens. In the cases of *Fol* and *R. solani* challenged sixty days old plants, an individual rise of 15.3 and 124 % have been monitored in the treatments Fol+Tv+JA and Rs+Tv+JA, respectively (Fig. 6.31 A). Moreover, in the case of ninety days old plants, priming tomato seeds with these biostimulants either individually or in combination does not bring any significant change in the APX activity in the case of *Fol*-stressed plants but a considerable increase in the case of *R. solani* diseased tomato plants (Fig. 6.31 B).

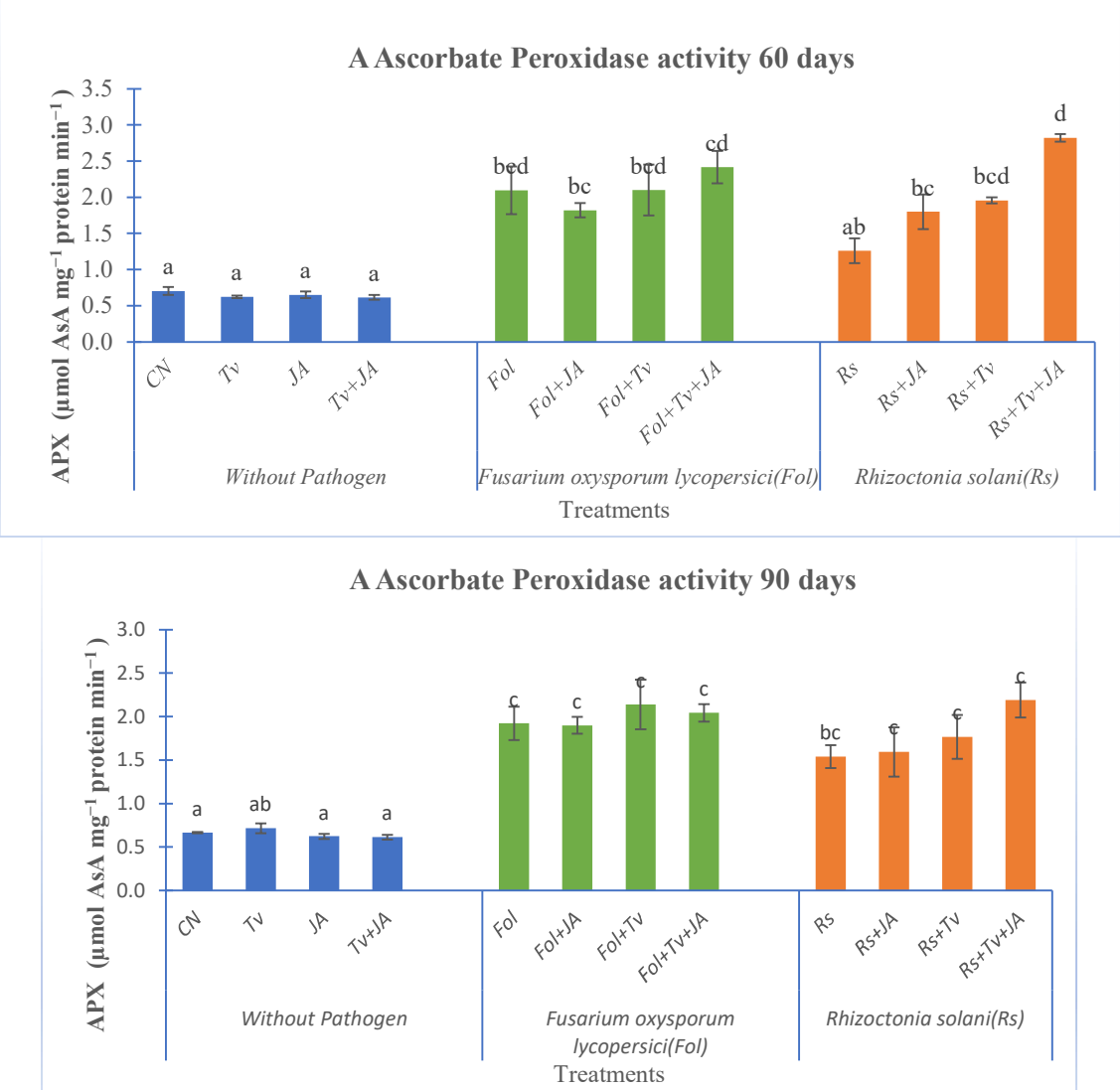
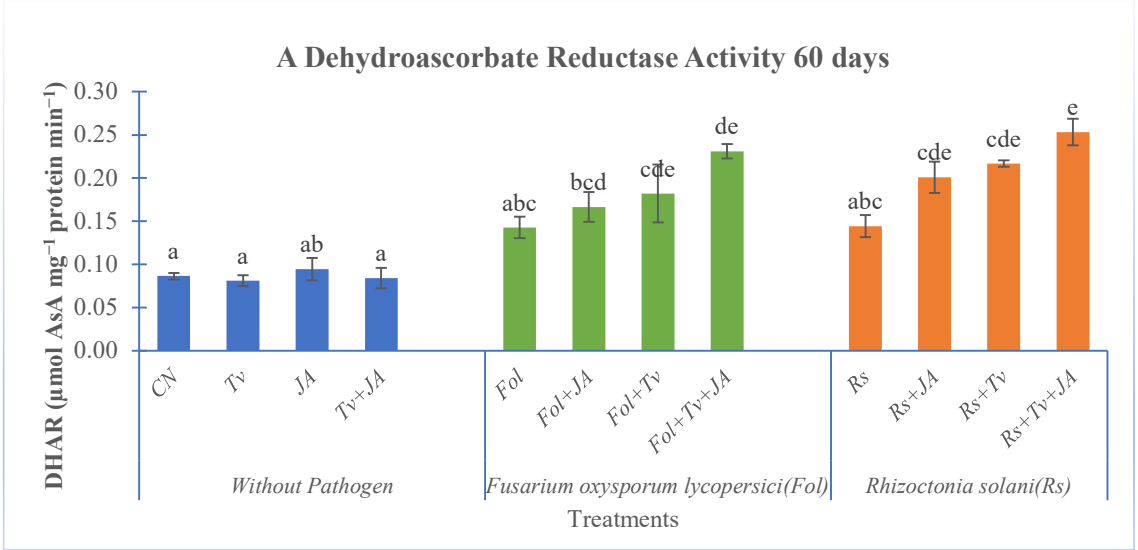


Fig. 6.31 Effect of Trichoderma virens (Tv) and Jasmonic acid (JA) on the enzymatic activity of APX enzyme in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters abc the column specify statistically significant variations amongst the treatmen corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

26. Dehydroascorbate reductase (DHAR) activity

The pathogenic infection elevated the DHAR activity in the stressed plants after thirty and sixty days of post pathogens inoculation. In the case of sixty days old plants, the highest DHAR activity was measured in the treatment Rs+Tv+JA followed by treatment Fol+Tv+JA which were respectively about 168 and 194 % higher than the control plants (Fig. 6.32 A). A similar DHAR activity enhancement trend has been monitored in the pathogenically stressed ninety days old tomato plants. Pre-treatment of tomato seeds with *T. virens* and Jasmonic acid combined increased the DHAR activity to the highest level, which was observed to be 62 and 76 % more than *Fol* and *R. solani* stressed plants, respectively (Fig. 6.32 B).



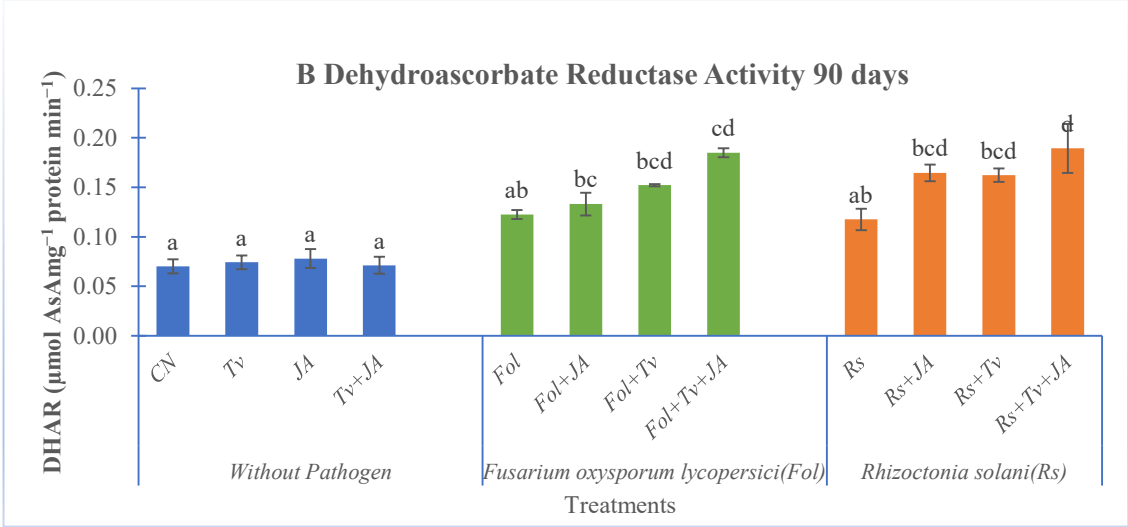


Fig. 6.32 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on the enzymatic activity of DHAR enzyme in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

27. Glutathione Peroxidase (GPX)

GPX activity was monitored after thirty and sixty days of pathogens inoculation in tomato plants. No significant time-dependent alteration in GPX activity has been observed in sixty- and ninety-day-old tomato plants under control as well as stressed conditions. In sixty days old plants, in comparison to the control, the highest activity of GPX was observed in the treatment Fol+Tv+JA (3.2- fold) followed by Fol+Tv (3.1- fold), and statistically, the values of these treatments were non-significant to each other. Furthermore, in the case of *R. solani* diseased plants, a marked increase of 41 % has been reported in the treatment Rs+Tv+JA (Fig. 6.33 A). Similarly, in the case of ninety days old non-inoculated plants, a significant enhancement and decrease in the activity of GPX have been monitored in the case of treatments Tv, JA and Tv+JA, respectively, when compared with control plants. Furthermore, in the case of *Fol* and *R. solani* stressed tomato plants significant increase in the activity of GPX

has been observed in the plants, in which priming of tomato seeds was done with these ameliorative agents before the pathogenic inoculations except in the treatment Fol+JA (Fig. 6.33 B).

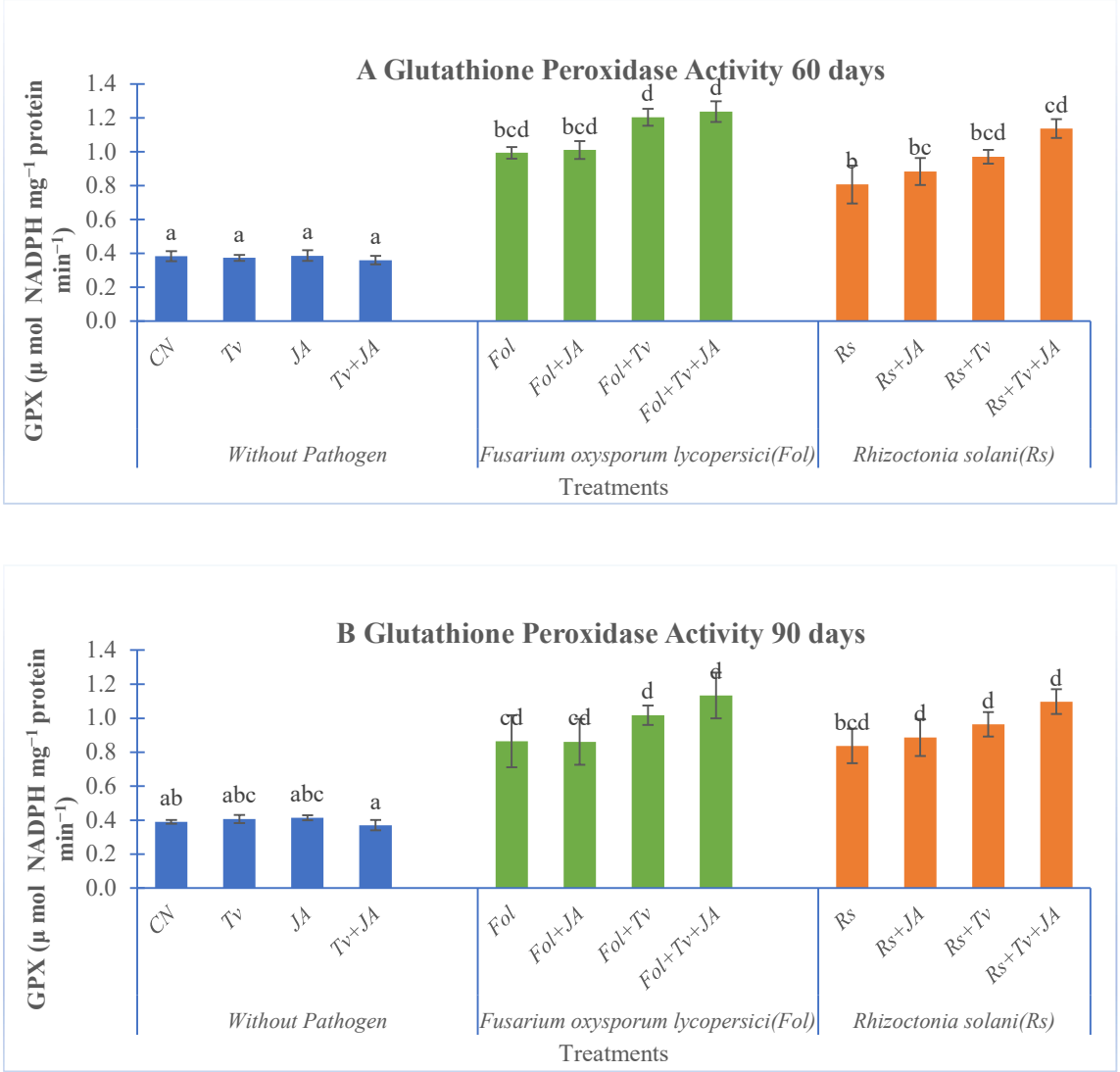
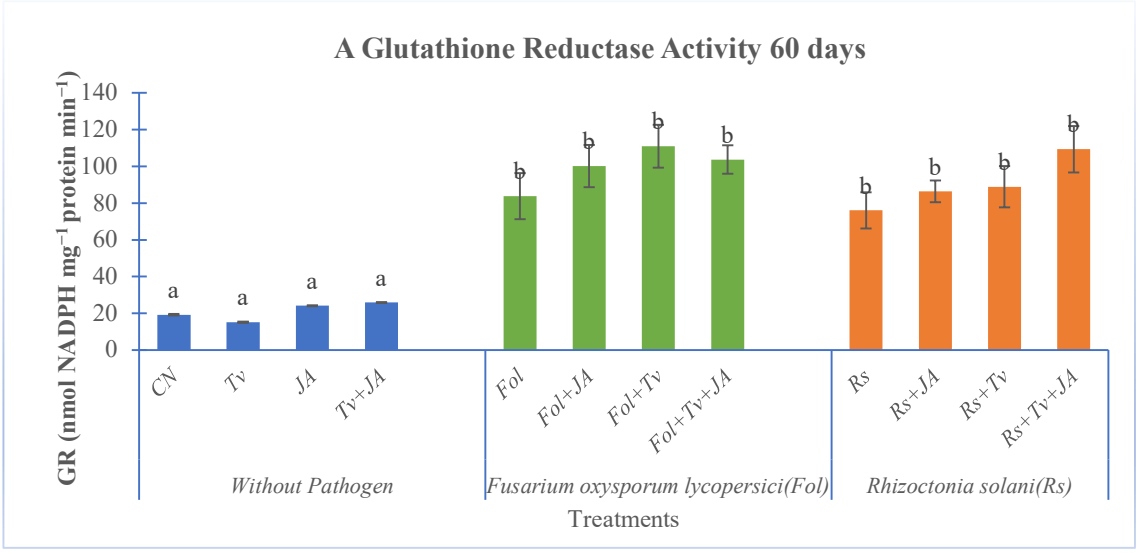


Fig. 6.33 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on the enzymatic activity of GPX enzyme in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

28. Glutathione Reductase (GR)

In comparison to the control plants, a significant elevation in GR activity has been reported in the pathogenically stressed plants. In contrast to the control, a 4.4- and 4-fold increase in the activity of GR enzymes has been observed in *Fol* and *R. solani* infected sixty days old tomato plants, respectively (Fig. 6.34 A). Similar findings have also been monitored in the ninety days old plants (Fig. 6.34 B). However, pre-treatment of tomato seeds with *T. virens* and JA alone or in combination did not induce any significant change in the activity of GR enzyme in pathogens-inoculated and non-inoculated tomato plants at both the observed growth stages (Fig. 6.34 A & B).



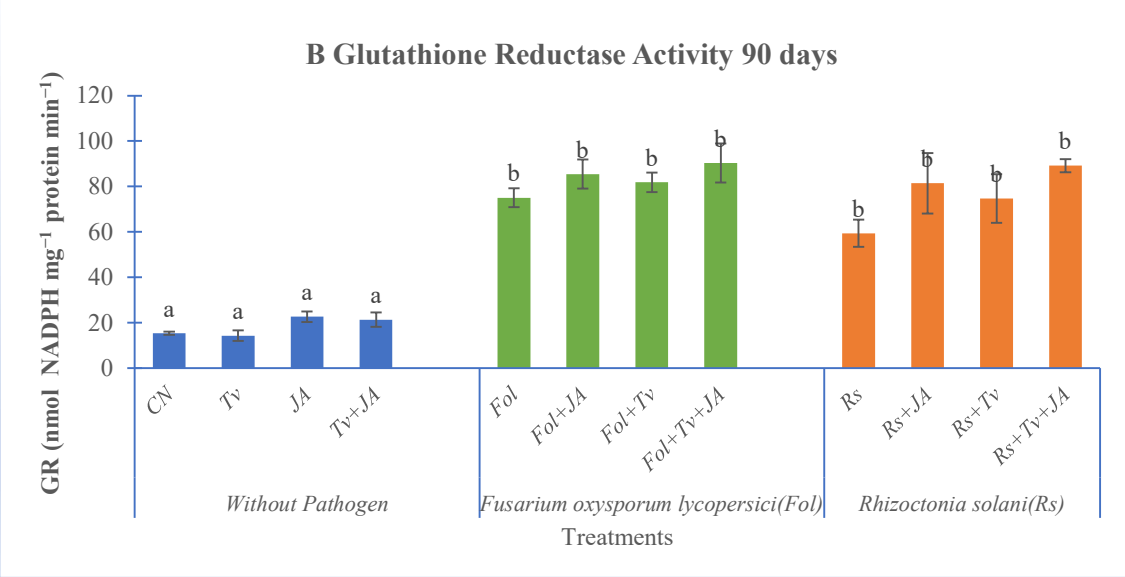
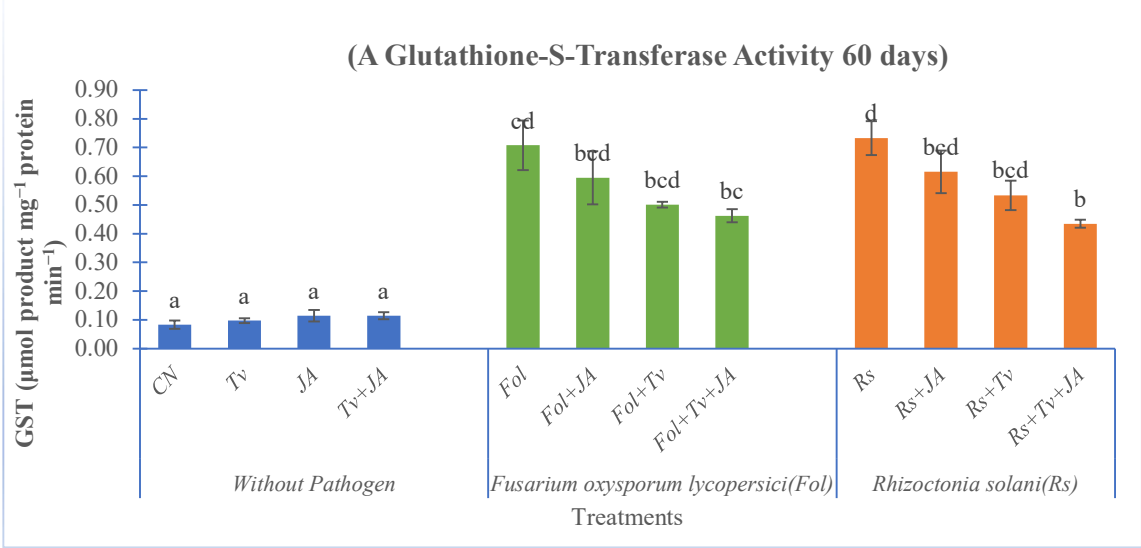


Fig. 6.34 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on the enzymatic activity of GR enzyme in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

29. Glutathione-S-Transferase (GST)

Quantifying GST activity by using CDNB substrate revealed that in comparison to control plants, pathogenically challenged plants exhibit a significant increase in GST activity. Furthermore, no remarkable alteration in the enzymatic activity has been reported in the case of sixty- and ninety-days old plants. Tomato seed priming with *T. virens* and Jasmonic acid, either alone or in combination, decreases the GST activity in both *Fol* and *R. solani* stressed plants at both the growth stages (Fig. 6.35 A and B). Maximum GST activity has been monitored in the treatments Rs (8.8- fold of control) and Fol (9.5- fold of control) for sixty- and ninety-days old plants, respectively.



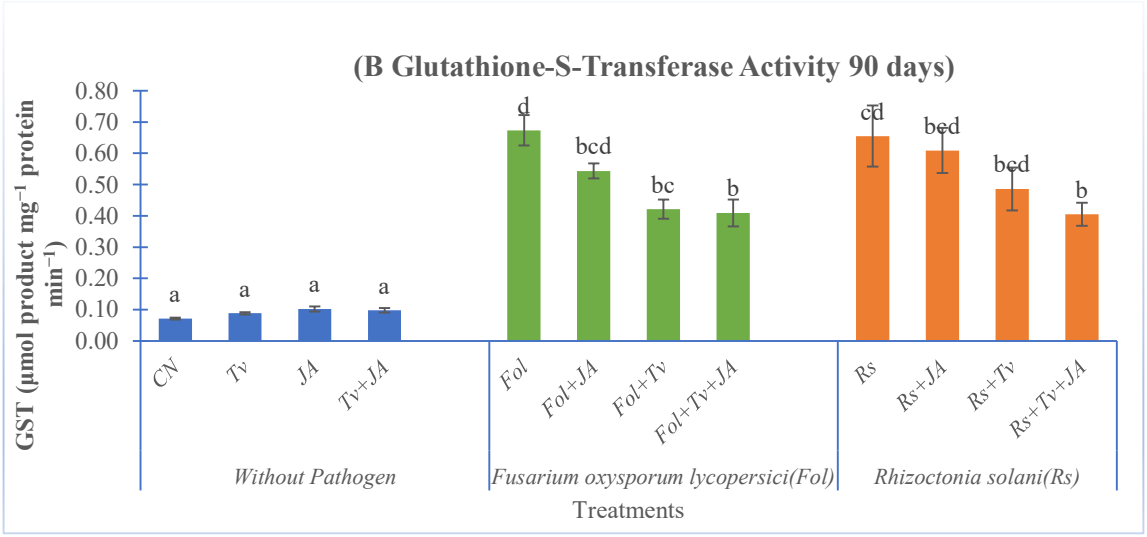


Fig. 6.35 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on the enzymatic activity of GST enzyme in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

30. Polyphenol Oxidase (PPO)

Owing to the pathogenic stress, an enhancement in the activity of PPO has been monitored in the primed as well as non-primed tomato plants. In the sixty days old plants, maximum PPO activity has been monitored in the Rs+Tv+JA. In comparison to control plants, a 140 and 104 % enhancement in the PPO activity has been detected in the *Fol* and *R. solani* stressed plants, respectively. Moreover, in the case of *Fol*-inoculated tomato plants, seed priming with *T. virens* alone and in combination with Jasmonic acid significantly raised the enzymatic activity to an equal extent, statistically. Similar findings have been observed in *R. solani* diseased, sixty-day-old tomato plants. As depicted in (Fig. 6.36 A) a percentage increase of 72.5 and 89.3 % has been observed in the tratments Rs+Tv and Rs+TV+JA in the case of infection with *R. solani*, respectively. Similarly in the case of ninety days old plants in comparison to control, pathogens inoculation elevates the activity of PPO enzyme in the stressed plants, but the pre-treatment of infected plants with JA and JA+Tv

exhibited the most significant influences on PPO activity in the case of both *Fol* and *R. solani* challenged plants (Fig. 6.36 B).

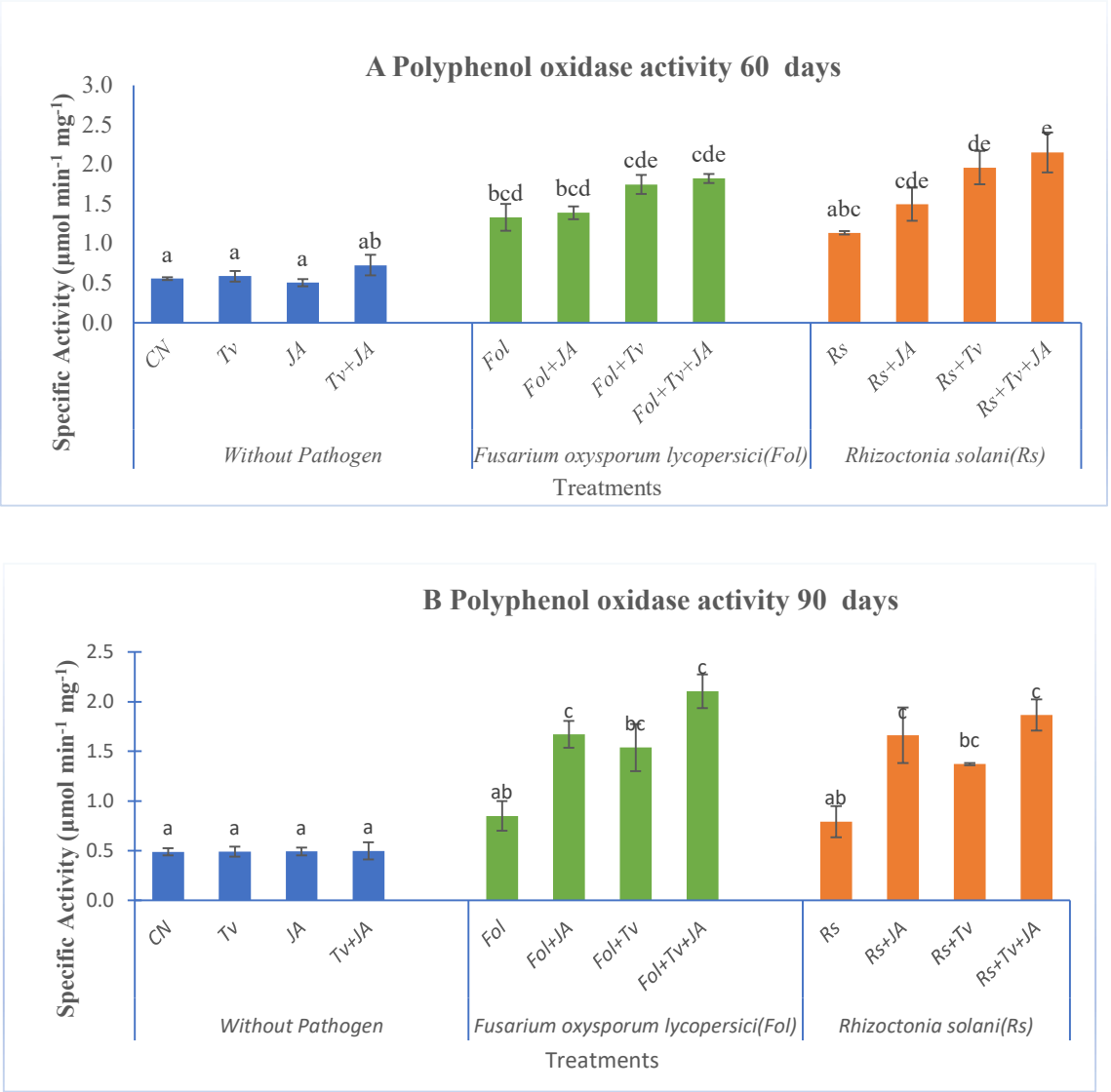
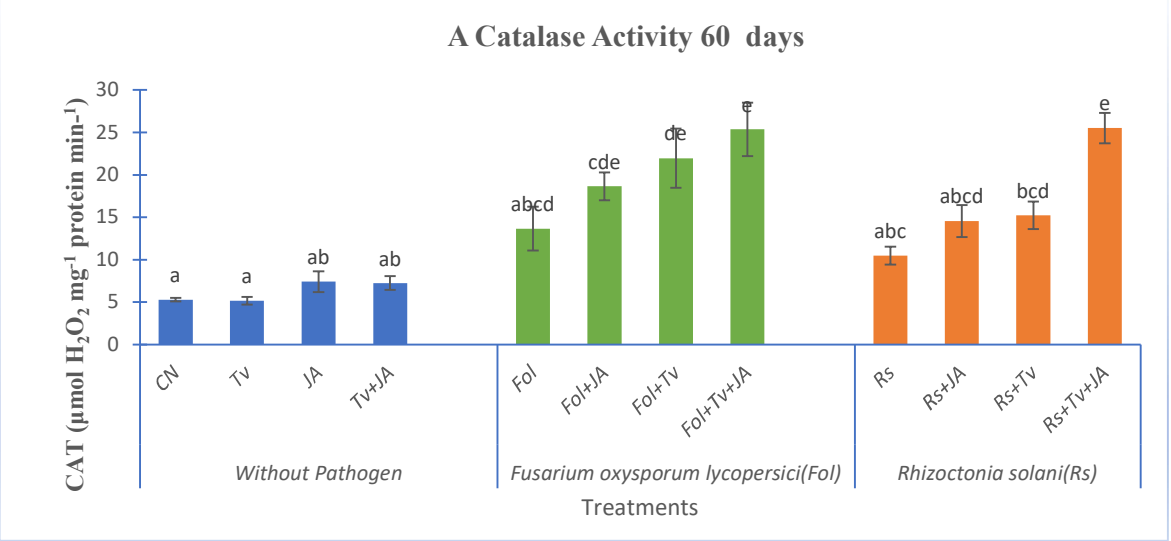


Fig. 6.36 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on the enzymatic activity of PPO enzyme in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

31. Catalase

Our findings further revealed that the pathogenic inoculations tended to increase not only the content of hydrogen peroxide but also lead to the increase in the activity of catalase enzyme at both the growth stages. Moreover, either alone or combined application of *T. virens* and Jasmonic acid further augmented the activity of this enzyme in the case of both diseased as well as non-inoculated sixty days old plants except in the treatment Tv. Consequently, compared to sixty days old plants, the enzyme activity decreased in ninety days old plants. In the case of sixty days old plants most significant increase in the catalase activity has been observed in the treatment Fol+Tv+JA in the case of *Fol*-stressed plants (Fig. 6.37 A). Likewise, in *R. solani*-challenged plants most remarkable increase in the catalase activity has been monitored in the treatment Rs+Tv+JA. Similarly, in the case of ninety days old plants, compared to the control, the maximum catalase activity has been observed in the treatment Rs+Tv+JA (3.05- fold) (Fig. 6.37 B).



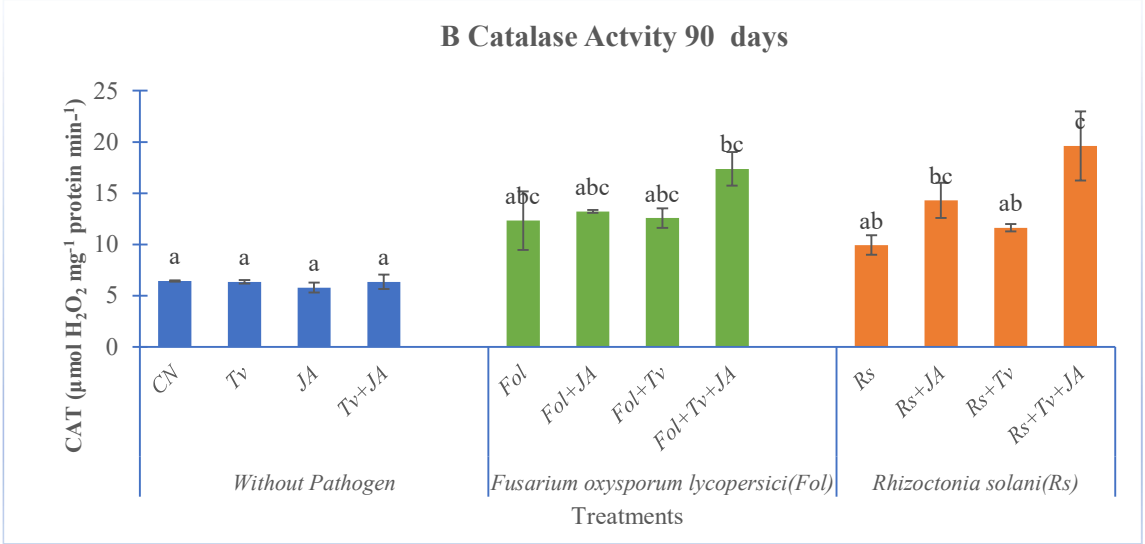


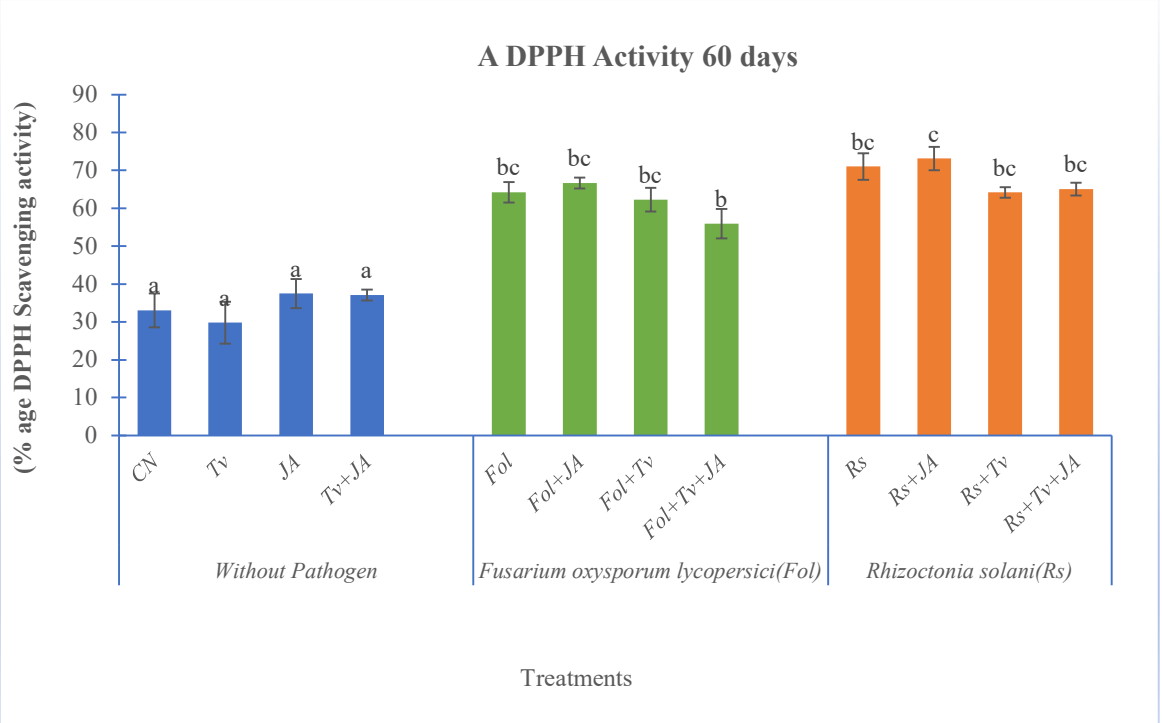
Fig. 6.37 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on the enzymatic activity of CAT enzyme in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

32. Free radical scavenging Activity

The antioxidant capabilities of tomato seedlings have been found to increase due to pathogen-induced damage and are exhibited in their inhibition activity to some radicals. The antioxidant activity of the methanolic extract of stressed and non-stressed tomato leaves was investigated through ABTS and DPPH methods. In sixty days, old tomato leaves, in comparison to control, the percentage increase in DPPH activity was measured to be 94.4 and 115 % in the case of *Fol* and *R. solani*, respectively. Further, a significant reduction in the DPPH activity has been observed in the treatment Fol+Tv+JA in *Fol* stressed tomato plants (Fig. 6.38 A). On the other hand, in the case of *R. solani* diseased plants, the most significant increase in the DPPH activity has been monitored in the treatment Rs+JA. As with DPPH activity, the percentage increase in ABTS activity in the case of *Fol (*106.6 %) and *R. solani* (161.4 %) has also been reported when compared with control plants. Our findings also revealed that the most significant enhancement in ABTS activity has been depicted in the diseased plants where seeds were primed with jasmonic acid before the exposure of the respective pathogen (Fig. 6.38 B). Similarly, in 90 days old

tomato plants challenged with *Fol* a significant reduction in DPPH activity has been monitored in the treatment Fol+Tv and Fol+Tv+JA, while in the case of *R. solani* infected tomato plants priming of tomato seeds with *T. virens* alone brought a significant reduction in DPPH activity under the treatment Rs+Tv (Fig. 6.39 A). Likewise, to DPPH, a similar trend of enhancement has been followed by ABTS activity in diseased plants. Furthermore, plants (disease free or diseased) have depicted maximum extent of ABTS activity in which tomato seeds were pre-treated with JA alone (Fig. 6.39 B).



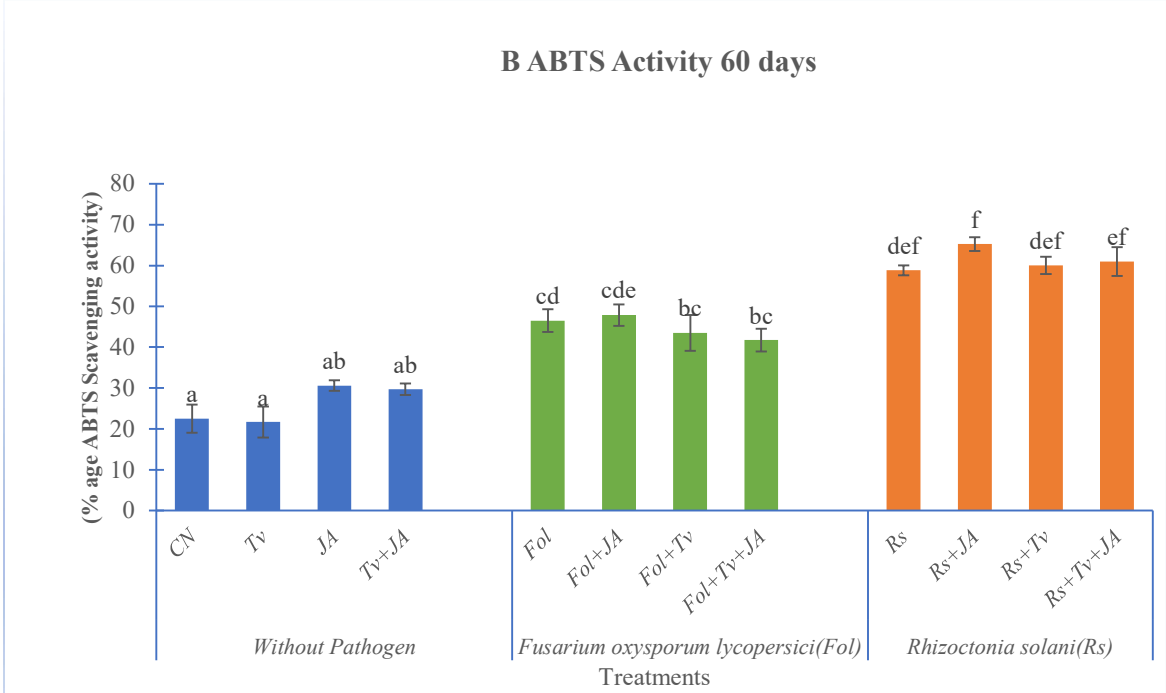
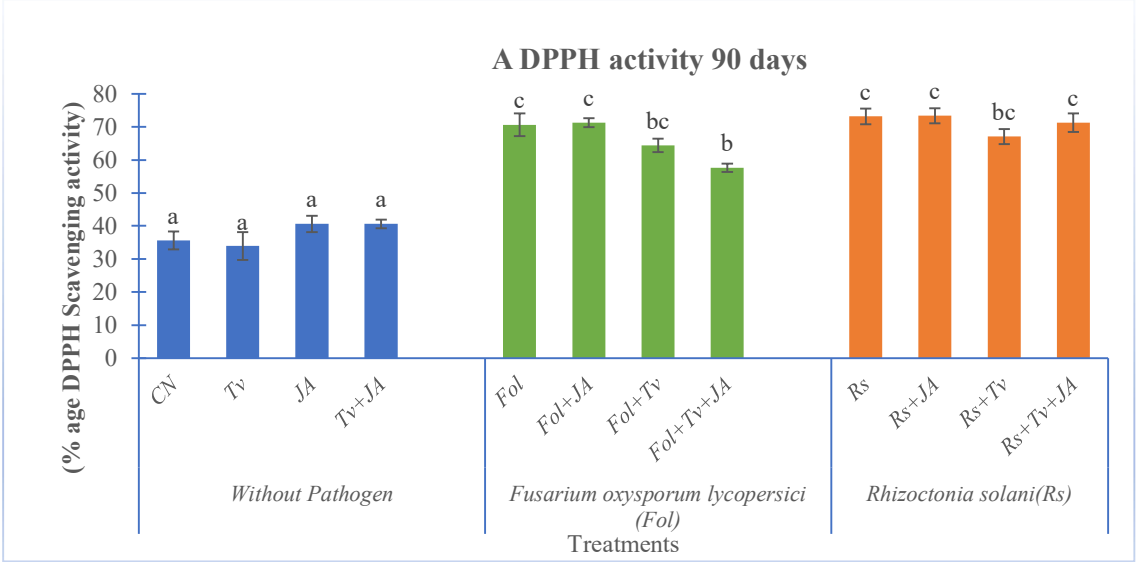


Fig. 6.38 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on the free radical scavenging activity through [A] DPPH and [B] ABTS assay in 60 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).



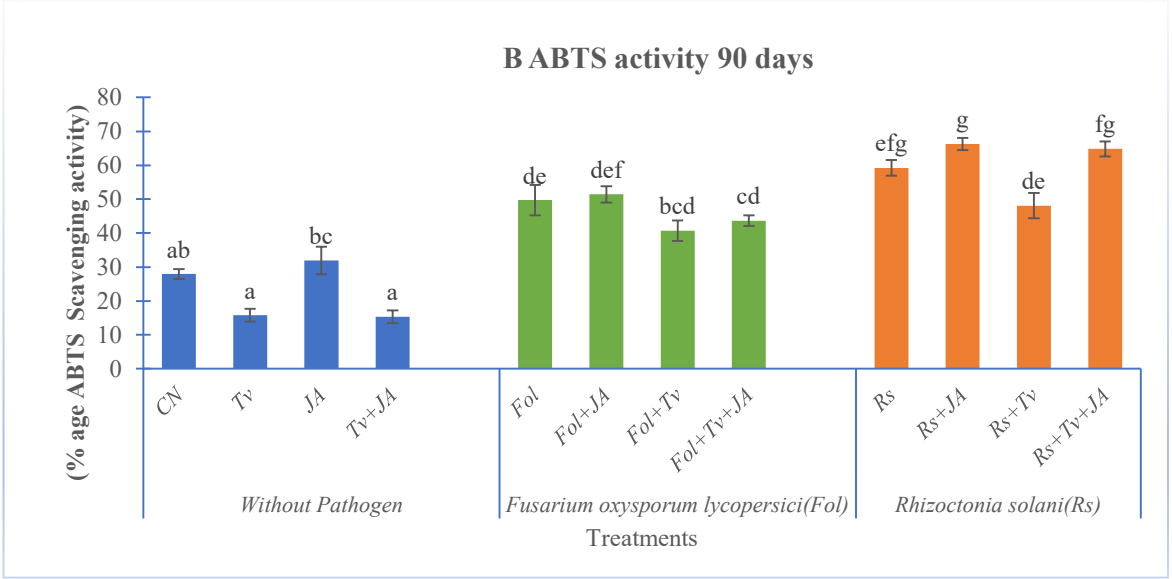


Fig. 6.39 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on the free radical scavenging activity through [A] DPPH and [B] ABTS assay in 90 days old tomato plants with and without pathogenic stress.

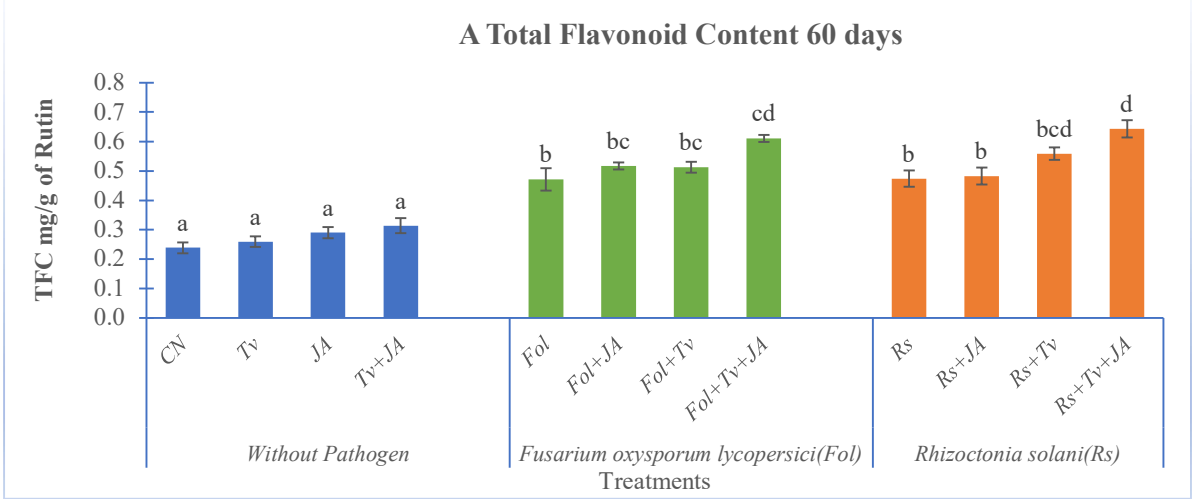
Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

Secondary metabolites

33. Total Flavonoid Content (TFC)

Flavonoid contents were observed to enhance because of pathogen-induced damage to tomato leaves, in sixty days old tomato plants, percentage increases in total flavonoid content were of the magnitude of 97.7 % in the case of *Fol* and 98.9 % in the case of *R. solani* when compared with non-infected counterparts. Further, the most significant increase in the total flavonoid content to 29.7 % (*Fol*) and 35.8 % (*R. solani)* was monitored when tomato seedlings were primed with JA and *T. virens* combinedly. The maximum flavonoid content has been reported in the treatment Rs+Tv+JA followed by treatment Fol+Tv+JA (Fig. 6.40 A).

A similar trend of increase in TFC has been monitored in ninety days old tomato plants after sixty days of pathogen contamination. Furthermore, the level TFC was reported to be more in the case of *R. solani* stressed plants than in comparison to *Fol* challenged one in ninety days old tomato plants. Maximum TFC was observed in the treatment, Rs+Tv+JA i.e., 0.63 mg/g FW followed by treatment Fol+Tv+JA which is measured to be 0.59 mg/g FW (Fig. 6.40 B).



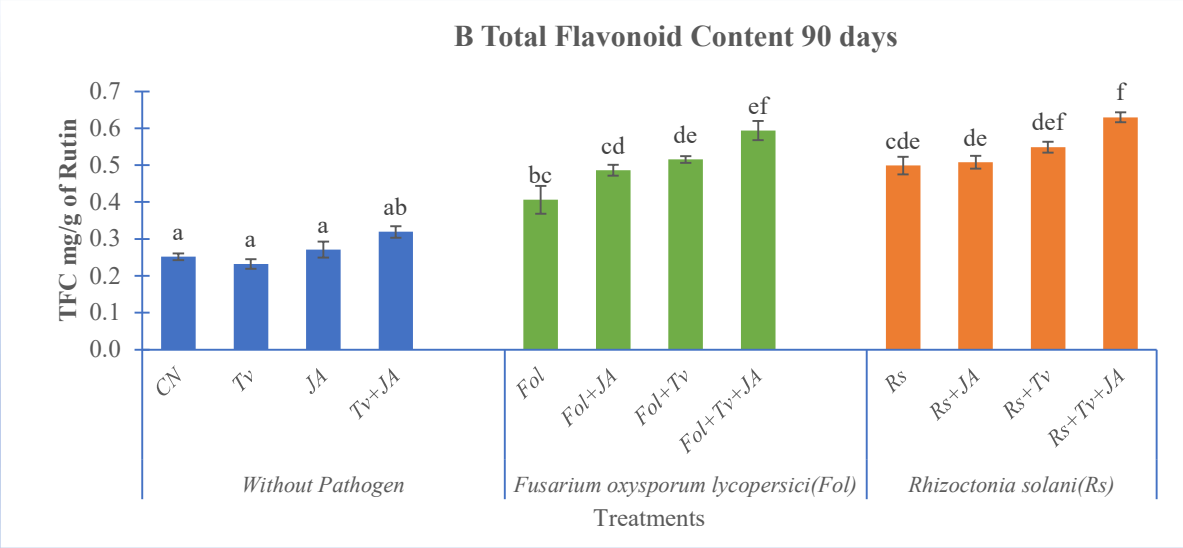
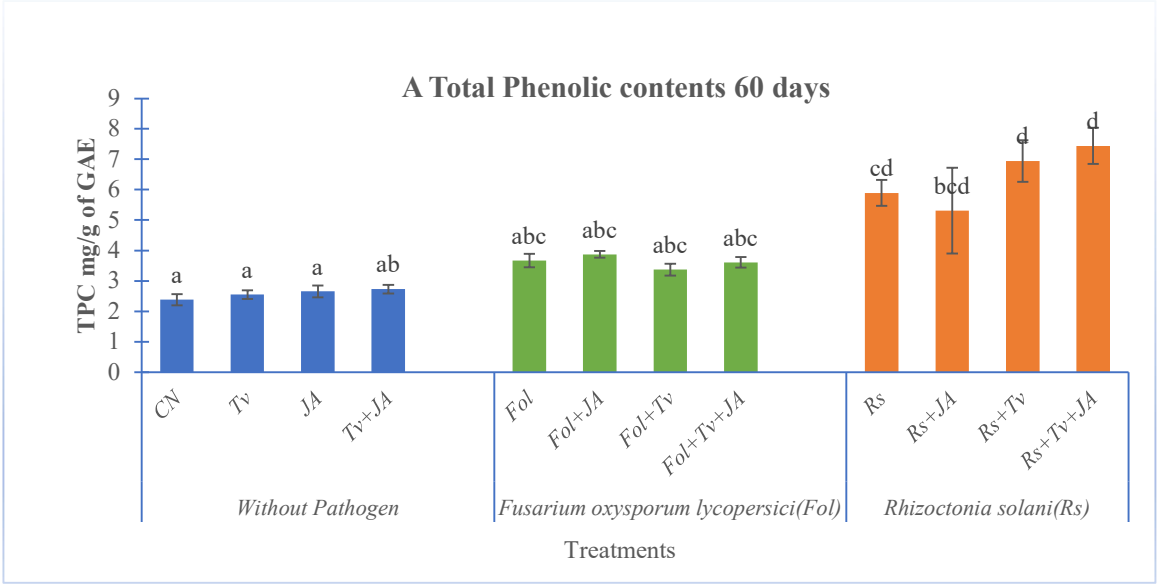


Fig. 6.40 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Total Flavonoid content in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

34. Total Phenolic Content (TPC)

TPC was calculated using the standard curve of Gallic acid. Pathogens inoculations has been observed to raise the levels of TPC in diseased plants. In the case of sixty days old plants, in contrast to the control, a significant increase in TPC (54 and 147 %) was perceived due to *Fol* and *R. solani* induced stresses, respectively. Moreover, pre-treatment of tomato seeds with JA, *T. virens* and JA+*T. virens* generated non-significant and significant changes in TPC in *Fol* and *R. solani* diseased plants, respectively. Statistically, the highest content of TPC was monitored in the treatment Rs+Tv+JA and Rs+Tv (Fig. 6.41 A). Likewise, TPC reported after sixty days of pathogen inoculation exhibited a similar trend of a substantial increase in TPC in *R. solani* infected plants when compared with non-inoculated, and *Fol*-inoculated plants. Pre-treatment of tomato seeds with *T. virens* and JA alone or in combination did not insert any significant influence in TPC accumulation in both *Fol* and *R. solani* diseased ninety days old tomato plants (Fig. 6.41 B).



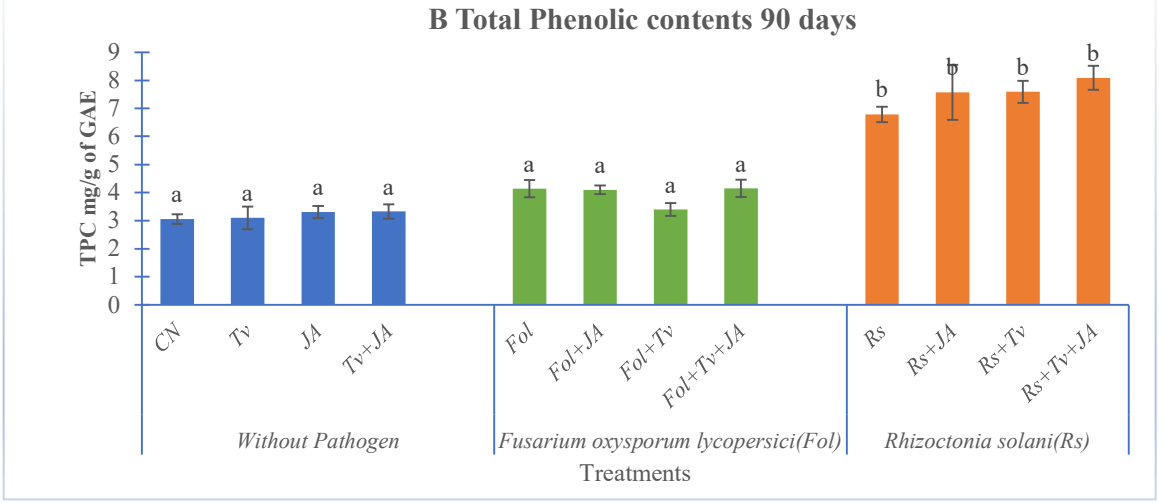


Fig. 6.41 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Total Phenolic content (TPC) in [A] 60 and [B] 90 days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=3]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$).

35. Molecular Aspects

To ascertain the effectiveness of biological control agent and chemical inducer in the enhancement of transcription of defence-related genes, the expression pattern of the two antioxidative genes, i.e., *Cu-Zn SOD* and *Catalase-I,* was investigated using quantitative RT-PCR from tomato leaves after thirty days of pathogen inoculation (Fig. 6.42). Consequently, compared to non-inoculated control, a considerable rise in the *catalase* gene expression has been observed. As depicted in (Fig. 6.43 A) in the *Fol*-challenged plants concerning the expression of *actin* gene, above 3- and 7-fold increase in the expression of *catalase* gene has been monitored in the treatments Fol and Fol+Tv+JA, respectively. Similar findings have been observed in the *R. solani*-challenged tomato plants.

In addition to *catalase,* expression of *SOD* gene has also been reported to be upregulated owing to the pathogens-induced stresses in the diseased plants. In reference to *actin* gene, a 2.5- and 6.8- fold increase in the expression of the *SOD* gene has been reported in the treatment Fol and Fol+Tv+JA, respectively, in the *Fol* challenged plants. Similarly, in the case of *R. solani* inoculated plants, a 2.9- and 7.3-fold increase in the level of expression of *SOD* has been reported (Fig. 6.43 B).

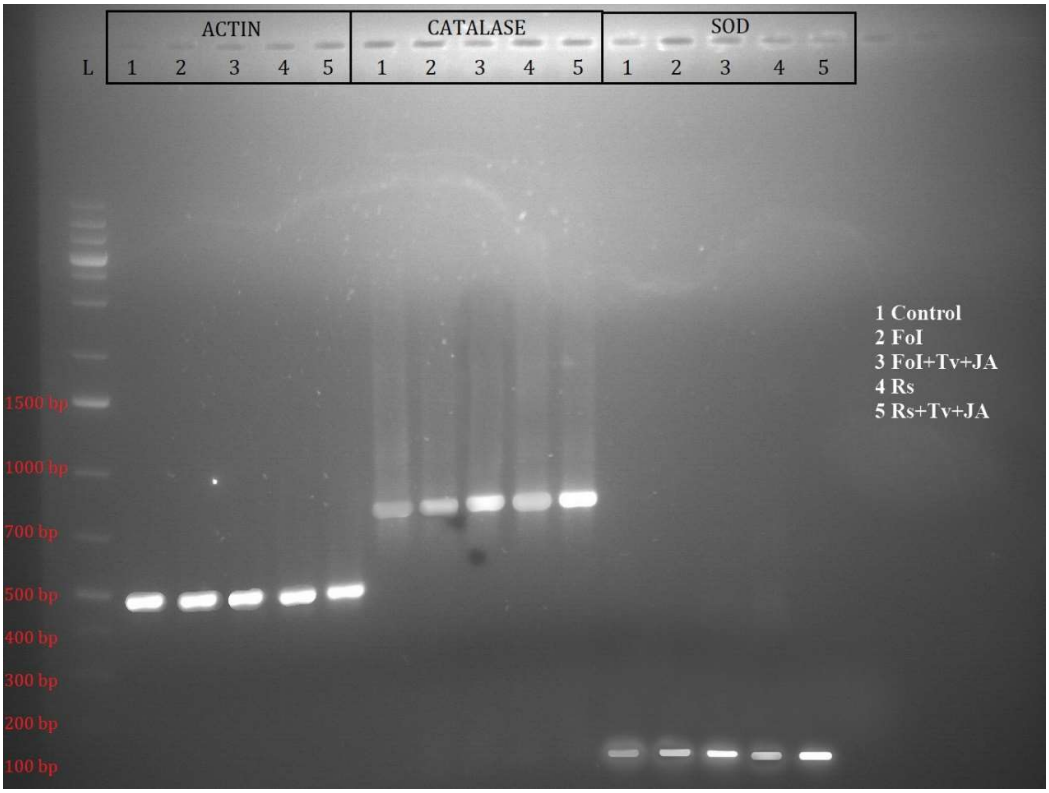
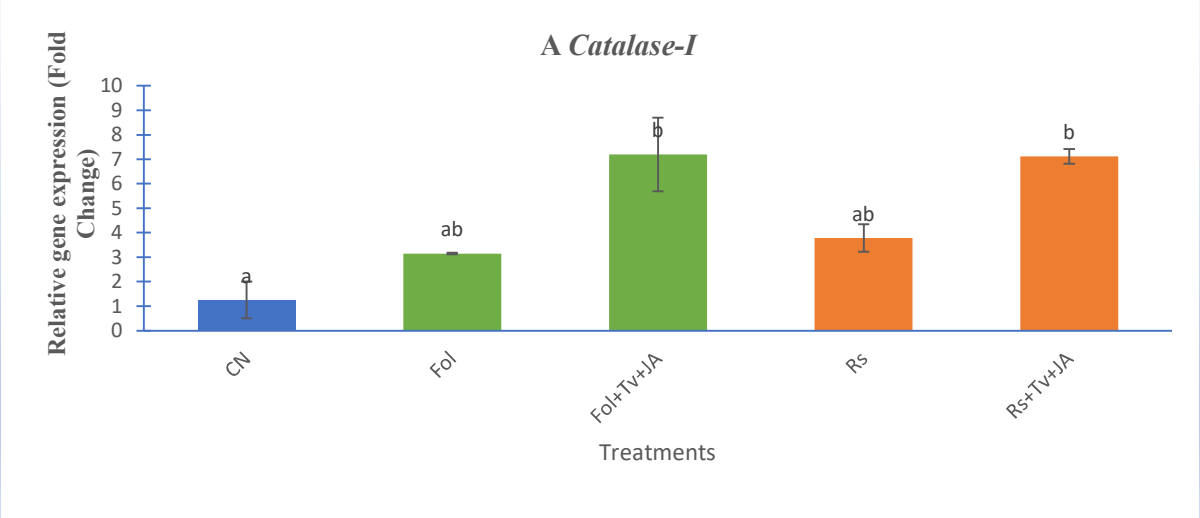


Fig. 6.42 Validation of specificity of primers used in Real Time *q*-RTPCR analysis. Contrast enhanced image of electrophoresis gel confirming amplicon size and primer specificity using RT-qPCR amplification product.

The sizes for nucleotide ladder are indicated to left of bands (100 to 1500 bp).



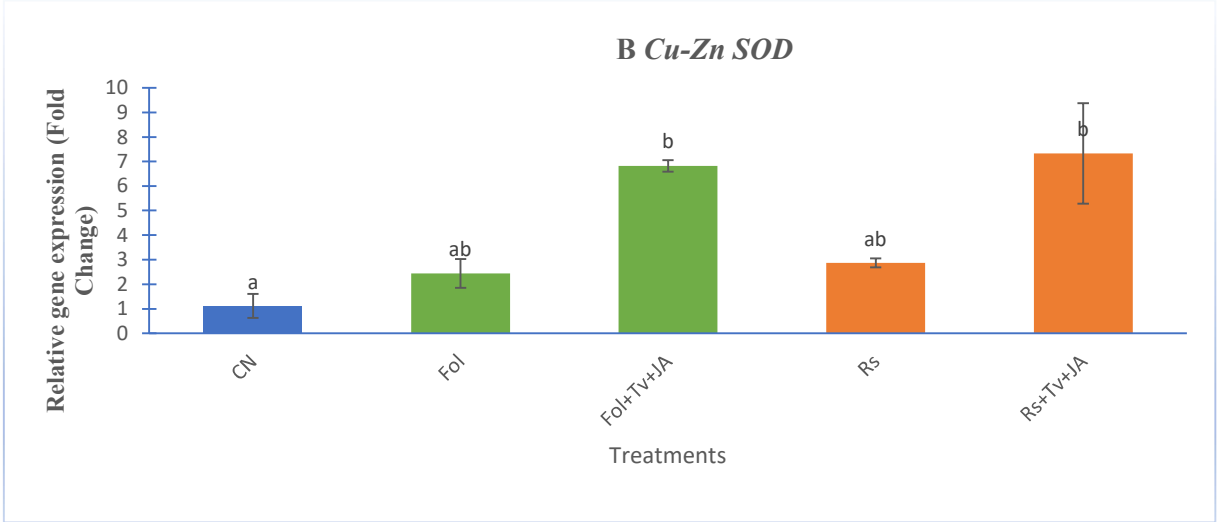


Fig. 6.43 Effect of *Trichoderma virens* (Tv) and Jasmonic acid (JA) on Relative fold change in the expression value of [A] *Cat-I* and [B] *Cu-Zn SOD* in sixty days old tomato plants with and without pathogenic stress.

Bars represent the Standard Error of Mean [n=2]. Different lower-case letters above the column specify statistically significant variations amongst the treatments, corresponding to Tukey's Multiple Comparison Test ($p < 0.05$)

Discussion

During different stages of growth and development, tomatoes are vulnerable to more than 200 types of diseases. Among these, soilborne pathogens represent a major element for longer persistence and a more comprehensive host range (Cheng et al., 2021; Singh et al., 2017). Amongst soil-borne pathogens, *Fol* has been considered one of the major critical risks to greenhouse and field-grown tomatoes globally, accounting for approximately 10-80 % yield reduction worldwide (Ma et al., 2023; Srinivas et al., 2019). Likewise, *Rhizoctonia solani*-induced infection causes many crops and yield loss at the early and later stages of growth of this horticulturally important vegetable crop (Ebrahimi-Zarandi et al., 2021; Taheri et al., 2024). Therefore, *Fol* and *R. solani,* the most devastating pathogens of tomato, were taken in the present investigation, which was identified through different methods. Consequently, the disease-producing capability of *Fol* was evaluated through the pathogenicity test by perceiving symptoms like vein clearing, reduced rate of photosynthesis and transpiration, wilting, leaf epinasty, vascular browning, interveinal necrosis, and cell death. Several previous investigations have supported our findings regarding the pathogenicity test of fusarium wilt in tomato plants (Adhikari et al., 2020; Hernández-Aparicio et al., 2021; Nirmaladeviet al., 2016). Similarly, the pathogenicity test in *R. solani*-infected tomato plants revealed rusty-brown, dry sunken lesions on plants near collar regions near the soil lines. Furthermore, the infected plants become stunted and yellow and may wilt with the degradation of lateral roots. Our findings agreed with several earlier reports (Abdelghany et al., 2022; Gondal et al., 2019).

Multiple management approaches have been employed to minimise the pathogens-induced damage and yield improvement in tomato crops, including the establishment of tolerant or resistant cultivars and the application of biological control agents (Meshram and Adhikari, 2024; Panth et al., 2020). Therefore, in pursuing sustainable agriculture, various spp. of the genus *Trichoderma* serves as economically significant biostimulants in plant growth promotion, induction of disease resistance and disease control. Several investigations have reported the antagonistic activities of *Trichoderma* spp. counter to several myco-pathogens of economically important crops through dual culture assay (Yao et al., 2023). In the present research work, screening

of two strains of *Trichoderma,* i.e., *T. virens* and *T. viride,* against *Fol* and *R. solani* was assessed through this technique. Maximum reduction in both pathogens' in vitro radial growth has been observed with *T. virens,* though, in the case of *R. solani*, both *Trichoderma* spp. exhibited statistically equal influence on radial growth inhibition. Developing an inhibition zone at the contiguous spot between pathogens and *Trichoderma* spp. in double culture assay might be ascribed to the synthesis and accumulation of mycoparasitic volatile and non-volatile metabolites along with the assembly of various extracellular hydrolytic enzymes by these species (Abbas et al., 2022). Antagonistic effects of *T. virens* in the radial growth of *R. solani* in dual culture assay have been registered by Halifu et al. (Halifu et al., 2020). Furthermore, the inhibitory action of *T. virens* against mycopathogens has been confirmed in previous investigations. In addition to this, synthesis and release of CWDE along with antibiotics are precious weapons used by *Trichoderma* spp. to reduce the pathogenicity of fungal pathogens in the host plants, as the cell wall of fungal pathogens is composed of chitin and β-1,3 glucan (Ghasemi et al., 2020). The present investigation also explores the biocontrol potential of *T. virens* and *T. virde* through chitinase activity in the chitinase detection media which could be participated in the disintegration of the pathogen’s cell wall during antagonism. Further, these fragments of pathogenic cell walls serve as ligands which, by binding with suitable receptors, initiate the downstream signalling cascades via the induction of mitogen-activated protein kinase (MAPK) and G proteins (Shobade et al., 2024; Sood et al., 2020).

Pathogen exposure disturbs the plant by interfering with plant vegetative growth and numerous physiological processes. For example, root rot disease of tomatoes triggered by *R. solani* infection results in a decline in total root length, a decrease in the number of root tips, as well as in the magnitude of root branching, which eventually makes plants incapable to access water from underground soil layers and consequently, lowers the shoot growth (Heflish et al., 2021). Similar findings were also obtained with *Phytophthora parasitica* causing infection in tomato seedlings (Larousse et al., 2017). Our study has also revealed the same results measured regarding root length, shoot length, fresh and dry weight, and the number of leaves. Furthermore, fusarium wilt diminishes plant growth by clogging and blocking the xylem vessels responsible for translocating water and essential minerals through the plants. In addition to this, chlorosis, stomatal closure, etc., are the reasons which might be accountable for the

pathogen-induced decrease in the growth of infected tomatoes (Chaturvedi et al., 2022). Similarly, *R. solani*-infected tomato plants display poor lateral growth of roots, indicating to less vigorous plants with decreased water and minerals uptake abilities. Consequently, these plants look chlorotic and stunted. Furthermore, with the subsequent decrease in approach to soil water and minerals, plants sooner or later wilt and die (Ajayi-Oyetunde and Bradley, 2018; Williamson-Benavides and Dhingra, 2021).

Recent information has uncovered the excellent efficacy of *Trichoderma* as an incredible plant growth-promoting fungus (PGPF) (Yao et al., 2023; Zin and Badaluddin, 2020). A substantial number of investigations revealed that *Trichoderma* augment the total plant health by establishing a good environment and assembly of a considerable number of secondary metabolites utilized by stressed plants during different metabolic processes. Our findings are supported by several previous investigations, which confirmed that the roots with a more extensive surface area due to *Trichoderma* spp. inoculation assists the root system in exploring a larger region of soil (Andrzejak and Janowska, 2022; Contreras-Cornejo et al., 2018), Subsequently, Cai et al. investigated that *T. harzianum* produced a secondary metabolite named harzianolide, which significantly stimulated the growing of tomatoes in soil/ hydroponic system even at low conc. of 0.1 and 1 ppm (Cai et al., 2013). Furthermore, this metabolite influences root development by directly improving the root length and tips. This eases the plant to absorb more micronutrients and macronutrients available in the rhizosphere, which in turn provide benefits to the plant when attacked by other harmful microorganisms to contest for the nutrients or when these are exhausted. In addition to this, *T. virens* and *T. atroviride* were observed to improve the level of IAA and associated substances. As a crucial phytohormone, IAA plays key roles in root development, cellular enlargement and division regulation, gravitropism and tissue differentiation (Contreras-Cornejo et al., 2014; González-Pérez et al., 2018; Ljung, 2013). Moreover, siderophore production by *Trichoderma* spp. might stimulate plant growth either directly by increasing the Fe (III) accessibility in the rhizosphere or simultaneously reducing the proliferation of pathogens by withdrawing them of the source of Fe (Sood et al., 2020; Tyśkiewicz et al., 2022).

Our findings further confirmed that priming tomato seeds with JA significantly enhanced the host growth (root-shoot length, fresh-dry weight and number of leaves in pathogenically challenged tomato plants. Our results agreed with the study of Ataei et

al. (Ataei et al., 2013). The growth-encouraging potential of the JA might be associated with the stimulation of variable responses in the affected plants, mainly including regulation of gene expression, synthesis of growth-related hormones, enhancement in crop production and protection against stresses (Ahmad et al., 2016; Sood, 2023). In addition to this antimicrobial potential of jasmonic acid against fungal phytopathogens has also been investigated in several previous investigations (Kępczyńska and Kępczyński, 2005; Macioszek et al., 2023; Singh et al., 2019; Švecová et al., 2013). These reports suggested that the exogenous applications of JA on the diseased plants not only stimulated the induced systemic resistance (ISR) in these plants but also radically diminished the mycelial growth and spore germination of fungal pathogens. Subsequently, our investigation further revealed that compared to non-primed sixty days old tomato plants, the combined application of *T. virens* and JA extensively lowered the percentage of disease incidence to the maximum extent in *Fol* infected plants while in the case of *R. solani*, treatments Rs+Tv and Rs+Tv+JA insert statistically equal significance on PDI in tomato plants. In other words, seed priming with these inducers improves plant health and vigour. Furthermore, it simultaneously decreases the magnitude of fusarium wilt and damping-off by directly limiting the invasion and proliferation of pathogens in the host plant rhizosphere. It might be because the application of JA increased the root colonization by *T. virens* in tomato plants, which further assists in the better survival of diseased plants in a pathogen-contaminated environment. Our observations are coincident with others (Singh et al., 2013; Singh et al., 2019).

The compatible osmolytes serve as significant metabolites by maintaining cellular homeostasis through various mechanisms like facilitation of driving gradient for water absorption, regulation of cell turgor through fine osmotic tuning, the establishment of cellular redox status by eliminating excess ROS and providing protection to cellular machinery from oxidative injury and osmotic stress (Zhou et al., 2023). Numerous stress signalling pathways, such as hormones, MAPK, and calcium-signalling cause the excessive formation of osmolytes in stressed plants. The most popular osmolytes that perform a key role in a plant's osmoregulation include proline, polyamines, glycine betaine and sugars (Chakraborty and Kumari, 2024; Sharma et al., 2019). Among these, proline serves as an important osmolyte which maintains the osmotic status of plants by stabilising and protecting macromolecules against the

damaging influences of ROS (Meena et al., 2019; Shan et al., 2015). Further several previous investigations have reported the fact that the exogenous application of JA helps in the enhancement of proline content in plants under different kinds of abiotic stresses (Ali et al., 2018; Farooq et al., 2016; Hosseinifard et al., 2022; Sirhindi et al., 2016). Inferences obtained from the present research work revealed that priming tomato seeds with jasmonic acid increases the proline content in ninety days old plants but decreases its accumulation in sixty days old plants challenged with *Fol* and *R. solani*. Similarly, pre-treated tomato plants under pathogen stress express a variable trend of proline accumulation after thirty and sixty days of pathogen inoculation. Our findings coincide with Scudeletti et al. (Scudeletti et al., 2021). A low level of proline accumulation in sixty days old plants might be linked to the low level of stress in *T. virens* and JA pre-treated plants. On the other hand, age-dependent higher accumulation of proline in ninety days old plants could lie in the fact that being a vital nitrogen source, proline accumulation increases the protein content and help the plants to recover from the stress and restore their growth (Karuppiah et al., 2019; Sousa et al., 2020).

In addition to proline, glycine betaine is also an effective compatible osmolyte that significantly induces stress tolerance in diseased plants. Besides maintaining intracellular osmolarity under stressful circumstances, glycine betaine, in coordination with defence-related enzymes and proteins, preserves the authenticity of membranes opposed to the destructive influences of several stresses (Zulfiqar et al., 2022; Sakamoto and Murata, 2002). Although in our study, priming of tomato seeds with either JA or *T. virens* did not insert any significant influence on glycine betaine content in pathogenically stressed plants, several previous reports emphasize the exogenous use of JA and *Trichoderma* spp. causes to an increase in the glycine betaine content and ultimately assists in mitigating different kinds of stresses. For instance, owing to the application of JA, a 45.4 % hike in GB content has been reported in Ni-treated soybean seedlings (Sirhindi et al., 2016). Similarly, as Ahmad described in salt and cadmium-stressed tomato plants, JA-induced enhancement in glycine betaine content leads to a remarkable improvement in plant growth (Ahmad et al., 2018).

Soluble sugars play a dual function in plant metabolism by directly participating in the various metabolic activities and regulating the expression of different genes, especially those implicated in sucrose metabolism, associated with photosynthesis and osmolyte production (Amombo et al., 2023; Rosa et al., 2009). Moreover, under

pathogenic contamination, sugar also performs a key role in plant defence as the sugar content is directly linked with disease response in most plants. Therefore, the higher sugar level corresponds to a higher degree of disease resistance in pathogenically challenged plants. Our findings have also revealed that under *Fol* and *R. solani* infection, a significant decrease in the sugar content has been observed. Our results are in co-incidence with the previous studies (Manghwar et al., 2021; Qian et al., 2015). It might be because, via the assistance of CWDEs or toxins, these pathogens may retard the translation rate. On the other hand, applying biostimulants to tomato seeds increases the soluble sugar content in infected plants. This might be correlated to the increase in the biosynthesis of sugar due to better growth parameters, higher absorption of water and mineral nutrients, better retention of chlorophyll and other pigments, and the increase in photosynthesis rate. In addition, the pre-treatment of tomato seeds with bioagent and chemical inducer in combination has promising effects in terms of an enhance in the TSS content under diseased conditions. These results might indicate a relationship between sugar regulation and stimulation of systemic resistance due to the upregulation of genes that participated in the sucrose catabolism and could relate to the augmented transcription of defence-linked genes.

Compared to the control, levels of total protein content significantly decreased in the plants diseased with pathogenic inoculations. Our findings coincide with previous research (Manghwar et al., 2021; Zehra et al., 2017). This significant decrease in the protein content might be attributed to its sequestration in light-harvesting complex protein or in some other events associated with the HR. Furthermore, as a consequence of pathogenic challenges, an increase in the level of ROS leads to protein modification in several approaches. Direct transformation entails alteration in the protein activity through carbonylation, glutathionylation, nitrosylation and formation of disulphide bonds. On the other hand, indirect modulation involves coupling with the collapsed resultants of fatty acid peroxidation. In addition, excessive ROS-induced site-specific modification of amino acids, peptide chain fragmentation and altered charge increases the vulnerability of protein to proteolysis (Hasanuzzaman et al., 2020; Sharma, 2012). Moller et al. have testified that tissue injury due to oxidative stress possesses carbonylated proteins to a greater extent which themselves serve as a marker for the oxidation of proteins (Møller and Kristensen, 2004). Further, owing to the fungal infection depletion in the synthesis of chief nitrogenous compounds may be associated

with a reduction in energy demand (Simón et al., 2020). It may be hypothesized that the harmful toxins produced by phytopathogens might act as uncouplers in ATP production (Duke and Dayan, 2011). Our findings indicated that the pre-treatment of JA+*T. virens* had more pronounced increasing effects on total soluble protein content in tomato plants than all other treatments. In Ni-stressed *Glycine max* seedlings, Sirhindi et al. reported that the exogenous use of JA before Ni-treatment protects the seedlings by regulating antioxidative machinery and protecting the DNA synthesis of total proteins (Sirhindi et al., 2016). Similarly, Azeem publicised that exogenous application of JA mitigates the harmful influences of oxidative stress on plant growth and biomass production and protein content in Ni-affected plants by further augmenting the antioxidative plant defence in *Zea mays* (Azeem, 2018). Moreover, Meldau et al. reported that JA and SA share a crucial regulator, glutaredoxin GRX480, which induces the redox regulation of proteins by their ability to catalyze disulfide transitions (Meldau et al., 2012).

As depicted in our results, plants exposed to pathogens showed marked chlorosis due to speedy chlorophyll deprivation causing total plant growth deferral (Mittelberger et al., 2017). Applying biocontrol agents and chemical inducer causes better chlorophyll and carotenoid content retention in diseased plants. As reported in a previous study, *Trichoderma* spp. pre-treatment causes better root growth, leading to superior mineral nutrients and water absorption from the soil. These nutrients are directly involved in chlorophyll synthesis. Furthermore, its treatment significantly improved Mg uptake, an essential chlorophyll constituent (Halifu et al., 2019). Similar observations were also reported in tomatoes (Vukelić et al., 2021) and Chickpea (Mishra and Nautiyal, 2018). Correspondingly, enhanced chlorophyll and carotenoid contents in JA acid pre-treated tomato plants might be because it activates the antioxidative defence system, which scavenges free radicals produced by stress-stimulated physiological damages. If not appropriately sequestered, these radicals can harm the chloroplasts and other cellular organelles (Bali et al., 2018). Our results agreed with Miclea et al. (Miclea et al., 2020).

Because of microbial infection, the host plant synthesises several secondary plant products. Due to their antimicrobial activities, these compounds are considered crucial components in the cellular defence system against several microbial phytopathogens. Generally, these secondary metabolites are called phytoalexins, which are vital in fighting off disease-causing organisms in the infected plants (Kaur

et al., 2022). These defensive chemicals mainly constitute simple phenolics, terpenoids, alkaloids, polyacetylenes, coumarins and many more (Erb and Kliebenstein, 2020). In this study, it has been demonstrated that phenolic components have increased due to pathogen inoculation. Previous research has suggested that phenolic compounds remove the fungal pathogens by changing cell membrane permeability, altering cell wall integrity, production of free radicals, decreasing enzymatic activities of pathogens, inhibiting protein synthesis and inducing DNA damage and suppressing virulence genes in plant pathogens and creation of signalling composites like SA (Khameneh et al., 2021). The present investigation also reports that in sixty days old tomato plants, the maximum accumulation of phenolic compounds has been observed in the treatments Rs+Tv+JA and Rs+Tv. The results obtained are supported by the fact that bioagents like *Trichoderma* spp. stimulate the latent plant defence system by inducing defence-related enzymes and synthesis of signalling phytohormones like SA and JA in response to the pathogenic attack (Yao et al., 2023; Contreras-Cornejo et al., 2011). Although among ninety days old tomato plants, pretreatment of tomato seeds with ameliorative agents did not significantly affect the TPC accumulation.

Like phenolic content in this study, it has also been deduced that biotic stress induced by both pathogens leads to enhanced accumulation of flavonoid content in tomato plants. Similar observations have also been observed in apple, lettuce, and *Arabidopsis* host plants diseased with Cedar-Apple rust, downy mildew disease and *Plasmodiophora brassicae*, respectively (Lu et al., 2017; McLay et al., 2020; Paesold et al., 2010). Moreover, pre-treatment of tomato seeds with *T. virens* and JA at both the growth stages further intensified the flavonoid contents in infected tomato leaves, except in the treatment Rs+JA in sixty days old plants, and such plants exhibited enhanced resistance against both soil-borne pathogens. Likewise, a sub-group of flavonoids, i.e., anthocyanins, are proven to be engaged in plant defence against fungal pathogens (Sicilia et al., 2021). The purple colour of tomato fruits due to the rise in anthocyanin contents corresponds to increased resistance and reduced oxidative damage due to *Botrytis cineria* (Zhang et al., 2013). Similarly, the peel of mango fruits exposed to sunshine acquires a red colour due to a higher accumulation of flavonoids and anthocyanin and exhibits more tolerance to cold injury and better resistance against *Colletotrichum gloeosporioides* infection (Sivankalyani et al., 2016). Similar findings related to the heightened anthocyanin contents in tomato

leaves exposed to *R. solani*-induced pathogenicity have also been observed in this investigation when compared to the control plants.

Antipathogenic properties of these substances are concealed in their antioxidative asset as an effectual sequester for ROS formed due to fungal infection. After synthesis, these flavonoids migrate to the infection spot, beginning HR and PCD. Furthermore, by regulating auxin action, they support the tightening of plant tissues by endorsing tissue differentiation, development of callus and tyloses and vascular system closure, to control the pathogen spread to healthy tissues (Beckman, 2000; Kurepa et al., 2023). Furthermore, they cause the deactivation of the pathogen's plant cell wall digesting enzymes (PCWDE) by directly binding to their chelating metals (Treutter, 2005). Moreover, their antifungal activities include inhibition of spore development, lessening of fungal mycelium, deactivation of microbial adhesion and membrane transporter and respiratory chain disorder (Al Aboody and Mickymaray, 2020; Naoumkina et al., 2010) also ward off pathogen spread to distant locations. Our findings have also demonstrated that JA and *T. virens* further enhanced the total flavonoid content in stressed tomato leaves. This might be because *Trichoderma* and JA induce resistance in diseased plants by upregulating the synthesis of secondary metabolites in them. In other words, *T. virens* and JA elicitation enhances the gene expression level of Phenylpropanoid-associated genes and the accumulation level of anthocyanin and flavonoid content. Our findings follow several previous reports (Kim et al., 2013; Mahmoud et al., 2021; Mayo-Prieto et al., 2019; Park et al., 2013).

Our investigation also revealed that the net photosynthetic rate of tomato leaves had been affected due to infection with *Fol* and *R. solani.* Lorenzini et al. also report a similar trend of a decrease in photosynthetic rate in tomato plants (Lorenzini et al., 1997). Likewise, in maize plants infected with pathogens photosynthetic inhibition is complemented by a sharp decline in chl. content (Pinto, 2000). Several investigations also report similar results in different plants infected with biotic stresses (Bonfig et al., 2006; Chen et al., 2015; Hu et al., 2020; Scharte et al., 2005; Swarbrick, 2006). After pathogen infection, generating and accumulating ROS due to disturbance in the ETC might be a severe cause of plant photosynthetic rate reduction (Guo et al., 2023; Scharte et al., 2005). However, priming tomato seeds with *T. virens* and JA specially in combination enhanced photosynthetic pigments and significantly

improved the photosynthetic rate. Enhancement in chlorophyll contents is attributed to increased enzymatic activities of genes such as proto-chlorophyllide reductase and α-aminolevulinic acid dehydratase associated with chlorophyll biosynthesis (Sirhindi et al., 2020.; Wu et al., 2018). These priming agents also activate some biochemical pathways, which convert these damaging ROS into less destructive molecules (Oljira et al., 2020; Sherin et al., 2022).

Present observation has depicted that exposure to both pathogens cause a significant reduction in transpiration rate, intercellular carbons and intercellular conductance at both the observed growth stages. Likewise, several reports have described various mechanisms of impairment in stomatal opening during pathogenesis. For instance, compared to green regions, stomata of the chlorotic area in maize leave infected with *Maize dwarf mosaic virus* exhibit a reduction in stomatal function (Lindsey, 1975). Respective reports proposed that the decline in chlorophyll contents owing to pathogen invasion is the leading cause of the decrease in stomatal conductance. A study on the leaves of common beans infected with *Colletotrichum lindemuthianum* reported that pathogen-induced destruction to the photosynthetic complex leads to stomatal closure in respected leaves (Meyer et al., 2001). Similarly, a toxin named Tenotoxin synthesized by *Alternaria alternata* deactivates chloroplastic ATPase and induces irretrievable stomatal closure (Dahse et al., 1990). In addition to these, many defensive compounds like nitric oxide (Mur et al., 2005.; Neill et al., 2002), phenolic compounds (Beguerisse-Diaz et al., 2012), salicylic acid (Chaerle et al., 2006), and phytohormones ABA and auxins (Grabov et al., 1998) accumulated as an outcome of pathogenesis cause a reduction in stomatal aperture size in plants. Reports on several pathosystems in the field revealed that decreased stomatal conductance reduces CO_2 assimilation and/or photosynthetic rate. For instance, grapevine-virus and eucalyptus-*Mycosphaerella* pathosystems depict that pathogen-imposed lessening in photosynthesis was due to diminished activity of RuBisCo (Pinkard et al., 2006; Sampol et al., 2003). Moreover, the generation of ROS serves as a signalling molecule in the process of stomatal closure because they trigger Ca channels in guard cells and participate in the osmotic reaction of stomata (Liu et al., 2022). Further, the accumulation of ROS causes the formation and deposition of callose to restrict the diffusion of H_2O (Sahu et al., 2022). Similarly, many pathogens have been observed to unsettle the stomatal control of transpiration, ultimately

affecting plant water relationships. Our study also revealed a significant improvement in transpiration rate, intercellular conductance and intercellular carbon due to priming of tomato seeds with *T. virens* and JA might be associated with less ROS generation and higher accumulation of secondary metabolite as well as improvement in photosynthetic pigments and apparatus.

Scanning electron micrographs presented in our study clearly showed in comparison to the control, the stomatal aperture of pathogenically stressed plants was either entirely or partially closed. Our findings indicate that plants have developed the capacity to regulate their stomatal apertures not only in response to phytohormones like ABA and to various environmental clues like light, CO_2 and humidity levels but also pathogens. Due to the fungal infection, these microorganisms variably disturb stomatal behaviours due to interplay among plant and fungal-derived compounds during host-pathogen interactions (Gudesblat et al., 2009; Meddya et al., 2023). Interestingly the recent observation suggesting the involvement of pathogen-associated molecular patterns (PAMP) in the stimulation of stomatal closure offers strong evidence that stomata act efficiently as a crucial part of the innate immunity of diseased plants (Melotto et al., 2017). As mentioned previously, pathogens-derived elicitors in diseased plants lead to the production of ROS. These ROS so generated serve as connecting links between the elicitor's recognition and activation of MPK3 in the guard cell. In correspondence to this assumption, it has been observed that the yeast-secreted elicitors and chitosan are efficient in stimulating plant defensive response and elevating the free cytosolic Ca^{2+} in the guard cell (Klüsener et al., 2002). Furthermore, this enhancement depends upon the occurrence of cytosolic NAD(P)H, which itself functions as a substrate for the enzyme NAD(P)H oxidase associated with ROS generation in plants. To sum up, we can say that augmentation in the level of both ROS and Ca^{2+} relates to the ABA-mediated closing of stomata in pathogenically stressed plants. Our observations are coincidental with several previous reports (Liu et al., 2019; Sun et al., 2017; Ye et al., 2020). Furthermore, our study revealed that the combined application of *T. virens*+JA partly reverted the pathogen mediated damages in the stressed plants by partially assisting in opening the stomatal aperture in *Fol* and *R. solani*-challenged plants. This might be directly linked to the factors like improved chlorophyll content, increase in photosynthesis and transpiration rates, improved growth, absorption and assimilation of mineral nutrients, decrease in the ROS

production and existence of a better enzymatic and non-enzymatic antioxidative defence system. For instance, Toum et al. have studied that coronatine, a toxin produced by *Pseudomonas syringae* mimics the phytohormone JA isoleucine and stimulates stomatal opening through inhibition of ABA-induced NADPH oxidase-dependent production of ROS (Toum et al., 2016).

Several reports supported the fact that the encounter of susceptible plants with soil-borne fungal pathogens advances the production of ROS in host plants (Berrios and Rentsch et al., 2022; Chialva et al., 2018; Foley et al., 2016; Jayamohan et al., 2018). Such ROS are hypothesized as the first line of the effective defence system in plants against most invading pathogenic organisms and may precisely serve as an antimicrobial agent (Li et al., 2021; Memar et al., 2018). In the present research, our results revealed that pathogen inoculation causes an upsurge in H_2O_2 content in diseased plants. In sixty days old tomato plants, the maximum accumulation of H_2O_2 is detected in treatment Rs followed by *Fol*. Enhancement in H_2O_2 contents in the presence of *R. solani* and *Fol* has also been testified in other previous investigations (Behiry et al., 2023; Meena et al., 2016; Nikraftar et al., 2013). Production of ROS like H_2O_2 during oxidative burst has been recognized as an important event in disease resistance activation. The direct participation of H_2O_2 in pathogen killing, cell wall strengthening, stimulation of HR and establishment of systemic resistance signalling has been documented in several investigations (Dumanović, 2021; Lavanya et al., 2022). Supplementary, in the presence of pathogens, minimal H_2O_2 was exhibited by plants in which seed priming was performed by combined treatment of *T. virens* and JA. Reduction in H_2O_2 levels in the treatments i.e., Fol+Tv+JA and Rs+Tv+JA might be linked with the enrichment in the performances of antioxidative enzymes, which diminish the H_2O_2 accumulation through oxidation or decomposition. Our findings agree with the previous investigation (Zehra et al., 2017). However, in the ninety days old plants, pre-treatment of biostimulants did not significantly alter the H_2O_2 accumulation in *Fol*-challenged plants.

Pathogen-induced degradation of membrane lipids leads to the production of free fatty acids, which themselves serve as a substrate for lipoxygenase enzyme (LOX), which via oxidative deterioration, causes lipid peroxidation of the cellular membrane. The act of membrane peroxidation ultimately generates various alkoxy and peroxy radicals (He and Ding, 2020). In sixty days old plants, our investigation

monitored the highest magnitude of MDA content in treatment Rs, followed by Fol-treated tomato leaves. But the combined application of *T. virens* and JA decreases the MDA content accumulation to the lowest extent in the case of both pathogens under both observed growth stages, except in the treatment Fol+Tv in 90 days old plants. Prior studies have also highlighted that suppression of MDA contents in *Trichoderma* and JA pre-treated plants might be due to the activation of Halliwell-Asada pathway members, viz. various antioxidative defensive enzymes and synthesis of other protective molecules participating in ROS elimination responsible for lipid peroxidation. Our results are supported by several such studies (Ji et al., 2021).

If the pathogen-induced generation of ROS in various cellular compartments is not detoxified, these ROS can damage and deactivate several significant biomolecules. The plants' antioxidative defence system detoxifies these ROS through enzymes or non-enzymatic antioxidants like GSH, AsA and Tocopherol (Huang et al., 2019; Zechmann, 2020). In the Plant system, AsA performs a significant function of redox buffering plant cells on their own or through the AsA-GSH cycle. AsA might be found either in AsA or two oxidized compounds. Owing to the pathogenic attack, a remarkable increase in ROS and AsA has been observed in *Solanum lycopersicon,* and *Brassica rapa* stressed with *Cucumber mosaic virus* and *Turnip mosaic virus (*TuMV)*,* respectively (Fujiwara et al., 2013; Tsuda et al., 2005). Analogous results have also been registered in our investigation. For example, the highest level of AsA content has been monitored in the treatment Rs+Tv+JA for sixty days old plants. Similarly, in the ninety days old plants, tomato seeds priming with ameliorative agents increased the AsA content in the inoculated and non-inoculated plants. These results suggest that disease resistance inducers in the presence of pathogens further intensify the AsA accumulation to initiate defence responses in stressed plants (Boubakri et al., 2016; Ding et al., 2022). In addition, some experiments evidenced that pre-treatment of AsA directly affects the pathogenic growth, as observed in of rice infected with *Magnaporthe oryzae* and *Arabidopsis* plants infected with *Alternaria brassicicola* (Botanga et al., 2012; Egan et al., 2007).

Our results further demonstrated that GSH contents have drastically and significantly increased in biotically stressed tomato leaves. It plays a dual role in minimizing pathogen-induced damage in plants; first by keeping the ROS levels under control and second by inducing the defensive pathways against pathogens in plants by mediating signal transduction among ROS, JA, SA and ethylene (Künstler et

al., 2019, 2020). Our findings agree with other investigations (Simon et al., 2013; Vanacker et al., 2000; Zechmann and Müller, 2008). In sixty days old plants, our investigation depicted that the highest accumulated level of GSH was monitored in those plants pre-treated with *T. virens* along with JA followed by pathogen contamination except in the treatment Rs+JA which exhibited equal significance to Rs+Tv+JA and Fol+Tv+JA. A similar enhancement trend in GSH content has also been perceived in ninety days old tomato plants. Being a signalling molecule, JA can boost the antioxidant machinery of biotically stressed plants by enhancing the genes transcription that participate in the creation and accumulation of antioxidant molecules in plants (Bali et al., 2020). Similarly, increased GSH content following priming of tomato seeds with *T. virens* individually or in combination with JA could have strengthened the antioxidant defence mechanisms against the free radicals produced because of pathogen-induced damages in diseased plants (Soliman., 2020; Zehra, 2017).

Tocopherol shows a significant task in cell communication by sending signals from the chloroplast to the nucleus to respond against various stresses (Nowicka et al., 2021). Changes in the levels of tocopherol are generally linked with the altered expression of genes associated with degradation-recycling pathways. Therefore, a general assumption is made regarding tocopherol accumulation, i.e., its enhancement ensures better stress tolerance and vice-versa. Our findings are also per this hypothesis; compared to the control, tocopherol contents have increased in pathogenically stressed plants under both the observed growth stages. The stress ameliorative potential of tocopherol might be due to its ability to preserve the integrity and fluidity of the membrane through neutralising lipid peroxy radicals and effective quenching of ROS (Sahu et al., 2022). Our results also demonstrate that tocopherol accumulation was further prompted by persuaders of disease resistance, which might be linked to signalling tectics that alter cell function. Several investigations support the fact that the presence of JA and MeJA enhanced the accumulation of tocopherol because of their ability to up-regulate the genes associated with tocopherol-biosynthetic pathways involving HPPD and HPT genes (Antognoni, 2009; Caretto et al., 2010; Gala et al., 2005).

Free radicals are short-lived and highly reactive due to unpaired electrons in their valence shell. These radicals, once generated due to various metabolic processes, elicit oxidative damage to essential biomolecules. The different metabolic processes

in plants are sensitive to biotic/ abiotic stresses, and it has been observed that under these circumstances, an approximate increase of 3-10-fold in free radical creation has been described in various studies (Kasote et al., 2015; Rai and Kaushik, 2023). Further, they interact with and are subsequently neutralized by the antioxidants, which donate electrons to them before or after the onset of damage initiation (Lalhminghlui and Jagetia, 2018). The present research monitored radical scavenging activity through DPPH and ABTS assays. In our investigation, in comparison to the control, free radical scavenging activity was increased in pathogen-stressed plants, which might be due to enhancement in the antioxidative defence system in these plants measured in terms of AsA, GSH and secondary metabolites like phenols and flavonoids, carotenoids etc. along with antioxidative enzymes. Similar findings have also been reported in *Botrytis cineria* stressed lettuce plants (Iwaniuk and Lozowicka, 2022). A relative increase in the free radical scavenging activities in both assays in JA pre-treated tomato seeds followed by pathogen inoculation might be due to the better strengthening of the antioxidative defence system due to the upregulation of associated genes as well as weakening in the pathogenicity of disease-causing organisms (Baek et al., 2021).

Besides non-enzymatic antioxidants, antioxidative enzymes also play a defensive response against differential oxidative stresses in plants. Iqbal et al. suggested that in plant leaves, the activities of defence-associated genes are directly correlated to the resistive attitude of the plant to the various kinds of biotic stresses (Iqbal et al., 2021). Subsequently, at the cellular level, numerous plant protein families are known to relate to the disposition of ROS under pathogenic infection. Among these SOD, APX, catalase, and peroxidases are most efficient in effectively regulating these ROS (Hasanuzzaman et al., 2020; Huang et al., 2019; Sharma et al., 2012). Our findings also demonstrated a significant enhancement in the actions of antioxidative enzymes detected in both *Fol* and *R. solani*-stressed plants. Our results are supported by several pieces of research which reported that an upsurge in enzymatic antioxidants had been observed in *F. oxysporum lycopersici* (Hashem et al., 2021; Zehra et al., 2023) and *R. solani* (Afzal et al., 2022; Manganiello et al., 2018) stressed tomato plants. The augmentation in antioxidant enzyme function is facilitated to alleviate the oxidative damage and detoxify the *Fol* and *R. solani*-triggered ROS.

SODs are ubiquitous metalloenzymes that catalyze the dismutation of O_2•− radicals to O_2 and H_2O_2. Enhancement in SOD activity is often associated with improved plant lenience against various oxidative stresses (Tyagi et al., 2019). Subsequently, the upsurge in SOD action in tomatoes has been reported under *Ralstonia solanacearum* (Li et al., 2008), *Fusarium oxysporum lycopersici* (Zehra et al., 2017), *Rhizoctonia solani* (Manganiello et al., 2018) etc. Like SOD, catalase prevents cellular oxidative damage by facilitating the decomposition of hydrogen peroxide into oxygen and water (Ransy et al., 2020). Thereby catalase assists in maintaining an optimum level of hydrogen peroxide in the cell, which is essential for signal transduction cascade. The subsequent importance of this enzyme can be evaluated from its direct or indirect contribution to many plant diseases. Augmentation in the activity of catalase has been reported in *Fusarium* stressed melon (Sadeghpour et al., 2022), tomato (Abbasi et al., 2019) and pea (Perincherry et al., 2021) plants. Likewise, a similar increase in catalase activity has also been reported in *Rhizoctonia*-challenged cotton (Kumar et al., 2009) and tomato (Al-Surhanee et al., 2021) plants. Guaiacol peroxidases (GPOX) are heme-bearing proteins which oxidize aromatic electron donors at the cost of hydrogen peroxide. Besides being widely acknowledged as a stress enzyme, GPOX is linked with numerous important plant biosynthetic processes. Compared to non-inoculated plants increase in the GPOX activity in diseased plants might be correlated with the higher rate of lipid peroxidation and H_2O_2 production in these plants. Several previous kinds of research support that the aid of H_2O_2 cell wall-linked peroxidase (POD) mediates the cross-linking between cell wall polymers. Consequently, this cross-linking reduces growth due to cell wall stiffening in stressed plants (Erofeeva, 2015; Meena and Samal, 2019).

Additionally, the AsA-GSH cycle operational in cell mitochondria, chloroplast and cytosol constitute the chief H_2O_2 detoxification system in oxidatively stressed plants. This cycle in plants comprises four enzymes and two antioxidants (Mishra et al., 2023; Pandey et al., 2015). APX detoxifies H_2O_2 by catalyzing the peroxidation of AsA to MDHA radical. MDHA so formed either changed to AsA again by MDHAR or non-enzymatic disproportionate to DHA and AsA. The next phase in the cycle entailed DHAR-catalyzed change i.e., DHA to AsA utilizing GSH. If not reduced by DHAR, DHA go through irretrievable hydrolysis to 2, 3-diketogulonic acid. Therefore, DHAR mediates in the revival of AsA and performs a significant role in

sustaining AsA reservoir (Greco et al., 2013). Similar to AsA, the renewal of GSH is also essential for the smooth going of this cycle. Subsequently, GSH is known to be revived from the GSSG by the action of NADPH-dependent GR (Gill et al., 2013; Vašková et al., 2023). Due to its ubiquitous existence in plant cell AsA-GSH cycle protects the plants from the damaging influences of ROS produced due to several biotic and abiotic stresses. Our findings demonstrate a significant alteration in the activity of the AsA-GSH pathway's enzyme due to the pathogen-induced production of ROS in diseased plants. Like our observations, pathogen mediated modifications in the level of activity of antioxidative enzymes have been reported in several types of research. For instance, in *Hordeum vulgare* leaves, fungal infection with *Blumeria graminis* resulted in a markable decrease in the activity of APX and GR enzymes in the resistant cultivar but no considerable alteration in the susceptible one. However, MDHAR and DHAR do not exhibit substantial changes in their respective activities under pathogen inoculation (Vanacker et al., 1998). Similarly, Kuzniak et al. have conveyed that in *L. esculentum,* fungal infection with *Botrytis cinerea* variably affects the expression of AsA-GSH gene families (Kuźniak and Skłodowska, 2005). Likewise, in *Sesamum orientale* plants, fungal inoculation with *Alternaria sesami* expressed an enhancement in APX, MDHAR and GR activity which is subsequently followed by a steady reduction in the related activities (Shereefa and Kumaraswamy, 2016). On the other hand, the decrease in MDHAR activity of *T. aestivum* corresponds to better resistance against *Puccinia striiformis* in these plants (Feng et al., 2014).

Our results further displayed the increase in the activity of GST enzyme in *Fol* and *R. solani*-challenged plants. GST and GSH played a pivotal role in effectively eliminating ROS and lipid peroxide in the infected tissue and consequently restraining the extreme spread of HR-linked apoptosis. In pathogenic fungi-infected plants, an important characteristic of GSH metabolism is the detoxification of mycotoxins by the host plant' GST. Interestingly, *Triticum aestivum* infected with barley powdery mildew caused by *Blumeria graminis* f.sp. *hordei* established a local and induced resistance counter to a subsequent infection with *Blumeria graminis* f. sp. *tritici* caused wheat powdery mildew. The establishment of this resistance might be associated with a 20- fold raise in the transcript quantity of the *GST* gene in *Blumeria graminis hordei* infected wheat leaves (Dudler et al., 1991). Similarly, upregulation of *GST I* gene has been reported in wheat plants due to infection *Puccinia recondita*

(Mauch and Dudler, 1993). Likewise, in *Colletotrichum destructivum* and *C. orbiculare* infected *Nicotiana benthamiana,* the expression of two *GSTs* encoding genes were upregulated significantly. Furthermore, silencing of these genes led to 67 per cent more fungal colonization and approximately 130 % more lesions development when compared with controlled plants (Dean et al., 2005).

Like GST, plants' GPX prevents PCD due to oxidative damage induced by stressful circumstances. The GPX protein family comprises various isozymes situated in distinct subcellular components and display expression patterns as per the type of tissue and growth phase of the plant (Bela et al., 2022; Gao et al., 2014). GPX show a crucial task in the AsA-GSH cycle by reducing the accumulation of H_2O_2 and phospholipid hydroperoxide at the cost of GSH (by oxidising GSH to the disulfide form GSSG) by utilizing thioredoxin (Trx) as an electron donor (Zhang et al., 2019). In contrast to animal GPX which relies on the oxidation of GSH due to three conserved non-selenium Cys residues at the active site, plant GPX oxidises Trx to reduce H_2O_2. Besides ROS detoxification, GPX protects redox homeostasis by preserving adequate thiol/ disulfide equilibrium and protein functions (Iqbal et al., 2006). Previous research supported the GPX catalysed oxidation of Cys-containing proteins implicated in cellular signalling like phosphatases, kinases and various transcription factors and eventually regulate the different important metabolic pathways in plants (Bela et al., 2015; Marinho et al., 2014). Moreover, the active participation of ER-located GPXL3 in oxidative protein folding, formation of disulfide bridge and/ or regeneration of the contestant enzymes, in addition to the effective disposal of ROS, has been a concern in previous reports (Attacha et al., 2017; Meyer et al., 2021). Therefore, as a summation, a boost in the action of GPX in *Fol* and *R. solani*-infected tomato plants might be connected to its numerous roles in stress tolerance as well as in development in pathogenically challenged plants.

Our interpretations from the present investigation have uncovered that the priming of tomato seeds with *T. virens* increases the activity of antioxidative enzymes compared to both pathogens inoculated and non-inoculated 60- and 90-days old tomato plants. Multiple preceding reports emphasize the lifestyle of *T. virens* as an important symbiotic endophyte in diverse host plants (Gan et al., 2022; Inayati et al., 2020; Morán-Diez et al., 2015; Nogueira-Lopez et al., 2018; Romão-Dumaresq et al., 2016). Besides maintaining the oxidative homeostasis of the host plant, an antioxidative defence mechanism is also essential to preserve the plant-microbe

symbiotic communications (Marschall and Tudzynski, 2016). This research work has studied *T. virens*-mediated elevation in the activity of ROS detoxification enzymes. The results obtained from this research work revealed the ability of *T. virens* primed tomato seedlings against oxidative damage might be accompanied by positive regulation of genes associated with the reduction of AsA and GSH as well as an increase in the activity of related enzymes required for the preservation of redox stage of GSH and AsA pool to reduced state. Consequently, these findings strongly support the fact that pre-treatment of *T. virens* altogether enhances the efficiency of the plant to scavenge and dispose of damaging quantities of ROS, $O_2^{\bullet-}$ and H_2O_2 (Mastouri et al., 2012). Likewise, through their findings, Gan et.al. revealed that pre-treatment of *T. virens,* intensified the activity of several genes viz., SOD, CAT, APX, POD, PAL and PPO implicated in plant growth promotion and disease resistance in *Agrostis stolonifera* against *Clarireedia homoeocarpa* infection (Gan et al., 2022). It has been beforehand stated that *Trichoderma* spp. stimulate systemic alterations in the gene expression because of a complex signal transduction cascade along with MeJA. Furthermore, MeJA itself persuade the expression of antioxidative enzymes encoding genes. In *Arabidopsis thaliana,* the presence of CGTCA-motif- ME-JA responsive element in the promotor of the majority of enzymatic antioxidative genes indicates that Me-JA plays a signalling role in the upregulation in the expression of these genes (Mastouri et al., 2012; Rouster et al., 1997).

In addition to *T. virens*, elevation in the activities of antioxidative enzymes has also been established in the JA-pre-treated tomato plants studied in the present research work. Our findings agree with some previous research work. For instance, in wheat plants infected with *Fusarium culmorum,* pre-treatment of Me-JA induced the activity of SOD, PPO, LOX, POX, and PAL antioxidative enzymes and raised TPC and callose content levels in stressed plants. MeJA reduced lipid peroxidation and H_2O_2 in all studied wheat cultivars (Motallebi et al., 2017). Similarly, in their investigation, Jin et.al. reported that post-harvest treatment of peach fruits with 1 μmol L^{-1} MeJA vapours alleviated the fungi induced diseases by upregulating the activity of defensive enzymes such as β 1,3 Glucanase, PAL, POD, SOD, CAT, APX etc. (Jin et al., 2009). These results strongly support the fact that, after pathogens exposure, JA-dependent defence signalling might increase the resistance against the pathogens by stimulating the activities of enzymatic and non-enzymatic antioxidants and phenolic compounds. Our data significantly revealed that *T. virens*-JA ISR is

based directly upon activating the host plant's basal resistance mechanisms via augmented stimulation of a multifaceted antioxidative defensive network under pathogen exposure. Furthermore, several-fold increases in the action of defence-linked enzymes have been recorded in plants pre-treated with *Trichoderma* spp. and JA in combination, contrasted to plants treated with either of these two ameliorative agents independently (Singh et al., 2019). In other words, in Tv+JA primed tomato plants, enhanced accumulation of antioxidants and defence-associated mediator molecules/ antioxidative enzymes leads to better survival and disease suppression in *Fol* and *R. solani* diseased plants. Our findings agree with other researchers (Singh et al., 2019; Zehra et al., 2017, 2023).

In addition to the determination of the enzymatic activity of different antioxidative enzymes, the present investigation also focussed on the expression pattern of *SOD* and *CAT* genes in sixty days old tomato plants inoculated with *Fol* and *R. solani*. consequently, the inferences obtained from the existing study exposed that the combined application of *T. virens* and JA as priming agent has a significant influence in the upregulation of the expression of both these antioxidative genes. Through their investigation, Perl et al. reported that overexpression of the tomato *Cu-Zn SOD* gene in potato plants expresses enhanced tolerance to oxidative stress in transgenic plants (Perl et al., 1993). Subsequently, the efficiency of *SOD* genes in enhancing tomato tolerance to various biotic and abiotic stresses has been evaluated through recent investigations (Aydin et al., 2014; Li, 2009; Soydam Aydin et al., 2013; Sreedevi et al., 2013). In tomatoes, Feng et al. reported the presence of nine *SOD* genes which were unevenly distributed on twelve chromosomes. Of these, four were *Cu/Zn SOD,* and three and one were *Fe SOD* and *Mn SOD*, respectively (Feng et al., 2016). Similarly, the expression of *CAT* also exhibits a similar pattern of upregulation in pathogenically challenged plants. For instance, in bacterial spot disease-infected tomato plants, a positive regulation of the expression of antioxidative genes viz., *CAT, APX* and *GR* ensure better survival of stressed plants under *Xanthomonas perforans* infection (Srinivasa et al., 2022). Due to the successful pathogenic invasion, pathogen-eliciting triggers by binding with suitable receptors lead to the altered Ca^{2+} content in the infected plant. Subsequently, Ca^{2+} is perceived by calmodulin, calmodulin-like proteins, as well as calmodulin-dependent protein kinases, which in turn cause the production of ROS and RNS. This change in cellular redox status initiates a signal transduction cascade which activates the variable

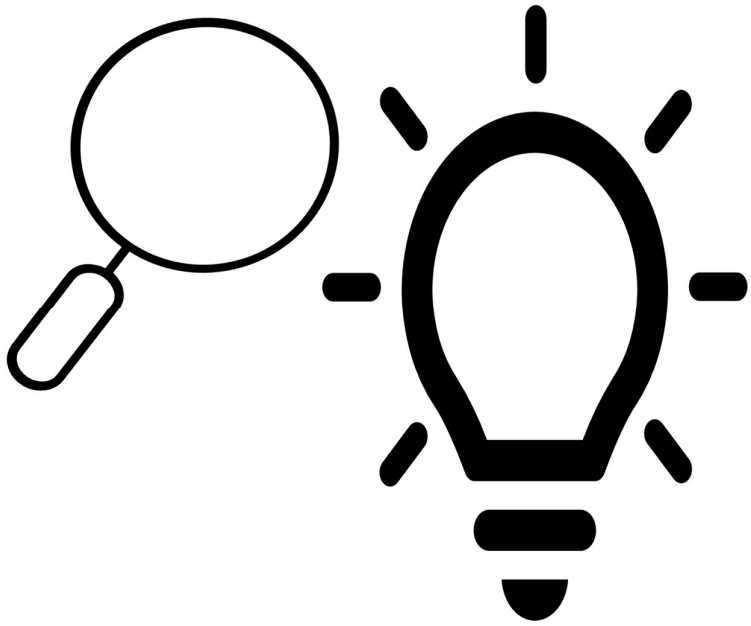
transcription factor and ultimately alters the expression of target genes (Frederickson Matika and Loake, 2014).

In a study, tomato seed priming with *T. erinaceum* leads to positive regulation of *SOD* gene expression in *Fol*-challenged tomato plants (Aamir et al., 2019). Similar findings have also been depicted in *T. velutinum* pretreated bean seed under the pathogenicity of *R. solani* (Mayo et al., 2016). In addition, previous research also documented the upregulation of the enzymatic antioxidative gene expression level against stresses due to treatment with *Trichoderma* spp. (Brotman et al., 2013; Inayat et al., 2022; Zhang et al., 2016). Furthermore, being an important chemical inducer JA serves as a signalling molecule. It is liable to the stimulation of ROS (secondary messengers) mediated signalling that could alter the expression of defence-related genes and might be the reason for better tolerance against pathogen-induced stress in diseased plants. Due to pathogenic attacks, multiple invader-derived or damage-associated plant-originated signals, which may be either chemical or physical, have been reported in local responses. These signals are later identified by PRR situated on the cell membrane. Consequent to this recognition event, the *de novo* formation of JA and JA-Ile (JA-Isoleucine) is initiated. The cytoplasmic enzyme JASMONATE RESISTANT 1 (JAR1) is known to be responsible for the conjugation of JA with Isoleucine to convert it into a biologically active form, i.e., JA-Ile (Sood, 2023; Staswick and Tiryaki, 2004). Several reports emphasize that for JA signalling, serves as the natural and direct ligand in the plant system (Yan et al., 2016). Numerous findings support the ameliorative potential of JA as a signalling component in the upregulation of expression of genes involved in antioxidative defence response in stressed plants (Bali et al., 2020; Zhu et al., 2015; Zhu and Tian, 2012). In the current research work under pathogenic stress, the enhance in the expression level of *CAT* and *SOD* genes due to combined pre-treatment with *T. virens* and JA might be correlated with the enhancement in the transcription level of these genes and also co-related with the increase in SOD and Catalase enzymatic activities; both these increases agreed with the trend of the activity of the associated enzymes.

In brief, the present research work describes the overall approaches included in the *T. virens* and JA-mediated disease control in soil-borne pathogens-tomato pathosystem. The combined application of this BCA and chemical inducer showed well-coordinated mechanisms during pathogen invasion, expression of defence-linked

molecules, enzymes etc., which ultimately leads to reduced pathogenic establishment and proliferation rate, disease development and enhanced plant growth and development under a biotically stressed environment.

Chapter 7
Summary & Conclusion



Chapter 7 Summary and Conclusions

Plants often face various stresses that disrupt their metabolism, inhibiting growth and reducing crop yield. During different growth stages, crops are attacked by fungi, bacteria, and nematodes. To survive, plants use mechanisms like acclimation and adaptation to maintain homeostasis. After pathogens invade, the metabolism of both the pathogen and the plant become interconnected, causing nutrient loss for the plant and benefiting the pathogen. This leads to downregulation of important metabolic pathways and upregulation of defence mechanisms in the plant. The extent of disease symptoms depends on how much the plant's physiology is altered. Defence is crucial for plant growth and involves quick pathogen recognition and the production of defence molecules. Tomato, the second most valuable horticultural crop, is widely consumed in various forms and is rich in minerals, vitamins, fibres, and proteins. Lycopene in tomatoes has health benefits as an antioxidant. Tomatoes are also a model plant for studying resistance mechanisms against pathogens. However, cultivated tomatoes have low genetic diversity and are susceptible to over 200 diseases, especially soil-borne pathogens like *Fol* and *R. solani*, which are difficult to manage due to their persistence in soil. These pathogens cause severe symptoms such as wilting, necrosis, and cell death. The current approach to disease-free agriculture heavily relies on chemical fungicides, which pose environmental and health risks. Biocontrol agents (BCA) are a better alternative as they target specific pathogens and have fewer negative effects on the ecosystem. BCAs also help plants allocate more resources towards growth. However, their use is limited due to a lack of technology transfer and economic recognition, especially in developing countries. Seed priming is a sustainable technique to enhance seed value, seedling strength, productivity, and resistance to stresses while reducing harmful chemical use. Our study shows that treating tomato seedlings with *Trichoderma virens* and Jasmonic acid before pathogen exposure improves growth and induces disease resistance mechanisms, such as the synthesis of secondary metabolites and osmolytes, enhanced photosynthesis, and activation of the antioxidative defence system.

Inferences drawn from the experimental findings of the whole study could be summarised through the following points

- ✓ The pathogens, i.e., *Fol* and *R. solani* were identified through morphological and cultural characteristics. Three kinds of asexual spores- microconidia, macroconidia and chlamydospores, are formed in the case of *Fol,* while *R. solani* was identified by the existence of perpendicular hyphal branches of mycelium.
- ✓ Pathogenicity test has been validated through the development of wilt symptoms like yellowing and wilting of leaves and stunting in *Fol* inoculated plants and occurrence of a brown-coloured, water-soaked lesion near collar region and dry sunken stem canker in *R. solani* infected tomato plants.
- ✓ Out of *T. viride* and *T. virens*, the latter shows better antagonistic activity against the tested pathogen as determined through dual culture and chitinase assay.
- ✓ Priming tomato seeds with *T. virens* and JA leads to improved vegetative growth and decreased disease incidence in *Fol* and *R. solani*-infected tomato plants.
- ✓ Pathogen-inoculated plants showed a significant decrease in Chlorophyll, carotenoids, photosynthesis rate and gaseous exchange parameters. Pre-treatment of tomato seeds with ameliorative agents, especially in combination, has a marked influence on the enhancement of pigments and a marginal impact on gaseous exchange parameters.
- ✓ SEM studies revealed that, compared to the control, the stomatal aperture of pathogenically stressed plants was either entirely or partially closed. However, priming tomato seeds with combined treatment of *T. virens* and JA in combination reduced the pathogen-induced damage to tomato leaves depicted in terms of partially recovered stomatal response and comparatively open stomatal aperture.
- ✓ The total protein and sugar content in tomato plants decreased after pathogenic infections. This reduction in protein and sugar content can be recovered through tomato seed priming with *T. virens* and JA.

- ✓ Pathogen inoculation leads to an increase in the content of compatible osmolytes, i.e., proline and glycine betaine. The application of seed-priming agents has variable influences on the proline and glycine betaine accumulation in diseased plants.
- ✓ A significant increase in the phenol, flavonoids and anthocyanin content has been observed in *Fol,* and *R. solani* challenged tomato plants. The application of BCA further enhances the range of these phenolic compounds in stressed plants.
- ✓ Exposure of tomato seedlings to fungal pathogens intensifies the ROS (MDA and H_2O_2) levels in diseased plants. However, pre-treatment of tomato seeds with *T. virens* and JA, either individually or combined, leads to further reduced ROS contents in plants affected by respective pathogens.
- ✓ In response to oxidative destruction perpetrated by these pathogens, plants' oxidative defence systems began through increased synthesis and accumulation of non-enzymatic antioxidants like ascorbic acid, glutathione and tocopherol. Combined treatment with priming agents further raises the magnitude of these antioxidants in pathogenically challenged plants.
- ✓ Owing to the pathogen infection, there was an increase in the activity of antioxidative enzymes, viz., SOD, CAT, GPOX, PPO, DHAR, APX, GR, GST and GPX. Like non-enzymatic antioxidants, combined treatment with *T. virens* and JA increases the activity of these enzymes in both *Fol* and *R. solani*-challenged plants.
- ✓ Compared to control, free radical scavenging activity monitored through DPPH and ABTS assay was increased in pathogen-stressed, primed tomato plants due to the establishment of a better antioxidative defence system.
- ✓ qRT-PCR studies revealed the upregulation of the expression of *Cu-Zn SOD* and *Cat-I* genes in stressed tomato plants due to combined pre-treatment with *T. virens* and JA.

Therefore, from the above analysis, we can conclude that in the present study, higher induction of ameliorative compounds in the plants pre-treated with *T. virens* as BCA in combination with JA as chemical inducer can be correlated to an improved defence response activated against damping off and fusarium wilt infection in tomato plants. Furthermore, priming of tomato seeds with these ameliorative agents leads to a

marked improvement not only in morphological parameters but also leads to biochemical changes through various mechanisms of disease suppression like reduction of growth and proliferation of pathogens, induction of SAR and ISR in the diseased plants through the synthesis and accumulation of secondary metabolites and osmolytes, improvement in pigments, photosynthesis rate and gaseous exchange as well as induction of antioxidative defence system in the stressed plants. Therefore, seed-priming with these agents, especially in combination, can be recommended as a cost-effective, environment and farmer-friendly approach to raising the productivity of this essential horticultural crop on a commercial scale.

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Appendix - I

Abbreviations

12-oxo-PDA	12-oxo-Phytodienoic Acid
$^1O_2$	Singlet oxygen
Ab.	Absorbance
ABA	Abscisic Acid
ABC	ATP-binding cassette
ABTS	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
ACC	Amino Cyclopropane Carboxylate
AGs	Anastomosis groups
ANOVA	Analysis of Variance
AOS	Allen oxide synthase
APX	Ascorbate peroxidase
AsA	Ascorbic Acid
ATP	Adenosine Triphosphate
BCA	Biocontrol Agent
BOD	Biological Oxygen Demand
Cat	Catalase
cDNA	complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
CE	Common Era
Chi	Chitinase
Chl	Chlorophyll
CHS	Chalcone Synthase
cm	centi-meter
CMC	Carboxy Methyl Cellulose
CO_2	Carbon Dioxide
COI-1	Coronatine Insensitive-1
CT	Cycle Threshold
CTR	Constitutive Triple Response
Cu-Zn	Copper-Zinc

CWDEs	Cell Wall Degrading Enzymes
DEPC	Diethyl pyrocarbonate
DHA	Dehydro Ascorbate
DHAR	Dehydroascorbate Reductase
DNA	Deoxyribonucleic acid
DNPH	2,4 dinitrophenyl hydrazine
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
DW	Distilled Water
e^-	Electron
EDTA	Ethylene Diamine Tetra Acetic acid
ER	Endoplasmic Reticulum
et al	et alia
ET	Ethylene
ETC	Electron Transport Chain
ETR	Ethylene Receptor
Eub	E3 Ubiquitin ligase complex
f.s	formae speciales
FC	Folin-Ciocalteu
Fol	Fusarium oxysporum lycopersici
FW	Fresh Weight
g	gram
GAA	Glacial acetic acid
GABA	Gamma-Aminobutyric Acid
GAE	Gallic acid Equivalents
GB	Glycine Betaine
GDHP	Guaiacol Dehydrogenation Products
GPOX	Guaiacol Peroxidase
GPX	Glutathione Peroxidase
GPXL3	Glutathione Peroxidase-Like Enzyme 3
GR	Glutathione reductase
GRX	Glutaredoxin

GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione Synthetase
H_2O_2	Hydrogen peroxide
HAHB4	Helianthus Annuus Homeo Box 4
HClO	Hypochlorous acid
HCN	Hydrogen Cyanide
HPLA	Hydroperoxylinolenic acid
HPPD	p-Hydroxyphenyl Pyruvate Dioxygenase
HPT	Homogentisate Phytyltransferase
HR	Hypersensitive Response
IAA	Indole Acetic Acid
IARI	Indian Agricultural Research Institute
IBM	International Business Machines
IBA	Indole Butyric Acid
IC	Intercellular Carbon
IDM	Integrated Disease Management
IR	Infrared
IRGA	Infra-Red Gas Analyzer
iso-JA	Isoleucine-Jasmonic Acid
ISR	Induced Systemic resistance
ITCC	Indian Type Culture Collection
IUPAC	International Union of Pure and Applied Chemistry
JA	Jasmonic Acid
JAR1	Jasmonate Resistant 1
JMT	Jasmonic acid carboxyl Methyl Transferase
KI	Potassium iodide
LA	Linolenic Acid
LDL	Low-density lipoprotein
LOX	Lipoxygenase
MAMPs	Microbe-Associated Molecular Patterns
MAPK	Mitogen Activated Protein Kinase

MAPS/PAMS	Microbe/Pathogen Associated Molecular Patterns
MDA	Malondialdehyde
MDHA	Monodehydro Ascorbate
MDHAR	Monodehydroascorbate Reductase
MED	Mediator
mg	milli-gram
mL	millilitre
mM	milli-Molar
MT/H	Metric Tonnes/Hectare
NADP	Nicotine Amide Adenine Dinucleotide Phosphate
NBT	Nitro Blue Tetrazolium
NCBI	National Center for Biotechnology Information
nm	nanometre
nM	nano-Molar
NMB	2- nitro- 5- mercaptobenzoic acid
NPR 1	Nonexpresser of Pathogenesis Related Genes 1
O^{2-}	Superoxide Radical
$^{\circ}C$	Degree Celsius
OD	Optical Density
OH^{-}	Hydroxyl radical
PAL	Phenylalanine Ammonia Lyase
PAMP	Pathogen-Associated Molecular Patterns
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PDI	Percentage of Disease Incidence
PGPF	Plant Growth-Promoting Fungus
POD	Peroxidase
PPB	Potassium phosphate buffer
PPFD	Photosynthetic Photon Flux Density
PPO	Polyphenol Oxidase

PR	Pathogenesis Related
PR	Photosynthetic Rate
PRR	Pattern Recognition Receptor
PS	Photosystem
q-RTPCR	Quantitative Reverse Transcription PCR
R. solani	Rhizoctonia solani
RBD	Random Block Design
RBO	Respiratory Burst Oxidase
RNA	Ribose Nucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
rpm	revolutions per minute
RSV	Rice stripe virus
RT	Room Temperature
RUBISCO	Ribulose Bisphosphate Carboxylase Oxygenase
SA	Specific Activity
SAM	S-adenosyl-L-methionine
SAR	Systemic Acquired Resistance
SC	Stomatal Conductance
SCF	Skp1/Cullin/F-box
SeCy	Selenocysteine
SEM	Scanning Electron Microscopy
-SH	Thiol
SMs	Secondary Metabolites
SOD	Superoxide Dismutase
Sp.	Species
SPSS	Statistical Package for the Social Sciences
SSA	Sulphosalicylic acid
T. virens	*Trichoderma virens*
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TFC	Total Flavonoid Content

TFs	Transcription Factors
TPC	Total Phenolic Content
TPI	Trypsin Protease Inhibitors
TPTZ	2,4,6-tripyridyl-S-triazine
TR	Transpiration rate
Trx	Thioredoxin
TSS	Total Soluble Sugar
TuMV	*Turnip mosaic virus*
UA	Unit Activity
UV-vis.	Ultra Voilet-Visible
w/v	Weight/Volume
XOD	Xanthine Oxidase
ZAJ	Jasmonate-Zim Domain
μM	micro-Molar

List of Publications

1. Sood, M., Sharma, S. S., Singh, J., Prasad, R., and Kapoor, D. (2020). Stress Ameliorative Effects of Indole Acetic Acid on Hordeum vulgare L. Seedlings Subjected to Zinc Toxicity. *Phyton*, *89*(1), 71.
2. Sood, M., Kapoor, D., Kumar, V., Sheteiwy, M. S., Ramakrishnan, M., Landi, M., ... and Sharma, A. (2020). Trichoderma: The "Secrets" of a Multitalented Biocontrol Agent. *Plants*, *9*(6), 762.
3. Sood, M., Pujari, M., and Kapoor, D. (2020). HEAVY METALS TOXICITY AND DETOXIFICATION STRATEGIES IN PLANTS. *Plant Archives*, *20*(2), 2641-2646.
4. Sood M., Kumar V., and Rawal, R. (2020). Seed biopriming a novel method to control seed borne diseases. *Biocontrol Agents and Secondary Metabolites: Applications and Immunization for Plant Growth and Protection*, 181.
5. Sood, M., Kapoor, D., Kumar, V., Kalia, N., Bhardwaj, R., Sidhu, G. P. S., and Sharma, A. (2021). Mechanisms of Plant Defense under Pathogen Stress: A Review. *Current Protein and Peptide Science*.
6. Sood M., Bhardwaj S., Kapoor B., Kapoor D (2021) AN INTRODUCTION TO SPECIFIC ATTRIBUTES OF BARLEY: CULTIVATION, PRODUCTION AND CONSUMPTION. *Hordeum vulgare:* Production, cultivation and uses.
7. Sood, M. Jasmonates: "The Master Switch" for Regulation of Developmental and Stress Responses in Plants. *J Plant Growth Regul* (2023). https://doi.org/10.1007/s00344-023-11047-3.
8. Sood, M., Kukreja, S., and Kumar, V. (2023). Identification of disease suppressive potential of *Trichoderma virens* and Jasmonic acid against fusarium wilt and damping-off in "Seed Primed" tomato plants. *Plant Science Today*. https://doi.org/10.14719/pst.2325.
9. Selwal, N., Goutam, U., Akhtar, N., Sood, M., & Kukreja, S. (2024). Elicitation:"A Trump Card" for Enhancing Secondary Metabolites in Plants. *Journal of Plant Growth Regulation*, 1-21.

List of Conferences

1. 106th Indian Science Congress at Lovely Professional University, Phagwara, Jalandhar, Punjab from 3-7th January 2019.
2. International Conference on Biosciences and Biotechnology (ICBB-2019) at Lovely Professional University, Phagwara, Jalandhar, Punjab from 4-5th November 2019.
3. National Symposium on Smart and Sustainable Agriculture (Agricon, 2019) at Lovely Professional University, Phagwara, Jalandhar, Punjab on 23rd November 2019.
4. International virtue conference on Plant Specialized Metabolism and Metabolic Engineering (PSMME-2020) CSIR-CIMAP from 14th-16th October 2020.
5. International Conference on Sustainability: Life on Earth 2021 (ICS-LOE 2021) held on 17-18 December 2021 organized by Department of Botany and Zoology, School of Bioengineering and Biosciences, and Institute of Forest Productivity, Ranchi, Jharkhand, at Lovely Professional University, Punjab.
6. 5th International Conference on Advances in Agriculture Technology and Allied Sciences (ICAATAS 2022) from 4th-5th June 2022.
7. 1st International Conference on "Global Initiatives in Research, Innovation and Sustainable Development of Agriculture and Allied Sciences (GIRISDA-2022)" organized by Just Agriculture, Guru Kashi University, Bathinda (ICAR Accredited) and AEEFWS, Punjab on 06-07-08 June 2022 at Guru Kashi University, Bathinda.

List of Workshops

1. Online Short-Term Course 'National Workshop on Statistical Analysis using SPSS' organized by Lovely Professional University, Phagwara, Jalandhar, Punjab w.e.f. 22-26th June 2020.
2. Short Term Course on Scientific Writing Using Typesetting Software LaTeX organized by Lovely Professional University w.e.f. October 17- November 01, 2020 (6 Days).
3. The one-week Short Term Training Program on **"**Academic Writing using LATEX" organized by Applied Mathematics and Humanities Department (AMHD), Sardar Vallabhbhai National Institute of Technology (SVNIT), Surat during January 04–08, 2021 in online mode.
4. The e-workshop on Data-Analysis with 'R' Programming held from 30th Sept.- 1st Oct. 2023, organized by comcad.