Are monocot C_4 plants able to utilize H_2S as a sulfur source?

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Sulfur assimilation is an essential pathway for the plant, primarily for the metabolism of sulfate providing cysteine for methionine and protein synthesis. It also functions as a source of reduced sulfur for the synthesis of numerous other essential metabolites. The enzymes catalyzing the first two steps of this pathway are confined to bundle sheath cells in several C_4 monocot species but are present in all mesophyll cells of C_3 species. C_3 species have been shown to be able to assimilate H_2S as a viable sulfur source. Knowing this difference in the sulfur assimilation pathway we tried to show if a C_4 plant is able to utilize H_2S as a viable sulfur source. We fumigated *Zea* mais with 0 or 0.5 µl l^{-1} H_2S and provided the plant with a nutrient solution with or without sulfur. During this treatment we monitored the biomass, amino acid amount, thiols and anions for the plants. The findings show that C_4 plants are able to utilize H_2S as a viable sulfur source and can even grow on H_2S as the sole sulfur source.

Sulfur is an essential macro-nutrient for a plant used for its proper growth and functioning. Sulfur is only present in minor quantities in the plant ranging from 0.1 to 6% of the dry weight(De Kok A.; Durenkamp, M.M; Stuiver, C.E.; Westerman, S.; Yang, L.; Stulen, I., 2002). The major sulfur source for the plant is sulfate and this is mainly taken up by the roots(Hawkesford and De Kok, 2006). Before the plant can assimilate sulfur, the sulfate which is taken up by the roots, must be reduced to sulfide. This reduction and assimilation pathway is highly coordinated and regulated and is specific for each plant species to require the exact amount of sulfur required for growth(De Kok A.; Durenkamp, M.M; Stuiver, C.E.; Westerman, S.; Yang, L.; Stulen, I., 2002; Hawkesford and De Kok, 2006).

When the sulfate is taken up by the roots it is stored in the vacuoles or assimilated trough the assimilatory reduction pathway. This pathway first converts the sulfate to adenosine 5'-phosphosultate (APS) using the ATP sulfurylase enzyme. The newly formed APS is subsequently reduced by the enzyme APS reductase to sulfite which in turn is reduced to sulfide using sulfite reductase. The sulfide can be incorporated in the amino acid cysteine using a reaction with *O*-acetylserine (OAS) using the catalyst *O*-acetylserine(thiol)lyase(De Kok A.; Durenkamp, M.M; Stuiver, C.E.; Westerman, S.; Yang, L.; Stulen, I., 2002; Hawkesford and De Kok, 2006; Hell, 1997; Kopriva, 2017; Saito, 2004; Takahashi et al., 2011). This process takes place in the plastids of the plant cells of both the shoots and the roots.

In addition to sulfate, plants are able to utilize atmospheric sulfur gasses like H_2S and SO_2 as a sulfur source. The sulfur gasses are taken up by the leafs and H_2S is directly metabolized to cysteine and SO_2 will be dissolved in the water of the cells and formed in to sulfite(Buchner, 2004; De Kok et al., 1997; Koralewska et al., 2008; Westerman et al., 2001). It has even be observed that plants can solely grow on atmospheric sulfur sources and grow healthy without pedospheric sulfur sources like sulfate(Stuiver and De Kok, 2001; Westerman et al., 2001).

Most of this research has been done on C_3 plants and found that all the cells in the mesophyll of the leaf are able to reduce and assimilate sulfur. However, C_4 plants differ from C_3 plants in metabolic pathways due to their difference in leaf makeup. The C_4 plants differ in leaf makeup by having the Kranz anatomy. This means that the bundle sheath cells, the cells around the vascular tissue, are very large. These bundle sheath cells have special metabolic features on carbon and nitrogen assimilation and also on the sulfur

assimilation(Burnell, 1984; Kopriva and Koprivova, 2005; Koprivova et al., 2001; Schmutz and Brunold, 1984).

It has been observed that the bundle sheath cells of monocot C_4 plants have a pivotal role in sulfur metabolism. Up until the formation of cysteine the sulfate reduction and assimilation of these plants exclusively takes place in the bundle sheath cells. After the cysteine is formed, the mesophyll cells have a role in making further products from the cysteine(Kopriva and Koprivova, 2005; Weckopp and Kopriva, 2014). This means that findings on the regulation of sulfur metabolism in C_3 plants cannot simply be extrapolated to monocot C_4 plants.

Hydrogen sulfide (H_2S) is quickly metabolized directly into cysteine after it is taken up from the atmosphere by the leaf stomata. This assimilation process can start directly in the mesophyll cells of the C_3 plants. Due to the difference in leaf makeup and spatial separation of the sulfur assimilation the C_4 plants need to transport the H_2S to the bundle sheath cells before the assimilation can begin. This initial transport to the bundle sheath cells could impact the effectiveness of the H_2S possibly to a point where H_2S is not viable sulfur source for the plant. Therefore, in this research project we are looking at the possibility for monocot C4 plants to utilize H2S as a sulfur source with Zea *mais* as a model.

Materials and methods

Plant material and growth conditions.

Zea *mays* were germinated on filtration paper and transferred to aerated aquaculture containers with tap water in a climate-controlled room. 10-day-old seedlings were transferred to an aerated 25% Hoagland nutrient solution at 0 mM sulfate (-S, sulfate-deprived; were the sulfate salt are replaced chloride salts) or 0.5 mM sulfate (+S, sulfate-sufficient)

During the seed-removal experiment half the plants had their seed removed and were grown for 13 days. The other half was grown as normal for 13 days. There were 4 containers, each with 18 sets of plants, 4 plants per set.

During the fumigation experiment *t*he plants were grown in 4 containers (12 sets of plants per container, 4 plants per set) in climate-controlled fumigation cabinets for 12 days and fumigated with 0 or 0.5 µl l^{-1} H_2S. These where 150 L cylindrical stainless-steel cabinets (0.6 m diameter) with a polymethyl methacrylate top. Sealing of the lid of the containers and plant sets prevented absorption of atmospheric H_2S by the solution. Day and night temperatures were respectively 24 and 20°C (±2°C), and relative humidity was 40–50%. The photoperiod was 14 h at a photon fluence rate of 300 ± 20 µmol m^{-2} s^{-1} (400–700 nm) at plant height.

Pigment content

Shoots were homogenized in 98% ethanol using an Ultra Turrax (10 mL g^{-1} fresh weight) and centrifuged at 800*g* for 20 min. The chlorophyll *a*, *b* and carotenoid contents were determined according to Lichtenthaler(Lichtenthaler, 1987).

Nitrate and sulfate content

Frozen material was homogenized in demineralized water (10 mL g^{-1} fresh weight) with an Ultra Turrax for 30 s at 0 °C. The homogenate was filtered through one layer of Miracloth and incubated at 100 °C in a water bath for 10 min. The filtrate was centrifuged at 30,000*g* for 15 min at 0 °C. The anions were separated by HPLC on an Ionosphere A anion exchange column (250×4.6 mm) and determined refractometrically according to Maas *et* al.(Maas et al., 1986) using a Knauer differential refractometer. 25 mM potassium biphthalate (pH 4.3) containing 0.02% NaN_3 (w/v) was used as a mobile phase. The HPLC apparatus consisted of a Separations high precision pump with a Rheodyne sample injector (loop volume 20 ml). The flow rate was 1 mL min^{-1} and the detector temperature was kept at 25 °C by a water bath. Peak analysis was performed with a Shimadzu Chromatopac C-R8A data processor.

Water-soluble non-protein thiols

For analysis of the water-soluble non-protein thiols, fresh plant material was homogenized in an extraction medium containing 80 mM sulfosalicylic acid, 1 mM EDTA, and 0.15% (w/v) ascorbic acid with an Ultra Turrax at 0 °C (10 mL g^{-1} fresh weight). Oxygen was removed from the solution by aspiration with N_2. The homogenate was filtered through one layer of Miracloth and the filtrate was centrifuged at 30,000*g* for 15 min at 0 °C. Total water-soluble non-protein thiol content was determined colorimetrically at 413 nm after reaction with 5,5′-dithiobis[2-nitrobenzoic acid] according to de Kok *et* al.(De Kok et al., 1988).

Amino acid content

The amino acids were extracted and deproteinized. The amino acid content for nitrate and sulfate was determined with the ninhydrin color reagent according to Rosen(Rosen, 1957) and measured colorimetrically at 578 nm on a spectrophotometer

Statistical analysis

Data from different experimental sets ware analyzed for statistical significance using an unpaired two-tailed *t*-test ($P < 0.01$).

Results

Seed removal experiment

Because the seed is the first source of nutrients for the plant, it was decided to remove the seed from the plant after germination. A small-scale experiment was set up to verify that this had the expected effect and that the removal of the seed does not impact the development of the plant.

Removing the seed had an impact on the plants after only 5 days. The plants with removed seeds showed a lagging growth where the S-group without seeds were already significantly lagging their growth compared to the groups with their seeds still attached (Fig. 1). The plants without seeds growing in a sulfurous medium showed a lagging growth but not significantly different from any group. The shoot to root ratios were the same over the four groups.

On day 9 and 13 the results were similar. The biomass production on the control group was higher compared to the other groups but only a significant difference in biomass production could be found between the control group and the sulfur deprived group with seed removed. The other two groups showed a similar growth that did not deviate significantly from any group.

However, when looking at the shoot to root ratio there was a gap created between the groups. The plants growing on the 25% Hoagland with 0.5 mM sulfate kept the same shoot to root ratio either with or without seeds attached. The plants growing on the 0 mM sulfate medium also had a comparable shoot to root ratio but is different from the other two groups.

The chlorophyll content shown in Fig. 2 shows that there was an overall decline in total chlorophyll content in these plants. However, there was no difference between the experimental groups nor in the total amount of chlorophyll nor in the chlorophyll A/B ratio. Despite that there was no difference in the chlorophyll content found between these groups there was a slight indication on day 13 that the groups on the 0 mM sulfate medium might be lagging compared to the other groups

The results of amino acids in the root and shoot give a clear indication that something was going on between the treatments and that the groups with the normal Hoagland medium had similar results and that there was an accumulation of amino acids in the sulfur deprived groups. However, the lack of significance in these differences make it impossible to make a clear statement on what these results show and mean (Fig. 3). However, the significant differences in amino acid content in the shoot on day 13 where the deprived sulfur groups showed statistically that there was an accumulation.

Fumigation experiment

Exposing *Zea mais* to H_2S had no effect in its biomass production for the first week but it did have an effect on the shoot to root ratio. Where the groups with the 0.5 mM sulfate 25% Hoagland had comparable S/R ratios, a difference occurred between the 0 mM sulfate medium groups. The group that had no sulfur source accessible (S-) had a slight decreased S/R ratio and the group with only atmospheric sulfur (S- + H2S) had a slight increased S/R ratio. Both did not greatly differ from the control group (S+) but they did from each other (Fig. 4).

By day 10 the sulfur deprived group started to show a clearly lagging growth especially compared to the groups that had access to 0.5 µl l^{-1} H_2S. By day 12 the plants with an atmospheric sulfur source and the control group all had a similar biomass production and the sulfur deprived group had a significant lagging biomass production.

The same pattern that the sulfur deprived group showed for its biomass can also be found in the shoot to root ratio. Although the S/R ratios of the other groups were similar on day 10 this was changed by day 12. The plants that only growed on atmospheric sulfur started to show a lower S/R ratio and the group with both sulfur sources a higher ratio. These changes in shoot to root ratio differed significantly from each other but not from the control group.

The amino acid content already showed clear results from day 7 (Fig. 5). The control group and the plants fumigated with 0.5 µl l^{-1} H_2S had a comparable concentration of amino acids ranging from 46 µmol/g fresh weight to 55 µmol/g fresh weight on day 7, 24 to 32 µmol/g fresh weight on day 10 and 10 to 24 µmol/g fresh weight on day 12 for the shoot. If this was compared to the amount of amino acid measured in the sulfur deprived group an accumulation could be found of 4, 8 and 25 times the amount found in the other groups respectively.

The same could be found in the root measurements although the spread between the control and fumigated groups was greater, it was not significant. The amount of amino acids measured in the root of the sulfur deprived group was way greater than the rest of the groups to an approximate increase of 4 to 15 times the amount measured in the roots of the other groups.

The concentration of water-soluble non-protein thiols measured on day 11 showed again a significant variation between the sulfur deprived plants and the rest of the experimental groups. Interestingly the plants with only the H_2S gas as a sulfur source had a comparable SH concentration with the control group and the plants with both sulfur sources did not. The plants with access to sulfate and H_2S gas had an increased SH concentration compared to the control group.

The amount of sulfate measured in the plant had a clear divide for the root, and on a smaller scale for the shoot, between the plants that had access to sulfate via the Hoagland medium and the plants that did not had access to sulfate (Fig. 7). This divide was for the root very clear on day 7 and reasonably clear on day 10. On day 12 however the control group seemed to decline in its sulfate concentration but the group with both sulfur sources kept the sulfate concentration on the same level.

For the shoot the same divide could be found but it is less clear compared to the root measurements. The S+ +H2S group had a slight increase in sulfate concentration compared to the control but never significant. The sulfur deprived group had the lowest amount of sulfate on all the measurement days but stayed overall the same. There was not even a statistical deviation between the amounts measured on the different days for the S- group. The nitrate concentration showed very different results. The nitrate concentration in all experimental groups was approximately the same over all days and even for the root and the shoot.

Seed-removal experiment

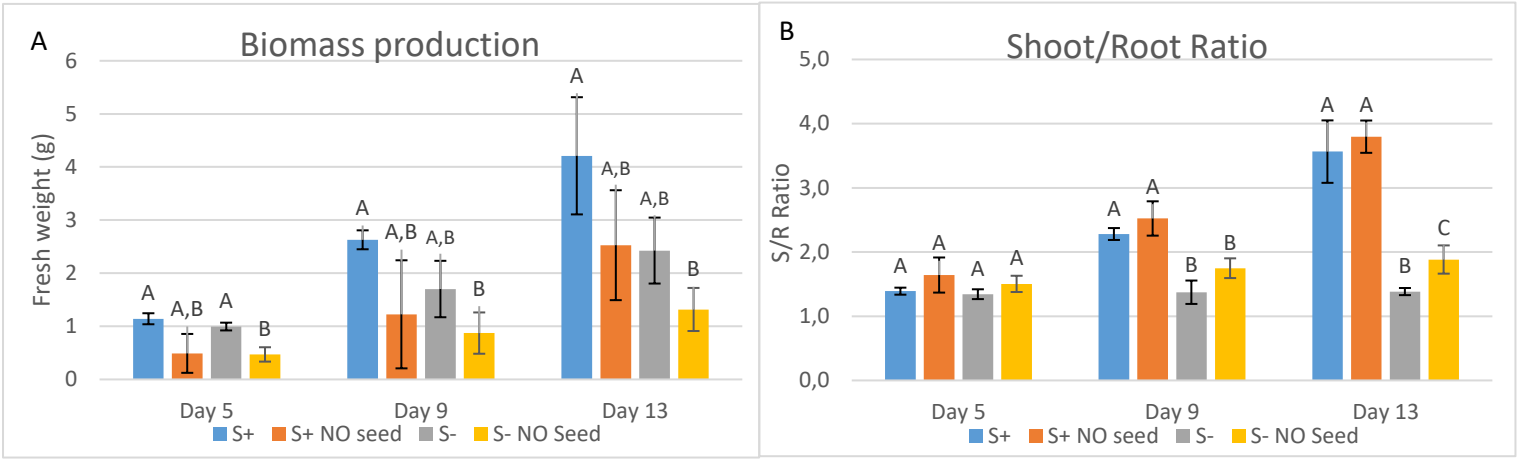

Fig. 1| The impact of seed removal and sulfur deprivation on biomass production of Zea *mais.* Ten-day-old seedlings were grown on an aerated 25% Hoagland solution at 0 (S-) or 0.5 mM sulfate (S+) for 5, 9 of 13 days. The initial weights of the plant were 0.37±0.19 g. The data on biomass production and shoot/root biomass ratio represents the mean of 4 measurements with 4 plants in each (±SD). Different letters indicate significant differences between treatments (P<0.01 student's *t*-test) for each day.

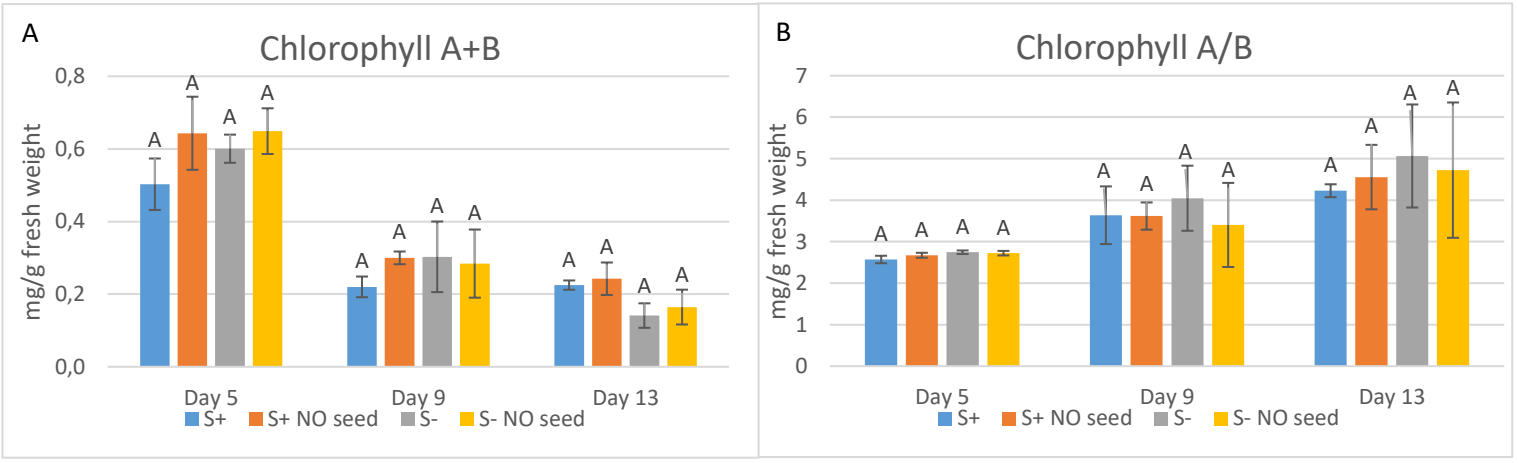

Fig. 2| The impact of seed removal and sulfur deprivation on chlorophyll content of Zea *mais*. For experimental details, see legends of Fig. 1. The data represents the mean of 3 measurements with 2 to 4 plants in each (±SD). Different letters indicate significant differences between treatments (P<0.01 student's t-test) for each day.

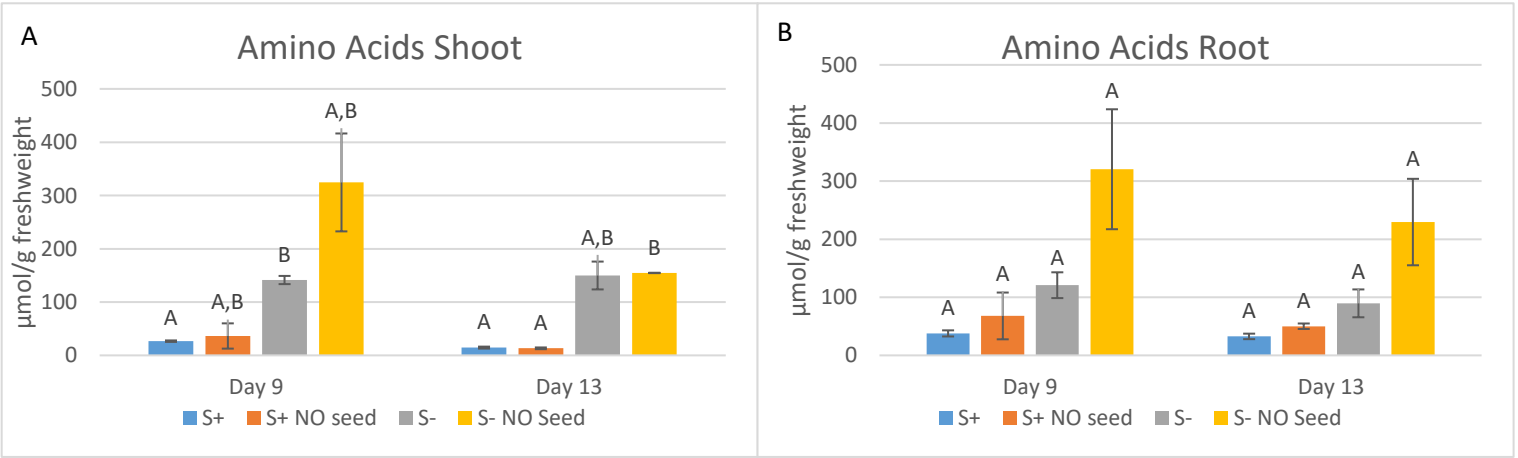

Fig. 3| The impact of seed removal and sulfur deprivation on the amino acid content of Zea *mais*. For experimental details, see legends of Fig. 1. The data represents the mean of 2 measurements with 2 to 4 plants in each (±SD). Different letters indicate significant differences between treatments (P<0.01 student's t-test) for each day.

Fumigation experiment

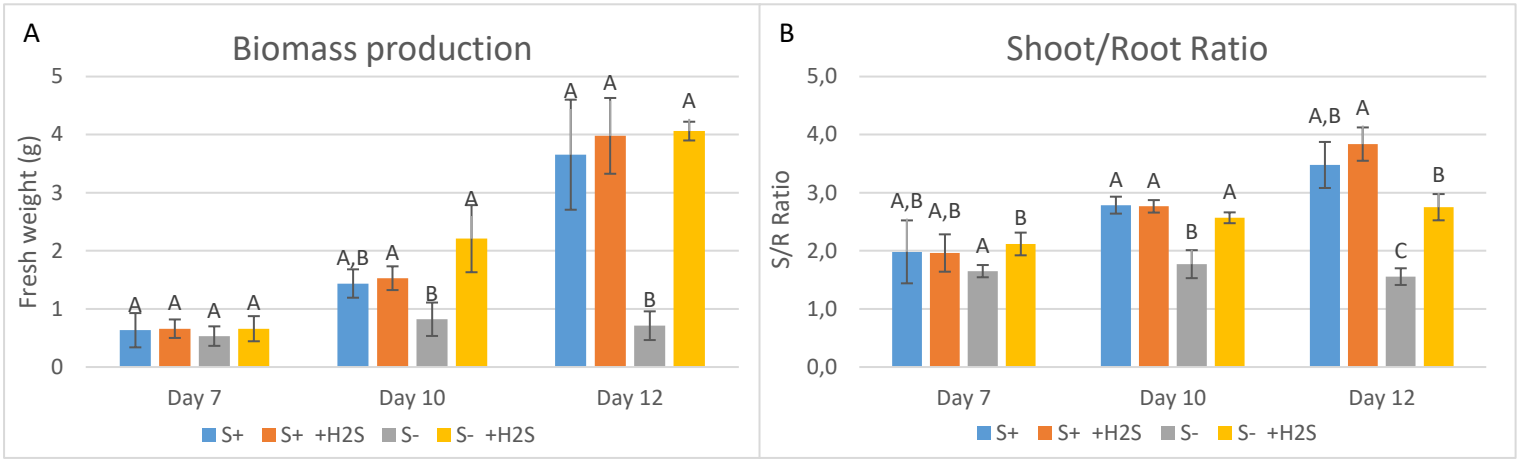

Fig. 4| The impact of H_2S and sulfate deprivation on biomass production of Zea *mais*. Ten-day-old seedlings were grown on an aerated 25% Hoagland solution at 0 (S-) or 0.5 mM sulfate (S+) and exposed to 0 or 0.5 µl l^{-1} H_2S (+H2S) for 7, 10 or 12 days. The initial weights of the plant was 0.32±0.10 g. The data on biomass production and shoot/root biomass ratio represents the mean of 2 experiments with 4 measurements and 4 plants in each (±SD). Different letters indicate significant differences between treatments (P<0.01 student's *t*-test) for each day.

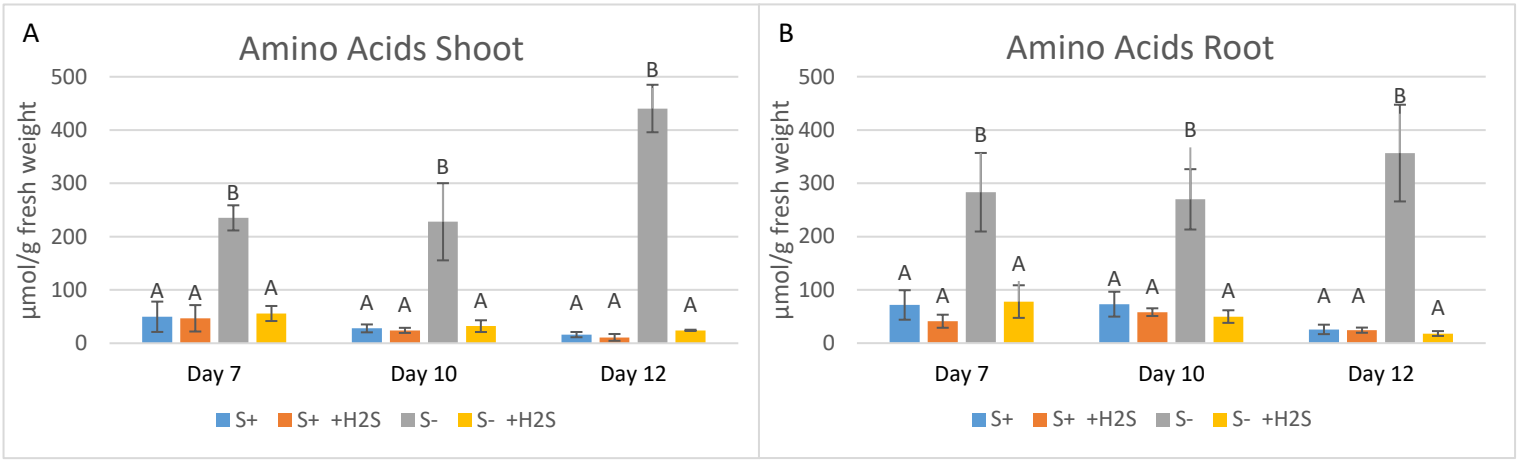

Fig. 5| The impact of H_2S and sulfate deprivation on the amino acid content of Zea *mais*. For experimental details, see legends of Fig. 4. The data represents the mean of 2 experiments with 3 measurements and 2 to 4 plants in each (±SD). Different letters indicate significant differences between treatments (P<0.01 student's t-test) for each day.

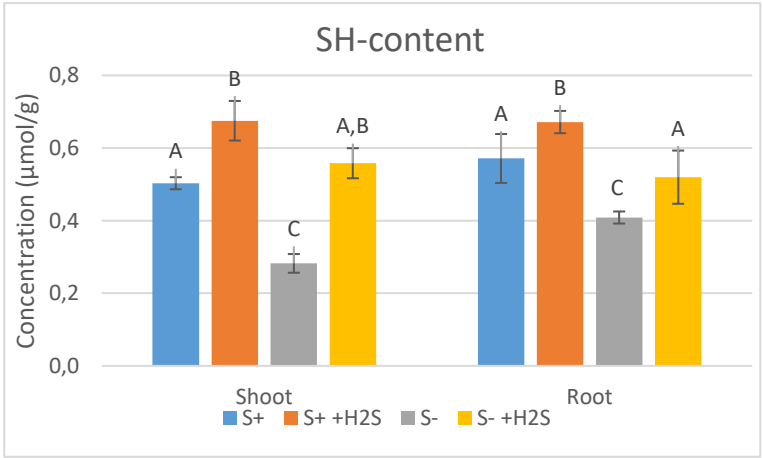

Fig. 6| The impact of H_2S and sulfate deprivation on water-soluble non-protein thiol content of shoot and root of Zea *mais*. For experimental details, see legends of Fig. 4. Samples for the SH-essay were taken on day 11. The data represents the mean of 2 experiments with 3 measurements and 2 to 4 plants in each (±SD). Different letters indicate significant differences between treatments (P<0.01 student's t-test) for root or shoot.

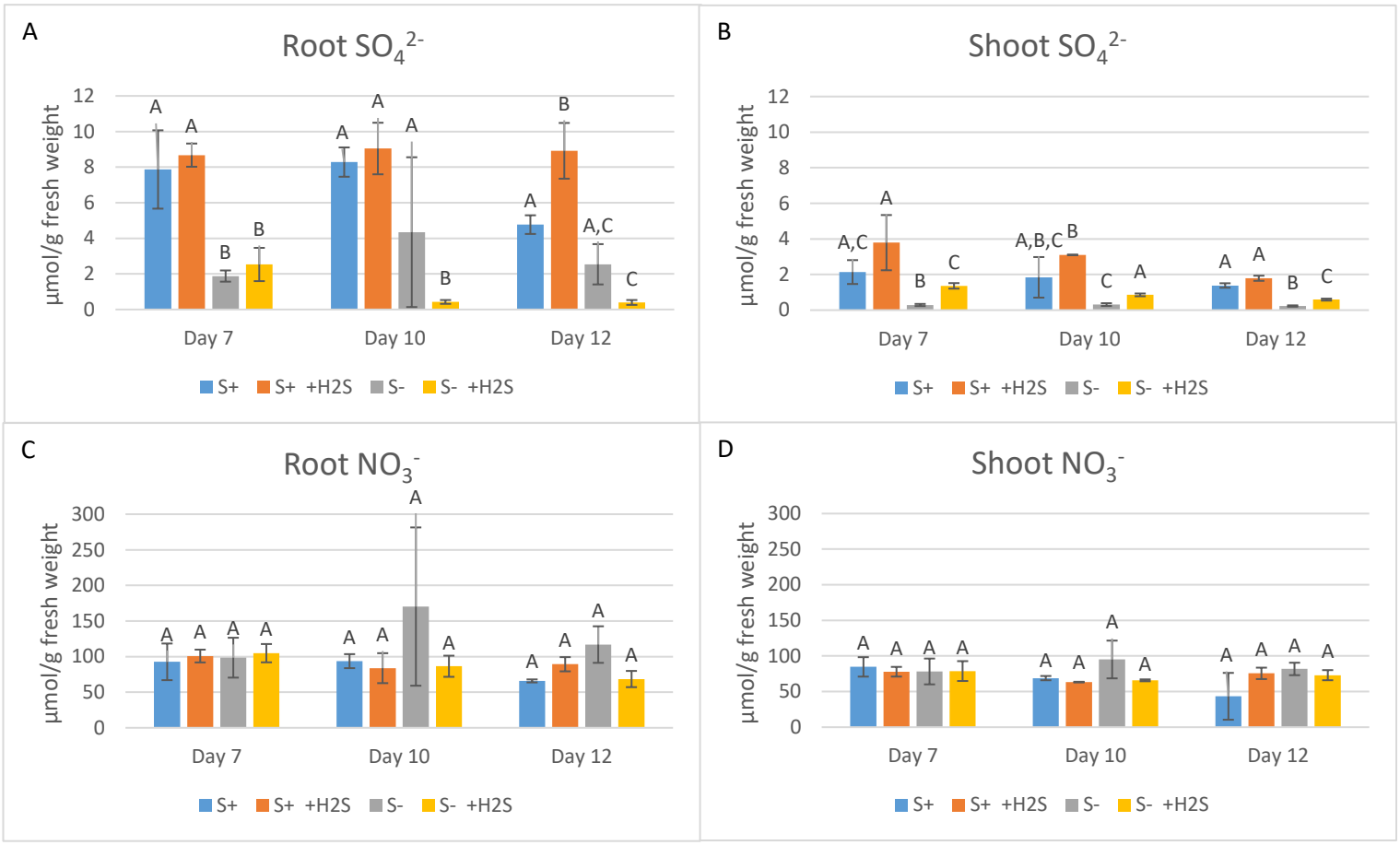

Fig. 7| The impact of H_2S and sulfate deprivation on the nitrate and sulfate content of shoot and root of Zea *mais*. For experimental details, see legends of Fig. 4. The data represents the mean of 2 experiments with 3 measurements and 2 to 4 plants in each (±SD). Different letters indicate significant differences between treatments (P<0.01 student's t-test) for each day.

Discussion

Removing the seed from a 10-day old seedling has an impact on the growth of a plant. This can be seen in Fig.1 where the biomass production is lagging compared to the control group (S+). This has also been shown by Cooper and MacDonald (Cooper and MacDonald, 1970). From their results it can be concluded that the first ten days are necessary for the plant to have the seedling for the first development and growth. After these first days the seed still has a role as nutrient source, but the plant can manage to supply its own energy.

The impact of the removal of the seed on the plant could be called minimal despite the lagging growth of the group with no seed growing on normal Hoagland solution (S+ NO Seed) compared to the control group. This because the Shoot to Root ratios between these two groups are similar which means a similar growth and health.

The high concentration of amino acids in the sulfur deprived groups indicate an accumulation of amino acids. This accumulation is most likely the result of a lack of cysteine in the plant. Where the plant cannot complete building proteins from different amino acids because of the lack of cysteine. The fact that these results are not significant is a result of the small sample size of the experiment. However, the big differences in amino acid concentration indicate that deprivation results in accumulation.

Growing a C4 plant solely on an atmospheric sulfur source is possible and proves that C4 plants can utilize H_2S as a viable source of sulfur. This however is not a completely normal growth compared to control groups. When

looking at the shoot to root ratio on the last day of the fumigation experiment (Fig. 4) the group growing solely on H2S has a smaller shoot/ root ratio. A difference in shoot to root ratio compared to the control would indicate that something is wrong with the plant.

This difference in shoot to root ratio could be a result of the plant growing out its roots to search for a viable sulfur source. Even though the plant does not seem to need a different sulfur source for its growth or health given the results of the amino acid contents (Fig. 5), water-soluble non-protein thiol content (Fig. 6) and the NO_3^- content (Fig. 7 C, D), all of these are the same with the plants that have SO_4^{2-} in their medium. The only difference between the control and the interest group (S- + H2S) is the lower concentration of SO_4^{2-} in shoot and root for the interest group. This difference is a direct result from the lack of SO_4^{2-} accessibility for these plants and the H_2S being immediately metabolized into cysteine.

Comparing these results to what is found in C3 plants(Aghajanzadeh et al., 2016; De Kok et al., 1997; Koralewska et al., 2008; Sue WESTERMAN et al., 2001), the results are similar at time of termination of the experiment. In Aghajanzedeh *et* al. (2016) the research on B. *juncea* and B. *Rapa* gave similar results as found in Z. *mais*. The S- +H2S group had a smaller shoot/root ratio than the control but a similar biomass production. The water-soluble non-protein thiols show the same results as does the sulfate. However, these similar results are found after only seven days and in this research after twelve days.

The other papers written on Brassica as a model C3 plant give the same results on thiol and anion concentration but all these experiments were terminated on day seven. While a lot of the results found on day seven can be extrapolated to show the same as on day twelve. This however, cannot be done for the biomass production results and shows that there is a difference in how a C3 plant or C4 plant handle H_2S as a sulfur source.

The difference in leaf makeup and sulfur metabolism in C4 plants compared to C3 plants could play a role in creating these results. The plant could be more efficient with the sulfur it has collected as is the case with the carbon assimilation(Christin et al., 2014; Keeley and Rundel, 2003; PEARCY and EHLERINGER, 1984; Sage and Zhu, 2011). Or the spatial separation in sulfur assimilation up to cysteine is the result for the differences in results and time scale where the effect can be observed.

The plant first needs to transfer H_2S to the bundle sheath cells before it can be assimilated in to cysteine. This has to be done because of the spatial separation of the sulfur assimilation. But due to the highly reactant nature of sulfide and thus phytotoxicity of H_2S this could be a risk for the plant(Kopriva et al., 2015; Riemenschneider et al., 2005). Therefore, the spatial differences as an answer is highly unlikely given the results. If this where true, the H_2S fumigated groups would show a more lagging growth compared to the control group and more akin to the sulfur deprived group.

The difference in results from C3 to C4 plants is likely due to the mechanism used in the sulfur assimilation due to the entanglement of the sulfur, carbon and nitrogen pathways where both carbon and nitrogen regulate the sulfur assimilation(Kopriva, 2017; Kopriva and Koprivova, 2005). The efficiency of the plants sulfur utilization is for now the best explanation for the temporal difference between the plant types. This is based on the sulfur deprived group showing similar results for at least the growth during the first week.

Conclusion

The results clearly show that Zea *mais* as a C4 plants can utilize H_2S as a sulfur source. The plants that had solely H2S as a sulfur source showed comparable results to the plants

grown under normal conditions. Sulfur deprivation shows a clearly distinct pattern to the rest of the groups to rule out any deprivation effects on the plant. The significance of the difference in leaf makeup on sulfur metabolism in this process needs to be evaluated further.

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