Arbuscular mycorrhiza influences carbon-use efficiency and grain yield of wheat grown under pre- and post-anthesis salinity stress

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Pre- and post-anthesis salinity stress in AM wheat

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Abbreviations: AM, Arbuscular mycorrhiza; AMF, Arbuscular mycorrhizal fungi; DAS, Days after sowing; C, Carbon; N, nitrogen; FW_R/DW_R, Fresh/dry root weight ratio; SWC, Soil water capacity; P_n, Rate of net photosynthesis; g_s, stomatal conductance; WUE_i, intrinsic water use efficiency

Abstract

- Soil salinity severely affects and constrains crop production worldwide. Salinity causes osmotic and ionic stress, inhibiting gas exchange and photosynthesis, ultimately, impairing plant growth and development. Arbuscular mycorrhiza (AM) have been shown to maintain light- and carbon use-efficiency under stress, possibly providing a tool to improve salinity tolerance of the host plants. Thus, it was hypothesised that AM will contribute to improved growth and yield under stress conditions.
- Wheat plants (*Triticum aestivum* L.) were grown with (AMF+) or without (AMF-) AM fungal inoculation. Plants were subjected to salinity stress (200 mM NaCl) either at pre, post-anthesis, or at both stages. Growth and yield components, leaf chlorophyll content as well as gas exchange parameters and AMF colonisation were analysed.
- AM plants exhibited a higher rate of net photosynthesis (P_n) and stomatal conductance (g_s), and lower intrinsic water use efficiency (WUE_i). Furthermore, AM wheat plants subjected to salinity stress at both pre-anthesis and post-anthesis maintained higher grain yield than non-AM salinity-stressed plants.
- These results suggest that AMF inoculation mitigates the negative effects of salinity stress by influencing carbon-use efficiency and maintaining higher grain yield under stress.

Introduction

Wheat (*Triticum aestivum*) is a major global food crop, covering more production area than any other crop (Curtis et al. 2002). With a global production of approximately 700 million tonnes, wheat provides 20% of the calories consumed by humans (FAO 2013). However, productivity has declined, mainly due to increase in biotic and abiotic ~~(a)biotic~~ stress events (de Bossoreille de Ribou et al. 2013). The increase in world population will generate higher demands for food, which need to be overcome by an increase in sustainable food production (IPCC 2017, 2019; United Nations 2019). However, increases in the frequency, severity, and duration of abiotic stress episodes are already affecting agriculture globally as crops are affected more than once and often by a combination of different stress types during growth and development, ultimately compromising production (Pandey et al., 2017).

Salinity, drought-, and heat stress events are major abiotic stress types that affect crop growth and development, and consequently biomass production, yield, and quality (Çiçek and Çakirlar 2008; Zhu et al. 2016). Salinity affects 20% of the global irrigated area (62 million hectares) worldwide and diminishes the productivity of food crops (Qadir et al. 2014). Thus, the development of new strategies for improving salinity tolerance and achieving higher yields is vital (Hanin et al. 2016). Salinity stress causes water deficit and excessive salinity accumulation, interfering with the acquisition and assimilation of carbon (C) and nitrogen (N) at different growth stages (Debouba et al. 2006; Cruz et al. 2017). N is an essential element for the synthesis of amino- and nucleic acids, as its deficiency reduces protein synthesis ultimately leading to inhibition of crop growth and development (Frechilla et al. 2001; Evelin et al. 2009; Abdel Latef and Miransari 2014). Furthermore, salinity stress leads to stomatal closure and thereby to inhibition of gas exchange and photosynthesis (Munns 2002; Yousfi et al. 2010). When the rate of photosynthesis is reduced, reactive oxygen species (ROS) start to accumulate, interfering with crucial cellular functions by severely damaging lipids, proteins and nucleic acids (Apel and Hirt 2004). There are various strategies for improving salinity tolerance and management requires multidisciplinary efforts (Wollenweber et al. 2005; Farooq et al. 2015; Hatfield and Walthall 2015; Hoang et al. 2016).

Arbuscular mycorrhizal fungi (AMF) establish symbiotic relationships with 72% of land plant species (Brundrett and Tedersoo, 2018). AMF provide increased nutrient acquisition and water uptake, receiving carbohydrates from the host-plant to use as an energy source (Smith and Read, 2008). AM symbiosis has been shown to contribute to plant tolerance to salinity stress, improving yield and nutritional quality of crops grown in soil with high salinity levels (Porcel et al. 2012; Estrada et al. 2013; Abdel Latef and Miransari 2014; Goicoechea and Antolín 2017).

Moreover, AM plants have been shown to have improved plant N and C accumulation and distribution under salinity stress (Evelin et al. 2009). In wheat plants, AM has been reported to alleviate the negative effects of salinity by improving nutrient uptake and translocation (Zhu et al. 2016, 2018a). For instance, studies have shown that AM can mitigate the negative effects of salinity stress in wheat plants, both in field and in greenhouse (Daei et al. 2009; Talaat and Shawky 2014; Mardukhi et al. 2015; Fileccia et al. 2017, Zhu et al., 2018b). This was found to occur via several mechanisms, such as enhanced water relations, stomatal conductance (Talaat and Shawky 2014, Zhu et al. 2018b), nutrient uptake (Daei et al. 2009, Mardukhi et al. 2015) and photosynthetic efficiency (Talaat and Shawky 2014). Furthermore, AM plants have been shown to have increased production of antioxidants (Chang et al., 2018) in response to abiotic stress (Lambais et al., 2003, Baslam and Goicoechea 2012), which might also constitute a mechanism to mitigate salinity stress. However, to date the effect of AMF inoculation on multiple salinity stress occasions has not been addressed. Since episodes of stress affecting the growth and development of plants are likely to increase in the future, it is important to observe possible priming effects on the alleviation of later stress (Wang et al. 2014).

Cereal crops have been shown to be more sensitive to salinity stress during pre-anthesis than post-anthesis growth stages, decreasing yield by reducing the number of spikes, tillers and grains per spike (Ugarte et al. 2007; Al-Ajlouni et al. 2016). Moreover, salinity stress has been shown to decrease the plants' capacity of photosynthetic recovery (Pérez-Pérez et al. 2007), as measured by an increase in leaf water potential following the recovery of stomatal conductance (Kirschbaum 1987; Kirschbaum 1988). Increases in the synthesis of photosynthetic proteins have been shown to contribute to photosynthetic recovery, following a drought stress event (Bogeat-Triboulot et al. 2007). The capacity for recovery depends on both the duration and intensity of stress as prolonged exposure to stress may irreversibly damage photosynthesis (Chaves et al. 2009). The consequences of multiple salinity stress events, at pre- and post-anthesis stages, on the recovery of photosynthetic capacity remain to be understood. Additionally, as AM symbiosis has been reported to increase photosynthesis (Zhu et al. 2012), its role in influencing the plants' recovery capacity needs more investigations.

By subjecting wheat plants to salinity stress at pre-anthesis, post-anthesis, both stages or none (control), the hypothesis that AM alleviates the negative effects of salinity in *Triticum aestivum* by increasing photosynthetic light- and carbon-use efficiencies was assessed. The objectives of the current study were to investigate the effects of AM symbiosis on: i) carbon assimilation, ii) plant growth iii) yield components, and iv) AMF structures in the roots, of plants grown under single or multiple salinity stress events.

Materials and Methods

Experimental Setup and Growth Conditions

A greenhouse experiment was conducted (16/8 h day/night; 18/16 °C; 50/60% humidity) with spring wheat (*Triticum aestivum* L.), cultivar 1110 (Kloka WM1353, IPK, Germany) from December 2017 to May 2018 at the Research Centre Flakkebjerg, Aarhus University, Denmark (55°19'N,11°24'E). The experiment followed a full factorial design: AMF inoculation (AMF+) or without (AMF-), two salinity treatments (0 mM and 200 mM NaCl). Plants were subjected to salinity treatment for 6 days, either at the pre-, post-anthesis, both growth stages, or none (Table 1). Each treatment consisted of five replicates.

The soil was heat sterilised twice at 80 °C for 48 h to obtain a growth substrate free of AMF propagules (Cabral et al., 2016). Nutrient solutions, growth substrate and pot filling were prepared as described in detail in ~~(~~Cabral et al., 2016~~)~~. Two seeds were sown per pot and then thinned to one. A low P clay soil used in the growth substrate, as described in Cabral et al., 2016. Each pot contained 2.30 kg of soil: sand mixture (1:1) mixed with 50 g of AMF inoculum (in AMF+) or the carrier of the inoculum (in AMF-). 100 g of growth substrate without AMF inoculation per pot created a top layer in pots to avoid spore propagation. AMF inoculum was acquired from Symbiom Ltd. (Lanskroun, Czech Republic) containing a mixture of five AMF (*Rhizophagus irregularis* isolate BEG140, *Rhizophagus irregularis*, *Funneliformis mosseae* isolate BEG95, *Funneliformis geosporum*, *Claroideoglomus claroideum*) in a carrier of expanded clay.

Pots were weighed and watered every other day, to maintain 65% soil relative water content (SRWC). Soil water content was determined as described in detail in Cabral et al.,(2016): three pots were weighed and saturated with excess water, sealed and drained for 24 h at room temperature and re-weighted to obtain water-saturated weight. For dry weight, the pots were dried 48 h at 80ºC. SRWC was calculated as described in Cabral et al., (2016). Until the pre-anthesis salinity treatment, 1.5 mL of 0.7M NH_4NO_3 was added to the pots once a week.

On-line measurements

Phenological recording of five biological replicated per treatment was performed from day 47 after sowing (DAS). ~~started taking place 47 days after sowing (DAS), according to (Zadoks et al. 1974) five biological replicates of each treatment were recorded.~~ The pre-anthesis salinity stress event lasted from Z32 (55 DAS) to Z43 (61 DAS), (Zadoks et al. 1974), and the post-anthesis salinity stress event lasted from Z60 (76 DAS) to Z68 (82 DAS). Starting at Z32 (55 DAS), photosynthetic carbon-use (CO_2 fixation) was measured on the penultimate leaves of

three biological replicates per treatment. Gas exchange parameters were obtained by using LI-6400 portable photosynthesis system (LI-COR Inc., Lincoln, Nebraska, USA), coupled to a 6400-02 red LED light source with the following settings: Leaf area 3.0 cm^2 (between 55 and 61 DAS) and 4.5 cm^2 (between 61 and 75 DAS); block temperature 20 °C; Flow rate 500 µmol s-1; Light 1500 µmol m^{-2} s^{-1}; reference CO_2 concentration: 400 µmol m^{-2} s^{-1}. Measurements were taken when P_n and g_s values were constant. After the post-anthesis stress treatment, from Z69 (86 DAS), the chlorophyll content was measured in both flag- and penultimate leaves (5 biological replicates) using a chlorophyll meter (SPAD-502, Minolta Co. Osaka, Japan).

Harvests and sampling

20 plants (from AMF-S+-, AMF- S- -, AMF+ S- -, AMF+ S+, 5 replicates) were harvested at Z43 (62 DAS), after the first stress event. After two weeks of a recovery (R) period, 10 plants (from AMF+ S+ and AMF- S+, 5 replicates) were harvested at Z60 (76 DAS). After the post-anthesis salinity stress treatment, plants were irrigated with water only until the grains were fully developed and then 5 replicates (AMF-S--, AMF-S+-, AMF-S-+, AMF-S++, AMF+ S--, AMF+ S+-, AMF+ S-+, AMF+ S++) were harvested at Z69 (132 DAS).

At each harvest occasion, tillers and leaves and roots were separated. The number of leaves on the main and side tillers, leaf area, tiller number, shoot height, fresh weights of leaves and shoots were recorded. The leaf area was measured using a LI-3100C area meter (LI-COR, Lincoln, Nebraska, USA). ~~At the final harvest, grains were collected~~Grains were collected at final harvest, the number of grains as well as grain weight were recorded. ~~and the number of grains and grain fresh and weight were recorded.~~ The obtained plant material and grains was frozen, freeze-dried and the dry weight recorded.

Root length and AMF colonisation

The root systems were separated from the plant stems and thoroughly rinsed in running water. These were ~~and~~ cut into 1 cm pieces~~.~~ and 0.7 g of root material ~~were~~ was randomly sampled ~~from each root system~~ to evaluate AMF colonisation. Samples were cleared in 10 % KOH, ~~and~~ stained in 5 % Sheaffer Skrip ink blue (adapted from Vierheilig et al. 1998) and stored in 85% glycerol. AMF colonization in roots was examined by microscopy (Giovanneti and Mosse 1980) at 16x amplification. Using the point-intersection method, AMF structures were classified in intra-radical hyphae, vesicles, and arbuscules (McGonigle et al. 1990). The number of colonised roots and each of the AMF structures were recorded and total root length was calculated. The ~~rest of the~~remaining material was freeze-dried, weighted and stored at -20 °C.

Statistical Analysis

Data analysis was done using the statistical program R (R Core Team, 2017). Linear models ~~linear models~~ followed by analysis of variance (ANOVA) were used to discern differences between factors. Relative values, as well as ratios were log-transformed to fit the assumptions of normality and homoscedasticity of the ANOVA. Fisher's LSD test was performed *post-hoc*, using R package *agricolae* (de Mendiburu 2019). To test the homogeneity of variances Bartlett's test was performed. For gas exchange parameters, to determine differences between treatments *p*-values were adjusted according to student t-test. Pairwise comparisons were obtained using the emmeans package (Lenth, 2018).

Results

Salinity stress influenced aboveground biomass, ear- and grain weight, AMF inoculation influenced the number of ears and grains

Aboveground biomass non-AM plants subjected to two salinity stress events (AMF-S++) was significantly lower than that of non-AM plants ~~and~~ subjected to only one salinity stress event (AMF-S+- and AMF-S-+). By contrast, there was a tendency to increased aboveground biomass of plants subjected to salinity-stress during either pre- (AMF-S+, AMF+S+) or post-anthesis (AMF-S-+, AMF+S-+) compared to plants without AM and salinity stress (AMF-S--) (Table 2). However, biomass decreased when non-AM plants were subjected to salinity stress at both periods (AMF-S++) (Table 2). There was no effect of AM alone on aboveground biomass.

The numbers of ears and grains were higher in AMF-inoculated plants subjected to salinity stress (Table 2). The number of grains was significantly higher in AMF-inoculated plants subjected to salinity stress at both stages (AMF+S++) (Table 2). There was no significant effect of AMF inoculation on ear- and grain weight. Salinity stress decreased ear- and grain weight in non-AM plants subjected to salinity stress at both pre- and post-anthesis (AMF-S++). These parameters increased in plants subjected to salinity stress at post-anthesis (AMF-S+-) as compared to plants without salinity stress (Table 2).

Root length and root dry-weight were not influenced by salinity stress in non-AM plants. However, when AMF-inoculated plants were salinity-stressed, fresh/dry root weight ratios increased (AMF+S+, AMF+S-+, AMF+S (Table 2).The applied salinity treatments had no significant effect on AMF root colonisation, as evidenced by the percentages of intraradical hyphae, arbuscules and vesicles (Table 3).

Effect of salinity stress and AMF inoculation on C and N concentrations

C concentrations in grains were not significantly different between treatments. In general, AMF inoculation significantly decreased N concentrations in grains. N in grains from non-AM plants showed a tendency to increase by post-anthesis stress (AMF-S-+) and was significantly increased when plants were subjected to salinity stress at both stages (AMF-S++) (Table 2). C/N ratio in grains were not affected by salinity stress in plants without AMF inoculation, but AMF-inoculated plants had higher C/N ratios (Table 2).

C concentration in roots significantly decreased in non-inoculated plants when salinity stress was applied at both stages (AMF-S++) and increased when applied at pre-anthesis (AMF-S+-). Neither salinity stress nor AMF inoculation had a significant effect on root N concentrations. There were no significant differences in root C/N ratios between treatments except for AMF+S+-, where AMF inoculation significantly decreased the C/N ratio as compared to plants without AM (AMF-S+-) (Table 2).

Effect of salinity stress and AMF inoculation on gas exchange parameters

In plants salinity-stressed at both pre- and post-anthesis, with and without AMF inoculation (AMF+S++, AMF-S++) leaf chlorophyll contents were significantly declined at 99 DAS, indicating the onset of senescence. Leaf chlorophyll contents of plants with AMF inoculation (AMF+S++) were higher than in non-AM plants stressed at both pre- and post-anthesis (AMF-S++) (Table 4, Table 5).

In general, AM plants had higher rates of net photosynthesis (P_n) and stomatal conductance (g_s) than non-AM plants (Table 6). Furthermore, non-AM plants showed higher intrinsic water use efficiency (WUE_i). Pre- and post-anthesis salinity stress tended to decrease P_n in non-AM plants (AMF+-, AMF-S-+), moreover ~~and with~~ salinity stress at both stages, significantly decreased P_n ~~was significantly decreased~~ (AMF-S++) (Table 6). In AM plants, there was no significant effect of salinity stress on P_n (Table 6). Salinity stress at pre-anthesis, at post-anthesis, and both stages significantly decreased g_s in plants without AMF (AMF-S+, AMF-S+-, AMF-S++) (Table 6), whereas salinity stress did not affect g_s in AM plants. AMF inoculation significantly decreased WUE_i in all treatments, whereas salinity stress had no significant effect on WUE_i (Table 6). After the recovery period, gas exchange parameters were similar to the values that had been observed before the exposure to salinity stress.

Discussion

AM increased the number of ears and grains

AMF inoculation increased grain and ear number in salinity-stressed wheat plants at both pre- and post-anthesis stages. This is corroborated by a previous study, where salinity stress increased grain number in AM plants as compared non-AM (Talaat and Shawky, 2012). In previous studies using the same cultivar under drought and heat conditions, AM plants had higher number of grains than non-AM plants, whereas plants grown without stress had produced similar amount of grains irrespective of AM status (Zhou et al. 2015, Cabral et al. 2016). Under heat-stress, the AM mitigating effects were related to shifts in plant nutrient allocation and composition, combined with a shift in source-sink relationships under stress conditions (Cabral et al., 2016), which may also have influenced grain yield in present study. In this study, the observed AM effects could be related to the enhanced water and nutrient uptake through increased stomatal conductance and higher gas exchange rates (Augé 2000; Hajiboland et al. 2010; Zhu et al. 2018a, 2018b). Nonetheless, the better agronomical performance observed in AM plants under stress may also have been related to increased photosynthetic capacity, as seen for AM wheat plants under drought stress (Zhou et al., 2015).

The timing of the stress events has been shown to affect source-sink relationships differently, dependent on whether these events strike prior to anthesis or afterwards (Wollenweber et al., 2003; Tewolde et al., 2006). Drought and heat stress were reported to have more severe effects on growth and yield parameters when occurring before than during- and after anthesis (Ugarte et al., 2007; Ma et al. 2017). This reflects in the current study, as non-AM plants had a lower number of ears and grains as well as lower ear- and grain weight. Conversely, in AM plants there were no significant differences between pre- and post-anthesis stress effects on grain- and ear weight.

In the present study, stress at both pre- and post-anthesis significantly reduced both above-ground biomass and grain- and ear number in salinity-stressed plants, especially in non-AM plants. This is in contrast to previous findings that indicated that pre-anthesis heat- and drought stress could have a 'priming' effect on wheat plants, alleviating the negative effects of more severe stress events applied at later growth stages (Wang et al., 2012, 2015). However, in this study, double exposure to saline conditions increased the severity of stress effects, which might be due to the accumulation of high concentrations of Na^+ and Cl^-, unbalancing the plant ion-balance and leading to toxicity (Munns and Tester 2008).

AM symbiosis influenced carbon-use efficiency under salinity stress

AM plants maintained higher P_n and g_s than non-AM plants upon salinity, and none of the gas exchange parameters (P_n, g_s, and WUE_i) of AM plants were negatively affected by salinity

stress. These results are in agreement with previous studies conducted on wheat and rice (Porcel et al. 2015, Zhu et al. 2018b). The positive effect of AM could be attributed to the increased stomatal conductance, enhanced water relations improved development, and changes in phytohormone levels of AM plants (Augé 2001; Sheng et al. 2008; Kaschuk et al. 2009; Hoeksema et al. 2010; Ruiz-Lozano and Aroca 2010; Porcel et al. 2015). Salinity stress at pre- and post-anthesis, and at both stages decreased P_n and g_s in non-inoculated plants, whereas P_n and g_s of AM plants exhibited no significant differences between control and salinity-stressed plants. Due to decreased stomatal conductance, the available CO_2 for carboxylation decreased, leading to the reduction in carbon-use efficiency in response to salinity (Zhu et al. 2018b, Sheng et al. 2008). On the other hand, AM decreased WUE_i at both stressed and non-stressed conditions. ~~I~~in contrast to previous studies reporting improved WUE_i by AM through minimized water loss and maintained carbon fixation of the host plant (Augé 2000; Ruiz-Lozano and Aroca 2010).In this study, AM plants had lower WUE_i than non- AM plants. Due to the relatively higher induction of g_s than P_n, WUE_i decreased in AM plants.

AM did not alter C/N ratios and decreased N concentration in grains

Salinity stress had no significant influence on C/N ratios in the current study. This contrasts with previous studies, where decreased C/N ratios were reported under drought and low-temperature stress (Yang et al., 2014; Zhu et al. 2015), and could be explained by significantly decreased N concentrations in grains in AM plants in all treatments, except for the pre-anthesis salinity stress treatment. AMF ~~have~~ has been reported to contribute to the acquisition of N (Johansen et al. 1994; Mäder et al. 2000), and its transfer to the host plant (Hodge and Fitter 2010; Hodge and Storer 2015). N has been reported to be higher in the roots of AM plants than in non-inoculated plants. Thus N might be conserved in the roots and not transferred to other organs of the plants (Johansen et al. 1994; Mäder et al. 2000, Cabral et al., 2016).

In non-AM plants, salinity stress at pre-anthesis decreased N concentrations in grains, and increased when plants were stressed at both pre- and post-anthesis stages. By contrast, a previous study conducted in durum wheat showed that N concentrations were higher in salinity-stressed plants than in non-stressed plants, and that AMF improved N accumulation (Fileccia et al. 2017). This was confirmed in studies showing that AM symbiosis could improve N assimilation of the host plant through additional N-uptake *via* the extraradical hyphal network of the AMF under salinity stress (Mardukhi et al. 2015). Higher N concentrations were also observed in shoots of AM wheat (Abdel-Fattah and Asrar, 2012). However, there are inconsistencies in stress responses of different genotypes (Chandrasekaran et al. 2014). In a study using the same

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cultivar of wheat and the same AMF inoculum as in this study, drought stress had no influence on N concentrations in general, and AM decreased the N concentration in the grains of drought-stressed plants (Zhu et al. 2015). In this way, both the combination of different plant- and AMF genotypes (Ravnskov and Larsen, 2016), and the severity and duration of the abiotic stress conditions could have influenced plant nitrogen-use efficiency. In conclusion, AM mitigated the negative effects of salinity stress by maintaining higher P_n and g_s of wheat plants subjected to salinity stress at both pre- and post-anthesis, or both growth stages. Higher grain yields were obtained in AM plants than in non-AM plants when subjected to salinity stress at both growth stages. Salinity stress caused the reductions in photosynthesis via stomatal limitations resulting in lower carbon assimilation. AM could ameliorate the adverse effects of salinity stress through enhanced stomatal conductance and rates of photosynthesis. AM plants subjected to salinity stress did not exhibit the negative effects of salinity stress, as evidenced by lower P_n and g_s at all three stress occasions. Thus, AMF inoculation could help plants to maintain higher grain yield under prolonged salinity stress conditions by influencing carbon-use efficiency. These results suggest that AM symbiosis might contribute to the alleviation of soil salinity.

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by C.E, C.C and H.T. The manuscript was written by C.E with contributions from all authors.

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