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Total Soluble Protein Mediated Morphological Traits in Mustard Treated with Thiourea and Salicylic Acid

Shipa Rani Dey*, Prasann Kumar*† and Joginder Singh**
*Department of Agronomy, School of Agriculture, Lovely Professional University, Phagwara-144411, Punjab, India
**Department of Botany, Nagaland University, Nagaland, India
†Corresponding author: Prasann Kumar; prasann0659@gmail.com

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ABSTRACT

The total soluble protein-mediated morphological traits in mustard treated with Thiourea and Salicylic acid were investigated. In addition, it tested the hypothesis that the growth regulator salicylic acid protects the photosynthetic apparatus by up-regulating morphological traits. Under natural environmental conditions, seeds were sown in the field, and seed emergence was recorded. For three days after the 15-day stage, plants in the area were treated with thiourea and salicylic acid and allowed to grow for 90 days. Plants were harvested to assess various morphological traits. A follow-up application of SA and Thiourea plants improved plant height, leaf area, internodal length, leaf number, and accelerated plant activity. The up-regulation of morphological traits may have occurred in SA and Thiourea-mediated plants. After treatments, the level of total soluble protein was estimated in the leaves at proposed day intervals.

INTRODUCTION

The continuous quest for sustainable agricultural practices and the mitigation of various biotic and abiotic stresses has driven significant research toward understanding the intricate interplay between plant physiology and exogenous agents (More et al. 2023; Sangwan et al. 2015; Vioratti Telles de Moura et al. 2023). Among the numerous signaling molecules and compounds that have emerged as promising candidates in this context, thiourea (TU) and salicylic acid (SA) have garnered considerable attention. These chemical elicitors are known to modulate plant responses, leading to enhanced tolerance against environmental challenges and improved growth patterns. These topics have always been under constant investigation and development because SA also plays a role in stress tolerance and the metabolic reprogramming of plants during biotic and abiotic stress responses. Mustard (*Brassica* sp.) is a vital oilseed crop with widespread global cultivation and economic significance. As mustard plants are exposed to many environmental factors, including pathogenic attacks, temperature fluctuations, and soil nutrient availability, unraveling the underlying mechanisms that govern their response to stressors is crucial for developing efficient strategies to enhance crop productivity and yield. Total Soluble Proteins (TSPs) mediate various physiological processes as critical players in signal transduction pathways and regulatory networks. The abundance and composition of TSPs within plant tissues are essential indicators of the plant's health and stress responsiveness (Kaya et al. 2023; Nabizade et al. 2023). This study aims to investigate the impact of Thiourea and Salicylic Acid treatments on mustard plants and the consequential alterations in Total Soluble Protein levels. By examining the morphological traits, growth patterns, and biochemical responses, this research sheds light on the intricate relationship between these chemical elicitors and mustard plants at the protein level. Depending on its concentration, mode of application, and plant type, it regulates plants' photosynthesis, water relations, and metabolic aspects. Moreover, SA is also involved in various developmental processes, such as floral induction, root initiation, and senescence, and is a significant component of hormone-derived signaling pathways. Besides leaf structure, it also affects stomatal closure, chlorophyll, rubisco activity, and ribulose bisphosphate carboxylase activity. In addition, SA also plays a critical role in plant defense, as it is involved in the induction of systemic acquired resistance by triggering the expression of pathogenesis-related (PR) genes. SA also confers tolerance to various abiotic stresses through ion exclusion and comparison, osmotic adjustments, lipid

peroxidation reduction, synthesis of protein kinases (SIPK), and regulation of the oxidative system. By regulating the oxidative system, SA helps stabilize the plant's internal environment and protects it from further damage caused by external stresses(Ashraf et al. 2016; Nazar et al. 2015). Seedling growth of Hedysarumcoronarium is facilitated by SA priming. Many studies have examined salicylic acid's response to abiotic stresses, such as salt, Cu, Cd, and Hg. However, little is known about salicylic acid's response to increasing Mn levels in Brassica juncea. The present study thus aimed to investigate the effects of Mn on the photosynthetic apparatus, plant growth, and the response of salicylic acid to Mn on antioxidant enzymes and Osmo protectants in mustard plants. Increasing crop productivity is significant in today's agricultural production. It is achieved through improved crop varieties, better irrigation systems, and the broader use of fertilizers and pesticides. By increasing the number of crops produced, farmers can feed more people at a lower cost, making food more accessible and affordable. In an earlier study, foliar application of thiourea (TU, a non-physiological thiol-based ROS scavenger) increased stress tolerance and yield of different crops. Additionally, using thiourea helps to increase the stress tolerance of crops, reducing the amount of crop loss that farmers experience due to harsh weather conditions. It helps them to produce higher yields and better quality food for their customers. The application of TU enhances the efficiency of PSI and PSII photosystems and vegetative growth. Thiourea helps to reduce the amount of oxidative stress on the crops, which can damage the crop and reduce yields. The crop can withstand harsh weather conditions and produce higher yields by reducing oxidative stress. Additionally, thiourea helps to increase photosynthesis efficiency, which allows the crop to use more energy from the sun for growth. The investigation is expected to yield crucial insights into the mechanisms through which Thiourea and Salicylic Acid influence mustard plants' TSPs, consequently influencing their growth, development, and stress tolerance. Furthermore, the findings from this study could pave the way for harnessing the potential of these elicitors as eco-friendly and sustainable alternatives to traditional agricultural practices, fostering improved crop resilience and productivity in mustard and potentially other crop species (Hasanuzzaman et al. 2022; Islam et al. 2023; Tanveer et al. 2023). Exploring Total Soluble Protein-mediated morphological traits in mustard plants subjected to Thiourea and Salicylic Acid treatments holds promise for broadening our understanding of plant stress responses and providing innovative agricultural advancements. By bridging the gap between molecular signaling and physiological outcomes, this research contributes to the ongoing efforts to create resilient agricultural systems that meet the challenges of a changing environment and ensure food security for the growing global population.

MATERIALS AND METHODS

Salicylic Acid Preparation

Salicylic acid (SA) was procured from Sigma Aldrich Chemicals Pvt. Ltd., India. SA solutions were prepared by dissolving the required quantity (300 ppm) (approximately Rec.) of SA in 5 ml of ethanol in 100 mL volumetric flasks, and the final volume was made up according to the mark by using Double Distilled Water as a solvent.

Source of Sulfur

Thiourea (CH_4N_2S) (1000 ppm) (Rec.) was used as a sulfur (S) source. The required S concentrations were prepared by dissolving the requisite amount in DDW.

Biological Material

Brassica seeds were obtained from Punjab Agricultural University, Punjab. The healthy-looking and uniform-size seeds were surface sterilized with 1% sodium hypochlorite solution for 5 min, followed by repeated washing with double distilled water (DDW).

Treatment Pattern

A field trial was conducted under a randomized block design at Lovely Professional University, Phagwara, Punjab. Field preparation and sowing of brassica seeds were performed according to the recommended package and practices for the Punjab region by the Indian Council of Agricultural Research. A total of twelve treatments and three replications were conducted in this study. The following were the treatments: T0- Control, T1- Sulfur Recommended, T2- Salicylic Acid Recommended, T3- Sulfur (Rec) + Salicylic acid (Recommended), T4- Sulfur (Reco+1/2th of Rec) + Salicylic acid (Recommended), T5-Sulfur (Rec) + Salicylic acid (Reco+1/2th of Rec), T6- Sulfur (Reco-1/2th of Rec) + Salicylic acid (Recommended), T7- Sulfur (Rec) + Salicylic acid (Reco-1/2th of Rec), T8- Sulfur (Reco-1/2th of Rec) + Salicylic acid (Double of Rec), T9- Sulfur (Double of Rec) + Salicylic acid (Reco-1/2th of Rec), T10- Sulfur (Double of Rec) + Salicylic acid (Double of Rec), T11- Sulfur (Reco-1/2th of Rec) + Salicylic acid (Reco-1/2th of Rec)

Each plant's foliage was sprinkled three times with salicylic acid (SA) and thiourea (TU) solution. The sprayer's nozzle was adjusted to pump out approximately one mL (approx.) in each sprinkle. As a result, 3 mL of SA + TU solution was applied to each plant's foliage. The 30th, 60th, and 90th days of word were conducted throughout all

the observations. The assays were repeated three times to confirm the results.

Statistical Analysis

Statistical data analysis was carried out, and the mean and the standard deviation (SD) were calculated. Analysis of variance (ANOVA) and Test of Homogeneity (DMRT) were performed on the data using SPSS (ver. 22.0 Inc., USA) to determine the least significant difference (LSD) for significant data to identify differences in the mean of the treatment. The treatment means were separated by LSD test. Data are presented as mean ± SD ($n = 12$). Factorial analysis of FTIR spectra was performed.

Morphological Traits

Periodic observations were done in mustard to assess the effects of the provided treatments concerning morphological attributes analysis. Five plants were tagged and marked from each treatment plot for periodical observation and assessment.

Plant height: At an interval of 30 days, the height of the mustard plants was measured with a ruler. All five tagged plants from each treatment plot were measured for height, and their average value was considered for each treatment.

Leaf area: After selecting a healthy plant from each plot, the leaves were counted and separated. Then, a leaf area meter was used to measure the area. The total leaf area is the sum of individual leaf areas. The leaf area measurement was done at 30-day intervals.

Analysis of Functional Groups Through FTIR

FTIR was used to analyze the compound's functional group following the protocol by Chamberlain et al. (1969). Fourier Transform Infrared Spectroscopy (FTIR) is a powerful analytical technique used to identify and characterize the functional groups in a wide range of organic and inorganic compounds. FTIR spectroscopy is based on measuring the absorption of infrared radiation by molecules sensitive to the functional groups' vibrational modes. As a part of the FTIR analysis, dried powders of each plant material were used. A dried extract powder of 10 mg was encapsulated in a pellet of 100 mg KBr to prepare translucent sample discs from the KBr pellet. Each powder sample of each plant specimen was loaded into an FTIR spectroscope (Shimadzu, IR Affinity 1, Japan), with a range of scans from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}. This protocol outlines the step-by-step procedure for analyzing functional groups using FTIR.

Materials:

1. FTIR spectrometer
2. Sample holder (e.g., potassium bromide (KBr) pellets or attenuated total reflection (ATR) accessory)
3. Pestle and mortar (if using KBr pellets)
4. Sample preparation tools (spatula, glass slides, etc.)
5. IR-grade KBr (if using KBr pellets)
6. Samples with known functional groups for calibration

Procedure:

1. Sample Preparation
   a. Grind Method (KBr Pellets): i. Thoroughly clean the pestle and mortar to avoid contamination between samples. ii. Prepare a small amount of KBr powder and the sample to be analyzed. iii. Mix the sample and KBr powder in a 1:100 ratio (sample: KBr) and grind them together gently to form a homogenous mixture. iv. Use a pellet press to compress the mixture into a thin, transparent pellet.
   b. ATR Method: i. Place a drop of the sample directly onto the ATR crystal or attach the sample using a suitable method (e.g., double-sided tape). ii. Spread the sample evenly on the ATR crystal to ensure good contact.
2. FTIR Instrument Setup
   a. Power on the FTIR spectrometer and allow it to warm up for the specified time. b. Before starting the analysis, calibrate the instrument using the appropriate background spectrum (e.g., air or pure solvent).
3. Data Acquisition
   a. For KBr Pellet Method: i. Place the prepared KBr pellet in the sample holder of the FTIR spectrometer. ii. Set the appropriate measurement parameters, such as resolution and scanning range (typically 4000-400 cm^{-1}). iii. Acquire the background spectrum using a clean KBr pellet in the same holder. iv. Measure the sample spectrum by subtracting the background spectrum from the sample spectrum.
   b. For ATR Method: i. Place the sample on the ATR crystal and ensure proper alignment. ii. Set the appropriate measurement parameters as in step 3a. iii. Acquire the background spectrum using clean ATR crystal or double-sided tape without any sample. iv. Measure the sample spectrum by subtracting the background spectrum from the sample spectrum.
4. Data Analysis
   a. Save the obtained FTIR spectra for further analysis. b. Identify the characteristic peaks corresponding to different functional groups by comparing them with

reference spectra or databases. c. Assign the peaks to the specific functional groups present in the sample.

5. Interpretation and Reporting
    a. Interpret the results, identifying the functional groups present in the sample based on the peak assignments. b. Prepare a report summarizing the findings and discussing the implications of the identified functional groups.

Note: It is essential to follow appropriate safety measures and guidelines while handling samples and using the FTIR spectrometer. Ensure proper disposal of specimens and cleaning of equipment after the analysis.

Determination of Protein in Mustard Leaves After Treatment

Analyzing protein content in plant tissues is paramount in understanding the physiological responses and metabolic changes induced by various treatments. Mustard leaves (Brassica spp.) serve as a model system for investigating the effects of external agents on protein levels due to their rapid growth and diverse biochemical responses. The Lowry method, a widely used colorimetric assay, is well-established for quantifying protein concentrations and offers high sensitivity and reproducibility. This protocol outlines the step-by-step procedure for determining protein content in mustard leaves after treatment using the Lowry method.

Materials:

1. Mustard leaves samples (treated and untreated)
2. Liquid nitrogen (for snap-freezing the leaves)
3. Extraction buffer (e.g., Tris-HCl or phosphate buffer with protease inhibitors)
4. Homogenizer (mechanical or ultrasonic)
5. Centrifuge
6. Bovine Serum Albumin (BSA) standard solution (1 $mg.mL^{-1}$)
7. Lowry reagents: a. Folin-Ciocalteu reagent b. 2% sodium carbonate solution c. 1% copper sulfate solution d. Distilled water
8. Cuvettes or microplates for colorimetric measurements
9. Spectrophotometer capable of measuring absorbance at 750 nm

Procedure:

1. Sample Collection and Preparation
    a. Harvest mustard leaves from treated and untreated plants at a similar growth stage. b. Quickly immerse the leaves in liquid nitrogen to snap-freeze them and preserve the protein content. c. Store the frozen samples at -80°C until further processing.
2. Protein Extraction
    a. Prepare the extraction buffer by dissolving protease inhibitors in the chosen buffer according to the manufacturer's instructions. b. Weigh the frozen mustard leaves and transfer them to a pre-cooled homogenizer. c. Add the extraction buffer to the leaves in a ratio of 1:10 (w/v). d. Homogenize the mixture on ice until a uniform and homogenous suspension is obtained. e. Centrifuge the homogenate at a suitable speed (e.g., 10,000 x g) and temperature (4°C) to pellet cellular debris. f. Transfer the supernatant (protein extract) to a new tube and keep it on ice.
3. Protein Quantification using the Lowry Method
    a. Prepare a standard curve using the BSA standard solution. Dilute the BSA standard solution to create a series of known concentrations (e.g., 0, 25, 50, 75, and 100 $\mu g.mL^{-1}$). b. Add 100 µL of each standard solution and 100 µL of the protein extract to separate cuvettes or wells in a microplate. c. Add 1 mL of the Lowry reagent (Folin-Ciocalteu) to each cuvette or well and mix gently but thoroughly. d. Incubate the cuvettes or microplate at room temperature for 30 minutes, protected from direct light.
4. Colorimetric Measurement
    a. After the incubation period, measure the absorbance of each sample at 750 nm using a spectrophotometer. b. Ensure the spectrophotometer is blanked using a blank sample (containing all reagents except the protein extract).
5. Protein Concentration Calculation
    a. Plot the absorbance values of the BSA standards against their corresponding concentrations to create a standard curve. b. Determine the protein concentration of the mustard leaf extract samples from the standard curve using their respective absorbance values.
6. Data Analysis and Interpretation
    a. Calculate the treated and untreated samples' mean protein concentration and standard deviation. b. Perform statistical analysis, if applicable, to assess the significance of the observed differences.

The Lowry method provides a reliable and sensitive means of quantifying protein content in mustard leaves after treatment. By following this protocol, researchers can assess the impact of various treatments on protein levels,

gaining valuable insights into the physiological responses of mustard plants to different external agents. The obtained data can contribute to a deeper understanding of the molecular mechanisms underlying stress responses, ultimately aiding the development of strategies to enhance crop resilience and productivity.

RESULTS AND DISCUSSION

Plant Height

Concerning control, the plant height was maximum in T2 at 30 days after sowing. In treatment T11, its maximum height was 60, 90, and 120 DAS. The 30 days older plant shows the maximum height in treatment T2, concerning control (T0). In contrast, the plant height in T2 reached its maximum at 30 DAS compared to the control. Nevertheless, later on, the height was significantly higher than the maximum at 60, 90 and 120 DAS (Fig. 1). The role of salicylic acid in the development and growth of the plant has essential physiological functions, including enhancing the plant's ability to survive and respond to any conditions (biotic or abiotic) by enhancing the resistance of the plant to System Acquired Resistance (SAR) by stimulating or changing the internal environment in the plant. Salicylic acid involves many biological processes, including growth and development, photosynthesis, and stress response. It is believed to act as a signal molecule and can induce the production of other compounds that can help the plant respond to stress and increase its tolerance. It can also help the plant produce more defense compounds to ward off pests and diseases. In contrast, sulfur generally makes plants grow taller. Sulfur also helps to increase the production of chlorophyll, which is necessary for photosynthesis (Barman & Kundu 2023; Li et al. 2023; Wang et al. 2024). It also helps to improve the uptake of minerals and increases the plant's overall health. Sulfur encourages cell division and increases elongation and expansion in plants. There was an analysis of the interaction on plant height in T11 in which it was observed that when sulfur and salicylic acid were applied to the foliar in the appropriate amounts at half the rate of the recommendation, the plant height was observed to be elevated as well as the internodal length elongation. This is because sulfur helps in the production of hormones that boost the growth of plants. It also aids in absorbing other essential nutrients such as nitrogen, potassium, and phosphorus. In addition, the presence of sulfur can help to reduce the amount of stress the plant experiences, which leads to an increase in overall health and vigor. A combination of the two interventions was effective after 60, 90, and 120 days. On the other hand, salicylic acid was found to be effective only for plants 30 days older (Fig. 1). The impact of Total Soluble Protein (TSP) on plant height in mustard treated with Thiourea (TU) and Salicylic Acid (SA) is a subject of significant interest in plant physiology and agricultural research. TSPs are crucial in various physiological processes, including growth and development, as they are involved in signal transduction, enzymatic activities, and stress responses. Understanding how these chemical elicitors (TU and SA) affect TSP levels and subsequently influence plant height in mustard plants can provide valuable insights into their growth-regulatory mechanisms and potential agricultural applications. Increased TSP levels in mustard plants treated with TU and SA may positively influence plant growth and development, enhancing plant height. TSPs are essential components in cell division, elongation, and differentiation, contributing to overall plant height. Elevated TSP levels may stimulate the synthesis of growth-related proteins, such as kinases and transcription factors, leading to accelerated cell elongation and promoting taller plant stature. TU and SA treatments can enhance enzyme activity in nutrient uptake and assimilation, thus increasing nutrient availability for growth processes. As TSPs regulate enzyme activities, higher TSP levels may improve nutrient utilization, increasing plant biomass and taller plants. TU and SA induce systemic acquired resistance (SAR) and priming responses, enabling mustard plants to cope better with biotic and abiotic stresses (Kaya et al. 2020; Parashar et al. 2014; Pirasteh-Anosheh et al. 2023). Higher TSP levels may contribute to synthesizing stress-responsive proteins, such as chaperones and defense-related proteins, thus enhancing stress tolerance and supporting uninterrupted growth, even under challenging environmental conditions. TU and SA treatments can influence hormone signaling pathways, including auxins, cytokinins, and gibberellins, directly impacting plant height. TSPs can modulate the biosynthesis and signaling of these hormones, leading to altered growth patterns and increased plant height. TSPs are involved in cell wall biosynthesis and remodeling, crucial for cell elongation and growth. Higher TSP levels may lead to modified cell wall composition, allowing increased cell expansion and, consequently, taller plant height in mustard. Elevated TSP levels may impact gene expression patterns, particularly those related to growth and developmental processes. TU and SA treatments can activate or repress specific genes involved in plant height regulation, and TSPs can potentially act as mediators in this process. The impact of Total Soluble Protein-mediated plant height in mustard plants treated with Thiourea and Salicylic Acid highlights the complex interplay between chemical elicitors, TSP levels, and growth-regulatory mechanisms. Combining TU and SA treatments may result in higher TSP content, enhancing growth, stress tolerance, and overall plant height in mustard. This knowledge can be harnessed to develop innovative

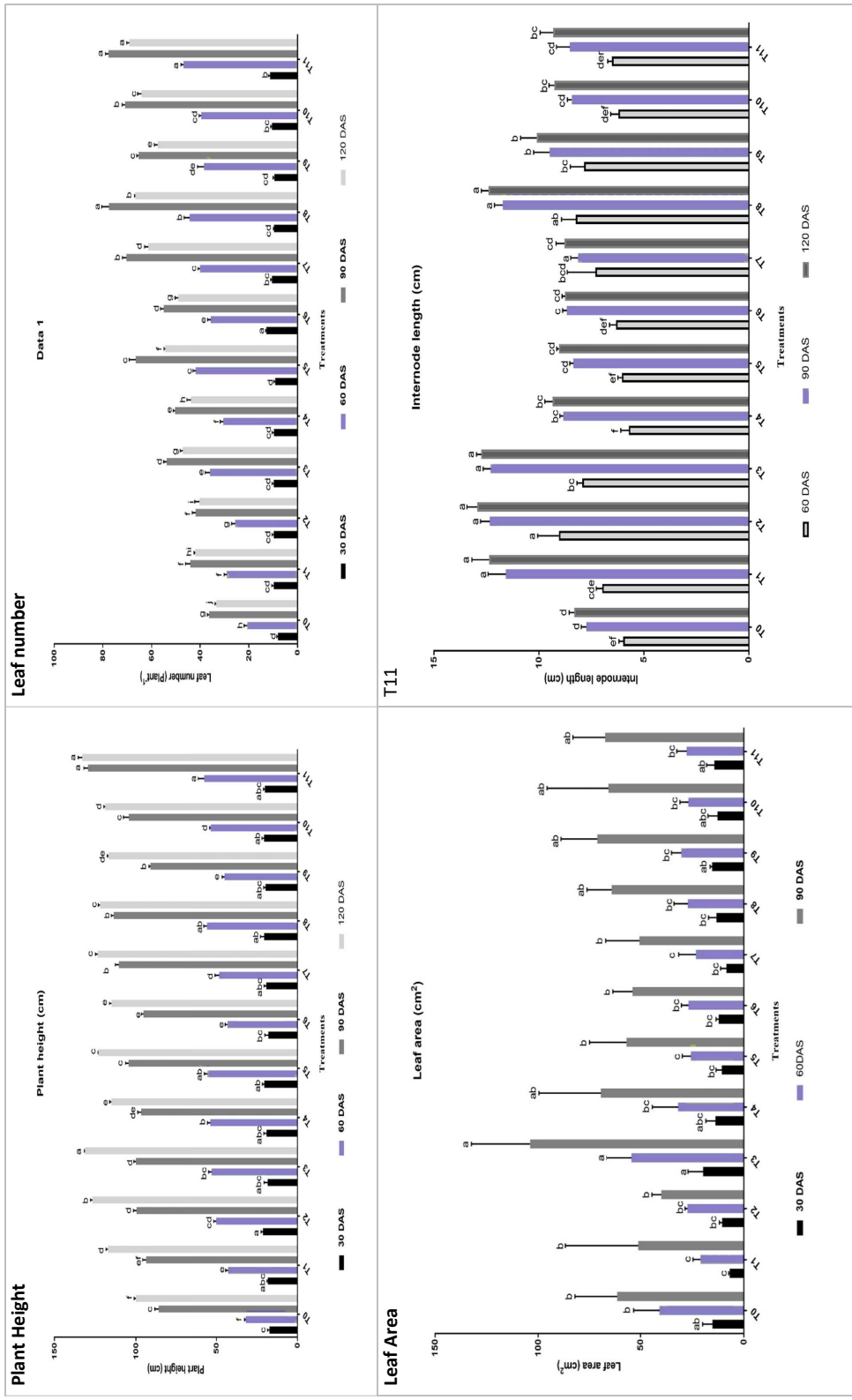

Fig. 1: Comparison of plant height, leaf number, leaf area, and internodal length of mustard crops under different treatments.

strategies for enhancing crop productivity and resilience, contributing to sustainable agricultural practices in the face of changing environmental conditions. Further research is essential to fully elucidate the molecular mechanisms involved in this intriguing phenomenon and its potential applications in crop improvement (Chakma et al. 2021; Panthi et al. 2024; Thepbandit et al. 2023).

Leaf Number

Concerning control, Sulfur (Recommended-1/2th of Rec) + Salicylic acid (Recommended) shows maximum leaf production at 30 days. However, at 60, 90, and 120 days, T11- Sulfur (Reco-1/2th of Rec) + Salicylic acid (Reco-1/2th of Rec) showed the highest number of leaves. In addition to interacting with plant nutrient S, SA interacts with phytohormones, polyamines, nitric oxide, and other nutrients. SA can also control the expression of specific genes, regulate the activity of enzymes, and induce resistance to certain diseases. In addition, SA is involved in plant acclimation to various environmental changes. Numerous reports report that S coordinated studies on auxins, gibberellins, cytokinins, abscisic acid, brassinosteroids, ethylene, nitric oxide, and salicylic acid in plants. These hormones affect plant growth, development, and stress response to environmental stimuli. SA has also been shown to improve plant stress tolerance and yield. The interaction between SA and nutrient S and other phytohormones contributed significantly to plant growth, metabolism, and stress tolerance. Salicylic acid (SA) interacts with other phytohormones and nutrients to transfer environmental signals to the plant cells. It helps plants to perceive environmental stimuli and activate the appropriate responses, such as growth and stress tolerance. The interaction between SA and other nutrients and phytohormones has improved plant growth and stress tolerance and increased yields. Higher GR activity and exogenous SA can result in a higher concentration of S/Cys-GSH. It helps to reduce oxidative damage to the plant and increases its tolerance to environmental stress. In addition, SA can improve the efficiency of photosynthesis and the absorption of other essential nutrients, leading to increased plant growth and productivity. An SA-mediated increase in GSH contents is due to increased ATP-S activity, serine acetyltransferase activity (SAT), and S and Cys contents. SA can also increase the expression of genes associated with photosynthesis and nutrient absorption, leading to more efficient photosynthesis and nutrient uptake (Devi et al. 2023; Hayat et al. 2012; Huang et al. 2024). In addition, SA can regulate the activity of enzymes in synthesizing glutathione (GSH) and other compounds, which increases GSH content and helps protect the plant from environmental stresses. Finally, SA regulates sulfotransferase (SOT12) and S-nitrosylation, which are essential for proper SA homeostasis and signaling (Fig. 1). The impact of Total Soluble Protein (TSP) on leaf number in mustard (Brassica spp.) treated with Thiourea (TU) and Salicylic Acid (SA) is an essential aspect of plant physiology and agricultural research. Enhanced Leaf Primordia Initiation and Proliferation: Increased TSP levels in mustard plants treated with TU and SA may promote the initiation and proliferation of leaf primordia. TSPs are involved in cell division and differentiation processes, critical for forming leaf primordia during early plant development. Higher TSP content can enhance the number of leaf primordia, ultimately increasing the total leaf number. TU and SA treatments can influence hormonal signaling pathways, including those of auxins, cytokinins, and gibberellins, which are crucial regulators of leaf development. TSPs can modulate the biosynthesis and signaling of these hormones, affecting leaf initiation and expansion. Altered hormonal balance may lead to changes in leaf meristem activity, affecting leaf number in mustard plants. TU and SA treatments can induce stress-responsive genes and signaling pathways, increasing stress tolerance in mustard plants. Higher TSP levels may contribute to the synthesis of stress-related proteins, enhancing plant resilience and facilitating leaf development even under adverse conditions. TSPs are vital in photosynthesis and carbon and nitrogen assimilation in plant tissues. Increased TSP content may positively impact photosynthetic rates and assimilate allocation to developing leaves, promoting leaf expansion and the formation of additional leaves.TSPs are involved in cell wall biosynthesis and remodeling, crucial for cell elongation and expansion during leaf growth. Higher TSP levels can lead to modified cell wall composition, facilitating increased cell elongation and ultimately contributing to more extensive and abundant leaves. Elevated TSP levels may impact gene expression patterns during leaf development(Ali et al. 2014; Ayyaz et al. 2022; Darvizheh et al. 2019). TU and SA treatments can influence gene expression in leaf initiation and expansion, and TSPs may act as mediators in this process. The impact of Total Soluble Protein-mediated leaf number in mustard plants treated with Thiourea and Salicylic Acid highlights the complex interactions between chemical elicitors, TSP levels, and leaf development processes. Combining TU and SA treatments may result in higher TSP content, promoting leaf primordia initiation, expansion, and resilience, ultimately increasing the mustard plants' total number. This knowledge can be applied to develop innovative strategies for enhancing leaf development and crop productivity, contributing to sustainable agricultural practices and improved yield in mustard and potentially other crop species. Further research is needed to fully elucidate the molecular mechanisms involved in this phenomenon and

its potential implications for crop improvement and stress tolerance.

Leaf Area

Concerning Control (To), the leaf area increased most at the combined dose of recommended sulfur and salicylic acid (T3) throughout the observation period. SA interacts with several prohormones, polyamines, nitric oxide, and plant nutrient sulfur. SA mediates the regulation of a wide range of physiological processes, such as growth, development, and stress responses. SA also protects plants from pathogens and other environmental stresses. SA is essential for plant growth and development. SA also interacts with different plant nutrients. SA is a key signaling molecule in plant stress responses, and its production is induced in response to a wide variety of environmental and biotic stimuli. SA is also involved in gene expression regulation and is crucial in abiotic stress responses (Jain et al. 2020; Wang et al. 2024; Zhou et al. 2024). In several reports, sulfur has coordinated studies on phytohormones in plants, such as auxins, gibberellins, cytokinins, abscisic acid, brassinosteroids, ethylene, nitric oxide, and salicylic acid. SA has been demonstrated to regulate various physiological processes, such as photosynthesis, root growth, and flowering. It has also been shown to play a role in plant defense against biotic and abiotic stressors. Furthermore, SA has been studied for its potential beneficial effects on plant growth and development. Salicylic acid (SA) is a hormone naturally present in plants. It has been shown to act as a signaling molecule, triggering a cascade of responses to help the plant better adapt to stressors. It includes regulating gene expression, triggering the production of defense compounds, and stimulating the growth of new cells. Additionally, SA and other phytohormones and S play a significant role in plant growth, metabolism, and stress tolerance. SA has been found to significantly affect plant hormones, such as gibberellic acid and auxin, which can lead to enhanced plant growth and development. SA has been found to regulate the expression of specific genes and proteins involved in stress response, defence mechanisms, and growth and development. It also plays a role in activating specific metabolic pathways and can modulate the levels of certain hormones, such as gibberellin acid and auxin. This makes it essential in plant development, growth, and stress tolerance. SA has also regulated cell wall development, essential for plant structure and stress tolerance (Fig. 1).

The impact of Total Soluble Protein (TSP) on leaf area in mustard (Brassica spp.) treated with Thiourea (TU) and Salicylic Acid (SA) is a crucial aspect of plant physiology and agricultural research. Leaf area is an essential trait that directly influences a plant's photosynthesis, transpiration, and overall biomass production capacity. Understanding how TU and SA treatments affect TSP levels and subsequently influence leaf area in mustard plants can provide valuable insights into the underlying mechanisms and potential applications for enhancing crop productivity (Conversa et al. 2024; Islam et al. 2023; Khan et al. 2023; Zulfiqar et al. 2023). Increased TSP levels in mustard plants treated with TU and SA may positively impact photosynthetic efficiency, leading to a more extensive leaf area. TSPs are essential components of the photosynthetic apparatus, playing a pivotal role in light harvesting, electron transport, and carbon fixation. Higher TSP content can enhance the photosynthetic capacity of mustard leaves, resulting in increased leaf area to capture more sunlight and assimilate carbon dioxide. Improved Stomatal Regulation: TU and SA treatments can influence behavior and regulate stomatal density and size.

TSPs may mediate the expression of genes involved in stomatal development and function. Enhanced stomatal regulation can affect water loss through transpiration, potentially leading to larger leaves with efficient water-use strategies.TSPs are critical in cell wall biosynthesis and remodelling, directly impacting cell expansion and differentiation. Higher TSP levels can increase cell division and expansion, contributing to larger leaf areas in mustard plants. TU and SA treatments can modulate hormonal signaling pathways, including auxins, cytokinin, and gibberellins, essential leaf growth regulators. TSPs may be involved in hormonal crosstalk and response, influencing leaf area through hormonal regulation (Ghassemi-Golezani et al. 2020; Ghassemi-Golezani & Farhangi-Abriz 2018; Huang et al. 2024; Shohani et al. 2023). TU and SA treatments can induce stress-responsive genes and signaling pathways, increasing stress tolerance in mustard plants. Higher TSP levels may contribute to synthesizing stress-related proteins, promoting better leaf health and expansion, even under challenging environmental conditions. Elevated TSP levels may impact gene expression patterns during leaf development. TU and SA treatments can influence gene expression in leaf expansion and differentiation, and TSPs may act as mediators in this process. The impact of Total Soluble Protein-mediated leaf area in mustard plants treated with Thiourea and Salicylic Acid underscores the complex interactions between chemical elicitors, TSP levels, and leaf development processes (Akhter et al. 2023; Haghighi et al. 2023; Hundare et al. 2022; SOOD et al. 2013). Combining TU and SA treatments may result in higher TSP content, promoting photosynthetic efficiency, stomatal regulation, cell expansion, and stress tolerance, contributing to increased leaf area in mustard plants. This knowledge can be harnessed to develop innovative strategies for enhancing leaf development and crop productivity, contributing to

sustainable agricultural practices and improved yield in mustard and potentially other crop species. Further research is needed to fully elucidate the molecular mechanisms involved in this phenomenon and its potential implications for crop improvement and stress resilience.

Internodal Length

A maximum internodal length was found in treatment T2 compared to control (T0) at all the observation dates, such as 30, 60, and 90 days after treatment. This suggests that the treatment T2 was effective in increasing the internodal length of the plants compared to the control. The longer internodal length indicates that the treatment T2 successfully promoted better plant growth and development. Besides its role in the growth and development of plants, salicylic acid also plays a fundamental physiological role in plants. Salicylic acid involves various physiological processes, including photosynthesis, respiration, and stomatal closure. It is also known to be a signaling molecule and can trigger systemic acquired resistance in plants. By increasing the levels of salicylic acid, the treatment T2 improved the growth and development of the plants, as evidenced by the increased internodal length. It justifies the observation that salicylic acid plays a fundamental physiological role in plants. This signaling molecule triggers a defense response that helps the plant protect itself from disease. It also helps to regulate the growth and development of the plant, as evidenced by the increased internodal length in the plants treated with the T2 treatment. It suggests that salicylic acid is vital to plants' health and well-being. It includes enhancing the plant's ability to survive and respond to stress conditions (biotic or abiotic) by improving its resistance to System Acquired Resistance (SAR) by stimulating or changing its internal environment to amplify its ability to withstand and respond to stress conditions (biotic or abiotic). By activating the salicylic acid pathway, research has shown that it increases the production of antioxidants and other defence-related compounds which help the plant to better cope with environmental stressors, such as drought, cold, or pests (Bano et al. 2023; Cheng et al. 2020; Farhangi-Abriz et al. 2019; Idrees et al. 2013; Sangwan et al. 2015). In addition, salicylic acid has also been found to reduce the symptoms of diseases caused by fungi, bacteria, and viruses. It is generally believed that sulfur, in contrast to nitrogen, tends to increase the height of plants. When used as a fertilizer, sulfur helps increase chlorophyll production, essential for photosynthesis. It improves the plant's health and allows it to withstand environmental stressors. Hence, it has been found that plants treated with sulfur tend to have higher yields and be more resistant to diseases. There is evidence that sulfur promotes cell division and elongation and stimulates the expansion of cells in plants. Sulfur helps to increase the availability of essential micro-nutrients, such as phosphorus and calcium, which also aids in plant growth. It also helps to increase the production of certain enzymes, which can increase rates of photosynthesis. Additionally, sulfur helps increase the availability of nitrogen, which is vital for synthesizing proteins and other essential compounds in the plant. When salicylic acid was applied to the foliage at the prescribed rate and in the recommended quantity in T2, an interaction analysis revealed that the average internodal length and the amount of salicylic acid applied to the foliage increased. This suggests that the application of salicylic acid and the availability of sulfur in the soil may play an important role in the synthesis of proteins and other essential compounds in the plant, leading to an increase in the average internodal length and overall growth. After 30, 60, 90, and 120 days of intervention, the intervention was effective. The results showed that the application of salicylic acid and the increased availability of sulfur in the soil positively affected the synthesis of proteins, increasing the internodal length of the plant and its overall growth. This suggests that salicylic acid and sulfur can be essential in plant development (Ahmad et al. 2011; Ali 2021; Ali et al. 2024; Ghahremani et al. 2023; Khalid et al. 2023; Osama et al. 2019).

FTIR Spectroscopy in Combination with Principle Component Analysis

The FTIR spectroscopy was performed on a leaf sample for the treatments T2, T3, and T11, as well as the control T0, to determine the effect of each treatment. FTIR spectroscopy involves the measurement of the infrared spectrum of a sample, which helps to identify the functional groups present in the sample. The effects of each treatment can be determined by comparing the ranges of the different treatments and the control. Results showed significant differences between the treatments and the control. There is no other reason this particular treatment was chosen besides the morphological output produced on the plants after these treatments have been applied. This morphological output is associated with a significant increase in the productivity of the plants. It is also believed that the treatment helps prevent disease and insect infestations. Therefore, it is the most effective and economical way of achieving the desired results. The spectral peak under different treatments is observed in Fig. 2. The results show that the spectral peak behavior is affected by the treatments. The figure also shows that the spectral peak increases with increasing treatments. It can be concluded that the treatments significantly impact the spectral peak behaviour (Devi et al. 2023; Dey et al. 2023; Hidangmayum et al. 2023; Kumar et al. 2023; Sharma et al. 2023; Sharma et al. 2023). As shown in Fig. 2, different types of functional groups are stimulated and affected after

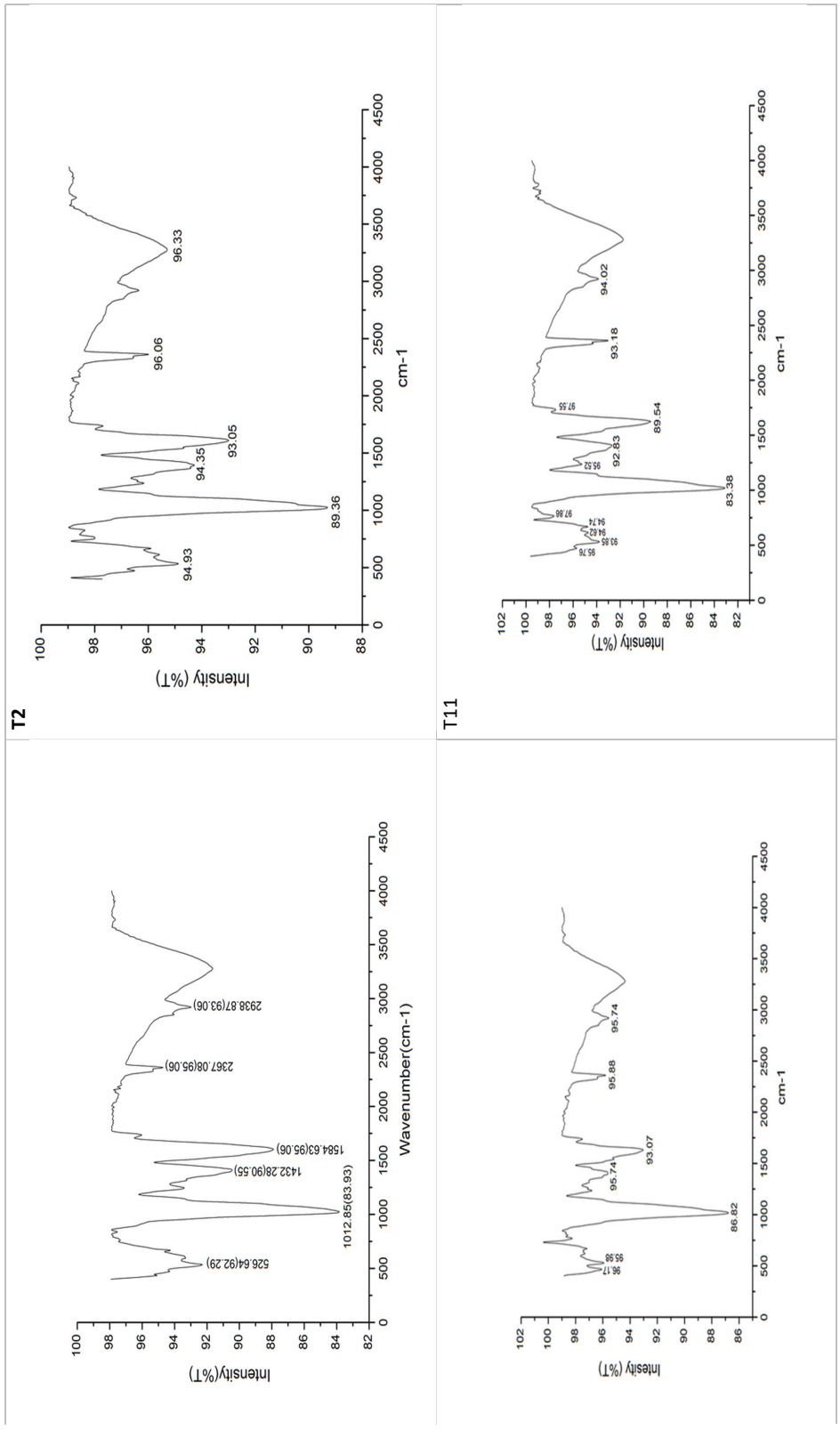

Fig. 2: Analysis of FTIR spectral patterns of leaves samples that were treated with a variety of treatments.

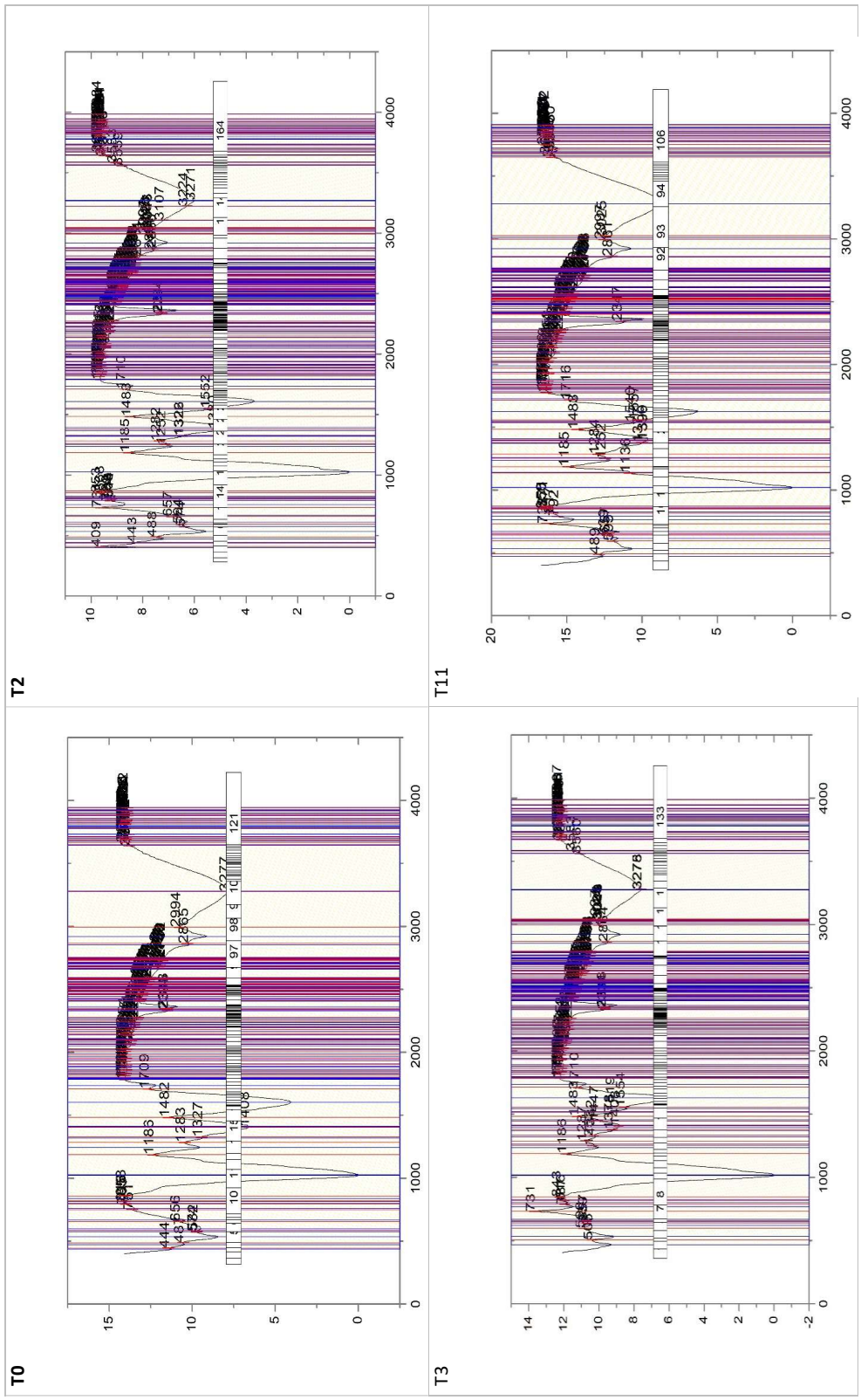

Fig. 3: The number of peaks obtained during FTIR analysis as a result of different treatments.

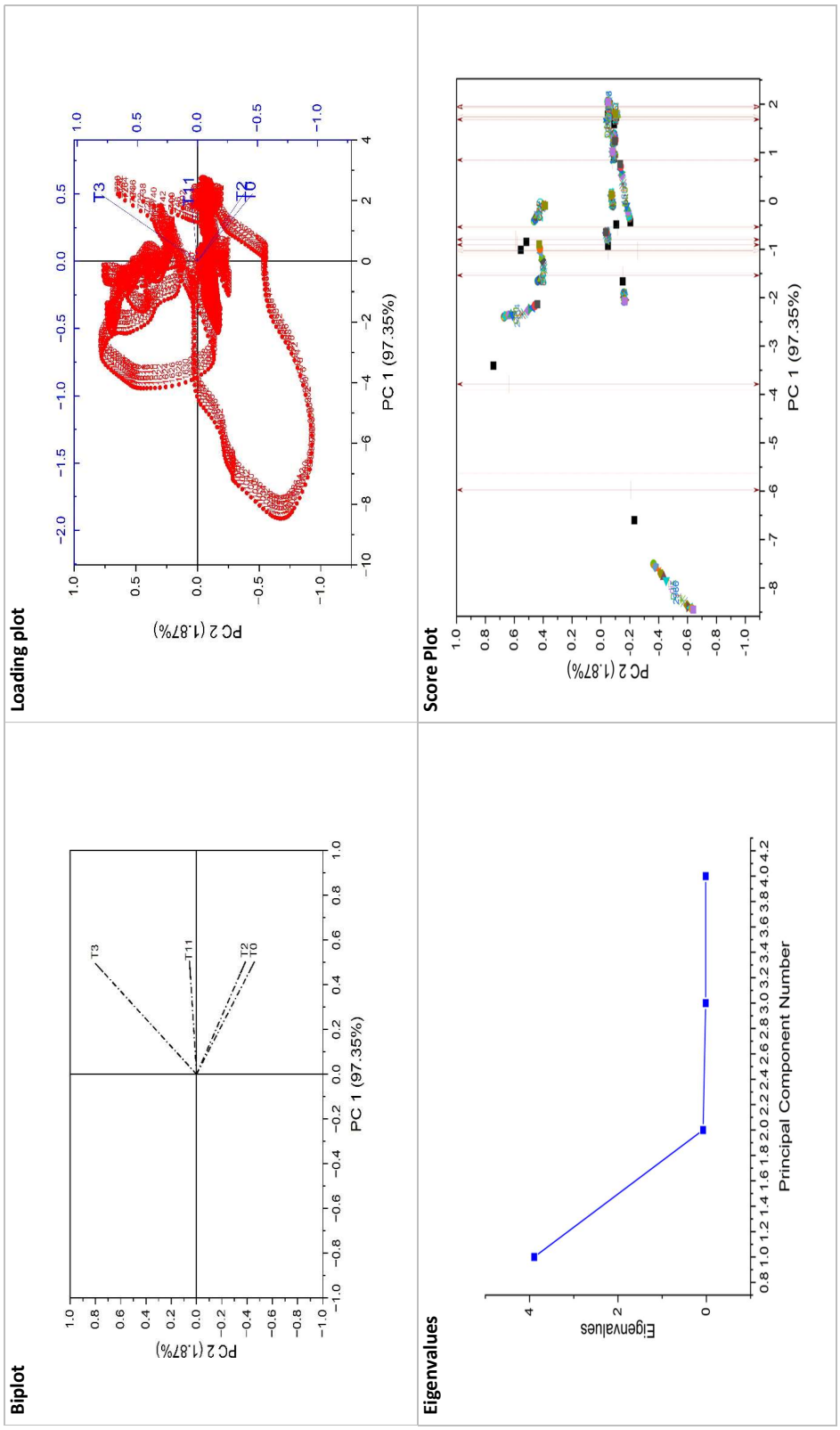

Fig. 4: A PCA analysis was carried out to analyze the FTIR spectra of mustard crops under different treatments.

treatment is applied. The results show that the functional groups were activated differently depending on the type of treatment used. All the treatments effectively started functional groups and influenced spectral peak behavior. As a result, plants that undergo such changes have differences in the types of metabolites in their cells. It leads to changes in the physical and chemical properties of the plants, affecting their growth and development. Such changes directly impact the yield and quality of the produce.

According to the peak analysis results, there were 121 peaks in T0o (Control), whereas there were 164 peaks in T2(164), 133 peaks in T3(133), and 106 peaks in T11(106) (Fig. 3). Most peaks were observed in T2 and T3, while the least was in T11. The peaks in the different time frames represented varying levels of activity. Interestingly, the peak numbers for T11 were lower than the other time frames. It suggests that the activity in T11 was lower than in the different time frames, and the lower peak numbers could indicate this.

Additionally, the varying peak numbers in the different time frames could be attributed to the different activity levels present in each time frame. A threshold value of % height was used to select the peaks, and when this threshold value was subtracted from the constant value of Y, the peaks were selected. The peak values were used to calculate the area under the curve. The area under the curve was then used to calculate the total amount of substance in the sample. This data was then used to determine the concentration of the substance in the sample. The threshold value was selected based on an analysis of the data and was used to identify the points on the graph where the substance was present in the most significant amount. The peaks were identified by subtracting this threshold value from the constant value of Y, and the area under the curve was calculated. This area was then used to determine the concentration of the substance in the sample, which allowed for a more precise measurement than would have been possible without the threshold value (Devi & Kumar 2023a, 2023b; Kumar et al. 2023; Sharma et al. 2023; Sharma et al. 2023; Sinam et al. 2023; Upadhyay et al. 2023).

Using the principal component analysis method, we calculated the factorial. The results showed that the principal component analysis could accurately capture the underlying structure of the T0, T2, T3, and T11 data. The factorial explained a large portion of the variance in the data, indicating that the method was successful. In Graph 4, we have shown the different analyses conducted. The analysis results provide a clear insight into the data structure and its relationships. We can conclude that the principal component analysis is an effective method of data analysis. Using the biplots graph, we were able to determine the distribution of PC1 (97.35%) and PC2 (1.87%) of treatments T0, T2, T3, and T11 in the different quadrants (Q1, Q2, Q3, and Q4). We also identified the most influential variables that significantly impacted the analysis results. The biplot graph was an effective tool for visualizing the data and understanding the relationships between the variables (here, the variables are % Transmittance and wavelength under T0, T2, T3, and T11). The observation also indicated a similarity in treatment. The biplot graph showed that the treatments were clustered in one quadrant, indicating similar effects on the data. It also showed the most influential variables, which were the ones that had the most substantial impact on the outcome of the analysis. It enabled us to understand better the relationships between the variables and the treatments, which helped us make better decisions and interpret the data. During the analysis, one of the essential eigenvalues (0 to 4) (Fig. 4) was calculated, and its distribution was nicely presented. This eigenvalue was used to measure the variance in the data explained by each variable (Principal component number from 0.8 to 4.2) (Fig. 4). By understanding this, researchers could identify which variables influenced the analysis outcome most, allowing them to make better decisions and interpret the data. According to Fig. 4, there is a score plot and a scattered plot based on the analysis of FTIR spectra between % T and wavelength after analyzing FTIR spectra. After treatments, there was a clear difference in outcomes. By analyzing the FTIR spectra, researchers could identify how different variables, such as wavelength, interacted with each other and which variables had the most influence on the outcome (Devi et al. 2023; Devi et al. 2023; Dey et al. 2023; Kumari et al. 2023; Saini et al. 2023). The score and scatter plots in Fig. 4 illustrate the differences in outcomes after treatments. This allowed researchers to make more informed decisions and interpretations of the data. A representation of the loading point of data under PC1 (97.35%) and PC2 (1.87%) can be found in Fig. 4.

Estimation of Proteins in mustard leaves

The detailed table of protein content under the treatment of TU and SA has been represented in another paper by the same author.

CONCLUSION

As a result of the SA application coupled with thiourea, mustard morphological characteristics were enhanced. Additionally, it increases the plant's performance by improving the morphological attributes of mustard. As a result of the spectral changes in the spectrum, it can be concluded that both compounds change the internal metabolites of the

plant and boost its growth and development. In most of the parameters, the treatment T11 [500 ppm Thiourea + 150 ppm Salicylic Acid] was mainly effective on all the days of the treatment. However, as far as the T2 and T3 treatments are concerned, they are most effective during the first months of the plant's growth and development.

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