Effects of Melatonin on *Pisum sativum* under Salinity Stress

Research Question:

What is the effect of 500µM melatonin solution on the stomatal density of Pea Plants (*Pisum sativum*) as a response to salinity stress induced by 100mM NaCl solution?

Introduction: 3 Key factors

Stomata

Stomata are found on the epidermis of plant leaves and allow for gasses such as carbon dioxide (CO_2) and water vapor to exchange with the environment. Stomatal density can be affected by intensity of light, humidity, temperature and water availability. A plant leaf's stomatal density will increase or decrease according to these environmental factors and how it affects the plants' need to conserve water in limiting transpiration. A smaller stomatal density indicates decreased CO2 intake, but higher control over water loss whereas a higher stomatal density would indicate increased CO2 intake—resulting in increased photosynthesizing abilities— but higher transpiration rates (McElwain, 2021). For this reason, it is crucial for a plant to adapt and develop the right stomatal densities according to environmental factors.

Figure 1: Microscope View of Stomata

Salinity Stress

The study of **salinity stress** on agriculture becomes increasingly relevant. This is attributed to rising temperatures induced by climate change, escalating the rates of evapotranspiration which results in a higher soil salinity. This consequently leads to an increased uptake in salt by plant roots (Grozeva et al., 2023). Excess intake of salt by plants can be detrimental to crop health as it causes ion toxicity and induces osmotic and oxidative stress. Osmotic stress is caused by salinity stress as the increase in salt concentration creates a lower osmotic potential in the soil and water is drawn out, along with other nutrients in the soil thereby inhibiting the capacity of water absorption. Similarly, ion toxicity is caused by the excess intake of sodium (Na^+) and chlorine (Cl^-) ions which compete with the intake of other nutrients such as potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}). The physiological effects of continued salinity stress include stunted plant growth and development (Hasanuzzaman et al., 2023).

Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is a chemical produced naturally in both vertebrates and plants. Though melatonin's role in humans is commonly known to regulate sleep patterns, melatonin's role in plants is lesser known. While plants have been shown to adopt circadian rhythms (Murch & A E Erland, 2021), recent discoveries concerning melatonin in plants have resulted in research on melatonin's endogenous function in plants in addition to exogenous roles. According to these studies, melatonin is associated with plant growth and development, antioxidant activity and responses to both biotic and abiotic stresses such as salinity (Zhang et al., 2021).

Additionally, melatonin acts as an antibiotic in the ROS scavenging system, protecting cells from oxidative damage (Li et al., 2019). The reactive oxygen species (ROS) of a plant are another factor affected by high saline concentrations. ROS act as signaling molecules which carry the ability to respond to biotic and abiotic stresses by regulating stress response pathways. When abiotic stressors such as salinity are high, ROS formation increases, resulting in oxidative damage to cell components (AbdElgawad et al., 2016). The oxidative stress caused by this excess production of ROS is alleviated through the cells' ROS scavenging system (Li et al., 2019).

Aim & Investigation

Investigation

Since melatonin has a role in abiotic stress resistance, changes in stomatal density will be investigated in this exploration as the stomatal density of a plant is variable to abiotic stresses such as salinity.

Independant Variable: Application of 500μM melatonin solution throughout germination and plant growth.

Dependant Variable: Stomatal density — obtained by inventory through microscope

The aim of this research is to investigate whether or not melatonin has an effect on the stress response of pea plants (*Pisum sativum*) to saline solution via stomatal density via 3 treatment groups:
Group 1 - Treated with melatonin and saline solution
Group 2 - Treated with saline solution
Group 3 - Control, untreated

Hypothesis

According to Yan et al. (2020) and Ahmad et al. (2023), it can be concluded that the osmotic stress caused by an increased intake of saline will lead to a decrease in stomatal density of plants as a response to the adaptation to saline stress. Furthermore, the application of melatonin should contribute to the antibiotic defense against abiotic stresses by regulating stomatal behavior and scavenging ROS.

Aim

Since melatonin has a role in abiotic stress resistance, changes in stomatal density will be investigated in this exploration as the stomatal density of a plant is variable to abiotic stresses such as salinity. The investigation of melatonin's effects on a plant's stress response has several valuable implications.

Since melatonin's more recent implementation as a supplement in the last 30 years, the amount of melatonin in domestic wastewaters is subject to increase. It is then vital in determining its consequent effects to the crops which are treated by domestic wastewaters, especially considering that 80% of global waste waters end up in the environment untreated (United Nations, n.d.).

Additionally, due to melatonin's role in abiotic stress resistance as well as nutrient uptake (Zhang et al., 2021), melatonin treatment can be utilized to optimize the nutrient uptake by plants in the constructed wetlands and serve as efficient wastewater treatments (Ye et al., 2022). In this regard, effects of melatonin for crops on an agricultural scale are important to investigate.

Lastly, the warming effects of climate change increase the impact of salinity stress on crops, and therefore the abiotic stress resistant qualities of exogenous melatonin is studied as a potential protective measure.

Method & Material

Materials

- 75 x Pisum sativum seeds
- Melatonin pills (1.9mg/0.22g)
- Table salt
- 25 x Plastic plant pots
- Tissue paper
- Mortar and pestle
- Weighing boat
- Volumetric flask± 100 cm^3
- Graduated cylinder ± 0.1 cm^3
- 15 x Petri dishes
- Storage bottle
- 3 cm^3 Pipette ± 0.25 cm^3
- LED plant light and clamps and stands

Method

1. Preliminary testing to test the effects of varying saline solution concentrations and method development to determine the effective melatonin application method
2. Preparing solutions
3. Expimerementaion: Germination of seeds in petri dishes in the dark, with Group 1 seeds germinated in melatonin solution.
4. Repotting germinated seeds once sprouted into tissue filled pots and setting up light system with 6 hour phtoperiod
5. Treating plants groups 1 and 2 with 30ml saline solution every 3 days, and watering all plants equally for a period of 9 days
6. Watering all plants and allowing development of stomata for a remaining 18 days
7. Mapping out sample plots for each plant
8. Obtaining stomata count for each sample leaf:
    a. Painting on clear nail varnish
    b. Peeling it off with clear tape and attaching to a microscope slide
    c. Counting stomata visible through x400 microscope view.
9. Calculating stomatal density (area of view was measured by using a 0.01mm graticule)

Figure 2: preliminary testing of experimental set up

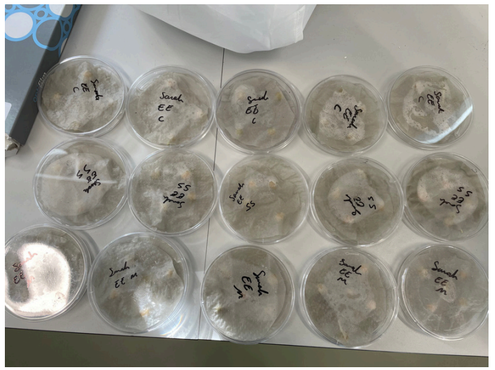

Figure 3: Germination of pea seeds in petri dishes: labeled by treatment group and covered in two layers of tissue paper soaked in 3 ml of water or 3 ml of melatonin solution (one tissue below and over the seeds to prevent drying)

Method & Material

Measuring and calculating stomatal density via sample plots

Figure 4: Visual of sample plot according the sampling method

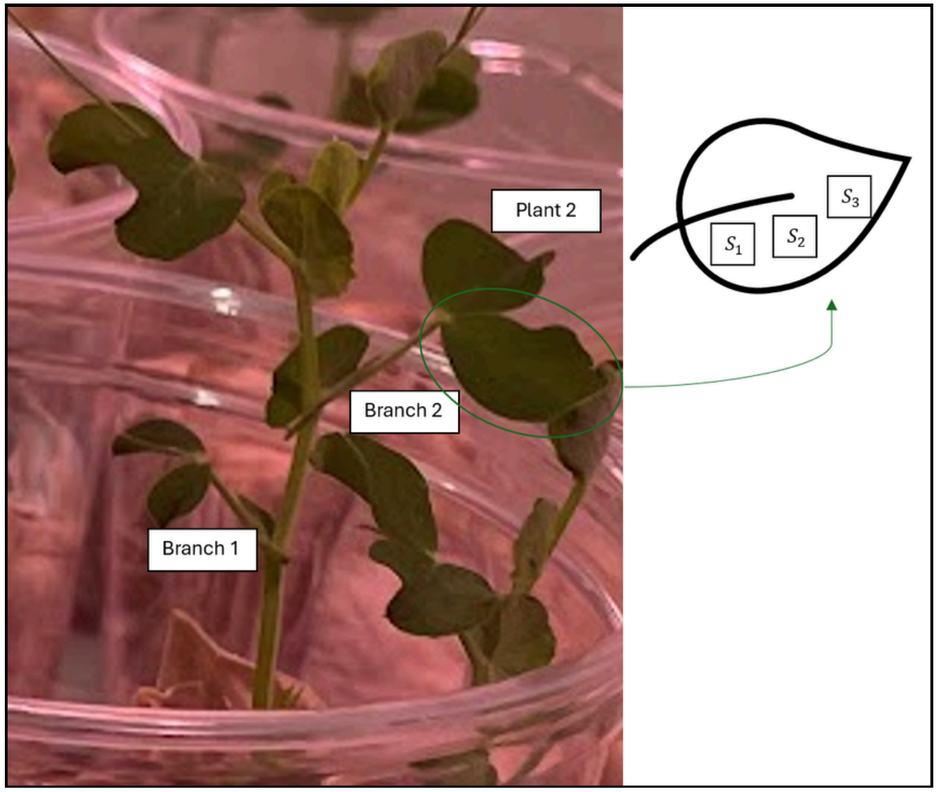

Figure 5: Diagram of sampling technique

Figure 6: Data collection set up

Results
following data processing

Table 1: Stomatal density for each group

Stomatal Density for pea plant (*Pisum Sativum*) Treatments	
	Average Stomata / 0.069mm²
*Group 1: Saline Stress and Melatonin Treatment	191
*Group 2: Saline Stress	160
Group 3: Control	211

*outliers removed in accordance to box and whiskers graph

As seen in stomatal density values in Table 1, both Groups 1 and 2 demonstrated a decreasing influence on stomatal density compared to that of the control Group.

The control group was not treated, therfore the average stomatal density of these plants can be considered a baseline value for stomatal density in the experimental enviroment.

Plants solely under 100 mM saline stress had the fewest stomata per unit area, indicating that salinity stress was a factor that decreased the stomatal density.

Plants under 100 mM salinity stress, which were also treated with 500 μM melatonin solution had a stomatal value higher than that of plants plainly under stress. This suggests that the application of melatonin mitigated the decrease in stomatal density value.

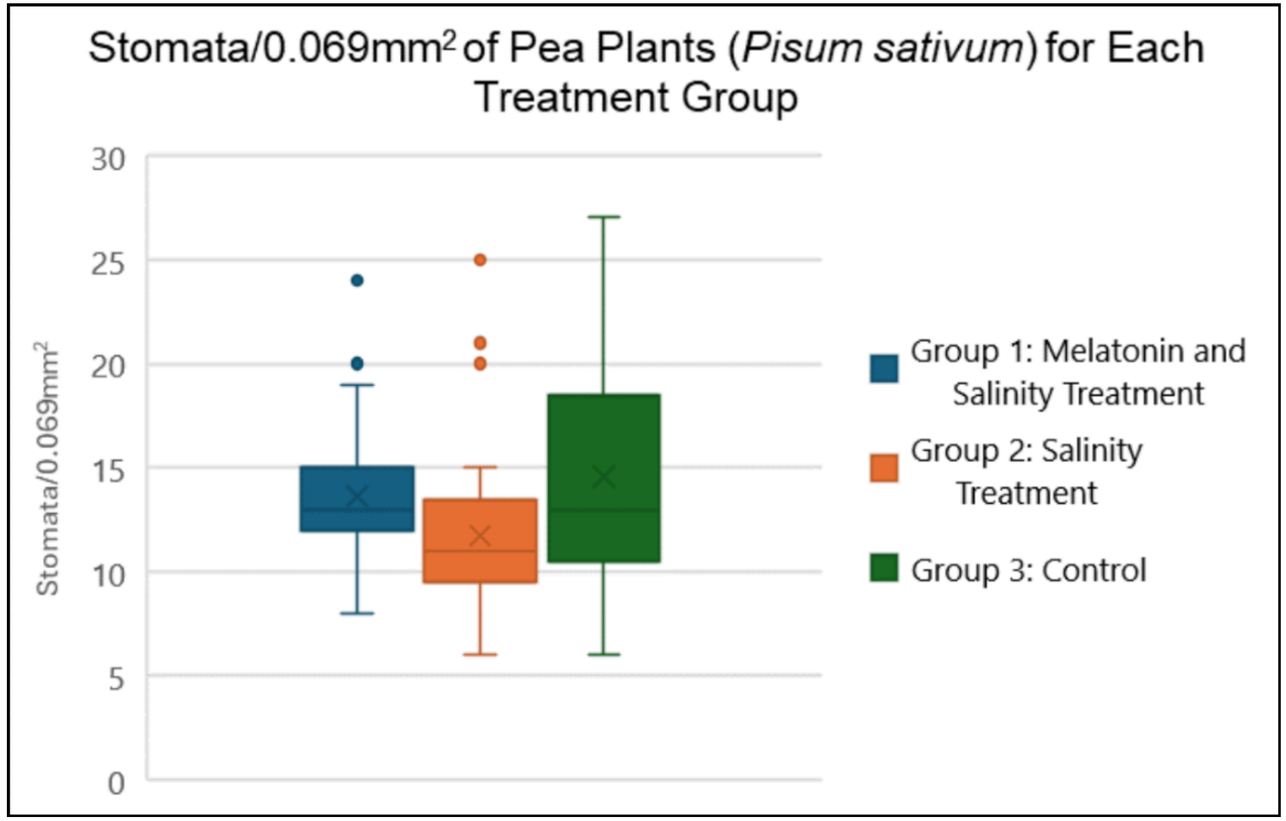

Figure 7: Stomatal density of pea plants (*Pisum sativum)* for each treatment group

Discussion

The results of this exploration demonstrate that pea plants under salinity stress treated with 500μM melatonin solution have an increased stomatal density in comparison to pea plants under salinity stress, indicationg stress alliviating qualities of melatonin. This can be deduced based on the following grounds:

- Salinity stress increases amounts of sodium chloride (NaCl) in plants, causing water to be drawn out and creating osmotic pressure.
  - The peas under salinity stress adapt to this osmotic pressure by the devolpment of fewer stomata to balance CO_2 intake eith eater conservatio and turgor pressure maintanance (Hasanuzzaman et al., 2023).

- Melatonin functions as a signaling molecule in plants, it responds to abiotic stresses as a part of the reactive oxygen species (ROS) scavenging system, protecting the plant cells from the oxidative damage caused by salinity stress.
  - Therefore, the exogenous application of 500μM melatonin solution increased endogenous concentration of melatonin in pea plants, which increased the plant's ability to neutralize harmful ROS, and alleviated the stress caused by salinity. Finally, this allows melatonin to regulate stomatal conductivity (Li et al., 2019)

Conclusion

Since pea plants under salinity stress treated with melatonin have a stomatal density closer to that of the control group, the results show that melatonin can be deemed an effective treatment in maintaining stomatal density under salinity stress. This is because the increase in stomatal density of melatonin treated plants indicates the ability of the pea plant to increase CO_2 intake and photosynthesize at higher rates without having to conserve as much water as pea plants solely under salinity stress. The results of this experiment were verified using the Mann-Whitney U statistical test to show that the differences between the stomatal density values of Group 1 and 2 were statistically significan

Evaluation & Limitations

Improvements to method:

- Increased germination sample size due to rotting
- Increased sample size in order to acheive more reliable results adn enesure an equale number of samples for each treatment group
- Light set up: the range of light intensity by the LED plant lights led to stunted plant growth and development, increasing the number of outliers, as smaller leaf sizes are shown to correlate with larger stomatal density values Kouwenberg et al. (2007).
- Straining of melatonin solution: the melatonin tablet was not pure melatonin, it consisted of melatonin, cellulose and magnesium stearate. While these components may not have dissolved fully in the solution, the final melatonin solution could have been strained to prevent external chemical absorbtion by the plants.

Figure 8: Molded pea seeds in germination process

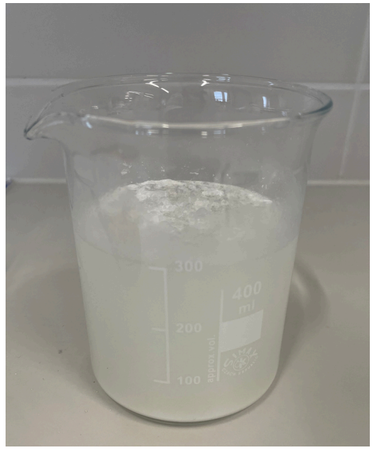

Figure 9: Melatonin solution

Limitations

- Variance in the temperature, humidity and light of the lab environment are factors that could not be under strict control, and thus may have inhibited the development of growing pea plants.
    - This is especially important as fluctuating humidity and temperature directly affects the stomatal density of plants leaves by influencing transpiration rates
- External contaminants may have led to an increase in the amount of moldy pea seeds during the germination process, thereby affecting sample size.