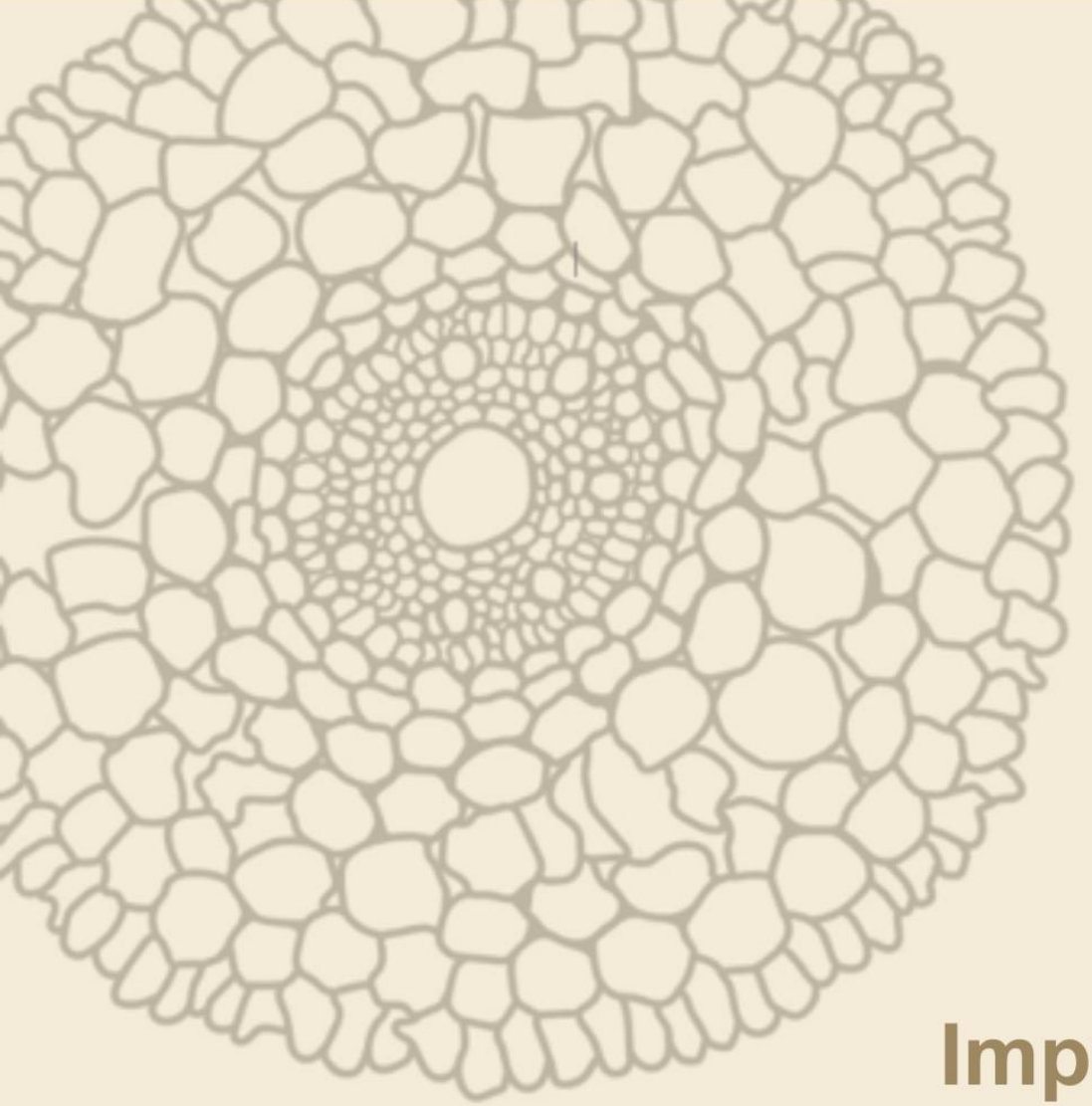

Improving the material potential of oats by promoting exodermal development

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Abstract

In recent years the world of science and design have been captivated by biomaterials. Now a new material has entered the stage: materials created from the roots of plants. *Avena sativa* has proven to have excellent material properties, mainly because of its rapid root development and architecture. However, currently these root-grown materials made from oats lack the mechanical strength to be considered as a proper material.

The key to enhancing the biomaterial potential of oats lies in promoting the exodermal development. Biopolymers suberin and lignin contribute to the structural stability of roots and by increasing their abundance, increasing the tensile strength might be achieved. The stress hormone abscisic acid (ABA) is known to play a role in the synthesis of the exodermis. This research aims to map the ABA-suberin-lignin interplay in oats, potentially bolstering root strength.

Besides manual application of ABA, this research will also explore the natural response of the exodermis to stress conditions. Drought and salt stress are known to correspond with the synthesis of ABA and are therefore included as a treatment. In addition, the effect of silica on the mechanical strength of oat roots is tested, since silica is known to contribute to the stability of grasses and improve Casparian band formation.

ABA appeared to have an effect on the exodermal development of *A. sativa, A. strigosa* and *A. sterilis*. When being treated with salt or drought stress *A. sativa* did not develop an exodermis, nor did it have an effect on the endodermis. However, when treated with both ABA and silica *A. sativa* showed strong increase in endodermal lignin and exodermal suberin. Therefore, it was expected that treatment of the root-based material would improve the mechanical strength of oats. However, results of the materials did not test positive for improvement of the root tensile strength when receiving different treatments. No correlation could be found between the exodermal development of oats and changes in tensile strength increase.

Layman's summary

In recent times, scientists and designers have been exploring the potential of biomaterials. This includes an interest in making materials from the roots of plants, including from a plant called *Avena sativa*, commonly known as oats. These root-based materials from oats have some suitable qualities, because oat roots grow quickly and have a structure which is very favorable for the production of a material. But the development of the material is faced with one problem: the root-material is not yet strong enough to replace our currently used materials.

To make these oat-based materials better, this research focused on a part of the roots called the exodermis. There are natural substances like suberin and lignin, known from cork and the bark of trees, that make the roots strong. By increasing these substances, it might be possible to make the roots of the oats stronger. This could potentially be done using a hormone called abscisic acid (ABA). This hormone is known to be involved in making the exodermis by increasing the amount of suberin an lignin in it. The goal is to understand how these processes could potentially make oat roots stronger.

This research will investigate the possibilities by applying 3 different methods. First, by manually applying ABA to the oat roots. Second, by investigating how the roots respond to stress, like drought and salt, because these stress conditions can also make ABA. And third, there will be tested if adding silica makes the roots stronger, as it does in some other plants.

Results showed that ABA affected the exodermis in different types of oats, but when the oats were stressed with salt or drought, they didn't develop an exodermis. However, when both ABA and silica were applied on oats, it increased the lignin and suberin in the roots. So, this could improve the strength of the root-based material. However, the results showed that the material's strength didn't get better, and there couldn't be found a connection between the exodermal development and strength improvement.

Contents

Introduction

In the last decade a new trend has emerged in science and design, where the development of biomaterials has received increased attention. The application and purpose of these materials is very diverse, but the overall aim is to acquire materials which are of no harm to the environment and help us engage in a symbiotic relationship to the natural world. This includes in their production as well as whenever they are discarded. Materials that are fully plant-based have the potential to fit our needs in the future of material development and production.

The plasticity of roots makes them very suitable to function as a biomaterial, therefor leading to an increase in their popularity. The first ever example of such root-based production were the living bridges in Meghalaya (Shankar, 2015). These bridges were grown over many years, eventually creating a natural, living structure of a bridge. This has been an inspiration to other artists who began to discover the art of root-bending. This has led to the creation of woven biomaterials that could function as fabrics and are naturally grown by the roots of plants. The primal species used for these materials is *Avena sativa*, also known as our domestic oats. These plants are favorable for material production due to their extremely fast root seedling development and their root architecture (Burr-Hersey et al., 2017; Scherer). Even though root grown materials have been developed, these do not yet have the properties to compete with the properties of conventional materials such as cotton fabrics. Their resistance to mechanical stress is still very insufficient and the material has a high tendency to break due to the fragility of the roots. Physiological properties of the roots would have to improve for the root-based material to have the potential to function as a fabric.

Whenever roots are considered as a possibility for biomaterial usage, the phenotypical aspects which influence their ability to function as a biomaterial should be accounted for. Therefore, an increase in a better physical resistance of roots would be favorable. In natural environments plants increase the physical strength of their roots in scenarios where belowground conditions are tough and soil structures are difficult to penetrate. As a response to such soils, plants have developed multiple traits to improve their fitness. One of such responses is changing the length and diameter of the roots (Potocka & Szymanowska-Pulka, 2018). In addition, the composition of their cell walls have a large effect on the physical attributes of the roots. Root cell wall composition is known to change in response to mechanical stress. The changes are mostly related to an increase in the amount of suberin, pectin and lignin, and a decrease in the amount of cellulose (Potocka & Szymanowska-Pulka, 2018). Increased suberin and lignin levels in the roots would therefore suggest an improved resistance to mechanical stress.

Suberin and lignin play an important role in the composition of the Casparian bands. The Casparian band is mainly composed of lignin, but suberin acts as a type of glue which binds the cells of woody plants together. Suberization of the Casparian band increases its membrane adhesion, giving the membranes of the plant more strength and creating a tighter bond to both the Casparian band and reinforcing the cell walls of the cells within that layer (Barros et al., 2015). In addition, lignin acts as a barrier from external harm, protecting the interior of the plant from external stresses. The function of suberin within the Casparian bands is to regulate the fluxes of nutrients flowing in and out of the plant system in cases of drought, salt or nutrient deficit stresses (Lu et al., 2022).

Both substances are primarily located in the endodermis and for some species in the exodermis of plant root systems. The endodermis serves as a semipermeable barrier to the vascular system of plants (G. Viana et al., 2022). This individual cell layer is located between the cortex and the vascular tissue of the plant roots. It has an important function in the structural stability of the root, but its main function is to regulate the in and outflow of

water and dissolved substances from the vascular system of the plant (Shukla & Barberon, 2021). This regulation is controlled by the Casparian strip which is located between the cells of the endodermis.

The exodermis of the root can also contain a Casparian strip, however this forms in a later developmental stage than the endodermis (Ma & Peterson, 2003). Where the endodermis is a specialized controller of the water and nutrient regulation of the plant, the exodermis acts as a barrier against the entry of soil pathogens, toxins and excess water. The presence of lignin in endodermal cell walls has a very high occurrence among species, but for the exodermis this is less homogeneous (Manzano et al., 2022). Having these biopolymers enforce the outermost cortex layer adds to the sturdiness of these cell layers, consequently resulting in resilience to mechanical stress. The adaptive character of the exodermis is determined in its genotype, but the precise construction of the exodermis is in response to its environment (Hose et al., 2001). Therefore, in the development of the root the plant may alter its phenotype to better fit its surroundings.

Suberin and lignin play an important role in plant root stress responses, due to their role in the protection of the plant against stresses induced by drought and a wide range of nutrient stresses (Lu et al., 2022). The hormone which is known to play an important role in this response system, activating these biopolymers, is abscisic acid (ABA). In many species ABA regulates the response of root suberization (Shiono et al., 2022). By repressing the biosynthesis of serotonin, suberization can take place in the cell walls, thickening the Casparian band through suberin deposition (Lu et al., 2022). Besides root suberization, ABA influences lignin biosynthesis. It has been shown that an increase in ABA is directly related to an increase in lignin accumulation and deposition in root cell walls (Singh, et al., 2019). In addition, abscisic acid is of importance to many processes in plant development, including plant growth and root and shoot development (Canales et al., 2021). The severeness of each response strongly depends on the species.

Besides manually applying ABA to enhance lignin and suberin production, the effect can also be achieved by stimulating a more natural response. According to Mu et al. (2015) *Avena sativa* is a species with a high tolerance to salt stress due to its adaptive properties. This is in accordance with earlier discoveries presenting increased cellulose and lignin depositions in plants which are confronted with higher salinity (Oliveira et al., 2020). Abscisic acid plays an important role in the reaction to salt stress in different species, so it may be of influence in oats as well (Kempa et al., 2008). In this more natural response, when enduring salt stress, the response system of the plant might lead to an increased root tensile strength as a defense system on its own. Comparing the natural to the manual stress effect will give more insight on the role of ABA in the salt stress response of oat species and increase the potential of finding a more resilient root development.

In addition to these stress responses Fleck et al. (2015) also showed how increased silicon promotes exodermal Casparian band development through the formation of complexes with phenols. Silicon is an abundant substance in global soils since it is one of the main substances in our crust. High silicon abundancy in the soil is correlated with an increased uptake of silicon through their roots by various plants (Fleck, et al., 2015). Poaceae species, including oats, are known to have a relatively large silicon content and it plays an important role in their growth and resilience to stresses (Fleck et al., 2015; Kumar et al., 2017). Increased silicon concentrations in the roots have various effects on the morphology of the roots. Silicification is found to be connected with various forms of plant strengthening against stresses and mechanical hardening of the root tissues (Lux, et al, 2020; Hattori, et al., 2003). This property makes silicon an interesting additional treatment to test for an increased tensile strength for oat roots.

However very little is known yet about its interaction with ABA.

ABA and silica and their effect on lignin and suberin quantities have shown to impact the physical strength of plant roots. Therefore, manually increasing the presence of ABA in the environmental conditions of the plants of interest, may increase the suberin and lignin quantities in the plant cells. These increased biopolymer quantities may result in a more suitable characteristics of the roots to function as a biomaterial. This research will investigate the potential of ABA manipulation on the biomaterial properties of plant roots. The research will test for the abundancy of lignin and suberin in different oat species and their response to increased ABA quantities. Consequently, an increase in lignin and suberin could lead to an increase in tensile strength of the roots, resulting in more favorable characteristics for biomaterial purposes. Therefore, the aim of this study is to define artificial ways to increase the biomaterial potential of oat species, inspired by their own natural response system.

Methods

Growth conditions

Prior to germination oat seeds were sterilized using ethanol and bleach, where seeds were washed in between with milliQ. After the washing step, the seeds were stored in a 50 mL tube filled with milliQ, wrapped in aluminum and stored for 7 days in 4 degrees Celsius (Appendix 1.1). After the 7 days, the seeds were removed from the tube and spread in a Petri dish. For germination large filter paper was used, drenched in ½ Murashige and Skoog (MS) On each paper approximately 10 oat seeds were placed in a line on one half of the filter paper. When all seeds were placed, the filter paper was folded once and then tightly rolled. All rolls were placed in a glass beaker, halfway filled with ½ MS. The beaker was stored in the phytotron in 20 degrees with a daylength of 16 hours. For the first two days the rolls remained wrapped with foil. After these days, foil was removed from the top of the rolls to enable shoot growth (Appendix 1.2).

After 5 days the seeds germinated and were ready to be transferred to the growth pouches. Per treatment 4 pouches were prepared, containing 3 plants per pouch. This research tested for 7 different treatments separated in two trials. The first trial contained two ABA treatments (2µM and 10µM), silica treatment (30mg/L), silica+ABA (2µM) treatment and a control. The second trial tested ABA (2µM), salt stress (100 mM), sorbitol stress (200mM) and control. Of each treatment 20 mL ½ MS was added to each pouch, containing the additional treatment if so the case. In the pouches the plants were left to grow for 3 more days (Appendix 1.3). Before harvesting the roots were scanned using an Epson V scanner. After the harvest the roots were fixed in PFA for 1 hour on vacuum and then saved on a shaker in ClearSee (Appendix 1.4).

Figure 1. Growth pouch used for treatment application.

Microscopy

In order to prepare the samples for microscope imaging the roots were sliced into 250 micrometer slices. These slices were made using the Leica Vibratome. The samples were embedded in 3% agar in order to generate slices suitable for the microscopy (Appendix 1.5). To analyze the roots a confocal microscope was used, for measuring both lignin and suberin. For lignin analysis a Basic fuchsin stain was used and for suberin a Fluorol yellow stain, contrasted with Analine Blue (Appendix 1.6 & 1.7).

Analysis

Microscopy pictures were analyzed using the Icy software. Per picture taken, all individual cell

layers were identified using the 2D polygon function. For each cross section the cell layers were labeled according to table 1. This software would determine the intensity of the channel signaling either the FY or BF. For the analysis of the scanned pouches the ImageJ software SmartRoot was used to identify the average root length and average number of lateral roots per plant.

EP	Epidermis
C1	First cortex layer
C2	Second cortex layer
C3	Third cortex layer
EN-1	Cell layer between the cortex and the endodermis
EN	Endodermis
EN+1	Cell layer between the xylem and the endodermis

Table 1. Terminology for the different cell layers used in Icy analysis.

Sample growth

In order to test the tensile strength of the biomaterial, multiple replicates of samples were grown. For each treatment 12 trays (185 x 33 x 35 mm) were used, a total of 48 trays per replicate. Each tray was layered in the right order with a silicon pattern, a mesh, a thin layer of soil, 4.7 grams of oat seeds and another thin layer of soil. Each tray was watered properly. After 5 days, shoots had emerged from the soil, so the treatments were applied. For each replicate, 4 groups with different treatments were tested, ABA, silica, silica+ABA and a control group. The group tested for its response to ABA received 10 µM ABA, the silica group received 30 mg/L SiO_2 and the silica+ABA group would receive the same quantities of both substances. On day 7 after sowing the samples were harvested by removing the grown roots from the shoots using a razorblade. After harvest the roots were left to dry on filter paper for 7 more days (Appendix 1.8).

Single root growth

When testing the tensile strength of single roots, germination protocols were identical to the ones used for pouch analysis. However, for single root testing the seedlings were grown on filter paper for a total of 10 days. Since the chances of infection increased during this longer growth period, the seedlings were transferred to new filter paper after 4 days and restored in the phytotron. On day 7 two 10L tanks were prepared with ¼ MS and to one of the two tanks 10 µM ABA was added. The seedlings were transferred from the filter paper to 0.6 cm mesh to enable the roots to enter the tank, but the shoots to stick out. The plants were grown like this for 2 days, after which the roots were separated from the shoots and lain out to dry on filter paper for 7 days (Appendix 1.9).

Sample testing

7 days after harvest the material samples and single roots had properly dried and are ready to be tested. Sample testing was done using an Instron with a 5 kN capacity fixture, for single root testing 10 kN capacity fixture was used (Appendix 1.10). Results from the Instron were analyzed with the Nexygen plus software which produced a graph visualizing the load per unit of time (figure 2).

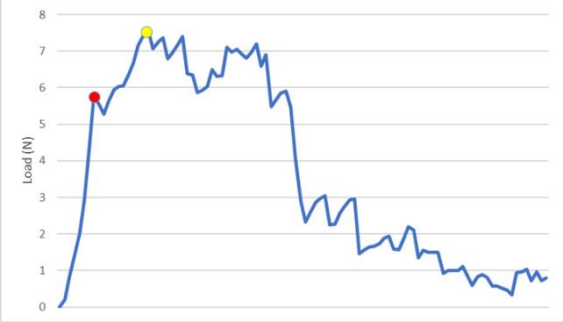

Figure 2. Results generated by the Instron of the material tests. Yellow dot representing the plasticity barrier, red dot representing the maximum load the material could carry.

Analysis of sample testing

Figure 2 demonstrates a stress-strain curve as could be plotted from the data received from the Instron measurements. The red dot indicates the plasticity barrier and the yellow dot the maximum tensile strength of the material. The maximum tensile strength was used for further analysis to determine the impact of the different treatments on the root-based material.

Results

This research aims to investigate the potential of the exodermis to improve the physical attributes of oats for the purpose of material development. By promoting exodermal formation, *A. sativa* levels of suberin and lignin should increase, which contributes to an improved physical resilience of the roots. In order to exclude the chance of the lack of an exodermis in *A. sativa*, or perhaps find another with better performance, the exodermal development of four other *Avena* species was investigated.

A hormone which is known to play an important role in the exodermal development of the roots in several species is abscisic acid (ABA). This hormone stimulates the synthesis of both suberin lamellae and Casparian bands in both the endodermis as well as the exodermis. However, not all plant species are known to develop an exodermis under ABA stimulation, so therefore these results will provide more information on the exodermal presence in the four different *Avena* species.

A small panel of closely related Oat species was created to map the differences in exodermal presence and composition. Observed differences could be future leads to continue to investigate the scope of root derived fabrics. This panel consists of the cultivated *Avena sativa* and its relatives *A. barbata*, *A. strigosa* and *A. sterilis*. Of none of these species the presence of the exodermis has yet been determined, nor its response to abscisic acid and whether this plant hormone might induce an exodermis. Therefore, these species were screened for an exodermis by determining the presence of lignin and suberin. These biopolymers were identified using a confocal microscope, staining the samples with Basic Fuchsin (BF) and Fluorol Yellow (FY), respectively. In all figures that contain heatmaps, plots marked with a star indicate the presence of a significant difference when compared to the control.

Exodermal diversity

The confocal images provide insights that show that in the control conditions of all oat species there was both suberin and lignin present in the endodermis (figure 3). In addition, lignin was clearly present in xylem vessels of the root vasculature. The Basic Fuchsin signal was also located in the epidermis of each of the cross sections. However, in controlled conditions this was not the case for the exodermis.

By taking a closer look at cortex layer 1 (C1), which represents the outer cortex layer of each cross section, no clear lignin signal was shown in control conditions in either of the sections. This was neither the case for a suberin signal, which lacked in both the epidermis as well as the exodermis. The endodermis did give a Fluorol Yellow signal in its cell walls. In order to test the presence of the exodermis statistically, the results of cortex layer 1 were compared with the signal in the cells of cortex layer 2 (C2), to test for a difference in signal intensity. When tested for significant differences, using a Wilcoxon test, only *A. sativa* showed a significant difference in lignin quantities between the two cortex layers (p-value = 0,016).

Testing the species on their response to ABA yielded a more distinct and pertinent result, enabling a more precise differentiation between them. For all four species the sections from the ABA treated plants were compared to the control (figure 3). Below the confocal images are the corresponding heatmaps, which illustrate the intensity of the signal measured in each cell layer of the root. Each star-marked cell layer indicates a significant increase in signal when treated with ABA. *A. strigosa* and *A. sativa* appeared to be the only species which had a higher abundance of both suberin and lignin in the first cortex layer. When looking at *A. sativa* it was visible how lignin was dominantly formed in between the cells of the cortex layer as strips, indicating the presence of the Casparian band. This suggests the presence of an exodermis for

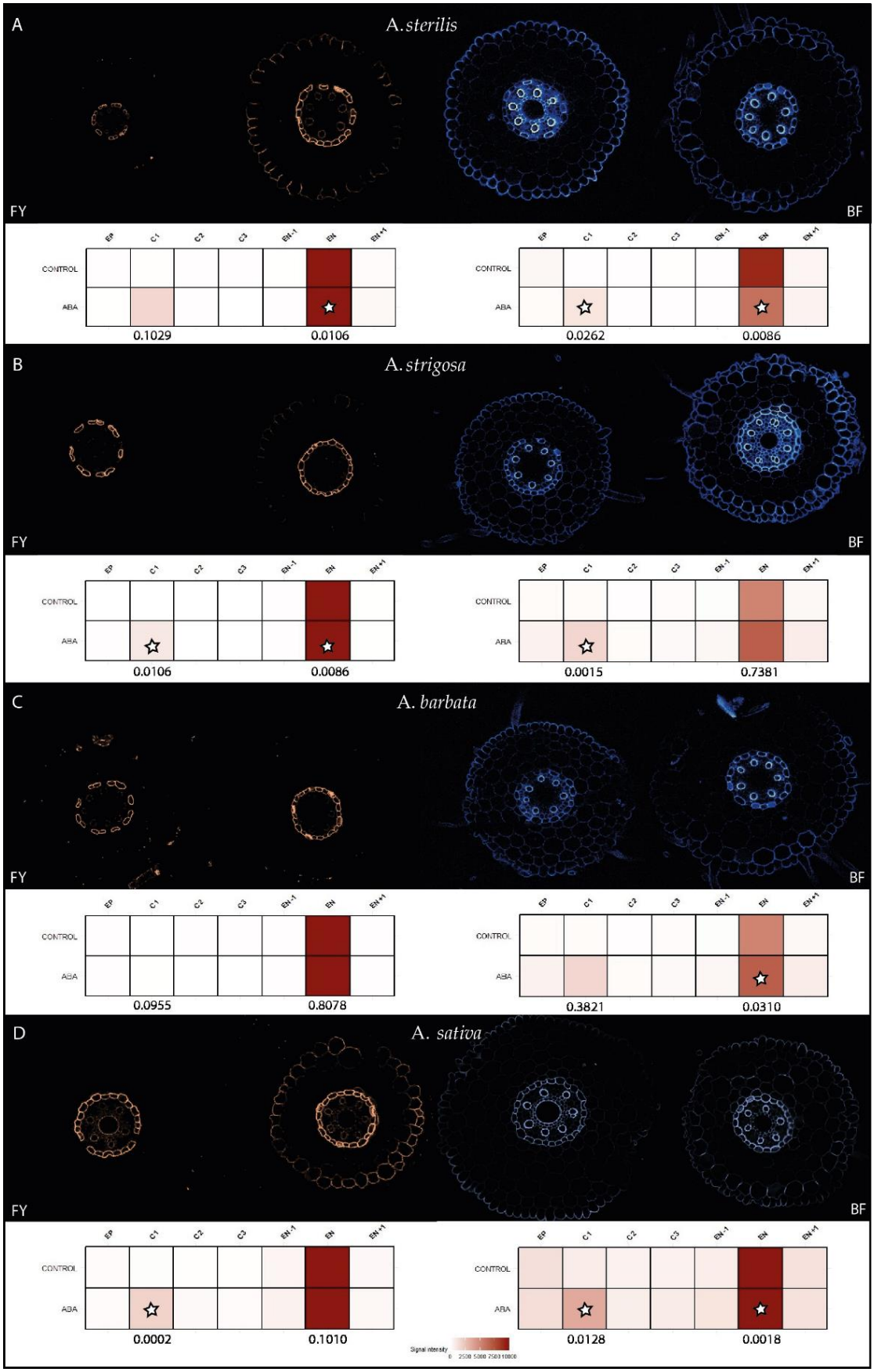

Figure 3. Confocal images presenting the exodermal diversity of four closely related Avena species and their effect when treated with ABA. The heatmaps present the signal value per cell layer, where stars indicate the presence of a significant difference between the ABA and control of that cell layer. Results are quantified by measuring the signal intensity of either the FY or BF signal.

both *A. sativa* and *A. strigosa*. Suberin was generally formed more throughout the entire cell wall of the first cortex layer. In contrast to *A. strigosa* and *A. sativa*, *A. barbata* had no signal in C1 for neither suberin nor lignin, implying the absence of an exodermis. However, in the endodermis lignin increased significantly in its abundancy. For *A. sterilis*, no increase in suberin content in C1 was observed but only an increase in lignin content (figure 3). Endodermal suberin and lignin abundancy increased almost for all species. However, only for the endodermal lignin of *A. strigosa* this increase was not significant nor for the endodermal suberin of both *A. barbata* and *A. sativa* (figure 3).

Stress specific response for exodermal development

The exodermis and endodermis function as a semipermeable barrier to protect the interior of the plant from external stresses. Abscisic acid is known to play an important role in the protection of the plant against drought stress, salt stress and possible regulation of this layer.

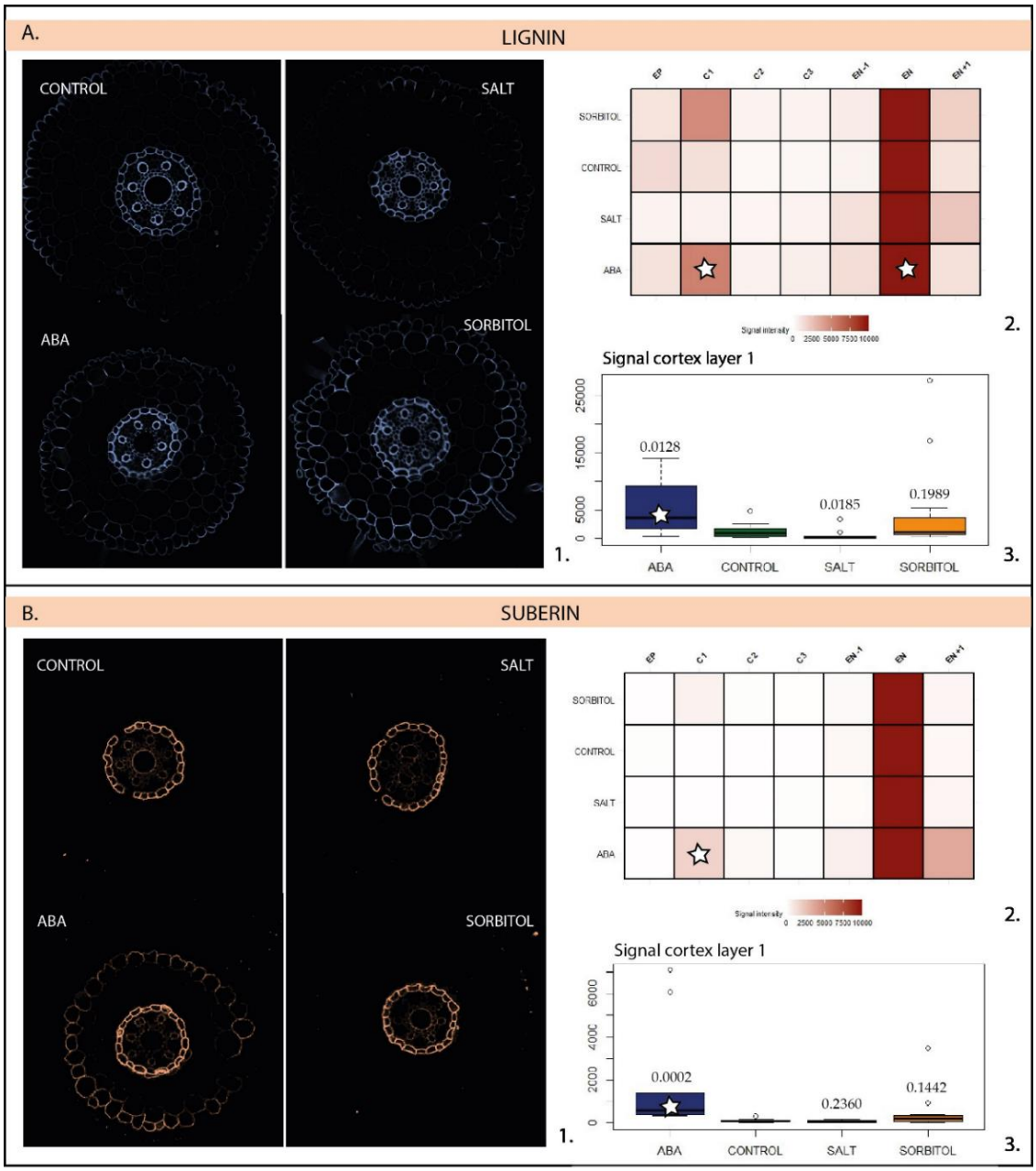

Figure 4. Confocal images from the cross sections of the roots tested on environmental stress responses. A) presenting the results of the lignin levels and B) of the suberin levels. The heatmaps show the signal values of each cell layer. Stars indicate the presence of a significant difference compared to the control. Corresponding p-values are mentioned in the boxplots of C1.

Sorbitol is a substance which can mimic the impact of drought stress whereas NaCl would induce a salt stress response. To get a more precise understanding of the role of ABA in the exodermis and endodermis of plants in these scenarios, tests were also performed where a treatment with salt or sorbitol was applied to the seedlings (figure 4).

Responses of the roots of *A. sativa* to salt and sorbitol treatments are presented in figure 4, A showing the effects on lignin levels and B on suberin. The ABA, salt and sorbitol treatments were all compared to the control of which their distributions are presented in the additional boxplots (figure 4 A3 and B3). All significantly different results are marked with a star in both the heatmaps and boxplots.

These graphs present the same results shown earlier of the effect of ABA (figure 3), increasing the level of lignin and suberin in the exodermis. Contrasting to ABA, salt stress showed no increase in the suberin and lignin quantities of the exodermis. Therefore, the low salt stress induced in this experiment had no direct effect on the synthesis of an exodermis. This does not confirm a role of ABA in the stress response against salt in the roots. However, when looking at the plots, the roots treated with sorbitol show an increase in lignin and slightly in their suberin abundancy. Nevertheless, this induction did not yield any significant difference when compared to the control group (figure 4 A3 and B3).

When focusing on the endodermis of the roots, none of the groups had a higher suberin content in their cell walls, compared to the control group. For lignin this was only the case for the ABA treated samples (p-value = 0.0106). Salt and sorbitol contents both did not increase.

The effects of silica application

Besides investigating the effect of salt and drought stress, *A. sativa* was also screened for the possible effect of silica on the exodermis and endodermis of the roots. Besides silica, the 2 µM ABA–contents (ABA2) was also increased to 10 µM (ABA10) in these experiments to study the effect when ABA contents differed. Adding merely silica had little effect on the lignin contents of the exodermis (figure 5). Some increase was observed when combined with ABA, but this was not significant (see figure 5 A3). Increasing the ABA dosage to 10 µM led to higher lignin contents, higher than in the group treated with 2 µM ABA, yielding a significantly different result (p-value = 0.005).

In the case of suberin it is notable that all ABA-treated groups showed a significantly higher suberin level in the exodermis. Of all three groups (ABA2, ABA10 and SI-ABA), SI-ABA showed the highest level of suberin. SILICA, however, did not differ from the control. The differences observed when increasing the level of ABA that we saw in the results for lignin, were not in line with the effects on suberin (figure 5B). The level of suberin measured in the exodermis of the roots treated with 10 µM ABA appeared to be slightly lower than in the roots treated with 2 µM ABA, however this difference was not significant (p-value = 0.4496).

Architectural characteristics

In addition to assessing the impact of suberin and lignin contents on improved material properties, root architectural characteristics were also examined. Specifically total root length and lateral root development, as these factors may influence the material properties of treated roots. It was evident that in both the silica as well as the salt experiment, the control groups showed the overall highest average root length (figure 6A). When tested on its significance, roots of ABA10 and SI-ABA were significantly different compared to the control group. From the salt experiment none of the treatments differed significantly from the control.

From the results of the abundance of lateral roots it can clearly be stated that ABA had a negative effect on the development of lateral roots (figure 6B). Each group that received ABA had a lower number of lateral roots then the groups without ABA.

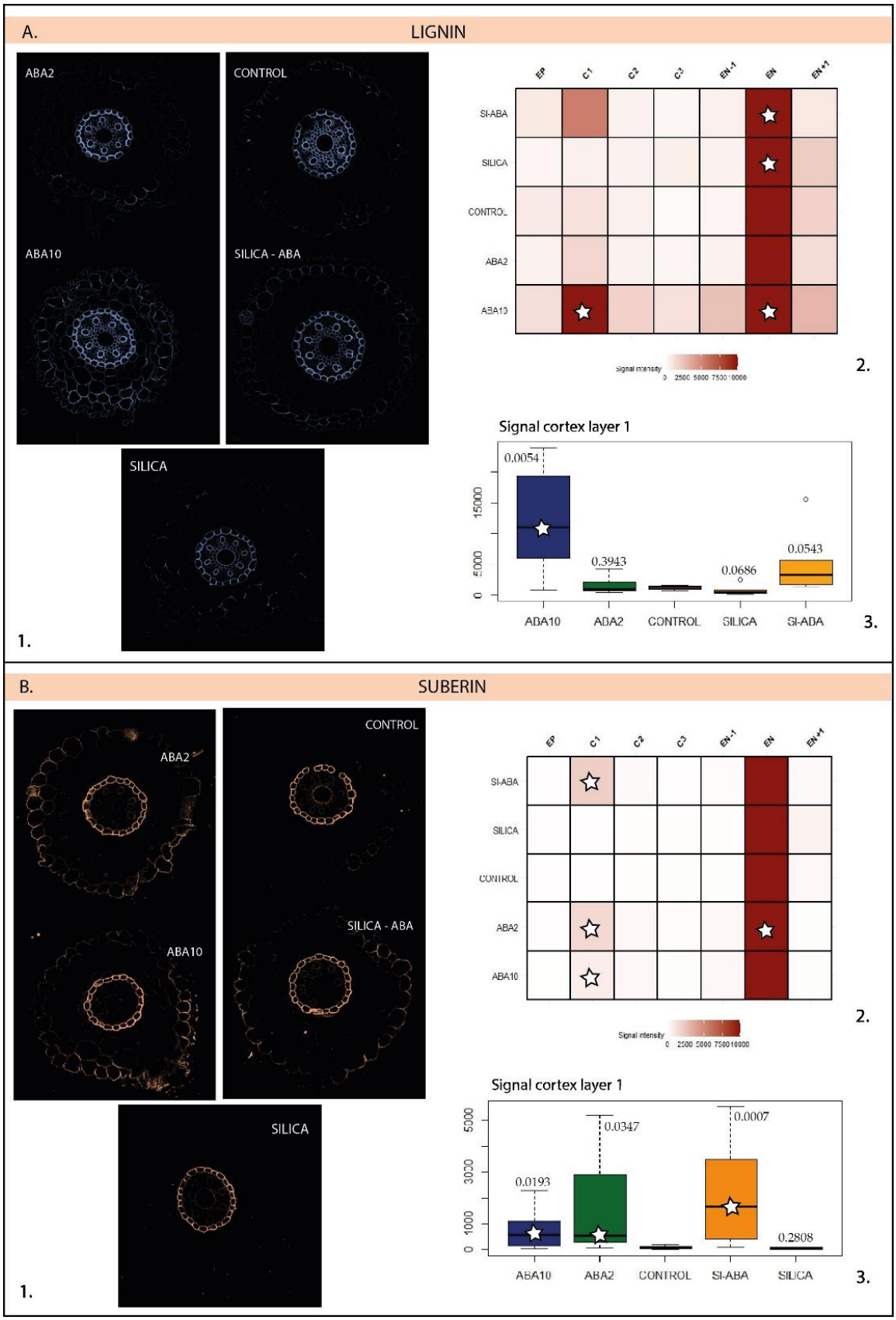

Figure 5. Confocal results when testing for the effect of silica and higher ABA dosages. A) presenting the results of the lignin levels and B) of the suberin levels. The heatmaps indicate the signal value per cell layer. Stars indicate the presence of a significant difference compared to the control. Corresponding p-values are mentioned in the boxplots.

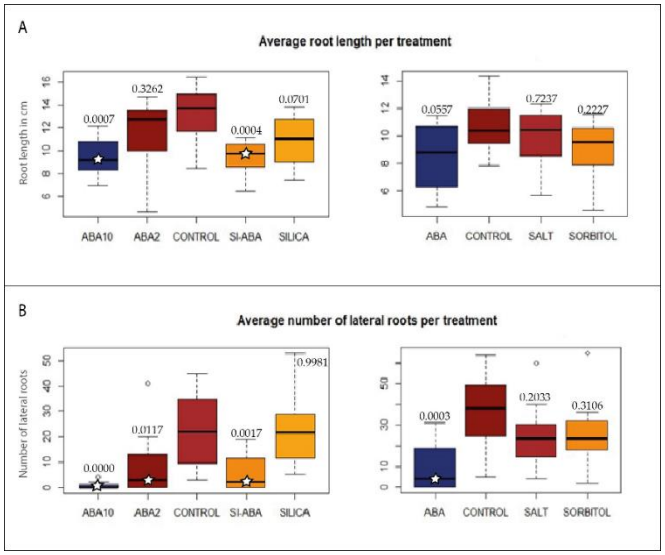

Figure 6. Architectural characteristics of the roots describing in A) the impact of the treatments on root length and in B) on lateral root development of the roots. Stars indicate a significant difference when compared to the control.

Within the silica experiment all groups had a significantly lower amount of lateral roots compared to the control, except for the SILICA group. Salt and sorbitol treated plants responded very similarly according to their lateral root growth, showing no significant difference to the control.

Material testing

In order to test whether there was a correlation between the tensile strength of the root-based material and the previously discussed characteristics, this section will present the results of the root material testing. The tensile strength was measured using the Instron, in four individual instances. Each of these replicates were conducted under identical conditions (figure 7).

The average tensile strengths varied for each series of measurements. In replicate 1 average tensile strengths ranged above 12 N, while replicates 2 and 3 (figures 7 B and C) displayed averages reaching no higher than 8 N. In addition, no specific trend could be observed amongst all four graphs. None of the treatments exhibited a definitive pattern in comparison to the others. These findings aligned with the outcomes of the statistical analysis. ANOVA tests were performed on all of the results, but none showed a significant difference between the treatments.

Single root testing

Besides testing on the root-based material, one test was performed measuring the tensile strength of single roots. Roots were either grown under control conditions, or with ABA treatment applied to the medium. The tensile strength of the control appears to be slightly higher than the strength of the ABA treated roots (figure 8). However, conducting a t-test led to no significant difference between the two groups.

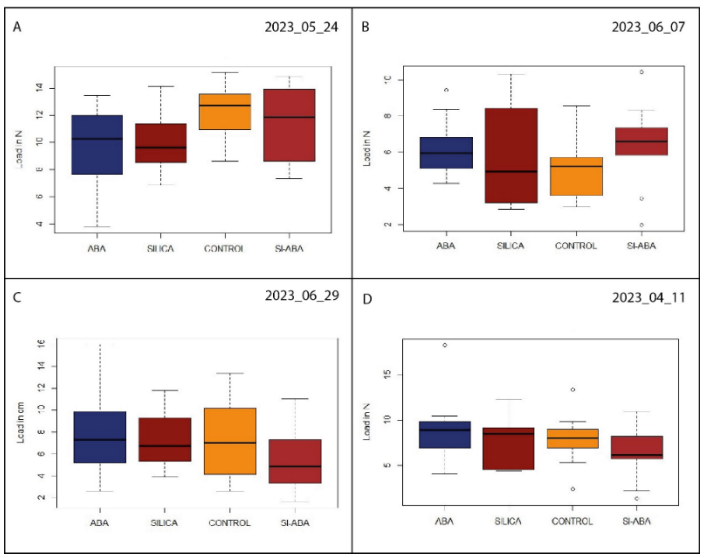

Figure 7. Results from the Instron tests. Each plot representing the results of one of the replicates, each labeled with the day on which they were tested. None of the plots showed significant differences

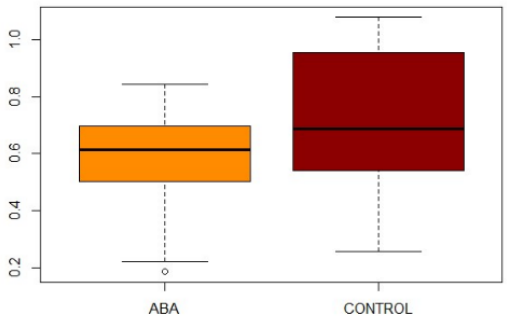

Figure 8. Results from the single root tests on the Instron, comparing the tensile strength of ABA and the control. P-value = 0.1471

Discussion

Avena sativa is a domesticated oat species, which has been bred to optimize its growth efficiency and ultimately increase its total yield. Its incredible growth speed and development are favorable for consumption purposes, but also for its usage as a biomaterial. In addition, oats are generally quite a resilient crop, well adapted to many environmental stresses, able to grow under marginal environmental conditions (Gao et al., 2022; Kutasy et al., 2022; Mu et al., 2015). These qualities make *A. sativa* a species very well suited for the purpose of a biomaterial. Taking all of these characteristics into account, a notable difference between *A. sativa* and its relatives is the localization of lignin in the first cortex layer, in unstressed conditions. Optimized abilities to protect its roots against mechanical stress is the main aim of this research, so therefore it was favorable to promote the exodermal development using an abscisic acid treatment.

Exodermis diversity of four closely-related oat species

A. sativa responded to the ABA treatment, inducing both a lignin and suberin signal in the exodermis (see figure 3D). In the endodermis only the lignin signal increased, whereas suberin did not. The processes of endodermal development have been broadly studied considering the formation of Casparian bands and suberin lamellae in *Arabidopsis thaliana* (Xu et al., 2022). It is described how endodermal development starts with the formation of Casparian bands, followed by synthesis of suberin lamellae. This could explain the insignificant suberin increase, suggesting that the suberin lamellae would form in a later developmental stage.

An interesting observation which can be made when looking at the suberin signal of all four species (figure 3), is how the FY signal in the endodermis appears to be patchy. When looking at the endodermis of the controls, one cell can clearly have suberin in its cell wall, when its neighboring cells lack any signal of suberin. When treated with ABA this inconsistency tends to be solved, indicating the presence of suberin in all of the cells in the endodermis. ABA treatment is known to enhance endodermal development, possibly showing this patchy suberization in lower parts of the root (Xu et al., 2022). Consistent suberin in the endodermis should be favorable for root resistance to mechanical stress. Lignin is present much more consistently throughout the entire endodermis, in both control and ABA treatments.

When looking at the other oat species, the only other species that showed clear induction of an exodermis when treated with ABA was *A. strigosa,* also known as Black oat. This cultivated species is used for the production of feed for cattle and as a covercrop in areas in Asia (Salgado et al., 2012). In *A. sterilis* the significant induction of lignin proposes the presence of Casparian strips in the first cortex layer, suggesting the presence of an exodermis in *A. sterilis*. *Avena sterilis* is the species closest related to *Avena sativa*, so here similar response could be expected (Loskutov, 2008). So, for both species it can be stated that external ABA application induces the development an exodermis.

Avena barbata was the only species of the four which showed no indication of an exodermis when treated with ABA. *A. barbata* is known to have an extensive adaptive response to drought stress according to (Sherrard & Maherali (2006), however apparently this does not include the synthesis of an exodermis, at least not with the dosage used in this research. ABA synthesis has been reported in the roots of *A. barbata*, but can apparently not be linked to lignin and suberin synthesis in its roots (Swarbreck et al., 2011). The fact that *A. barbata* was the only species of the four that did not form an exodermis might be explained by the evolutionary pathway of the four species. According to (Loskutov, 2008) *Avena barbata* is the only truly “wild” oat species of the four, having no close trace of origin for *A. sativa.*

Considering the ABA response of all four species, it would be appropriate to favor *Avena sativa* as the species most suitable for material development. *A. strigosa* is similarly suitable, however, due to its excessive availability in Europe, *A. sativa* seems most appropriate for the purpose of a material.

Exodermal responses to natural stressors

Besides investigating the response of *A. sativa* to ABA, two ways of generating a more natural stress responses were induced in this study. One way to do so was by inducing salt stress to the plants. For many species the ABA pathway is correlated to the salinity response of a plant (Kempa et al., 2008). Hai et al (2022) demonstrated how ABA plays a role in the salt-stress response of oats, presenting increased levels of ABA under salt stressed conditions. However, very little is known about the effect of salt stress on the endodermis and exodermis of oats. In order to better understand the role of ABA in the salt stress response of oats, this research tested for the potential of exodermal development, in saline conditions for *A. sativa*. However, no correlation can be found when looking at the tests conducted in this research (figure 4). Salt stress did not induce the presence of an exodermis, nor suggest an ABA synthesis as a result. However, this does not suggest that ABA was not formed in the roots at all, just that no exodermis was formed as a result. This can be stated from the fact that presence of ABA clearly resulted in the formation of an exodermis, when salt did not. Additional tests would have to be performed to better understand the ABA synthesis in salt-stressed environments and its relation to exodermal development. In this study not too high salt concentrations were applied to avoid inhibiting the root growth. The concentration of 100 mM was based on various articles using a similar concentration (GAO et al., 2014; Legocka & Sobieszczuk-Nowicka, 2012; Mu et al., 2015).

In addition to salt stress, the effect of drought stress was studied in relation to exodermal development. Sorbitol was used to apply osmotic stress in many plant species. Osmotic stress is closely related to the effect that drought stress can induce. Where some uncertainties exist in the role of ABA in the salt stress response, the role of ABA in the drought stress response for oat species is much better described. Drought resistance in oat species involves an ABA-mediated response, and also heavily impacts root development (Canales et al., 2021). This response includes root suberization and lignification, two responses which were not priorly stated for salt stress (Canales et al., 2021; Kim et al., 2022). A sorbitol treatment slightly increased the levels of lignin in the exodermis, but not significantly (figure 4A). However, in comparison to the response the plants had to a salt treatment, this response to sorbitol was much stronger. This suggests an effect of sorbitol on the synthesis of ABA, but this cannot clearly be concluded from these results. Further research would have to be conducted testing perhaps with higher sorbitol and salt levels for more concrete results. However, too much drought stress can strongly damage the cortex layers of the roots, including the exodermis (Canales et al., 2021). Therefore, excessive stress might damage the root architecture too much and thereby negatively influence the root tensile strength. In conclusion, it might be preferred to directly apply ABA to the plants instead of stimulating the natural response through stress, since this might affect other favorable traits in the roots.

The impact on root architecture

In order to measure the effects of the applied levels of salt and drought stress, the impact on root length and lateral root development was also measured (figure 6). For both salt and sorbitol no significant impact on the root length was found in this study. Both treatments appear to yield a slightly lower average root length, but the difference is very slim. The same goes for the average amount of lateral roots. Both salt and sorbitol show a slight decrease in the number of lateral roots, but not significant when compared to the control. The levels of

stress induced in this research were thus not detrimental to root architecture.

Silica as strength enhancer

The goal of strengthening the roots of oats could also be achieved by using the additional application of silica. Not all species are known to accumulate silica in their roots, however plants belonging to the Poaceae family are known to be high silicon accumulators, including oats (Toledo et al., 2012). When accumulated the silica contributes to the mechanical hardening of the root tissues, so thereby would improve the potential of the root-based material (Lux et al., 2020).

The lignin results from the silica trial, show how roots treated with ABA10 clearly had the highest level of lignin in the exodermis (figure 5A). This suggests that the lignin level is dependent on the level of ABA in the roots, higher ABA levels promoting lignification. In addition, some effect when treated with silica was observed. The signal for lignin of the SI-ABA treatment in the exodermis was much stronger than that of the control and the ABA2 treatment. This might be similar to results known to happen in rice, where silica promotes the formation of Casparian bands in the exodermis through lignification (Fleck et al., 2015). The SI-ABA received an ABA dosage of 2 µM, equal to ABA2, so the fact that the SI-ABA group had a stronger signal than ABA2 suggests a potential influence of the interaction between the ABA and silica enforcing lignification. Fertilization of foliar silica is known to influence the drought resistance of oats, which can potentially also be caused by the promoted exodermal formation induced by an ABA signal (Kutasy et al., 2022; Mehrabanjoubani et al., 2019). However, since this result is not significant in this report, it is difficult to conclude this based on these results. It is also important to state how there were some faults in the fixation of the silica trial. Many of the sections were heavily damaged when observing them with the confocal microscope. In particular from the ABA2 and SI-ABA group very few samples were appropriate for analysis, resulting in sample sizes of only 5 samples. This was sufficient for analysis, however this made the results less reliable since sample sizes between the groups were uneven. The other groups had a sample size of 8 samples per treatment. However, the observations found are in line with other published literature, making it relevant to study the observed effects further.

The beforementioned improved drought resistance when treated with silica can also be correlated with the changes occurring in the endodermis. Apart from the effect on the exodermis, silica significantly influenced the lignin abundancy in the endodermis of the *A. sativa* species. Both groups that were treated with silica showed an increase in lignin levels (figure 5A). According to Mehrabanjoubani et al., (2019) silica plays a role in cell wall thickening, increasing the lignification rate in the endodermis. This is in correspondence with the results found in this report. Even though most of the absorbed silicon is transported to the leaves of plants, the silicon accumulating in the roots play a role in strengthening the root mechanical barrier (Hattori et al., 2003).

When focusing on the effect of silica on suberin, changes can be observed primarily in the exodermis. Results showed an increased level of suberin in the exodermis when treated with SI-ABA, ABA2 and ABA10 (figure 5B). In the endodermis the level of suberin increased only when treated with 2 µM ABA, not when treated with SI-ABA or ABA10. This is in line with results from research conducted by Fleck et al. (2011), where additional silica treatment resulted specifically in suberization of the exodermis, by stimulating the development in an earlier developmental stage in root growth. Interestingly, mere silica application appears not to be sufficient for suberization since this did not yield an increase in FY signal, where SI-ABA gave the highest level of suberin of the experiment (figure 5 B3). Only when stimulated with an ABA signal the silica seems to have an effect on exodermal suberin. In addition, the higher dosage of ABA in this experiment did not

yield an increased effect on the level of suberin in the exodermis. Where lignin clearly increased with the higher amount of ABA, this did not happen for suberin. The FY signal in ABA10 seems even to be lower than the signal observed in ABA2. Therefore, we can suggest that higher dosage of ABA, does not yield a higher level of suberin in neither the exodermis, nor the endodermis.

Silica impacting root architecture

It can be expected for silica treated plants to show patterns of root elongation (Stadnik et al., 2023). However, this is not in line with the results seen in this report, since no increase in average root length was observed for none of the silica treated groups. SI-ABA even showed an average root length that was significantly lower than the control. This can also have been caused by the ABA present in this treatment, since ABA10 also showed significantly shorter roots. Interesting to state is though that ABA2 did not decrease significantly in root length. This is in line with Canales (2021) which illustrates how a moderate ABA increase promotes root growth in *Avena sativa*. The results of this study illustrate why ABA should not be applied excessively, since this would negatively affect the root architectural development.

In addition, investigating oats physiological characteristics when treated with ABA showed how ABA strongly decreased the amount of lateral roots. Each of the groups which received ABA in its treatment had a much lower average amount of lateral roots, compared to the treatment that did not receive ABA (figure 6). This might be an unfavorable trait for material development, since more lateral roots could increase the amount of linkages which can be made between different root structures. The impact of lateral roots as not yet been tested in relation to material stability, however it has in relation to soil adhesion (Saifuddin et al., 2022). The same could be stated for the root length, since longer roots increase the range of where a single root can contribute to provide stability.

The effects of manipulation on the material potential of oats

Considering all previously mentioned results, silica might be able to influence the tensile strength of root-based material. Namely the SI-ABA group could have the potential to increase root tensile strength, since both lignin and suberin levels increased particularly in the exodermis in the roots treated with both silica and ABA. The additional mechanical support that silicon provides through its deposition in the roots would also improve the strength of the material. This conclusion however cannot be drawn from the results of the Instron material tests since in none of the replicate showed a significant difference (figure 7). This could perhaps have been influenced by the negative effect observed on the architectural characteristics of the treatment on the material. The shorter root length and diminished lateral root growth could have impacted the tensile strength of the SI-ABA treated material. Moreover, after harvest it could often be observed how the growth of the material was not very consistent over the different replicates. When comparing the trials, the materials grew very irregularly even though growth conditions were identical. For some the overall root growth was limited, or the growth was skewed, impacting the even distribution of the strength of the material. However, if a treatment would have a dominant effect on the tensile strength, such a result would still have been observed, since the uneven growth was constant over all treatments. As a result, it cannot be concluded that promoting exodermal development has a positive effect on the material potential of oats. In order to detect errors in the root growth between treatments it is recommended to weigh the dry biomass of the samples before testing them with the Instron. This could confirm material impacted by irregular root growth.

When reperforming this experiment a suggestion on the methodology would also be to spread the dosage of the treatment, applying some of the treatment on day 5 and the other

on day 6. This would create a more realistic condition for the plant, as it would receive abscisic acid more spread out, instead of receiving it all at once. In addition, it would be interesting to include tests for different ABA concentrations, since now only one was tested (10 µM ABA) on the materials. However, to much ABA would negatively affect other traits of the roots such as root length. Therefore, testing a range of ABA concentrations would give more insight on the effects of ABA on the material. Furthermore, a more extensive research into the effect of silica is suggested, since these results provoke an interest in the detailed effect of silica on root reinforcement. This would include the application of different dosages of silica to the roots. In addition, the plants should be tested after harvest on the true amount of silica deposited in the roots.

Apart from the tests performed on the grown samples, tensile strength was also measured for the single roots. The results from comparing ABA treated roots to the control yielded no difference between the two (figure 8). It would have been preferred to do multiple replicates of this test, since these tests would have demonstrated more about the effect of exodermal development in the individual root. However, when setting up the experiment many times faults occurred, such as contamination of the water basin, making the roots unsuitable for further testing.

Conclusion

Of all the four *Avena* species discussed, *Avena sativa* appears to be most appropriate for material development. When subjected to an abscisic acid treatment, *A. sativa* exhibited a significant enhancement in the exodermal formation, enforcing the structural stability of its root system. This species is also the only species that showed presence of an exodermis in control conditions.

Besides its response to ABA, *A. sativa* was also investigated for its ability to develop an exodermis when subjected to salt or drought stress. Under both stress conditions, there was no substantial increase in suberin and lignin levels in either the exodermis or the endodermis. The application of sorbitol resulted in a slight increase in lignin in the exodermis, however results were not significant. Therefore it can't be concluded that salt nor drought stress have a large influence on the exodermal development of oat roots.

The treatment that was most promising to enhance the tensile strength of the roots was the treatment where the plants received both silica and ABA (SI-ABA). This treatment led to an increase in suberin within the exodermis and lignin in the endodermis. Lignin was also abundant in the exodermis, but did not yield a significant increase. Only applying silica as treatment did not result in exodermal or endodermal development. Comparing the SI-ABA to the ABA2 results showed that SI-ABA had a higher increase in both suberin and lignin, even though both samples received the same amount of ABA. This suggests the positive correlation between silica and ABA. In addition, when comparing the different levels of ABA it was observed that lignin levels in the root were higher when treated with a higher dosage of ABA.

Despite its favorable impact on exodermal development, ABA had a negative impact on the development of lateral roots. Furthermore, both SI-ABA and ABA10 showed a decrease in the average root length, compared to the control. These factors do not influence the strength of the individual roots, but they do in the development of the developed material, by decreasing the total root mass of the material.

As a result, none of the treatments resulted in the higher tensile strength of the material. Among all the four replicates that were conducted, none of the treatments demonstrated a tensile strength structurally higher than the others. Therefore it can't be concluded that an improved exodermal development enhances the mechanical strength of the roots of oats.

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Appendix 1

1.1 Washing Avena seeds

Wash with ethanol for 5 min

Put the required amount of seeds into a 50mL tube. The seeds will increase in size because they will saturate with water, therefor the tube should not be filled more than halfway. When more seeds are required, take a second tube. Fill the tube with ethanol until all seeds are soaked in ethanol. Let it rest for 5 minutes.

Washing with water

Rinse the seeds 6 times with autoclaved milliQ

Wash with bleach for 5 min

After the seeds are washed, soak the seeds with a 50% bleach solution. Be aware not to shake the tube, since this will greatly increase the amount of bubbles which will make the washing step more difficult. After 5 minutes, pour the bleach from the tube and move on to the next step.

Washing with water

Rinse the seeds 7 times with autoclaved milliQ

1.2 Germination protocol

Materials

- A4 sized filterpaper
- 1 liter ½ MS
- 2 Beaker
- Pincet
- Petridish

Perform the germination protocol in a flowhood to ensure the filterpaper from getting infected. Properly disinfect the table using alcohol and fill one of the beaker with ½ MS.

Step 1

Fold the filterpaper in the long side and properly wet the filterpaper with the ½ MS. Unfold the filterpaper on the table and lay the oat seeds in a zigzagged line on one side of the filterpaper. Make sure to use a sterilized pincet to place the seeds on the paper.

Step 2

Refold the paper and tightly role the filterpaper and put it in the beaker, which is filled with ½ MS. When the beaker is fully filled with filterpaper rolls, wrap it with aluminum foil and store it in the climate chamber in 20 degrees, with 16 hour light days.

Step 3

On day 2 in the climate chamber make sure to remove the aluminum foil from the beaker to enable the shoots to grow. On day 5 the seeds are ready to be transferred to the growth pouches.

1.3 Growth pouches

Step 1

Start by preparing the treatments. For each pouch a total solution of 20 mL will be applied, so make sure to prepare at least 100 mL to be able to fill at least 5 pouches. Depending on the treatments required for the experiment, use the following concentrations:

Solution concentrations

ABA low dosage: 2 mM
ABA high dosage: 10 mM
Silica: 30 mg/L
Silica + ABA: 30mg/L + 2 mM
Salt: 100 mM NaCl
Sorbitol: 200 mM Sorbitol

Step 3

Unroll the filterpaper rolls one by one and replace the seedlings from the filterpaper roll to the growth pouches. Per pouch place 3 avena seeds in a row. Make sure to place the seedlings in the pouches by inserting them with the shoot through the holes of the growth pouch. Make sure to prewet the pouch papers slightly to make sure the roots are not to badly damaged by the dry air and to let the roots stick to the paper which makes it easier to put them back into the pouch.

Step 4

When the paper is reinserted into the pouch, the 20 mL solution can be poured into the bags. Make sure to place the pouches vertically in the climate chamber in a rack that prevents the roots from getting to much light.

Step 5

After 3 days the plants are ready to be harvested.

1.4 Harvesting and fixing the roots

Step 1

After the roots are grown in the pouches for 3 days the roots are ready to be harvested.

Step 2

First scan the roots before they are harvested on a resolution of 400 dpi.

Step 3

After the scan is completed the roots can be cut, but try to keep the roots of each plant together, so you can identify later on which roots belong to the same plant. Place them in a 6 hole tray with pfa. Place them with the lit removed in a vacuum for 1 hour.

Step 4

After the hour, remove the pfa with the pipet and soak the roots in clearsee.

1.5 Slicing the roots

Step 1

The roots are removed from the upper part of the plant and the roots are washed and stored in clearsee. Make sure that they remain properly separated per species and per treatment during this entire step. Properly mark all used equipment.

Step 2

Start with making the 3% agar. 3% suggestes 30 g per liter. Preferably make 500 mL, so first put 15g in a liter bottle. Add 500mL of milliQ and place the bottle in the microwave until all agar is fully diluted into the water. In the meantime set up the machine and collect all required materials:

Materials

- Extra tray for slices containing clear see
- Pinchet
- Knife
- Tray to fill with agar
- Bucket of ice

Cut the root in such a way that you maintain the part of the root that contains the 75% of the root. Collect the melted agar and fill a tray with about a centimeter of agar. Place the root in the agar and try to position it as straight as possible. Place the tray in the bucket of ice to let the agar harden.

Step 4

When solid, remove the agar block from the tray. Remove surplus agar, and safe the part which contains the root. From this part separate the 75% part of the root from the rest and place this on the black circle which is to be placed in the slicer. Glue the cube to the black circle. To speed up the process glue different cubes with the root parts together on the black circle.

Step 5

Fill the slicer with ice and water to keep it cool and place the circle in the proper position and install the slicer. Install the slice thickness of the Vibertome on 250 micrometer.

Step 6

Collect all slices, but throw the first 3 away, these might have been damaged. Store all other slices the extra tray which is filled with clearsee. Slice each species and treatment separately to prevent slices from being mixed.

1.6 Staining the roots for microscopy

Lignin staining

Start by preparing the Basic Fuchsin solution in a 50 mL tube wrapped in aluminum foil to prevent light on the solution. Weigh 0,25 grams of basic fuschsin and dilute it with clearsee. Select two slices of each of the roots and place them in a new empty 6 hole tray. Spread them over 3 of the holes to make sure same roots are not scanned double. Add enough of the BF solution so that the slices are completely soaked. Cover the tray with aluminum foil and place on the shaker for 30 minutes. After 30 minutes remove the BF from the tray and wash the slices 3 times with Clearsee. For each washing step let the slices shake for 30 minutes in the new clearsee, before washing them again. After the three washing steps, add glycerol to the slices until you move to the confocal.

Suberin staining

Start by preparing a solution of Fluorol Yellow by mixing 0.01 g with 1 mL of DSO in a Eppendorf tube wrapped in aluminum. Transfer 0.5 mL to a 50 mL tube wrapped in aluminum and mix with 96% alcohol. Select two slices of each of the roots and place them in a new empty 6 hole tray. Spread them over 3 of the holes to make sure same roots are not scanned double. Add enough of the FY solution so that the slices are completely soaked. Wrap the tray in aluminum and place on the shaker for 30 minutes. In the meantime make sure to prepare the Aneline Blue. For the Aneline Blue weigh ... g in a 50 mL tube, add MilliQ and wrap it with aluminum. Store it in the freezer untilt the 30 minutes of the FY are over.

Remove the FY from the tray with a pipet and add AB to the tray. Rewrap the tray and put it back on the shaker for 30 minutes. Wash the samples twice with MilliQ, where the slices are washed for 20 minutes per step.

1.7 Confocal microscopy

Step 1

After staining the root slices they can be taken to the convocal microscope. Make sure that they stay as much out of the light as possible after they have been stained, otherwise the stain works out. Take them to the convocal, along with:

Materials

- Pinchet
- Pipet + tips
- Glycerol
- Dekglaasjes
- Optical glassware

Step 3

Turn on the microscope by turning on all 3 switches. Start the progamm, this may take a while. In the mean time, prepare the slides by putting glycerol on the slide. Lay the root slices on the glycerol, no more then 5/6 roots in total. Add the dekglaasje and add as much glycerol as feels necessary between both pieces of glass. Place the glass in the holder.

Step 4

Set the lens to 25x, and add a drop of glycerol on the lens. Place the holder and focus the lens. In the program, use the function 'position' to focus. When in focus switch to capture and use the live function to properly aim. Save the snapped image to both the computer and the harddrive.

1.8 Material preparation

Step 1

In order to make the material, prepare 12x4 frikandelbakjes with at the bottom the silicon mat and on top of it the gaas. Start with a small layer of soil after which 4,7 grams of seeds are divided over the soil. Cover the seeds with a small layer of soil and wet the trays. Store the samples in the climate chamber on 20 degrees with a light day of 16 hours. After 5 days, the seeds will have properly germinated and the treatments can be applied to the soil. There are 4 types of treatments.

Treatments

- Control
- ABA = 10 mM per tray
- Silica = 30mg/L per tray
- ABA+Silica (see above)

Step 2

For each treatment a total volume of 35 mL is poured into the tray. A total of 12 samples should be prepared per treatment. On day 7 the trays are ready and the material is ready to be harvested.

1.9 Single root preparation

Step 1

For single root testing the seeds are left to germinate for 10 days in the filterpaper rolls. Since this might increase the chances of the filterpaper getting infected the filterpaper should be refreshed once, on day 5.

Step 2

On day 10 the seedlings are ready to be transferred to the tank for the ABA treatment. To prepare the treatment properly wash two 10L tanks with both soap and alcohol. Fill both tanks with 1/4 MS and insert an aquarium aeration. Make sure not to turn it on too hard.

Step 3

Take the seedlings from the filterpaper and one by one try to place the roots through the 0.6 cm wide gaas. With the roots at the bottom of the gaas hang the roots in the tank so that the roots are fully covered by the medium.

Step 4

After two days in the tank the roots can be harvested. Remove the roots from the shoots and lay the roots individually out to dry on filterpaper for 7 days before they are ready to be tested using the Instron.

1.10 Material testing

Step 1

Prior to testing the material, the material is grown for 7 days and then harvested. The harvesting is done using either a razorblade or a stanley knife. From the top of the gaas the plants are separated from the roots and the gaas and silicon frame are carefully removed from the roots. The roots are then left to dry for at least 7 days of dry filterpaper.

Step 2

The material is tested using the Instron. Make sure to properly set the material in the machine. The speed setting is set to 3 mm/min for the material testing. 1 mm/min for single root testing. Use the 5 kN lever for the material testing and the 10 kN lever for single root testing. When changing the lever make sure to correctly change the settings of the program. If the material did not break within 3 minutes the test can be stopped. The results are automatically loaded into an excel sheet, where the value of load represents the amount of Newton necessary to break the material. When analyzing the results the maximum value of the load of each sample represents its strength.