ROLE OF MELATONIN AND PLANT GROWTH PROMOTING RHIZOBACTERIA IN DEGRADATION OF THIAMETHOXAM BY *BRASSICA JUNCEA*

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY
in

Biotechnology

By

Sadaf Jan

Registration Number: 11719353

Supervised By
Dr Rattandeep Singh (19383)
Department of Biotechnology (Assistant Professor)
Lovely Professional University

Co-Supervised by
Professor Renu Bhardwaj
Department of Research (Director)
Guru Nanak Dev University

Dr Parvaiz Ahmad
Department of Botany (Associate Professor)
GDC Pulwama, Jammu & Kashmir

Department of Botany & Microbiology
King Saud University, Riyadh, Saudi Arabia

LOVELY PROFESSIONAL UNIVERSITY
PUNJAB
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Declaration

I hereby asseverate that the thesis "**ROLE OF MELATONIN AND PLANT GROWTH PROMOTING RHIZOBACTERIA IN DEGRADATION OF THIAMETHOXAM BY *BRASSICA JUNCEA***" submitted for the award of degree of **Doctor of Philosophy** is based on my individual research work and all the sources used in this research effort have been comprehensively acknowledged.

Sadaf Jan

11719353

Department of Biotechnology

School of Bioengineering and Biosciences,

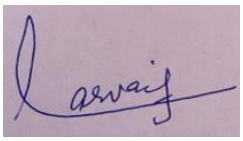
Lovely Professional University, Punjab, India.

Date: 23-May-2022

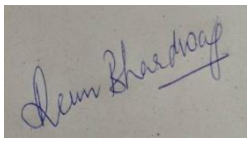
Certificate

Certified that **Sadaf Jan (11719353)** has executed the research work presented in this thesis entitled **"ROLE OF MELATONIN AND PLANT GROWTH PROMOTING RHIZOBACTERIA IN DEGRADATION OF THIAMETHOXAM BY *BRASSICA JUNCEA*"** for the award of **Doctor of philosophy** from Lovely Professional University, Punjab, under our joint supervision. The thesis embodies results of original work, and studies are carried out by the scholar herself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University.

Signature
Supervisor
Dr Rattandeep Singh
School of Bioengineering and Bioscience,
Lovely Professional University, Punjab

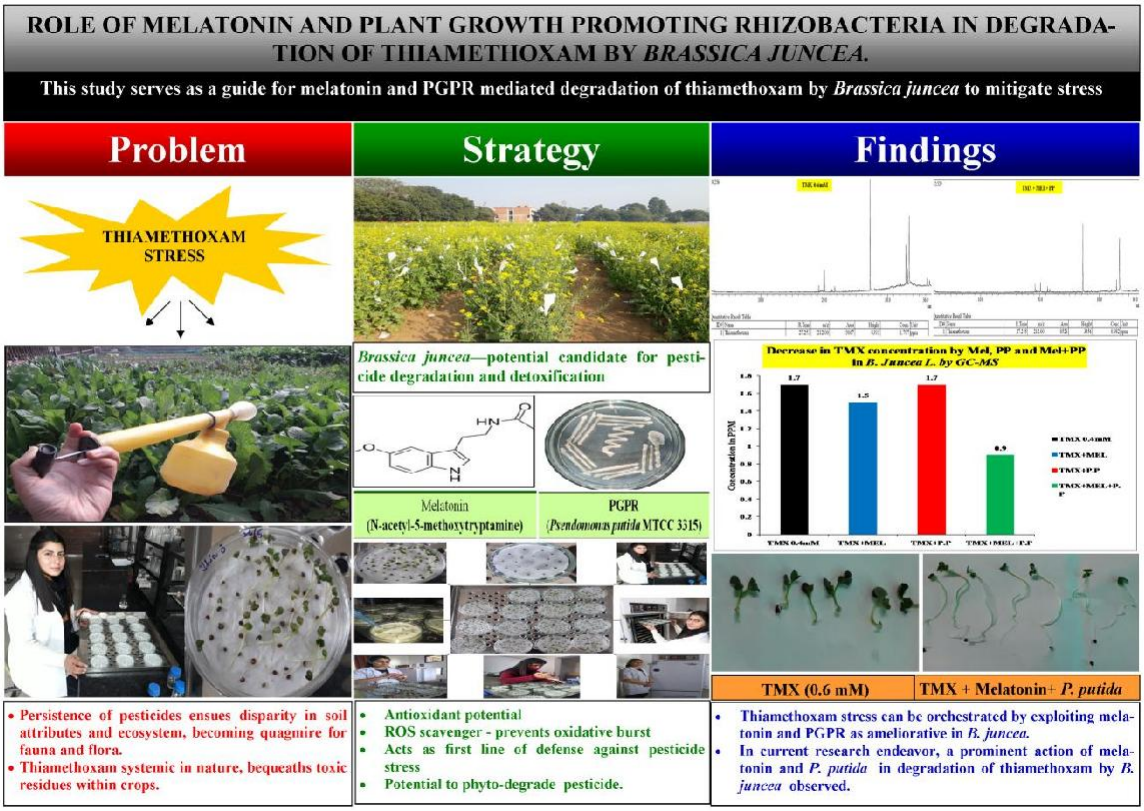


Signature
Co-supervisor
Dr Parvaiz Ahmad
Department of Botany
GDC, Pulwama, J&K, and
Department of Botany and Microbiology
King Saud University, Riyadh, Saudi Arabia



Signature
Dr Renu Bhardwaj
Department of Botanical
and Environmental Sciences
Guru Nanak Dev University,
Amritsar

GRAPHICAL ABSTRACT



Role of Melatonin and Plant Growth Promoting Rhizobacteria in Degradation of Thiamethoxam by *Brassica juncea.*

ABSTRACT

Pesticides play an indispensable role in controlling pest manifestation. Yet, the aftermath of indiscriminate and inadequate use is contamination of food cycle, thereby becoming jeopardy to humankind. Promiscuous exploitation of one such compound that is thiamethoxam augments its presence and accumulation in crops, soils and water bodies. Accumulation of such persistent, less biodegradable toxic compounds ensue disparity in soil attributes and consequently to the ecosystem, as it becomes quagmire for the fauna and flora. Pesticides utilized in agriculture is not entirely consumed by plants, but in lieu leached to the soil and groundwater. Mode of action exerts toxic effects not only to specific organisms but also to non-target species.

Pesticide prevalence within ecosystem is emanating and is the most significant environmental issue, drawing the emphasis and focus of scientific community. Demand of pesticides and lack of efficient detoxification and remediation approaches indicates that the predicament is exacerbating, thereby bringing this subject to forefront in the immediate future. Thiamethoxam (TMX) has been subject to certain scrutiny, due to its accumulation in plant parts and high persistence rate in soil, it poses risks to the non-target species. Besides insecticidal control, thiamethoxam bequeaths considerable toxic residues within crops which beget potential danger to living beings. After-effects of pesticides on plants are dose-dependent causing disruption and alteration of various physiological and biochemical processes, ultimately reducing growth and productivity.

Conversely, *Brassica juncea* have ability to counteract pesticide stress via detoxification system and metabolize pesticides through enzyme-mediated pathways. Melatonin a pleiotropic molecule is accountable for invigorating multifarious physiological processes. The cardinal role of phyto-melatonin is to provide first line of defense against oxidative stress that befalls due to unfavorable conditions. Phyto-melatonin has a significant role in ROS scavenging, enhancing antioxidant potential and preventing oxidative stress. The role of melatonin in plant protection and defense mechanism renders a direct way for attenuating the pesticide stress and reducing pesticide residues in food crops. Plant

growth promoting rhizobacteria (PGPRs) play an imperative role in overall development of plant by providing tolerance to hostile conditions, shaping rhizosphere, enhancing nutrient absorption and biomass. PGPRs are known to withstand abiotic stresses and enhance stress tolerance in plants, they produce metabolites which alter defense responses and mitigate damages caused by ROS. *Pseudomonas putida* provides utmost tolerance against pesticide and has degrading potential. Pesticides act as a sole source of carbon for microbes, constructing a way for pesticide degradation and transformation into non-toxic form. Tremendous quantum leap has been made in comprehending, how plant growth regulators and PGPRs safeguards plants against abiotic stress. Therefore, thiamethoxam stress and degradation can be orchestrated by exploiting melatonin and *P. putida* as ameliorative in *B. juncea.*

Herein, we demonstrate the efficiency of seed priming with melatonin, a powerful antioxidant and *P. putida,* a plant growth promoting rhizobacteria for reduction of TMX residues in *B. juncea* seedlings. TMX residues in seedlings were analyzed and quantified using Gas Chromatography–Mass Spectrometry (GC-MS). Melatonin primed seeds were inoculated with *P. putida* and further dosed with 0.6 mM TMX. Results proclaim that melatonin treatment decreased TMX residues by 13.33%, whereas, combinatorial application of melatonin and *P. putida* showed 88.88% reduction as compared to investigational TMX treated *B. juncea* seedlings. The current analysis shows a prominent action of melatonin and *P. putida* in TMX degradation, which further can be maneuvered to phyto-degrade the xenobiotic compounds. In current research endeavor, relative organic acid content of intermediates of TCA cycle were analyzed by HPLC technique. Our findings revealed that relative organic acid content was considerably higher in TMX + melatonin + *P. putida* treated seedling as compared to the control plant. The accretion of organic acids (intermediates of TCA cycle) may correspond to meet energy generation requirement to combat the thiamethoxam mediated stress.

Our findings indicate that melatonin and *P. putida* alone as well as synergistically boost the resilience of *B. juncea* seedlings under thiamethoxam stress. The association of

melatonin and *P. putida* with plants in up-regulating levels of metabolites under TMX toxicity has been explored. Morphological analysis asserts that effect of TMX stress is more on FW, DW and seedling length and combinatorial approach of melatonin and *P. putida* can significantly reduce the TMX induced stress on plant's morphology. Increment in the photosynthetic components viz, chlorophyll, carotenoid, anthocyanin and flavonoid collaborates with the plant growth. Higher plant growth is related to the photosynthetic area. The ample photosynthetic area, in-turn, elevates photo-assimilates, which results in enhanced growth. Osmo-protectants possess defensive properties and acts as stress indicator. In present investigation, application of TMX increases the osmo-protectant content, while the distinct application of melatonin and *P. putida* was able to retain the osmo-protectant content in TMX treated *B. juncea* seedlings, bestowing membrane stabilization, better osmotic balance and protection during unfavorable conditions. In current strive, elucidation of osmolyte content and their role in context to pesticide toxicity serves as a first of a kind suggesting their role in shielding plants against pesticide stress.

Pesticide mediated phytotoxicity produces ROS which causes an imbalance in redox homeostasis and antioxidative defense system, eliciting oxidative stress, lipid peroxidation and ultimately compromising the growth, development and crop yield. ROS is considered as an index to depict cell damage. An enhanced level of MDA in oxidative stress conditions acts as a marker in measuring cellular damage. The accretion of activated oxygen molecule is caused by erratic creation and irregular detoxification cycle of ROS which is the repercussion of abiotic stress conditions. Our results depicts that contents of $O^{\cdot-}_2$, H_2O_2, and MDA were much higher in TMX treated plants, howbeit, diminution in oxidative stress marks was observed in plants amended with melatonin and *P. putida* under pesticide stress. Our findings also unveil that melatonin and PGPR aids in decreasing MDA levels in plants during pesticide exposure indicating their ameliorative role towards membrane impairment. In response to ROS plants regulate their antioxidative defense system to neutralize oxidative stress mediated by unfavorable factors. We discerned variation in antioxidative activity dynamics during TMX exposure,

whilst, increase in antioxidants (glutathione, tocopherol, ascorbic acid and phenol content) and higher antioxidant activity (SOD, CAT, POD, GPOX, APOX, GR, DHAR and GST) upon supplementation of melatonin and *P. putida* was recorded. Contemporary study proposes that melatonin and *P. putida* stimulates expression of genes encoding detoxifying enzymes viz (*P450, CXE, NADH, POD* and *GST*). In light of this, exogenous melatonin and PGPR has proven excellent in degrading thiamethoxam and coping against thiamethoxam stress.

This unfolds new areas of research to ascertain precise molecular mechanisms involved in regulatory processes fostered by application of melatonin and *P. putida* in stressed plants. Moreover, phytoremediation is an eco-friendly, and in situ resilient technique for remediating contaminated soil. Primarily it retains the soil fertility, despite detoxifying and eliminating toxic compounds. In future, this study can be extended to fields that are pesticide contaminated and with assistance of phytohormones and PGPRs the *B. juncea* will render such soils contamination free. As *B. juncea* has potential to assimilate and disintegrate pesticides into less toxic state. Due to high biomass and colossal produce *B. juncea* is considered as a suitable candidate for pesticide degradation. Our study breakthroughs can also be extrapolated to other crops for which melatonin and *P. putida* can be exploited as natural bio-stimulating agents in order to palliate the negative impact of multifarious abiotic stresses.

Acknowledgement

On earth, nothing is possible without the will of the 'Creator' of this universe, Who created man and stood by to learn from nature, and taught to learn from cradle to grave by using wisdom.

First and Foremost, I would like to mention my eternal gratitude to Almighty Allah, most beneficent and merciful, the greatest helper of all, who blessed me with patience and strength to accomplish this research endeavor.

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My experience during the course of research was extremely invigorating.

Sadaf Jan Date: 23-May-2022

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CHAPTER 1

1. Introduction

1.1 Scenario of Pesticide Production and Usage

Any natural or synthetic compound used to annihilate, prevent or metamorphose life cycle of any pest is denominated as pesticide. Pesticides are an extensive part of agriculture, ensuring crop fortification and augmenting crop yield (Jan *et al.,* 2020). Specific pests include weeds, microorganism, insects, birds, rats and mice (Singh *et al.,* 2018). Pesticides are categorized on the bases of their roles (insecticides, rodenticides, herbicides, fungicides etc), chemical nature (organophosphates, organochlorine, carbamates, pyrethroids, triazines) (Rani *et al.,* 2017), mobility (systemic and non-systemic), target rates (selective or broad spectrum) (Rodrigo *et al.,* 2014) Further they can also be classified on the basis of toxicity and mode of entry (Singh *et al.,* 2018). Owing to, limited agricultural land and rapidly growing population there is need to escalate crop production to meet the demand of people and ensure food security. Consequently, initiatives are taken to scale up the crop yield by minimizing crop loss from pest damage. In an effort to protect crops, pesticides are extensively exploited by humans to destroy the pests (Morillo and Villaverde 2017).

Keeping in view the infestation of sucking insect pests, thiamethoxam came into picture. Thiamethoxam [3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene (nitro) amine], launched as second generation neonicotinoid was developed by Ciba-Geigy (now Syngenta) crop protection in 1991 and marketed since 1998 under trademarks Actara for foliar treatment, Cruiser for seed priming and Platinum for soil treatment (Maienfisch *et al.,* 2001). It is board-spectrum insecticide, which offers control over wide-array of pests including aphids, flea beetles, hoppers, thrips, wireworms, whiteflies and lepidopteran species. Being systemic in nature, it translocates throughout the plant system and is eventually delivered to the pests feeding on it (Mohapatra *et al.,* 2019). Thiamethoxam renders both systemic and contact activity against pests, as

dissolved thiamethoxam is assimilated along soil water and acropetally transported into the xylem by plants (Hilton *et al.,* 2016). Thiamethoxam acts on central nervous system of insects by working as an agonist of nicotinic acetylcholine receptors (nAChRs), thereby causing nervous receptor blockage, paralysis and ultimately death (Kou *et al.,* 2021). In recent years, thiamethoxam has been subject to certain scrutiny, due to its accumulation in plant parts and high persistence rate in soil, it poses potential risks to the non-target species (Wang *et al.,* 2020; Gui *et al.,* 2019; Thompson *et al.,* 2019). From collective data it is apparent that thiamethoxam has longer half-life in soil with DT_{50} ~ 229 days (Hilton *et al.,* 2019) and high leaching potential with water solubility = 4,100 mg/L (Klarich *et al.,* 2017).

Thiamethoxam is exploited world-wide, and the formulated products are registered for use in over 130 countries, including, India, Australia, Canada, Europe, Brazil, Russia and the United states (Hilton *et al.,* 2018). Thiamethoxam production in India has increased steadily, as documented by Ministry of Chemical and Fertilizers, Department of Chemical and Petro-Chemicals (Figure 1.1). As per the report from Directorate of Plant Protection, Quarantine and Storage, India, the consumption of indigenous and imported thiamethoxam shows colossal trend from year 2016-2021 as depicted in figure 1.2.

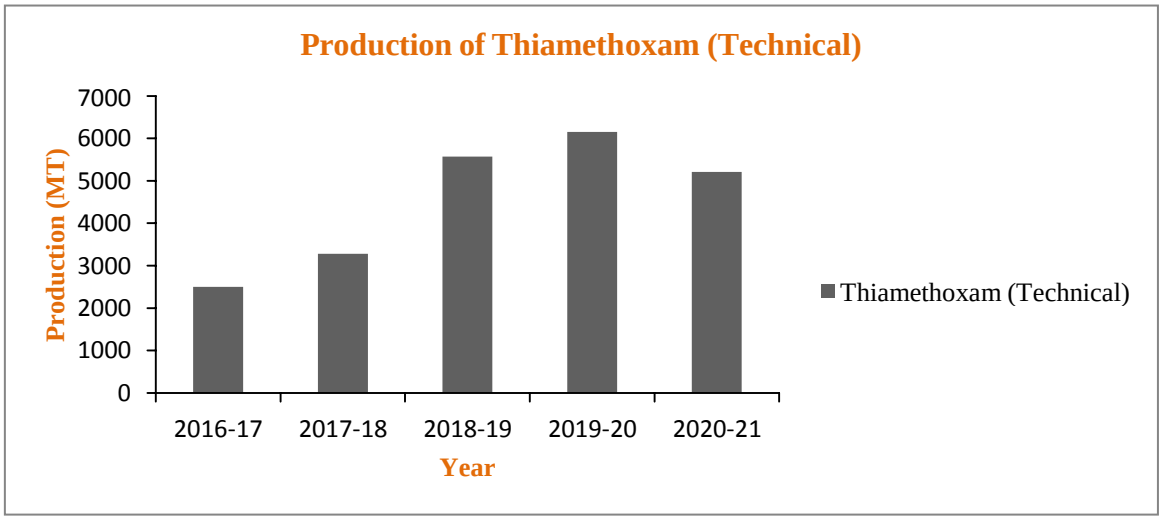


Figure 1.1: Yearly production of thiamethoxam in India.

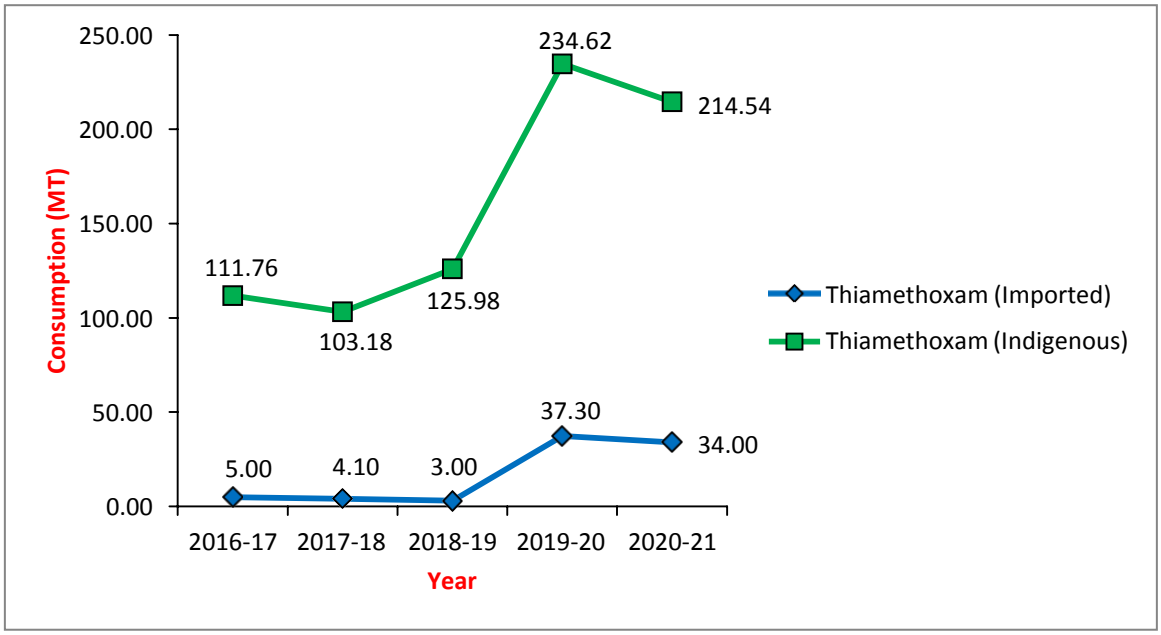


Figure 1.2: Trends in Consumption of Thiamethoxam in India.

1.2 Thiamethoxam toxicity and its metabolism

Besides insecticidal control, thiamethoxam bequeaths considerable toxic residues within crops which beget potential dangers to living beings (Han *et al.*, 2018; Flores *et al.*, 2021). Vetting the accretion of thiamethoxam is imperative for redressing utilization rates and risk management. Environmental protection agency (EPA, 2020) established tolerance levels for thiamethoxam residues and its metabolites viz, 4.5 ppm in seed and stem of *Brassica* spp. and 3.0 ppm in leafy greens of *Brassica* spp. After-effects of pesticides on plants are dose-dependent causing disruption and alteration of various physiological and biochemical processes, ultimately reducing growth and productivity. Conversely, plants have ability to counteract pesticide stress via detoxification system. Pesticides are metabolized by plants through enzyme-mediated pathways. This detoxification system is three-phased process. Phase I: activation of pesticide through hydrolysis, oxidation or reduction, induced by enzymes – carboxylesterases, cytochrome P450 monooxygenase and peroxidase. Phase II: activated pesticide is conjugated with amino acids, glucose or glutathione. The conjugation is catalyzed by enzymes glutathione

S-transferase and UDP-glycosyltransferase. Phase III: lastly, less toxic pesticide conjugates are carried to vacuoles/apoplasts or bind to cell wall/lignins (Figure 1.3) (Jan et al., 2020).

In plants thiamethoxam undergoes intense metabolism which generates various metabolites. During absorption and distribution process, thiamethoxam encounters multifarious enzymes and ergo, transformed to another compounds and derivatives via conjugation, hydrolysis, oxidation or reduction (Karmakar *et al.,* 2009). The heterogeneity in metabolic pathways pivots upon plant species, metabolic enzymes and chemical configuration of pesticide. In different plant species the same pesticide may disintegrate to unlike metabolic products (Eerd *et al.,* 2003). In cotton plants the precursor thiamethoxam cleaves to another potent neonicotinoid namely clothianidin (Nauen *et al.,* 2003). Whilst in tomato plant the thiamethoxam metabolites are nitro-guanidine (neonicotinoid), nitroso product and urea (Karmarkar *et al.,* 2009). It is corroborated that *Pseudomonas* sp. 1G, transforms thiamethoxam to desnitro (=NH), nitosoguanidine (=N-NO) and urea metabolites in *in-vitro* condition (Pandey *et al.,* 2009). The anecdotal corroboration suggests that, thiamethoxam treatment affects plant vigor. The cytotoxic and genotoxic effect of thiamethoxam on plant has been documented. Thiamethoxam treatment eventuates oxidative stress especially lipid peroxidation, affects anti-oxidative defense system and triggers chromosomal abnormalities in plants. The frequency of cell-cycle retardation and mitotic aberration augments with increasing concentration of thiamethoxam in *Helianthus annuus* L. (Georgieva and Vassilevska-Ivanova 2021). Thiamethoxam treated onion plants portended alteration in germination rate, biomass and root length. It evinced increased lipid peroxidation, MDA levels and mito-depressive effect on mitosis. Some anatomical impairment including necrotic cell death, cell deformation, unclear vascular tissue and epidermis layer were also discerned (Cavusoglu *et al.,* 2010).

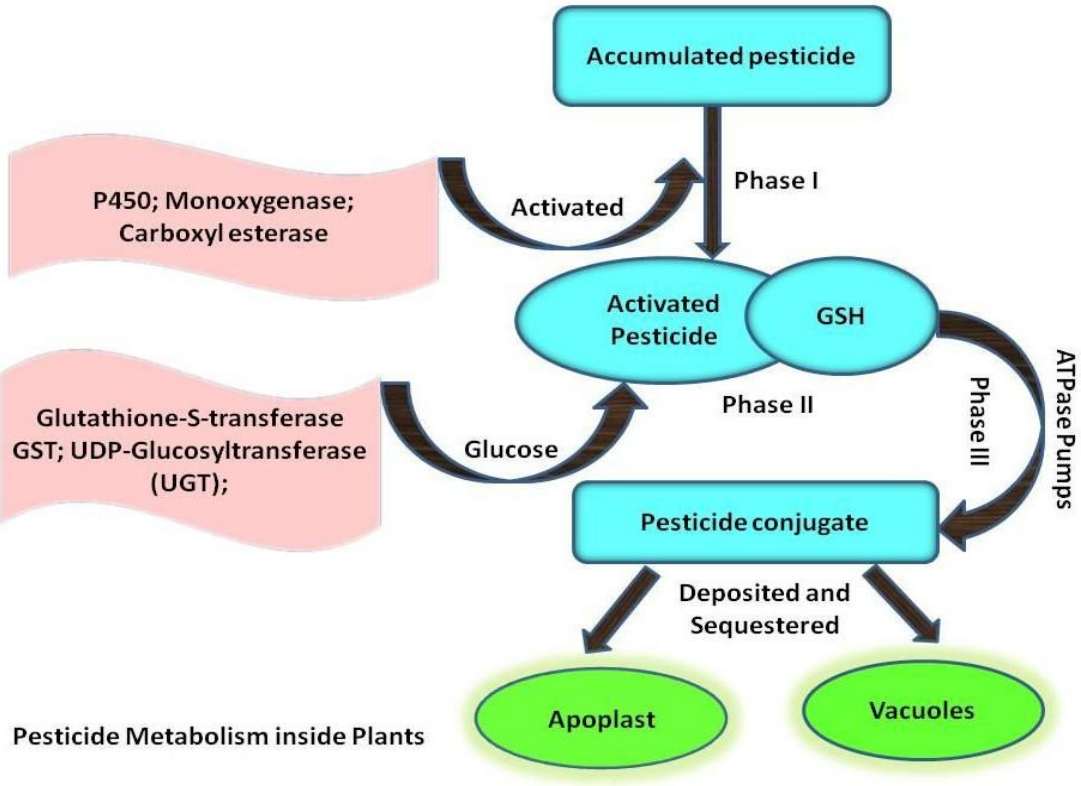


Figure 1.3: Mechanism of Pesticide Detoxification.

1.3 Model plant for study:

Taxonomic classification of *Brassica juncea*	
Kingdom	Plantae
Sub-kingdom	Tracheobionta
Super-division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Sub-class	Dilleniidae
Order	Capparales
Family	Brassicaceae
Genera	*Brassica*
Species	*Brassica juncea*

Brassica juncea, a member of Brassicaceae family is generally recognized as Indian mustard. The estimated mustard yield is 72.42 million tones with productivity of 1980 kg/ha in 2018-19 in India (ICAR, 2018). This oil seed crop is usually invaded by various arthropods such as aphids. The pest manifestation radically decreases the crop-yield of mustard cultivars (Yadav *et al.,* 2019). More than 43 species of insects infest *Brassica juncea* crop in India causing up-to 96% of yield loss (Pradhan *et al.,* 2020). In an effort to protect crop and scale up the yield insecticides are used indiscriminately (Jan *et al.,* 2020). Insecticide toxicity has eminent reverberation on plants and subsequently affects the ecosystem. Insecticide accretion causes phytotoxicity and poses detrimental impact on food safety. The aftermath of insecticide use is oxidative stress, plant growth reduction, photosynthesis impairment and reactive oxygen species (ROS) generation. Conversely, *B. juncea* has ability to counteract insecticide stress via detoxification system. Being a hyperaccumulator, it is also well-known for skirmishing toxic effect of insecticides. Enzymatic transformations serve as a radical route for detoxifying insecticides (Sharma *et al.,* 2018).

1.4 Melatonin and *Pseudomonas putida* as Amelioratives

Melatonin (*N*-acetyl-5-methoxy-tryptamine), derivative of tryptophan, manifested as a conserved domain, which is ubiquitously apportioned from bacteria to higher organisms extending to fungi and algae as well (Lerner *et al.,* 1958). Melatonin is entailed in umpteen developmental processes of plants, including stress responses (Figure 1.4). The pleiotropic impact of melatonin in regulating transcripts of manifold genes validate it's imperative contribution as multi-regulatory substance. Albeit, the progressive research regarding plants is yet prelusive in contrast to orthodox melatonin physiology in animals. This reinforces the exigency for comprehensive reassessment pertaining to its potential in biochemical and physiological processes, anti-stress response against abiotic stimulators; pesticides (Liu *et al.,* 2021), heavy metals (Kaya *et al.,* 2019), drought (Khan *et al.,* 2020), salinity (Siddiqui *et al.,* 2020), detoxification mechanism, and its other salubrious effect (Bose and Howlander, 2020). Abiotic stress induces melatonin synthesis and this

redeeming upsurge in melatonin succors plant to thrive under stress conditions. Tremendous quantum leap has been made in comprehending, how melatonin safeguards plants against abiotic stress. Here, focus will be on mechanistic basis of melatonin mediated protection to abate insecticide stress.

Plant growth promoting rhizobacteria (PGPR), an omnipresent root microbiome is enormously exploited as biocontrol agents (Sharma *et al.,* 2020). They are capable of enhancing plant growth by colonizing plant roots that can benefit the plant. Various PGPR's such as *P. aeruginosa, B. gladioli, P. pseudoalcali* are known to withstand biotic and abiotic stresses (Khanna *et al.,* 2019; Yasmin *et al.,* 2020). To enhance stress tolerance in plants, PGPR has been known to produce metabolites which alter the defense response of plants by mitigating the damage caused by ROS (Rashid *et al.,* 2020). PGPR confer vital functions in host plants for example releasing many phytohormones (IAA, gibberellins, cytokinins, ethylene, salicylic acid), enzymes, siderophore production, phosphate solubilization and nitrogen fixation (Yasmin *et al.,* 2020). *P. putida* exhibits degradation of thiamethoxam without leaving any detectable metabolites (Rana *et al.,* 2015). *Pseudomonas putida* has shown utmost tolerance against insecticides and possess degrading capability (Jan *et al.,* 2022). Therefore, insecticide stress in plants can be orchestrated by *Pseudomonas putida*.

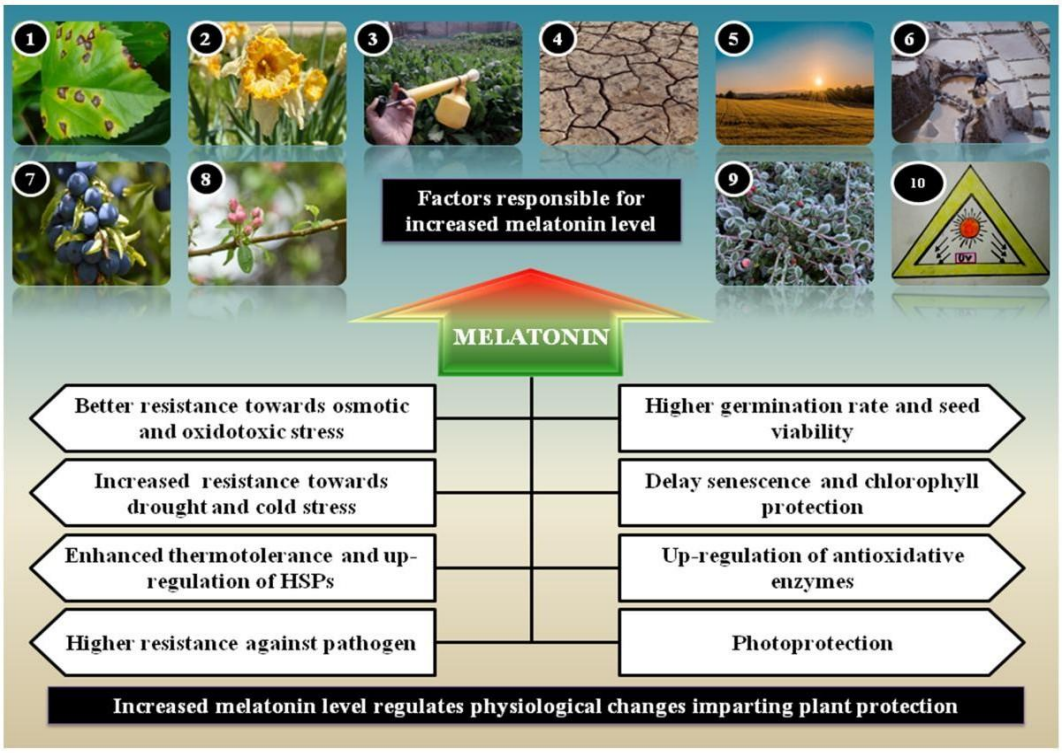


Figure 1.4: Increase in Melatonin level and their corollary for plant protection and stress tolerance is presented in pictorial form. (1) Pathogen attack (2) senescence (3) Chemical stressors (4) Drought (5) Heat (6) Salinity (7) Developmental stage - Fruit ripening (8) Developmental stage - Flower bud (9) Cold (10) Intense UV radiation. 1 – 10 correspond to the increased melatonin content. HSPs; Heat shock proteins.

CHAPTER 2

2. Review of Literature

2.1 Background of Pesticide Use

Predicament of pesticide contamination is progressively becoming ubiquitous. Prevalence of pesticides in circumambient is an indicative environmental issue. 1000 BC back pesticides came into use, when Chinese began to use sulfur as a fungicide. Following that, sulfur and arsenic were brought into practice as pesticides for centuries. Synthetic pesticides came into existence after World War II. Two arsenic compounds were highly considerable: arsenic trioxide (used as herbicide) and lead arsenate (used as an insecticide). History makes it evident, why several agricultural and industrial lands surplus with pesticides till today. After World War II, arsenic derived pesticides were gradually replaced by new organic pesticides. The first synthetic pesticide introduced to world was dichlorodiphenyl – trichloroethane (DDT), having broad effectiveness. Till 1970's, DDT was immensely used to fight mosquitoes and exterminate malaria until its negative effects were detected and were relinquished due to toxicity and resistance towards pests (Handford *et al.,* 2014). Currently, wide-arrays of chemical are being used as pesticides in agricultural realms. Pesticides benefit farmers by producing greater yield, in two ways: i) eradicating problems caused by insects, fungi, viruses, etc. ii) substitute as plant growth regulators or inhibit growth of weeds. The rate of pesticide production in 20^{th} century has reached 600000 tons in comparison to early 40's producing 140 tons only (Bakshi *et al.,* 2021). In modern era, the farming has expanded significantly in order to guarantee food supply for the growing populace. Crop cultivation is made feasible by prodigious utilization of pesticides, thereby becoming a significant measure in agribusiness.

2.1.1 Production and utilization of pesticide in India

A sizeable majority of population in India is occupied in farming and is consequently exposed to various pesticides, employed in agriculture. At present, consumption of

pesticides in India is 0.6 kg/ha while UK and China retains highest position, using 5-7 kg/ha and ~ 13 kg/ha respectively (Sharma *et al.*, 2019). Consumption and production of pesticides in India is illustrated in table 2.1. As on 30th October 2016, India commercialized 275 pesticides, out of which nearly 255 are toxic compounds. A survey by PAN India publicized that more than 115 pesticides among 275 pesticides are profoundly dangerous (Yadav and Dutta, 2019).

Table 2.1: Trends of pesticides production and consumption in India.

CONSUMPTION OF INDIGENOUS INSECTICIDES (2017-18 TO 2021-22)					
Unit: M.T. (Tech. Grade)					
INSECTICIDES	**2017-18**	**2018-19**	**2019-20**	**2020-21**	**2021-22**
Acephate	169	330.60	405.91	356.90	350.95
Acetamiprid	15	98.36	114.42	100.85	105.40
Alphacypermethrin	17	51.65	35.25	32.00	35.00
Bifenthrin	19	27.06	19.89	47.48	50.11
Buprofezin	37	392.00	126.00	79.00	125.00
Carbofuran	45	198.83	206.76	214.75	180.33
Carbosulfan	3	11.70	27.99	31.13	25.90
Cartap Hydrochloride	208	365.30	358.19	374.41	371.55
Chlorantraniliprole	37	116.47	105.00	135.34	140.44
Chlorpyriphos	478	1105.61	1430.62	1036.69	1170.90
Cypermethrin	176	308.54	674.65	343.91	340.98
Deltamethrin	11	45.49	49.67	24.59	52.58
Diclorvos	287	344.05	537.05	35.42	116.17
Dimethoate	92	323.64	367.51	209.59	216.36
Emamection Benzoate	20	95.00	97.00	124.73	190.55
Ethion	21	50.04	36.83	79.14	118.63
Fenvalerate	57	467.14	667.33	149.73	288.97
Fipronil	131	548.12	444.05	256.83	379.56

Imidacloprid	135	309.42	371.99	317.17	323.74
Indoxacarb	26	71.06	72.00	112.00	106.01
Lindane	0.60	0.20	0.50	-	-
Malathion	103	656.41	647.14	305.41	516.18
Phorate	480	723.70	641.49	19.86	4.00
Profenophos	300	401.79	425.00	433.40	457.18
Quinalphos	242	509.84	564.61	412.60	442.29
Thiamethoxam	103	125.98	234.62	214.54	248.36

Source: Government of India, Ministry of Agriculture & Farmers Welfare, Department of Agriculture, Cooperation & Farmers Welfare. Directorate of Plant Protection, Quarantine & Storage.

2.2 Discovery and Pioneering of Thiamethoxam

A research stratagem on neonicotinoids was commenced by Ciba in 1985. Novel variation in imidacloprid (nitroimino-heterocycle) were explored, which ensued in synthesis of 3 compounds viz., 2-nitroimino-hexahydro-1,3,5-triazine, 4-nitroimino-1,3,5-oxadiazinane and 4-nitroimino-1,3,5-thiadiazinane. Among aforementioned compounds, 4-nitroimino-1,3,5-oxadiazinane (Thiamethoxam) showed better insecticidal activity. It is second generation neonicotinoid and is member of thianicotinyl sub-class. Thiamethoxam synthesis began in 1991. The physico-chemical attribute of thiamethoxam includes crystalline, inodorous compound with melting point 139.1 °C. It has low volatility, high solubility and polarity (Maienfisch *et al.*, 2001).

2.3 Exigency for Pesticide Removal

Pesticides have boosted the agrarian realms globally by conferring protection to economically important plants through battling out wide-array of malignant pests. But the indiscriminate and repeated application of pesticides turns them into a menace thereby, threatening the well being of living organisms and disturbing the balance of ecosystem

(Bakshi *et al.,* 2021). The synthetic pesticides are intended to possess intricate chemical composition and are impervious to biodegradation. These chemical compounds emigrate from treated field to air, land and water bodies via drift, runoff, drainage and leaching, causing severe ecological issues. These ecological problems can end up in human health concerns due to their bio-accumulating tendency (Jan *et al.,* 2020).

Neonicotinoids are most immensely used insecticides since 2010 further, annual consumption is escalating rapidly in current years (Wang *et al.,* 2019). The extensive application of thiamethoxam has inflated concern pertaining to its residues. Without proper trials and safeguards it has led contamination of natural resources mainly soil and from there it venture further to pollute water bodies including surface and groundwater, eventually culminating their journey to defile biota (Main *et al.,* 2014; Jones *et al.,* 2014). It is anticipated that bulk of thiamethoxam remains in top soil layers, unless assimilated by plants or bio-degraded by microbes. Thiamethoxam tend to persist for long duration with DT_{50} upto 229-353 days in the soil (Goulson *et al.,* 2013). In Indian alluvial soil the half-life of thiamethoxam ranges from 46 to 301 days, depending upon the moisture content (Gupta *et al.,* 2008). Sparse data is available regarding thiamethoxam degradation under field or laboratory condition.

Thiamethoxam is under scrutiny because of its acute and chronic effect on colony survival and progression of bees and other pollinators. Insect pollinators like *Bombus* (bumble bees) and *Apis mellifea* L. (honey bees) are highly sensitive to thiamethoxam (Whitehorn *et al.,* 2012). In an investigation, honey bee colonies were affected by exposure to crops which were seed primed by thiamethoxam, and corroborated presence of residues within pollen and nectar in concentration <1-7 μg kg^{-1} (Piling *et al.,* 2013). Decline in honey bee colonies has befallen globally which is attributed to the pesticide application (Wood *et al.,* 2020). Concerns in Europe have been raised over risks for bees that forage on seed treated crops and crops cultivated on those fields which have been previously treated with thiamethoxam (EFSA, 2018). In 2013, European Commission proposed a restriction on use of thiamethoxam for 2 years (EU, 2013). Foragers also

come in contact with these chemicals through guttation or dust released during application (Krupke *et al.,* 2012). In bees the high mortality caused by thiamethoxam is because of homing failure that leads to colony collapse disorder. Homing impairment by thiamethoxam intoxication was detected by radio-labeled foragers (Mason *et al.,* 2013). Honey bees play indispensable role in crop production and functioning of whole ecosystem, besides precious apiculture products. The degree of persistence in soil escorts thiamethoxam into the rotational crops, sown on previously treated lands and consequently bringing long term exposure to non target species (Xiao *et al.,* 2022).

Pesticide utilized in agriculture is not entirely consumed by plants, but in lieu leached to the soil and groundwater. Mode of action exerts toxic effect not only to specific organisms but also to non-target species. Thiamethoxam deposits in soil have adverse effect on soil health (Jyot *et al.,* 2015), microbial community, altering their activity, characteristics and compromising their overall performance (Gupta *et al.,* 2008). Decrease in the phosphate activity and drastic decline in nitrifying bacteria by thiamethoxam has been documented (Filimon *et al.,* 2015). Inevitable tenacity of thiamethoxam and its movement into aquatic environment induces threat to susceptible aquatic invertebrates upon which vertebrates rely for food. It is evidenced that neonicotinoids can elicit immune suppression in fishes (Mason *et al.,* 2013). A study conducted by Stark and Banks (2003) revealed acute-toxicity data on population of water flea exposed to thiamethoxam. Experiments, demonstrate that thiamethoxam causes histopathological, hematological and genotoxic alteration in fresh water fishes (Ghaffar *et al.,* 2019). Moreover, birds are also sensitive to thiamethoxam exposure, by directly consuming treated seeds and via aquatic food chain contamination (Mineau and Palmer, 2016). Acute toxicity was observed in South American eared dove by thiamethoxam exposure. The intoxication in birds caused by thiamethoxam eventuates in blackout and ultimately deaths (Addy-Orduna *et al.,* 2019). Thiamethoxam accumulation in edible crops has undoubtedly increased the complications in residue removal by surface rinsing. Human exposure to thiamethoxam is associated with serious afflictions viz., tumor, hepatotoxicity, neurotoxicity and cell apoptosis (Green *et al.,* 2005; Green *et al.,* 2005a;

Han *et al.,* 2018). Pesticide prevalence within ecosystem is emanating and is the most significant environmental issue, drawing the emphasis and focus of scientific community. Demand of pesticides and lack of efficient remediation approaches indicates that the predicament is exacerbating, thereby bringing this subject to forefront in the immediate future.

2.4 *Brassica juncea* – Paragon for Phytodegradation

Plants assimilate pesticides and translocate to edible parts, rendering a feasible route for these chemicals to infiltrate humans via food chain and food consumption (Kim *et al.,* 2017). Exposure to noxious pesticides induces umpteen aliments including cancer, neurotoxicity, hepatotoxicity, headache, asthma, nausea etc (Akoto *et al.,* 2013; Bhandari *et al.,* 2019). Therefore it is essential to eliminate accrued pesticide residues from edible crop tissues for food safety. *Brassica juncea* commonly known as Indian mustard is a vegetable and oil bearing crop and often attacked by various insects like aphids. The thrust perpetrated by insects drastically reduces crop yield (Razaq *et al.,* 2011). In India, mustard crop is cultivated over 5.76 million hectare area with production of 6.82 million tones and yield of 1184 kg/hectare in year 2016 (Directorate of Economics & Statistics, DAC & FW, 2016). The vulnerability to insect infestation deems low crop production. In India, nearly 50 insect species infest mustard crop. In order to deal with this menace, thiamethoxam is used indiscriminately (Bawaskar *et al.,* 2017).

Pesticide pollution being major environmental issue thereby its remediation is indispensable. Among various available methods plant assisted remediation is most propitious approach. Lately, phytoremediation and phytodegradation has gained eminence as eco-friendly manoeuvre for environmental clean-up (Kumar *et al.,* 2019). Brassicaceae family comprises 80 Brassica spp. such as *Brassica rapa*, *Brassica juncea*, *Brassica napus* etc with hyperaccumulation ability (Kanwar *et al.,* 2012; Rathore *et al.,* 2013; Sharma *et al.,* 2017; Cheng *et al.,* 2022). *Brassica juncea* is a potential candidate for pesticide degradation and detoxification as numerous studies have reported for its beneficial effects in neutralizing pesticide surge (Sharma *et al.,* 2018; Bakshi *et al.,* 2021;

Jan *et al.,* 2022). An investigation demonstrated that *Brassica juncea* notably degrades the herbicide by gradually decreasing its concentration in the soil sample with course of time (Khan and Gaikwad 2013). In another study, *Brassica juncea* along with microbe (*Aspergillus sydowii*) effectively degraded the insecticide indicating that plant-microbe association accelerated the degradation of xenobiotic compound (Zhang *et al.,* 2020). Withal, hairy root culture of *Brassica juncea* remarkably uptakes and degrades potent insecticide, suggesting endogenous root enzymes play a crucial role in break-down of insecticide (Suresh *et al.,* 2005). Exploiting *Brassica juncea* for pesticide contamination will act as a feasible approach to address such catastrophe.

2.5 Amelioratives for Pesticide Stress Management

2.5.1 Melatonin

Melatonin was identified in bovine pineal gland during late 1950s (Lerner *et al.*, 1985). It acquired its name due to accretion of melanin, a pigment granule within chromatophores of amphibian skin cells also serotonin being an intermediate compound during its conversion from tryptophan (Wurtman *et al.,* 1963) (figure2.1). This is a pleiotropic molecule and is evolutionarily conserved, exists ubiquitously in fauna and flora (Hardeland *et al.,* 2011). The simultaneous identification of melatonin in plants was explored (Dubbels *et al.,* 1995) howbeit, the term phytomelatonin was coined in 2004 (Blask *et al.,* 2004). Melatonin is distributed in various plant parts and is accountable for invigorating multifarious physiological process. The antioxidant potential of phytomelatonin may elucidate its few physiological functions viz, to reinforce plants exposed to abiotic stresses like heat, drought, pesticides, UV radiations, cold and salinity, rendering melatonin as remarkable candidate to countermeasure field crops (Bose and Howlander, 2020). The enzymes involved in melatonin biosynthesis are up-regulated by stress stimuli. External factors certainly affect the melatonin level in plant tissues. Plants raised indoor under controlled conditions possess less melatonin content comparatively to field grown plants under more variable conditions. Plants cultivated in sunlight have 3 and 2.5 times more melatonin in roots and leaves respectively than those grown under in

vitro conditions (Yu *et al.,* 2018). Apart from endogenous synthesis, plants can absorb exogenous melatonin and accumulate it within tissues (Zhang *et al.,* 2021). The impact of exogenously supplied melatonin varies from being restorative to ineffective or even toxic. The concentration is the actual cause of this marginal difference.

Stressors likely produce reactive nitrogen species (NOS) and reactive oxygen species (ROS), the aftermath of this is oxidative stress in plants. As long as oxidative stress is not addressed properly, it results in cell damage and death. To thrive in hostile conditions, plants have in-built mechanisms to combat the oxidative damage. These mechanisms include antioxidative defence system and higher production of antioxidants. Melatonin acts as potent free radical scavenger countering hazardous reactive molecules including reactive oxygen species (ROS) and reactive nitrogen species (RNS). Additionally, melatonin receptors perform independently and their bioactive compounds mediate the replacement of ROS and RNS with melatonin (Arnao and Hernandez Ruiz, 2019). Melatonin also has a significant role in non-receptor mediated actions for instance, ROS scavenging, enhancing antioxidant potential, and preventing tissues from oxidative stress (Lee and Back, 2020). Melatonin controls ROS levels in two peculiar ways: (A) chemical interaction between melatonin and ROS which causes their inactivation (Arnao and Hernandez-Ruiz, 2019) (b) melatonin mediated activation of redox enzymes viz., SOD, POD APX, CAT and GPX which leads to ROS detoxification (Khan *et al.,* 2020). Melatonin controls hydrogen peroxide burst in plants, probably by scavenging superfluous ROS and improves antioxidative enzyme activity and capacity of ascorbate glutathione cycle (Liang *et al.,* 2018). It is assessed that, through cascade reaction, one melatonin molecule scavenges 10 ROS species, which is in contrast with typical antioxidants, as they normally detoxify one free radical per molecule (Balaji and Varadarajan, 2021). Seed priming with melatonin assuages lipid peroxidation and electrolyte leakage caused by malondialdehyde content during plant stress response. Lately the role of melatonin in reducing oxidative stress has been evaluated in context to phyto-toxic agents. In *Arabidopsis,* subjected with paraquat (herbicide) the melatonin up-regulated catalase and ascorbate peroxidase activity further controlling the production of

hydrogen peroxide and superoxide (Wang *et al.*, 2015). In another study exogenous melatonin effectively eliminated ROS, enhanced antioxidant defense system and degraded the insecticide which was applied in *Cucumis sativa* (Liu *et al.*, 2021). Promulgating the advancement of melatonin in recent years, the action of melatonin in plants is explored extensively and thoroughly. Ergo, it is concluded that fundamental role of melatonin in living forms is to boost antioxidant system and represent as a first line of defense against stress conditions (Arnao and Hernandez-Ruiz, 2019).

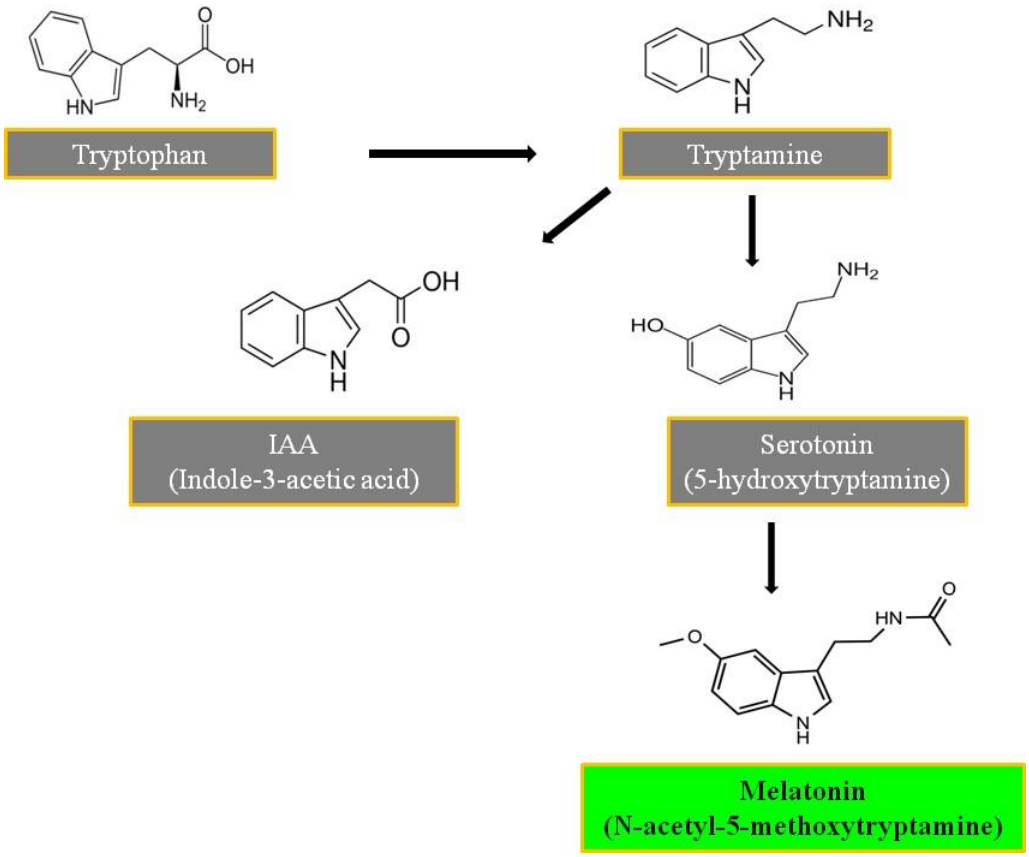


Figure 2.1: Biosynthetic pathway of melatonin.

2.5.2 PGPR

In pursuance to, mitigate the effect of pesticides on health and environment, for polluted site remediation, for management of pesticide residues, various approaches have been

established including physical treatment, chemical treatment, etc (Figure 2.2). These orthodox physicochemical techniques are generally uneconomical and remediation process often generates more toxic metabolites from parent compounds (Singh *et al.,* 2009). Another pesticide treatment technique with significant global reverberation is bioremediation or bacterial assisted phytoremediation. The approach relies on the potential of microbes to convert toxicants into non-toxic forms in non-hazardous and cost-effective way (Zhan *et al.,* 2021). Bacterial assisted phytoremediation offers effective and economical option for destruction and degradation of pesticides (Table 2). Microorganisms have the ability to biotransform pesticides as it has been observed that soil biota can swiftly degrade various pesticides. Pesticides acts as a sole source of carbon for certain soil microbes, constructing a way for pesticide treatment (Qiu *et al.,* 2007). Persistence and development of plant in unfavorable conditions can be enhanced by PGPR. The PGPRs plays imperative role in overall development of plant by providing tolerance to hostile conditions, shaping rhizosphere, enhancing nutrient absorption and biomass (Kroll *et al.,* 2017). In addition, microorganisms promote plant growth and development either directly or indirectly during stress conditions. Biochemical and molecular means are used by microorganisms to stimulate growth and development of plants. Treatment of PGPR, promotes plant development by modulating hormonal and nutritional stability, synthesizing phytohormones and providing resistance to toxins and pathogens (Spence & Bais, 2015). Multifarious bacterial species viz., *Aspergillus, Bacillus, Enterobacter* and *Pseudomonas* have been chronicled for degrading contaminants. Now-a-days, microorganisms have been effectively inoculated in plants for pesticide remediation (Nurzhanova *et al.,* 2021). Microbial stimulated metabolism of thiamethoxam has been demonstrated by few species namely, *Ensifer adhaerens* (Zhou *et al.,* 2013), *Bacillus, amyloliquefaciens, Bacillus Pumilus, Bacillus subtilis* (Myresiotis *et al.,* 2012), *Pesudomonas* (Pandey *et al.,* 2009), *Acinetobacter and Sphingomonas* (Wang *et al.,* 2011). Transformation of thiamethoxam by catabolic pathways yields nitrosoguanidine, desnitro and urea metabolites (Pandey *et al.,* 2009). However, meager attempts regarding degradation of thiamethoxam by plant-bacterial consortium has been

documented. Application of PGPRs in pesticide polluted soil is most promising approach for pesticide remediation (Dar *et al.*, 2019). Different bacterial species have been identified viz., *Bacillus* (El-Helow *et al.*, 2013), *Burkholderia* (Baksi *et al.*, 2021), *Pesudomonas* (Rana *et al.*, 2015) and *Klebsiella* (Ghanem *et al.*, 2007) possessing pesticide degrading ability. The ameliorative effect of various PGPRs on plant growth via up-regulating defence mechanism during stress condition has also been documented (Khanna *et al.*, 2019). PGPRs assist in plant growth by decreasing oxidative stress, ROS scavenging and increasing antioxidative enzyme activities under stress conditions (Islam *et al.*, 2014). Prominently, PGPRs improve plant vigor by strengthening defence mechanism and mitigates pesticide stress in plants (Pozo *et al.*, 2015).

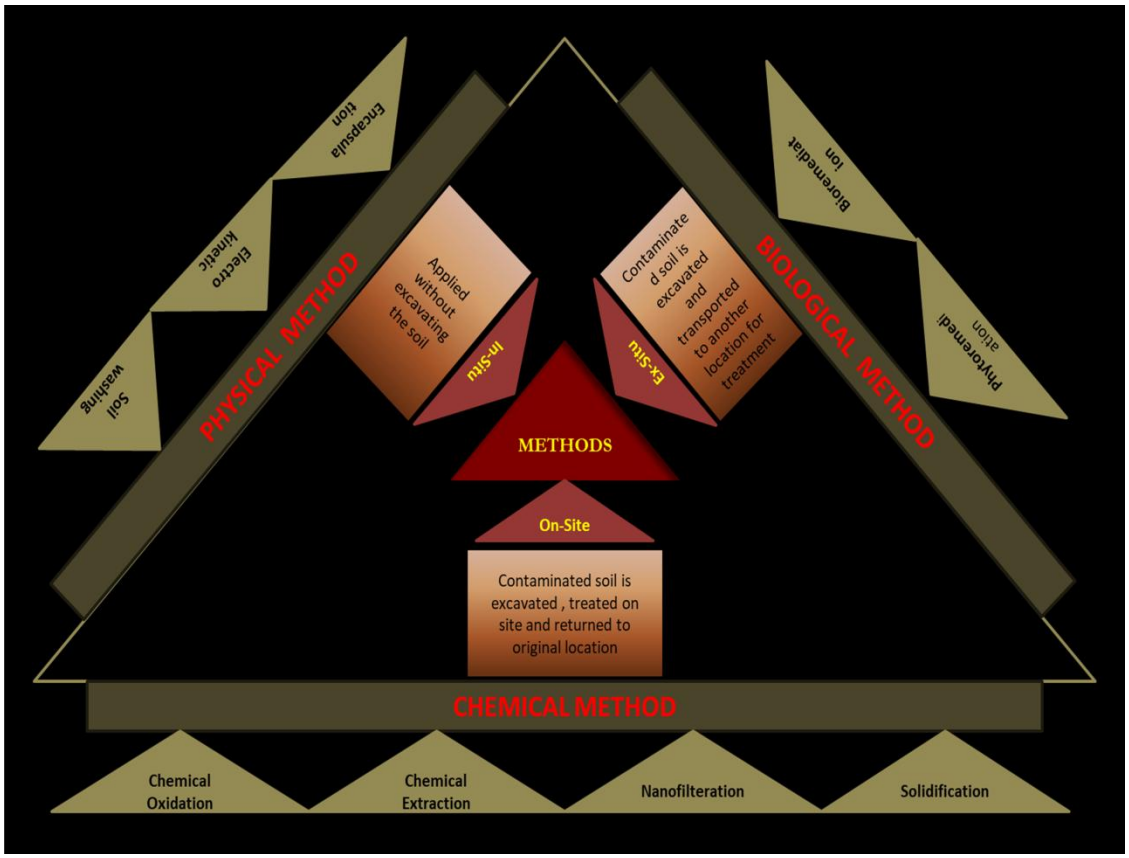


Figure 2.2: Illustration of various remediation techniques.

Table 2.2: List of microbial assisted phytoremediation of pesticides

Microbial inoculants	Plants	Pesticides	References
Pseudomonas putida	*Pisum sativum*	2,4-dichlorophenoxyacetic acid	Germaine *et al.*, 2006
Pseudomonas nitroreducens	*Lolium multiflorum*	Chlorpyrifos	Korade and Fulekar, 2009
Staphylococcus cohnni	*Withania somnifera*	Lindane	Abhilash *et al.*, 2011
Pseudomonas aeruginosa	*Vigna radiata*	Tebuconazole	Ahemad and Khan, 2012
Rhodococcus erythropoli	*Cytisus striatus*	Hexachlorocyclohexane	Becerra-Castro *et al.*, 2013
Bacillus megaterium	*Nicotiana tabacum*	Quinclorac	Liu *et al.*, 2014
Streptomyces	*Saccharum officinarum*	Atrazine	Mesquini *et al.*, 2015
Mezorhizobium	*Lolium multifolrum*	Chlorpyrifos	Jabeen *et al.*, 2016
Sphingomonas	*Allium tuberosum*	Chlorpyrifos	Feng *et al.*, 2017
Pseudomonas fluorescens	*Glycine max*	Fenamiphos	Romeh and Hendawi, 2017
Pseudomonas	*Lolium perenne* and *Festuca arundinacea*	DDT	Wang *et al.*, 2017
Bradyrhizobium japonicum	*Vigna radiata*	Hexachlorocyclohexane	Shahid and Khan, 2018
Enterobacter	*Brassica chinensis*	Thiamethoxam	Wang *et al.*, 2020

Ochrobactrum haematophilum and *Rhizobium*	*Lotus corniculatus*	Glyphosate	Massot *et al.*, 2021
Bacillus vallismortis and *Bacillus aryabhattai*	*Cucurbita pepo* and *Xanthium strumarium*	DDT	Nurzhanova *et al.*, 2021
Enterobacter cloacae	*Oryza sativa*	Thiamethoxam	Zhan *et al.*, 2021

2.5.3 Detoxification and Attenuation of Oxidative stress and Modulation of Gene Expression

Existing as static life form, plants cannot circumvent stresses by relocation rather they acquire an internal mechanism to assuage stressful conditions by various physiological and biochemical processes. Absorption of pesticide occurs via roots and leaves, further metabolized by intrinsic detoxification system or amassed in different parts of plant. The repercussions of pesticide use are production of ROS and adversely affecting growth and development of plant. Albeit, the internal mechanism – antioxidative defense system of plant is triggered to combat pesticide stress (Sharma et al. 2018). Plants have inbuilt capability to break, transform and store pesticide, augmenting their intrinsic detoxification system would assist to guard plants as well as the ecosystem from pesticidal damage (Wang et al. 2017). Plant growth regulators play a fundamental role in perceiving and signaling varied stressful conditions including pesticide toxicity thereby eliciting various responses viz, modulate gene expression, activation of various stress signaling pathways in response to pesticide toxicity (Ali et al. 2020) (Table 2.3).

Table 2.3: Role of PGRs in eliciting various responses in plant subjected to stress conditions.

PGR	Pesticide	Negative impact of pesticide	Mechanism of alleviating pesticide induced toxicity	Reference
Castasterone	Imidacloprid	• Generating oxidative stress • Decreased plant growth • Decline in chlorophyll content • Down- regulation of key genes	• Enhanced content of chlorophyll, carotenoids, anthocyanins, xanthophylls • Activation of antioxidative defense system • Biosynthesis of organic acids and phenolics	Sharma et al. (2019)
Cytokinin	Glyphosate	• Induced oxidative stress • Decreased growth and pigment content	• Protective action by rendering hardiness in antioxidant defense system • Increased levels of glutathione	Sergiev et al. (2006)
Epibrassinolide	Chlorothalonil	• Negative effect on soluble protein • Raise malondialdehyde content	• Enhance osmoregulation • Remarkably facilitate manifestation of antioxidant genes.	Wang et al. (2017)
Epibrassinolide	Chlorpyrifos	• Lowers photosynthesis and quantum yield of photosystem II	• Stimulate antioxidant defense system • Improves pesticide degradation by switching on vital genes like P450	Xia et al. (2009)
Epibrassinolide	Imidachloprid	• Hampers plant growth • Lowers chlorophyll content • Reduction in photosynthesis • Lowers concentration of vigorous	• Reduction in IMI toxicity • Enhance plant growth • Improves pigment storage • Improves photosynthesis and stomatal modulation • Promotes production	Sharma et al. (2017)

		biomolecule	of various phytochemicals	
Epibrassinolide	Phenanthrene and Pyrene	• Prevent photosynthesis • Formation of H_2O_2, OH^-, $O2^-$ • Prevents seed germination • Prevents pigment storage • Deformation of leaves	• Ameliorate seed germination • Improves morphological features • Improves chlorophyll emission • Augment plant biomass • Enhance pesticide detoxification network	(Ahammed et al. 2012)
Epibrassinolide	Polychlorinated biphenyl	• Reduction in biomass • Lowers chlorophyll content • Obstruct photosynthesis • Inhibits stomatal conductance	• Enhances plant growth and development • Mitigates photo inhibition • Regulates oxidative stress • Lowers lipid peroxidation	Ahammed et al. 2013)
Epibrassinolide	Terbutryn	• Decreases carbon dioxide assimilation • Reduces quantum yield of photosystem II. • Lowers non photochemical quenching	• Increases plant biomass • Enhances growth and development of plants • Improves florescence and carbon dioxide assimilation	Pinol and Simon (2009)
Jasmonic acid	Imidacloprid	• Production of ROS species leading oxidative burst • Reduction in plant growth • Reduced photosynthetic efficiency	• Modulates antioxidant defense system • Reduction in oxidative stress • Regulates biochemical and physiological processes	Sharma et al. (2018)
Melatonin	Paraquat	• Mitochondrial dysfunctioning • ROS generation	• Activation of autophagy • Up-regulation of genes responsible for ROS scavenging	Wang et al., 2015

Melatonin	Inidacloprid	• Compromised photosystem II • ROS generation • Oxidative stress	• Enhanced enzymatic activities • ROS scavenging • Increased photosynthetic capacity	Liu et al., 2021
Salicylic acid	Glyphosate	• Obstructing EPSPS enzyme, leads to less carbon supply to important pathways • Lowers the activity of nitrate reductase • Enhanced MDA content, proline and ROS species • Damages plant physiology	• Improves seedling height, growth and biomass • Scavenges ROS species • Increases carotenoids, chlorophyll and pigment content • Boost the photosynthetic rate • Increases protein content and synthesizes new proteins	Singh et al. (2017)
Salicylic acid	Glyphosate	• Growth inhibition • Accumulation of H_20_2, O^-_2.	• Improved growth • Lowered amount of H_20_2	Spormann et al. (2019)
Salicylic acid	Napropamide	• Reduction in crop quality and yield • Substantial formation of $O^{\cdot-}_2$, H_20_2 and peroxides • Oxidative damage in plasma membrane	• Improves plant tolerance • Inhibits the accumulation of ROS species • Protects cell membrane against lipid peroxidation • Restores oxidative damage	Cui et al. (2010)
Salicylic acid	Paraquat	• Induce oxidative damage by producing oxygen radicals • Impedes photosynthesis, production cation radicals and inhibiting the production of NADPH	• Improved antioxidant capacity • Inhibits pesticide penetration into cell • Synthesizes proteins responsible for plant stress management • Enhances stress tolerance by triggering detoxifying enzymes	Ananieva et al. (2004)

		• Damages membrane stability		
Salicylic acid	Thiram	• Oxidative stress • Lowered total chlorophyll and carotenoids levels	• Decreade in $H_20_{2,}$ and MDA levels • Increased photosynthetic pigments • Regulation of antioxidant enzymes	Yuzbasioglu and Dalyan (2019)

Abiotic stress elevates ROS and MDA levels, which directly affects cell membrane, causing increased electrolyte leakage in plants. Melatonin facilitates stress tolerance by regulating redox homeostasis in plants, which is elucidated as a survival stratagem to eradicate oxidative stress in cell organelles (Bai et al., 2020). Evidences show that besides ROS scavenging melatonin also improves antioxidant levels viz, glutathione. For example, hydrogen peroxide regulates melatonin mediated GSH redox status and resulting in oxidative stress tolerance in *Cucumis sativus* (Li et al., 2016). A dynamic synchronization was discerned between melatonin and nitric oxide in regulating glutathione levels and glutathione reductase activity in *Helianthus annuus* (Kaur and Bhatla, 2016). Data manifest that melatonin interacts with wide array of signaling molecules including nitric oxide (NO) and it is well accepted that nitric oxide produces S-nitrosylation and NO_{2-} which are regarded as prominent antioxidant proteins. This is a vital action for maintaining antioxidant potential of AsA/GSH cycle during nitro oxidative conditions (Pardo-Hernandez et al., 2020). Also, it has been discerned that ROS has an ability to up-regulate melatonin biosynthesis pathway gene which leads to upsurge of endogenous melatonin levels in plants (Zhan et al., 2019).

Studies acclaim that phytohormones protect plants from oxidative damage caused by pesticides and simultaneously reduces pesticide residues in plants (Sharma et al., 2015). It is documented that brassinosteroids when applied exogenously enhances the expression of genes viz, *P450, CAT, GST, POX, CXE, NADH,* SOD and *GR* leading to pesticide detoxification (Sharma et al., 2019). Since melatonin is a ROS scavenger and assuages

oxidative damage induced by stressors in plants, concurrently, melatonin improves antioxidant capacity of organelles and regulates the expression of stress responsive genes such as C-repeat binding factors (CBFs) to increase plant resistance when subjected to stressful conditions (Huang et al., 2020). Mechanistic studies divulge that aforementioned response occurs at gene expression level. A study confirms, during pesticide stress, application of exogenous melatonin up-regulates transcript levels encoding *GPX*, *GST1*, *GST2* and *GST3* which indicates melatonin mediated degradation of pesticide (Liu et al., 2021). Another study corroborates that in *Brassica juncea* the supplementation of phytohormone considerably increased the fold expression of *CXE*, *NADH*, *P450*, *POD* and *GST1* genes under pesticide stress suggesting reduction of pesticide residues by detoxification gene transcripts (Sharma *et al.*, 2019).

CHAPTER 3

3. Research Gap

Pesticide prevalence within ecosystem is emanating and is the most significant environmental issue, drawing the emphasis and focus of scientific community. Demand of pesticides and lack of efficient remediation approaches indicates that the catastrophe is exacerbating, thereby bringing this subject to forefront in the immediate future.

Predicament of pesticide contamination is progressively becoming ubiquitous. In modern era, the farming has expanded significantly by conferring crop protection via, battling out wide-array of malignant pests in order to meet the food demand for growing population. Crop cultivation has achieved extensive proliferation by prodigious utilization of pesticides. Despite their usefulness, pesticides can pose potential risk to food safety and ecosystem (Jan *et al.,* 2020). Concern about the impact of repeated pesticide use has prompted research into the environmental fate of these agents, which can emigrate from treated fields to air, far-lands and water bodies (Bakshi *et al.,* 2021). Howbeit, evidences of potential risks to non target species including humans (Han *et al.,* 2018) and pollinating insects (Whitehorn *et al.,* 2012) has become quagmire across the globe. The availability of thiamethoxam for agricultural purposes, opened a new era for pest control. Thiamethoxam is preferred for its broad-spectrum insecticidal activity (Mir *et al.,* 2013). The degree of persistence in soil escorts thiamethoxam into the rotational crops, sown on previously treated lands and consequently bringing long term exposure to non target species (Xiao *et al.,* 2022). Sparse data is available pertinent to thiamethoxam degradation under field or laboratory conditions. Lately, phytoremediation and phytodegradation has gained eminence as eco-friendly manoeuvre for environmental clean-up (Kumar *et al.,* 2019). *Brassica juncea* is a potential candidate for pesticide degradation and detoxification, as umpteen studies have been reported for its beneficial role in neutralizing pesticide surge. The vast bioactive compounds evoke strong antioxidant response in plants subjected to pesticide stress (Jan *et al.,* 2022). Past studies divulge that plant growth regulators (PGR) and plant growth promoting rhizobacteria

(PGPR) enhances the degradation of pesticides by stimulating enzymatic detoxification system. Using melatonin and *Pseudomonas putida* as an adjunct in phytodegradation via seed priming and exogenous application can activate plant defense system against pesticide stress by modulating gene expression, decreasing oxidative stress in *Brassica juncea* seedlings. We prognosticate, a novel in-vitro approach - plant, PGR and PGPR consortium will likewise intensify the degradation process.

CHAPTER 4

4.1 Objectives

The potential harmful effects of thiamethoxam and its toxic effects on plants and other non-target species need to be understood and elucidated. The interplay between melatonin and *Pseudomonas putida* in phytodegradation and underlying mechanism of this relationship still remains unknown. On this account, contemporary study aims to determine the role of exogenously supplemented melatonin and PGPR in *Brassica juncea* seedlings under the influence of thiamethoxam.

1. To study role of PGPR in the degradation of thiamethoxam.
2. Investigating the effect of melatonin (PGR) in degradation of thiamethoxam by *Brassica juncea.*
3. Growth and biochemical analysis (enzymatic and non-enzymatic assay) of grown seedlings.
4. Molecular analysis of seedlings by qRT-PCR.

The present research endeavor in its modest will help to comprehend the role of melatonin and *Pseudomonas putida* in phytodegradation of thiamethoxam.

CHAPTER 5

MATERIALS & METHODS

5.1 Study Material

5.1.1 Seeds

Certified seeds of *Brassica juncea* (Variety PBR 357) were acquired from Punjab Agricultural University (PAU), Ludhiana, Punjab, India.

Figure 5.1: Field of *Brassica juncea* at Punjab Agriculture University, Ludhiana, India.

5.1.2 Thiamethoxam (TMX)

TMX was purchased from Sigma-Aldrich, St. Louis, USA for standard preparation pertinent to TMX residue analysis. For experimental purpose, TMX 25% WG was purchased from ADAMA India Private Limited, Hyderabad, Telangana, India. TMX concentrations for experiments were selected on the basis of IC_{50} (inhibitory

concentration) values. In total three concentrations were selected for experimental work: one lower to IC_{50} (0.2 mM), IC_{50} (0.4 mM) and one higher to IC_{50} (0.6 mM).

5.1.3 Melatonin

Melatonin was purchased from Himedia laboratories Pvt. Ltd, Mumbai, India. A stock solution of 1 mM was prepared by dissolving melatonin in analytical grade methanol. Different concentrations of melatonin (50 µM and 100 µM) were prepared by serial dilution of stock. For current study, the concentration of melatonin was selected on the basis of effective concentration. Accordingly, 50 µM concentration was chosen for the experimental work.

5.1.4 PGPR

Plant growth promoting rhizobacteria (PGPR) namely; *Pseudomonas putida* (MTCC 3315) was procured from CSIR-IMTECH, Mohali, Punjab, India. The lyophilized strain was cultured in sterile 50 ml nutrient broth media (NB media; $13gL^{-1}$). The culture flask was kept at 28 °C (24 to 48 h) in BOD incubator (Calton Deluxe Automatic, New Delhi, India) for proliferation. After optimum growth, culture was subjected to sub-culturing to maintain it for future use. For experiments, 1 mL of grown culture was added in 50 mL of NB media and allowed to grow at 28 °C (24 to 48 h) in BOD incubator. Further, it was centrifuged (Plasto Crafts, Rota 4R-V/Fm) at 10000 rpm, 4 °C for 20 minutes to collect pellet. Obtained pellet was rinsed with double distilled water and therefore resuspended to acquire 10^9 cells/ml Jan et al., (2022).

Figure 5.2: Pellet extraction and streaked plate.

5.2 Raising of Plants

5.2.1 Combinations of treatments

Combinations of TMX, Melatonin and PGPR used for experiments are referred in table 5.1.

Table 5.1: Combinations of TMX and Melatonin selected for experiments.

S. No	TMX (mM)	Melatonin (µM)	PGPR (10^9 cells/ml)
1.	0	0	0
2.	0	50	0
3.	0	0	10^9
4.	0.2	0	0
5.	0.4	0	0
6.	0.6	0	0
7.	0.2	50	0
8.	0.4	50	0
9.	0.6	50	0
10.	0.2	0	10^9
11.	0.4	0	10^9
12.	0.6	0	10^9
13.	0.2	50	10^9
14.	0.4	50	10^9
15.	0.6	50	10^9

5.2.2 Seed sterilization

Uniform sized viable seeds were surface sterilized by using 0.01% mercuric chloride ($HgCl_2$) for 1-2 minutes followed by thorough rinsing with double distilled water.

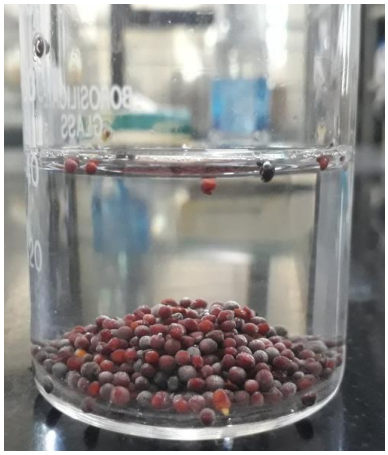


Figure 5.3: Surface sterilization of seeds in 0.01% mercuric chloride solution.

5.2.3 Cultivation of seedlings *in vitro*

Surface sterilized seeds were immersed in freshly prepared melatonin solution (50 µM) for 7 hours. The melatonin dosed seeds were swilled with double distilled water, blotted dried and placed in dark at room temperature, until returning to their initial weight (over-night). Autoclaved petri-plates were layered with Whatman (Grade 1) filter paper and added with thiamethoxam solution (0.2 mM, 0.4 mM and 0.6 mM). Subsequently, primed seeds were sown in thiamethoxam supplemented petri-plates and simultaneously microbial suspension (10^9 cells/ml) was inoculated into petri-plates containing seeds. The petri-plates were kept in seed germinator (Caltan, NSW 191-192) under controlled condition (light intensity – 175 µmol m^{-2} s^{-1}; temperature – 25 ± 0.5 °C, photo-period 16 hours). After 10 days sowing, the seedlings were harvested for further analysis Jan et al., (2022).

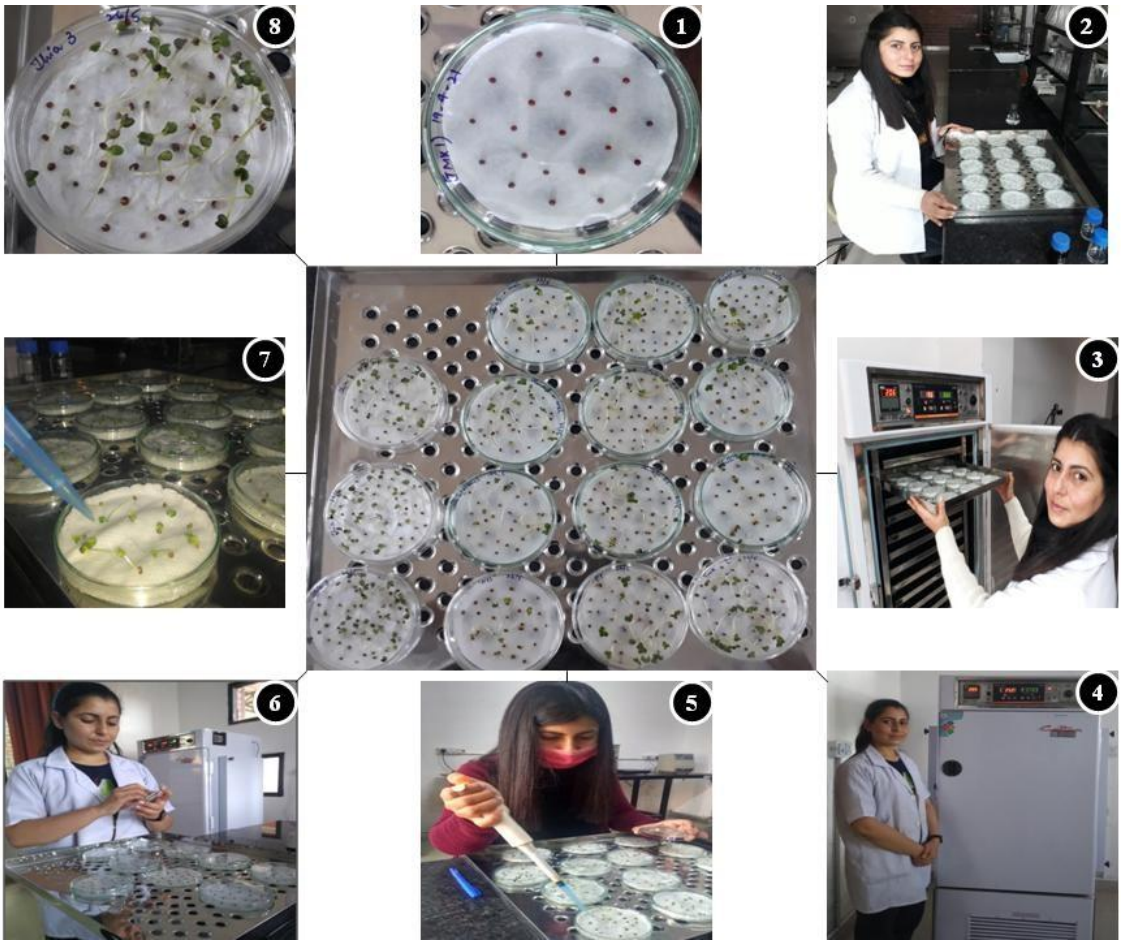


Figure 5.4: Pictorial form of *in-vitro* seed cultivation: (1-4) Seed sowing (5-7) Nurturing seedling (8) Final growth of seedlings.

5.3 Melatonin and PGPR Assisted Degradation of Thiamethoxam in *Brassica juncea*

5.3.1 Sample preparation for TMX residue analysis

For TMX residue analysis, AOAC official method 2007.01 (2007) was followed using fresh plant material. Extraction process encompasses: pulverizing 1 g fresh plant material in 1 ml acetonitrile (containing 1% acetic acid). To this extract, 0.5 g anhydrous magnesium sulfate ($MgSO_4$) and sodium acetate ($C_2H_3NaO_2$) in ratio of 4:1 w/w was added and thoroughly mixed, proceeded with 1 minute of centrifugation at 10000 rpm. Thereafter, dispersive solid-phase extraction was carried out. To 1 mL the upper layer,

150 mg of anhydrous $MgSO_4$ and primary amine sorbent in ratio of 3:1 w/w was added and shaken vigorously. Further centrifugation at 10,000 rpm for 2 minutes was done to obtain the supernatant.

5.3.2 Analysis by Gas Chromatography – Mass Spectrometry (GC-MS)

To determine pesticide residues, the obtained supernatant (8 µL) was injected into auto-sampler vials of Shimadzu GC-MS - TQ8040 NX.

Figure 5.5: GC-MS (Shimadzu – TQ8040 NX) setup.

Instrument conditions **(GC):** Carrier gas used was helium. The initial column oven temperature was kept at 40 °C for 2 minutes. Further the temperature was raised to 250 °C at the rate of 7 °C/min and with hold time of 5 minutes. Finally sample injection was carried out at 250 °C. Subsequently, the instrument was synchronized to: injection mode – splitless, sampling time – 1 minute, flow control mode – linear velocity (47.0 cm/sec), pressure – 95.4 kPa, total flow – 13.2 mL/min, column flow – 1.70 mL/min, purge flow – 3.0 mL/min, analytical column – DB 5ms Sharma et al., (2019).

Instrument conditions **(MS):** Ion source temperature – 250 °C, interface temperature – 260 °C, solvent cut time – 2 minutes and detector gain mode – relative.

5.4 Morphological Analysis of *Brassica juncea* Seedlings

The incremental measurements of seedlings were attained by centimeter scale. Fresh weight and dry weight were recorded using shimadzu analytical balance (AUX-120). For dry weight analysis the samples were oven dried (Narang Scientific Works, India, NSW-143) at 70 °C for 48 hours. 10 days old plants of *Brassica juncea* were assessed for aforementioned growth parameters.

Figure 5.6: Evaluating length and fresh weight of seedlings.

5.5 Photosynthetic Pigment Analysis

The content of photosynthetic pigments, viz. total chlorophyll, chlorophyll-a, chlorophyll-b, carotenoid, anthocyanin and total flavonoid were estimated using UV-Visible PC Based Double Beam Spectrophotometer (Shimadzu UV-1800).

5.5.1 Chlorophyll content

Chlorophyll content was determined according to Arnon (1949).

Reagents

Acetone – 80%

Procedure

0.2 g of fresh plant sample was homogenized in chilled pestle-motor using 4 mL acetone (80%). The macerated material was subjected to centrifugation at 12,000 rpm for 20 minutes at 4 °C. The supernatant obtained was further used for quantification of carotenoid content and chlorophyll content. The absorbance was taken at 645 nm and 663 nm.

Calculations

Below stated equations were used to calculate the content of total chlorophyll, chlorophyll-a, chlorophyll-b and were expressed as mg/g FW.

$$\textit{Total Chlorophyll} = (20.2 \times \text{Abs}_{645}) + (8.02 \times \text{Abs}_{663}) \times \left(\frac{V}{1000 \times W}\right)$$

$$\textit{Chlorophyll-a} = (12.7 \times \text{Abs}_{663}) - (2.69 \times \text{Abs}_{645}) \times \left(\frac{V}{1000 \times W}\right)$$

$$\textit{Chlorophyll-b} = (22.9 \times \text{Abs}_{645}) - (4.68 \times \text{Abs}_{663}) \times \left(\frac{V}{1000 \times W}\right)$$

Where, V = extract volume (mL)

W = plant sample weight (g)

5.5.2 Carotenoid content

Carotenoid content were determined according to Maclachlan and Zalik (1963).

Reagents

Acetone – 80%

Procedure

0.2 g of fresh plant sample was homogenized with 80% acetone (4 mL) using pre-chilled pestle-motor. The extract was subjected to centrifugation at 12,000 rpm for 20 minutes at 4 °C. The obtained supernatant was used for carotenoid content estimation and the absorbance was taken at 480 and 510 nm respectively.

Calculations

Following is the equation for calculation of carotenoid content and expressed as mg/g FW.

$$\textit{Carotenoid content} = (7.6 \times \text{Abs}_{480}) - (1.49 \times \text{Abs}_{510}) \times \left(\frac{V}{1000 \times W}\right)$$

Where, V = extract volume (mL)

W = plant sample weight (g)

5.5.3 Anthocyanin content

Anthocyanin content was evaluated according to Mancinelli (1984).

Reagents

Absolute methanol

Hydrochloric acid (HCL)

Distilled water

Procedure

0.35 g of fresh plant tissue was pulverized in chilled pestle-motor using 3 ml extraction mixture (2.37 mL methanol, 0.6 mL distilled water, 0.03 mL HCl). The homogenized material was centrifuged at 12,000 rpm for 20 minutes at 4 °C. Subsequently, absorbance of supernatant was taken at 530 nm and 657 nm to reckon the anthocyanin content.

Figure 5.7: Obtained supernatant to reckon anthocyanin content.

Calculations

Below quoted equation was used to calculate the anthocyanin content and expressed as µg/g FW.

$$A = (Abs_{530}) - (0.25 \times Abs_{675})$$

$$\textit{Anthocyanin content} = [A \times \text{Mol. Wt. (std.)} \times DF \times 1000] / \varepsilon$$

Here standard used = cyanidin-3-glucoside

Where Mol. Wt. = 449.2

DF = dilution factor

ε = molar absorptivity (cyaniding-3-glucoside – 26900)

5.5.4 Total flavonoid content

Total flavonoid content is assessed according to Kim *et al*. (1999).

Reagents

Sodium hydroxide (NaOH) – 4%

Aluminium chloride ($AlCl_3$) – 10%

Distilled water

Absolute alcohol

Sodium nitrite ($NaNO_2$) – 5%

Rutin (standard) = 1 mg mL^{-1}

Plant extract preparation

Fresh plant sample (0.35 g) was crushed in 3 mL of absolute methanol using chilled pestle-motor. The extract was subjected to centrifugation at 12,000 rpm for 20 minutes at 4 °C. The acquired supernatant was further used for reckoning of total flavonoid content.

Procedure

To 1 mL of supernatant, 4 mL of distilled water, 0.3 mL $NaNO_2$ and 0.3 mL $AlCl_3$ were added and allowed to incubate for 5 minutes. After occurrence of reaction, pink colour appeared followed by the addition of 2 mL NaOH. Further, mixture was diluted with 2.4 mL of distilled water and absorbance was recorded at 510 nm.

Figure 5.8: Pink colour formation during flavonoid content determination.

Calculations

A graph between absorbance and concentration of rutin (std.) was plotted. The content of total flavonoid was determined by equation generated from the graph and is expressed as mg g^{-1} FW.

5.6 Analysis of Osmolytes

5.6.1 Trehalose content

Trehalose content was measured as per Trevelyn and Harrison (1956).

Reagents

Anthrone reagent – 0.1 g/50 mL sulphuric acid (95%)

Ethanol – 80%

Trichloroacetic acid (TCA) – 2.5 M

Sulphuric acid (H_2SO_4) – 95%

D-glucose (Standard)

Procedure

10 mg oven dried plant material was crushed in ethanol (80%) and centrifuged at 5,000 rpm for 15 minutes at 4 °C. To 100 µl of supernatant, anthrone reagent (4 mL) and TCA (2 mL) was added. A yellow colour complex was developed whose absorbance was taken at 620 nm.

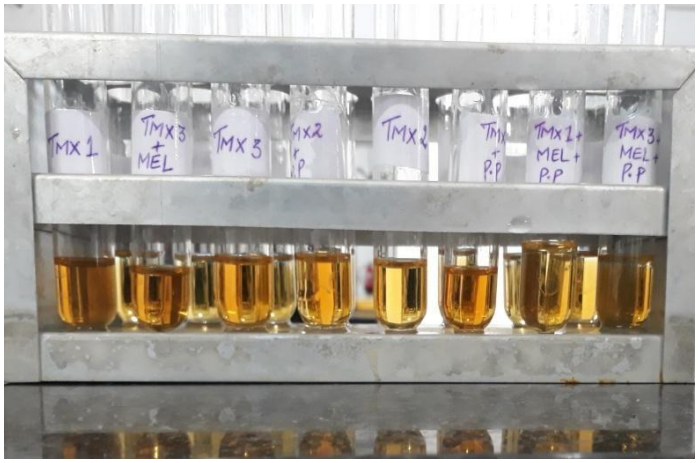


Figure 5.9: Formation of yellow coloured product.

Calculations

Graph between absorbance and D-glucose concentration was plotted and the standard equation acquired was used to estimate the trehalose content and expressed in mg g^{-1} dry weight.

5.6.2 Glycine-Betaine content

Glycine-Betaine content was quantified according to Grieve and Grattan (1983).

Reagent

Hydrochloric acid (HCl) – 2N and 1N

Toulene – 0.05%

Potassium tri-iodide (PI_3)

1,2-dichloromethane

Betaine hydrochloride (standard)

Procedure

10 mg oven dried plant material was crushed in 5 mL mixture of toluene (0.05%) and distilled water. The amalgam was incubated (24 hours) and filtered. To 0.5 mL of plant extract, 1 mL of 2N hydrochloric acid and 0.1 mL potassium tri-iodide was added. Thorough mixing was done and subjected to ice cold conditions for 90 minutes. Further, chilled distilled water (2 mL) and 1,2-dichloromethane (10 mL) were added to it. Rigorous mixing of reaction tubes was done till two separate layers were formed. Upper layer was discarded and absorbance of pink coloured lower layer was recorded at 365 nm.

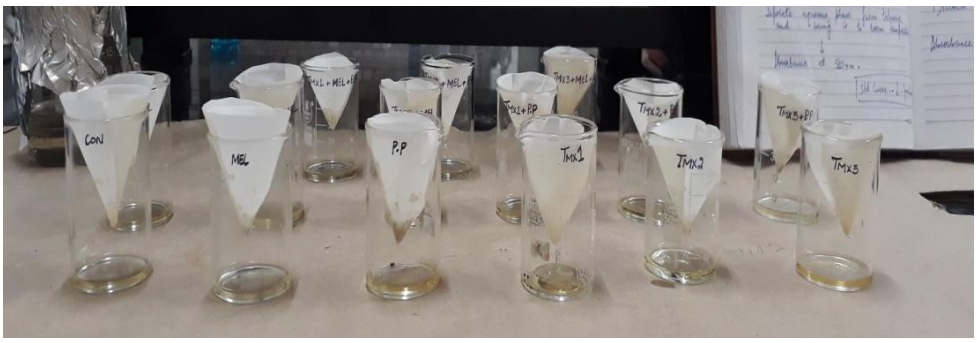


Figure 5.10: Filtration of incubated plant extract.

Calculations

Standard curve of betaine hydrochloride was plotted and used for assessing the glycine-betaine content and expressed in mg g^{-1} dry weight.

5.6.3 Proline content

Proline content estimation was carried as per Bates *et al*. (1973).

Reagents

Sulphosalicylic acid – 3%

Glacial acetic acid

Ninhydrin

L-proline (standard)

Procedure

250 mg plant sample was pulverized in 10 mL sulphosalicylic acid (3%) and subjected to centrifugation 10,000 rpm for 10 minutes at 4 °C. In 2 mL supernatant, 2 mL ninhydrin and 2 mL glacial acetic acid were added and kept in water bath (100 °C) for 1 hour. The reaction was terminated by shifting the mixture to ice bath. Toluene (4 mL) was added to the reaction blend and stirred for 50-60 seconds. The red colour toluene layer was separated and absorbance was noted at 520 nm.

Figure 5.11: Rusty red colour complex formation upon reaction of proline with ninhydrin.

Calculations

Standard curve of L-proline was plotted and used for assessing the proline content and expressed in mg g^{-1} fresh weight.

5.7 Analysis of Oxidative Stress Markers

Contents of oxidative stress markers viz superoxide anion ($O^{\cdot-}_2$), malondialdehyde and hydrogen peroxide (H_2O_2) were assessed in regard to oxidative burst.

5.7.1 Superoxide anion ($O^{\cdot-}_2$) content

Superoxide anion levels were quantified according to Wu *et al*. (2010).

Reagents

1-napthylamine – 7 mM

3-amino benzene sulphonic acid – 58 mM

Hydroxylamine hydrochloride – 10 mM

Phosphate Buffer (pH-7.8) – 65 mM

Polyvinylpyrrolidone (PVP) – 1%

Sodium nitrate (standard)

Procedure

500 mg fresh plant tissue was homogenized in 4 ml phosphate buffer (65 mM, pH-7.8) containing 1% PVP. The mixture was centrifuged at 12,000 rpm for 15 minutes at 4 °C. In 0.5 mL of supernatant, 0.5 mL phosphate buffer and 0.1 mL hydroxylamine hydrochloride (10 mM) were added and mixed thoroughly. The amalgam was incubated for 30 minutes at room temperature. Following incubation, 3-aminobenzenesulphonic acid (58 mM) and 1-napthylamine (7 mM) 1 mL each were added to the mixture and again incubated for 20 minutes at room temperature. Absorbance was recorded at 520 nm.

Calculation

Standard curve of sodium nitrate ($NaNO_2$) was plotted and further used to determine the superoxide anion production and expressed as µmole/g FW.

5.7.2 Hydrogen peroxide (H_2O_2) content

Hydrogen peroxide content was reckoned as per Velikova *et al*. (2000).

Reagents

Potassium phosphate buffer (pH-7.0) – 10 mM

Potassium iodide (KI) – 1M

Trichloroacetic acid (TCA) – 1M

Procedure

Fresh plant material (500 mg) was pulverized in 2 mL trichloroacetic acid (1M) and centrifuged at 12,000 rpm for 15 minutes at 4 °C. Thereupon, 0.5 mL of potassium phosphate buffer (10 mM) and 1 mL KI (1M) were added to 0.5 mL of supernatant and mixed well. Absorbance was recorded at 390 nm.

Calculations

Standard curve of hydrogen peroxide was plotted to determine the concentrations in plant samples and expressed as µmole/g FW.

5.7.3 Malondialdehyde (MDA) content

Malondialdehyde content was reckoned as per Heath and Packer (1998).

Reagent

Trichloroacetic acid (TCA) – 0.1%

Thiobarbituric acid (TBA) – 0.5%

Procedure

350 mg of plant sample was macerated 0.1% trichloroacetic acid (5 mL) and centrifuged at 5,000 rpm for 15 minutes at 4 °C. Then, 1 mL supernatant was mixed with 20% trichloroacetic acid (4 mL, containing 0.5% thiobarbituric acid) and incubated for 30 minutes in water bath at 95 °C, followed by ice bath. The mixture was again centrifuged at same conditions and the absorbance of obtained supernatant was noted at 532 nm and

600 nm. Correction of un-specific turbidity was achieved by subtracting value of absorbance at 532 nm from 600 nm.

Calculations

MDA = Abs × total volume × 1000/sample volume × plant weight × ε

(Extinction co-efficient, ε = 155 Mm^{-1} cm^{-1}).

5.8 Biochemical Analysis

Biochemical analysis was determined in terms of protein content, antioxidative enzymes activity, content of antioxidants and organic acid profiling. 10 days old *in vitro* grown seedlings were used for the analysis.

5.8.1 Protein content

Protein content was quantified according to Lowry *et al*. (1951).

Reagents

Reagent A = sodium carbonate (Na_2CO_3) (2%) in sodium hydroxide (NaOH) (0.1N)

Reagent B = copper sulphate ($CuSO_4$) (0.5%) in potassium sodium tartarate (1%)

Reagent C = reagent A (100 mL) and reagent B (2 mL)

Reagent D = Folin-Ciocalteau reagent (FC)

Potassium phosphate buffer – 50 mM, pH-7.0

BSA (bovine serum albumin) – standard (50 mg/50 mL)

Procedure

Plant tissue (0.5 g) was in levigated in potassium phosphate buffer (3 mL) and centrifuge at 12,000 rpm for 20 minutes at 4 °C. 0.1 mL supernatant and prepared standard was transferred into the series of test tubes and distilled water (0.9 mL) was added to make

the final volume. 1 mL distilled water containing tube served as blank. Thereupon, reagent C (5 mL) was added to each tube and vortexed, followed by incubation for 10 minutes at room temperature. Then, reagent D (0. 5 mL) was poured in each tube and again incubated in dark for 30 minutes at room temperature. Incubated samples appear blue in colour. Subsequently, absorbance was recorded at 660 nm.

Calculations

Graph of standard (abs vs conc.) was prepared to calculate the amount of protein in the desired samples and was expressed in mg g^{-1} FW.

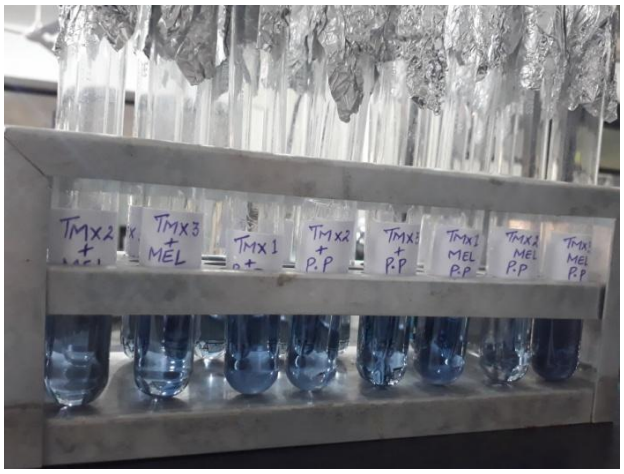


Figure 5.12: Termination of reaction by formation of blue colour.

5.8.2 Enzymatic antioxidants

For evaluating activities of enzymatic antioxidants, fresh plant material (0.5 g) was pulverized in potassium phosphate buffer (3 mL, 100 mM, pH-7.0). Homogenate was subjected to centrifugation at 12,000 rpm for 20 minutes at 4 °C. The obtained supernatant was stored at -20 °C for further use to determine the activities of CAT, POD, DHAR, APOX, GPOX, GR and GST. For SOD activity, the plant tissue was levigated in sodium carbonate buffer (3 mL, 50 mM, pH-10.2) and centrifuged under same condition to acquire the supernatant.

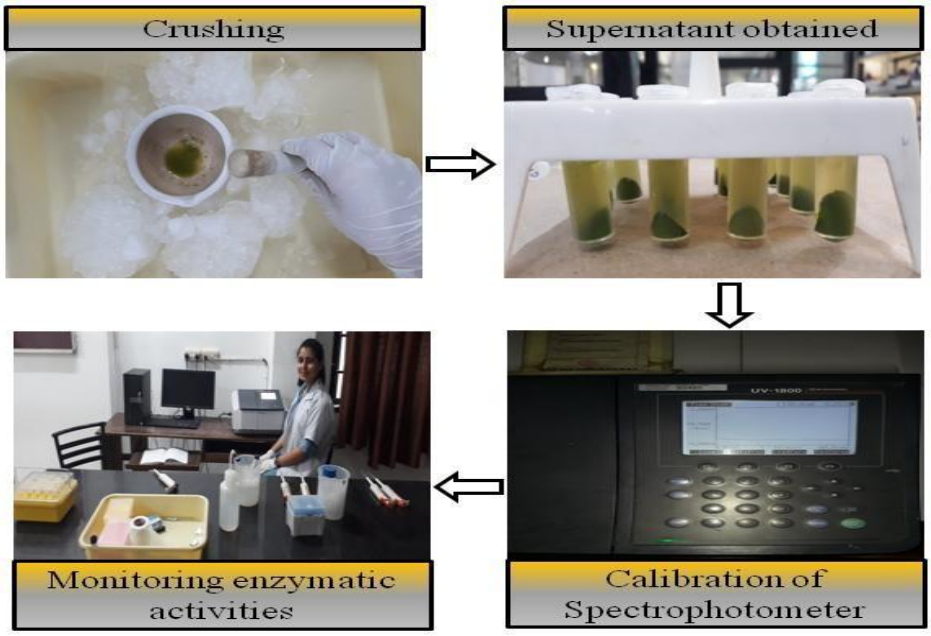


Figure 5.13: Schematic representation of process involved in determination of enzymatic activities.

5.8.2.1 Catalase (*CAT*) activity

Catalase activity was calculated as per Aebi (1984) with some modifications

Reagents

Potassium phosphate buffer – 100 mM, pH 7.0

Hydrogen peroxide – 15 mM

Procedure

Reaction amalgam was prepared in cuvette comprising, 1.5 mL potassium phosphate buffer, 0.93 mL hydrogen peroxide and 0. 07 mL sample. Absorbance was recorded at 240 nm.

Calculations

Activity was calculated by following equations

$$\textit{Unit Activity}\ (\textit{Unit min}^{-1}\ g^{-1}\ \textit{FW}) = \frac{\text{Change in Abs/min} \times \text{Total Vol. (ml)}}{\text{Ɛ} \times \text{Sample Vol. (ml)} \times \text{Wt. of tissue (g)}}$$

Where, Extinction coefficient (Ɛ) = 39.4 Mm^{-1} cm^{-1}

$$\textit{Specific Activity}\ (\mu\textit{mol min}^{-1}\ \textit{mg}^{-1}\ \textit{protein}) = \frac{\text{Unity activity (Unit min}^{-1}\ \text{g}^{-1}\ \text{FW)}}{\text{Protein content (mg g}^{-1}\ \text{FW)}}$$

5.8.2.2 Guaiacol peroxidase (*POD*) activity

Guaiacol peroxidase activity was evaluated in line with Putter (1974).

Reagents

Potassium phosphate buffer – 50 mM (pH, 7.0)

Guaiacol – 20 mM

Hydrogen peroxide – 12 mM

Procedure

A reaction blend was prepared in a cuvette, comprising potassium phosphate buffer (2.35 mL), guaiacol (0.05 mL), hydrogen peroxide (0.03 mL) and sample (0.07 mL). The absorbance was recorded at 436 nm.

Calculations

$$\textit{Unit Activity}\ (\textit{Unit min}^{-1}\ g^{-1}\ \textit{FW}) = \frac{\text{Change in Abs/min} \times \text{Total Vol. (ml)}}{\text{Ɛ} \times \text{Sample Vol. (ml)} \times \text{Wt. of tissue (g)}}$$

Where, Ext. coefficient (Ɛ) = 25 mM^{-1} cm^{-1}

$$\textit{Specific Activity}\ (\mu\textit{mol min}^{-1}\ \textit{mg}^{-1}\ \textit{protein}) = \frac{\text{Unity activity (Unit min}^{-1}\ \text{g}^{-1}\ \text{FW)}}{\text{Protein content (mg g}^{-1}\ \text{FW)}}$$

5.8.2.3 Ascorbate peroxidase (*APOX*) activity

Ascorbate peroxidase activity was assessed according to Nakano and Asada (1981).

Reagents

Potassium phosphate buffer – 50 mM (pH 7.0)

Ascorbate – 0.5 mM

Hydrogen peroxide – 1 mM

Procedure

To compose a reaction mixture, potassium phosphate buffer (2.13 mL), ascorbate 0.2 mL), hydrogen peroxide (0.1 mL) and sample (0.07 mL) were used. Absorbance was recorded at 290 nm.

Calculations

$$\textit{Unit Activity}\ (\textit{Unit min}^{-1}\ \textit{g}^{-1}\ \textit{FW}) = \frac{\text{Change in Abs/min} \times \text{Total Vol. (ml)}}{\text{Ɛ} \times \text{Sample Vol. (ml)} \times \text{Wt. of tissue (g)}}$$

Where, Ext. coefficient (Ɛ) = 2.8 mM^{-1} cm^{-1}

$$\textit{Specific Activity}\ (\mu\textit{mol min}^{-1}\ \textit{mg}^{-1}\ \textit{protein}) = \frac{\text{Unity activity (Unit min}^{-1}\ \text{g}^{-1}\ \text{FW)}}{\text{Protein content (mg g}^{-1}\ \text{FW)}}$$

5.8.2.4 Glutathione peroxidase (*GPOX*) activity

Glutathione peroxidase activity was reckoned in line with Flohe and Gunzler (1984).

Reagents

Potassium phosphate buffer – 50 mM (pH, 7.0)

Reduced glutathione (GSH) – 1mM

Hydrogen peroxide – 0.15 mM

Sodium azide (NaN_3) – 1 mM

Ethylene diamine tetra acetic acid (EDTA) – 0.5 mM

Nicotinamide adenine dinucleotide phosphate (NADPH) – 0.15 Mm

Procedure

A reaction blend included potassium phosphate buffer (1.18 mL), EDTA (0.25 mL), NADPH (0.25 mL), reduced glutathione (0.25 mL), hydrogen peroxide (0.25 mL), sodium azide (0.25 mL) and sample (0. 07 mL). The absorbance was recorded at 340 nm.

Calculations

$$\textit{Unit Activity}\ (\textit{Unit min}^{-1}\ \textit{g}^{-1}\ \textit{FW}) = \frac{\text{Change in Abs/min} \times \text{Total Vol. (ml)}}{\text{Ɛ} \times \text{Sample Vol. (ml)} \times \text{Wt. of tissue (g)}}$$

Where, Ext. coefficient (Ɛ) = 6.22 mM^{-1} cm^{-1}

$$\textit{Specific Activity}\ (\mu\textit{mol min}^{-1}\ \textit{mg}^{-1}\ \textit{protein}) = \frac{\text{Unity activity (Unit min}^{-1}\ \text{g}^{-1}\ \text{FW)}}{\text{Protein content (mg g}^{-1}\ \text{FW)}}$$

5.8.2.5 Dehydroascorbate reductase (*DHAR*) activity

Dehydroascorbate reductase activity was analyzed as specified by Dalton *et al.*, (1986).

Reagent

Potassium phosphate buffer – 50 mM (pH, 7.0)

Reduced glutathione (GSH) – 2.5 mM

Ethylene diamine tetra acetic acid (EDTA) – 0.1 mM

Dehydroascorbate – 0.2 mM

Procedure

A reaction amalgam constituted potassium phosphate buffer (1.33 mL), dehydroascorbate (0.3 mL), GSH (0.5 mL), EDTA (0.3 mL) and plant sample (0.07 mL). Absorbance was recorded 265 nm.

Calculations

$$\textit{Unit Activity}\ (\textit{Unit min}^{-1}\ \textit{g}^{-1}\ \textit{FW}) = \frac{\text{Change in Abs/min} \times \text{Total Vol. (ml)}}{\text{Ɛ} \times \text{Sample Vol. (ml)} \times \text{Wt. of tissue (g)}}$$

Where, Ext. coefficient (Ɛ) = 14 mM^{-1} cm^{-1}

$$\textit{Specific Activity}\ (\mu\textit{mol min}^{-1}\ \textit{mg}^{-1}\ \textit{protein}) = \frac{\text{Unity activity (Unit min}^{-1}\ \text{g}^{-1}\ \text{FW)}}{\text{Protein content (mg g}^{-1}\ \text{FW)}}$$

5.8.2.6 Glutathione-*S*-Transferase (*GST*) activity

Glutathione-*S*-Transferase activity was measured in line with Habig *et al.*, (1974).

Reagents

Potassium phosphate buffer – 50 mM

Reduced glutathione (GSH) – 10 mM

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

Procedure

A reaction mixture composes, potassium phosphate buffer (1.93 mL), GSH (0. 25 mL), cDNB (0. 25 mL) and plant extract (0. 07 mL) was its absorbance was recorded at 340 nm.

Calculations

$$\textit{Unit Activity}\ (\textit{Unit min}^{-1}\ \textit{g}^{-1}\ \textit{FW}) = \frac{\text{Change in Abs/min} \times \text{Total Vol. (ml)}}{\text{Ɛ} \times \text{Sample Vol. (ml)} \times \text{Wt. of tissue (g)}}$$

Where, Ext. coefficient (Ɛ) = 9.6 mM^{-1} cm^{-1}

$$\textit{Specific Activity}\ (\mu mol\ min^{-1}\ mg^{-1}\ protein) = \frac{\text{Unity activity (Unit min}^{-1}\text{ g}^{-1}\text{ FW)}}{\text{Protein content (mg g}^{-1}\text{ FW)}}$$

5.8.2.7 Glutathione reductase (*GR*) activity

Glutathione reductase activity was reckoned as per Carlberg and Mannervik (1975).

Reagents

Potassium phosphate buffer – 50 mM (pH, 7.6)

Ethylene diamine tetra acetic acid – 3 mM

Oxidized glutathione (GSSG) – 1 mM

NADPH – 0.1 mM

Procedure

A reaction blend contained potassium phosphate buffer (1.53 mL), NADPH, GSSG, EDTA (0.3 mL) each and plant extract (0.07 mL). The absorbance was recorded at 340 nm.

Calculations

$$\textit{Unit Activity}\ (\textit{Unit min}^{-1}\ g^{-1}\ \textit{FW}) = \frac{\text{Change in Abs/min} \times \text{Total Vol. (ml)}}{\text{Ɛ} \times \text{Sample Vol. (ml)} \times \text{Wt. of tissue (g)}}$$

Where, Ext. coefficient (Ɛ) = 6.22 mM^{-1} cm^{-1}

$$\textit{Specific Activity}\ (\mu mol\ min^{-1}\ mg^{-1}\ protein) = \frac{\text{Unity activity (Unit min}^{-1}\text{ g}^{-1}\text{ FW)}}{\text{Protein content (mg g}^{-1}\text{ FW)}}$$

5.8.2.8 Superoxide dismutase (*SOD*) activity

Superoxide dismutase activity was calculated in line with Kono (1978).

Reagents

Sodium carbonate buffer (Na_2CO_3) – 50 mM (pH, 10)

Hydroxylamine hydrochloride (NH_2OH HCl) – 1 mM (pH, 6.0)

Nitroblue tetrazolium (NBT) – 24 µM

EDTA – 0.1 mM

Triton X-100 – 0.03%

Procedure

A reaction amalgam was prepared by adding sodium carbonate buffer (1.63 mL), NBT (0.5 mL), triton X-100 (0.1 mL), EDTA (0.1 mL), hydroxylamine hydrochloride (0.1 mL) and plant sample (0.07 mL). The absorbance was recorded at 560 nm.

Calculations

The percentage of inhibition in NBT reduction was reckoned by equation quoted below:

$$\textit{Percentage inhibition } (x) = \frac{\text{Change in Abs/min (Blank)} \times \text{Change in Abs/min (Test)}}{\text{Change in Abs/min (Blank)} \times 100}$$

Where, (x) = percent inhibition induced by plant sample (70 µL)

Therefore, 50% inhibition is calculated as $= \frac{50 \times 70}{x}$ = z mL of sample

$$\textit{Unit Activity } (\textit{Unit min}^{-1}\ \textit{g}^{-1}\ \textit{FW}) = \frac{\text{Total Vol. (ml)}}{\text{z mL of sample} \times \text{Wt. of tissue (g)}}$$

$$\textit{Specific Activity } (\mu\textit{mol min}^{-1}\ \textit{mg}^{-1}\ \textit{protein}) = \frac{\text{Unity activity (Unit min}^{-1}\ \text{g}^{-1}\ \text{FW)}}{\text{Protein content (mg g}^{-1}\ \text{FW)}}$$

5.8.3 Non-enzymatic antioxidants

To estimate the content of non-enzymatic antioxidants viz, ascorbic acid, glutathione, tocopherol and total phenol, fresh plant tissue (0.7 g) was pulverized in tris buffer (3 mL, 50 mM, pH-10). The mixture was subjected to centrifugation at 12,000 rpm for 20 minutes at 4 °C. The supernatant was collected for analysis.

5.8.3.1 Ascorbic acid content

Ascorbic acid (*Vitamin C*) amount was determined in lines with Roe and Kuether (1943).

Reagents

Tri chloro acetic acid (TCA) – 50%

2,4-dinitrophenylhydrazine (DNPH) reagent

Activated charcoal

Sulphuric acid (H_2SO_4) – 65%

Ascorbic acid (standard) – 1 mg/100 mL

Procedure

A blend was prepared comprising plant extract (0.5 mL), distilled water (4 mL) and TCA (0.5 mL). To this mixture activated charcoal (100 mg) was added and mixed thoroughly. Thereupon, it was filtered using Whatman filter paper (Grade 1). DNPH reagent (0. 4 mL) was added to obtained filtrate and kept for 3 hour incubation at 37 °C, followed by immediate cooling in ice bath. Then chilled sulphuric acid (1.6 mL) was added and again incubated for 30 minutes at room temperature. Absorbance was recorded at 520 nm.

Figure 5.14: Addition of activated charcoal to amalgam and filtration process using Whatman filter paper #1.

Calculations

$$\textit{Ascorbic acid content (mg/g FW)} = \frac{\text{Abs. (sample)} \times \text{Std. conc.} \times \text{Total vol.}}{\text{Abs. (Std)} \times \text{Vol. (sample)}}$$

5.8.3.2 Glutathione (*GSH)* content

Glutathione content was reckoned as specified by Sedlak and Lindsay (1968).

Reagents

Absolute methanol

Tris buffer – 0.2 M (pH, 8.3)

5,5-dithiobis,2-nitrobenzoinc acid (DTNB) – 0.1 mM

Glutathione (standard) – 1mg/100mL

Procedure

To 0.1 mL of plant sample, tris buffer (1 mL), DTNB (0.05 mL) and absolute methanol (4 mL) were added. The blend was incubated for 15 minutes at room temperature and centrifuged at 3,000 rpm for 15 minutes at 4 °C. The supernatant with yellow colour pigment was obtained and absorbance was recorded at 412 nm.

Calculations

$$GSH\ content\ (mg/g\ FW) = \frac{\text{Abs. (sample)} \times \text{Std. conc.} \times \text{Total vol.}}{\text{Abs. (std)} \times \text{Vol. (sample)}}$$

5.8.3.3 Tocopherol content

Tocopherol (*Vitamin E*) content was measured as per Martinek (1964).

Reagents

Absolute ethanol

Ferric chloride – 0.12% (0.12 g in ethanol)

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

Xylene

Tocopherol (standard) – 1mg/100mL

Procedure

In plant sample (0.5 mL) absolute ethanol (0.5 mL, consisting 0.12% ferric chloride) and distilled water (0.5 mL) was added and vortexed until protein precipitates were produced. To this mixture xylene (0.5 mL) was added and further subjected to centrifugation at 3,000 rpm for 10 minutes at 4 °C. Thereupon, TPTZ (0.5 mL) was mixed with 0.5 mL of obtained upper layer and absorbance was recorded at 600 nm.

Calculations

$$Tocopherol\ content\ (mg/g\ FW) = \frac{\text{Abs. (sample)} \times \text{Std. conc.} \times \text{Total vol.}}{\text{Abs. (std)} \times \text{Vol. (sample)}}$$

5.8.3.4 Total Phenol content

Total phenol content was quantified in lines with Singleton and Rossi (1965).

Reagents

Folin-Ciocalteu (FC) reagent

Ethanol – 60%

Sodium carbonate (Na_2CO_3) – 7.5%

Gallic acid (standard)

Procedure

Oven dried plant sample (0.2 g) was crushed in ethanol (5 mL) and allowed it to incubate for 10 minutes at 60 °C. Then, extract was filtered by Whatman filter paper and diluted with ethanol (100 mL, 60%). Thereafter, FC reagent (10 mL) and sodium carbonate (2 mL) was added to 2 mL extracted mixture. The amalgam was again incubated at room temperature for 2 hours and then absorbance was recorded at 765 nm.

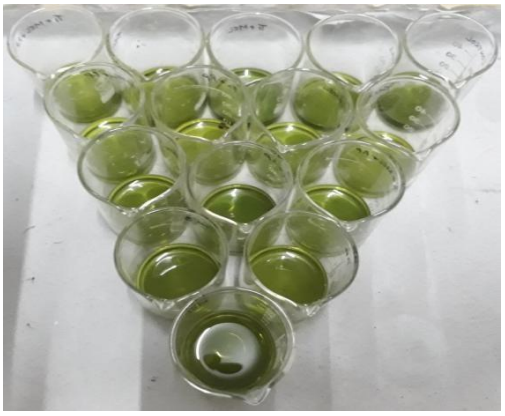


Figure 5.15: Formation of green colour upon adding sodium bicarbonate to reaction mixture.

Calculations

A graph of standard was plotted and total phenol content was measured from the equation generated and expressed in mg/g dry wt.

5.8.4 Organic acid profiling by High-Performance Liquid Chromatography (HPLC)

Sample Preparation

For organic acid profiling the samples were prepared according to Arnetoli *et al.*, 2008. 1 g of frozen plant sample was homogenized in 10 mL of distilled water using chilled motor-pestle. The homogenate was subjected to centrifugation at 10,000 rpm for 20 minutes at 4 °C. The obtained supernatant was kept at -20 °C for further use. Before performing organic acid analysis the supernatant was filtered through nylon syringe filter with pore size 0.22 µm.

Figure 5.16: Sample preparation for organic acid analysis by HPLC

Instrument conditions

HPLC analysis of all the samples was carried out on Perkin-Elmer 200 series HPLC system. The stationary phase of the system constitutes of C-18 reverse phase column and KH_2PO_4 (pH- 2.5) was used as mobile phase. 15 µl of sample injection volume was used and the respective chromatogram for each sample was obtained at 210 nm wavelength.

Figure 5.17: HPLC (Shimadzu LC-20AP) setup.

Calculations

Detection of the organic acids in test samples was confirmed by matching their retention time with the standard organic acids retention time. The quantification of organic acids was carried out based on comparing the peak area of standard with respective treatments.

5.9 Molecular Study

Molecular analysis was conducted in 10 days old *Brassica juncea* seedlings to study the gene expression.

5.9.1 Gene Expression Analysis by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA isolation and cDNA synthesis

Trizol protocol was followed to extract total RNA from 200 mg of *B. juncea* seedlings. Further, from the 1 microgram total RNA processed by reverse transcriptase to obtain cDNA by Takara primeScript RT-PCR kit (containing reverse transcriptase, 5X primeScript buffer, RNase inhibitor, dNTP mixture, random 6 mers, oligo dT primer, and

RNase free distilled water. The primers pertaining to considered genes were retrieved from NCBI (Table 5.2). By considering the uniform apex expression level of *actin* gene during vegetative state of plants, it was taken as an endogenous control for present investigation.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was executed using the QuantStudio ^{TM} 5 System and SYBR Green PCR Master Mix (ThermoFisher SCIENTIFIC). Inbuilt statistical package was utilized after the completion of every cycle in order to produce a melting temperature plot of the sample which validated the specificity of the query sample being amplified. The quantification of the messenger RNA in the sample was done by following the methodology given by (Livak and Schmittgen, 2001). The ΔCt values for all the test samples were obtained by deducting the Ct of endogenous control from the query samples. Further ΔΔCt values were obtained by deduction of the ΔCt value of control from the ΔCt value of treated samples. Finally, the results were expressed as number of folds increase/decrease by taking $2^{-\Delta\Delta Ct}$ signifying normalized fold change values.

Table 5.2: Primer Sequence for quantitative real time polymerase chain reaction (qRT-PCR).

Gene	Primer Sequence
actin	Forward primer 5’ CTTGCACCTAGCAGCATGAA 3’ Reverse primer 5’ GGACAATGGATGGACCTGAC 3’
CHLASE	Forward primer 5' GAATATCCGGTGGTGATGCT 3' Reverse primer 5' TCCGCCGTTGATTTTATCTC 3'
P450	Forward primer 5' CATTTGTTCTCACCCACACG 3' Reverse primer 5' CACAACCGAGTTCGTGAATG 3'

POD	Forward primer 5' TTCGAACGGAAAAAGATGCT 3' Reverse primer 5' AACCCTCCATGAAGGACCTC 3'
CXE	Forward primer 5' GGCGCTAACATGACTCATCA 3' Reverse primer 5' CTCCCAGAGTTGAGCGATTC 3'
GST-1	Forward primer 5'CGTCGTCGAAGAAGAAGAGG 3' Reverse primer 5'TTTTTGGTGGGAGTTCCAAG 3'
NADH	Forward primer 5'CTCGGCCTTTCTCAACAGAC 3' Reverse primer 5'CATTTCCCAAGTTTCCCAGA 3'
Target genes: Total six number of genes to be targeted viz, ***CHLASE*** (Chlorophyllase); ***P450*** (Cytochrome P450 monooxygenase); ***POD*** (guaiacol peroxidase); ***CXE*** (Carboxylesterase); ***GST*** (Glutathione-S-transferase); ***NADH*** (NADH-ubiquinone oxidoreductase). **Endogenous gene: *actin***	

5.10 Statistical evaluations

The data retrieved from various analyses (Morphological and Biochemical) and analytical techniques (HPLC and qRT-PCR) were subjected to statistical evaluation by employing General Linear Model Univariate ANOVA (Analysis of variances) pertaining to significance validation of the treatments on *Brassica juncea* plant. The quantitative data was represented as a mean of triplicates ± standard error. The significance of mean difference for each treatment was analyzed by Tukey's HSD (Honestly Significant Difference) test at $P<0.05$ significance level. All the analyses were performed by using the SPSS statistical package (version 17.0).

CHAPTER 6

RESULTS & DISCUSSION

6.1 Melatonin and PGPR Assisted Degradation of Thiamethoxam in *Brassica juncea*

Herein, we demonstrate the efficiency of seed priming with melatonin and *Pseudomonas putida* (PGPR) inoculation for reduction of TMX residues in *Brassica juncea* seedlings.

6.1.1 TMX residue analysis

In order to proceed with TMX residue analysis, initially the calibration curve for the TMX was generated using its various concentrations namely 0.2, 1.0, 5.0 and 10.0 ppm respectively. The calibration curve for the standard TMX has been shown in Figure 6.1. The r^2 value allows us to estimate the closeness of the observed values to the linear regression line in a standard plot, with higher value (maximum of 1) corresponding to higher efficiency of the line to represent observed values (Willett and Singer, 1988). The observed r^2 value of 0.99 in the present investigation implies the correctness of the linear regression line in representing the observed concentrations (ppm) in the plot (Figure 6.1). Thus, this calibration curve was further employed to determine the concentration of the control and treated samples respectively. The Figure 6.2 represents the chromatogram of TMX treated seedlings. The retention time of 27.25 minutes was observed for the TMX having m/z ratio of 212. Concentration of 1.7 ppm (TMX) was observed in case TMX treated seedlings. The chromatogram of TMX treated seedlings under the application of melatonin has shown in figure 6.3 and concentration of 1.5 ppm (TMX) was evaluated from the standard plot. Further, in figure 6.4, concentration of 1.7 ppm (TMX) was seen in the TMX treated seedlings grown under the influence of *Pseudomonas putida*. Finally, the chromatogram of combinatorial application of melatonin and *Pseudomonas putida* in TMX treated seedlings is shown in Figure 6.5, in which TMX concentration of 0.98 ppm was observed. A decline in TMX residues was observed in 10 days old *B. juncea* seedlings treated with melatonin and combination of *P. putida* and melatonin raised in TMX amended substratum. Melatonin treatment decreased TMX residues by 13.33%,

whereas, combinatorial application of melatonin and *P. putida* showed 88.88% reduction as compared to investigational TMX treated *B. juncea* seedlings (Table 6.1). In case of TMX stressed, *P. putida* treated *B. juncea* seedlings no effect on concentration was observed.

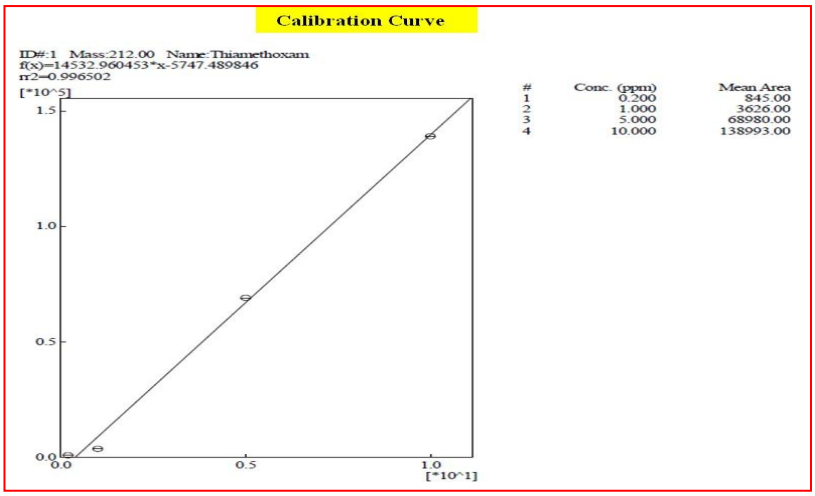
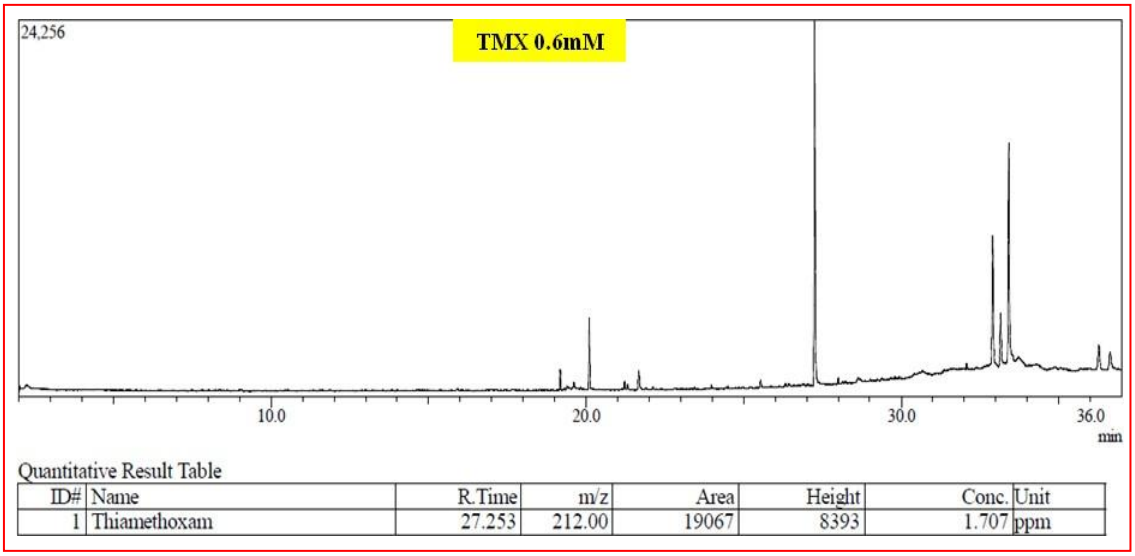


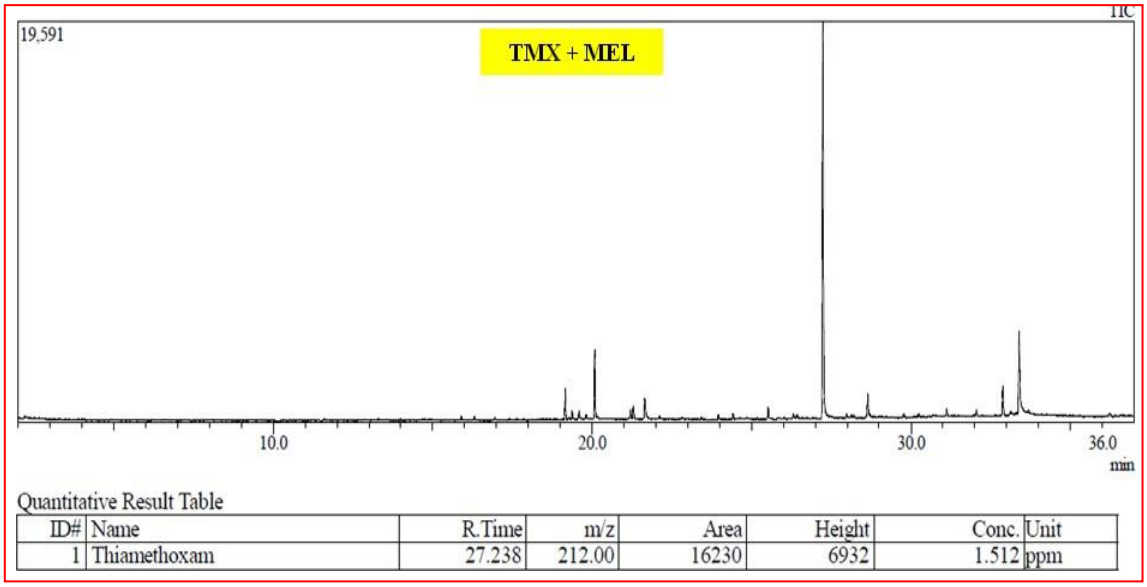
Figure 6.1: Standard chromatogram of Thiamethoxam using 0.2, 1, 5 and 10 ppm respectively.



Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	Conc.	Unit
1	Thiamethoxam	27.253	212.00	19067	8393	1.707	ppm

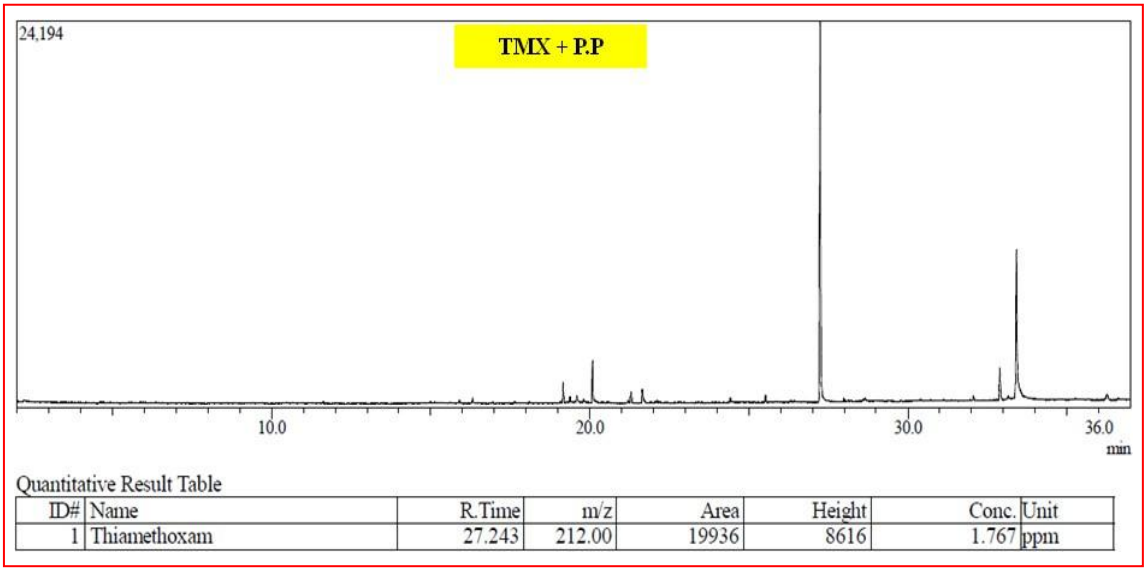
Figure 6.2: GC-MS Chromatogram of TMX treated seedlings.



Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	Conc.	Unit
1	Thiamethoxam	27.238	212.00	16230	6932	1.512	ppm

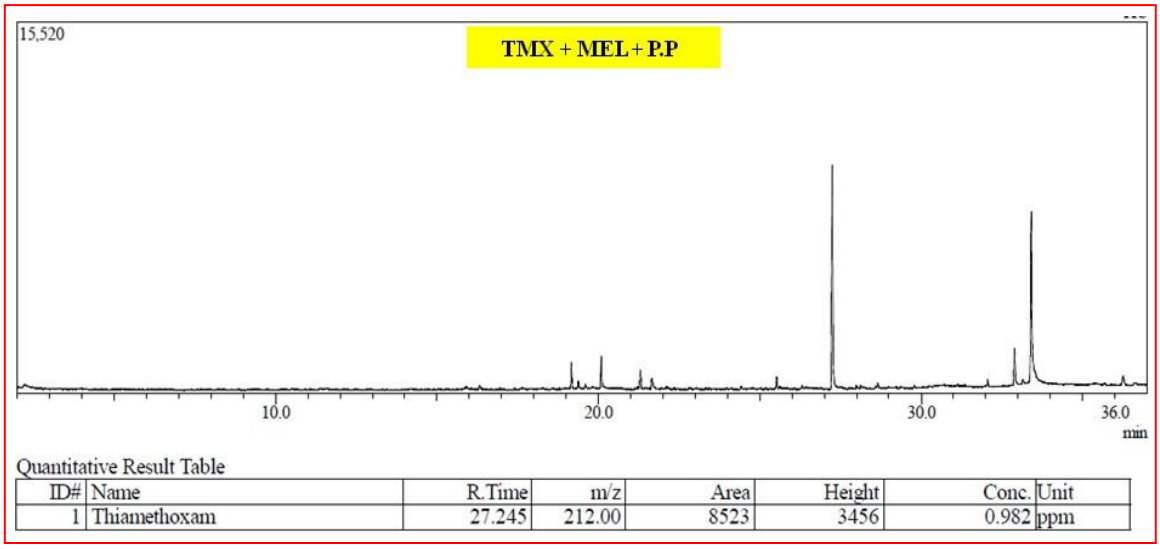
Figure 6.3: GC-MS Chromatogram of Melatonin treated *B. juncea* seedlings amended with TMX.



Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	Conc.	Unit
1	Thiamethoxam	27.243	212.00	19936	8616	1.767	ppm

Figure 6.4: GC-MS Chromatogram of *P. putida* inoculated *B. juncea* seedlings amended with TMX.



Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	Conc.	Unit
1	Thiamethoxam	27.245	212.00	8523	3456	0.982	ppm

Figure 6.5: GC-MS Chromatogram of melatonin treated and *P. putida* inoculated *B. juncea* seedlings amended with TMX.

Table 6.1: Depicting percent decrease of TMX in *Brassica juncea* seedlings treated with melatonin and *Pseudomonas putida* (N.E= No effect).

Sample	Concentration (ppm)	Percent Decrease
TMX	1.7	-
TMX + Mel	1.5	13.33
TMX + P.P	1.7	N.E
TMX + Mel + P.P	0.9	88.88

Persistence of pesticide in crops is a major concern for consumers. Moreover, meager information is available regarding pesticide metabolism and mechanisms involved for pesticide detoxification, yet few experiments have been executed for decreasing pesticide residue in living plants (Zhaou *et al.*, 2015). Following translocation, pesticides are degraded into less toxic compounds (Cherian and Oliveira, 2005) and three phased detoxification system mediated by enzymes is accountable for pesticide degradation in plants (Coleman *et al.*, 1997). Melatonin is involved in regulating manifold genes

associated with pesticide detoxification and also is well recognized to modulate the physicochemical activities of abiotic stressed plants (Kaya *et al*., 2019; Jan *et al.,* 2022). A recent study demonstrated that exogenous application of melatonin promotes the degradation of imidacloprid in *Cucumis sativus L*. (Liu *et al*., 2021). The increased activity of GST enzyme was also observed which reinforced pesticide degradation activity of the plant (Liu *et al*., 2021). Similar trend of TMX degradation was observed in the present investigation when melatonin was applied exogenously to the TMX treated *Brassica juncea* seedlings. Studies also depict that *P. putida* effectively degrades TMX and utilizes its degraded constituents as a source of carbon and energy (Rana *et al.,* 2015). The study corroborates with our present research where we observed that the combinatorial application of melatonin and *P. putida* was able to degrade TMX residues with higher rate as compared to non-combinatorial treatments. Hence, by pesticide residue analysis we unravel the potential of melatonin and combinatorial application (melatonin + *P. putida*) for degradation of TMX residues in *Brassica juncea* seedlings.

6.2 Morphological analysis

Various morphological parameters like fresh weight (FW), dry weight (DW) and seedling length (SL) were considered to study the impact of TMX and ameliorative in *Brassica juncea* seedlings. All the parameters were evaluated in triplicates and further their respective values are represented as mean ± standard deviation (S.D.) as shown in table 6.2. Graphical representation of the evaluated parameters is shown in Figure 6.6.

The univariate ANOVA test statistics revealed that FW of TMX3+Mel treated *Brassica juncea* seedlings was significantly higher ($p<0.05$) by 2.82 mg/seedling as compared to TMX3 treated seedlings. A significant mean difference ($p<0.05$) of 2.78 mg/seedling was observed while comparing the FW content of TMX3+PP treated seedlings with TMX3 inoculated *Brassica juncea* seedlings. The FW content of TMX3+MEL+PP was observed to be 5.90 mg/seedling ($p<0.05$) higher as compared to TMX3 treated *Brassica juncea* seedlings. From the above, we can conclude that individual application of melatonin and *P. putida* was able to retain the FW content of *Brassica juncea* seedlings, while their

combinatorial application retained the highest FW content in thiamethoxam treated seedlings respectively.

The DW content of TMX3 treated seedlings was observed to be significant lower ($p<0.05$) than TMX3+Mel treated seedlings by 1.11 mg/seedling. TMX3+PP treated seedlings showed statistically significant ($p<0.05$) increased DW content than TMX3 treated seedlings by 0.99 mg/seedling. Finally, the TMX3+MEL+PP treated *Brassica juncea* seedlings were having significant ($p<0.05$) increase in DW content by 1.98 mg/seedling as compared to TMX3 treated seedlings. Hence, we can say that combinatorial application was observed to exhibit highest impact on DW content of thiamethoxam treated *Brassica juncea* seedlings.

The seedling length of TMX3+Mel treated *Brassica juncea* seedlings was observed to be 5.87 cm ($p<0.05$) larger than the TMX3 treated seedlings, whereas the seedling of TMX3+PP treated seedlings were having significant ($p<0.05$) mean difference of 7.05 cm on comparison to TMX3 treated seedlings. The combinatorial application (TMX3+MEL+PP) of melatonin and *P. putida* treated seedlings showed significant mean difference ($p<0.05$) in seedling length as compared to TMX3 treated seedlings by 10.12 cm per seedling.

Table 6.2: Morphological features estimation of thiamethoxam treated *Brassica juncea* seedlings enriched by melatonin and *P. putida* alone as well as in combination respectively. The values are estimated in triplicates and presented as mean ± SD.

Treatments	Fresh Weight (mg/seedling±SD)	Dry Weight (mg/seedling±SD)	Seedling length (cm±SD)
Control	9.61 ± 0.16	2.47 ± 0.13	9.3 ± 0.37
MEL	11.18 ± 0.32	3.40 ± 0.33	10.11 ± 0.20
P.P	10.72 ± 0.17	2.7 ± 0.32	10.22 ± 0.27
TMX1	8.66 ± 0.25	2.43 ± 0.37	8.76 ± 0.28
TMX2	7.77 ± 0.19	1.49 ± 0.33	5.65 ± 0.31

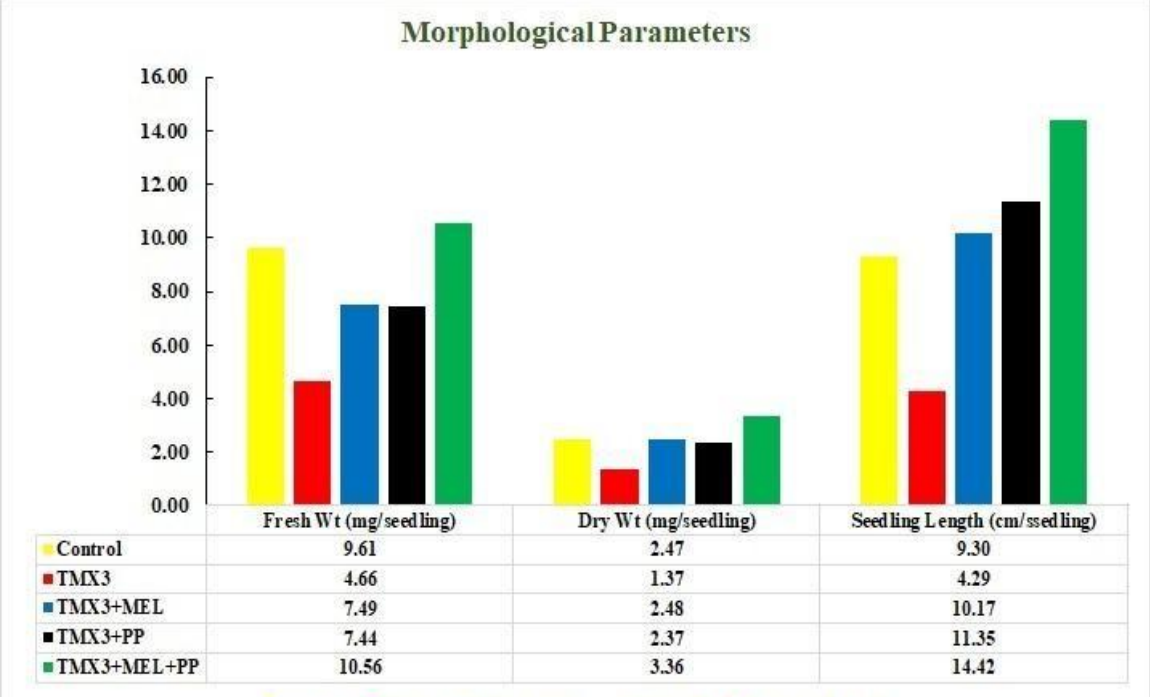
TMX3	4.66 ± 0.22	1.37 ± 0.42	4.29 ± 0.24
TMX1+MEL	11.18 ± 0.32	2.58 ± 0.20	15.30 ± 0.29
TMX2+MEL	8.39 ± 0.39	2.37 ± 0.23	13.56 ± 0.39
TMX3+MEL	7.49 ± 0.46	2.48 ± 0.42	10.16 ± 0.26
TMX1+PP	11.32 ± 0.41	2.45 ± 0.29	14.54 ± 0.44
TMX2+PP	9.23 ± 0.26	2.59 ± 0.33	13.55 ± 0.39
TMX3+PP	7.44 ± 0.28	2.36 ± 0.28	11.34 ± 0.32
TMX1+MEL+PP	12.28 ± 0.38	3.48 ± 0.42	21.15 ± 0.17
TMX2+MEL+PP	10.22 ± 0.25	2.50 ± 0.38	18.20 ± 0.26
TMX3+MEL+PP	10.56 ± 0.15	3.35 ± 0.25	14.42 ± 0.35
Univariate ANOVA Test Statistics	**FW Vs Treatments**	**DW Vs Treatments**	**SL Vs Treatments**
	F = 149.291	**F** = 9.660	**F** = 600.108
Control Vs TMX3	**HSD** = 4.950*	**HSD** = 1.110*	**HSD** = 5.873*
TMX3+MEL Vs TMX3	**HSD** = 2.826*	**HSD** = 1.096*	**HSD** = 5.006*
TMX3+PP Vs TMX3	**HSD** = 2.783*	**HSD** = 0.993*	**HSD** = 7.053*
TMX3+MEL+PP Vs TMX3	**HSD** = 5.903*	**HSD** = 1.983*	**HSD** = 10.126*

*Note: *Represents the significant mean difference at p< 0.05 level*

The observed morphological parameters in the present investigation pertaining to thiamethoxam treated *Brassica juncea* seedlings revealed that application of melatonin and *P. putida* proved as an efficient treatment strategy to diminish the pesticidal impact on seedlings. We analyzed that combinatorial application (MEL + *P. putida*) produced highest positive impact on thiamethoxam treated *Brassica juncea* seedlings suggesting this approach to be most efficacious. The immense exploitation of pesticides like thiamethoxam ensue their persistence in plants, water and soil sources (Jan *et al*., 2022; Wang *et al*., 2020a; Wang *et al*., 2020b). Numerous studies have reported the negative impact of thiamethoxam application on biochemical constituents, growth and development of *Helianthus annuus* (Sunflower), *Allium cepa* (onion), *Brassica chinensis* (Chinese cabbage) and *Solanum lycopersicum* (Tomato) (Georgieva and Vassilevska-

Ivanova, 2021; Çavuşoğlu *et al*., 2012; Wang *et al*., 2020b; Yıldıztekin *et al*., 2015). In present study, a decline in seedling length, fresh weight and dry weight was observed in TMX treated *B. juncea* grown in petri plates. The study carried out by (Çavuşoğlu *et al*., 2012), evaluated the impact of thiamethoxam application in *Allium cepa* where significant reduction in germination percent, length of roots and weight of seeds was observed in thiamethoxam treated seedlings as compared to experimental controls (Çavuşoğlu *et al*., 2012). Similarly, in contemporary study we have observed a statistically significant ($p<0.05$) reduction in the FW, DW and SL of the thiamethoxam (TMX3) treated seedlings compared to experimental untreated seedlings as shown in table 6.2. The FW content of TMX3 treated seedlings was 4.95 mg/seedling ($p<0.05$) less than control plants whereas, DW was 1.11 mg/seedling ($p<0.05$) reduced than investigational controls. A significant ($p<0.05$) reduction of 5.87 cm was observed in TMX3 treated seedlings as measured with control seedlings. Thus, we can conclude that in the present investigation thiamethoxam (TMX3) application had negative impact on FW, DW and SL content of *Brassica juncea* seedlings. The molecular mechanisms pertaining to negative impact of thiamethoxam are induced by obstruction in the cellular interactions resulting in deficiency of nutrient availability to the tissues and producing noxious compounds which hinders the protein and carbohydrate production by tweaking the associated macromolecular machineries (Çavuşoğlu *et al*., 2012; Siddiqui and Ahmed, 2006; Siddiqui and Ahmed, 2000). Seed priming with melatonin and inoculation of *P. putida* in presence of TMX resulted in recovery of morphological parameters that were earlier negatively affected by TMX application. Due to systemic nature of TMX, it accumulates within plant tissues causing toxicity to plants. Reduction in plant biomass and growth is due to impairment in chlorophyll synthesis, photosynthesis, nutrient uptake, hormone and water balance (Jan *et al.,* 2020). Decrease in dry weight is due to depletion of chloroplast which leads to chlorosis caused by stress conditions (Chaudhary *et al.,* 2020). In *B. juncea* reduction in growth and biomass was observed as an aftereffect of pesticide toxicity (Sharma *et al*., 2019). Similar observations, that is reduced growth in *Allium cepa* (Cavusoglu *et al.,* 2010) and altered morpho-physiological traits in *Coffea*

arabica subjected to TMX were reported (Leite *et al.,* 2022). Melatonin mediated recovery of affected growth, might be due to ability of regeneration, promotes plant growth and enhances proliferation of vitro cells also it is recognized as pleiotropic molecule with efficient anti-stress activity (Sarropoulou *et al.,* 2012). During stress conditions reinforcement in growth characteristics was reported in *Oryza sativa* inoculated with *P. putida* (Awasthi *et al.,* 2018). It is speculated that improvement in growth is primarily due to phytohormone production and plant growth promoting traits possessed by microorganisms that enable plants to grow under stress condition (Khanna *et al.,* 2021). The findings of current study are in accordance with the aforementioned studies.



	Fresh Wt (mg/seedling)	Dry Wt (mg/seedling)	Seedling Length (cm/ssedling)
Control	9.61	2.47	9.30
TMX3	4.66	1.37	4.29
TMX3+MEL	7.49	2.48	10.17
TMX3+PP	7.44	2.37	11.35
TMX3+MEL+PP	10.56	3.36	14.42

Figure 6.6: Graphical representation of estimated morphological parameters of control, TMX3, TMX3+MEL, TMX3+PP and TMX3+MEL+PP treated *Brassica juncea* seedlings respectively.

6.3 Photosynthetic parameter analysis

Photosynthetic parameters estimation allowed us to infer the effect of thiamethoxam, melatonin and *P. putida* on *Brassica juncea* seedlings. Photosynthetic parameters including total chlorophyll, chlorophyl a, chlorophyl b, carotenoid, flavonoid and anthocyanin contents were analyzed in the present investigation.

6.3.1 Total chlorophyll content

Total chlorophyll content of the *Brassica juncea* seedlings grown under the influence of thiamethoxam, melatonin and *P. putida* is referred to Table 6.3. The statistical analysis of the observed content in triplicates revealed that total chlorophyll content of experimental untreated *Brassica juncea* seedlings was 25.91 mg/g FW (significant at $p<0.05$) higher than thiamethoxam (TMX3) treated seedlings suggesting the negative impact of thiamethoxam on total chlorophyll content. The observed difference in total chlorophyll content of TMX3+MEL treated seedlings compared to TMX3 treated seedlings by 17.43 mg/g FW ($p<0.05$) suggests the ameliorative impact of melatonin in enhancing the total chlorophyll content. Similarly, TMX3+PP treated seedlings were also observed to show elevated total chlorophyll content compared to TMX3 treated seedlings by 18.01 mg/g FW implying the remedial role of *P. putida*. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to have highest retrieval of total chlorophyll content when compared with TMX3 treated seedlings by 19.49 mg/g FW ($p<0.05$) signifying the positive role of combinatorial application in reviving total chlorophyll content of thiamethoxam stressed *Brassica juncea* seedlings.

6.3.2 Estimation of Chlorophyll a content

Chlorophyll a content of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.3. The chlorophyll a content of untreated experimental *Brassica juncea* seedlings was observed to be significantly ($p<0.05$) higher than TMX3 treated seedlings by 19.31 mg/g FW which signified the role of thiamethoxam in decreasing chlorophyll a content of *Brassica juncea* seedlings. The ameliorative impact of melatonin was observed as chlorophyl a content of TMX3+MEL

treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 8.22 mg/g FW. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of chlorophyll a content by 7.69 mg/g FW when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to retain the chlorophyll a content in *Brassica juncea* seedlings by 12.19 mg/g FW as compared to TMX3 treated seedlings implying that combinatorial application significantly restored the chlorophyll a content of thiamethoxam treated *Brassica juncea* seedlings.

6.3.3 Estimation of Chlorophyll b content

Chlorophyll b content of the *Brassica juncea* seedlings grown under the influence of thiamethoxam, melatonin and *P. putida* is referred to Table 6.3. The statistical analysis revealed that chlorophyll b content of experimental untreated *Brassica juncea* seedlings was 2.55 mg/g FW (significant at $p<0.05$) higher than thiamethoxam (TMX3) treated seedlings suggesting the negative impact of thiamethoxam on chlorophyll b content. The observed difference in chlorophyll b content of TMX3+MEL treated seedlings compared to TMX3 treated seedlings by 1.21 mg/g FW ($p<0.05$) suggested the ameliorative impact of melatonin in enhancing the chlorophyll b content. Similarly, TMX3+PP treated seedlings were also observed to show elevated chlorophyll b content as compared to TMX3 treated seedlings by 0.78 mg/g FW. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to have highest revival of chlorophyll b content when compared with TMX3 treated seedlings by 1.59 mg/g FW ($p<0.05$) signifying the positive impact of combinatorial application in restoring chlorophyll b content of thiamethoxam stressed *Brassica juncea* seedlings.

Table 6.3: Total chlorophyll, chlorophyll a and chlorophyll b estimation of thiamethoxam treated *Brassica juncea* seedlings enriched by melatonin and *P. putida* alone as well as in combination respectively. The values are estimated in triplicates and presented as mean ± SD.

Treatments	**Total chlorophyll (mg/g FW± SD)**	**Chlorophyll a (mg/g FW± SD)**	**Chlorophyll b (mg/g FW± SD)**
Control	36.54 ± 0.03	31.55 ± 0.02	3.62 ± 0.05
MEL	49.03 ± 0.02	36.94 ± 0.02	4.87 ± 0.03
P.P	44.22 ± 0.03	33.62 ± 0.01	3.78 ± 0.04
TMX1	17.24 ± 0.03	18.07 ± 0.02	1.44 ± 0.04
TMX2	14.03 ± 0.02	13.97 ± 0.02	1.27 ± 0.04
TMX3	10.63 ± 0.03	12.24 ± 0.04	1.07 ± 0.02
TMX1+MEL	32.64 ± 0.02	27.34 ± 0.03	3.08 ± 0.04
TMX2+MEL	29.23 ± 0.02	24.57 ± 0.02	2.55 ± 0.04
TMX3+MEL	28.07 ± 0.02	20.47 ± 0.02	2.29 ± 0.04
TMX1+PP	31.75 ± 0.03	26.07 ± 0.02	2.55 ± 0.04
TMX2+PP	29.14 ± 0.01	22.97 ± 0.03	2.16 ± 0.03
TMX3+PP	28.64 ± 1.15	19.94 ± 0.02	1.85 ± 0.04
TMX1+MEL+PP	35.94 ± 0.03	29.56 ± 0.34	3.66 ± 0.03
TMX2+MEL+PP	34.14 ± 0.02	28.82 ± 0.03	2.95 ± 0.04
TMX3+MEL+PP	30.13 ± 0.02	24.43 ± 0.02	2.66 ± 0.03
Univariate ANOVA Test Statistics	**Total Chlorophyll Vs Treatments**	**Chlorophyll a Vs Treatments**	**Chlorophyll b Vs Treatments**
	F = 3559.610	**F** = 18279.163	**F** = 2748.338
Control Vs TMX3	**HSD** = 25.910*	**HSD** = 19.310*	**HSD** = 2.550*
TMX3+MEL Vs TMX3	**HSD** = 17.433*	**HSD** = 8.223*	**HSD** = 1.216*
TMX3+PP Vs TMX3	**HSD** = 18.010*	**HSD** = 7.696*	**HSD** = 0.780*
TMX3+MEL+PP Vs TMX3	**HSD** = 19.493*	**HSD** = 12.190*	**HSD** = 1.590*

*Note: *Represents the significant mean difference at p< 0.05 level*

6.3.4 Estimation of Carotenoid content

Carotenoid content of *Brassica juncea* seedlings treated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.4. The carotenoid content of untreated *Brassica juncea*

seedlings was observed to be significantly ($p<0.05$) higher than TMX3 treated seedlings by 3.00 mg/g FW which signified the role of thiamethoxam in decreasing carotenoid content of *Brassica juncea* seedlings. The ameliorative impact of melatonin was observed as carotenoid content of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 2.57 mg/g FW. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of carotenoid content by 1.39 mg/g FW when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to retain the carotenoid content in *Brassica juncea* seedlings by 3.29 mg/g FW as compared to TMX3 treated seedlings implying that combinatorial application significantly restored the carotenoid content of thiamethoxam treated *Brassica juncea* seedlings.

6.3.5 Estimation of Anthocyanin content

Anthocyanin content of the *Brassica juncea* seedlings grown under the influence of thiamethoxam, melatonin and *P. putida* is shown in Table 6.4. The statistical analysis revealed that anthocyanin content of untreated *Brassica juncea* seedlings was 1.21 mg/g FW (significant at $p<0.05$) higher than thiamethoxam (TMX3) treated seedlings suggesting the negative impact of thiamethoxam on anthocyanin content. The observed difference in anthocyanin content of TMX3+MEL treated seedlings compared to TMX3 treated seedlings by 0.90 mg/g FW ($p<0.05$) suggested the ameliorative impact of melatonin in enhancing the anthocyanin content. Similarly, TMX3+PP treated seedlings were also observed to show elevated anthocyanin content as compared to TMX3 treated seedlings by 1.70 mg/g FW. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to have highest revival of anthocyanin content when compared with TMX3 treated seedlings by 3.11 mg/g FW ($p<0.05$) signifying the positive impact of combinatorial application in restoring anthocyanin content of thiamethoxam stressed *Brassica juncea* seedlings.

6.3.6 Estimation of Flavonoid content

Flavonoid content of *Brassica juncea* seedlings treated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.4. The flavonoid content of untreated *Brassica juncea* seedlings was observed to be significantly ($p<0.05$) higher than TMX3 treated seedlings by 0.26 mg/g FW. The flavonoid content of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 0.25 mg/g FW. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of flavonoid content by 0.54 mg/g FW when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to retain the flavonoid content in *Brassica juncea* seedlings by 0.90 mg/g FW as compared to TMX3 treated *Brassica juncea* seedlings.

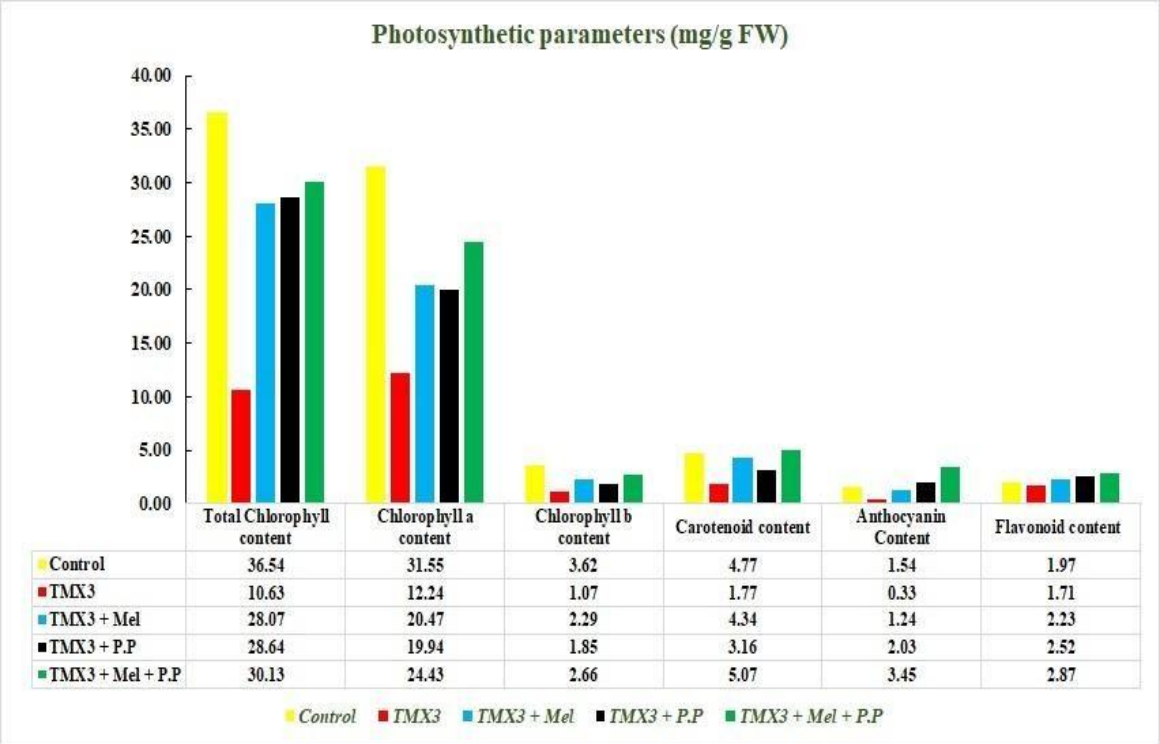
The estimation of photosynthetic parameters in the present investigation revealed that application of thiamethoxam (TMX3) leads to decrease in photosynthetic content of treated *Brassica juncea* seedlings while the distinct application of melatonin (MEL) and *P. putida* (PP) was able to retain the photosynthetic content of thiamethoxam treated *Brassica juncea* seedlings. The highest revival of photosynthetic content in all of the estimated parameters was observed in combinatorial application of melatonin and *P. putida* implying this strategy to be most effective in rejuvenating thiamethoxam stressed *Brassica juncea* seedlings. The graphical representation of the estimated photosynthetic content among various treatments is shown in Figure 6.7.

Table 6.4: Carotenoid, Anthocyanin and Flavonoid content estimation of thiamethoxam treated *Brassica juncea* seedlings enriched by melatonin and *P. putida* alone as well as in combination respectively. The values are estimated in triplicates and presented as mean ± SD.

Treatments	**Carotenoid (mg/g FW± SD)**	**Anthocyanin (mg/g FW± SD)**	**Flavonoid (mg/g FW± SD)**
Control	4.77 ± 0.02	1.54 ± 0.03	1.97 ± 0.02
MEL	4.94 ± 0.03	1.65 ± 0.03	2.16 ± 0.01
P.P	4.22 ± 0.03	1.55 ± 0.02	2.03 ± 0.02

TMX1	2.86 ± 0.02	1.05 ± 0.03	1.86 ± 0.01
TMX2	2.94 ± 0.03	0.92 ± 0.02	1.78 ± 0.02
TMX3	1.77 ± 0.02	0.33 ± 0.02	1.71 ± 0.02
TMX1+MEL	3.93 ± 0.02	1.98 ± 0.02	2.74 ± 0.01
TMX2+MEL	4.27 ± 0.02	1.58 ± 0.02	2.45 ± 0.01
TMX3+MEL	4.34 ± 0.55	1.24 ± 0.03	2.23 ± 0.02
TMX1+PP	3.73 ± 0.02	1.46 ± 0.02	1.99 ± 0.01
TMX2+PP	3.86 ± 0.03	1.72 ± 0.02	2.37 ± 0.01
TMX3+PP	3.16 ± 0.03	2.03 ± 0.02	2.52 ± 0.01
TMX1+MEL+PP	5.15 ± 0.03	2.13 ± 0.02	3.43 ± 0.01
TMX2+MEL+PP	5.53 ± 0.02	2.65 ± 0.02	3.08 ± 0.01
TMX3+MEL+PP	5.07 ± 0.02	3.45 ± 0.02	2.87 ± 0.02
Univariate ANOVA Test Statistics	**Carotenoid Vs Treatments**	**Anthocyanin Vs Treatments**	**Flavonoid Vs Treatments**
	F = 155.019	**F** = 3309.141	**F** = 5218.734
Control Vs TMX3	**HSD** = 3.003*	**HSD** = 1.210*	**HSD** = 0.260*
TMX3+MEL Vs TMX3	**HSD** = 2.570*	**HSD** = 0.903*	**HSD** = 0.253*
TMX3+PP Vs TMX3	**HSD** = 1.390*	**HSD** = 1.700*	**HSD** = 0.546*
TMX3+MEL+PP Vs TMX3	**HSD** = 3.296*	**HSD** = 3.113*	**HSD** = 0.900*

*Note: *Represents the significant mean difference at p< 0.05 level*



	Total Chlorophyll content	Chlorophyll a content	Chlorophyll b content	Carotenoid content	Anthocyanin Content	Flavonoid content
Control	36.54	31.55	3.62	4.77	1.54	1.97
TMX3	10.63	12.24	1.07	1.77	0.33	1.71
TMX3 + Mel	28.07	20.47	2.29	4.34	1.24	2.23
TMX3 + P.P	28.64	19.94	1.85	3.16	2.03	2.52
TMX3 + Mel + P.P	30.13	24.43	2.66	5.07	3.45	2.87

Figure 6.7: Graphical representation of estimated photosynthetic parameters of control, TMX3, TMX3+MEL, TMX3+PP and TMX3+MEL+PP treated *Brassica juncea* seedlings respectively.

In contemporary study the application of TMX reduced the chlorophyll content and pigments viz, anthocyanin, carotenoids and flavonoids. Further the contents of all parameters have shown prominent increase in melatonin and *P. putida* treated seedling which were subjected to TMX. Drop in chlorophyll content can be due to higher chlorophyllase activity, chloroplast degradation and chlorophyll oxidation lead by ROS (Harpaz-Saad *et al.*, 2007). Moreover, it is documented that TMX decreases chlorophyll content in algae (Al-Badri and Suaad, 2020), affects the photosynthetic pigment and activity of photosynthetic apparatus in *Zea mays* (Todorenko *et al.*, 2020). However, chlorophyll content was improved by application of melatonin and *P. putida*. The melatonin plays a role in up-regulation transcription and translation processes (Arnao and Hernandez-Ruiz, 2015). Studies also suggest that exogenous application of melatonin alleviates chlorophyll degradation and improves photosynthetic rate thereby quashing the

pernicious effect of abiotic stresses (Zhang *et al.,* 2013). Also PGPRs are known to enhance pigment content in plants during stressed condition. The strains of *Pseudomonas* drastically increased the chlorophyll content in abiotic stressed *Lycopersicum esculentum* (Khanna *et al.,* 2019). *Pseudomonas* bacteria increases plant mineral uptake (phosphorus), due to its extreme potential to dissolve insoluble phosphate. Thereupon, phosphorus acts as energy transporter, enhances uptake and movement of nitrogen to leaves, resulting in higher production of chlorophyll and also stimulates ATPase protein pump activity (Vatanpour *et al.,* 2020). In current study, the content of carotenoid and anthocyanin decreased under the influence of TMX, whereas, no effect in the content of flavonoid was observed, which suggests that TMX has negative impact on carotenoid and anthocyanin photosynthetic pigments. Withal, seed priming with melatonin enhanced the photosynthetic pigment content which is supported by the study (Wei *et al.,* 2015) where melatonin up-regulated the expression of photosynthetic genes. Similar evidences were reported where melatonin imparted protective role on photosynthetic pigments in abiotic stressed *Triticum* (Zhang *et al.,* 2021) and *Solanum lycopersicum* (Altaf *et al.,* 2021). Likewise, contents of photosynthetic pigments were augmented when *P. putida* and melatonin were used in combination in seedlings subjected to TMX. Past studies proclaim that *P. putida* significantly increases the carotenoid content in *Daucus carota* under stressed conditions (Ahamad and Zaki, 2021). Similarly, increments in photosynthetic pigment (carotenoid) by P. putida were observed in Gossypium under abiotic stress (He et al., 2017) and in Triticum aestivum under pesticide stress (Munir *et al.,* 2019).

6.4 Osmo-protectant parameter analysis

Estimation of osmoprotectant parameters allowed us to infer the effect of thiamethoxam, melatonin and *P. putida* on *Brassica juncea* seedlings. Osmoprotectant parameters including trehalose, glycine betaine and proline contents were analyzed in the present investigation.

6.4.1 Estimation of Trehalose content

Trehalose content of the *Brassica juncea* seedlings grown under the influence of thiamethoxam, melatonin and *P. putida* is shown in Table 6.5. The statistical analysis revealed that trehalose content of thiamethoxam (TMX3) treated seedlings was 8.75 mg/g DW (significant at $p<0.05$) higher than untreated *Brassica juncea* seedlings suggesting the impact of thiamethoxam on trehalose content. The observed difference in trehalose content of TMX3+MEL treated seedlings compared to TMX3 treated seedlings by 3.85 mg/g DW ($p<0.05$) suggested the ameliorative impact of melatonin in enhancing the trehalose content. Similarly, TMX3+PP treated seedlings were also observed to show elevated trehalose content as compared to TMX3 treated seedlings by 5.19 mg/g DW. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to have highest revival of trehalose content when compared with TMX3 treated seedlings by 10.03 mg/g DW ($p<0.05$) signifying the positive impact of combinatorial application in restoring trehalose content of thiamethoxam stressed *Brassica juncea* seedlings.

6.4.2 Estimation of Glycine-betaine content

Glycine-betaine content of the *Brassica juncea* seedlings grown under the influence of thiamethoxam, melatonin and *P. putida* is referred to Table 6.5. The statistical analysis of the observed content in triplicates revealed that glycine-betaine content of thiamethoxam (TMX3) treated seedlings was 2.29 mg/g FW (significant at $p<0.05$) higher than experimental untreated *Brassica juncea* seedlings suggesting the impact of thiamethoxam on glycine-betaine content. The observed difference in glycine-betaine content of TMX3+MEL treated seedlings compared to TMX3 treated seedlings by 2.72 mg/g FW ($p<0.05$) suggests the ameliorative impact of melatonin in enhancing the glycine-betaine content. Similarly, TMX3+PP treated seedlings were also observed to show elevated glycine-betaine content compared to TMX3 treated seedlings by 3.01 mg/g FW implying the remedial role of *P. putida*. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to have highest retrieval of glycine-betaine content when compared with TMX3 treated seedlings by 7.68 mg/g FW ($p<0.05$) signifying the

positive role of combinatorial application in reviving glycine-betaine content of thiamethoxam stressed *Brassica juncea* seedlings.

6.4.2 Estimation of Proline content

Proline content of *Brassica juncea* seedlings treated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.5. The proline content of TMX3 treated seedlings was observed to be significantly ($p<0.05$) higher than untreated *Brassica juncea* seedlings by 4.12 mg/g FW. The proline content of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 1.43 mg/g FW. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of proline content by 1.97 mg/g FW when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to retain the proline content in *Brassica juncea* seedlings by 5.41 mg/g FW as compared to TMX3 treated *Brassica juncea* seedlings.

The estimation of osmo-protectant parameters in the present investigation revealed that application of thiamethoxam (TMX3) leads to increase in osmo-protectant content of treated *Brassica juncea* seedlings while the distinct application of melatonin (MEL) and *P. putida* (PP) was able to retain the osmo-protectant content of thiamethoxam treated *Brassica juncea* seedlings. The highest revival of osmo-protectant content in all of the estimated parameters was observed in combinatorial application of melatonin and *P. putida* implying this strategy to be most effective in rejuvenating thiamethoxam stressed *Brassica juncea* seedlings. The graphical representation of the estimated osmo-protectant content among various treatments is shown in Figure 6.8.

Table 6.5: Trehalose, Glycine-betaine and Proline content estimation of thiamethoxam treated *Brassica juncea* seedlings enriched by melatonin and *P. putida* alone as well as in combination respectively. The values are estimated in triplicates and presented as mean ± SD.

Treatments	Trehalose (mg/g DW± SD)	Glycine betaine (mg/g FW± SD)	Proline (mg/g FW± SD)
Control	15.72 ± 0.13	2.82 ± 0.07	1.62 ± 0.02
MEL	18.46 ± 0.16	3.52 ± 0.05	2.13 ± 0.01
P.P	19.46 ± 0.19	3.87 ± 0.06	2.66 ± 0.01
TMX1	21.19 ± 0.14	4.08 ± 0.04	3.88 ± 0.10
TMX2	22.20 ± 0.11	4.80 ± 0.06	5.27 ± 0.10
TMX3	24.48 ± 0.12	5.12 ± 0.05	5.75 ± 0.14
TMX1+MEL	23.58 ± 0.11	5.71 ± 0.08	4.41 ± 0.14
TMX2+MEL	25.49 ± 0.17	6.25 ± 0.04	6.41 ± 0.12
TMX3+MEL	28.33 ± 0.12	7.84 ± 0.06	7.18 ± 0.16
TMX1+PP	24.85 ± 0.10	6.68 ± 0.44	5.84 ± 0.10
TMX2+PP	27.58 ± 0.09	6.60 ± 0.14	7.25 ± 0.25
TMX3+PP	29.67 ± 0.11	8.14 ± 0.06	7.72 ± 0.14
TMX1+MEL+PP	31.86 ± 0.09	8.73 ± 0.05	8.63 ± 0.10
TMX2+MEL+PP	32.13 ± 0.06	10.37 ± 0.06	9.42 ± 0.09
TMX3+MEL+PP	34.51 ± 0.11	12.80 ± 0.06	11.16 ± 0.08
Univariate ANOVA Test Statistics	**Trehalose Vs Treatments**	**Glycine betaine Vs Treatments**	**Proline Vs Treatments**
	F = 5817.021	**F** = 1339.681	**F** = 1578.704
TMX3 Vs Control	**HSD** = 8.756*	**HSD** = 2.296*	**HSD** = 4.123*
TMX3+MEL Vs TMX3	**HSD** = 3.853*	**HSD** = 2.723*	**HSD** = 1.433*
TMX3+PP Vs TMX3	**HSD** = 5.193*	**HSD** = 3.016*	**HSD** = 1.976*
TMX3+MEL+PP Vs TMX3	**HSD** = 10.033*	**HSD** = 7.680*	**HSD** = 5.410*

*Note: *Represents the significant mean difference at p< 0.05 level*

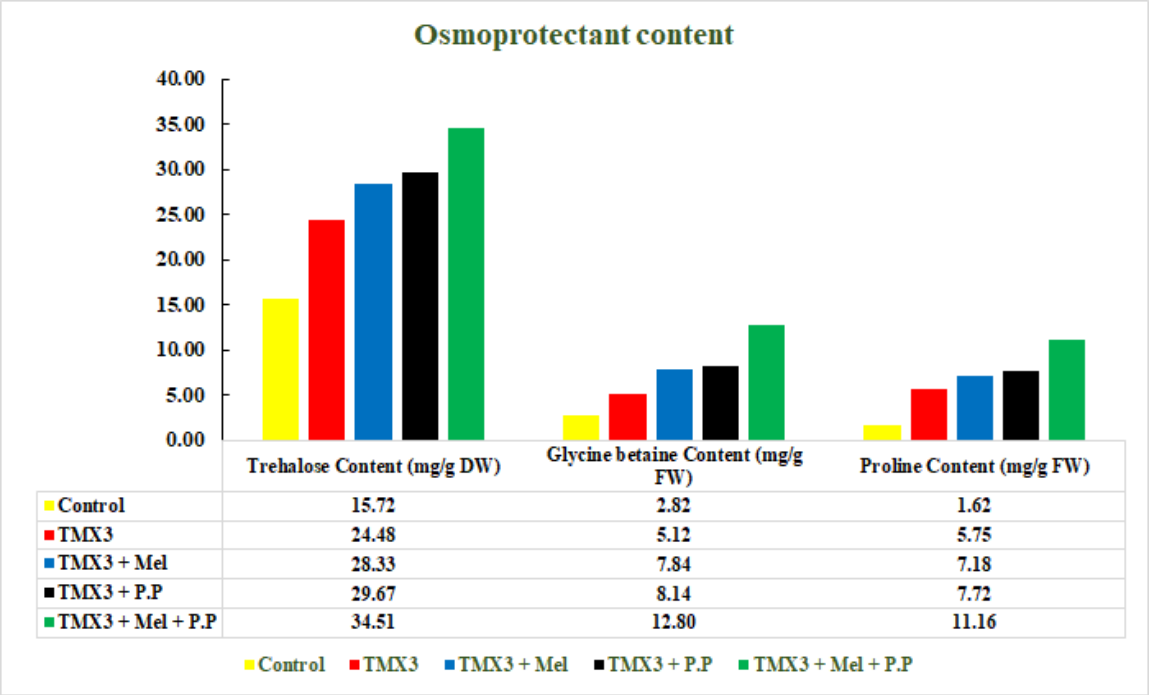


Figure 6.8: Graphical representation of estimated osmoprotectant parameters of control, TMX3, TMX3+MEL, TMX3+PP and TMX3+MEL+PP treated *Brassica juncea* seedlings respectively.

Osmo-protectants possess defensive properties. Increment in osmolyte content viz, glycine-betaine, trehalose and proline induced by ameliorative under TMX stress was investigated in current study. Findings are in line with Homayoonzadeh *et al.* (2021) which states that osmolyte content increases during pesticides toxicity and acts as a stress indicator. Increase in proline content in pesticide exposed plants is attributed to cellular-dehydration as reported by Yildiztekin *et al.* (2015). Proline is regarded as vital metabolite and in response to oxidative stress it accumulates within plant tissues in order to protect from damage. The main role of proline is to detoxify ROS, stabilize enzymes, acts as osmo-protectant and protect cell membrane of stressed plants (Boaretto *et al.*, 2014). It is reported, overdosing of pesticide causes an increase in proline level in *Solanum lycopersicum*. Enhanced level of proline in pesticide dosed plants reveals its protective role under unfavorable conditions (Shakir *et al.*, 2018). Further, in our

investigation, seed priming with melatonin elevated the levels of proline in TMX treated seedlings which suggests better osmotic balance during TMX toxicity and assists plant to maintain normal functioning and growth. It is in accordance with the study conducted, which proclaims that melatonin promotes proline accumulation in stressed rapeseed plant (Khan *et al.,* 2020; Sharma *et al.,* 2020). Similarly, in *Medicago sativa* upsurge in proline content was noticed suggesting its role in protecting cellular damage and scavenging ROS (Antoniou *et al.,* 2017). The role of *P. putida* in increasing the proline content in *Medicago sativa* under pesticide stress has also been reported (Munir *et al.,* 2019) which corroborates with our present study where analogous results were achieved (Table6.5 and Figure 6.8). PGPRs are not only known for promoting plant growth, degrading pesticide residue, but also mitigate the signs of stress .A study divulges, that PGPR, enhances proline production under pesticide stress in *Vigna unguiculata* thereby imparting tolerance against stress conditions (Inthama *et al.,* 2021). The role of *P. putida* under stress conditions was studied, where increase in proline content was observed in stressed chickpea, suggesting its impact on restoration and recovery of cultivars (Tiwari *et al.,* 2016).

In current study the role of osmolytes has been elucidated in *B. juncea* under TMX toxicity. It serves as a first of a kind, where impact of TMX on glycine-betaine (GB) and trehalose content is investigated. Antecedent reports suggests that GB and trehalose content is crucial for maintaining osmotic balance, membrane stability (Aldesuquy and Ghanem, 2015), improving photosynthetic rates (Manaf, 2016), detoxifying ROS and protecting plants from stresses (Kerchev *et al.,* 2020). Trehalose an imperative carbohydrate acts as an osmolyte and helps plant to maintain their cellular integrity during stress conditions (Jain and Roy, 2009). It is documented that trehalose aggregation provides protection to plants under stressed conditions (John *et al.,* 2017) and elevation in GB levels enables plants to combat stress and provides tolerances against toxicity (Kohli *et al.,* 2018). There is a report which states that biofertilizer cyanobacterium produces more osmolytes as a defensive strategy against pesticide stress (Habib *et al.,* 2011). The upsurge in GB content is linked with reduction in lipid peroxidation in plants (Cushman,

2001) also it is suggested that GB acts as signaling molecule that induces expression of stress responsive genes (Rontein *et al.,* 2002). Our study corroborates with Wei *et al.* 2014, where melatonin promoted trehalose synthesis under abiotic stress and (Zhang *et al.,* 2021) reports that melatonin significantly elevated the contents of GB in stressed sugar beet. Moreover melatonin treatment enhances accumulation of osmolytes in order to shield plants against abiotic stress, as observed in *Zea mays* (Li *et al.,* 2019) and *Actinidia deliciosa* (Liang *et al.,* 2018) seedlings subjected to abiotic stress conditions. Melatonin mediated osmolyte accumulation is associated with overall development of plant and reducing ROS induced oxidative stress (Rehaman *et al.,* 2021). Protective role of PGPRs against stress is in accordance with our current study, which unravels that *P. putida* plays a significant role in increasing the contents of osmolytes in *B. juncea* under stress conditions. PGPRs have the potential to improve biosynthesis of GB and trehalose which in turn promotes stress tolerance in plants (Gou *et al.,* 2015; Khanna *et al.,* 2019). These beneficial microbes lead to the upliftment of osmo-protectants in plants during stress conditions.

6.5 Oxidative burst parameter analysis

Estimation of oxidative burst parameters allowed us to infer the effect of thiamethoxam, melatonin and *P. putida* on *Brassica juncea* seedlings. Osmoprotectant parameters including superoxide anion, hydrogen peroxide and MDA contents were analyzed in the present investigation.

6.5.1 Superoxide anion content estimation

Superoxide anion content of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.6. The superoxide anion content of TMX3 treated seedlings was observed to be significantly ($p<0.05$) higher than untreated experimental *Brassica juncea* seedlings by 3.68 µmol/g FW, implying accumulation of superoxide anions under thiamethoxam stress. Application of melatonin in thiamethoxam treated *Brassica juncea* seedlings was observed to significantly ($p<0.05$) reduce the

superoxide anion content in TMX3+MEL treatment as compared to TMX3 treated seedlings by 2.40 µmol/g FW which signified the superoxide anion neutralization potential of the melatonin in the present investigation. Similarly, TMX3+PP was also observed to reduce the superoxide anion content by 1.57 µmol/g FW as compared to TMX3 treated seedlings suggesting the superoxide anion counteract activity of the *P. putida.* The combinatorial application TMX3+MEL+PP, showed highest activity by reducing the superoxide anion content by 3.15 µmol/g FW on comparison to TMX3 treated seedlings which corresponds to synergism among melatonin and *P. putida* to restrain superoxide anion content in thiamethoxam treated *Brassica juncea* seedlings. The graphical representation of the estimated superoxide anion content among various treatments is shown in Figure 6.9.

6.5.2 Hydrogen peroxide content estimation

Hydrogen peroxide content of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.6. The hydrogen peroxide content of TMX3 treated seedlings was observed to be significantly ($p<0.05$) higher than untreated experimental *Brassica juncea* seedlings by 8.67 µmol/g FW, implying accumulation of superoxide anions under thiamethoxam stress. Application of melatonin in thiamethoxam treated *Brassica juncea* seedlings was observed to significantly ($p<0.05$) reduce the hydrogen peroxide content in TMX3+MEL treatment as compared to TMX3 treated seedlings by 2.07 µmol/g FW which signified the hydrogen peroxide neutralization potential of the melatonin in the present investigation. Similarly, TMX3+PP was also observed to reduce the hydrogen peroxide content by 0.55 µmol/g FW as compared to TMX3 treated seedlings suggesting the hydrogen peroxide counteract activity of the *P. putida.* The combinatorial application TMX3+MEL+PP, showed highest activity by reducing the hydrogen peroxide content by 3.12 µmol/g FW on comparison to TMX3 treated seedlings which corresponds to synergism among melatonin and *P. putida* to restrain hydrogen peroxide content in thiamethoxam treated *Brassica juncea* seedlings.

The graphical representation of the estimated hydrogen peroxide content among various treatments is shown in Figure 6.9.

6.5.3 MDA content estimation

MDA content of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.6. The MDA content of TMX3 treated seedlings was observed to be significantly ($p<0.05$) higher than untreated experimental *Brassica juncea* seedlings by 2.56 µmol/g FW, implying accumulation of MDA under thiamethoxam stress. Application of melatonin in thiamethoxam treated *Brassica juncea* seedlings was observed to significantly ($p<0.05$) reduce the MDA content in TMX3+MEL treatment as compared to TMX3 treated seedlings by 1.51 µmol/g FW which signified the MDA neutralization potential of the melatonin in the present investigation. Similarly, TMX3+PP was also observed to reduce the hydrogen peroxide content by 1.34 µmol/g FW as compared to TMX3 treated seedlings suggesting the MDA counteract activity of the *P. putida*. The combinatorial application TMX3+MEL+PP, showed highest activity by reducing the MDA content by 2.05 µmol/g FW on comparison to TMX3 treated seedlings which corresponds to synergism among melatonin and *P. putida* to restrain MDA content in thiamethoxam treated *Brassica juncea* seedlings. The graphical representation of the estimated MDA content among various treatments is shown in Figure 6.9.

Table 6.6: Superoxide anion, Hydrogen peroxide and MDA content estimation of thiamethoxam treated *Brassica juncea* seedlings enriched by melatonin and *P. putida* alone as well as in combination respectively. The values are estimated in triplicates and presented as mean ± SD.

Treatments	Superoxide Anion (µmole/g FW)	Hydrogen peroxide (µmole/g FW)	MDA (µmole/g FW)
Control	1.65 ± 0.03	6.66 ± 0.02	3.43 ± 0.12
MEL	1.43 ± 0.01	6.43 ± 0.01	3.15 ± 0.12

P.P	1.75 ± 0.01	6.75 ± 0.01	3.57 ± 0.11
TMX1	4.04 ± 0.01	12.26 ± 0.03	5.13 ± 0.11
TMX2	4.41 ± 0.41	13.86 ± 0.04	5.34 ± 0.13
TMX3	5.33 ± 0.02	15.33 ± 0.02	5.99 ± 0.11
TMX1+MEL	2.27 ± 0.02	10.27 ± 0.02	4.13 ± 0.10
TMX2+MEL	2.59 ± 0.02	12.06 ± 0.02	4.33 ± 0.08
TMX3+MEL	2.92 ± 0.01	13.26 ± 0.03	4.47 ± 0.11
TMX1+PP	2.17 ± 0.02	11.17 ± 0.02	4.25 ± 0.12
TMX2+PP	2.73 ± 0.01	12.15 ± 0.02	4.35 ± 0.13
TMX3+PP	3.76 ± 0.01	14.77 ± 0.02	4.64 ± 0.12
TMX1+MEL+PP	1.94 ± 0.01	11.94 ± 0.01	2.85 ± 0.06
TMX2+MEL+PP	1.86 ± 0.03	11.86 ± 0.03	3.42 ± 0.11
TMX3+MEL+PP	2.17 ± 0.05	12.21 ± 0.02	3.94 ± 0.09
Univariate ANOVA Test Statistics	**Superoxide Anion Vs Treatments**	**Hydrogen peroxide Vs Treatments**	**MDA Vs Treatments**
	F = 339.432	**F** = 46422.774	**F** = 185.350
TMX3 Vs Control	**HSD** = 3.680*	**HSD** = 8.670*	**HSD** = 2.560*
TMX3+MEL Vs TMX3	**HSD** = -2.406*	**HSD** = -2.070*	**HSD** = -1.516*
TMX3+PP Vs TMX3	**HSD** = -1.570*	**HSD** = -0.556*	**HSD** = -1.346*
TMX3+MEL+PP Vs TMX3	**HSD** = -3.153*	**HSD** = -3.120*	**HSD** = -2.050*

*Note: *Represents the significant mean difference at p< 0.05 level*

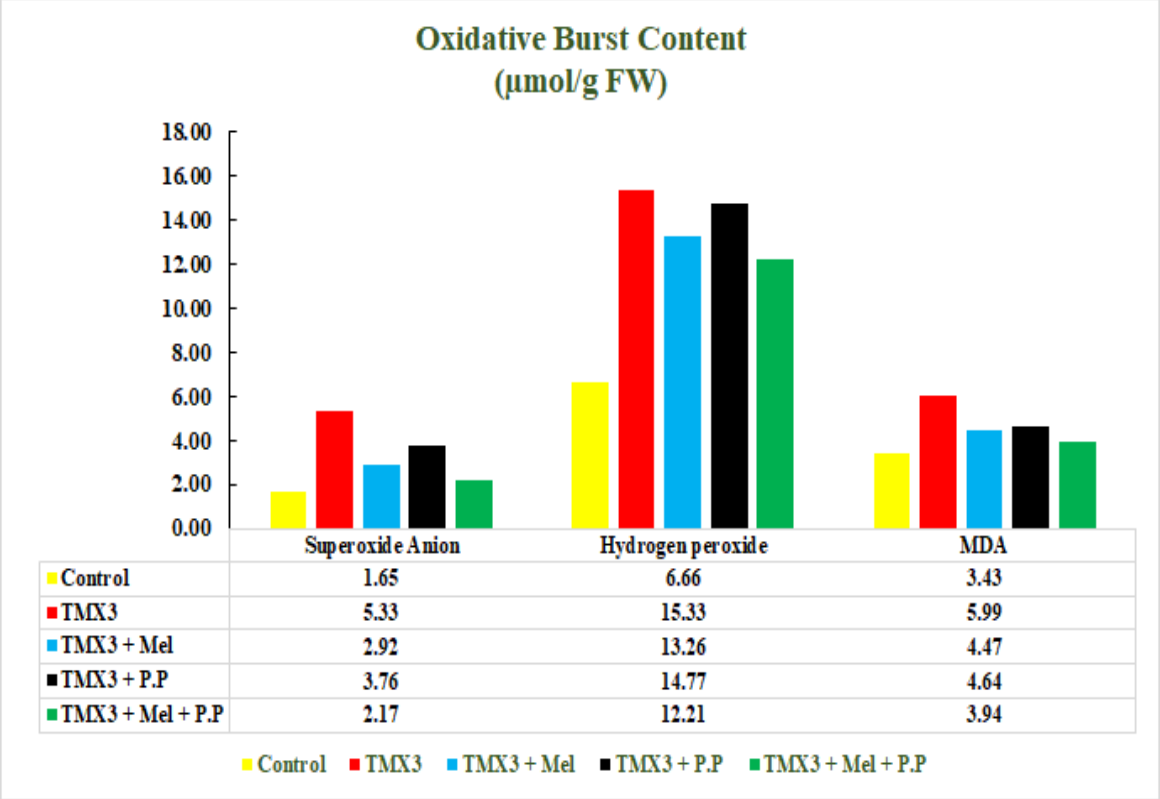


Figure 6.9: Graphical representation of estimated oxidative burst content of control, TMX3, TMX3+MEL, TMX3+PP and TMX3+MEL+PP treated *Brassica juncea* seedlings respectively.

Pesticide stress elicits oxidative stress and lipid peroxidation by generation of ROS viz, superoxide anion ($O^{\cdot-}_2$) and hydrogen peroxide (H_2O_2) and escalation in malondialdehyde (MDA) levels (Bakshi *et al.,* 2021). The accretion of activated oxygen molecule is caused by erratic creation and irregular detoxification cycle of ROS (Demidchik, 2015). Oxidative burst is the repercussion of abiotic stress conditions because it disturbs the antioxidative defense system (Mittler, 2002). In present investigation, the contents $O^{\cdot-}_2$, H_2O_2 and MDA elevated in TMX treated *B. juncea* seedlings which syncs with the observations made by Sharma *et al.,* (2017) where significant increase in $O^{\cdot-}_2$ and H_2O_2 was recorded in *B. juncea* under pesticide stress. Previous studies have reported an increase in the ROS proportions in tomato and bitter gourd plants exposed to pesticide stress (Mishra *et al.,* 2009; Shakir *et al.,* 2018). Similar observations have been reported

in the present investigation pertaining to elevated ROS levels of hydrogen peroxide and superoxide anion in TMX3 exposed *B. juncea* seedlings as compared to investigational untreated seedlings as depicted in Table 6.6 and Figure 6.9 respectively. Also similar study depicts that under pesticide stress there is an increment in the contents of ROS and MDA in *O. sativa* (Sharma *et al.,* 2015). Production of MDA content occurs due to deterioration poly-unsaturated fatty acids and serves as an indicator lipid peroxidation, which is an onset of cellular damage induced by pesticide toxicity (Parween *et al.,* 2012; Schmid-Siegert *et al.,* 2016). Besides, lipid peroxidation disrupts various membrane properties such as membrane integrity, ion transport, membrane fluidity, increased cell leakage etc and lipid peroxidation generating higher MDA content results in loss of cell viability (Mahapatra *et al.,* 2019). Rise in the levels of $O^{\cdot-}_2$, H_2O_2 and MDA during pesticide stress has been demonstrated in *Pisum sativum* (Singh *et al.,* 2016), *Cucumis sativus* (Liu *et al.,* 2021) and *Solanum lycopersicum* (Yildiztekin *et al.,* 2019). In current examination, diminution in H_2O_2 and $O^{\cdot-}_2$ was noticed in *B. juncea* seedlings when amended with melatonin and *P. putida* under pesticide stress. Our findings also unveil that melatonin and PGPR aids in decreasing MDA levels in plants during pesticide exposure indicating their ameliorative role towards membrane impairment. Concomitantly, Park *et al.,* (2013) revealed that supplementation of melatonin in rice plants provides resistance against herbicide triggered oxidative stress. Melatonin efficiently lowers H_2O_2 and MDA levels in herbicide treated rice seedlings. In another study, pesticide induced $O^{\cdot-}_2$, H_2O_2 and MDA generation was redressed by exogenous application of melatonin (Liu *et al.,* 2021). The main site of melatonin biosynthesis is mitochondria and chloroplast and in parallel ROS generation also takes place in these organelles therefore the ROS produced during adverse conditions can be cleared effectively. Moreover melatonin has powerful ability to scavenge ROS (Zhang *et al.,* 2017). The role of PGPR against pesticide mediated oxidative stress has been studied earlier which signifies that microbial inoculation alleviates the ROS mediated severity in plants via degrading the pesticide and lowering the production of ROS (Wang *et al.,* (2020). It is in accordance with our current study demonstrating that inoculation of *P.*

putida in TMX treated *B. juncea* can remarkably reduce the contents of ROS and MDA. Our results are also supported by the study carried out by Bourahla *et al.,* (2018) which reveals that *P. putida* eases phytotoxic effects generated by herbicides in durum wheat seedling and significantly reduced the levels of MDA.

6.6 Biochemical analysis

Biochemical analysis was determined by calculating protein content, antioxidative enzymes activity, content of antioxidants and organic acid profiling.

6.6.1 Protein content estimation

Protein content of the *Brassica juncea* seedlings grown under the influence of thiamethoxam, melatonin and *P. putida* is shown in Table 6.7. The statistical analysis revealed that protein content of untreated *Brassica juncea* seedlings was 2.60 mg/g FW (significant at $p<0.05$) higher than thiamethoxam (TMX3) treated seedlings suggesting the negative impact of thiamethoxam on protein content. The observed difference in protein content of TMX3+MEL treated seedlings compared to TMX3 treated seedlings by 5.55 mg/g FW ($p<0.05$) suggested the ameliorative impact of melatonin in enhancing the protein content. Similarly, TMX3+PP treated seedlings were also observed to show elevated protein content as compared to TMX3 treated seedlings by 6.58 mg/g FW. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally retain the protein content when compared with TMX3 treated seedlings by 8.21 mg/g FW ($p<0.05$) signifying the positive impact of combinatorial application in restoring protein content of thiamethoxam stressed *Brassica juncea* seedlings. The estimated protein content of various treatments is represented graphically in Figure 6.10.

Table 6.7: Protein content estimation of thiamethoxam treated *Brassica juncea* seedlings enriched by melatonin and *P. putida* alone as well as in combination respectively. The values are estimated in triplicates and presented as mean ± SD.

Treatments	**Protein Content (mg/g FW)**
Control	6.75 ± 0.09
MEL	8.34 ± 0.13
P.P	8.93 ± 0.07
TMX1	5.05 ± 0.09
TMX2	4.54 ± 0.10
TMX3	4.15 ± 0.08
TMX1+MEL	18.44 ± 0.13
TMX2+MEL	11.92 ± 0.09
TMX3+MEL	9.71 ± 0.03
TMX1+PP	20.25 ± 0.07
TMX2+PP	12.57 ± 0.12
TMX3+PP	10.74 ± 0.09
TMX1+MEL+PP	24.06 ± 0.10
TMX2+MEL+PP	19.43 ± 0.11
TMX3+MEL+PP	12.37 ± 0.12
Univariate ANOVA Test Statistics	**Protein content Vs Treatments**
	F = 12007.446
Control Vs TMX3	**HSD** = 2.600*
TMX3+MEL Vs TMX3	**HSD** = 5.556*
TMX3+PP Vs TMX3	**HSD** = 6.586*
TMX3+MEL+PP Vs TMX3	**HSD** = 8.213*

*Note: *Represents the significant mean difference at p< 0.05 level*

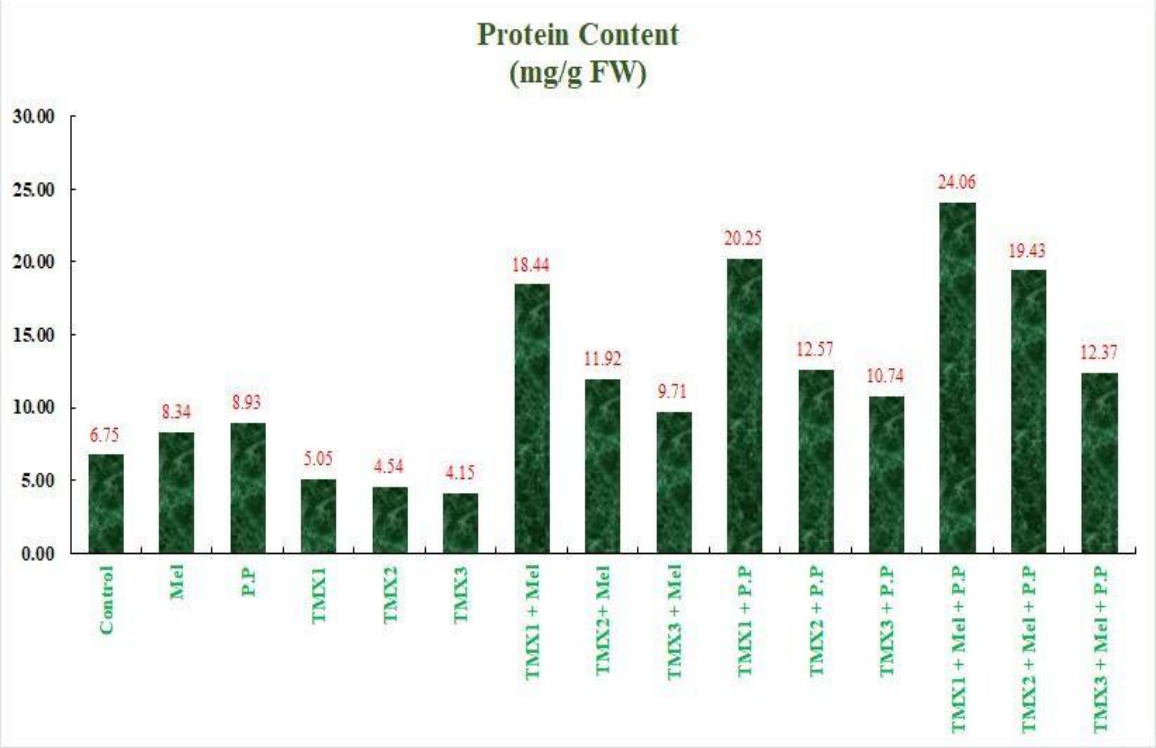


Figure 6.10: Graphical representation of estimated protein content of various treatments in *Brassica juncea* seedlings respectively.

6.6.2 Enzymatic antioxidant activity

The activities of SOD, CAT, POD, DHAR, GST, GR, APOX and GPOX were evaluated.

6.6.2.1 SOD activity

SOD activity of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.8. The SOD activity of untreated experimental *Brassica juncea* seedlings was observed to be significantly ($p<0.05$) higher than TMX3 treated seedlings by 6.30 µmol/min/mg, which signified the role of thiamethoxam in causing disparity in antioxidant enzyme activity. The ameliorative impact of melatonin was observed, as SOD activity of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 10.63 µmol/min/mg. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of

SOD activity by 12.90 µmol/min/mg, when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally induce the SOD activity in *Brassica juncea* seedlings by 13.83 µmol/min/mg, as compared to TMX3 treated seedlings, implying that combinatorial application significantly enhanced the SOD activity of thiamethoxam treated *Brassica juncea* seedlings.

6.6.2.2 CAT activity

CAT activity of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.8. The CAT activity of untreated experimental *Brassica juncea* seedlings was observed to be significantly ($p<0.05$) higher than TMX3 treated seedlings by 0.95 µmol/min/mg. The ameliorative impact of melatonin was observed, as CAT activity of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 1.83 µmol/min/mg. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of CAT activity by 1.40 µmol/min/mg, when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally induce the CAT activity in *Brassica juncea* seedlings by 4.27 µmol/min/mg, as compared to TMX3 treated seedlings, implying that combinatorial application significantly enhanced the CAT activity of thiamethoxam treated *Brassica juncea* seedlings.

6.6.2.3 POD activity

POD activity of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.8. The POD activity of untreated experimental *Brassica juncea* seedlings was observed to be significantly ($p<0.05$) lower than TMX3 treated seedlings by 1.43 µmol/min/mg, implying activation of antioxidant enzyme activity to counteract thiamethoxam stress. The ameliorative impact of melatonin was observed, as POD activity of TMX3+MEL treated *Brassica juncea* seedlings was significantly

($p<0.05$) higher than TMX3 treated seedlings by 1.55 µmol/min/mg. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of POD activity by 1.50 µmol/min/mg, when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally induce the POD activity in *Brassica juncea* seedlings by 3.45 µmol/min/mg, as compared to TMX3 treated seedlings, implying that combinatorial application significantly enhanced the POD activity of thiamethoxam treated *Brassica juncea* seedlings.

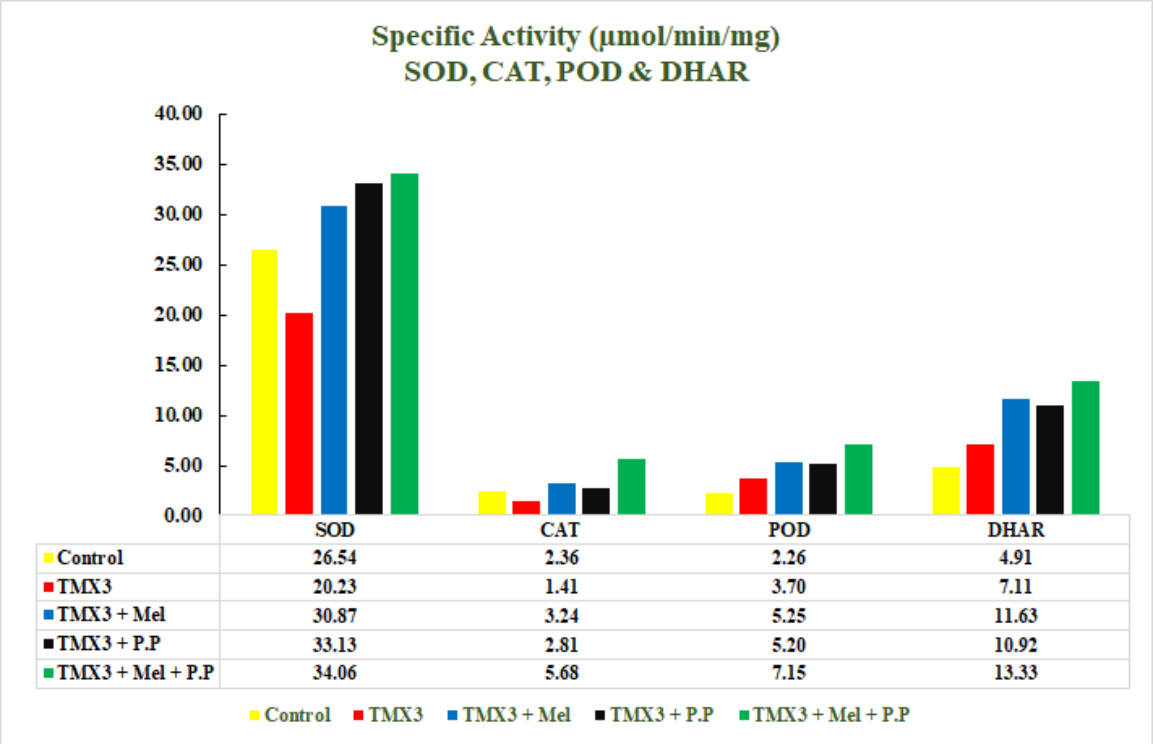
6.6.2.4 DHAR activity

DHAR activity of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.8. The DHAR activity of untreated experimental *Brassica juncea* seedlings was observed to be significantly ($p<0.05$) lower than TMX3 treated seedlings by 2.20 µmol/min/mg, implying activation of antioxidant enzyme activity to counteract thiamethoxam stress. The ameliorative impact of melatonin was observed, as DHAR activity of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 4.52 µmol/min/mg. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of DHAR activity by 3.80 µmol/min/mg, when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally induce the DHAR activity in *Brassica juncea* seedlings by 6.22 µmol/min/mg, as compared to TMX3 treated seedlings, implying that combinatorial application significantly enhanced the DHAR activity of thiamethoxam treated *Brassica juncea* seedlings. The graphical representation of the estimated SOD, CAT, POD and DHAR activity among various treatments is shown in Figure 6.11.

Table 6.8: SOD, CAT, POD and DHAR activity estimation of thiamethoxam treated *Brassica juncea* seedlings enriched by melatonin and *P. putida* alone as well as in combination respectively. The values are estimated in triplicates and presented as mean ± SD.

Treatments	SOD (µmol/min/mg)	CAT (µmol/min/mg)	POD (µmol/min/mg)	DHAR (µmol/min/mg)
Control	26.54 ± 0.06	2.36 ± 0.06	2.26 ± 0.03	4.91 ± 0.06
MEL	26.97 ± 0.06	3.27 ± 0.09	3.18 ± 0.05	5.76 ± 0.05
P.P	25.84 ± 0.03	2.78 ± 0.05	2.72 ± 0.07	5.27 ± 0.06
TMX1	26.71 ± 0.07	4.22 ± 0.06	3.89 ± 0.06	5.32 ± 0.06
TMX2	24.08 ± 0.06	3.62 ± 0.07	4.40 ± 0.08	8.62 ± 0.08
TMX3	20.23 ± 0.06	1.41 ± 0.06	3.70 ± 0.07	7.11 ± 0.06
TMX1+MEL	33.86 ± 0.06	6.44 ± 0.07	5.83 ± 0.05	10.30 ± 0.05
TMX2+MEL	31.75 ± 0.053	4.59 ± 0.07	5.35 ± 1.79	13.21 ± 0.06
TMX3+MEL	30.87 ± 0.06	3.24 ± 0.06	5.25 ± 0.13	11.63 ± 0.06
TMX1+PP	32.92 ± 0.07	6.35 ± 0.08	5.67 ± 0.12	9.17 ± 0.06
TMX2+PP	30.51 ± 0.06	3.80 ± 0.07	6.11 ± 0.08	11.87 ± 0.06
TMX3+PP	33.13 ± 5.71	2.81 ± 0.07	5.20 ± 0.05	10.91 ± 0.05
TMX1+MEL+PP	38.52 ± 0.07	8.61 ± 0.05	8.07 ± 0.06	12.44 ± 0.04
TMX2+MEL+PP	35.52 ± 0.06	7.32 ± 0.07	8.65 ± 0.11	13.44 ± 1.21
TMX3+MEL+PP	34.06 ± 0.05	5.68 ± 0.06	7.15 ± 0.09	13.33 ± 0.05
Univariate ANOVA Test Statistics	**SOD Vs Treatments**	**CAT Vs Treatments**	**POD Vs Treatments**	**DHAR Vs Treatments**
	F = 32.815	**F** = 2868.468	**F** = 47.599	**F** = 299.439
Control Vs TMX3	**HSD** = 6.306*	**HSD** = 0.956*	**HSD** = -1.436*	**HSD** = -2.200*
TMX3+MEL Vs TMX3	**HSD** = 10.636*	**HSD** = 1.833*	**HSD** = 1.550*	**HSD** = 4.523*
TMX3+PP Vs TMX3	**HSD** = 12.900*	**HSD** = 1.403*	**HSD** = 1.500*	**HSD** = 3.806*
TMX3+MEL+PP Vs TMX3	**HSD** = 13.830*	**HSD** = 4.270*	**HSD** = 3.450*	**HSD** = 6.223*

*Note: *Represents the significant mean difference at p< 0.05 level*



	SOD	CAT	POD	DHAR
Control	26.54	2.36	2.26	4.91
TMX3	20.23	1.41	3.70	7.11
TMX3 + Mel	30.87	3.24	5.25	11.63
TMX3 + P.P	33.13	2.81	5.20	10.92
TMX3 + Mel + P.P	34.06	5.68	7.15	13.33

Figure 6.11: Graphical representation of estimated SOD, CAT, POD and DHAR activity of control, TMX3, TMX3+MEL, TMX3+PP and TMX3+MEL+PP treated *Brassica juncea* seedlings respectively.

6.6.2.5 GST activity

GST activity of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.9. The GST activity of TMX3 treated seedlings was observed to be significantly ($p<0.05$) higher than untreated experimental *Brassica juncea* seedlings by 7.98 µmol/min/mg, implying activation of antioxidant enzyme activity to counteract thiamethoxam stress. The ameliorative impact of melatonin was observed, as GST activity of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 3.22 µmol/min/mg. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of GST activity by 1.02 µmol/min/mg, when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to

optimally induce the GST activity in *Brassica juncea* seedlings by 10.63 µmol/min/mg, as compared to TMX3 treated seedlings, implying that combinatorial application significantly enhanced the GST activity of thiamethoxam treated *Brassica juncea* seedlings. The graphical representation of the estimated GST activity among various treatments is shown in Figure 6.12.

6.6.2.6 GR activity

GR activity of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.9. The GR activity of TMX3 treated seedlings was observed to be significantly ($p<0.05$) higher than untreated experimental *Brassica juncea* seedlings by 3.98 µmol/min/mg, implying activation of antioxidant enzyme activity to counteract thiamethoxam stress. The ameliorative impact of melatonin was observed, as GR activity of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 3.73 µmol/min/mg. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of GR activity by 1.32 µmol/min/mg, when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally induce the GR activity in *Brassica juncea* seedlings by 8.97 µmol/min/mg, as compared to TMX3 treated seedlings, implying that combinatorial application significantly enhanced the GR activity of thiamethoxam treated *Brassica juncea* seedlings. The graphical representation of the estimated GR activity among various treatments is shown in Figure 6.12.

6.6.2.7 APOX activity

APOX activity of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.9. The APOX activity of TMX3 treated seedlings was observed to be significantly ($p<0.05$) higher than untreated experimental *Brassica juncea* seedlings by 5.39 µmol/min/mg, implying activation of antioxidant enzyme activity to counteract thiamethoxam stress. The ameliorative impact of melatonin was observed, as

APOX activity of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 10.93 µmol/min/mg. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of APOX activity by 7.88 µmol/min/mg, when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally induce the APOX activity in *Brassica juncea* seedlings by 16.58 µmol/min/mg, as compared to TMX3 treated seedlings, implying that combinatorial application significantly enhanced the APOX activity of thiamethoxam treated *Brassica juncea* seedlings. The graphical representation of the estimated APOX activity among various treatments is shown in Figure 6.12.

6.6.2.8 GPOX activity

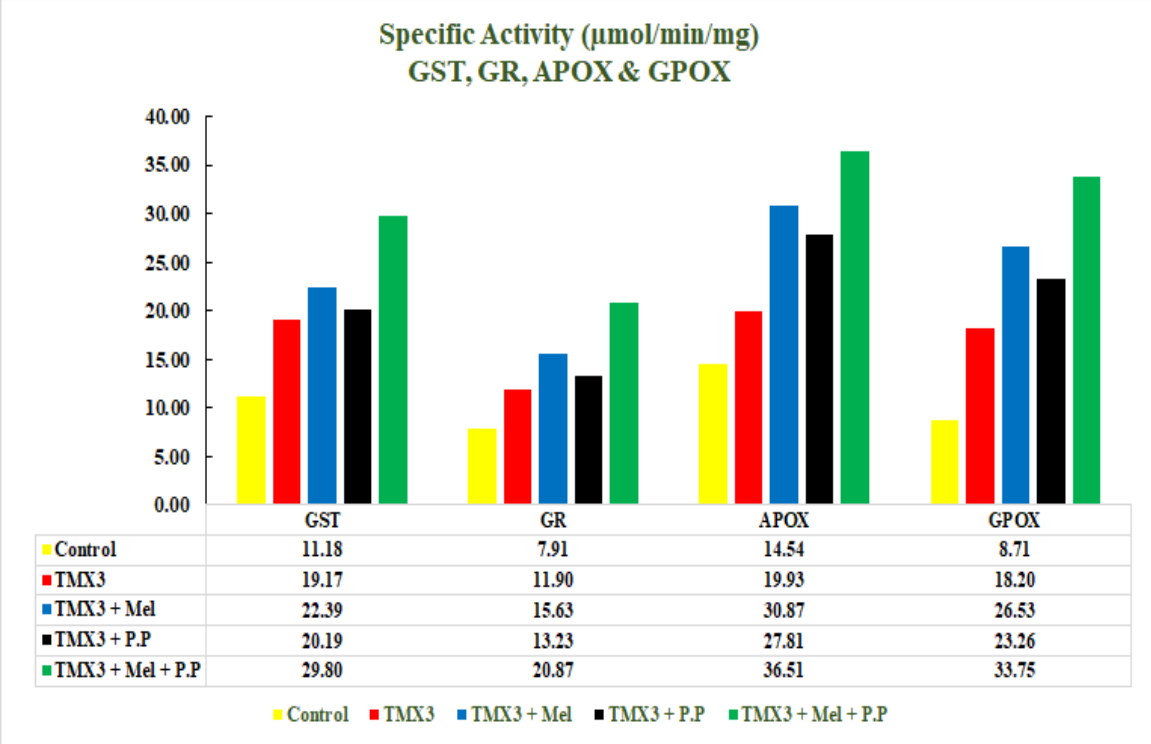
GPOX activity of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.9. The GPOX activity of TMX3 treated seedlings was observed to be significantly ($p<0.05$) higher than untreated experimental *Brassica juncea* seedlings by 9.48 µmol/min/mg, implying activation of antioxidant enzyme activity to counteract thiamethoxam stress. The ameliorative impact of melatonin was observed, as GPOX activity of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 8.33 µmol/min/mg. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of GPOX activity by 5.05 µmol/min/mg, when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally induce the APOX activity in *Brassica juncea* seedlings by 15.55 µmol/min/mg, as compared to TMX3 treated seedlings, implying that combinatorial application significantly enhanced the GPOX activity of thiamethoxam treated *Brassica juncea* seedlings. The graphical representation of the estimated GPOX activity among various treatments is shown in Figure 6.12.

Table 6.9: GST, GR, APOX and GPOX activity estimation of thiamethoxam treated *Brassica juncea* seedlings enriched by melatonin and *P. putida* alone as well as in combination respectively. The values are estimated in triplicates and presented as mean ± SD.

Treatments	GST (µmol/min/mg)	GR (µmol/min/mg)	APOX (µmol/min/mg)	GPOX (µmol/min/mg)
Control	11.18 ± 0.06	7.91 ± 0.07	14.54 ± 0.06	8.71 ± 0.06
MEL	15.04 ± 0.06	9.50 ± 0.54	15.91 ± 0.06	9.54 ± 0.52
P.P	14.07 ± 0.06	9.36 ± 0.06	15.19 ± 0.08	8.75 ± 0.12
TMX1	21.13 ± 0.06	14.37 ± 0.05	18.77 ± 0.06	14.71 ± 0.08
TMX2	23.17 ± 0.06	14.67 ± 0.06	21.81 ± 0.06	20.45 ± 0.08
TMX3	19.17 ± 0.05	11.90 ± 0.05	19.93 ± 0.05	18.20 ± 0.08
TMX1+MEL	25.92 ± 0.07	18.31 ± 0.05	28.89 ± 0.06	21.45 ± 0.09
TMX2+MEL	28.52 ± 0.07	18.91 ± 0.06	33.08 ± 0.06	31.02 ± 0.05
TMX3+MEL	22.39 ± 0.05	15.63 ± 0.08	30.87 ± 0.05	26.53 ± 0.06
TMX1+PP	24.09 ± 0.03	16.80 ± 0.07	24.93 ± 0.06	21.05 ± 0.03
TMX2+PP	27.77 ± 0.06	17.06 ± 0.05	27.51 ± 0.07	27.14 ± 0.10
TMX3+PP	20.19 ± 0.06	13.23 ± 0.06	27.81 ± 0.08	23.26 ± 0.09
TMX1+MEL+PP	31.07 ± 0.04	21.61 ± 0.08	32.11 ± 0.06	32.16 ± 0.09
TMX2+MEL+PP	33.08 ± 0.05	22.76 ± 0.06	38.53 ± 0.06	42.73 ± 0.07
TMX3+MEL+PP	29.80 ± 0.06	20.87 ± 0.07	36.51 ± 0.06	33.75 ± 0.07
Univariate ANOVA Test Statistics	**GST Vs Treatments**	**GR Vs Treatments**	**APOX Vs Treatments**	**GPOX Vs Treatments**
	F = 42887.294	**F** = 2763.730	**F** = 51264.019	**F** = 12236.578
TMX3 Vs Control	**HSD** = 7.983*	**HSD** = 3.986*	**HSD** = 5.390*	**HSD** = 9.486*
TMX3+MEL Vs TMX3	**HSD** = 3.220*	**HSD** = 3.730*	**HSD** = 10.936*	**HSD** = 8.333*
TMX3+PP Vs TMX3	**HSD** = 1.026*	**HSD** = 1.326*	**HSD** = 7.883*	**HSD** = 5.056*

TMX3+MEL+PP Vs TMX3	**HSD** = 10.633*	**HSD** = 8.973*	**HSD** = 16.580*	**HSD** = 15.550*

*Note: *Represents the significant mean difference at p< 0.05 level*



	GST	GR	APOX	GPOX
Control	11.18	7.91	14.54	8.71
TMX3	19.17	11.90	19.93	18.20
TMX3 + Mel	22.39	15.63	30.87	26.53
TMX3 + P.P	20.19	13.23	27.81	23.26
TMX3 + Mel + P.P	29.80	20.87	36.51	33.75

Figure 6.12: Graphical representation of estimated GST, GR, APOX and GPOX activity of control, TMX3, TMX3+MEL, TMX3+PP and TMX3+MEL+PP treated *Brassica juncea* seedlings respectively.

6.6.3 Estimation of non-enzymatic antioxidants

The activities of non-enzymatic antioxidants namely tocopherol, ascorbic acid, glutathione and phenol content were evaluated to observe the effect of thiamethoxam, melatonin and *P. putida* on *Brassica juncea* seedlings.

6.6.3.1 Tocopherol content

Tocopherol content of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.10. The tocopherol content of TMX3 treated seedlings was observed to be significantly ($p<0.05$) higher than untreated experimental *Brassica juncea* seedlings by 0.45 mg/g FW, implying activation of antioxidant enzyme activity to counteract thiamethoxam stress. The ameliorative impact of melatonin was observed, as tocopherol content of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 0.84 mg/g FW. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of tocopherol content by 0.29 mg/g FW, when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally induce the tocopherol content in *Brassica juncea* seedlings by 1.62 mg/g FW, as compared to TMX3 treated seedlings, implying that combinatorial application significantly enhanced the tocopherol content of thiamethoxam treated *Brassica juncea* seedlings. The graphical representation of the estimated tocopherol content among various treatments is shown in Figure 6.13.

6.6.3.2 Ascorbic acid content

Ascorbic acid content of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.10. The ascorbic acid content of TMX3 treated seedlings was observed to be significantly ($p<0.05$) higher than untreated experimental *Brassica juncea* seedlings by 1.87 mg/g FW, implying activation of antioxidant enzyme activity to counteract thiamethoxam stress. The ameliorative impact of melatonin was observed, as ascorbic acid content of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 1.37 mg/g FW. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of ascorbic acid content by 1.19 mg/g FW, when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally induce the ascorbic acid content in *Brassica juncea* seedlings by 2.28 mg/g FW, as compared to TMX3 treated seedlings,

implying that combinatorial application significantly enhanced the ascorbic acid content of thiamethoxam treated *Brassica juncea* seedlings. The graphical representation of the estimated ascorbic acid content among various treatments is shown in Figure 6.13.

6.6.3.3 Glutathione content

Glutathione content of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.10. The glutathione content of TMX3 treated seedlings was observed to be significantly ($p<0.05$) lower than untreated experimental *Brassica juncea* seedlings by 5.89 mg/g FW. The ameliorative impact of melatonin was observed, as glutathione content of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 5.98 mg/g FW. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of glutathione content by 5.69 mg/g FW, when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally induce the glutathione content in *Brassica juncea* seedlings by 8.07 mg/g FW, as compared to TMX3 treated seedlings, implying that combinatorial application significantly enhanced the glutathione content of thiamethoxam treated *Brassica juncea* seedlings. The graphical representation of the estimated glutathione content among various treatments is shown in Figure 6.13.

6.6.3.4 Phenol content

Phenol content of *Brassica juncea* seedlings inoculated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.10. The phenol content of TMX3 treated seedlings was observed to be significantly ($p<0.05$) lower than untreated experimental *Brassica juncea* seedlings by 1.82 mg/g DW. The ameliorative impact of melatonin was observed, as phenol content of TMX3+MEL treated *Brassica juncea* seedlings was significantly ($p<0.05$) higher than TMX3 treated seedlings by 4.66 mg/g DW. Likewise, *P. putida* application (TMX3+PP) also showed significant ($p<0.05$) revival of phenol content by 4.51 mg/g DW, when compared to TMX3 treated *Brassica juncea* seedlings. The

combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally induce the phenol content in *Brassica juncea* seedlings by 5.92 mg/g DW, as compared to TMX3 treated seedlings, implying that combinatorial application significantly enhanced the phenol content of thiamethoxam treated *Brassica juncea* seedlings. The graphical representation of the estimated phenol content among various treatments is shown in Figure 6.13.

Table 6.10: Tocopherol, ascorbic acid, glutathione and phenol content estimation of thiamethoxam treated *Brassica juncea* seedlings enriched by melatonin and *P. putida* alone as well as in combination respectively. The values are estimated in triplicates and presented as mean ± SD.

Treatments	Tocopherol (mg/g FW)	Ascorbic acid (mg/g FW)	Glutathione (mg/g FW)	Phenol (mg/g DW)
Control	1.07 ± 0.05	4.86 ± 0.05	10.85 ± 0.03	12.23 ± 0.02
MEL	1.46 ± 0.04	5.66 ± 0.05	15.23 ± 0.02	15.99 ± 0.01
P.P	1.26 ± 0.03	5.22 ± 0.03	12.24 ± 0.02	12.79 ± 0.02
TMX1	1.33 ± 0.04	6.33 ± 0.06	9.56 ± 0.03	11.64 ± 0.37
TMX2	1.64 ± 0.03	6.83 ± 0.05	6.95 ± 0.03	11.74 ± 0.02
TMX3	1.52 ± 0.03	6.74 ± 0.03	4.96 ± 0.03	10.41 ± 0.05
TMX1+MEL	2.21 ± 0.05	7.15 ± 0.04	13.18 ± 0.03	13.24 ± 0.02
TMX2+MEL	2.55 ± 0.03	7.65 ± 0.04	11.84 ± 0.03	13.87 ± 0.02
TMX3+MEL	2.37 ± 0.03	8.15 ± 0.04	10.95 ± 0.04	15.07 ± 0.01
TMX1+PP	1.68 ± 0.04	6.96 ± 0.04	12.77 ± 0.03	12.54 ± 0.04
TMX2+PP	1.94 ± 0.03	7.59 ± 0.09	10.24 ± 0.03	13.28 ± 0.01
TMX3+PP	1.81 ± 0.04	7.93 ± 0.04	10.66 ± 0.04	14.92 ± 0.02
TMX1+MEL+PP	2.87 ± 0.02	7.75 ± 0.03	14.76 ± 0.04	13.73 ± 0.02
TMX2+MEL+PP	3.44 ± 0.03	9.06 ± 0.03	13.53 ± 0.04	13.97 ± 0.02
TMX3+MEL+PP	3.14 ± 0.03	9.02 ± 0.03	13.04 ± 0.05	16.33 ± 0.02
Univariate ANOVA Test	**Tocopherol Vs**	**Ascorbic acid Vs**	**Glutathione Vs**	**Phenol Vs**

Statistics	Treatments	Treatments	Treatments	Treatments
	F = 1341.741	**F** = 2102.002	**F** = 21959.077	**F** = 859.622
TMX3 Vs Control	**HSD** = 0.456*	**HSD** = 1.876*	**HSD** = -5.890*	**HSD** = -1.826*
TMX3+MEL Vs TMX3	**HSD** = 0.843*	**HSD** = 1.376*	**HSD** = 5.983*	**HSD** = 4.663*
TMX3+PP Vs TMX3	**HSD** = 0.290*	**HSD** = 1.193*	**HSD** = 5.693*	**HSD** = 4.516*
TMX3+MEL+PP Vs TMX3	**HSD** = 1.620*	**HSD** = 2.280*	**HSD** = 8.076*	**HSD** = 5.923*

*Note: *Represents the significant mean difference at p< 0.05 level*

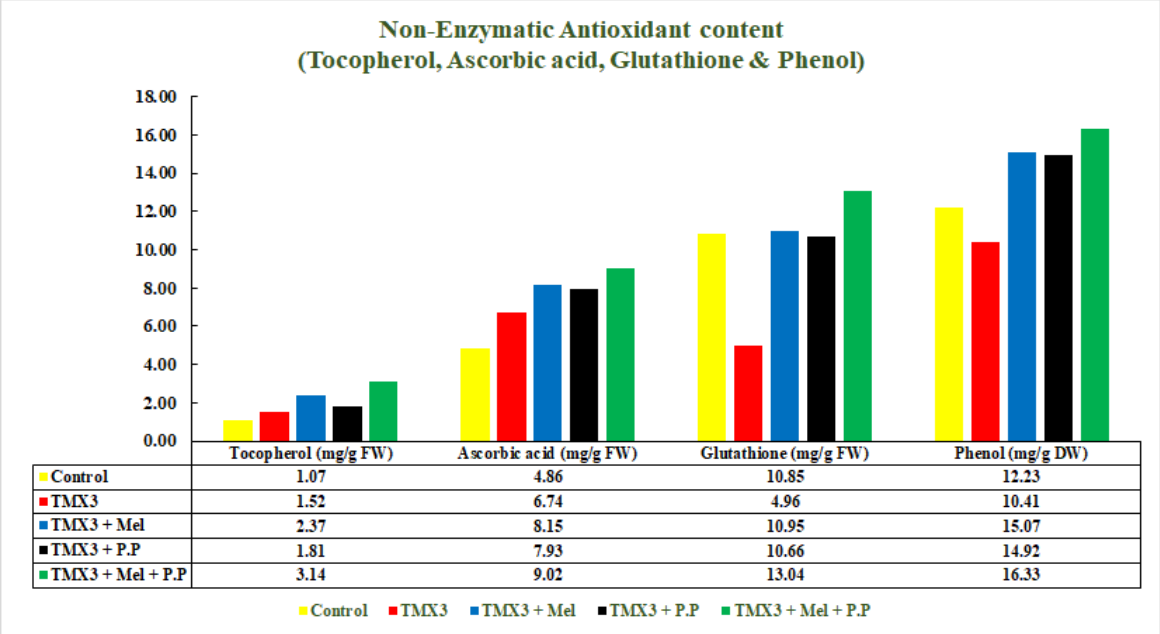


Figure 6.13: Graphical representation of estimated non-enzymatic antioxidant content of control, TMX3, TMX3+MEL, TMX3+PP and TMX3+MEL+PP treated *Brassica juncea* seedlings respectively.

Plants in response to ROS, regulate their antioxidative defense system to neutralize oxidative stress mediated injuries (Das and Roychoudhury, 2014). The antioxidative defense system includes enzymatic components like SOD, POD, CAT, DHAR, GST, GR, APOX and GPOX enzymes respectively (Ahmad *et* al., 2010; Das and Roychoudhury, 2014; Liu *et al*., 2014; Gullner *et al*., 2018). On the other hand, non-enzymatic

antioxidants are tocopherol, ascorbic acid, glutathione and phenol (Ahmad *et al*., 2010; Das and Roychoudhury, 2014).

The enzymatic antioxidant SOD is responsible for the conversion of O_2^- to H_2O_2. Higher expression of SOD has been observed to mitigate the oxidative stress mediated by abiotic factors (Ahmad *et* al., 2012; Ahmad *et* al., 2010). POD antioxidant enzymes regulate the transformation of H_2O_2 into H_2O (Caverzan *et al*., 2012). POD has been reported to play a crucial role in maintenance of antioxidant defense system in plants during the various stress conditions (Caverzan *et al*., 2012). The enzymatic antioxidant CAT is responsible for the conversion of two molecules of H_2O_2 into two molecules of H_2O and one molecule of O_2 (Ahmad *et al*., 2010). Another enzymatic antioxidant DHAR revives the ascorbic acid in cells by reducing dehydroascorbate (Das and Roychoudhury, 2014). Previous studies have reported the elevated action of DHAR in plants experiencing abiotic stresses (Nadarajah, 2020; Hossain *et al*., 2010). The increased activity of antioxidant enzyme GST is associated with providing protection from ROS damage by enhancing osmotic balance (Nadarajah, 2020). In genetically modified tobacco plant higher activity of GST from alfalfa was observed to safeguard against stress induced ROS (Du *et al*., 2019). The enzymatic antioxidant GR is required for the conversion of disulfide glutathione to reduced glutathione by performing catalysis of the disulfide bond in disulfide glutathione reservoir (Nadarajah, 2020). By this GR has been recognized as an important enzyme in ASCC-GSH cycle in order to retain higher proportion of GSH/GSSG in cells and enhance plants activity to combat various stress conditions (Das and Roychoudhury, 2014; Yadav and Sharma, 2016). The enzymatic antioxidant APOX performs the reduction of H_2O_2 into H_2O (Ahmad *et al*., 2010). It has been recognized as crucial component of plants antioxidant defense system because of its higher binding potential for H_2O_2 than POD and CAT (Ahmad *et* al., 2010; Davletova *et al*., 2005). The enzymatic antioxidant GPOX is responsible for the conversion of hydrogen peroxide and hydroperoxide into alcohols (Ahmad *et al*., 2010).

The non-enzymatic antioxidant tocopherol is an effective neutralizer of the ROS and lipid radicals and thus provide defense to the cellular membranes of organisms (Ahmad *et al*., 2010). Another non-enzymatic antioxidant ascorbic acid is found plentiful and has been examined vastly (Ahmad *et al*., 2010). Ascorbic acid has the ability to provide electrons to various enzymatic and non-enzymatic processes and acts as primary barrier to combat the ROS induced injuries (Ahmad *et al*., 2010). The non-enzymatic antioxidant glutathione has multifarious functions including regulation of the genes which corresponds to stress conditions (Ahmad *et al*., 2010). It has strong reduction potential against $^{1}O_2$, $OH^{\bullet}$, and $O_2^{\bullet -}$ species and produces disulfide as derivative compound (Ahmad *et al*., 2010). Glutathione also acts as an important factor to rejuvenate ascorbic acid to produce disulfide glutathione (Ahmad *et al*., 2010). This disulfide glutathione is further transformed to GSH, which in turn prevent formation of cellular ROS compounds (Ahmad *et al*., 2010). Phenol compounds includes wide array of secondary metabolites including flavonoid, tanins and lignins and are found in plentiful amount plant system (Ahmad *et al*., 2010). The conformation of phenols has been found favourable to combat the free radical species more effectively than ascorbate and tocopherols (Schroeter *et al*., 2002; Ahmad *et al*., 2010).

Previous study has reported a variation in the antioxidant activity dynamics of the tomato plants exposed to pesticide (Shakir *et al*., 2018). The application of pesticide in tomato plants leads to increase in the antioxidant activity of SOD, POD, CAT, GR, APOX and proline as compared to untreated plants (Shakir *et al*., 2018). Similar observations have been recorded in the present study in which application of thiamethoxam lead to increased activities of antioxidants namely GR, APOX, proline, GST, POD, DHAR, GPOX, tocopherol and ascorbic acid in *B. juncea* seedlings as compared to investigational untreated seedlings (Table 6.8, 6.9, 6.10) respectively.

A recent study has shown that application of melatonin in cucumber under pesticide stress was able to elevate the activities of GST, GR and DHAR resulting in degradation of the pesticide by utilization of glutathione-ascorbic acid pathway (Liu *et al*., 2021). In

the present investigation we have also observed that application of melatonin in thiamethoxam treated *B. juncea* seedlings resulted in increased activities of GST, GR and DHAR (Table 6.8, 6.9) on comparison to thiamethoxam treated seedlings. These increased activities as per the literature (Liu *et al*., 2021), in our case, may corresponds to the neutralization of thiamethoxam by the *B. juncea* seedlings.

Study has shown that application of *Pseudomonas putida* (P3-57) resulted in increased activities of CAT and APOX enzymes to combat against the stress condition in cucumber (Kafi *et al*., 2021). Similarly, we have also seen that application of *P. putida* as compared to thiamethoxam treated seedlings enhanced the activities of CAT and APOX enzymes to provide defense against the thiamethoxam pesticide (Table 6.8, 6.9).

A review study in 2019, has discussed the role of melatonin and PGPR in enhancing the plant growth and development during stress conditions (Asif *et al*., 2019). The combinatorial application employing melatonin and *P. putida* in the present investigation comparatively achieved the highest enzymatic and non-enzymatic antioxidant activity in thiamethoxam treated *B. juncea* seedlings.

6.6.4 Organic acid Profiling by HPLC

The present study investigates the relative organic acid content (oxalic acid, malic acid, ascorbic acid, citric acid, succinic acid and fumaric acid) variation observed in B. juncea plant subjected to TMX stress and to evaluate the role of melatonin and *P. putida* used as ameliorative in regulating the organic acid levels. With the help of HPLC technique the organic acids were detected in control and treated samples and their relative content were analyzed based on peak area percent. The HPLC peaks obtained for the control, TMX, TMX + melatonin, TMX + *P. putida* and TMX + melatonin + *P. putida* treated plants are shown in figure (6.14) below.

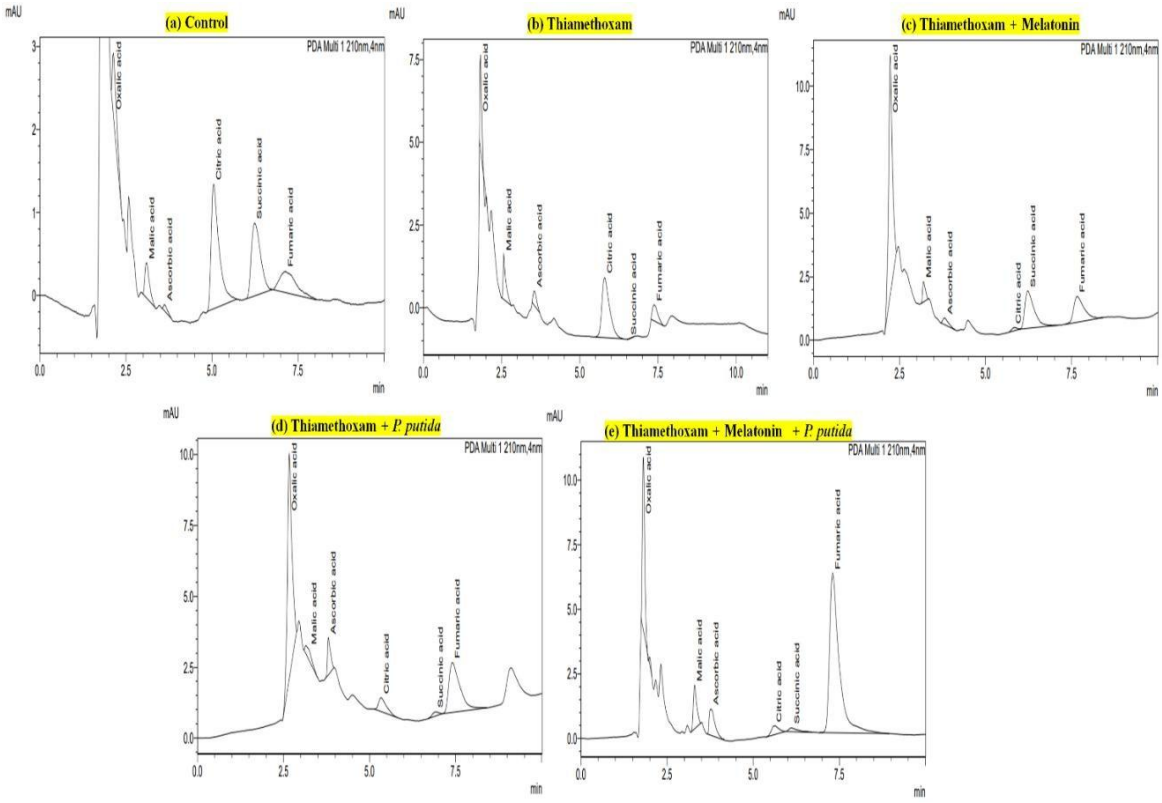


Figure 6.14: HPLC chromatogram obtained for the control untreated plant (a); TMX treated plant (b); TMX + melatonin treated plant (c); TMX + *P. putida* treated plant (d); TMX + melatonin + *P. putida* treated plant respectively. The horizontal axis in the chromatogram represents the retention time (time taken by the analyte to reach detector), whereas, vertical axis represents milli absorbance units (mAU).

Comparing the relative organic acid content among control (untreated) and pesticide treated plants the relative organic acid (oxalic acid, malic acid, ascorbic acid and citric acid) content of pesticide treated plants were found to be increased as referred in Table (6.11). Similar trend of increased relative organic acid content was observed for oxalic acid, ascorbic acid and fumaric acid in TMX + melatonin, TMX + P. putida and TMX + melatonin + P. putida treated plants on comparison to control (Table 6.11). On the other hand, peak area percent of citric acid and succinic acid were increased in control sample in contrast to TMX + melatonin, TMX + P. putida and TMX + melatonin + P. putida treated plants (Table 6.11).

Table 6.11: Peak area percent of various organic acids detected in *B. juncea* subjected to numerous treatments (TMX, TMX + melatonin, TMX + *P. putida* and TMX +melatonin + *P. putida.*

Organic Acid	Peak Area Percent				
	Control (%)	**TMX (%)**	**TMX + Melatonin (%)**	**TMX+ *P. putida* (%)**	**TMX + Melatonin + *P. putida* (%)**
Oxalic acid	11.27	17.06	55.32	53.44	17.24
Malic acid	6.37	13.59	3.55	3.51	6.39
Ascorbic acid	1.07	6.00	2.30	6.05	6.34
Citric acid	39.74	53.26	0.99	5.92	2.79
Succinic acid	27.01	0	21.38	1.42	1.46
Fumaric acid	14.53	10.08	16.45	29.66	65.78

6.6.4.1 Quantification of organic acids in various treatments

The quantification of organic acids for various treatments carried out in triplicates are shown in Table (6.12). An increase in the amount of organic acids, malic acid (110%), citric acid (170%), succinic acid (81%), fumaric acid (40%) and ascorbic acid (55%) was observed in TMX treated plants in comparison to the investigational untreated plants. The exogenous application of melatonin in TMX treated plants resulted in up-regulation of malic acid, citric acid, succinic acid, fumaric acid and ascorbic acid by 81%, 0.94%, 11%, 21% and 6% respectively. Further, the organic acids content of TMX treated plants inoculated with P. putida were elevated by 161% (malic acid), by 14% (citric acid), by 33% (succinic acid), by 30% (fumaric acid), by 100% (oxalic acid) and decreased by 10% (ascorbic acid). Finally, the combinatorial approach, involving the application of melatonin and *P. putida* in TMX treated plants, resulted in substantial upsurge of malic acid by 165%, citric acid by 10%, succinic acid

by 69%, fumaric acid by 42%, ascorbic acid by 3% and oxalic acid by 100% respectively.

Table 6.12: Organic acid quantification of the various treatments subjected to the HPLC analysis. The quantified value represents the mean ± standard error for three replicates of each sample. The * sign represents that mean difference for each treatment are statistically significant at p<0.05 according to Tukey HSD test.

Treatment	Malic Acid (Mean ± Std error)	Citric Acid (Mean ± Std error)	Succinic Acid (Mean ± Std error)	Fumaric Acid (Mean ± Std error)	Ascorbic Acid (Mean ± Std error)	Oxalic Acid (Mean ± Std error)
Control	1.48 ± 0.01*	1.95 ± 0.09*	0.53 ± 0.03*	0.30 ± 0.04*	0.20 ± 0.06*	0.01 ± 0.001*
Thiamethoxam	3.11 ± 0.03*	5.27 ± 0.08*	0.96 ± 0.01*	0.42 ± 0.001*	0.31 ± 0.001*	0.01 ± 0.001*
Melatonin	1.68 ± 0.001*	4.20 ± 0.03*	0.89 ± 0.02*	0.32 ± 0.001*	0.23 ± 0.01*	0.01 ± 0.0007*
P. putida	6.01 ± 0.15*	5.13 ± 0.07*	0.98 ± 0.04*	0.43 ± 0.0007*	0.21 ± 0.006*	0.02 ± 0.0005*
Thiamethoxam + Melatonin	5.63 ± 0.16*	5.32 ± 0.09*	1.07 ± 0.01*	0.51 ± 0.004*	0.33 ± 0.008*	0.01 ± 0.00006*
Thiamethoxam + *P. putida*	8.12 ± 0.23*	6.01 ± 0.09*	1.28 ± 0.03*	0.55 ± 0.003*	0.28 ± 0.007*	0.02 ± 0.0004*
Thiamethoxam + Melatonin + *P. putida*	8.25 ± 0.39*	5.81 ± 0.02*	1.63 ± 0.06*	0.60 ± 0.003*	0.32 ± 0.006*	0.02 ± 0.0004*

Previous literature studies which ascertain that enhanced level of organic acids in plant correlates with their defence against the onset of biotic or abiotic stress (Bali et al., 2018) Organic acids also participate in the plant's defence mechanism thereby controlling the osmotic pressure and ionic balance during abiotic stress (Zeng et al., 2008; Sharma et al., 2017). Similar observations were noticed in current study corresponding to elevated level of organic acids, malic acid, citric acid, succinic acid, fumaric acid and ascorbic acid were observed in TMX treated plants. In a recent study, exogenous application of melatonin in rice resulted in elevated levels of organic acids which enhanced the mitochondrial respiration so as to meet the energy requirement to combat the arsenic stress (Samanta et al., 2020). Application of melatonin in soybean has been found to up-

regulate the expression of gene translating acetyltransferase NSl-like, reduced H202 generation and upregulated efflux of organic acids to mitigate the Aluminium toxicity (Zhang et al., 2017). The current study also demonstrates the role of melatonin in combating the plant stress, thereby up-regulating the organic acids level (Table 6.12). Equivalently, TMX treated B. juncea plants in present investigation by the exogenous application of melatonin were observed to up-regulate the malic acid, citric acid, succinic acid, fumaric acid and ascorbic acid by 81%, 0.94%, 11%, 21% and 6% respectively (Table 6.12), suggesting their role in alleviating the TMX stress. The plant growth promoting rhizobacteria P. putida has been observed previously in effective degradation of TMX by utilizing its degraded constituents as a source of carbon and energy (Rana et al., 2015). Similar trend of TMX degradation was observed in the current study on the basis of elevated levels of organic acids (by 161% (malic acid), by 14% (citric acid), by 33% (succinic acid), by 30% (fumaric acid), by 100% (oxalic acid) in treated seedlings (Table 6.12). The combinatorial approach involving application of melatonin and P. putida has also proved to be effective against alleviating the TMX stress in B. juncea seedlings as inferred by the upregulated levels of organic acids (malic acid by 165%, citric acid by 10%, succinic acid by 69%, fumaric acid by 42%, ascorbic acid by 3% and oxalic acid by 100%) on comparison to the TMX treated plants (Table 6.12).

6.6.5 Gene Expression Analysis by qRT-PCR

The gene expression for all the treatments were evaluated using the qRT-PCR analysis and finally normalized results were evaluated for the genes *CHLASE*, *CXE*, *GST*, *NADH*, *P450* and *POD* respectively. The results performed in duplicates are expressed as normalized fold change values ± SD.

6.6.5.1 *CHLASE* gene expression analysis

CHLASE gene expression analysis of *Brassica juncea* seedlings treated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.13. The *CHLASE* expression of TMX3 treated seedlings was observed to be increased to 443.86 folds as compared to

untreated experimental *Brassica juncea* seedlings suggesting the upregulation of *CHLASE* under thiamethoxam treated seedlings. *CHLASE* gene expression of TMX3+MEL treated *Brassica juncea* seedlings was lowered to 87.94 folds as compared to TMX3 treated seedlings. Likewise, *P. putida* application (TMX3+PP) also reduced the expression of *CHLASE* gene to 195.13 folds when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally reduce the *CHLASE* gene expression to 0.020 folds in *Brassica juncea* seedlings as compared to TMX3 treated seedlings, implying that combinatorial application significantly alleviated the thiamethoxam induced *CHLASE* gene expression in *Brassica juncea* seedlings. The graphical representation of the estimated fold change for *CHLASE* gene among various treatments is shown in Figure 6.15.

6.6.5.2 *CXE* gene expression analysis

CXE gene expression analysis of *Brassica juncea* seedlings treated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.13. The *CXE* gene expression of TMX3 treated seedlings was observed to be downregulated to 0.38 folds as compared to untreated experimental *Brassica juncea* seedlings. The ameliorative impact of melatonin was observed as *CXE* gene expression of TMX3+MEL treated *Brassica juncea* seedlings was upregulated to 1.18 folds as compared to TMX3 treated seedlings. Likewise, *P. putida* application (TMX3+PP) also upregulated the expression of *CXE* gene to 0.82 folds when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally upregulate the *CXE* gene expression to 3.35 folds in *Brassica juncea* seedlings as compared to TMX3 treated seedlings. The graphical representation of the estimated fold change for *CXE* gene among various treatments is shown in Figure 6.15.

6.6.5.3 *GST* gene expression analysis

GST gene expression analysis of *Brassica juncea* seedlings treated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.13. The *GST* gene expression of TMX3 treated seedlings was observed to be downregulated to 0.29 folds as compared to untreated experimental *Brassica juncea* seedlings. The ameliorative impact of melatonin was observed as *GST* gene expression of TMX3+MEL treated *Brassica juncea* seedlings was upregulated to 1.72 folds as compared to TMX3 treated seedlings. Likewise, *P. putida* application (TMX3+PP) also upregulated the expression of *GST* gene to 4.36 folds when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally upregulate the *GST* gene expression to 7.85 folds in *Brassica juncea* seedlings as compared to TMX3 treated seedlings. The graphical representation of the estimated fold change for *GST* gene among various treatments is shown in Figure 6.15.

6.6.5.4 *NADH* gene expression analysis

NADH gene expression analysis of *Brassica juncea* seedlings treated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.13. The *NADH* gene expression of TMX3 treated seedlings was observed to be downregulated to 0.09 folds as compared to untreated experimental *Brassica juncea* seedlings. The ameliorative impact of melatonin was observed as *NADH* gene expression of TMX3+MEL treated *Brassica juncea* seedlings was upregulated to 6.98 folds as compared to TMX3 treated seedlings. Likewise, *P. putida* application (TMX3+PP) also upregulated the expression of *NADH* gene to 1.52 folds when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally upregulate the *NADH* gene expression to 31.54 folds in *Brassica juncea* seedlings as compared to TMX3 treated seedlings. The graphical representation of the estimated fold change for *NADH* gene among various treatments is shown in Figure 6.15.

6.6.5.5 *P450* gene expression analysis

P450 gene expression analysis of *Brassica juncea* seedlings treated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.13. The *P450* gene expression of TMX3 treated seedlings was observed to be slightly upregulated to 1.12 folds as compared to untreated experimental *Brassica juncea* seedlings. The ameliorative impact of melatonin was observed as *P450* gene expression of TMX3+MEL treated *Brassica juncea* seedlings was upregulated to 1.16 folds as compared to TMX3 treated seedlings. Likewise, *P. putida* application (TMX3+PP) also upregulated the expression of *P450* gene to 1.18 folds when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally upregulate the *P450* gene expression to 2.27 folds in *Brassica juncea* seedlings as compared to TMX3 treated seedlings. The graphical representation of the estimated fold change for *P450* gene among various treatments is shown in Figure 6.15.

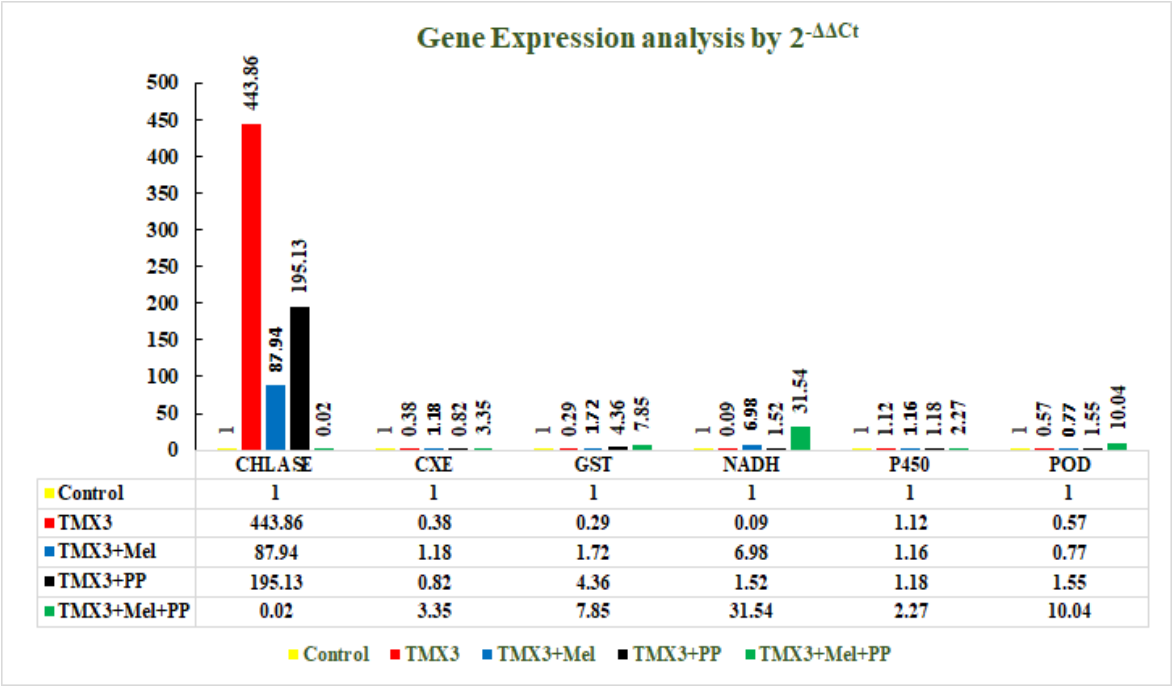
6.6.5.6 *POD* gene expression analysis

POD gene expression analysis of *Brassica juncea* seedlings treated with thiamethoxam, melatonin and *P. putida* is shown in Table 6.13. The *POD* gene expression of TMX3 treated seedlings was observed to be down-regulated to 0.57 folds as compared to untreated experimental *Brassica juncea* seedlings. The ameliorative impact of melatonin was observed as *POD* gene expression of TMX3+MEL treated *Brassica juncea* seedlings was upregulated to 0.77 folds as compared to TMX3 treated seedlings. Likewise, *P. putida* application (TMX3+PP) also upregulated the expression of *POD* gene to 1.55 folds when compared to TMX3 treated *Brassica juncea* seedlings. The combinatorial application of melatonin and *P. putida* (TMX3+MEL+PP) was observed to optimally up-regulate the *POD* gene expression to 10.04 folds in *Brassica juncea* seedlings as compared to TMX3 treated seedlings. The graphical representation of the estimated fold change for *POD* gene among various treatments is shown in Figure 6.15.

Table 6.13: Gene expression of the various treatments subjected to the qRT-PCR analysis. The quantified expression values are represented as mean ± SD for duplicates of each sample.

Gene Name	Control ($2^{-\Delta\Delta Ct}$)	TMX3 ($2^{-\Delta\Delta Ct}$)	TMX3+Mel ($2^{-\Delta\Delta Ct}$)	TMX3+PP ($2^{-\Delta\Delta Ct}$)	TMX3+Mel+PP ($2^{-\Delta\Delta Ct}$)
CHLASE	1 ± 0.00	443.86 ± 1.38	87.94 ± 0.46	195.13 ± 1.15	0.020 ± 0.33
CXE	1 ± 0.00	0.38 ± 1.42	1.18 ± 0.92	0.82 ± 2.50	3.35 ± 0.72
GST	1 ± 0.00	0.29 ± 0.21	1.72 ± 0.90	4.36 ± 0.39	7.85 ± 0.00
NADH	1 ± 0.00	0.09 ± 0.55	6.98 ± 0.58	1.52 ± 0.40	31.54 ± 0.00
P450	1 ± 0.00	1.12 ± 0.46	1.16 ± 1.10	1.18 ± 0.59	2.27 ± 1.16
POD	1 ± 0.00	0.57 ± 0.14	0.77 ± 0.09	1.55 ± 0.27	10.04 ± 0.56

Figure 6.15: Graphical representation of the normalized fold change expression values of the various treatments for *CHLASE, CXE, GST, NADH, P450* and *POD* genes respectively.



The enzyme *CHLASE* is associated with the pathways which triggers the degradation of the chlorophyll and contributes to the cellular senescence in plants (Aiamla-or *et al*., 2012). Previous study has reported an increase in the *CHLASE* gene expression in plants under pesticide stress (Sharma *et al.*, 2016), which corresponds with our observation of increase in *CHLASE* expression exposed to thiamethoxam stress as compared to untreated *B. juncea* seedlings (Table 6.13). Further, a study by (Sharma *et al.*, 2016) reported that application of plant growth hormones reduces the expression of *CHLASE* enzyme in plants under pesticide stress. Similar observation of decrease in *CHLASE* activity by melatonin application in the present investigation confers the role of melatonin in down-regulating the *CHLASE* expression in thiamethoxam treated *B. juncea* seedlings (Table 6.13). In contemporary study, TMX residues were decreased in melatonin and combined melatonin and *P. putida* amended seedlings grown under TMX toxicity. As described in the introduction section, the three phased enzyme-mediated detoxification system is responsible for pesticide degradation in plants (Jan *et al.*, 2020). In the current investigation, activities of *POD* and *GST* enzymes, which are involved in three phased detoxification system, were observed to increase with the TMX application and further got enhanced by supplementation of melatonin and *P. putida.* Additionally, the gene expression of phase-1 enzymes viz., *P450*, *POD*, *CXE* and phase-2 enzymes *GST* was also observed to increase upon melatonin and *P. putida* application under TMX toxicity and gene expression of *NADH* was also elevated upon melatonin and *P. putida* inoculation under TMX toxicity which is supported by the study conducted by Sharma *et al.*, (2017) where similar observation were recorded in pesticide treated plants (Sharma *et al.*, 2017). Since the expression of genes encoding pesticide detoxification enzymes was regulated by melatonin priming and inoculation of *P. putida*, this can be the possible reason for reduction of TMX residues in *B. juncea* seedlings.

CHAPTER 7

SUMMARY & CONCLUSIONS

Prevalence of pesticide within crops, soils and water bodies is emanating and is most alarming environmental issue, drawing the emphasis and focus of scientific community. Demand of pesticides and lack of efficient detoxification and remediation approaches indicates that the predicament is exacerbating, thereby bringing this subject to forefront in the immediate future. Exploring new techniques to deal with this quagmire is a subject of relevance. In current research, the intrinsic potential of *Brassica juncea* along with ameliorative melatonin and *Pseudomonas putida* have been exploited to degrade thiamethoxam and was accomplished, suggesting it as a promising approach for addressing pesticidal menace. Pesticides induce toxicity to plants by producing ROS, breakdown of photosynthetic component and altering antioxidative defense system. Plant growth regulators play a crucial role in pesticide detoxification by mitigating toxicity, protecting and imparting resistance to plants in response to pesticide toxicity. Seed priming with plant growth regulators can proficiently recuperate plant growth by controlling the plant's cellular processes. In presence of pesticide toxicity, plant growth regulators induce tolerance primarily by activating plant defense mechanism, which involves enzymatic and non-enzymatic antioxidants.

Melatonin is a nascent molecule possessing pleiotropic effects with multifarious functions in plants. It is a growth stimulator and simultaneously acts as a stress alleviator in plants. The cardinal role of phyto-melatonin is to provide first line of defense against oxidative stress that befalls due to unfavorable conditions. The role of melatonin in plant protection and defense mechanism renders a direct way for attenuating the pesticide stress and reducing pesticide residues in food crops. The PGPRs play an imperative role in overall development of plant by providing tolerance to hostile conditions, shaping rhizosphere, enhancing nutrient absorption and biomass. PGPRs assist plant by escalating antioxidative defense system and scavenging ROS under stress conditions. *P. putida* has the ability to swiftly transform thiamethoxam to non-toxic form. Pesticides act as a sole

source of carbon for microbes, constructing a way for pesticide degradation, as after effective degradation of pesticides by microbes, the degraded constituents are utilized for energy. Briefly, PGPRs strengthen the plants defense mechanism and improve the plant health by alleviating the effect of pesticidal stress.

In summation, our findings indicate that melatonin and *P. putida* alone as well as synergistically boost the resilience of *B. juncea* seedlings under thiamethoxam stress. The association of melatonin and *P. putida* with plants in up-regulating levels of metabolites under TMX toxicity was explored. Morphological analysis asserts that effect of TMX stress is more on FW (reduced by 4.96 mg/seedling), DW (1.10 mg/seedling) and seedling length (5.01 cm) and combinatorial approach of melatonin and *P. putida* can significantly reduce the TMX induced stress on plant's morphology. Increment in the photosynthetic components viz, chlorophyll, carotenoid, anthocyanin and flavonoid by melatonin and *P. putida* treatment corroborates with the plant growth under TMX stress condition. Higher plant growth is related to the photosynthetic area. The ample photosynthetic area, in-turn, elevates photo-assimilates, which results in enhanced growth (Figure 6.7). Osmo-protectants possess defensive properties and acts as stress indicator. In present investigation, application of TMX lead to increase in osmo-protectant content (Trehalose 8.76 mg/g DW; Glycine-Betaine 2.3 mg/g FW; Proline 4.13 mg/g FW), while the distinct application of melatonin and *P. putida* was able to retain the osmo-protectant content in TMX treated *B. juncea* seedlings, bestowing membrane stabilization, better osmotic balance and protection during unfavorable conditions. In current endeavor, elucidation of osmolyte content and their role in context to pesticide toxicity serves as a first of a kind suggesting their role in shielding plants against pesticide stress.

Pesticide mediated phytotoxicity produces ROS which causes an imbalance in redox homeostasis and antioxidative defense system, eliciting oxidative stress, lipid peroxidation and ultimately compromising the growth, development and crop yield. ROS is considered as an index to depict cell damage. An enhanced level of MDA in oxidative stress conditions acts as a marker in measuring cellular damage. The accretion of

activated oxygen molecule is caused by erratic creation and irregular detoxification cycle of ROS which is the repercussion of abiotic stress conditions. Our results depicts that contents of $O^{\cdot-}_2$, H_2O_2, and MDA were much higher in TMX treated plants, whereas, diminution in oxidative stress marks was observed in plants amended with melatonin and *P. putida* under pesticide stress. Our findings also unveil that melatonin and PGPR aids in decreasing MDA levels in plants during pesticide exposure indicating their ameliorative role towards membrane impairment (Figure 6.9). In response to ROS plants regulate theirantioxidative defense system to neutralize oxidative stress mediated by unfavorable factors. We discerned variation in antioxidative activity dynamics during TMX exposure, whilst, increase in antioxidants and antioxidant activity upon supplementation of melatonin and *P. putida* was recorded (Figure 6.11 and 6.12). Organic acids have been recognized to combat theabiotic stress conditions by regulating the osmotic pressure level and ionic balance. In current research endeavor, relative organic acid content the intermediates of TCA cycle (Oxalic acid, Malic acid, Ascorbic acid, Citric acid, Succinic acid and Fumaric acid) were analyzed by HPLC technique. Our findings revealed that relative organic acidcontent in treated plants was considerably higher as compared to the control plant. The accretion of organic acids may correspond to meet energy generation requirement to combat the TMX mediated stress.

GC-MS results of pesticide residue analysis unravel the potential of melatonin and combinatorial application of (melatonin + *P.* putida) for degradation of TMX residues in *B.* juncea seedlings (melatonin and melatonin + *P.* putida decreased TMX by 13.33 and 88.88% respectively). Prominent action of melatonin and PGPR in TMX degradation was depicted. Contemporary study proposes that melatonin and *P. putida* stimulates expression of genes associated with detoxifying enzymes viz *P450, CXE, NADH, POD, GST*. In light of this, exogenous melatonin has proven excellent in degrading thiamethoxam and coping against thiamethoxam stress.

This unfolds new areas of research to ascertain precise molecular mechanisms involved in regulatory processes fostered by application of melatonin and *P. putida* in stressed plants. Moreover, phytoremediation is an eco-friendly, and in situ resilient technique for remediating contaminated soil. Primarily it retains the soil fertility, despite detoxifying

and eliminating toxic compounds. In future, this study can be extended to fields that are pesticide contaminated and with assistance of phytohormones and PGPRs the *B. juncea* will render such soils contamination free. As *B. juncea* has potential to assimilate and

disintegrate pesticides into less toxic state. Due to high biomass and colossal produce *B. juncea* is considered as a suitable candidate for pesticide degradation. Our study breakthroughs can also be extrapolated to other crops for which melatonin and *P. putida* will be exploited as natural bio-stimulating agents in order to palliate the negative impact of multifarious abiotic stresses.

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List of Publications

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