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Research Article

The effects of theophylline on some physiological and biochemical aspects of *Lactuca sativa* L.

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Abstract

Theophylline is a well-known plant compound belonging to the purine alkaloids group of plant secondary metabolites called methylxanthines. It is commonly found in plants such as tea and coffee. This study aimed to investigate the allelopathic effects of theophylline on lettuce seeds from various physiological and biochemical perspectives using different concentrations of theophylline alongside a control group. Initially, lettuce seeds were placed on plates lined with Whatman paper and cultured with distilled water as the control and a stock solution of theophylline, as well as different concentrations of theophylline (0.1 and 0.05 mg/ml) as treatments, in the incubator. After germination, the seeds were transferred to pots containing perlite. Up to the seven-leaf stage, they were treated with Hoagland solution containing theophylline at 0.1 and 0.05 mg/ml concentrations, with a 0 mg/ml concentration as the control group. The results revealed the allelopathic properties of theophylline. This substance significantly decreased seed germination rates (22% decrease) and resulted in reductions in fresh (44 and 47% decrease in shoot and root, respectively) and dry (63% decrease in shoot) weights, shoot (26% decrease) and root (90% decrease) lengths, photosynthetic activity (54% decrease), and alterations in the activity of antioxidant enzymes (30% decrease in ascorbate peroxidase and 24% increase in polyphenol oxidase). Moreover, changes in band density were observed in the protein electrophoresis pattern, indicating significant effects of theophylline on the expression of certain genes involved in protein synthesis. There is a growing interest in compounds exhibiting allelopathic properties, particularly in agricultural systems. Such studies aim to develop natural herbicides to replace synthetic counterparts.

Keywords: Allelopathy, Allelochemical, Theophylline, Lettuce, Electrophoresis pattern

Abbreviations
ABA: Abscisic acid, BSA: Bovine serum albumin, ROS: Reactive oxygen species, SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide, Tris-HCl: Tris-hydrochloric acid, CAT: Catalase, H_2O_2: Hydrogen peroxide, APX: Ascorbate peroxidase, PPO: Polyphenol oxidase, PRO: Protease, SOD: Superoxide dismutase, TEMED: Tetramethylethylenediamine, KD: kilo Daltons.

Introduction

Theophylline is a well-known plant compound belonging to the purine alkaloids group of plant secondary metabolites, also known as methylxanthines. Purine-based compounds are widely distributed in taxonomically unrelated plants such as *Camellia sinensis* (green and black tea), *Coffea arabica* (coffee), and *Theobroma cacao* (cacao, chocolate). Derived from nucleic acids, theophylline is a methylxanthine that serves as an authorized medicine for treating respiratory diseases, including asthma, bronchitis, emphysema, and other lung diseases (Alamgir and Alamgir, 2018). This

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compound, similar to caffeine, acts to open airways and alleviate shortness of breath. Several studies have indicated that theophylline found in tea may reduce the risk of various types of cancers (Romero-Martinez *et al*., 2021). As a potent bioactive chemical, theophylline has been traditionally employed in medicine for asthma treatment since the 1930s and has been utilized for decades in managing respiratory conditions such as asthma and chronic obstructive pulmonary disease (Monteiro *et al*., 2019).

Despite its significant pharmaceutical usage, improper disposal of tea grounds or waste containing theophylline has led to the leakage of theophylline into water sources, posing risks to human health and causing poisoning in various environmental organisms. Numerous studies have reported the presence of theophylline in aquatic ecosystems, prompting its classification as an emerging contaminant of concern (Vystavna *et al*., 2012). The toxicity of theophylline has been documented to affect certain plant and fish species, impacting their reproduction, growth, and behavior. It can disrupt the normal physiological processes of plants, aquatic invertebrates, and other organisms within aquatic ecosystems, or it can enter farms through water sources, affecting crops. Moreover, research suggests that theophylline can remain active for extended periods, ranging from days to months, depending on environmental conditions. Consequently, it exhibits prolonged persistence in water and has significant potential for bioaccumulation in aquatic organisms (Vystavna *et al*., 2012).

In theophylline-bearing plants, the compound serves an ecophysiological role and acts as an allelopathic or allelochemical agent. It may inhibit insect feeding and can function as a pesticide at concentrations found in plants. Furthermore, at lower concentrations, theophylline acts as a potent synergist for other pesticides (Sanchez-Hernandez *et al*., 2022). Studies have demonstrated that methylxanthines, including theophylline, can inhibit seed germination in certain plants such as *Cicer arietinum* (Mohanpuria and Yadav, 2009).

In 1973, a German botanist named Molich first proposed the term "allelopathy" to describe the secretion of biochemical effects—both positive and negative—between plants and microbes (Staudinger, 2018). By secreting biochemical substances known as allelopathic substances, plants engage in a form of biochemical competition between species (Kong *et al*., 2019). Allelochemicals are secondary metabolites that exert allelopathic effects on other plants and organisms (Latif *et al*., 2017). These compounds derive from the metabolism of carbohydrates, lipids, and amino acids via the acetate or shikimate pathway. While allelochemicals serve as biosynthetic and storage substances within plant cells without affecting plant cell activity, upon release from the plant cell, they begin to affect other organisms (Scavo *et al*., 2019). Present in nearly all plant tissues, including leaves, roots, stems, fruits, rhizomes, seeds, flowers, pollen grains, and buds, the concentration of allelochemicals varies according to the type of organ (Lalrindiki, 2021). The toxicity of allelochemical compounds depends on factors such as concentration, plant age, metabolic stage, season, climate, and environmental conditions. Furthermore, their production fluctuates not only throughout the year but also in terms of age, variety, and type of organ in both quantity and quality (Tlak Gajger and Dar, 2021). In general, allelochemical compounds released from plants represent one of the environmental stresses affecting plant growth and physiology. They influence various physiological processes, including cell division, plant hormone production and balance, water relations, membrane stability and permeability, ion absorption, pollen grain growth, mineral absorption, stomatal movement, pigment synthesis, photosynthesis, respiration, nitrogen fixation, lipid peroxidation, and enzyme activity, thereby impacting the growth and development of agricultural plants (Ain *et al*., 2023).

Allelopathy encompasses any direct or indirect inhibitory or stimulating effect of a plant on other plants through the production of chemical compounds released into the environment. Exploiting this phenomenon, weeds can alter environmental conditions favorably for their growth, leading to a quantitative and qualitative decline in the performance of other plants (Latif *et al.*, 2017). In agricultural ecosystems, plants are more prone to interfering with each other rather than engaging in symbiotic relationships. When weed density is sufficiently high during critical stages of crop growth, it typically results in reduced growth and yield. There are at least two mechanisms by which plants interact with each other: competition for resource absorption and the release of toxic substances into the environment, known as allelopathy (Cheng and Cheng, 2015). Allelopathy represents a natural defense mechanism in plants against natural enemies and competing plants (Hickman *et al.*, 2021). According to the International Association of Allelopathy, any process in which plants produce secondary metabolites that affect the growth and development of other biological systems, regardless of whether the effects are negative or positive, is termed allelopathy (Zhang *et al.,* 2021). Interactions between plants have been a subject of interest for many years.

Lettuce, scientifically known as *Lactuca sativa*, belongs to the Asteraceae family. It is an annual plant native to Asia and serves as a suitable candidate as a model plant for evaluating the allelopathic or phytotoxic potential of natural chemicals (Chu *et al*., 2022). Lettuce is rich in minerals including iron, potassium, calcium, phosphorus, and sodium, as well as trace amounts of sulfur and magnesium (Delaide, 2017). Its high cellulose content contributes to increased intestinal and stomach motility, aiding in the expulsion of substances within the digestive tract. Lettuce contains lactucarium, a substance recognized as a sedative and is also used in ophthalmology to dilate the pupil (Ismail and Obeid, 2023). Additionally, among the metabolites

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present in lettuce, tridas stands out for its analgesic and anti-pain properties (Chu *et al*., 2022). Moreover, lettuce serves as a bioreactor for the production of recombinant proteins and oral vaccines due to its biomass and consumption method (Upadhyay and Singh, 2023). It is also a source of vitamin C, carotenoids, antioxidants, caffeic acid, and flavonols (Hernandez *et al*., 2021), with higher nutrient content found in the darker green outer leaves. Lettuce is low in calories, with each head containing approximately 65 to 70 kilocalories (Carmello and Cardoso, 2018). The nutrients present in lettuce play a crucial role in human health, aiding in the reduction of the risk of cardiovascular diseases and certain cancers (Shi *et al*., 2022).

Secondary metabolites are organic compounds with diverse compositions produced by plants and serve no direct function in plant growth and development (Tiwari and Rana, 2015). Unlike primary metabolites such as amino acids, nucleotides, carbohydrates, and free lipids, secondary metabolites have a limited distribution among plant species, often being found in a single plant species or related groups of species. These compounds serve to protect plants from being eaten by herbivores and contaminated by microbial pathogens (Tiwari and Rana, 2015). The purpose of this research was to investigate the allelopathic effect of theophylline on lettuce seeds from various physiological and biochemical aspects. This investigation is considered one of the effective strategies for the biological control of pathogens and even weeds in different countries. Depending on its mode of action, if theophylline acts as an inhibitor in nature, it can be utilized as a pesticide; Conversely, if it acts as an enhancer, it can promote faster growth of agricultural products in a short period of time.

Materials and methods

The plant cultivation and treatments: The lettuce seeds (*Lactuca Sativa* cv. siahoo) were obtained from Flat Iran Company, while theophylline and other necessary chemical compounds were acquired from Sigma Company (Iran).

This research encompassed both cultivation and laboratory investigation stages. Laboratory cultivation involved operations from the germination stage to planting and transferring seedlings into pots placed in the germinator. The examination of physiological and biochemical traits took place in the physiology laboratory of the Department of Biology, Faculty of Science, at Mohaghegh Ardabili University (Iran). The lettuce seeds underwent initial disinfection with a 1% sodium hypochlorite solution for three minutes, followed by three washes with distilled water. Subsequently, plates lined with filter paper were sterilized in an autoclave at a temperature of 120ºC and a pressure of one atmosphere for 15 minutes (Carmello and Cardoso, 2018).

To prepare various concentrations of theophylline, 10 mg of theophylline was weighed using a sensitive balance (accuracy 0.001 g) and transferred into a test tube. The volume was adjusted to 10 cc using five drops of Tween 20 and distilled water. The substance was completely dissolved using heat, resulting in a concentration of 1 mg/ml. Subsequently, this solution was diluted to obtain other desired concentrations (0.1, 0.05, and 0.001 mg/ml) (Stahl and Wermuth, 2002). To determine the optimal theophylline concentration for further testing, four plates were assigned to the control group and four plates for each tested concentration (1, 0.1, 0.05, and 0.001 mg/ml). In each plate, 20 lettuce seeds were evenly spaced. The prepared solutions, 5 cc each, were added to the respective plates, while 5 cc of distilled water was added to the control group. All plates were then placed in an incubator set at 25ºC. Daily, the number of germinated seeds was recorded. After seven days, the length of the seedlings' stems and roots was measured and analyzed using SPSS20 software. The optimal concentrations of 0.1 and 0.05 mg/ml were identified (Takla and Shawky, 2023) (Figure 1A). Once the cotyledons turned green, the seedlings were carefully extracted using tweezers and transplanted into plastic pots filled with a mixture of peat (coco peat and perlite) at a depth of 2 cm, ensuring that half of the stem remained exposed. For each of the control groups and optimal concentrations, 6 pots were considered (the number of treatments and repeats) (Figure 1B).

The control group was watered with 50% Hoagland's solution, while the treatment group received 50% Hoagland's solution supplemented with theophylline at concentrations of 0.1 and 0.05 mg/ml every two days. Subsequently, both groups were placed in a germinator equipped with a moonlight lamp, set at a temperature of 25ºC, with a lighting period of 16 hours and a dark period of 8 hours, maintaining a humidity level of 80% (Figure 1B). It's worth noting that six pots were allocated for both the control group and the optimal concentration treatments. After 37 days, upon reaching the 4-leaf stage, the plants were harvested for morphological, molecular, and biochemical analyses (Figure 1C).

Physiological parameters: Some physiological traits, including chlorophyll fluorescence, chlorophyll content, photosynthesis, and the fresh and dry weight of roots and shoots, were measured. Molecular studies were conducted following protein extraction in laboratory conditions.

In this phase of the research, the effect of theophylline on chlorophyll fluorescence was measured using a PEA model fluorimeter. Twenty minutes after the leaves were placed in the dark with special clamps, measurements were taken from the middle of the leaf, between the central vein and the edge of the leaf. The evaluated parameters included F0 (minimum fluorescence), Fm (maximum fluorescence), and FV/FM (photochemical efficiency of photosystem II). These parameters were measured at a wavelength of 650 nm (Legendre *et al*., 2021). Chlorophyll content was measured and recorded using an SPAD chlorophyll

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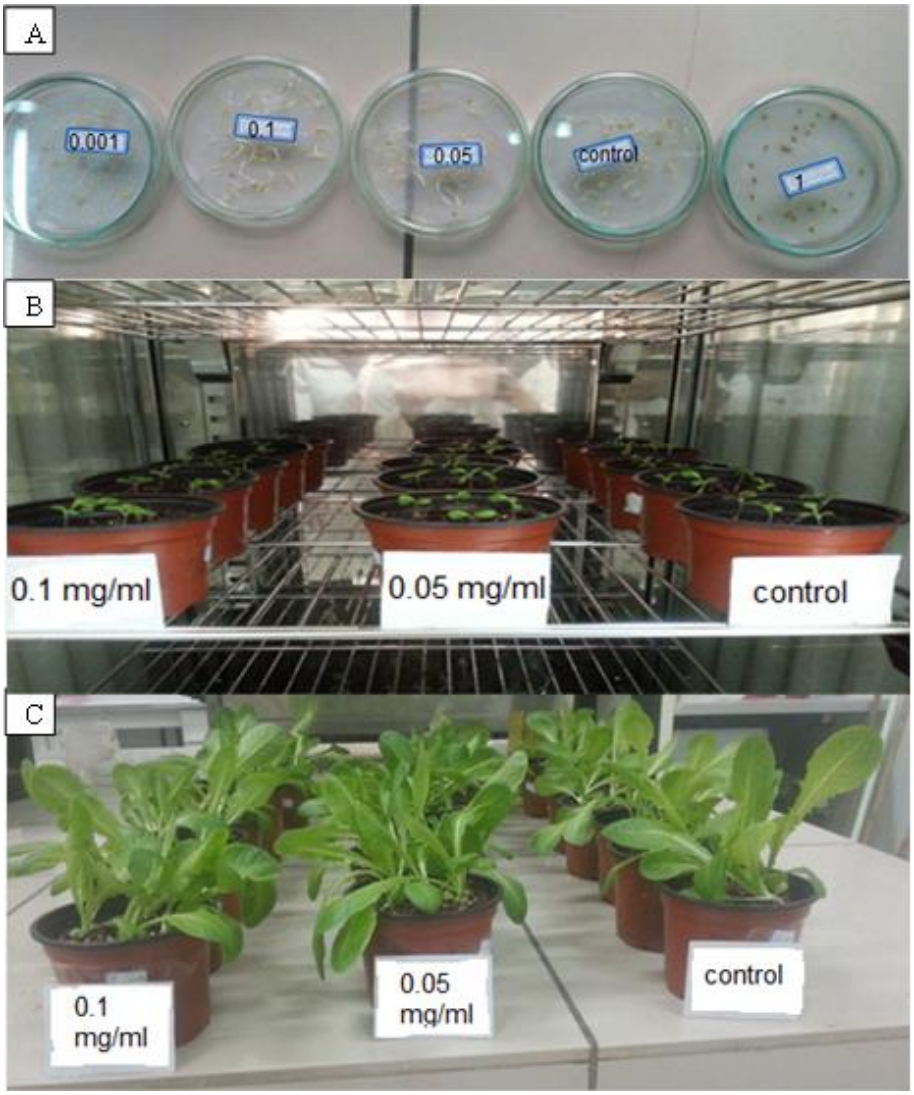

Figure 1. Germination test of lettuce seeds and determination of the optimum concentration of theophylline (A). The germinator setup displaying plant samples treated with concentrations of 0.1 and 0.05 mg/ml theophylline alongside control samples (B). Plant samples in the 7-leaf stage (C).

meter. Measurements were taken from the middle of the leaf, between the central vein and the edge of the leaf (Sub *et al*., 2015). The amount of oxygen released through photosynthesis in the leaf was measured using a photosensitometer, which includes a light and oxygen sensor.

Oxygen inside the chamber was adjusted to 21%, and then a leaf sample was placed inside the chamber. CO_2 gas, necessary for photosynthesis, was injected into the chamber until the oxygen level inside reached 15%. During this process, the device measured and recorded the percentage of oxygen released from the leaf at various time intervals. The light intensity and duration were kept constant (4000 lux and 1000 seconds, respectively). Additionally, the cross-sectional area of the leaf was measured, and the intensity of photosynthesis was determined using a specific formula (Gu, 2023).

$$\text{Oxygen level} = \frac{10000}{\left[\frac{273+T}{273} \times 22.413\right]} \times \%O_2$$

$\%O_2$= percentage of oxygen measured by the device, T= 25ºC

Growth parameters: After harvesting the lettuce seedlings in the 4-leaf stage, the weight of the aerial parts was measured using a scale with an accuracy of 0.001 g. Subsequently, these aerial parts were dried in individual envelopes inside an oven at 120°C for 20 minutes, and the dried weight was measured using a scale with the same accuracy. The roots were then carefully separated and washed, and their dry weight was measured using a scale with an accuracy of 0.001 g (Abd Ghani *et al*., 2023). The length of both the aerial parts and roots of the samples (control, 0.05 mg/ml theophylline, and 0.1 mg/ml theophylline treatments) was measured using a ruler (Abd Ghani *et al*., 2023) (Figure 2).

Biochemical parameters, proteins and enzymes extraction: To extract proteins for total protein measurement, initially, 0.6 g of Tris was weighed and dissolved in 100 ml of distilled water, with its pH

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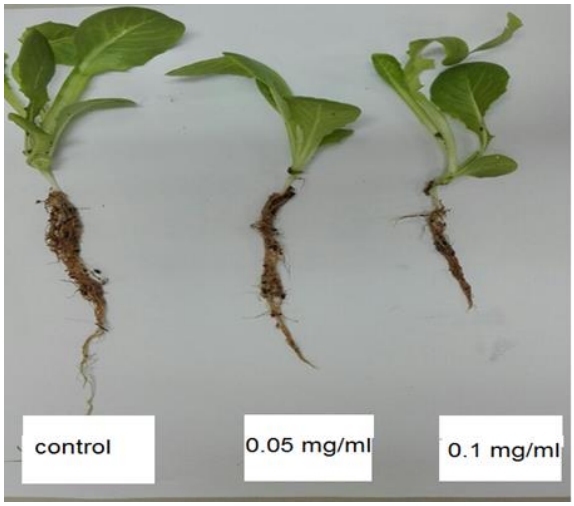

Figure 2. The difference in shoot and root length of lettuce plants in the control and theophylline treatment groups.

adjusted to 5.7 using concentrated hydrochloric acid. Next, to prepare 1M of Na_2EDTA, 3.7 g of Na_2EDTA was dissolved in 100 ml of distilled water. For the protein extraction buffer, 5 ml of 50 mM Tris-HCl (pH 7.5) was mixed with 200 µl of 1 M Na_2EDTA and 33 µl of 0.04% mercaptoethanol, and then distilled water was added to achieve a final volume of 100 ml (Nugraha *et al*., 2021). For protein extraction intended for electrophoresis, 1 g of fresh leaf sample from the control group and both treatment groups was weighed and completely powdered in a mortar using liquid nitrogen. Subsequently, 1.2 ml of protein extraction buffer was added, and the samples were pounded until thoroughly powdered. The powdered samples were then transferred into microtubes and centrifuged for 20 minutes at a speed of 12,000 rpm. Following centrifugation, the supernatant solution was separated. This supernatant was then centrifuged again at 10,000 rpm for 15 minutes, and the resulting supernatant was utilized for electrophoresis (Yadav *et al.,* 2020).

For enzyme extraction, 0.5 g of plant leaves were initially placed in a mortar and pounded with liquid nitrogen until they were finely powdered. Subsequently, 6 ml of 0.01 M phosphate buffer was added to the powdered leaves, and the mixture was transferred into 2 ml microtubes. All steps were performed inside an ice bath to maintain low temperatures. The microtubes were then immediately placed on ice and centrifuged using a refrigerated centrifuge at 13,000 rpm for 15 minutes at 4°C. Following centrifugation, the upper phase was carefully separated and transferred into separate microtubes, which were then stored in a -80°C freezer until further use (Aygun *et al.,* 2022). These samples were utilized for measuring enzyme activity and determining the protein content using the Bradford method (He, 2011).

Quantitative evaluation of proteins: To prepare the Bradford reagent, 100 mg of Coomassie Blue G was dissolved in 50 ml of methanol. The resulting solution was then added to 100 ml of 85% H_3PO_4 and diluted with 200 ml of distilled water. This process resulted in a dark red solution with a pH of approximately 0.01. The usable reagent was obtained by diluting one volume of the stock solution with four volumes of distilled water, resulting in a final pH of 1.1 (Chang and Zhang, 2017). To prepare the standard protein solution, 100 mg of bovine serum albumin (BSA) was weighed and dissolved in 10 ml of distilled water. From this solution, stock solutions with concentrations of 0.25, 0.05, 0.1, and 0.2 mg/ml were prepared. Subsequently, 0.1 cc of the protein extract was mixed with 5 cc of Bradford's reagent, and after vortexing, the mixture was left at room temperature for 15 minutes. The absorbance of the solution was then measured using a spectrophotometer at a wavelength of 595 nm. Based on the absorbance values obtained, the protein concentration was calculated using the standard curve prepared from concentrations ranging from 0 to 0.25 mg/ml of BSA (Bhuiyan *et al*., 2023) (Figure 3).

To assess the total protein content, 0.1 cc of the protein extract, previously stored at -80ºC, was combined with 5 cc of Bradford's reagent. The absorbance of all solutions was measured using a spectrophotometer at a wavelength of 595 nm. Subsequently, based on the equation derived from the standard curve of BSA, the protein content of the leaf samples in each concentration of both the treatment and control groups was calculated (Bradford, 1976).

The activity of enzymes: To assay catalase (CAT) activity, 2.5 ml of 0.05 M phosphate buffer with pH = 7 was combined with 0.3 ml of 3% H_2O_2, followed by the addition of 0.2 ml of enzyme extract, all within an ice bath. The absorbance of the solution was then measured at a wavelength of 240 nm using a spectrophotometer. By applying Beer-Lambert's law, the specific activity of the enzymes was determined (Sharma *et al*., 2023). To measure ascorbate peroxidase (APX) activity, a mixture of 2 ml of 0.05 M phosphate buffer with pH = 6.5, 0.2 ml of 3% H_2O_2, and 5 mmol of 0.2% ascorbic acid was prepared in an ice bath. Subsequently, 0.1 ml of enzyme extract was promptly added to the mixture. Absorbance was then recorded at a wavelength of 290 nm. The

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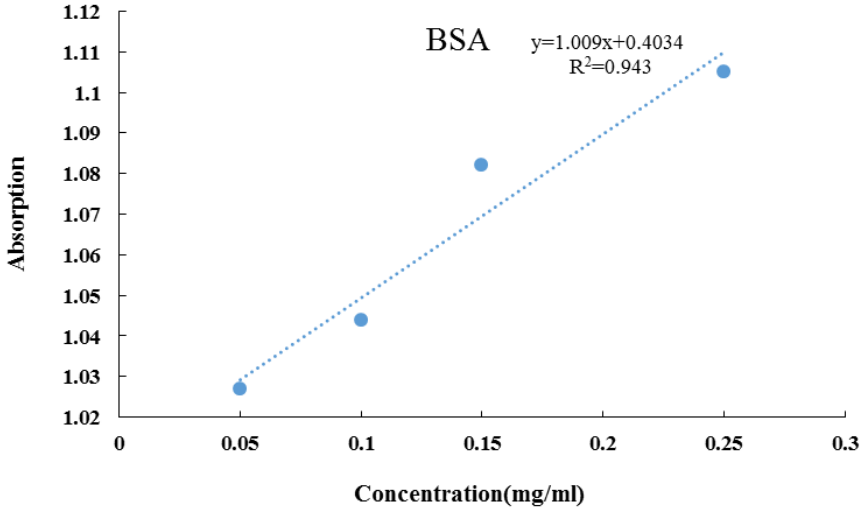

Figure 3. Protein standard curve

activity of the APX enzyme was determined using Beer-Lambert's law (Yasar and Uzal, 2023). To assess polyphenol oxidase (PPO) activity, 2.5 ml of 0.2 M phosphate buffer with a pH of 6.8 was dispensed into test tubes, followed by the addition of 0.2 ml of pyrogallol. The tubes were then heated to 40°C using a bain-marie. Subsequently, 0.2 ml of enzyme extract was introduced into the tubes, and the absorbance changes at a wavelength of 430 nm were monitored using a spectrophotometer. The specific activity of the PPO enzyme was determined using Beer-Lambert's law (Zhang, 2023). To assess protease activity, 2 ml of 0.1% hydrolyzed casein and 0.4 ml of enzyme extract were combined and incubated at 45°C for one hour. Following incubation, 0.4 ml of 40% trichloroacetic acid was added to halt the reaction, and absorbance was promptly recorded at a wavelength of 280 nm (Anwer *et al*., 2023).

Determination of proteins' electrophoretic pattern: To prepare the sample for SDS-PAGE, it was mixed with sample buffer in a specific ratio. The mixture was then heated in boiling water at a low temperature for a few minutes, causing complete protein precipitation due to the presence of SDS and the reducing agent (Mondal and De, 2023).

The electrophoresis procedure was conducted in several stages:

1. The glass plates were thoroughly cleaned. Depending on the required thickness of the gel, a spacer of appropriate size was placed between the glass plates. The glass mold was then secured with clamps and positioned vertically on a plate to prevent leakage of the gel solution from the gap between the glasses. Approximately 0.2 ml of hot agarose solution was placed at the inner edge of the glass mold.

2. The lower gel solution (separating gel) components were prepared according to Table 1. Initially, all components of the lower gel, except for TEMED, were mixed in a suitable container. Then, TEMED was added, and after quick mixing, the solution was poured into a glass mold using a syringe, ensuring a suitable height was maintained, leaving approximately 3 cm of space for the upper gel.

3. Once the lower gel was coagulated, the solution for the upper gel (condensing gel) was prepared following Table 1. After thoroughly mixing the ingredients (with TEMED added last), it was poured to the appropriate height on top of the lower gel. Subsequently, the comb was submerged into the upper gel solution.

4. The upper and lower spacers were carefully removed from between the glass plates. The glass mold was then securely attached to the electrophoresis tank using multiple clamps. Electrode buffers were added to both the upper and lower tanks, filling them to the appropriate levels.

5. One volume of sample buffer was mixed with four protein samples and placed in a container of boiling water for 5 min.

6. The cables were connected to the respective electrodes. For electrophoresis at a constant electric current, a current intensity of 28 milliamps with a voltage of 145 volts was applied. Once the indicator dye reached the bottom of the gel, the electric current was cut off. The gel staining process involved immersing the gel in a staining solution for 2 h followed by an 8 h immersion in a decolorizing solution. Decolorization was achieved using a shaker device (Shcherbak *et al*., 2023).

Statistical analysis: The experimental method followed a completely randomized design with four replications. All statistical analyses were conducted using SPSS software, and the significance of the data was assessed using Duncan's test and standard error at a probability level of $P \leq 0.05$.

Results

The results of growth parameters: The results of this study indicated a significant impact of theophylline on reducing the growth of both roots and stems of seedlings at the 4-leaf stage. Notably, at the highest concentration of theophylline, the root size of plants in

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Table 1. Amount (ml) of materials used for upper and lower gel in electrophoresis

	Upper gel (ml)	Lower gel (ml)
Upper gel buffer	2.5	--
Lower gel buffer	--	2.5
Acrylamide stock solution	1.65	3.3
Distilled water	5.65	4
SDS 10%	0.1	0.1
Ammonium persulfate 10%	0.1	0.1
TEMED 10%	0.025	0.02

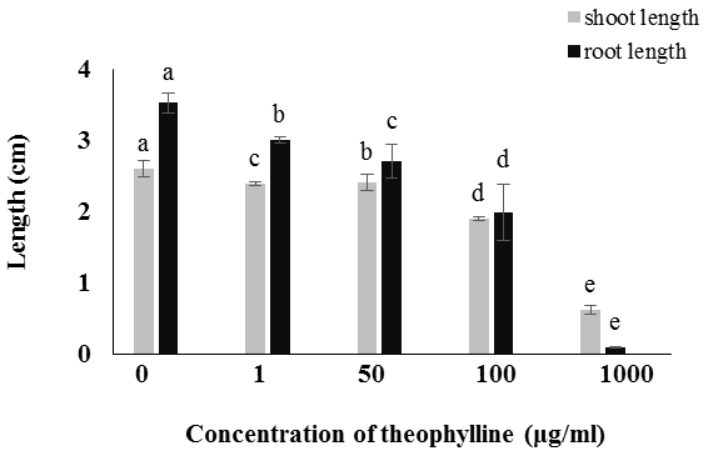

Figure 4. Effects of theophylline on shoot and root length. Similar letters in each column indicate the absence of significant differences based on Duncan's test at the 5% probability level.

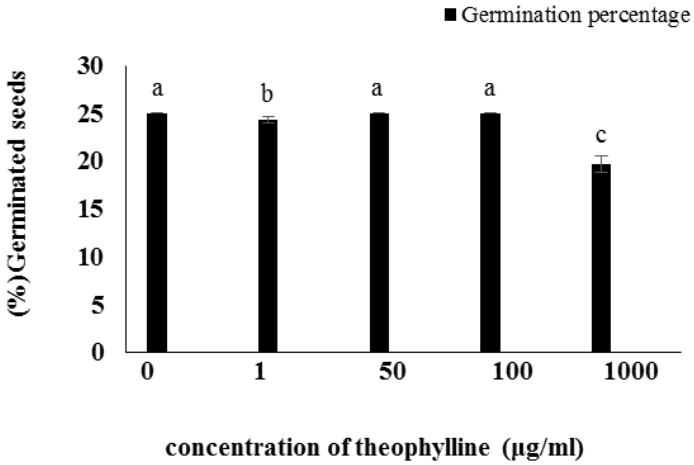

Figure 5. Effects of theophylline on germination. Similar letters in each column indicate the absence of significant differences based on Duncan's test at the 5% probability level.

the treatment group decreased by approximately one-sixth compared to the control group (90% decrease) (Figure 4). Nonetheless, the impact of theophylline on lettuce seed germination was notable, particularly at high concentrations, specifically at 1000 µg/ml (22% decrease) (Figure 5).

The results of measuring the fresh and dry weight of shoot and root showed that the effect of theophylline on the fresh and dry weight of shoot is significant (Figures 6 and 7). The fresh weight of the shoot was more affected by theophylline (44% decrease). While the greatest change in the fresh weight of the root was observed at the concentration of 100 µg/ml theophylline (47% decrease).

The results of physiological traits: The results obtained from the formula for measuring the intensity of photosynthesis showed that the amount of photosynthesis in the treatment group is significantly lower than in the control group (Figure 8). The greatest decrease was observed in the treatment of 100 µg/ml theophylline (54% decrease).

The results of biochemical traits: BSA was used as a standard solution to measure the amount of protein, and according to Bradford's standard curve, the amount of aerial parts proteins in different concentrations was calculated, based on the analysis of the data obtained

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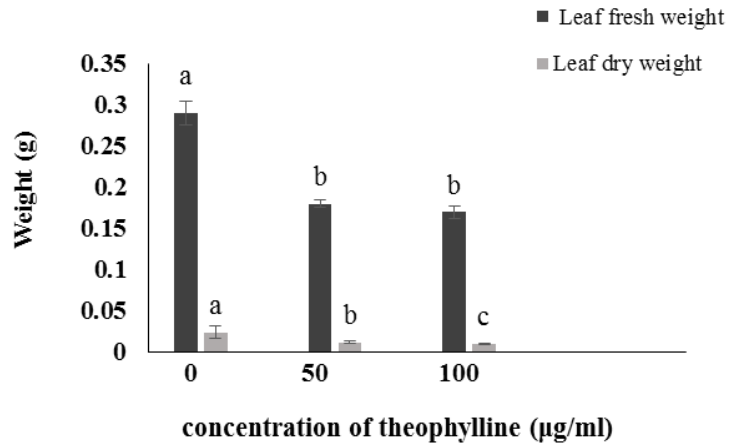

Figure 6. Measurement of the fresh and dry weight of plant leaves in the 4-leaf stage. Similar letters in each column indicate the absence of significant differences based on Duncan's test at the 5% probability level.

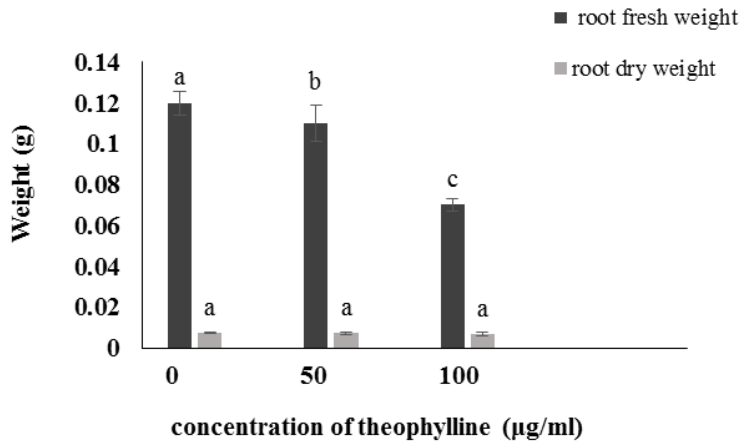

Figure 7. Measurement of the fresh and dry weight of plant roots in the 4-leaf stage. Similar letters in each column indicate the absence of significant differences based on Duncan's test at the 5% probability level.

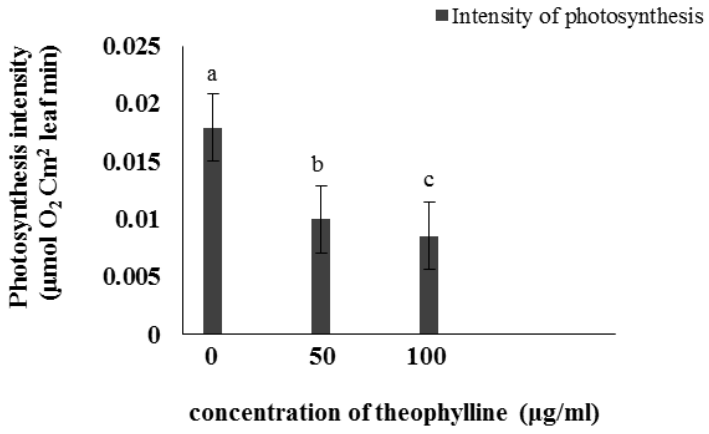

Figure 8. Evaluation of the intensity of photosynthesis. The same letters in each column indicate the absence of significant differences based on Duncan's test at the 5% probability level.

from measuring the amount of protein, different concentrations of theophylline had no significant effect on the amount of soluble protein in the whole shoot.

The measurement of enzyme activity showed that with the increase in theophylline concentration, the activity of APX enzymes decreased significantly (30% decrease), and the activity of PPX enzymes increased significantly (24% increase). However, no significant change was observed in the activity of CAT and PRO enzymes (Figure 9).

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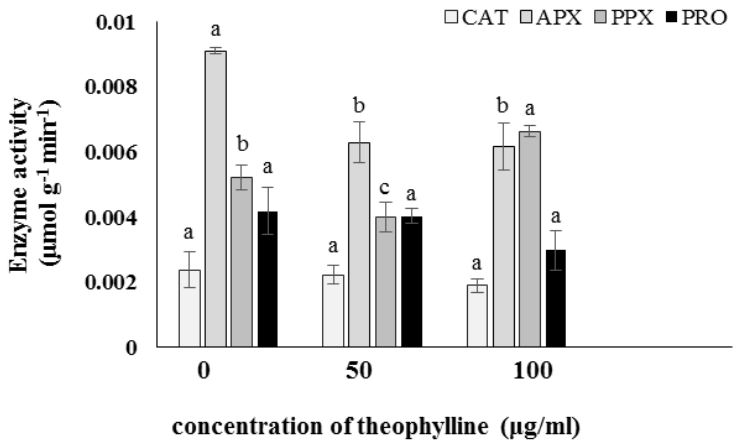

Figure 9. Specific activity of enzymes in control and theophylline-treated samples. Similar letters in each column indicate the absence of significant differences based on Duncan's test at the five percent probability level.

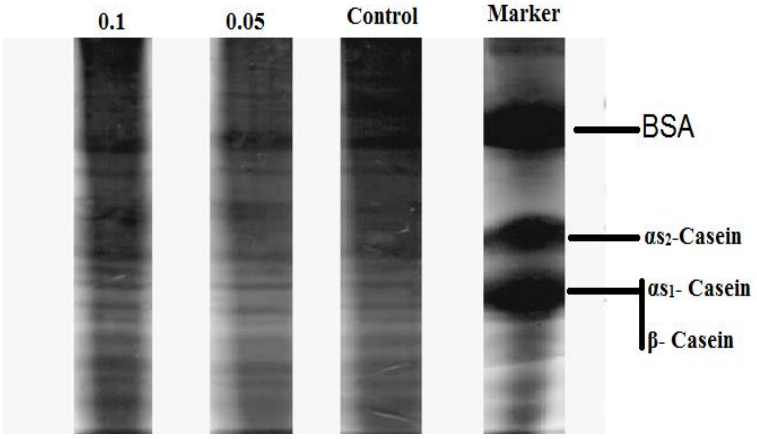

Figure 10. Protein electrophoresis in the control sample and two concentrations of theophylline.

Protein bands of BSA and casein protein appeared during the work process. According to the molecular weight of BSA of 66 KD, casein protein bands with a molecular weight of 25 KD in αs_2, the sum of the αs_1 band with a molecular weight of 22 KD and the β band with a molecular weight of 24 KD formed a band with a molecular weight of 23 KD. The results obtained from electrophoresis showed that the density of protein bands increased with increasing theophylline concentration. The increase of this density was more evident in proteins with molecular weights lower than 25 KD; this increase can be related to the expression of some genes involved in protein synthesis (Pham *et al*., 2024). The indicator band aligned with the protein band of BSA did not change under the effect of theophylline (Figure 10).

Discussion

Allelopathic compounds pose significant biological stress on plants, leading to considerable and sometimes irreparable damage while greatly diminishing agricultural yields. This phenomenon, known as allelopathy, becomes particularly evident during germination and initial growth stages. As concentrations of allelochemicals rise, so do their inhibitory effects (Zhang *et al*., 2021). The results of this experiment confirm, in many cases, other researchers' findings on the allelopathic effects of theophylline on seed germination. The mechanism underlying reduced seed germination due to allelochemicals likely involves a decrease in the activity of enzymes such as alpha-amylase, crucial for the germination process (Cheng and Cheng, 2015). Benarab *et al*. (2020) demonstrated how allelochemical compounds, specifically plant essential oils, hinder wheat seed germination. Through meticulous investigation, they revealed that essential oils penetrate the embryo, impeding germination (Benarab *et al*., 2020). Factors contributing to decreased seed germination include reduced mitotic divisions in root meristems, diminished enzyme activity, and disruptions in mineral ion absorption in the presence of allelochemicals (Scavo *et al*., 2018). Nasrollahi *et al*. (2018) reported thymol's adverse effects on lettuce germination percentage root and stem length, and it should be noted that lettuce seed is more sensitive to allelochemicals. Another study investigated the allelopathic impact of aqueous walnut extract on wheat, onion, and lettuce seedlings, highlighting its detrimental effects on shoot and root length, germination

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percentage, and seedling dry weight (Lal and Biswas, 2023). Cheng and Cheng, (2015) proposed the reduction of root growth as an escape mechanism to avoid allelopathic substance absorption. Scavo *et al.* (2018) examined chalcone's allelopathic effect on lettuce leaves, resulting in decreased aerial parts' dry weight. Similarly, Araniti *et al*. (2020) explored thymol's impact on lettuce leaves, noting reduced shoot dry weight and photosynthesis rates. Our research revealed a reduction in both the fresh and dry weight of lettuce shoots when exposed to theophylline. This finding, along with previous studies, suggests that most allelopathic substances inhibit germination and hinder rhizome and stem growth. This inhibition likely stems from their impact on hydrolytic conductivity, leading to insufficient water availability crucial for cell growth during the early stages of development. Our research revealed a reduction in all the growth parameters in the treatment with theophylline. This is probably due to the role of theophylline in disrupting hormonal balance and the effect of its compound on growth regulators biosynthetic pathways. It may be due to a hormonal balance disturbance between ABA and ethylene in germination seeds, inhibition of water uptake and a reduction in nutrient uptake in plant roots resulting a decrease in leaves relative water contents (Mirmostafaee *et al*., 2020).

Allelopathic compounds exert their influence on photosynthesis by impacting oxygen absorption rates, chlorophyll synthesis, and the electron transfer chain (Xu *et al*., 2024). This interference often results in fundamental disruptions between electron donors (PSII) and acceptors (PSI) (Li *et al.,* 2023). Diaz-Tielas *et al*. (2017) demonstrated the inhibitory effect of chalcone on photosynthesis rates. Similarly, our research observed a decrease in photosynthesis rates in response to theophylline. Chlorophyll, a pivotal protein complex in photosynthesis, is significantly affected by such stressors (Wang and Grimm, 2021). Studies across various agricultural plants have shown that increased stress, including exposure to allelopathic substances, leads to reduced nitrogen levels in aerial plant parts, consequently impacting chlorophyll content and synthesis (Otusanya *et al*., 2015). Allelochemicals influence chlorophyll accumulation through three main avenues: inhibiting synthesis, affecting mobility, and diminishing activity (Ghimire *et al*., 2020). Treatment with allelochemical compounds commonly results in reduced stomatal conductivity, often attributable to decreased turgor pressure (Scavo *et al*., 2018). The stomatal function is influenced by multiple factors, including water content, potassium concentration, and ABA levels (Li *et al*., 2020). Given that roots typically encounter allelochemical compounds first, impairments in water and ion absorption, along with increased ABA levels, constitute significant mechanisms of action (Cheng and Cheng, 2015). Our results regarding the decrease in the intensity of photosynthesis by theophylline were consistent with the findings of others. It could be related to blocking chlorophyll biosynthesis or induction of chlorophyll degradation through allelochemicals (Li *et al*., 2021). A decrease in photosynthetic pigments in allelochemical stress tends to a low photosynthetic rate. These alterations could be caused by a reduction in plant growth at allelochemical stress conditions. Hence, it could be inferred that the high concentrations of theophylline have an inhibitory effect on growth and photosynthesis through a decrease in chlorophyll synthesis (Li *et al*., 2023).

Plants have their own defense mechanisms in response to the generation of ROS by induction of antioxidant enzymes and nonenzymatic antioxidants. The activity of antioxidant enzymes is significantly influenced by allelochemicals, as evidenced by various researchers (Gulzar and Siddiqui, 2017; Li *et al*., 2020; Liu *et al.,* 2018). Studies have shown that allelochemicals can both decrease and increase the activity of enzymes such as peroxidase, PPO, CAT, SOD, auxin oxidase, and APX when exposed to aqueous extracts of different plants and allelochemical compounds (Ghimire *et al.,* 2020). In barley plants, for example, the presence of a 10% aqueous barley extract decreased the activity of APX and CAT enzymes but increased PPO enzyme activity (Dawood *et al*., 2022). Similarly, Razavi (2015) found that coumarin reduced APX enzyme activity while increasing PPO enzyme activity in lettuce. According to Soln *et al*. (2022), the decrease in antioxidant enzyme activity and cell membrane damage induced by allelopathic compounds could be a primary cause of reduced seedling growth in target plants under allelopathic substance influence. The present work revealed that some antioxidant enzymes, such as APX, decreased in response to theophylline. The reason for the decrease in the activity of the enzyme is related to the fact that the stress caused by theophylline in the lettuce plant did not induce the glutathione-ascorbate cycle, and the ROS scavenging process is carried out by other pathways, for example, by PPX (Razavi, 2015).

Protein metabolism is integral to the functionality of all living organisms (Konieczny *et al*., 2023). Allelochemicals play a significant role in protein synthesis, influencing the quantity of protein synthesized. For instance, phenolic compounds, a subset of allelochemicals, impede amino acid synthesis and their conversion into proteins, thereby reducing the rate of protein synthesis (Mahmoud *et al*., 2022). The results of lettuce leaf electrophoresis, depicting protein bands of BSA and casein, serve as confirmation of the proper preparation of electrophoresis gels and the integrity of involved components. Hence, factors hindering desired bands can be disregarded, and any alteration or emergence of bands may stem from theophylline-induced changes in gene sequences. Considering the molecular weights of BSA and casein, it can be inferred that most lettuce plant proteins have a molecular weight below 25 kDa. Notably, in the presence of theophylline, the density of these protein bands increased

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proportionally with theophylline concentration. This increase can be related to the expression of some genes involved in protein synthesis (Pham *et al*., 2024).

Conclusion

The findings of this study indicate that theophylline exhibits allelopathic properties. This substance has been observed to reduce seed germination rates and affect various parameters, including fresh and dry weight, seedling shoot and root length, photosynthesis, and chlorophyll fluorescence in lettuce. Additionally, theophylline alters the activity of antioxidant enzymes. Changes in band density observed in protein electrophoresis patterns suggest significant effects of theophylline on the expression of certain genes involved in protein synthesis.

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