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Salinity stress protection of Fusarium sp. in Puccinellia maritima

And Festuca rubra & Lolium perenne

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Abstract

Soil salinization is a big problem for agriculture because it decreases crop yields in a world where food demand is ever-growing. It would be of great help if we could somehow make crops more resistant to salinity stress so soil salinization doesn't affect crop yields as much. A fungus (*Fusarium* sp.) has been found to help protect Red Fescue (*Festuca* rubra) against salinity stress on a saltmarsh on the Dutch Wadden sea coast. We let the fungus interact with the roots of other grasses (*Puccinellia* maritima and *Lolium* perenne) to see if it was able to colonize other grasses. And to follow if it had the same growth-stimulating effect on saline soil as had been seen within *F.* rubra. We also look at what adaptations the fungus inhibits within the grasses that make them better protected against saline stress.

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Introduction

The food demand of our growing population is increasing. To meet this increasing demand the production of crops needs to grow as well. More and more agricultural land is salinizing. The salinization of soil causes a loss in agricultural areas. The crops that are still able to grow on these saline soils have a growth reduction and a decreased yield. So there is a decrease in food production. In general, grasses are more adapted to salinity stress, thus more resistant than crop-producing plants (Munns, 2008).

The NaCl in the saline soil has an osmotic stress effect on plants. It disrupts the osmotic regulated transport system in the plants. So the plants can't get the nutrients where they want anymore. Furthermore, Na+ and Cl- ions have a toxic effect on plants as it competes in the uptake of necessary K+ ions (Hussain, 2019).

If we would be able to prevent these salinity stress effects in plants it would increase crop yields in saline environments. And make a more saline environment usable as agricultural land. This would greatly aid in the solution to the ever-growing demand for food.

The use of salinity protection inducing fungi can potentially provide an effect in this solution. A species of Fusarium that grew in the roots of Festuca rubra on a saltmarsh has been found to have a growth-promoting effect on saline soil (Wang, unpublished). It is worth looking if this species of Fusarium would be able to colonize the roots of other plant species and induce the same growth-promoting behavior. So crop plants can be better protected against salinity stress.

Fusarium is a large genus of fungi. The genus is well known in agriculture as a pathogen as some Fusarium species are among the most important pathogens for plants.

The Fusarium sp. found in F. rubra helps protect the grass against salinity stress by inducing adaptations to the plant so it becomes better adapted for saline environments. The adaptations are especially seen in the roots. Where it makes them shorter but thicker. And it might affect the suberin it the roots. Where it makes it thicker so it forms a thicker barrier (Motos, 2017).

Suberin is hydrophobic material in plant roots that prevents water and nutrients to just enter the plants' transport system. Instead, the nutrients and water must bypass the endodermis, and here the plant can select what it wants to take up and whatnot. This way it can protect from taking up to much salt (Kolattukudy, 1984).

We hypothesize that Fusarium sp. can successfully colonize other grass species and will have a positive effect on the performance of P. maritima in saline environments. It will however have a light penalty on performance under non-saline circumstances. We also expect changes from the Fusarium sp. in the root structure and the suberin within the roots.

Materials and methods

Experimental setup

Inocula ECOstyle PT-Mix/ExSol P, containing several Bacillus species, was donated by ECOstyle BV, Oosterwolde, The Netherlands, and was applied as supplied.

Fusarium sp., originally isolated from Festuca rubra plants grown on a salt marsh near Noordpolderzijl, The Netherlands (53.43 N; 6.58 E) (Ausma, T. 2017. Master thesis, University of Groningen, the Netherlands), was grown for 48 hrs on potato dextrose broth supplemented with 50 µg/ml ampicillin and 50 µg/ml streptomycin at 28 °C on a rotary shaker (140 rpm).

Plant growth Seeds of Festuca rubra ssp rubra cv Rafael, Aurich, Germany; Lolium perenne, Vreeken's zaden, Dordrecht, The Netherlands; Puccinellia maritima, Western Regional PI Station, Pullman, United States; were germinated on vermiculite. After 26 days seedlings were transferred to potting soil. Puccinellia maritima was seeded later and treated with a germinating solution, incubated in 5 mL of 1 M KNO3 supplemented with 50 µL of 0.1 M gibberellic acid (Roth) and 25 µL of 0.5% (v/v) Tween 20 (Merck) at 4°C overnight (Schmidt, 2006). after 11 days these seedlings were transferred to potting soil. Roots of the

Fusarium sp. and ECOstyle -treated plants were dipped in the inoculum just before being planted in potting soil. Control plants were given identical treatment, but without *Fusarium* sp. being present in the solutions.

Growth conditions Plants were grown at 20/18 °C, RH 40%, light intensity ≥ 350 µmol.m-2.s-1, 14/10 hrs Light/Dark cycle in the greenhouse for another 14 days on soil, occasionally watered with tap water.

After 3 days on the potting soil, the salinity treatment was started: control plants were flushed with 0.11 l of demi water and 0.11 l of 25% Hoagland solution, and NaCl-treated plants were flushed with 0.11 l of demi water (to prevent the build-up of higher NaCl levels) and 0.11 l of 25% Hoagland + 100 mM NaCl solution. This was repeated weekly for 2 weeks. Twice a week the maximum shoot length was measured with a ruler. The PAM value of a selection of the plants was measured weekly, to determine stress the amount of stress.

At the end of the experiment, the plants were removed from the pots and the roots were gently washed under running water to remove adhering soil. The length of the shoot and roots was measured with a ruler. Fresh weight (immediately after harvest) of shoot and root were determined. (Wang, unpublished)

Species: **Treatment:**	***Puccinellia* maritima**	***Festuca* rubra**	***Lolium* perenne**
NaCl+ *Fusarium* sp. (NF)	n=14	n=3	n=2
NaCl (NX)	n=14	n=3	n=2
Fusarium sp. (XF)	n=14	n=3	n=3
Control (XX)	n=14	n=3	n=2

Table 1: The used grass species and the sample seize of each treatment

Root colonization

Successful infection of the plants by *Fusarium* sp. was checked. by staining of the fungal hyphae with 0.05% trypan blue in lactic acid, following the method of Michal Johnson et al. (2011) and incubation in 10% KOH for 10 minutes. Root sections were observed under the microscope.

Stomata

Negative prints were made of the leaves' surface so a clear nail polish positive could be used under a microscope to count the number of stomata per µm of surface area.

Suberin

In order to determine the presence and the location of suberin in the roots. The roots are fixated in agarose so sections can be cut using a microtome. The roots are stained overnight with berberine and the day after washed and counterstained with aniline blue. This way the Suberin in the roots will fluoridate using a fluorescent microscope.

PAM

The activity of the photosystems was measured with a PAM fluorometer, from which maximum yield was measured. From each grass species, multiple individuals were taken and put in the dark for 5 minutes. Now all the energy is gone from the photosystems, and the F0 can be measured. After this measurement, a saturating pulse of light is given so the Fm can be measured. With these data the effective yield can be calculated with the following equation (Krause, 1991):

$$YIELD = \frac{F_m - F_0}{F_m} = \frac{F_v}{F_m}$$

F_m = maximum fluorescence

F_0 = minimum fluorescence

F_v = maximum variable fluorescence

$\frac{F_v}{F_m}$ = ratio between variable fluorescence and maximal fluorescence

Statistical analysis

Data were analyzed with RStudio using two-way ANOVA, Barlett test of homogeneity of variances and Shapiro-Wilk normality test

Results

Root colonization

After analyzing the roots stained with 0.05% trypan blue in lactic acid under the microscope it was concluded that, for all the plants dipped in the inoculum with *Fusarium* sp., the colonization with *Fusarium* sp. was successful. And for all the plants where *Fusarium* sp. was not introduced there was no colonization.

Shoot growth

The greatest length of the shoots where measured nondestructively with a ruler as a measure for shoot growth to determine the effect that *Fusarium* sp. and 100mM NaCl has on the shoot growth of *P. maritima.* This growth curve was then expressed as a linear line (y= a + bx) without a fixed value (a) for the intersect. The different slopes (b) where analyzed using a Two-way ANOVA. This slope showed a significant negative effect of the salt treatment (p=0.040) and figure 1. shows a trend of a positive effect of *Fusarium* sp. with salt treatment compared to the salt only treatment.

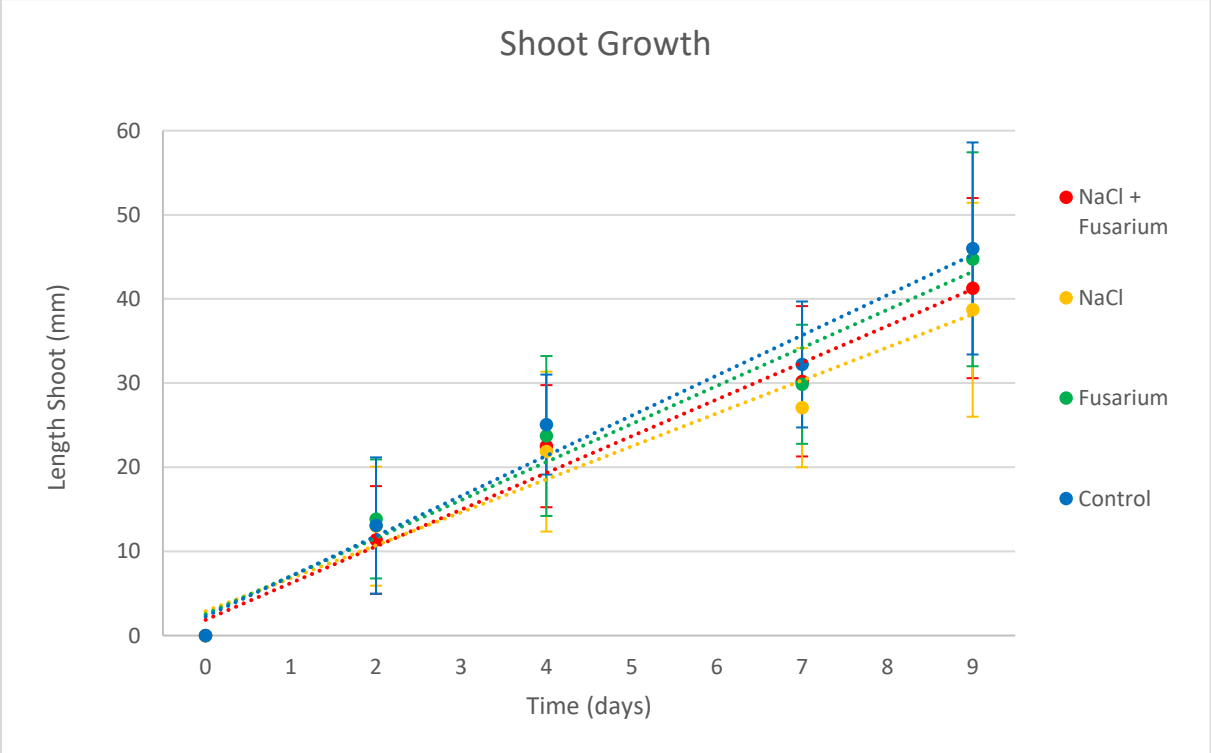

Figure 1. The effect of 100mM NaCl and *Fusarium* sp. treatment on the average shoot growth of *P. maritima.*

PAM

The F_v/F_m values that where measured with the PAM fluorometer where analyzed with a Two-way ANOVA. (fig 2.) this analysis show that *Fusarium* sp. Has a significant positive effect on the F_v/F_m ratio (p=0.0247) and NaCl shows a trend of a positive effect (p=0.06029).

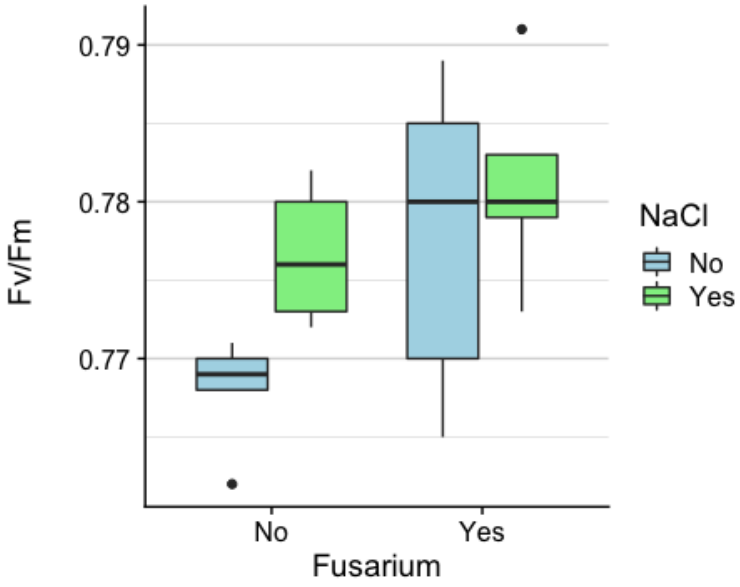

Figure 2. Effect of 100mM NaCl and *Fusarium* sp. treatment on F_v/F_m ratio in *P. maritima.*

Fresh weight

The total fresh weight where analyzed with a Two-way ANOVA. (fig 3.) this showed a significant Negative effect of 100mM NaCl treatment on the total fresh weight of *P. maritima* (=0.04235) and no effect of *Fusarium* sp. treatment (p=0.65536).

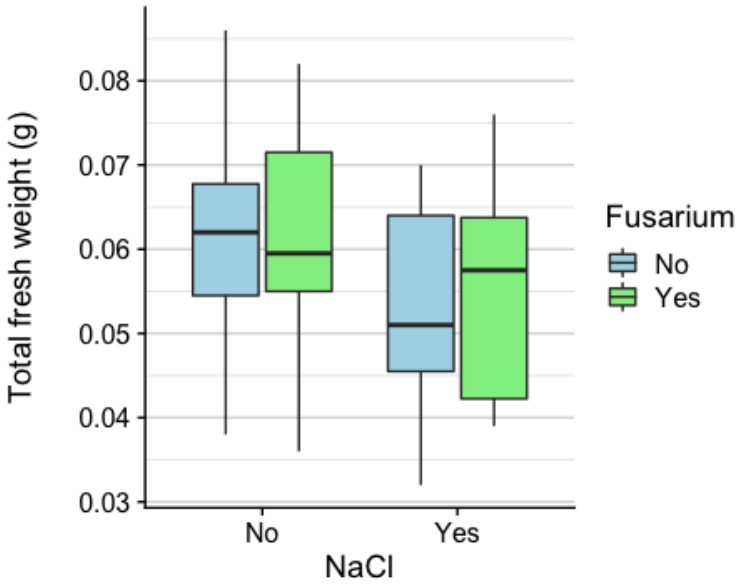

Figure 3. Effect of 100mM NaCl and *Fusarium* sp. treatment on total fresh weight of *P. maritima*

The roots fresh weight where analyzed with a Two-way ANOVA. (fig 4.) both *Fusarium* sp. and 100mM NaCl treatment did not show a significant effect (p= 0.8878 and p=0.5263 respectively).

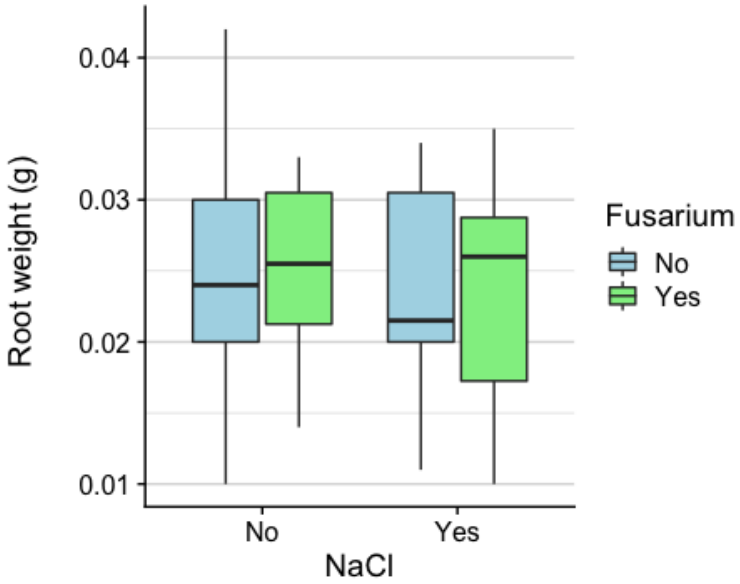

Figure 4. Effect of 100mM NaCl and *Fusarium* sp. treatment on fresh weight of the roots of *P. maritima*

Root length

The greatest length of the roots where measured with a ruler after harvest and analyzed with a Two-way ANOVA. (fig 3.) this showed a significant Negative effect of 100mM NaCl treatment on the greatest length of the roots of *P. maritima* (p= 0.000397) and no effect of *Fusarium* sp. treatment (p=0.570923).

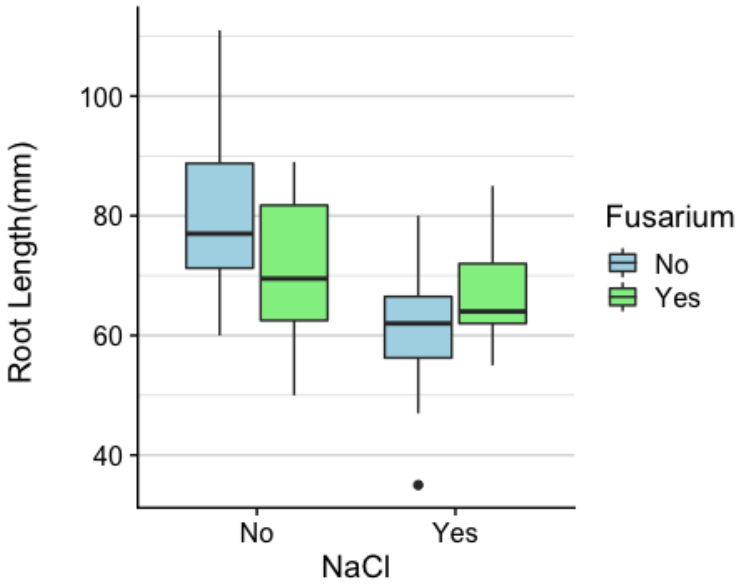

Figure 5. Effect of 100mM NaCl and *Fusarium* sp. treatment on root length of *P. maritima*

Root/Shoot ratio

The root length/shoot length ratio where analyzed with a Two-way ANOVA. (fig 6.) this showed a significant Negative effect of 100mM NaCl treatment on the root length/shoot length ratio of *P. maritima* (p=0.01337) and no effect of *Fusarium* sp. treatment (p= 0.72420).

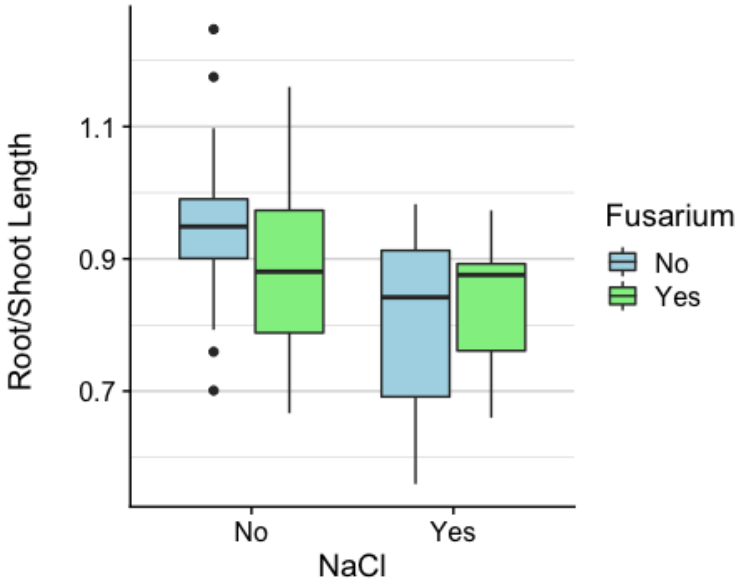

Figure 6. Effect of 100mM NaCl and *Fusarium* sp. treatment on root/shoot length ratio of *P. maritima*

The root weight/shoot weight ratio where analyzed with a Two-way ANOVA. (fig 7.) this showed a significant Positive effect of 100mM NaCl treatment on the root weight/shoot weight ratio of *P. maritima* (p=0.03909) and no effect of *Fusarium* sp. treatment (p=0.66745)

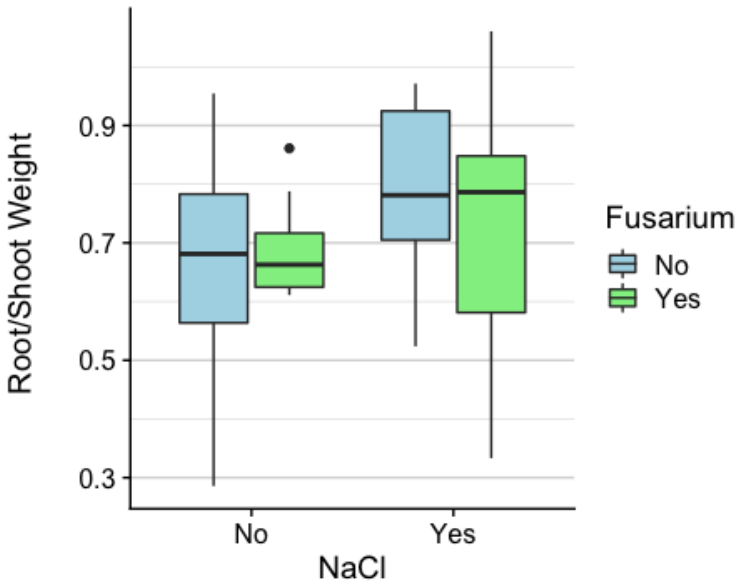

Figure 7. Effect of 100mM NaCl and *Fusarium* sp. treatment on root/shoot weight ratio of *P. maritima*

Suberin

Pictures taken with the fluorescent microscope of the root sections. The green/yellow colors in the pictures indicate suberin is present in that part of the root. This is a selection of pictures taken where pictures of the different treatments of the same species are placed next to each other. And pictures of the same treatments of the different species next to each other. No further measurement or statistical analyzes was performed on these pictures.

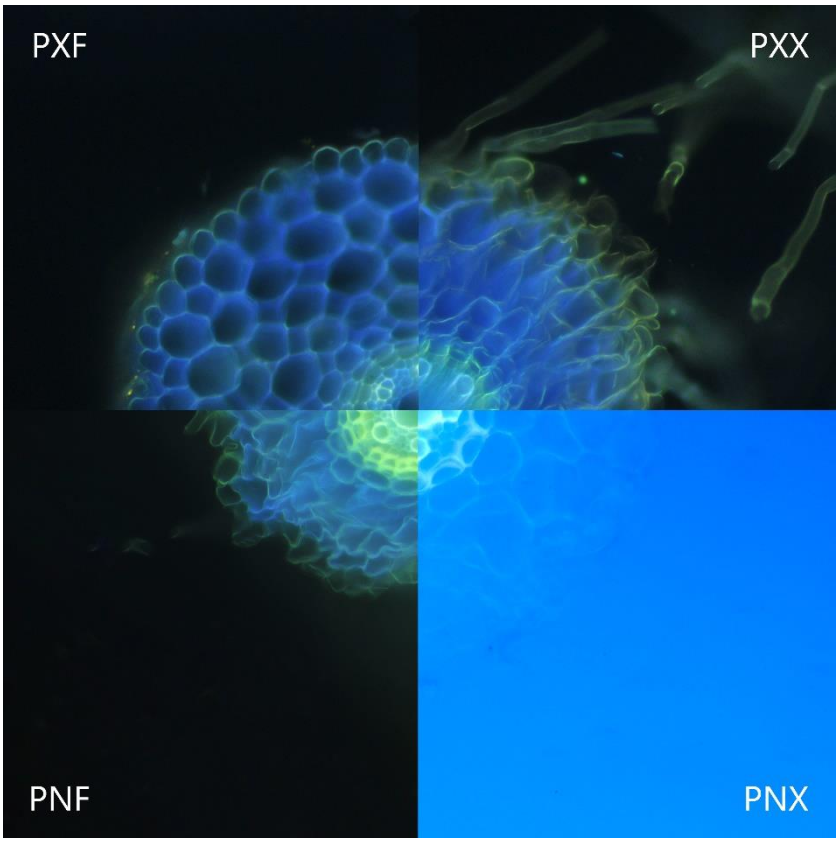

Figure 8. Root sections of *P. maritima* with the four treatments, NaCl + *Fusarium* sp. (NF), NaCl (NX), *Fusarium* sp. (XF) and control (XX).

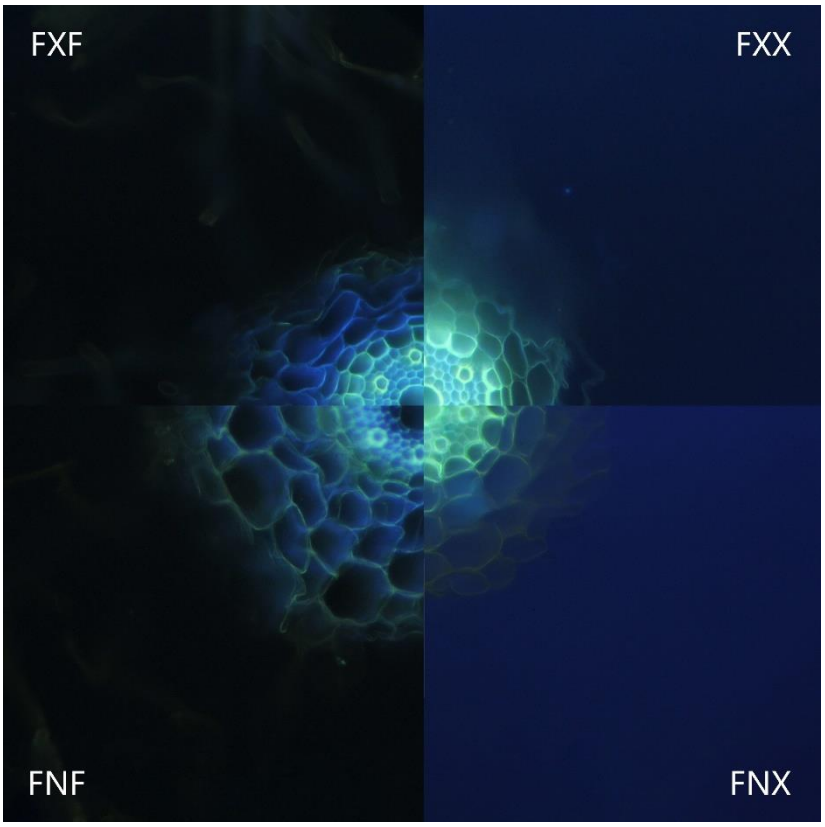

Figure 9. Root sections of *F. rubra* with the four treatments, NaCl + *Fusarium* sp. (NF), NaCl (NX), *Fusarium* sp. (XF) and control (XX).

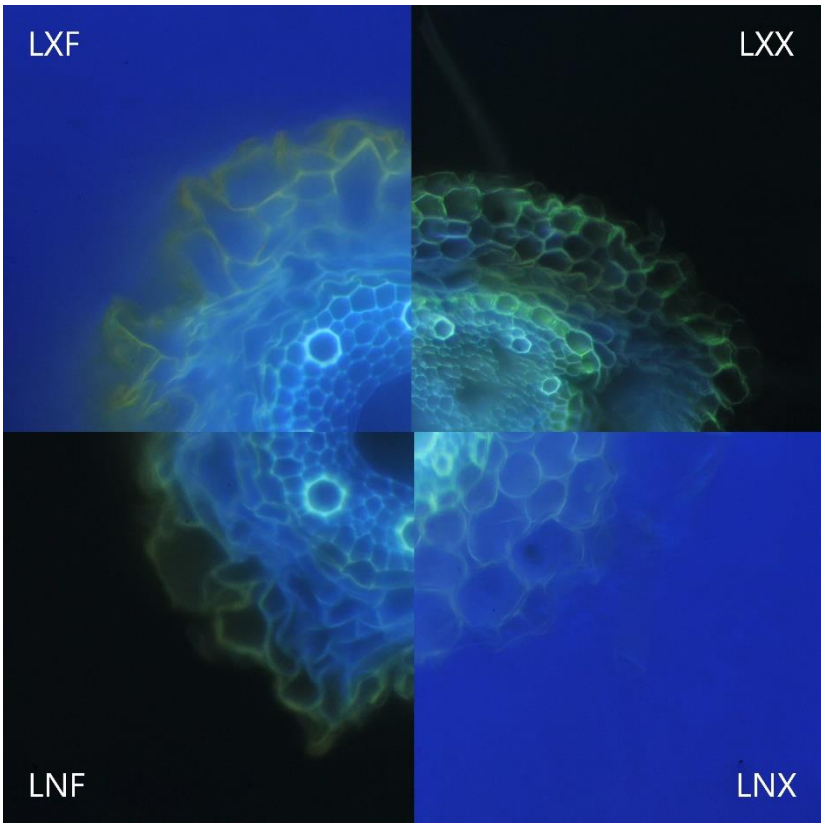

Figure 10. Root sections of *L. perenne* with the four treatments, NaCl + *Fusarium* sp. (NF), NaCl (NX), *Fusarium* sp. (XF) and control (XX).

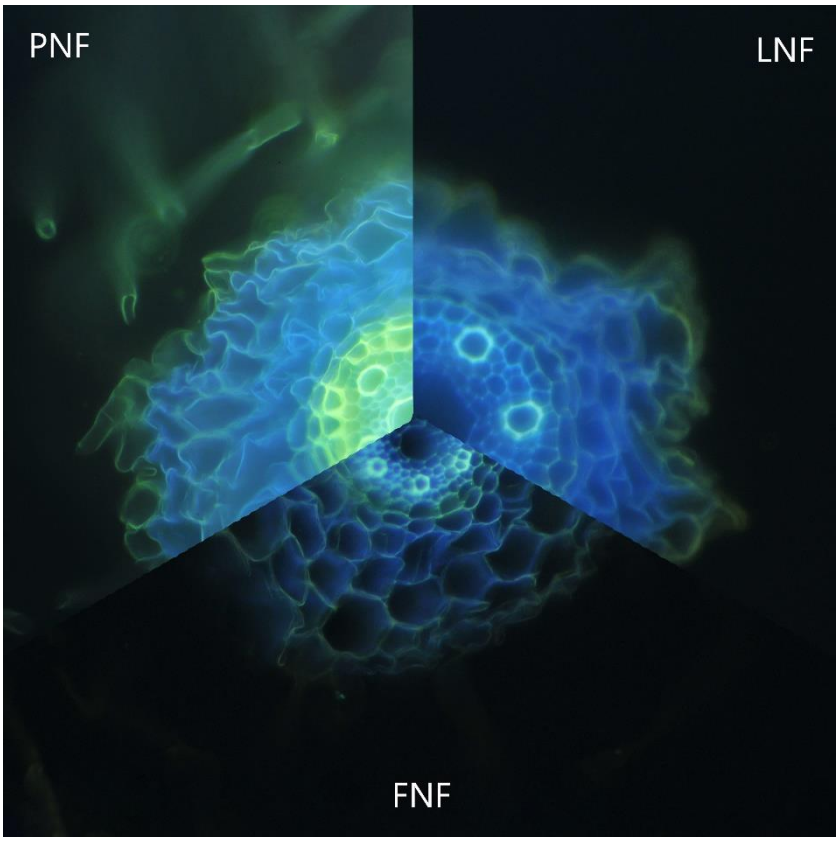

Figure 11. Root sections of *P. maritima*, *F. rubra* and *L. perenne* with treatment NaCl + *Fusarium* sp. (NF).

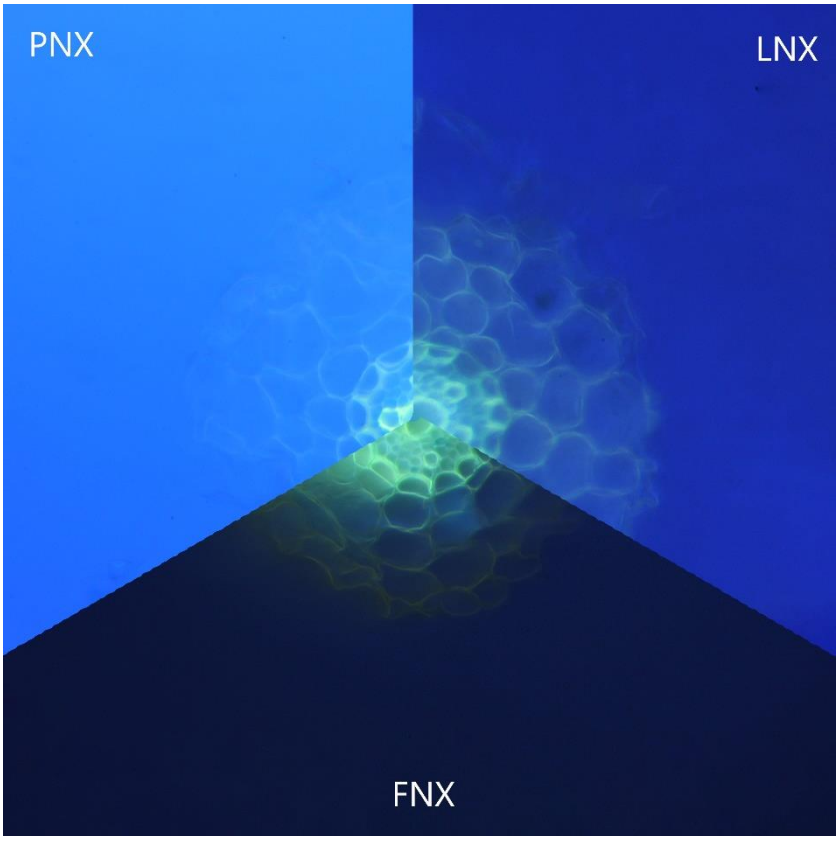

Figure 12. Root sections of *P. maritima*, *F. rubra* and *L. perenne* with treatment NaCl (NX).

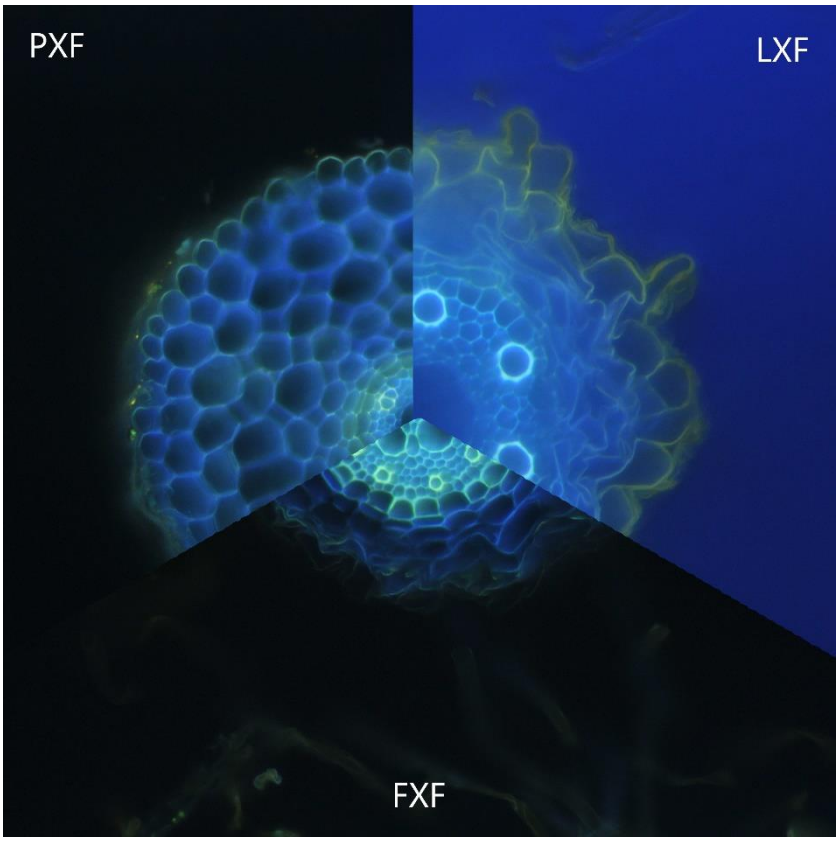

Figure 13. Root sections of *P. maritima*, *F. rubra* and *L. perenne* with treatment *Fusarium* sp. (XF).

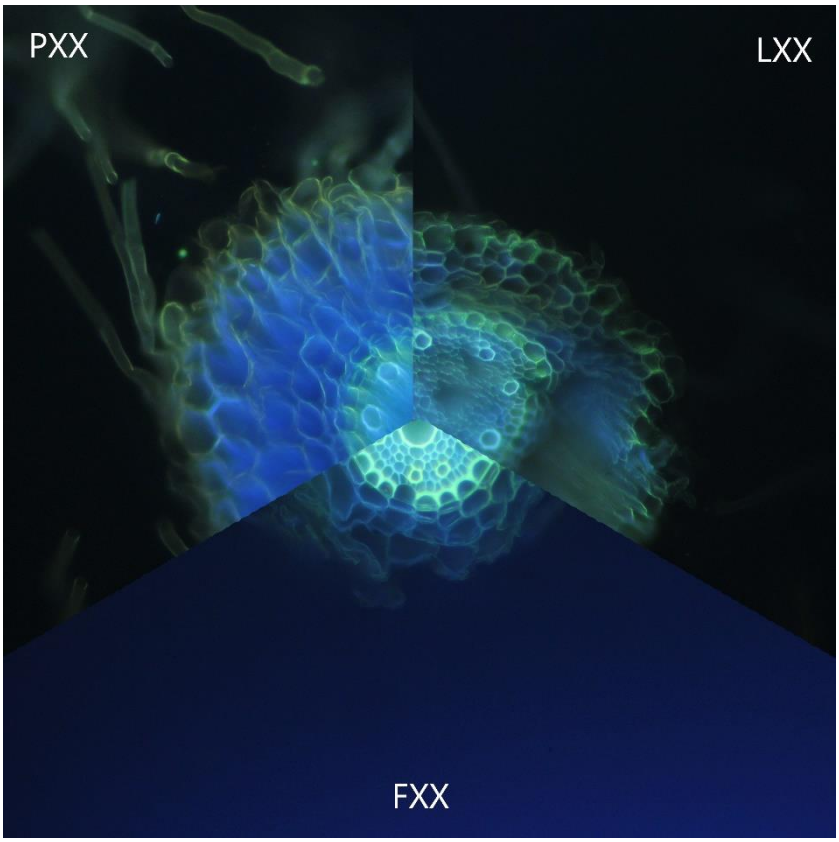

Figure 14. Root sections of *P. maritima*, *F. rubra* and *L. perenne* with treatment control (XX).

Discussion

Fusarium sp. is expected to have a slight negative effect in the grasses it colonizes as it is a parasite. However the changes it induces in the grasses root structure are expected to be beneficial for the grasses in saline environments.

We had a very small number of germinated plants of *F. rubra* and *L. perenne* so the results described and conclusions drawn in this discussion are on the basis of *P. maritima*.

Root colonization

To be able to say something about the effect of *Fusarium* sp. on the grasses we need to know if the fungus successfully colonized the grasses' roots. If not the effects seen couldn't be as result of *Fusarium* sp.. Wang found *Fusarium* sp. successfully colonize *F. rubra*. Expect is that *Fusarium* sp. will also successfully colonize *P. maritima*, *F. rubra* and *L. perenne* in this experiment.

Successful colonization of *Fusarium* sp. in the roots was found in all the species, *P. maritima*, *F. rubra* and *L. perenne*. This means that *Fusarium* sp. can not only successfully colonize *F. rubra*, as discovered by Wang (Wang, unpublished) but can also successfully colonize other grass species: *P. maritima* and *L. perenne*. This aligns with the expectations, the hypothesis can be accepted. Further findings in this experiment can be concluded as a result of *Fusarium* sp..

Shoot growth

As *Fusarium* sp. is a parasite we expect to see a small penalty on shoot growth in the grasses with *Fusarium* sp. compared to the control group without *Fusarium* sp.. On the soil where salt was added we expect the shoot growth to be the worst of all groups as saline soil has a negative effect on plant growth. However on this saline soil we expect *Fusarium* sp. to have a positive effect on the shoot growth as it induces changes in the plant that help it cope better with saline stress.

The shoot growth data (fig. 1) show that salt has a significant negative effect on shoot growth. The effects of Fusarium sp. on the shoot growth were not found to be significant, however, two trends can be seen:

1. *Fusarium* sp. has a negative effect on the shoot growth compared to the control group.

2. *Fusarium* sp. has a positive effect on the shoot growth compared to the group without *Fusarium* sp. when both groups are in saline environments.

This means that salt has a very negative effect on the shoot growth and suggests that in a non-saline environment *Fusarium* sp. has a small negative effect on the shoot growth of *P.* maritima. However, in saline environments, it suggests that *Fusarium* sp. reduces the negative effect that the salt has on the shoot growth.

While the results not all being statistically significant a trend that shows is in line with what we expected. How come *Fusarium* sp. has the effect we see? What are the changes *Fusarium* sp. induces that explain these results?

PAM

From the PAM results we expected to see the same image as seen in the shoot growth result. So the control group would have the best performance and the *Fusarium* sp. only group have a little worse performance. The groups on saline soil would have the worst performance as sign of salinity stress where the one with *Fusarium* sp. performed a bit better as it should help protect against salinity stress.

The fluorescence data (fig. 2) show that there is a significant positive effect of *Fusarium* sp. and suggest a positive effect of salt on the Fv/Fm ratio of *P. maritima*. Meaning the plants in these groups were the least stressed.

However, it's considered that above a Fv/Fm ratio of 0.7 a plant is healthy and not stressed. And bellow a Fv/Fm ratio of 0.7 a plant is considered stressed.

With this in mind, we can consider all the groups not stressed at all. And even though there was a statistically significant difference between the groups, the differences in values are all very small on a relatively big scale and are negligible (fig. 15).

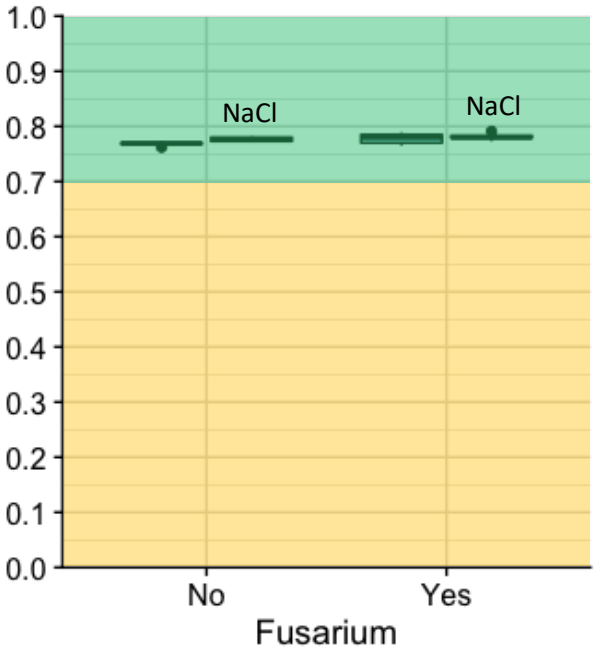

Figure 15. Effect of 100mM NaCl and *Fusarium* sp. treatment on F_v/F_m ratio in *P. maritima* with 0.7 threshold marking.

This is not in line with our expectations. Expected is that plants will have more stress in saline environments. So one could argue about the validity of this experiment as according to the Fv/Fm ratio all of these plants didn't grow under stress conditions. So how could *Fusarium* sp. potentially protect *P*. maritima against stress that wasn't there? Increasing the salt concentration for *P*. maritima could lead to actual noticeable stress and result in a more valid experiment.

Another explanation for these results is that the Fv/Fm ratio is not affected by salinity stress. That the photosystems don't feel a penalty from growing in saline environment and work as in normal circumstances. Then what is different in the plants with *Fusarium* sp. compared to the ones without that explains the shoot growth results?

Root and shoot performance

Fresh weight

As the shoot length is longest in the control group and shortest on saline soil we expect the fresh weight to be biggest in the control group and smallest on the saline soil. Here also we expect the *Fusarium* sp. to have a little negative effect on the fresh weight however on the saline soil we expect it to have a less negative effect then the salt only group.

Salt has a significant negative effect on the total fresh weight and *Fusarium* sp. does not affect the total fresh weight of *P*. maritima (fig. 3). Both *Fusarium* sp. and salt do not affect the fresh weight of the roots of *P*. maritima (fig. 4).

In saline environments, *P*. maritima has less total fresh weight and no change in root fresh weight, meaning it has less shoot fresh weight ($p=0.001412$). So as expected saline environment has a negative effect on the total fresh weight, however *Fusarium* sp. doesn't have any effect on the total fresh weight. Interesting is that the root fresh weight is similar between the different treatments. This suggests plants grown on saline soil have more roots relative to the plants on normal soil.

Root length

As described above we expect plants grown on saline soil to have relative more roots then plant grown on normal soil, *Fusarium* sp. doesn't have an effect on the roots weight so we don't expect it to have on its length.

Salt has a significant negative effect on the root length and *Fusarium* sp. does not affect the root length of *P*. maritima (fig. 5).

In saline environments, *P*. maritima has shorter roots. This is not what we expected as the weight of the roots was higher in saline environment relative to normal environments. So if the roots are shorter but the weight is relatively higher, the roots should be thicker in saline environments. As expected *Fusarium* sp. did not have any effect on the root length.

Root/shoot ratio

With the results seen above we expect the root/shoot length ratio to be negatively affected by saline environment and the root/shoot weight ratio to be negatively affected. *Fusarium* sp. shouldn't have any effect on both root/shoor ratios.

Salt has a significant negative effect on the root/shoot length ratio and *Fusarium* sp. does not affect the root/shoot length ratio of *P*. maritima (fig. 6). Salt has a significant positive effect on the root/shoot weight ratio and *Fusarium* sp. does not affect the root/shoot weight ratio of *P*. maritima (fig. 7).

As expected *P*. maritima has relatively shorter but heavier roots in saline environments. Meaning the roots get thicker. *Fusarium* sp. does not have an effect on the root/shoot ratio.

In reaction to NaCl, *P*. maritima gets thicker but shorter roots. This adaptation has a negative effect on the shoots. *P*. maritima allocates its biomass into the root system as a result of salinity stress. No significant effect of *Fusarium* sp. has been observed in this part of the experiment.

Suberin

As observed in previous part of the experiment we see that on saline soils the roots of the plants get thicker. We expect the roots get thicker as a result of a thicker suberin layer as a form of protection against salinity stress. Originally we expected this to be induced by *Fusarium* sp., however in the previous part of the experiment we couldn't find any significant prove to expect this. However we might still see a thicker suberin layer in the roots with *Fusarium* sp. in this part of the experiment.

In figures 8, 9, and 10 you see root sections of *P*. maritima, *F*. rubra, and *L*. perenne respectively in which each different treatment is shown. There is no visual difference in suberin when comparing the different treatments in the same species. Following the hypothesis it was expected to see a thicker suberin layer in the treatments with saline soil and also the ones with *Fusarium* sp.. As it was expected that *Fusarium* sp. would induce a thicker suberin layer in the roots as a form of protection against salinity stress. This hypothesis is rejected.

However there is something interesting we observed. In figures 11, 12, 13, and 14 you see the treatments NaCl + *Fusarium* sp. (NF), NaCl (NX), *Fusarium* sp. (XF), and control (XX) respectively in which each of the three grass species is shown. There is a visual difference in suberin and overall root structure when comparing the different species within the same treatment. There seems to be a difference in root structure between the species. So we can conclude that the different species have independently of their environment a different root structure building plan. This might also explain why in general one species (*P*. maritima) performs better in saline environments than the other (*L*. perenne) as its physiology is better adapted to these saline environments.

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