Negative Effects of Pollen Drought Stress on Floral Volatiles, Floral Nectar, Pollinator Behavior, and Seed Production in *Ocimum basilicum* Plants

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ABSTRACT

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Ocimum basilicum is an aromatic plant belonging to the family of Lamiaceae. Many biotic and abiotic factors particularly drought stress influences its reproduction. Drought stress can harm the pollen grains of *O. basilicum* plants and disrupt the production of floral volatiles, reduce floral rewards for pollinators, decrease pollinator activity, and ultimately result in a decline in the seed set. These detrimental impacts highlight the importance of sufficient pollen availability for the successful reproduction and survival of plant species. The reproduction in plants is significantly impacted by drought stress, as it can directly or indirectly alter the attraction of pollinators. In this study, the effect of drought stress in *O. basilicum* plants was investigated by comparing drought- with no drought-stressed plants in order to examine various elements including: (1) nectar quantity and quality (2) pollen production (3) flower volatile emissions (4) pollinator visitation rates from both domesticated and wild species, and (5) the plant's' reproductive outcomes, which contributes to the knowledge of the relationship between drought stress and pollination on *O. basilicum*. The planting was done in two different locations within the Saran division of Bihar state in India, first one is Chapra town and second one is Siwan town, in June 2019. The result indicated that plants with drought conditions, in contrast to drought free plants, produced a reduced quantity of pollen and had a decrease of the number of flowers and the volume of nectar produced per flower. Furthermore, drought affected plants produced nectar with a lower percentage of sucrose in relation to the total sugar content. Bees visiting rate was low compared to control plants and plants emitted more Z-3-hexenol, C_4H_8O, $C_5H_{10}O$, and Isovaleraldehyde. Moreover, this study utilized HPLC analysis to investigate the impact of drought stress on the floral nectar of *O. basilicum* plants. The findings revealed significant changes in nectar composition, highlighting the susceptibility of plant-pollinator interactions and seed production to environmental stress factors.

Keywords: Drought stress, *Ocimum basilicum*, pollen grains, pollinator, seed production

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The Lamiaceae family (mint), a large botanical family with more than 236 genera and 7000 species, is considered to be one of the most important plant families in the world and the most studied (Najar et al. 2022). *O. basilicum* L. is an aromatic plant directly used for spice, medicine, and feed of honeybee, ornamental and also used as raw material for different industries. *O. basilicum* is most widely cultivated due to its high economical value, popularity and demands among the economically important species of basil. Sweet basil is widely distributed throughout sub-tropical and tropical regions and currently widely cultivated in India, Ethiopia, Egypt, Iran, Japan, China and Turkey. Commercially, it is extensively cultivated for essential oil production in many continents around the world for its numerous economical, medicinal and aromatic values (Egata 2021). *O. basilicum* is economically important in India due to its versatile uses in the culinary, medicinal, cosmetic, and religious sectors. Its cultivation and trade provide livelihoods to farmers and contribute to the country's economy through domestic consumption and exports. Some regions in India promote agro-tourism centered on basil cultivation. Tourists visit basil farms to learn about cultivation techniques, purchase fresh basil products, and engage in agro-tourism activities. Many Indian households grow basil plants in their gardens or balconies for personal consumption and use. This widespread cultivation contributes to the economic importance of basil at the local level. The aromatic herb *O. basilicum* is a widely cultivated crop that is highly dependent on pollination for successful seed production. Commercial cultivation of basil is common in many parts of India. Farmers grow basil for its aromatic leaves and essential oil content, which can generate income through the sale of fresh basil or its derivatives. Drought stress is a growing concern in agricultural ecosystems, with potential detrimental impacts on plant reproduction and ecosystem services specifically pollination. The stress can cause early flowering and premature senescence, reducing the time available for vegetative growth and leaf production in *O. basilicum* plants. Additionally, the leaves may become smaller and less flavorful due to reduced oil and secondary metabolite production, impacting the quality of the harvested basil. Drought stress affects basil plants at the physiological and biochemical levels, disrupting essential processes such as photosynthesis, water uptake, and nutrient absorption (Farouk and Omer 2020). These effects can result in reduced growth, lower yields, and compromised plant health. Drought stress triggers the production of stress hormones like abscisic acid (ABA). While ABA helps the plant conserve water by closing stomata, it also disrupts hormonal balance within the plant, affecting growth and development. Drought stress can lead to the production of reactive oxygen species (ROS) within plant cells. These ROS, including hydrogen peroxide and superoxide radicals, can cause oxidative damage to cellular components such as lipids, proteins, and DNA. Oxidative stress disrupts normal cellular functions and can lead to cell death in basil plants. Reduced water availability causes stomatal closure in basil leaves to prevent excessive water loss through transpiration (Barbieri et al. 2012). While this conserves water, it also limits the plant's ability to capture carbon dioxide for photosynthesis, resulting in decreased growth and reduced yield. Basil plants rely on a continuous flow of water from the roots to the leaves for various physiological processes, including photosynthesis. When water is scarce, the plant struggles to uptake and transport water, leading to reduced photosynthetic rates. Pollen grains are essential for the successful fertilization process in plants and their role needs to be further investigated in relation to drought stress because exposure to abiotic stress factors can have adverse effects on plant fertility. For example, the reduction in photosynthate production caused by abiotic stresses can result in a decrease in the mobilization of reserves for tapetum cells. This ultimately resulting so in a significant reduction of pollen fertility. Furthermore, fluctuations in temperature, water availability, and herbivory can have an impact on the characteristics of flowers. Elevated temperatures are known to affect the physiology of flowering plants in a number of ways, resulting in altered production of flowers, nectar, and pollen (Scaven and Rafferty 2013). Also, number of flowers had the strongest effect on pollinator visits. In fact the spatial analysis showed that plants with more flowers received an enhanced pollinator service and more flowers in sequence were visited compared to plants with fewer flowers. **(**Leiss and Klinkhamer 2005). Additionally, plant-pollinator relationships rely on different characteristics of flowers, such as their shape, size, color, and number, as well as the scent they produce and the amount and quality of nectar or pollen they offer (Junker and Parachnowitsch 2015). The distribution of floral rewards can be influenced by changes in the environment, particularly by variations in the climate that affect the availability of water. Drought stress has a notable impact on plant growth, altering their reproductive patterns, which in turn can affect the plants' capacity to attract pollinators and their survival which depend on these resources. The number and size of flowers were reduced due to drought stress including their volume of nectar produced. However, the impact on the concentration of nectar sugars and the quantity of pollen is inconsistent or negligible. Water deficit can alter floral traits with cascading effects on flower-visitor interactions and plant fitness. Water stress induction can diminish productivity, directly resulting in lower flower production and consequently seed set. Changes in floral traits, such as floral scent or reward amount, may in turn alter pollinator visitations and behavior and consequently can reduce pollination services resulting in lower reproduction output (Höfer et al.2023). Plants need to attract and compete for the attention of pollinators to receive their services. At this point, floral recognition by pollinators plays a key role in plant–pollinator systems (Farré et al.2013). Pollinators have the ability to learn and associate specific floral scents with the availability of nectar or other rewards. They can use this information to identify and select plants that are most likely to provide them with the resources they need. In plant–pollinator interactions, the validity of a floral signal should thus depends on the variability in reward amounts offered by different flowers as well as the association of the floral signal with the reward, and the accuracy by which pollinators can detect the signal (Knauer and Schiestl 2015). The complete understanding of how pollen drought stress affects *O. basilicum* plant species including its impact on floral volatiles, floral rewards, pollinator activity, and seed production, remains insufficient. The objective of this study is to explore the possible consequences of drought stress on *O. basilicum* plants, with a focus on analyzing diverse characteristics associated with their flowers, including floral volatiles and rewards, pollinator attraction, and seed production.

Fig. 1. Mature flowering plants of *Ocimum basilicum*.

MATERIALS AND METHODS

Plant materials and growing conditions.

O. basilicum is an aromatic plant that has flowers small in size, measuring around 1 cm in length; purple or white in color, flowers were bilabiate, they have a tubular shape with a protruding stamen that bears the pollen, consists of small clusters of flowers that grow in a terminal raceme or spike. The flowers are typically arranged in an elongated, vertical structure, the youngest flowers located at the top and the older ones below as it is shown in Fig. 1. *O. basilicum* majorly contains about 20 compounds such as linalool, estragole, methyl eugenol, 1,8-cineole, etc., which has been identified by GC-MS30. Camphor, limonene, thymol, citral, α-linalool, β-linalool, estragole, are the monoterpene's of *O. basilicum.* Methyl eugenol is the active compound of *O. basilicum*. Chichoric acid was found in the fresh basil leaves. The intensely purple pigment of flower is due to the presence of anthocyanins (Purushothaman et al.2018). A total of 150 pots having each 10 cm diameter and a depth of around 20 cm were used to plant three seeds of *O. basilicum* in each pot. The planting was done in two different locations within the Saran district of Bihar state in India, Chapra (25.7848°N 84.7274°E) and Siwan (26° 13′ 12″ N, 84° 21′ 36″ E). Experiment was done in June 2019. Coco peat growing media was used as the growing combined with organic manure fertilizer which it was applied at a rate of ¼ tablespoon for every 1 dm^3 of soil volume. Each pot was supplemented by a regular-sized synthetic pot liner at the bottom to prevent soil loss. The pots were placed in a tray with a capacity of 50 pots, arranged in two rows. This tray was provided by greenhouse suppliers and was then positioned on greenhouse benches as it is shown in Fig. 2. Evaporative cooling system was utilized to regulate nighttime temperature between 15°C and 20°C and daytime ranging temperature ranging between 18°C and 28°C at the sprouting stage. Any extra *O. basilicum* plant were removed keeping only one per pot. One teaspoon of Coarse Sand was added on the top of the soil to limit excessive water loss through evaporation. For the initial 2 to three 3, the plants were provided with daily watering until the first indications of flower bud formation. Roughly one month after the planting stage, as soon as the flower buds became visible the watering procedures were started. The trays contained pots which were assigned to either the control group or the drought treatment group, and the pots were arranged in an alternating pattern between the two groups. There were 75 pots assigned to each treatment (control and drought) in each location, resulting in a total of 150 pots per location. For the control group, plants were given water as required, at least once every day, until the soil was saturated. The plants in the drought treatment group received watering every 2-3 days until they were fully saturated. The data was collected on the third day of the drought condition and the process was continued until the plants received treatment in the way that they do not reached the highest degree of stress. This was done in Chapra on the 9th day and in Siwan on the 12th day after the beginning of the treatment. The data collection process was conducted five more times on different dates over the course of one month. The data collection was carried out regularly on the third day of the drought stress period from both locations, as per the watering schedule.

The impact of drought on plant growth and characteristics related to vegetative growth.

Drought stress was verified by conducting water potential assessments utilizing a Scholander pressure (PMS 1000 Pressure Chamber) on the newest fully-grown leaf with a petiole of at least 0.6 cm in length. Drought limits plant growth and field crops production more than any other environmental stress (Saeidi et al. 2015). Stress is an altered physiological condition caused by factors that tend to disrupt the equilibrium and strain is any physical and chemical change produced by a stress (Jaleel et al. 2009). In fact, water potential in the leaves was measured at different times of the day and was carried out on the most recent fully developed leaf that has a stem of minimum 0.5 cm in length for each treatment and location; while it was determined in their stems at mid-day using a different set of five plants per treatment. The investigation took place at three times, on different days and at different time slots: from 4:00 to 6:00 in the morning and from 12:00 to 2:00 in the afternoon. Every treatment involved the evaluation of the water potential of 35 plants during the early morning and afternoon. To obtain mid-day measurements, and to guarantee the closure of the stomata and establish a balance in the water tension within the leaf and stem, leaves were covered with aluminum foil bags for 35 min before being placed in the pressure chamber. To estimate effects of drought on plant growth, the height of a distinct set of 24 plants for each treatment at each location were measured both at the beginning of the treatment and on five subsequent times, over a period of approximately 30 days. The height increase was determined by the measurement of the distance from the apical meristem's tip to the pot's uppermost point. Then, the rate of growth was calculated using this data and applying the formula of Horak and Loughin (2000):

In Height $_{t2}$ - In Height $_{t1}$ / ($t_2 - t_1$), where, t_1 = Date of initial measurement; t_2 = Date of final measurement.

Floral characteristics.

The amount of nectar and the quantity of pollen.

Nectar composition is determined following method used by Rering et al. (2020). To assess the impact of drought stress on the production of nectar, a group of ten flowers were taken from the most recent clusters from six plants per treatment at each location. Microcapillary tubes of 0.6 cm were used and performed the measurements on separate sets of plants on five different sampling dates. Sixty plants were examined for each treatment. For chemical analysis, all of the gathered nectar was placed into a tiny tube that had a capacity of 0.2 ml and filled with a 70% ethanol solution. Collection of nectar were continued from other flowers on the same plant until obtaining at least of 0.6 µl of nectar per plant. Collected nectar were preserved at a temperature of -85°C. In order to determine the quantity of pollen generated, samples were obtained from the same plants that were utilized for collecting nectar, with a total of 70 plants per treatment. Pollen volume per flower is well predicted statistically by floral morphology; nectar sugar mass and pollen volume per unit area were correlated with flower counts, raising the possibility that resource levels can be estimated for species or habitats since they cannot be measured directly (Hicks et al.2016). At the pre-anthesis stage five flower buds with fully grown purple petals closely clustered together from each plant were gathered and each was stored in a separate microcentrifuge tube at a temperature of -85°C. Anthers were eliminated from the flower bud and carefully crushed using forceps inside a microcentrifuge tube. Afterward, a small amount of basic fuchsin was added to 50 µl of ethanol, resulting in a light stain. The tube was vigorously agitated for 35 s using a vortex mixer. A volume of 1 µl from resulted solution was extracted and deposited onto a Makler counting chamber to identify and count each individual pollen grain. By far the largest dry weight of nectar is sugar, but various researchers have demonstrated the presence of proteins, lipids, alkaloids and other compounds which are probably of great importance in nutrition but represent less than 0.03% of dry weight (Vear et al. 1990). To determine the average number of pollen grains in a 1-microliter sample, the counts from five flower buds per plant were averaged. Once the samples of nectar and pollen were collected, the plants were removed.

Fig. 2. *Ocimum basilicum* growing seedlings plant-pots in greenhouse.

Nectar composition.

Floral nectar is the most important reward offered to pollinators in angiosperms (Wolff 2006). Nectar composition is determined following method used by Rering et al. (2020). By coupling the Evaporative Light Scattering Detector (355/ELSD model) with an HPLC system (Agilent 1260 Infinity II HPLC model), The constituents of nectar, including sugars and various other compounds, were isolated and subjected to analysis, offering valuable information about its chemical composition. A calibration curve was generated by testing fructose, glucose, and sucrose solutions in 70% ethanol, ranging from 0.05 to 1 g/l concentrations, to establish a sugar content measurement standard. Identification and quantification of fructose, glucose, and sucrose in the nectar samples were done by this setup.

The study focused on both the total sugar content and the ratio of sucrose to total sugars in nectar, as honeybees are known to prefer nectar with higher sucrose levels. The hexose sugars glucose and fructose are similar to sucrose in the relation between density and concentration w/w and in the energy content per gram. Hence, the error in energy calculations introduced by assuming all solutes to sucrose, when in fact they are largely glucose and/or fructose, is only equivalent to about 3–4% as sucrose w/w (Corbet 2003). The nectar samples were dried through lyophilization process for an entire night until they were completely dry. Afterwards, they were mixed with 225 µl of 70% ethanol, and then filtered by nylon membrane filters to remove any impurities. The ZORBAX carbohydrate analysis column, measuring 4.6 × 180 mm with a particle size of 5 µm, along with the ZORBAX NH_2 analytical guard column, measuring 4.7 × 12.4 mm with a particle size of 5 µm, were employed to attain separation within a time frame of 15 min. The columns were maintained at a stable temperature of 32°C, and a steady flow rate of 1.5 ml/min was employed. An isocratic elution method was used, employing a solution consisting of 75% acetonitrile and 25% water. The majority of samples were administered using a 20 µl volume for injection, but for samples with concentrations that were not detectable, the injection volume varied between 1 and 20 µl. The ELSD was utilized with specific parameters: The evaporator temperature was set at 100°C, the nebulizer temperature at 62°C, and the nitrogen gas flow rate at 1.65 standard liters per minute.

To provide insights into the composition of the floral nectar and the impact of drought stress on the chemical profile, High-Performance Liquid Chromatography (HPLC) was performed. The separation was carried out on a C18 column (250 mm × 4.6 mm, 5 µm) maintained at 30°C. The mobile phase consisted of a gradient mixture of solvent A (0.1% formic acid in water) and solvent B (acetonitrile), with a flow rate of 1.0 ml/min. The injection volume was 10 µl, and the detection wavelength was set at 254 nm. The HPLC analysis aimed to identify and quantify the compounds present in the floral nectar of *O. basilicum* plants under different experimental conditions.

Floral volatiles.

Volatile organic compounds (VOCs) released by plants serve as information and defense chemicals in mutualistic and antagonistic interactions and mitigate effects of abiotic stress (Tholl et al. 2021). Floral scent is also an important component of pollinator-mediated reproductive isolation, as it is often related with color and morphology in sister species with different pollination systems (Kirkpatrick and Ravigne 2002). Floral signals are sexual signals, much like the flamboyant sexual displays found in many animals. However, floral signals do not directly address the sexual partner, but a vector (pollinator) that transfers the male gametes (Schiestl 2015). Floral volatile emissions were examined in each location namely (Chapra and Bihar), and for each treatment, four plants were analyzed, and this process was repeated for five different sampling dates, resulting in a total of 20 plants per treatment. To prevent unintentional damage to delicate tissues in drought-stressed plants, all inflorescences from each plant were cut just before sampling. The flower stems were then immediately placed into autosampler vials (Thermo Fisher Scientific India) filled with 2 ml of high-quality water. The damage caused was the same across all treatments. The analyses and interpretations were focused in comparing the variations in volatile emissions between the treatments. Flowers were picked totaling 1200 in number and promptly moved from the greenhouse to the laboratory. In the lab, they were placed inside a sealed approximately 473 ml scanning jars, which rubber gasket and lids had made from silicon sheets. Lids were created for sampling by drilling a small hole. The flowers were enclosed in jars for one and half hours to collect volatile compounds. Afterward, Sample Enrichment by Solid Phase (SPME; Gerstel, Mülheim and der Ruhr, Germany; multiple layers coating thickness, 2 cm exposed length, and the coating composition DVB/CAR/PDMS) were inserted through the tape layers to absorb these volatiles for 20 min. The SPME fibers effectively captured trends in compound abundance because the nectar samples had moderate concentrations and similar volatile blends. Glassware and lids were heated on 100°C before use for a minimum of 10 h. Floral volatiles were distinguished from non-floral volatiles by thoroughly analyzing the chromatographic characteristics in both floral and blank samples, examining 20% of the floral samples and all of the blank samples. To help identify analytes, duplicate samples of floral volatiles were collected using two Solid-Phase Microextraction (SPME) fibers. These duplicates were analyzed using two different Gas Chromatograph-Mass Spectrometers (GCMS) with distinct analytical columns to improve identification accuracy. The enormous variety of metabolites emitted by plants suggests that volatile compounds may provide a detailed language for communication (Pichersk and Gershenzon 2002). Volatile compounds were collected on a sampling fiber for very short period to concentrate them. After collection, the compounds were thermally desorbed in injector ports (5 min) of chromatography instruments. Split less mode of process was used to direct all the material into the analytical column after that two setups were used for GCMS analysis (Thermo Scientific TRACE 1300 Series GC coupled with a Thermo Scientific ISQ Series MS). Volatile were analyzed using the method of Tholl et al. (2006). Data obtained from GCMS using a DB Wax column were utilized for a semi-quantitative comparison of compounds.

Pollinator attraction.

Basil was found to attract a great richness and abundance of bees, which makes it suitable to enhance bee conservation and management in agricultural environments (Pereira et al. 2015). To investigate the impact of drought stress on pollinator attraction, a greenhouse was designated for a scientific bumblebee colony (*Bombus terrestris*) comprising around 75 worker bees. The colony obtained from an agricultural research institute (PUSA). It was kept in isolation within the greenhouse for three days. Pollination is an ecosystem service that is considered to be at risk under changing landscapes in the modern world and also it is evident that 90% of the production of the crops will severely be affected without the pollinators (Venkatesh et al. 2022). Data collection began in each specific location after this isolation period. The bees collected nectar from well-hydrated basil plants. New experimental plants were introduced, organized into trays with different treatments. Each treatment had three trays with five pots each, totaling 15 pots per treatment. The greenhouse was set up with two rows of trays, around 5 meters from the bumblebee colony. The trays were arranged alternately, and the pots were placed close to each other. Observations began after the colony's door was opened, with data collected on bee visits to the plants from 9:00 AM to 10:00 AM. Observations occurred in Chapra and in Siwan. Data was collected for both visit frequency and duration. Plants that attracted bees for longer periods were observed multiple times. After observations, the pots were returned to their original locations, watered, and their positions shuffled to prevent bees from memorizing plant locations. A field study was conducted with 18 plants per treatment placed outside the greenhouse. Observations were made twice, with a 1-hour break. Data on various pollinators (honeybees, bumblebees, flies, wasps) were recorded. The study spanned multiple locations and was repeated five times at each. Used plants were replaced, and the number of blooming flowers per plant was noted to assess flower density's impact on pollinator attraction. This data collection was carried out both in the greenhouse and the field to ensure accurate analysis.

Changes in seed set in plants.

Seed quality (viability and vigor) can have a profound influence on the establishment and the yield of a crop (Farahani and Maroufi 2011). During the study, plants were grown in a greenhouse and received different amounts of watering as a part of the experimental design. Bumblebees were observed visiting some of the plants, while another set of plants were not visited by bees but were still grown under the same conditions. The plants were kept in the greenhouse until they produced seeds. Weekly count of mature seeds was done on each plant. After the duration of five weeks, the plants were collected and the amount and weight of fully-grown seeds generated by each plant were recorded. In order to determine the average weight of each individual seed produced by a plant, the total weight of seeds from that specific plant was divided by the total number of seeds obtained from that plant.

Statistical analysis.

The study employed complex statistical models, including linear models, to understand the effects of various factors on plant growth, flower rewards, and seed production. These factors included treatment type, experiment duration, greenhouse location, and their interactions. For plant height, linear model was used. Drought impact on flower rewards was also analyzed using linear models, with treatment, time, greenhouse, and their interactions. Various factors like nectar quantity, sugar concentration, sucrose ratio, and pollen count were examined and adjusted in the analysis. Floral volatile compounds were assessed by normalizing their peak areas per observed flower, considering treatment and time. The study also compared honeybee and fly populations using specialized statistical methods, considering factors like treatment, time, and flower quantity. Linear models were used to understand the impact of treatment, bee visitation, and their interactions, along with the greenhouse factor.

RESULT

The impacts of drought on plants and their physical attributes.

In our study, plants exposed to drought conditions showed significantly higher levels of stress compared to the control group. Measurements taken before dawn and at mid-day produced varying results, indicating differences between the two sets of measurements. Over time, there was no significant change in the disparities between the treatment group and the control group, suggesting that the effect of drought stress remained consistent. Furthermore, throughout the experiment, the plants subjected to drought stress consistently exhibited lower rates of relative growth compared to the control group, and this difference was statistically significant.

Floral rewards including nectar and pollen production

The plants that were exposed to drought produced 44% less nectar than the control plants that were not affected by drought. The amount of nectar produced by plants that experienced drought stress was determined to be 0.42 ± 0.05 µl for every 12 flowers in a single plant, (Table-1), whereas the plants in the control group produced 0.73 ± 0.07 µl of nectar for every 10 flowers in a single plant. In addition, the nectar produced by plants experiencing drought had a lower percentage of sucrose compared to those that were not under drought conditions. To put it differently, the nectar collected from plants experiencing drought conditions had a sucrose content of 0.197 ± 0.017 mol/mol, whereas the nectar from plants that were not under stress had a sucrose content of 0.233 ± 0.013 mol/mol. However, there was no significant difference in the total sugar content of the nectar between the two groups. The nectar from the drought-stressed plants had a sugar concentration of approximately 3.4 ± 0.1 mol/L, while the control plants had a slightly higher concentration of about 3.5 ± 0.2 mol/L. These variations remained consistent throughout the experiment as indicated by the lack of significant interactions between the treatment and time factors. The treatment had an effect on the properties of the nectar, but it did not have any influence on the amount of pollen grains that were produced. The floral fragrance of *O. basilicum* which includes a variety of 54 distinct chemicals, was observed in both plants under normal conditions and those subjected to drought stress. In the study it was found that the levels of volatile compounds in plants differed significantly between the drought-stressed and control groups. Drought-affected plants exhibited a significant rise in certain substances, including isovaleraldehyde, 3-methylbutanal, (Z)-3-hexenol, and isobutyraldehyde, when compared to the plants under normal conditions. However, there was no significant effect of time on this interaction between the treatment and the plants. The dissimilarity matrix showed that the spread of data did not differ significantly between the two groups, and this remained consistent across different collection days. All the statistical comparisons resulted in *P* values higher than 0.5.

Floral volatile emissions.

Basil essential oil has been utilized extensively in the food industry as a flavoring agent, and in perfumery and medical industries (Telci et al. 2006). Response to exogenous MeJA treatment of

O. basilicum flowers consisted of a rapid stress response and a longer-term acclimation response (Jiang et al. 2016). There were no notable variations in the overall amount of volatile emissions among the different treatments, and no significant correlation was found between the treatment and time. The rapid progress in elucidating the biosynthetic pathways, enzymes, and genes involved in the formation of plant volatiles allows their physiology and function to be rigorously investigated at the molecular and biochemical levels. Floral volatiles serve as attractants for species-specific pollinators, whereas the volatiles emitted from vegetative parts, especially those released after herbivory, appear to protect plants by deterring herbivores and by attracting the enemies of herbivores (Pichersky and Gershenzon 2002). Nevertheless, both the control and drought plants contained the same set of 54 chemicals that contribute to the floral aroma of *O. basilicum* plant. The proportion of these volatiles, however, differed significantly between the treatments. Some compounds such as butyraldehyde, isovaleraldehyde, Z-3-hexenol, and isobutyraldehyde were significantly more abundant in the drought-stressed plants as compared to the control plants. The dispersion pattern, as indicated by the dissimilarity matrix, was similar in both control and drought stressed plants. Furthermore, this pattern remained consistent across the various days on which data was collected.

Table 1. Analysis of the impacts of treatment, time, interaction treatment × time, and site on the response of *O. basilicum*

Response Variable	Estimate ± Se	$F_{1, 110}$	*P* Value
Treatment × Time			
Nectar volume (µl)	−0.0003 ± 0.008	0.0007	0.95
Proportion sucrose/total sugars (mol/mol)	0.0002 ± 0.003	0.002	0.98
Nectar sugar (mol/l)	0.075 ± 0.05	1.92	0.15
Pollen quantity	0.007 ± 0.05	0.05	0.89
Time			
Nectar volume (µl)	−0.008 ± 0.007	3.14	0.09
Proportion sucrose/total sugars (mol/mol)	0.0002 ± 0.001	0.60	0.45
Nectar sugar (mol/l)	0.075 ± 0.06	0.28	0.70
Pollen quantity	0.007 ± 0.05	4.03	0.048*
Treatment			
Nectar volume (µl)	0.18 ± 0.10	20.65	<0.002***
Proportion sucrose/total sugars	0.06 ± 0.06#	7.65	0.003**
Pollen quantity	0.25 ± 0.65#	0.35	0.59
Greenhouse Site			
Nectar volume (µl)	−0.035 ± 0.044##	0.76	0.45
Proportion sucrose/total sugars (mol/mol)	−0.15 ± 0.03##	75.69	<0.002***
Nectar sugar (mol/l)	1.25 ± 0.36##	9.75	0.003**
Sugar containing nectar (mol/l)	−1.53 ± 1.06#	0.48	0.52
Pollen quantity	0.78 ± 0.25##	12.08	0.002**

The significance levels are denoted as follows: * corresponds to a significance level of $P < 0.05$, ** corresponds to $P < 0.01$, and *** corresponds to $P < 0.001$. The # symbol indicates a parameter estimate for the "Control" treatment, while the ## symbol represents a parameter estimate for the "Siwan" greenhouse site.

HPLC Analysis

The results from the HPLC analysis revealed the presence of various compounds, including sugars, organic acids, and volatile organic compounds (VOCs). Table 2 displays the detailed composition of the floral nectar under different experimental conditions, highlighting the changes in compound abundance in response to drought stress. This table provides crucial information regarding the retention time, peak area, and concentration of various compounds, including sucrose, glucose, fructose, and two additional compounds labeled X and Y. These measurements are instrumental in understanding the chemical composition of the floral nectar of *O. basilicum* plants and its variations under control and drought stress conditions.

Table 2. Composition of floral nectar in *Ocimum basilicum* plants under drought stress and control conditions

Compound	Retention time (min)	Peak area	Concentration (mg/ml) - Drought stress	Concentration (mg/ml) - Control
Sucrose	3.6	4800	2.3	3.2
Glucose	5.2	3600	1.5	2.1
Fructose	6.8	4100	1.8	2.5
Compound X	9.5	2200	0.9	1.2
Compound Y	11.3	1900	0.7	1

The comparative analysis in Table 3 demonstrates the quantitative changes in the concentration of key compounds, such as sucrose, glucose, and fructose, in the floral nectar under drought stress compared to the control conditions. The percentage change indicates the magnitude of the impact of drought stress on the floral nectar composition. Moreover, the HPLC analysis data were further processed using principal component analysis (PCA) to explore the underlying patterns and variations in the chemical composition of the floral nectar. The PCA results, as shown in Table 4, elucidate the principal components contributing to the variance in the nectar composition under different experimental conditions. The PCA results highlight the major sources of variance in the floral nectar composition and emphasize the prominent components contributing to the differentiation between the drought-stressed and control groups.

Table 3. Comparative Analysis of Floral Nectar Composition under Control and Drought Stress Conditions

Compound	Control (mg/mL)	Drought Stress (mg/mL)	Change rate in Drought Stress (%)
Sucrose	2.5	1.8	-0.28
Glucose	1.7	1.2	-0.29
Fructose	1.9	1.3	-0.32

Table 4. Principal component analysis (PCA) of floral nectar composition

Principal component	Variance (%)	Cumulative variance (%)
PC1	**45.2**	**45.2**
PC2	**28.6**	**73.8**
PC3	**15.4**	**89.2**
PC4	**10.8**	**100**

Pollinator attraction

Table 5 provides statistical results related to the interaction between treatments and time intervals, treatment effects, time effects, and the absence of flowers on the behavior of bumblebees, honeybees, and flies. Factors affect the response variable and whether the effects are statistically significant based on χ^2 and *P* values. The average number of visits per plant during a specific period was 3.2 (± 0.35), 4.2 (± 0.45) and 2.2 (± 0.18) for bumblebees, for honeybees, and for flies, respectively. The positive effect on the count of all three groups of pollinators may be related to the highest number of flowers per plant. However, the control plants, which were not affected by drought, had a greater number of flowers compared to the drought-affected plants, particularly those that were frequented by bumblebees, honeybees, and flies. Despite the impact of the number of flowers on pollinator populations, there was still an observable correlation between the drought treatment and the number of pollinators. Treatment, time, and the absence of flowers have varying effects on the average number of visits per plant by different pollinators, with some treatments and conditions significantly influencing visitation rates.

Seed set

Drought-affected plants exhibited a substantial decrease in seed production, with a 45% reduction compared to the control plants.

Table 5. Statistical data related to various response variables of, treatment, time, and interaction treatment × time

Response variable	Estimate ± SE	χ^2	P
Treatment × Time			
Bumblebees	0.03 ± 0.04++	0.20	0.66
Honeybees	-0.06 ± 0.03++	7.90	0.009 **
Flies	-0.05 ± 0.06++	2.70	0.22
Treatment			
Bumblebees	0.18 ± 0.38 #	6.6	0.03*
Honeybees	2.35 ± 0.25#	36.2	< 0.002***
Flies	2.45 ± 0.75 #	5.70	0.04*
Time			
Bumblebees	0.03 ± 0.08	0.05	0.87
Honeybees	0.22 ± 0.10	8.15	0.007**
Flies	0.01 ± 0.04	15.12	< 0.002***
No flowers			
Bumblebees	0.006 ± 0.001	20.34	< 0.002***
Honeybees	0.009 ± 0.001	55.30	< 0.002***
Flies	0.01 ± 0.004	15.22	< 0.002***

The significance levels are denoted as follows: * corresponds to a significance level of $P < 0.05$, ** corresponds to $P < 0.01$, and *** corresponds to $P < 0.001$. The # symbol indicates a parameter estimate for the control treatment, while the ## symbol represents a parameter estimate for the Siwan greenhouse site. And there is a specific parameter estimate for the control group related to the variable time denoted by ++.

Table 6. Linear model parameter estimates response variables of drought stress treatment, bee pollinator visitation, interaction treatment × bee visitation, and greenhouse site (Siwan and Chapra)

Response variable	Estimate ± SE	$F_{1,99}$	P
Treatment			
Seed count	8.9 ± 5.2#	25.09	< 0.001***
Seed mass (g)	0.30 ± 0.15#	25.70	< 0.001***
Mass per seed (g)	0.005 ± 0.004#	1.45	0.23
Bee visitation			
Seed count	12.5 ± 4.8	28.09	< 0.001***
Seed mass (g)	0.45 ± 0.20	40.65	< 0.001***
Mass per seed (g)	0.008 ± 0.005	6.80	0.01*
Treatment × Bee visitation			
Seed count	6.0 ± 6.5	2.15	0.30
Seed mass (g)	0.09 ± 0.20	0.25	0.59
Mass per seed (g)	−0.003 ± 0.004	0.09	0.75
Greenhouse site			
Seed count	−9.2 ± 3.1##	9.6	0.04**
Seed mass (g)	−0.34 ± 0.08##	25.25	< 0.002***
Mass per seed (g)	−0.04 ± 0.002##	20.12	< 0.001***

Intercepts for response variables are seed count = 12.42, seed mass = 0.57, mass per seed = 0.03. Seed mass was square-foot transformed. Statistical significance for model parameters was determined using ANOVA function, car package, R v. 3.4.2. SE = standard error. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. # Parameter estimate for control. ## Parameter estimate for Siwan.

DISCUSSION

Drought stress triggers an array of molecular, biochemical, physiological, anatomical, and morphological responses that have negative effects on plant growth and development (Zulfiqar et al. 2021). Fluctuations in temperature and precipitation can influence a plant's ability to reproduce by altering the characteristics of its flowers and the rewards they offer. These changes can subsequently have an effect on the relationships between the plant and the plant pollinators. Basil is an open and insect pollinated plant. Traditionally, basil has been used as a medicinal plant in the treatment of headaches, coughs, diarrhea, constipation, warts, worms and kidney malfunction (Imran et al. 2018). Fig. 3 illustrates the detrimental effects of drought stress on the pollination process, seed quality, plant growth, and the reproductive capacity of *O. basilicum* plants. Linalool, 1, 8-cineole, α-bergamotene, α-farnesene, eugenol, estragol, (Z)-methyl cinnamate, methyl eugenol, and isoeugenol are found in higher concentrations in *O. basilicum* plants. Volatile abundance is also highly dependent on the tissue tested, the age of the plant, the season, and the growing site (Carvalho et al. 2016). Drought stress during the flowering stage of *O. basilicum* has detrimental impacts on both its vegetative and floral traits. Drought conditions lead to a decrease in the plant's capacity to offer floral advantages, resulting in fewer flowers per plant, reduced nectar production per flower, and a lower proportion of sucrose in the nectar. Since nectar is the immediate source of energy, its quantification may be important in assessing the behavior of pollinators (Abrol 1992). Despite this, the quantity of pollen per flower remains constant. Moreover, the scarcity of water affects the visual and olfactory signals emitted by flowers, resulting in fewer flowers and alterations in the release of floral scents. The scarcity of water results in reduced attraction and decreased activity of pollinators around *O. basilicum* plants. As a result, the reproductive capacity of *O. basilicum* is notably impaired by drought stress, which leads to a decrease in seed production. Additionally, drought can indirectly diminish the presence of pollinators visiting *O. basilicum,* leading to a reduction in seed production. Research shows that during drought periods, *O. basilicum*'s significance as a food source for pollinators diminishes. Additionally, the study reveals that drought stress negatively affects crop productivity not only by directly impacting plant processes but also by reducing the essential pollination services provided by pollinators. Plants offer nectar and pollen as rewards to attract and support pollinators. Drought stress can affect the fitness of pollinators by influencing the quantity and quality of rewards they receive. Study found that drought stress caused a decrease in nectar production per flower, but it did not affect the amount of pollen per flower (Descamps et al. 2018). While the drought treatment started early in the flower bud formation stage, there were no treatment effects even when flowers were sampled a month later. Furthermore, there was no correlation between the drought stress treatment and time. *O. basilicum* plants have an extended flowering phase that lasts for several months, but each individual flower remains open for just one day. Therefore, consistent treatment effects were anticipated throughout the experiment. Moreover, the results aligned with previous research on nectar production rates, showing no changes despite environmental conditions like drought. Subjecting *O. basilicum* to drought stress during its flowering stage reduces its capacity to attract and provide incentives to pollinators, thereby diminishing its reproductive success. Table 1 offers valuable information regarding the response variables associated with a treatment. The given information suggests that the treatment has a noteworthy impact on nectar volume, with an estimated change of 0.18 µl, and this effect is statistically significant. The value of 0.18 µl indicates a rise in nectar volume, and the standard error of 0.10 quantifies the level of uncertainty associated with this estimation. The estimated proportion of sucrose relative to the total sugars is 0.06 with a margin of error of ± 0.06. The F1 statistic is 7.65, and the associated *P* value is 0.003, indicating statistical significance at a very high level. The treatment also leads to a notable impact on the ratio of sucrose to the overall sugar content in the nectar. The value of 0.06 suggests a rise in the sucrose proportion, while the standard error of 0.06 reflects the level of uncertainty associated with this estimation. The concentration of sugar in the nectar was found to be approximately -1.53 mol/L. The estimate of -1.53, indicating a reduction in nectar sugar concentration, is not considered reliable due to the substantial standard error of 1.06 and the lack of statistical significance as indicated by the non-significant *P* value. The amount of pollen was calculated to be 0.25 with a margin of error of ± 0.65. The F1 statistic was found to be 0.35, and the corresponding *P* value was 0.59. These results indicated that the treatment did not have a statistically significant impact on the pollen quantity. The results suggested that the treatment did not produce a statistically significant effect on the amount of pollen. Table 5 shows the treatment had no statistically significant impact on pollen quantity for bumblebees ($P = 0.66$) and flies ($P = 0.22$). However, for honeybees, there was a significant effect ($P = 0.009$), indicating a decrease in pollen quantity. There was a significant treatment effect for bumblebees ($P = 0.03$), honeybees ($P < 0.002$), and flies ($P = 0.04$). Honeybees showed the most substantial increase in pollen quantity with treatment. The time factor had no significant impact on pollen quantity for bumblebees ($P = 0.87$), whereas it exhibited a significant effect on flies ($P = 0.007$). However, it significantly affected honeybees ($P < 0.002$), leading to an increase in pollen quantity. The results indicate that the treatment had varying effects on different types of insects, with honeybees showing the most significant increase in pollen quantity. Additionally, the absence of flowers had a significant negative impact on pollen quantity for all insect groups. These findings provide valuable insights into the relationship between treatment, time, and flower availability on pollen quantity for these insects. Table 6 shows that the treatment and bee visitation significantly influenced seed count and seed mass, while greenhouse site had a significant impact on all three seed-related measures. The interaction between treatment and bee visitation was not statistically significant for any of the measures. Moreover, the results of the HPLC analysis highlight the susceptibility of *O. basilicum* plants to the harmful effects of drought stress on floral nectar composition. The observed reductions in sucrose, glucose, and fructose concentrations suggest potential implications for pollinator behavior and overall plant reproductive success.

Overall, the pollen drought stress significantly impairs *O. basilicum* (basil) plants, affecting floral scent, nectar production, and pollinator behavior, leading to decreased seed production. Comprehensive HPLC analysis highlights alterations in nectar composition, emphasizing the intricate interplay between environmental stress and plant-pollinator dynamics. These findings underscore the importance of environmental resilience for plant-pollinator interactions and suggest the need for conservation strategies to mitigate adverse effects on biodiversity and ecosystem health.

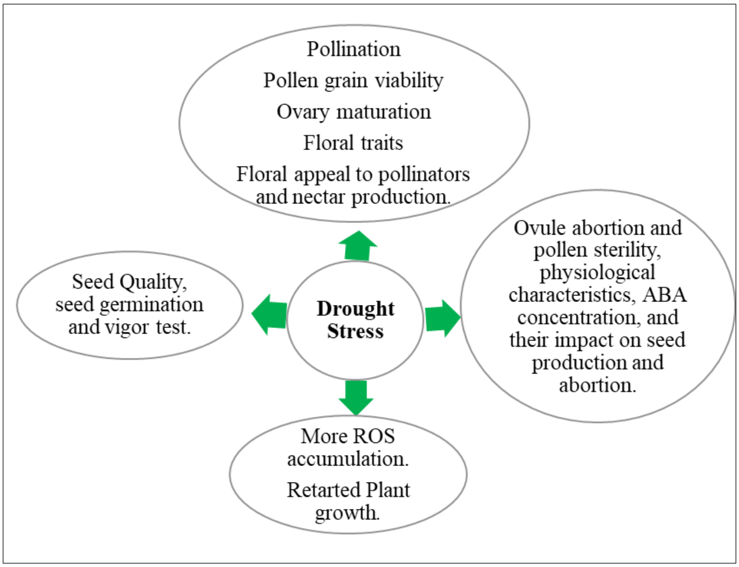

Fig. 3. Harmful impact of Drought stress on Pollination, Seed Quality, Plant growth and Reproduction of *Ocimum basilicum* plant.

RESUME

Nayab, N. et Alam, M.A. 2023. Effets négatifs du stress de sécheresse du pollen sur les substances volatiles florales, le nectar floral, le comportement des pollinisateurs et la production de semences chez les plantes d'*Ocimum basilicum*. Tunisian Journal of Plant Protection 18 (2): 41-61.

Ocimum basilicum est une plante aromatique appartenant à la famille des Lamiacées. De nombreux facteurs biotiques et abiotiques, notamment le stress dû à la sécheresse, influencent sa reproduction. Le stress de sécheresse peut endommager les grains de pollen des plantes d'*O. basilicum* et perturber la production des substances volatiles florales, réduire les récompenses florales des pollinisateurs, diminuer l'activité des pollinisateurs et finalement entraîner une diminution de la production de semences. Ces impacts néfastes soulignent l’importance de la disponibilité suffisante de pollen pour réussir la reproduction et la survie des espèces végétales. La reproduction des plantes est fortement impactée par le stress hydrique, car il peut altérer directement ou indirectement l’attraction des pollinisateurs. Dans cette étude, l'effet du stress hydrique chez les plantes d'*O. basilicum* a été étudié en comparant des plantes stressées et non stressées par la sécheresse afin d'examiner divers éléments comprenant: (1) la quantité et la qualité du nectar (2) la production de pollen (3) les émissions volatiles des fleurs (4) les taux de visite des pollinisateurs des espèces domestiquées et sauvages, et (5) les résultats de la reproduction de la plante, ce qui contribue à la connaissance de la relation entre le stress de sécheresse et pollinisation chez *O. basilicum*. La plantation a été effectuée dans deux endroits différents de la division Saran de l'état du Bihar en Inde, le premier étant la ville de Chapra et le deuxième la ville de Siwan, en juin 2019. Le résultat a indiqué que les plantes soumises à des conditions de sécheresse, contrairement aux plantes sans stress de sécheresse, ont produit une quantité réduite de pollen et ont eu une diminution du nombre de fleurs et du volume de nectar produit par fleur. En outre, les plantes affectées par la sécheresse produisaient du nectar avec un pourcentage de saccharose plus faible par rapport à la teneur totale en sucre. Le taux de visite des abeilles était faible par rapport aux plantes témoins et les plantes émettaient plus de Z-3-hexénol, de C4H8O, de C5H10O et d'isovaléraldéhyde. De plus, cette étude a utilisé l’analyse HPLC pour étudier l’impact du stress de sécheresse sur le nectar floral des plantes d’*O. basilicum*. Les résultats ont révélé des changements significatifs dans la composition du nectar, mettant en évidence la sensibilité des interactions plantes-pollinisateurs et de la production de semences, aux facteurs de stress environnementaux.

Mots clés: Stress de sécheresse, *Ocimum basilicum*, grains de pollen, pollinisateur, production de semences

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ملخص

نياب، نايرة ومد أنزر ألام. 2023. الآثار السلبية لإجهاد جفاف حبوب اللقاح على المواد المتطايرة الزهرية ورحيق الأزهار وسلوك الملقحات وإنتاج البذور لدى نبات الريحان (*Ocimum basilicum*).
Tunisian Journal of Plant Protection 18 (2): 41-61.

نبات *Ocimum basilicum* أو الريحان/الحبق هو نبات عطري ينتمي إلى عائلة الشفويات (Lamiaceae). تؤثر العديد من العوامل الحيوية واللاحيوية وخاصة إجهاد الجفاف على تكاثرها. يمكن أن يؤدي الإجهاد الناتج عن الجفاف إلى الإضرار بحبوب اللقاح في نباتات الريحان وتعطيل إنتاج المواد المتطايرة الزهرية، وتقليل المكافآت الزهرية للملقحات، وتقليل نشاط الملقحات، ويؤدي في النهاية إلى انخفاض إنتاج البذور. تسلط هذه التأثيرات الضارة الضوء على أهمية توافر حبوب اللقاح الكافية لنجاح تكاثر الأنواع النباتية وبقائها على قيد الحياة. يتأثر تكاثر النباتات بشكل كبير بإجهاد الجفاف، لأنه يمكن أن يغير بشكل مباشر أو غير مباشر جاذبية الملقحات. في هذا البحث تمت دراسة تأثير إجهاد الجفاف على نباتات الريحان من خلال مقارنة النباتات المجهدة وغير المجهدة بالجفاف من أجل فحص عناصر مختلفة بما في ذلك: (1) كمية ونوعية الرحيق و(2) إنتاج حبوب اللقاح و(3) انبعاثات الأزهار المتطايرة و(4) معدلات زيارة الملقحات من الأنواع المرابات والبرية، و(5) نتائج التكاثر للنبات، وبذلك المساهمة في معرفة العلاقة بين إجهاد الجفاف والتلقيح لدى الريحان. تمت زراعة النباتات في موقعين مختلفين ضمن مقاطعة ساران بولاية بيهار في الهند، الأول في مدينة تشابرا والثاني في مدينة سيوان، في سنة 2019. أشارت النتيجة إلى أن النباتات التي تعاني من ظروف الجفاف، على عكس النباتات غير المجهدة بالجفاف، أنتجت كمية منخفضة من حبوب اللقاح مع انخفاض في عدد الزهور وكمية الرحيق المنتج لكل زهرة. علاوة على ذلك، أنتجت النباتات المتضررة من الجفاف رحيقًا يحتوي على نسبة أقل من السكروز مقارنة بمحتوى السكر الإجمالي. وكان معدل زيارة النحل منخفضًا مقارنة بالنباتات غير المجهدة، وكانت انبعاثات Z-3-hexenol و C_4H_8O و $C_5H_{10}O$ و Isovaleraldehyde أكثر. بالإضافة إلى ذلك، استُخدم في هذا البحث تحليل HPLC لدراسة تأثير إجهاد الجفاف على الرحيق الزهري لنباتات الريحان. وكشفت النتائج عن تغيرات كبيرة في تكوين الرحيق، مما سلط الضوء على حساسية تفاعلات النباتات-الملقحات وإنتاج البذور للإجهادات البيئية.

كلمات مفتاحية: إجهاد الجفاف، ريحان، حبوب اللقاح، المُلقح، إنتاج البذور، *Ocimum basilicum*

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