Increasing the Desiccation Tolerance of *Eragrostis tef* through Exogenous Application of Abscisic Acid to Ensure Food Security

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ABSTRACT

With the world population expected to reach almost ten billion by 2050 and the large loss of agricultural land due to climate change, food security has become one of the largest issues for governments and farmers today. *Eragrostis tef* is a gluten-free, highly nutritious staple crop for Ethiopia that has the potential to ensure food security. It is drought tolerant, but only partially desiccation tolerant, meaning that it will still die if it loses much of its water. Abscisic acid (ABA), a plant hormone, has been known to confer desiccation tolerance when applied exogenously to seeds. The purpose of this study was to increase the desiccation tolerance of *E. tef* through exogenous application of ABA so that more countries with little rainfall can use it as a food source. This experiment was conducted in two stages: an optimization and proof of concept, and an experimental procedure. Due to the fact that *E. tef* was a precious resource, the model organism *Arabidopsis thaliana* was used in the optimization stage. It was determined that seeds were not the ideal tissue for ABA application because of low germination rates, possibly due to a barrier created by the seed coat. Therefore, in the experimental stage, calli, or masses of undifferentiated cells, of *E. tef* were used to ensure that ABA was taken up. The hypothesis that ABA would increase the desiccation tolerance of *E. tef* calli was supported, as ABA-treated calli had a higher relative water content, lower electrolyte leakage, and higher overall chlorophyll and carotenoid content.

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INTRODUCTION

With the world population expected to reach almost ten billion by 2050 and the large loss of agricultural land due to climate change, food security has become one of the largest issues for governments and farmers today. This loss of viable farming land is due to drought or lack of rainfall, which causes crop death. Drought has now become the most expensive and destructive natural disaster for farmers in developing countries (FAO, 2017). Between 2005 and 2015, drought cost the developing world agriculture industry $29 billion (FAO, 2017). In the United States, $10 to $14 billion are lost each year to drought (Kuwayama, 2019). Currently, about 11 percent (1.5 billion hectares) of land on Earth is used for farming (FAO, 2015). The United Nations Environment Programme (UNEP) published a study that found that arable land is being lost at 30 to 35 times the historical rates. Meanwhile, the world population continues to increase, consequently increasing food demand. Up to 52 percent of the land used for agriculture is moderately or severely affected by soil degradation (UN Sustainable Development Goals, 2018). Additionally, the world loses 23 hectares of arable land worldwide every minute to drought and desertification; yearly, this adds up to 12 million hectares lost (UNEP, 2018). To ensure that the growing population will have sufficient food, the world will need to see a 69 percent increase in food calories production, but it is becoming harder and harder for farmers to increase their yield as more and more land is lost to drought (Ranganathan, 2013).

Eragrostis tef, the focus species of this study, is a tall grass and a staple crop for Ethiopians. It is primarily found in Ethiopia and in certain parts of South Africa. It is gluten free as well as highly nutritious, making it valuable for agriculture. However, it is an orphan crop, meaning that it isn't internationally traded; therefore, it gets less research funding and has not been as extensively studied as other crops (Mechael, 2015). It is drought tolerant, meaning it can survive with low environmental water availability while maintaining high internal water content (Ntuli, 2012). However, it is only partially desiccation tolerant, defined as the ability of a living structure to survive drying to equilibrium with low (< 50%) relative humidity and maintain low intracellular water concentrations (Ntuli, 2012). A drought-tolerant organism that is not desiccation-tolerant will die if it loses much of its water, whereas a desiccation-tolerant organism

will survive under the same conditions (Ntuli, 2012). Increasing its desiccation tolerance facilitates greater survival in areas with little water.

Given the social and economic impacts of climate change, more research is being done to explore how to increase desiccation of crops and promote food security; especially in developing nations. For example, Costa *et al.* (2017) studied the desiccation characteristics of orthodox seeds (which are desiccation tolerant seeds) in comparison to those of resurrection plants, the latter being plants that can sustain a prolonged period of severe drought. They found that the desiccation process of both share key traits. For example, orthodox seeds acquired desiccation tolerance during their developmental stage, but lost that ability during germination. But if those seeds were soaked in an abscisic acid (ABA) solution before planting, the seeds maintained the desiccation tolerance property as mature plants, instead of losing it. Additionally, the study compared a resurrection plant (*E. nindensis*) leaf cell to a dehydrated orthodox seed (*C. pumilum*) cell, and found that the orthodox seed structurally altered itself to resemble the resurrection plant cells, which allowed them to withstand drought better. This, along with the finding that resurrection plants and orthodox seeds were found to share similar traits in their mechanisms for desiccation tolerance, contributed to the understanding that resurrection plants are the best model to increase desiccation tolerance in crops. And, if ABA could be a viable way to increase the desiccation tolerance of orthodox seeds, it may also increase the desiccation tolerance of other crops.

Similarly, Bano *et al*. (2018) studied a drought susceptible wheat cultivar and a drought tolerant wheat cultivar. They had two groups for each cultivar, one with seeds pre-soaked in a 10^{-6} mol/L solution of ABA for 18 hours, and the other with no pre-soaking. The ABA treatment helped the plants reverse the effects of drought stress. The plants with seeds that had been pre soaked recovered better from drought stress and had a higher relative water content than seeds that hadn't been pre soaked.

Both Costa *et al.* (2017) and Bano *et al.* (2018) found that soaking seeds in ABA before germination increased the desiccation tolerance of the plants. As *Eragrostis tef* is an important staple crop for several developing nations, increasing its desiccation tolerance could provide food security for millions of people.

The purpose of this study was to increase the desiccation tolerance of *Eragrostis tef* so that it can provide food security to more countries with little rainfall or to areas of aridity. No studies have examined the effect of exogenous application of ABA to *Eragrostis tef* calli. In this study, it was hypothesised that ABA applied exogenously to *Eragrostis tef* calli will increase its desiccation tolerance. Since *E. tef* is a precious resource, a proof of concept and optimization study using the model organism *Arabidopsis thaliana* was conducted initially. Then, the experimental procedure was carried out using *E. tef*.

METHODOLOGY

This study consisted of two parts: an optimization phase and an experimental phase. The optimization stage was conducted by soaking *Arabidopsis thaliana* seeds in six different ABA solutions, and then exposing the seeds to seven days of desiccation stress. The experimental stage consisted of inducing calli from *Eragrostis tef* seeds, which were then exposed to two different treatments: either a 20 μM ABA solution or water, and then drying them under a Laminar Air Flow. Tissue that did not receive any exogenous treatment served as a control. After exposure to treatment, the calli were transferred to regeneration media to induce seedling growth. Three different stress assays were performed on the regenerated seedlings: Relative Water Content, Electrolyte Leakage, and Chlorophyll and Carotenoid Content.

Focus Species

Eragrostis tef is a drought tolerant resurrection plant. The seeds were provided by the University of Cape Town. Due to its value as an orphan crop and a precious resource, access to the species was limited. In total, 100 seeds were cultured and used in the experiment. Each treatment had 30 replicates and the remaining seeds were cultured as extra replicates to use in the case of in vitro contamination of the material.

Hormone Selection

Abscisic acid is a plant hormone that regulates stress response as well as dormancy, germination, and seed development, and plays a role in conferring drought tolerance in plants.

Plant somatic embryos usually lack desiccation tolerance. It has been demonstrated that they may acquire such a tolerance upon preculture in the presence of abscisic acid (ABA), followed by slow drying (Hoekstra *et al.,* 2001). It is upon this premise that ABA was selected as an appropriate exogenous treatment for calli of *Eragrostis tef.*

Part A: Proof of Concept and Optimization of Experiment

Model Organism

Due to the precious nature of *Eragrostis tef*, the model organism *Arabidopsis thaliana* was used for the first part of this investigation. It is a genetic model organism for most plants and crop species, due to its relatively small and completely sequenced genome. Additionally, many proteins between *A. thaliana* and other crops are similar in shape and function. This means that experimental treatments applied to *Arabidopsis thaliana* might also work on crops, since they so closely resemble each other. Four different phenotypes of *A. thaliana* were used; three as controls and one as an experimental group. A wildtype strain (WT) was the regular control. The ABA deficient (ABAd) strand was a positive control, since it can't produce its own ABA. The negative control was ABA resistant (ABAr) due to its resistance to the hormone. The experimental group was a drought resistant strand (DR) which is hypersensitive to ABA. Ten seeds were used for each treatment, for a total of 60 seeds.

Choice of Treatment and Concentrations

The methodology of this experiment was modeled off of Bano *et al.* (2018), where two different wheat cultivars were studied. The seeds were soaked because it was a quick, easy, and reliable way to ensure all seeds received the treatment. Six different concentrations were used in the experiment. Bano, *et al.* (2018) used a concentration of 1 μM in the study. This experiment's purpose was to identify a concentration that was most beneficial to the plants, so values higher than the regular concentration were used. With the understanding that ABA cannot be harmful to the plant in higher concentrations, the highest concentration tested was 1 mM, and 0 μM were

used as a control (Dr. C Naidoo, personal communication, 2019). The rest increased from 0 by 5µM until 20µM: 5µM, 10µM, 15µM, and 20µM.

Model Organism (*Arabidopsis thaliana*)	Choice of Plant	Choice of Treatment (Soaking)
• genetic model • small, sequenced genome • knowledge can be applied to crops • proteins: similar shape & function	• Wild type [WT]: regular control • ABA deficient [ABAd]: (+) control (can't produce ABA) • ABA resistant [ABAr]: (-) control (ABA no effect) • Drought Resistant [DR]: hypersensitive to ABA	• quick & easy • cost effective

Figure 1: A brief depiction of the optimisation phase

Part 1: Solution Preparation

A 1mM stock solution (0.22824 g of ABA/L of solution) was prepared and the experimental solutions were created by diluting the stock solution. These were prepared according to the proportions shown in Table 1.

Table 1: Dilution of stock solutions.

Concentration (µM)	Stock Solution (mL)	Water Added (mL)
5	1	199
10	1	99
15	1	66
20	1	49

Part 2: Soaking and Planting

Before starting the treatment, the *Arabidopsis thaliana* seeds were stratified by placing the seeds in a freezer at a temperature of four degrees Celsius for four days to break their dormancy. All four strands were separated into six groups of ten seeds. The seeds were placed in

petri dishes on top of four filter papers. Specific concentrations of ABA were added to each petri dish, containing ten seeds. Using a pipette, 10 mL of solution was applied to each dish to fully soak the seeds. After the first 24 hours, another 10 mL of solution was added to the dishes to ensure that the seeds would not dry out. After 48 hours of soaking, the seeds were planted in pots and watered.

Part 3: Experimental Conditions

The seeds were watered every 2-3 days to maintain moisture, and were kept at a 16 hour light/8 hour dark cycle. A sheet of ClingWrap was put over each pot to maintain relative humidity. They were kept on a light cart in a classroom. Each strand of *Arabidopsis thaliana* was divided into two groups with an equal number of plants, either Control or Drought, and labeled accordingly. After two weeks of germination, the Control group received its regular watering as per the initial schedule, while the Drought group received no water.

Part 4: Data Collection

Data was collected to determine percent germination for each seed strain and percent germination of all seeds per each concentration. This was done after two weeks to ensure maximum stand count and seedling establishment. An additional comparison between the expected germination rates and observed germination rates was calculated. Two weeks after germination, the surviving plants of each strain and from each concentration of ABA were counted.

Part B: Experimental Procedure

Germplasm Selection

A callus is a mass of undifferentiated cells; therefore any treatment applied to it would be more evenly distributed and hence consistently taken up. The ideal tissue would be a seed since it carries genetic variation, but callus was chosen because *Eragrostis tef* seeds are small and they were difficult to manipulate without causing any physical damage. Additionally, there was a potential barrier in the form of the seed coat that could impede the uptake of ABA.

Choice of Treatments

Three different treatments were administered during the experiment. The negative control had no treatment added, the positive control had 1 mL of water, and the experimental group had 1 mL of a 20 μM ABA solution. The chosen concentration was based on Costa *et al.* (2017) where a solution of 10 μM was used on seedlings. It was doubled in this experiment to account for the large mass of a callus compared to that of a seed. A stock solution of 0.2M was created and then diluted by using 1 mL of stock solution in 10 mL of water.

Part 1: Culturing the Seeds

For each of the three groups, six petri dishes were used, with five replicates in each dish. After the preparation of 800 mL of callus-inducing 2.4-D medium in the Laminar Air Flow, it was evenly dispensed into 18 petri dishes and left to cool for 20 minutes. The seeds were then decontaminated. The petri dishes were clearly labeled with the date, the name of the species, and the treatment; a different colored sticker was used for each treatment (green for control, pink for water, orange for ABA). After closing the petri dishes and using Parafilm to ensure the environment remained sterile, the dishes were wrapped in foil and left in the dark for four weeks. This was done to inhibit light inducing reactive oxygen species reactions. On the third day, the plates were checked for contamination before being wrapped in foil again.

Part 2: Application of Treatments

After four weeks of induction, the treatments were added to the calli. To apply the treatments, a 1 mL pipette and tip were used. For each dish, 1 mL of treatment (water or ABA) was applied to the calli and medium. The control group was left unopened, since they received no treatment. The dishes were sealed shut with Parafilm and then wrapped in foil again and placed in the Conviron. They were left in treatment for 72 hours, then transferred to petri dishes containing regeneration medium. The calli were allowed to develop into seedlings and grow for a week in the Conviron. Then, all seedlings were placed in the Laminar Air Flow and dried for 48 hours.

Part 3: Stress Indicator Assays and Data Collection

For each treatment, three different assays were run: relative water content, electrolyte leakage, and chlorophyll and carotenoid content. These measured how well the seedlings were reacting to drought stress. An ANOVA was conducted to determine whether there was significance within the two treatment groups, and a follow-up Tukey analysis was done to determine where in the treatment the significance was found.

Relative water content: After dehydration under the Laminar Air Flow, one seedling was soaked in water so that it could reach full turgor. This was used as a comparison for the rest of the seedlings. The rest of the seedlings were first weighed and then placed in tin foil envelopes and left in an oven at 80°C for 24 hours. The calli were then reweighed to obtain their dry mass. An absolute water content calculation (AWC = (fresh mass - dry mass) ÷ dry mass) was done to calculate the relative water of the calli (RWC = (AWC sample ÷ AWC full turgor) x 100).

Electrolyte leakage: The CM100 (conductivity reading machine) well plates were filled with 750 µL of ultra-pure water. The machine was calibrated to ensure there could be no false readings. A blank well was filled. This well did not have tissue material in it and the reading was used in the final calculations. Each seedling was placed in an individual well. The wells were clearly marked to delineate the control, water and ABA treated tissue. Conductivity was read every minute for a 100 minutes to allow sufficient time for all electrolytes to leak out. After the readings were taken, the seedlings were blotted dry and placed in foil envelopes. These were then left in an oven at 80 degrees Celsius for 48 hours to obtain their dry mass. The rate of electrolyte leakage was calculated on a dry mass basis.

Chlorophyll and carotenoid content: The seedlings were placed in an eppendorf tube with 500 µl of 100% acetone and ground using a pestle. They were then wrapped in foil and left for 12 hours. Thereafter, the test tubes were centrifuged and 100 µM of the solutions were dispensed into a 96 well plate which was placed in a spectrophotometer. Three different readings were taken: at 662 nm, 645 nm, and 470 nm.

RESULTS

Part A: Proof of Concept and Optimization of Experiment

As seen in Fig. 1, for each seed type, there was significantly less germination than expected compared to established rates by the Ohio State University *Arabidopsis* Biological Resource Center ($p<0.01$). All seeds were expected to have high germination rates. The ABAd were expected to have rates similar to the WT but had significantly lower observed rates ($p<0.01$). The ABAr were not supposed to be affected by the ABA at all but had significantly lower rates than expected ($p<0.01$). The DR were also expected to have the highest germination rate, but the observed germination rate was equal to that of the WT.

Seed Type	Expected Germination Rate (%)	Observed Germination Rate (%)
WT	98	77
ABAd	86	13
ABAr	98	25
DR	100	77

Figure 2: Expected germination results (courtesy of *Arabidopsis* Biological Resource Center, OSU, 2019) compared to observed germination results for each type of *Arabidopsis* seed. In all seeds, a significantly lower germination was observed ($p<0.01$).

The results for percent germination at each concentration showed few consistent trends. For example, as shown in Fig. 2, the WT were expected to have increased germination rates as the concentration increased. There was a consistent germination rate of 90 percent at 10 µM, 15 µM, and 1 mM, while the 20 µM only had 60 percent, although this difference was not significant. As expected, the ABAd had significantly higher germination rates at 1 mM than any other concentration ($p<0.05$). On the other hand, at the 10 and 15 µM, no ABAd seeds

germinated. These should have looked more like the WT, since these plants had an ABA deficit and therefore had a low endogenous level of ABA. The ABAr was expected to have consistent germination rates based on the assumption that the ABA should not have had an effect on them since the strand is resistant to the hormone, but the observed rates were not. There were significantly more seeds germinating at 20 µM than at both 15 µM and 1 mM ($p<0.01$). The DR had increasing germination rates from 0 µM to 10 µM, then decreasing rates from 10 µM to 1 mM. There were significantly less seeds germinated at 1mM than at 10 µM ($p<0.01$).

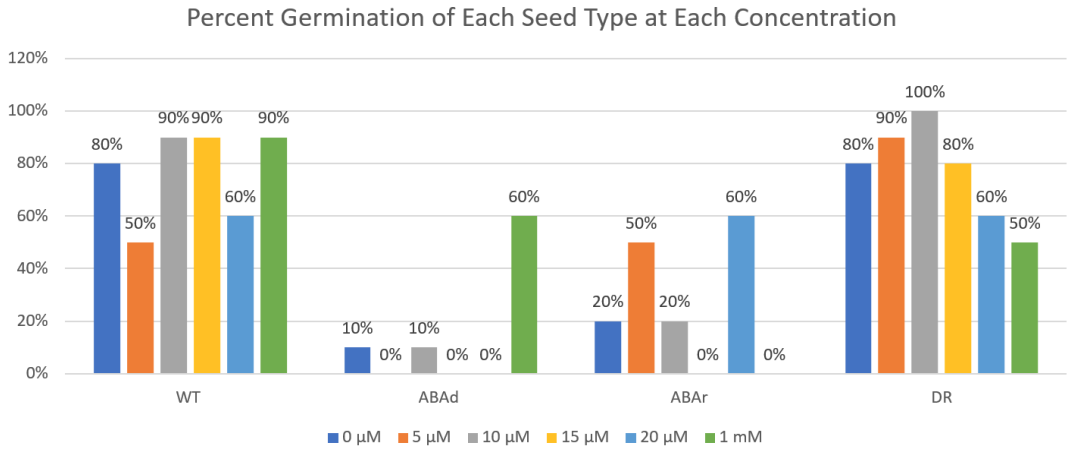

Figure 3: Percent germination of each seed type at each concentration. Germination rates were inconsistent as seeds were soaked in ABA.

All plants except the Drought Resistant mutant strand died either shortly after planting or during the growth period. As seen in Table 2, there were significantly more plants that survived in the lower concentrations (5 µM and 10 µM) for the DR ($p<0.05$).

Table 2: Percent of seedlings alive 14 days post germination.

Seed Type	0 µM	5 µM	10 µM	15 µM	20 µM	1 mM
WT	10%			10%		
DR	70%	80%	70%		10%	20%

Part B: Experimental Procedure

Relative water content measures the amount of water in a cell. The more water a cell has during drought, the better it is managing and surviving. If a cell has a low relative water content, then the cell will not survive for long. The relative water content of ABA-treated calli was higher than both the control and water treatments. There was significance between the Control and ABA treatments as well as between the Water and ABA treatments ($p<0.05$). However, there was no significance between the Control and Water treatment.

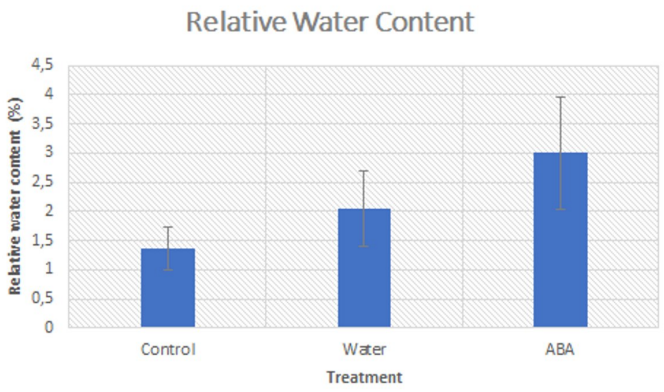

Figure 4: Relative Water Content of *E. tef* calli in the three different treatments: Control, Water, and ABA treatment. Significance was found between ABA and Control treated-calli and the ABA and Water-treated calli ($p<0.05$).

Electrolyte leakage quantifies the amount of electrolytes that leaked through the cell membrane, indicating plant health. The higher the electrolyte leakage, the more damage there is to the cell which increases the likelihood of cell death. Unexpectedly, there was a significant difference between the amount of electrolyte leakage in the ABA treated calli and the control ($p<0.05$). However, there was no significant difference between the water-treated calli and those treated with ABA.

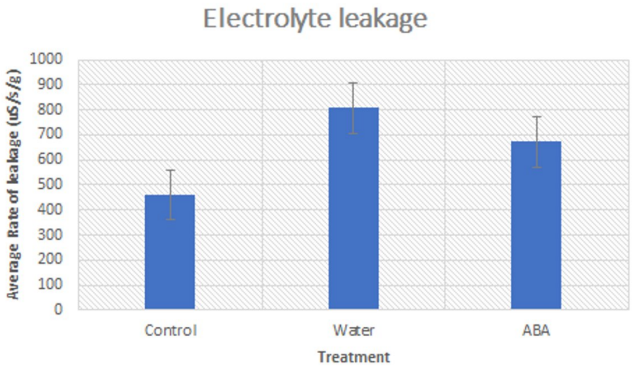

Figure 5: Electrolyte leakage of entire *E. tef* calli in the three different treatments: control, water, and ABA treatment. No significant difference existed between the ABA and water treated calli.

A chlorophyll and carotenoid content analysis was also performed to test the effect drought had on the photosystems of the cells. The higher the chlorophyll and carotenoid pigment concentration, the higher the rate of photosynthesis, which indicated how well the plant was managing stress. The ABA-treated calli had the highest overall pigment concentration and there was significantly higher chlorophyll b content in the ABA than in the Water-treated calli ($p<0.05$).

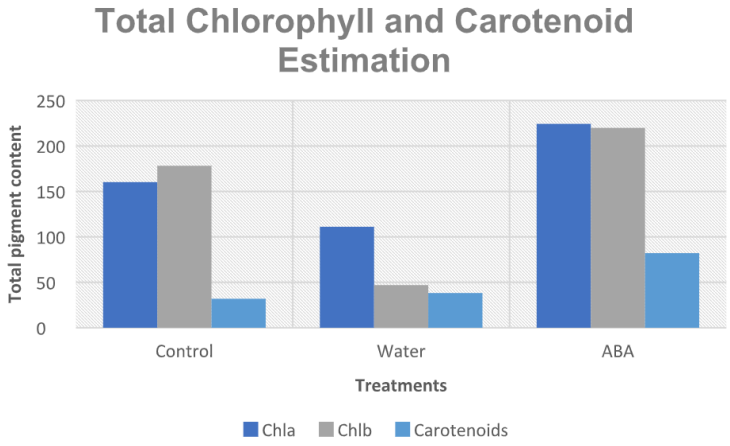

Figure 6: Total chlorophyll and carotenoid estimation of *E. tef* calli in the three different treatments: Control, Water, and ABA treatment. Significance was found in chlorophyll b content between the Water and ABA-treated calli.

DISCUSSION

The exogenous delivery of ABA by soaking seeds proved to be mostly unsuccessful. The DR seeds, hypersensitive to ABA and already more drought resistant than the other strains, should have survived both normal and drought conditions but did not. At the two-week mark, very few plants survived at the 20 μM and 1 mM concentrations (10 and 20 percent, respectively), while plants in the lower concentrations (5 μM and 10 μM) had the highest survival rate (80 and 70 percent, respectively).

These results could be due to the limitations of using a seed as the tissue for delivering the ABA. The seed coat could have prevented the ABA from entering the seed, and therefore could not help the seedling survive drought. This result is in contrast to the findings of Costa *et al.* (2017), which found that soaking orthodox seeds in ABA before germination would prevent the loss of desiccation tolerance that the seeds have prior to germination. Due to the poor germination rates and survival rates associated with the soaked seeds, it was decided that a better germplasm type to receive application of ABA would be calli.

The hypothesis that ABA would confer desiccation tolerance to seedlings regenerated from treated *Eragrostis tef* calli was supported. The ABA-treated calli had higher relative water content compared to the Water or Control treatments, additionally there was significantly more water in the ABA-treated calli than the control and water-treated calli. This indicated that the ABA helped the seedling retain water during desiccation, therefore increasing its probability of survival.

Although the ABA treatment had a higher electrolyte leakage than the Control, it was still lower compared to the Water treatment. This indicates that the ABA had a protective effect on the cell membranes and their structures, since less electrolytes leaked out in the ABA treatment than in the Water treatment and Control, indicating a less damaged and stronger cell membrane. The high electrolyte leakage rates in the Water treatment might be due to possible structural damage caused by manipulation of the calli when receiving treatment; therefore the calli treated with water or ABA would both have higher leakage rates than the control.

Overall, there was a higher total pigment concentration in the ABA treated calli than in the other treatments. Although there was no significance between any of the groups for any of

the pigments, it can be inferred that ABA had a protective effect on the photosystems during desiccation. Since the pigment concentration was highest in ABA-treated calli, the ABA protected the photosystems during drought. The lack of significance except for chlorophyll b can be explained as a consequence of fewer replicates due to *Eragrostis tef*'s nature as a precious resource.

Evaluation of Study

None of the 100 *Eragrostis tef* seeds that had been cultured in callus-inducing medium were contaminated. Additionally, there was no contamination after the application of ABA or water treatments. Due to *Eragrostis tef* being a precious resource, the number of seeds available and consequent sample sizes were limited. The concentration of ABA was chosen from the study Bano *et al.* (2018) performed and doubled to account for the calli's larger mass compared to a seed. Since the outcomes of the experiment were largely unknown (as it was the first study of its kind on the specific germplasm type chosen) and due to limited seed material, only one concentration was used.

For future research, different manipulations to confer desiccation tolerance to *Eragrostis tef* will be explored. For example, different ABA concentrations could be tested, as well as different developmental stages of the callus. Another possibility could be to increase the period of calli incubation in ABA. This study generated fundamental information to inform future projects which include identification and manipulation of the *Eragrostis tef* genes responsible for desiccation tolerance.

Conclusion

This was one of the first studies to explore the exogenous effect of ABA on *Eragrostis tef* calli. The results of this experiment indicate that more work should be done regarding the role of ABA in increasing the desiccation tolerance of *Eragrostis tef* as a way to provide food security for the future. Additionally, ABA did improve the desiccation tolerance of *Eragrostis tef.* The manner of ABA application as well as the germplasm type is important; seeds were not an effective way to deliver ABA due to the barrier created by the seed coat that prevented uptake of

ABA. On the other hand, calli was shown to be a more responsive germplasm type as exemplified by the significant results obtained.

As a staple crop for Ethiopia and a vital component in African agriculture, *Eragrostis tef* feeds millions of people and has the potential to become even more valuable as a food crop. Increasing the desiccation of *Eragrostis tef* will increase the ability of the plant to inhabit other parts of the world where land is being lost to drought. This will allow for the production of crops in otherwise unusable land and ensuring food security.

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