Arbuscular mycorrhizal fungi-mediated modulation of maize secondary metabolism under drought conditions

Inaugural dissertation
of the Faculty of Science,
University of Bern

presented by

Sheharyar Ahmed Khan

Supervisors of the doctoral thesis
Prof. Dr. Christelle AM Robert, Institute of Plant Sciences, University of Bern
Dr. Natacha Bodenhausen, Forschungsinstitut für biologischen Landbau

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Accepted by the Faculty of Science.

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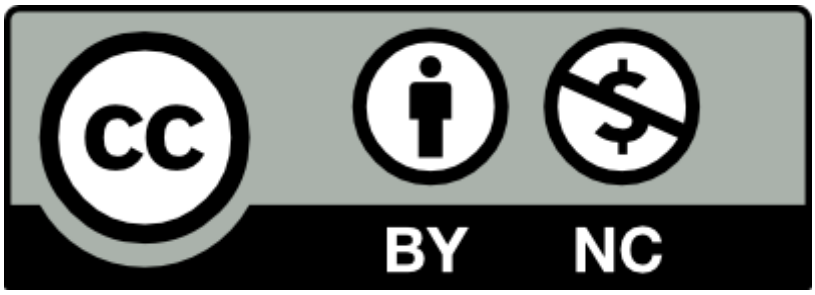

Contents

GENERAL INTRODUCTION

1. General background and challenges

Increasing world population and climate change are one of the key problems faced by humans during the 21st century. According to the United Nations Department of Economic and Social Affairs Population Division, the world population was about 8.2 billion in 2020 and will reach 10.3 billion in 2050 (World Population Prospects, 2024). In contrast to most models predicting population growth, a report by United Nations predicted population decline or stabilization in certain regions of the world at the end of the current century. This could have implications for food security and agricultural needs in the future (United Nations, 2019). For the year 2024/2025, annual cereal production is projected at approximately 2.853 billion tonnes. To feed this growing population, annual production needs to be increased by 50 percent by the year 2050 (FAO, 2025). Maize, wheat and rice are the staple crops and contribute significantly to caloric intake for a large proportion of the world's population (FAO, 2025). In particular, maize is a key crop due to its high productivity, food resourcefulness, and wide geographic range. Climate change can affect functioning of the natural and agricultural ecosystems including a drop in plant yield (Zhao et al., 2017). Drought is currently recognized as the one of the most devastating abiotic stresses that affects agriculture. Due to climate change, the frequency and intensity of drought is increasing rapidly (Trenberth et al., 2014). To improve plant resilience to drought, one of the most promising avenues could be plant-microbe interactions. These interactions help the plant not only with enhanced water and nutrient uptake but can also modulate stress responses (Begum et al., 2019). Drought results in altered plant physiological and biochemical processes, this can affect plant defence responses to the herbivores either increasing plant resistance or susceptibility to herbivory by insects (Hu et al., 2018). To increase the food production, we need not only to understand the impact of drought on agricultural ecosystems but also develop strategies to enhance food production under the climate change scenario (FAO, 2025).

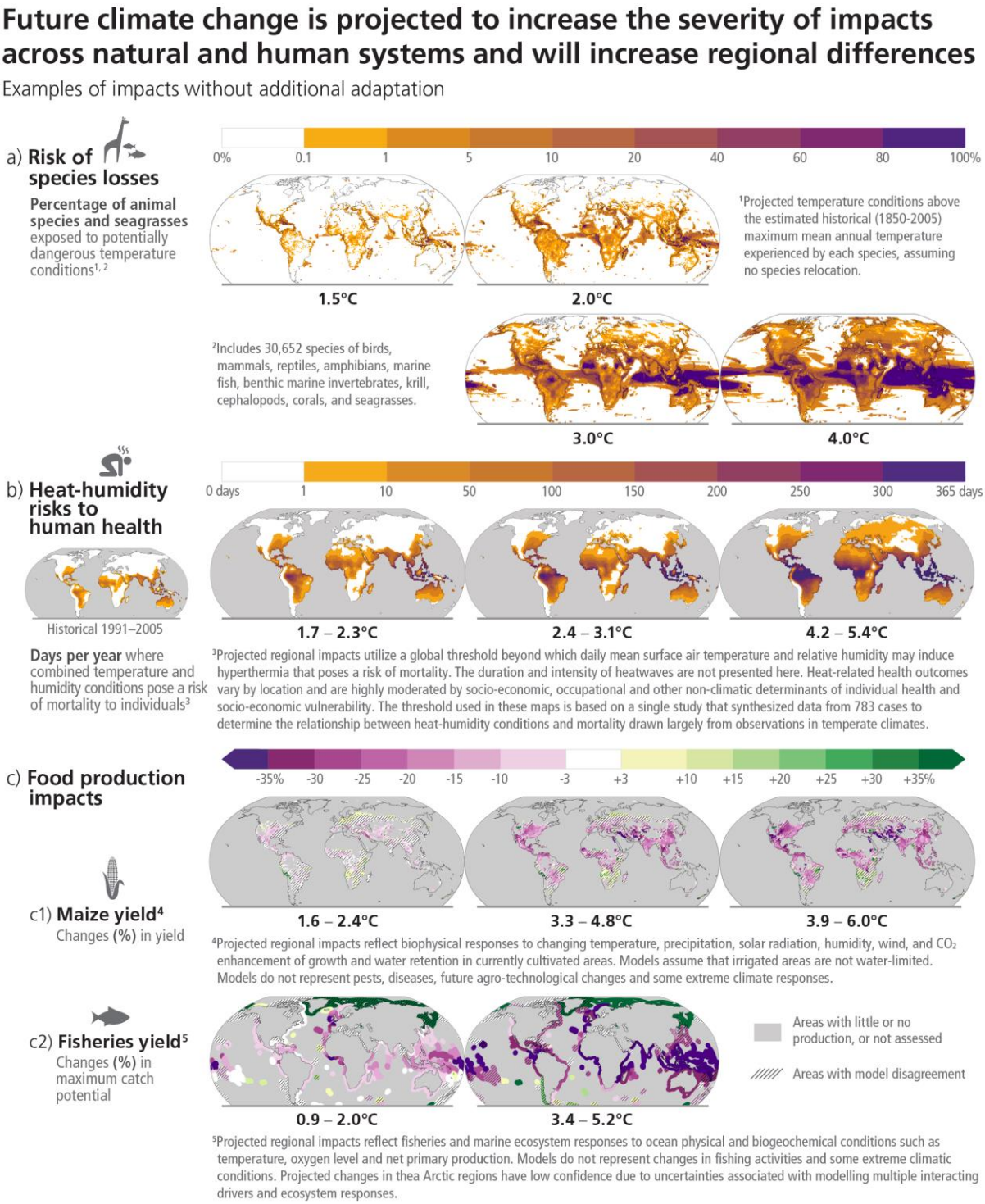

Figure 1. Impacts of climate change on species losses, human health, and food production (Reproduced from IPCC, 2023. Licensed under CC BY 4.0.).

2. Plant responses to drought

A wide range of physiological, biochemical, and molecular responses are exhibited by plants to cope with drought stress, which is being intensified under climate change. These changes include reduced water uptake, changes in hormonal balance and plant metabolism (Buragohain et al., 2024; Raza et al., 2025). For example, plants regulate stomatal opening to minimize

water loss, together with a reduction in photosynthetic activity and growth inhibition (Xu et al. 2025), eventually limiting production of carbohydrates (Bistgani et al., 2017). Under drought stress, plant accumulates osmoprotectants including proline, sugars and solutes, accompanied by upregulation of antioxidant defences to mitigate damages caused by the reactive oxygen species (ROS) (Per et al., 2017). The effect can also be antagonistic, as the concentration of sugars, amino acids, and nucleosides content was decreased in shoot of two grass species under drought stress (Gargallo-Garriga et al., 2015). Secondary metabolites such as phenolics, flavonoids, and benzoxazinoids (BXDs) are altered under drought stress and contribute to stress resilience and defence strategies. Drought also modulates BXDs profile in maize plants resulting in enhanced biotic interactions and plant resilience including herbivory (Hu et al., 2018). A pivotal role is played by the abscisic acid (ABA) in regulating these responses through hormonal signalling (Aslam et al., 2022). As highlighted in recent multi-omics studies, plants adapt to drought stress through complex regulatory networks revealing strategies associated with genotype specificity (Singh et al., 2023). It is vital to understand these integrative responses to develop crops that are well suited to varying climatic conditions.

Plants are the producers of energy in an ecosystem and are expected to lower damages caused by both biotic and abiotic stress eventually providing optimal yield. Drought has a negative impact on plants growth and productivity, thus leading to reduced biomass production (Ahmad et al., 2018). Drought also impairs plant's ability to assimilate nitrogen leading to downregulation of nitrate reductase and glutamine synthetase enzymes. Therefore, protein synthesis is reduced leading to poor levels of seed filling (Liu et al., 2022). Drought triggers a shift in metabolic energy of plants from growth and reproduction to survival mode resulting in significantly lower yields.

3. Plant responses to herbivory

3.1. Plant resistance

A wide array of compounds is produced by plants that are crucial for defences against environmental stresses and herbivores. These compounds are secondary metabolites and include phenolics (e.g., flavonoids, tannins), terpenoids, alkaloids, and benzoxazinoids, each playing distinct roles in mitigating damage or deterring herbivory (Erb & Kliebenstein, 2020; Dixon & Dickinson, 2024). Secondary metabolites act as toxins, feeding deterrents and can also enhance indirect defences by attracting natural enemies of herbivores through signalling molecules (Mithöfer & Boland, 2022). Through advances in metabolomics, studies reveal that the accumulation of these compounds are regulated by both biotic and abiotic factors with

implications for plant fitness and crop protection strategies (Alami et al., 2024). For example, the concentration of secondary metabolites in root latex of *Taraxacum officinale* was associated with low precipitation and higher temperature highlighting the potential role of secondary metabolites in resistance to abiotic stress (Bont et al., 2020). Plant secondary metabolites such as benzoxazinoids, the phytohormone abscisic acid (ABA), salicylic acid (SA) jasmonic acid (JA), and volatiles (VOCs) are all modulated under drought stress (Vaughan et al., 2018).

Plants have evolved their defence mechanisms against herbivore attack through the production of secondary metabolites. Jasmonic acid (JA) acts as a key hormone in mediating these responses and is involved in the production of diverse classes of secondary metabolites including phenolics, alkaloids, terpenoids and benzoxazinoids. Meta-analysis confirmed the increased phenolic levels in plants that are infected by pathogens or insects (Wallis et al., 2020). Plants infection by herbivores leads to enhanced production of phenolic compounds such as lignin, coumarins, furanocoumarins, flavonoids, and tannins (Gantner et al., 2019). Glutathione, glucosinolates, phytoalexins are sulphur containing compounds known for their important defensive role in plants. Glutathione is actively involved in plant-herbivore interactions by regulating both signalling and detoxification reactions (Künstler et al., 2020). In soybean plants, glutathione mediated generation of H_2O_2 leading to reduced nematodes accumulation (Chen et al., 2020). Alkaloids, cyanogenic glycosides, and non-protein acids are the nitrogen-containing compounds that are effective in plant defence mechanisms against herbivores. Pyrrolizidine alkaloids (PAs) such as jacobine and erucifoline are also actively involved in plant defence against insect herbivory (Liu et al., 2017).

Benzoxazinoids

Benzoxazinoids are maize secondary metabolites and originate from indole-3-glycerol phosphate localized in the chloroplasts. They undergo transformation by benzoxazinless (BX) into 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HBOA), which is further hydroxylated into 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) (Niculaes et al., 2018). In some cases, DIBOA can be hydroxylated and methylated into 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA). DIMBOA undergoes a methylation by *ZMBX10*, *ZMBX11*, *ZMBX12*, and *ZMBX14*, to form the methyl hydroxamate HDMBOA (Frey et al., 2009; de Bruijn et al., 2018). These compounds can be glycosylated by UDP-glucosyltransferases (UGT) into DIMBOA-xGlc or HDMBOA-xGlc which biologically inactivates them, preventing autotoxicity within the producing plant (Frey et al., 2009; de Bruijn et al., 2018). The resulting glucosides (DIMBOA-xGlc and HDMBOA-xGlc) can be stored in the vacuole and released upon tissue disruption, such as insect or herbivore attack (Robert and Mateo, 2022). This

ensures that toxicity is released only when the metabolite is hydrolyzed by glucosidases within the herbivore's system. Plant secondary metabolites play key role in plant defences, resilience and signalling.

Benzoxazinoids under drought

The synthesis of these compounds is often regulated under varying environmental conditions. Benzoxazinoids production is altered in maize plants when subjected to drought conditions. Under drought stress in seven days old seedlings, maize roots and leaves exhibited production of DIMBOA-2Glc, DIMBOA-3Glc, HMBOA-2Glc, HMBOA-3Glc, and HDMBOA-2Glc. This effect was consistent across various maize lines (Sutour et al., 2024). BXDs altered composition under drought conditions underscores the plant strategy to better adapt to changing environmental conditions. The expression of *ZMBX12* gene was enhanced under drought stress, resulting in the increased production of DIMBOA-Glc (Robert & Mateo, 2022). As specific BXDs are modulated in maize plants under drought, this could point towards their potential role in plant resilience.

Role of UDP-glycosyltransferases (UGTs)

UGTs catalyse the glycosylation of a wide range of compounds including phytohormones and secondary metabolites, therefore changing their solubility, stability, and bioactivity (Zhang et al., 2022). A critical role is played by UGTs in regulating plant stress responses by shifting levels and activities of secondary metabolites. For example, UGT UGT85E1 catalyses the glycosylation of abscisic acid, this influences ABA availability during drought stress and ultimately affects stomatal closure and water preservation (Gharabli et al., 2023). Despite the indicated significance of UGTs, they remain unexplored in production of maize secondary metabolites under drought stress (Liu et al., 2021). UGTs characterization in maize plants under drought stress can unravel their roles during stress adaptation and pave way for metabolic engineering to enhance drought tolerance.

3.2. Plant tolerance

Plants tolerance to herbivory is the ability to withstand or recover from damages caused by the herbivores without significantly reducing fitness. This allows the plants to maintain growth, reproduction and survival while sustaining damage at the same time. This can be achieved through various strategies involving increased photosynthetic rates, mobilization of stored resources and compensatory growth. Plants can also exhibit tolerance through reallocation of carbon and nitrogen resources or with regenerative capacity involving basal meristems (Strauss and Agarwal, 1999). Recent studies conducted on maize identify the role of genotypes,

environmental stress factors and herbivore specificity in conferring tolerance (Tůmová et al., 2018; Fontes-Puebla et al., 2020). Tolerant plants possess to ability to grow rapidly after damage occurrence and can even overcompensate at times. The tolerant plants might become more vulnerable under added layer of drought as environmental stressor. Water limited plant's ability to tolerate herbivory is constrained under limited resources needed for compensatory growth. Plants have been shown to form AMF associations that boost water and nutrient uptake to enhance tolerance under stress conditions (Ahmed et al., 2025). These findings show that tolerance is a context-dependent trait that evolves under environmental pressures. In the evolutionary perspective, growth rate and reproductive allocation are the trade-offs for plant tolerance to herbivory. Plants that are investing heavily in tolerance will consequently have less energy to spend on resistance, seed production and defence signalling. This is evident in the agricultural ecosystems where in pursuit of high yield crops, breeders compromise resistance making tolerance a more susceptible trait (Bergelson & Purrington, 1996).

4. Plant responses to combined drought and herbivory stresses

Plant responses become more complex when drought and herbivory occur simultaneously. Stress hormones ABA and JA are intensified during drought and therefore modify metabolite levels and composition (Kumari et al., 2023). In case of herbivory, drought stress enhances damage by tomato russet mite (TRM) by altering the plants defense responses (Ximénez-Embún et al, 2017). Under drought, TRM population grows faster as key defence pathways such JA gene is downregulated, thus reducing activities of defensive enzymes. In addition, interaction of drought and TRM results in increased levels of free sugars and salicylic acid, resulting in better pest performance (Ximénez-Embún et al, 2017). Plant responses to interaction of drought and insect herbivory can be herbivore species specific, for example drought reduced performance of generalist beet armyworms (BAW) but not that of specialist Colorado potato beetles (CPB) in *Solanum dulcamara* with differences explained by hormonal signalling (Nguyen et al., 2018). Additionally, findings of field experiments demonstrated that small-mammal herbivory on *Artemisia tridentata* seedlings in spring significantly increased summer mortality under drought stress reducing survival by up to 60%. These findings highlight that drought can enhance herbivore attack by compromising plant defenses and can eventually lead to pest outbreaks.

5. AMF: Potential plant allies under drought stress (interactions)

5.1. Plant and AMF association

Plants are at the first trophic level of the ecosystem and are actively involved in producing energy for the whole trophic chain. Plants also form mutualistic symbiotic association with the arbuscular mycorrhizal fungi (AMF). AMF are beneficial to plants as they enhance plant access to water and nutrients with the use of extraradical hyphal networks (Smith & Read, 2008). Extensive root-hyphal network can penetrate deep into the soil improving the soil structure (Dias et al., 2018) and mobilize elements synergistically to promote plant growth (Xu et al., 2024). The enhanced ability to absorb nutrients helps plant to tolerate different biotic and abiotic stresses (Qin et al., 2019). AMF has the ability to boost plants resistance to extreme environmental factors that include microplastics, and heavy metals. The ability to cope with biotic factors such as pathogens and insects is also enhanced (Nie et al., 2024)

AMF: classification, structure and functional roles

Arbuscular mycorrhizal fungi (AMF) belong to the phylum Glomeromycota and forms mutualistic symbiotic association with about 80% of the land plants including agricultural crops (Berruti et., al 2015). The AMF provides the plants with nutrients and water and in return get photosynthetic products (Smith and Read, 2008). The fungal hyphae are thin and can penetrate deeper into the soil pores than roots and therefore have access to nutrients at more soil volumes (Allen, 2011). The nutrient exchange between fungal hyphae and plant roots takes place with the help of specialized structures called arbuscules which develop inside the cortical cells of the roots (Balestrini & Lumini, 2018). The AMF can thus alleviate the nutrient deficiency of the plants (Nouri et al., 2020). The earliest land plant fossils (400 MYA) contained the tree like structures arbuscules highlighting that the AMF spread parallel to the plants colonization of land or even preceded that (Remy et al., 1994). It is also hypothesized that AMF facilitated the colonization of plants on lands as a liverwort belonging to the most ancient extant clade upon association with AMF exhibits significant uptake of photosynthetic carbon, growth, and reproduction (Humphreys et al., 2010).

Development of symbiosis

Arbuscular mycorrhizae development can be characterized into distinctive steps with the first step being the pre-contact stage also known as the pre-symbiotic stage. Mutual recognition involves plant derived Strigolactones (SLs) eliciting fungal branching responses (Akiyama et., al 2005) and diffusible fungal signalling molecules inducing gene expression in plants (Steinkellner et al., 2007). Cutin monomers produced by the plant determine subsequent

hyphopodium formation at the root surface (Murray et al., 2013). Fungal hyphopodia emits mechanical and chemical signals leading to the formation of an intracellular structure by the plant, the pre-penetration apparatus (PPA) (Genre et al., 2005). This apparatus facilitates the entry of intracellular fungi into the deeper cell layers. After entering the cortex, hyphae of the fungi progress in the longitudinal direction along the apoplast and eventually initiate the formation of arbuscules in the cortical cells of the roots (Genre et al., 2008).

Chemicals at the interface between AMF and roots

Plant roots release strigolactones (SLs) under conditions of limiting inorganic phosphate. SLs are carotenoid-derived plant hormones and stimulate the branching and elongation of fungal hyphae (Akiyama et al., 2005; Besserer et al., 2006). This extensive branching promotes the chances of encountering of the fungal hyphae with the host. Exposure to SLs increase the fungal mitochondrial metabolism including the organelle division, ATP production and gene expression (Besserer et al., 2008; Lanfranco et al., 2018). AMF release of chitin oligomers was enhanced by the SLs treatment which act as signalling molecule on the plant (Sun et al., 2015). fungal genes required for symbiosis are also induced by the strigolactones (Tsuzuki et al., 2016; Kamel et al., 2017). The critical importance of strigolactones for the formation of symbiosis is clear as plants that fail to biosynthesize or exude SLs exhibit a lower level of colonization whereas arbuscules development is normal (Waters et al., 2017; Lanfranco et al., 2018). Plants modify its defense mechanism to facilitate the controlled penetration of the fungal hyphae into the root cortical cells. To avoid killing of the fungal cells, plant immune responses including the jasmonic and salicylic acid are downregulated (Pozo & Azcón-Aguilar, 2007). In addition to sugars, lipids and sterols are also required by the AMF to sustain their metabolism. This transfer of lipids and sterols from the plants to the fungi is regulated by the terpenoids (Luginbuehl et al., 2017). Under nutrient-deficient conditions, strigolactones are crucial in regulating the early stages of AMF symbiotic association. However, AMF response to strigolactone production can be significantly influenced by drought stress.

In leguminous plants, phenolic compounds act as chemoattractants for AMF and enhance plant-fungal communication (Lone et al., 2024). The access of AMF to the cortical cells of the root is enhanced by the phenolic compounds through increased root exudation (Lone et al., 2024). On the other hand, certain chemicals alkaloids, isoflavonoids, tannins and saponins can negatively impact colonization of the host plant by the AMF. Alkaloids suppress colonization by disrupting the hyphal growth while saponins and tannins exhibit antifungal properties (Thomspon et al., 2015; Elgharbawy et al., 2020). Secondary metabolites can stimulate antagonistic microbes such as Trichoderma that can compete with the AMF for root space

(Contreras-Cornejo et al., 2106). Certain secondary metabolites such as phytoalexins also create unfavourable conditions for AMF establishment (Morandi et al., 1996).

Nutrient acquisition

AMF can play a crucial role in plant growth by acquiring nutrients from adverse environments such as arid and low fertility soils. The hyphae from AM fungi absorbs phosphorus, nitrogen, potassium, sulphur, calcium, zinc, copper and translocate them from soil into the associated roots (Gildon and Tinker, 1983). The immobile nutrients such as phosphorus, zinc and copper, determined by the rate of diffusion are reported to have significant improvements because of AMF symbiosis. In the absence of adequate amount of nutrients, plants increase their rate of absorption at a pace which is more than the rate at which nutrients are being diffused thus creating a zone of depletion. Mycorrhizal roots extend beyond this depletion zone to explore and absorb nutrients as compared to non-mycorrhizal roots which have less explorative capacity (Cornejo et al., 2017).

P acquisition

The role of AMF in acquiring P is reported in all soils globally but particularly with P deficient soils (Smith and Read, 2008; Seguel, 2015). Massive quantities of phosphorus as polyphosphates are protected and stored by the AMF structures. Phosphorus in polyphosphates is transferred from the soil's depletion zone and from non-colonized roots to the colonized roots. This P is hydrolysed to inorganic phosphorus before transferring to the plant cell (Hijikata et al., 2010). The AMF symbiosis has molecular implications for the plant as it can modify the mechanisms related to P uptake and the production of phosphatases (Mitra et al., 2023) that have high affinity for the P transporters (Biber et al., 2013). It is assumed that the association with AMF facilitates the uptake of phosphorus and mycorrhizal phosphate uptake (MPU) pathway is believed to be separate from the normal pathway involving root epidermal cells.

N acquisition

The most abundant mineral nutrient required by plants is nitrogen, indicating nitrogen fertilization in soil determines the crop productivity. Huge amounts of energy are invested in the production and application of nitrogen fertilizers consequentially increasing agricultural production costs (Xie et al., 2022). To ensure sustainability, it is therefore essential to increase nitrogen efficiency of the plants. AMF preferably acquire NH_4^+ instead of NO_3^- from the soil as NO_3^- must be reduced to NH_4^+ for incorporation into organic compounds and this process requires energy (Tanaka and Yano, 2005). AMF can degrade organic material such as grass leaves and accelerate the nitrogen acquisition by promoting the activity of bacteria in the

rhizosphere (Tanaka and Yano, 2005; Leigh et al., 2009). AMF can also uptake different amino acids such as arginine, glutamine and glycine (Whiteside et al., 2012). The AMF pathway for the uptake of nitrogen is not clearly understood.

Micronutrient acquisition

Micronutrients play a key role in plant growth, development and overall health. In soil, they are poorly mobile and available in limited quantities to the plants. AMF through their extended hyphae explore large volume of soil and mobilize key micronutrients including zinc, copper, iron and manganese through acidification, chelation and enzymatic solubilization. Bioavailability of zinc is increased by the AMF through secretion of low molecular weight compounds and promoting expression of Zn transporter genes (Zhang et al., 2025). AMF also enhances Fe acquisition through the stimulation of siderophores and a reduction in reductase activity near the root surfaces (Smith and Smith, 2011). Similarly, inoculation of Sorghum with AMF significantly enhanced micronutrients status, the total and bioavailable iron and zinc increased from 36.3% and 35.8% to 40.6% and 40.3%, respectively (Elsafy et al., 2024). Micronutrient acquisition mediated by the AMF supports plant productivity and stress resilience.

Factors affecting AMF colonization

The colonization of the plant by the AMF depends on various factors such as soil properties, plant species and genotype, fungal diversity, competition and environmental conditions. Plants rely on the AMF partner for the uptake of phosphorus, therefore its high availability in the soil or excessive use of chemical fertilizers containing phosphorus can limit colonization the colonization process (Treseder, 2004, Smith and Read, 2008). AMF thrive better in neutral to slightly acidic soils as extreme pH conditions of the soil results in reduced colonization (Van Aarle et al., 2002). Seasonal pattern also plays a crucial role in colonization with some species colonizing more in warmer seasons (Ruotsalainen et al., 2002). AMF spores germinate properly in the soils that are loose and well aerated as this allows for the hyphae to extend in broader regions (Ghorui et al., 2025). Plant species are also crucial in the onset of this association as different plant species have different compatibilities with AMF. Legume plants have higher rates of colonization due to their abilities to fix nitrogen, while members of the family Brassicaceae do not form this association (Maherali and Klironomos, 2007, Brundrett, 2009). Species composition of the AMF and the competition that exist between them can also impact the rates of colonization and eventual benefit to the plants (Bever at., 2001). A wide range of factors can affect AMF colonization; it is therefore crucial to understand specific molecules that lay the foundation for communication between plant and AMF.

Effect of drought on AMF colonization

AMF are considered beneficial to plants under drought stress, but drought can also limit the colonization efficiency due to reduced allocation of photosynthate from the host plant as functioning of the fungi depends on the carbon dependent growth (Augé, 2001; Jayne & Quigley, 2014). Drought induces changes in the soil structure and community composition, this can affect the viability of the fungal propagules and competitive dynamics (Company et al., 2010). AMF colonization increases under moderate drought as plant look to for support while extreme drought on the other hand can completely break down this association, this reflects that the mutualism between plant and amf is context dependent driven by environmental severity and host genotype (Ruiz-Lozano et al., 2016). Under drought stress, AMF colonization can be disrupted because of altered root exudation and modified signalling pathways. The question how root exuded metabolites including the secondary metabolites are shifted under drought changes and how does that impact colonization efficiency remains unanswered. Additionally, the timing of drought induction is an area that is not well understood as most studies induce drought along with inoculum induction.

5.2. AMF and drought

Drought is the most devastating environmental stress that strongly reduces soil biota and can restrict plant growth and yield. Drought affects the plant-microbe interactions both individually and at different levels (Bhattacharyya et al., 2021). The AMF enhances plant's ability to acquire nutrients particularly phosphorus, which is scarce under drought stress and can therefore support plant growth and metabolism (Smith and Smith, 2011). By the modulation of physiological and biochemical responses such as enhanced antioxidant enzyme activities, osmotic adjustment and regulation of stress-responsive hormones like abscisic acid, AMF can contribute to alleviating drought stress (Fitter, 2013; Porcel et al., 2016). AMF can alter the plant secondary metabolism potentially producing defensive compounds that can mitigate the oxidative stress produced because of drought (Ruiz-Lozano et al., 2012). The multifaceted benefits provide by the AMF makes it a critical component of agricultural practices to improve crop performance in environments affected by drought (Miransari. 2010).

5.3. AMF and herbivory

Insect herbivores can substantially damage plants leading to lower yields and altered plant metabolism. AMF can remarkably enhance the plant resistance to above ground feeding by the herbivores. This involves utilizing a wide range of mechanisms from plant defence priming, altered nutrient allocation and secondary metabolism. Upon association with the plant roots,

AMF can increase nutrient uptake particularly phosphorus, which is responsible for enhancing plant vigor and resilience to herbivore damage. AMF also induces systemic changes in the plants such as production of compounds like phenolics, alkaloids ad terpenes which have defence related functions. In potato plants, inoculation with AMF enhanced the levels of phenolics and glycoalkaloids, such as α-solanine and α-chaconine, in leaves. This resulted in reduced performance of above-ground herbivore of *Phthorimaea operculella*. Similarly, the association of strawberry plants with the AMF *Rhizophagus irregularis* decreased herbivore performance of *Spodoptera littoralis* Boisduval (Roger et al., 2013). The role of AMF in mediating above-ground plant defenses is highlighted through different studies but how this association will work under drought stress needs to be properly addressed.

5.4. AMF, drought and herbivory

Drought stress can directly affect herbivore performance and had indirect effects by altering plants nutrients (McKenzie et al., 2013). AMF can modulate nutrient allocation, secondary metabolism and gene regulation therefore altering plant quality for the insect herbivores (Pozo & Azcón-Aguilar, 2007; Bennett et al., 2009). AMF colonization in certain cases can reduce herbivore performance or feeding by enhancing plant resilience through jasmonic acid mediated defenses (Jung et al., 2012). In contrast, AMF can increase nitrogen and phosphorus in the plant tissues making them preferable for generalist herbivore feeding (Gange & West, 1994). The impact and magnitude of effect of AMF on herbivore feeding depends on the host plant, fungal species and the herbivore feeding highlighting a complex interplay between nutrient supply and defense signalling (Kempel et al., 2010). Drought weakens plant defences against herbivore feeding, how this will shape under association with AMF remains to be properly explored.

6. Thesis outline:

6.1. Overall aim of the thesis

This thesis aims to elucidate the interactions between arbuscular mycorrhizal fungi (AMF) and drought on maize secondary metabolism and whether these metabolic modulations can affect the performance of the larvae, *Spodoptera exigua.* Another vital component of this work is to explore the role of benzoxazinoids in forming association with the AMF and the effect of kinetic drought on the AMF symbiotic efficiency. The study also illustrates the function and enzymatic activity of the UDP-glucosyltransferases (UGTs), Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) involved in the production of double hexoses

benzoxazinoid in maize plants under drought stress. All together, these findings will advance our understanding how stresses along with symbiotic associations can shape plant chemical defences and ecological outcomes.

6.2. Model system

Climatic conditions

The Swiss Central Plateau (German: Schweizer Mittelland; French: plateau suisse; Italian: altopiano svizzero) is located between the Swiss Alps and the Jura Mountains and represents one of the three main landscapes in Switzerland. The Swiss Central Plateau is partly flat but mostly hilly and covers almost 30% of the surface area of Switzerland. It is located within a transition zone between the humid oceanic climate and the continental temperate climate making proving it with a special climatic condition (The National Centre for Climate Services NCCS, CH-8058 Zurich, Switzerland). In addition to being centre of economy and transportation, Swiss central plateau is also the most densely populated area by far. There is an important implication associated to do research on drought in Swiss Central Plateau as climate change is predicted to decrease precipitation leading to severe drought events. This will intensify during the summer period in the agricultural ecosystems.

Representative Concentration Pathway (RCP) scenarios

To stimulate future climate conditions, the current study incorporates two Representative Concentration Pathway (RCP) scenarios, RCP 8.5 and RCP 2.6 to establish drought conditions. RCP 8.5 represents a trajectory with high greenhouse gas emission leading to a severe warming and increased drought frequency and intensity. RCP 2.6 projects limited warming by the year 2100 given the emissions are reduced (IPCC, 2023).

Maize (Zea mays)

Maize (*Zea mays*) was chosen for the current model system study owing to its global agricultural importance as a staple crop and its well-characterized physiological and biochemical responses to abiotic and biotic stressors. Maize yield and quality is limited worldwide due to high susceptibility to drought stress (Casali et al., 2018; Kumar et al., 2024). Moreover, maize produces a diverse suite of specialized metabolites, such as benzoxazinoids, which are known to play crucial roles in defense against insect herbivores (Robert and Mateo, 2022). Maize is an ideal system to investigate the interactive effects of drought, arbuscular mycorrhizal fungi (AMF), and insect herbivory on plant secondary metabolism and defense.

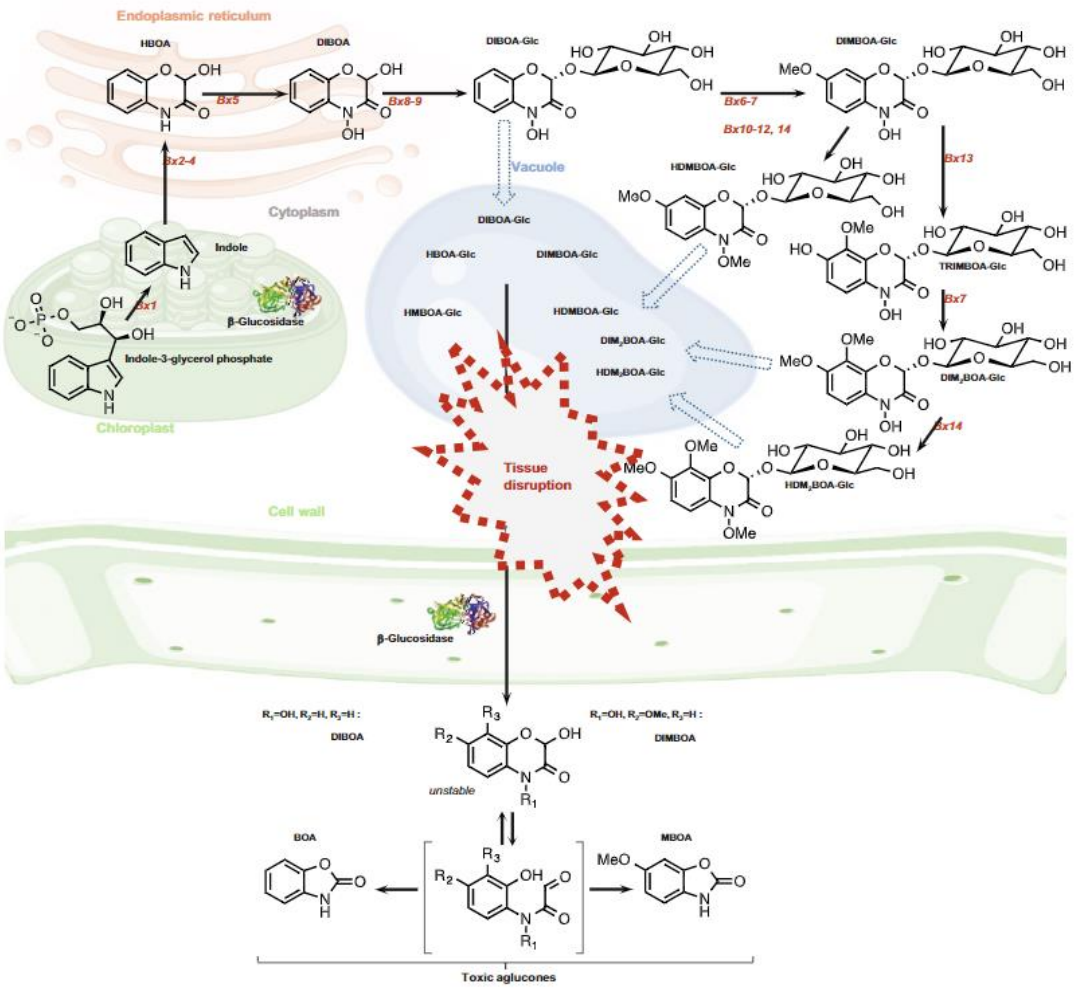

Figure 2. Known pathways involved in benzoxazinoid biosynthesis (Reproduced from Robert and Mateo, 2022. *Chimia*, Licensed under CC BY 4.0).

Arbuscular Mycorrhizal Fungi Species: Rhizophagus irregularis

The AMF species *Rhizophagus irregularis* was selected for its symbiotic relationship with maize and widespread distribution. *R. irregularis* exhibited the ability to improve host plant nutrient uptake and tolerance to biotic and abiotic stresses. *R. irregularis* potentially mitigates drought stress through enhanced water and phosphorus acquisition facilitated by the extensive hyphal networks (Fresno et al., 2023; Anandakumar et al., 2025). Additionally, plant secondary metabolism and defence pathways are modulated by the AMF, and impact plant-herbivore interactions (Jung et al., 2012). These features make *R. irregularis* well-suited to be used in this study where biotic and abiotic stresses are applied together.

Herbivore Species: Spodoptera exigua

Spodoptera exigua, also known as the beet armyworm, is a polyphagous lepidopteran herbivore and a common maize pest worldwide (Rabelo et al., 2022). Life cycle and feeding behaviour of *S. exigua* is well-characterized making it an excellent model for studying plant-insect

interactions under stress conditions. *S. exigua* larvae can cause significant yield loss by feeding on maize leaves. Studying the performance of *S. exigua* under drought and AMF treatments allows us to assess how these factors influence herbivore resistance mediated by plant secondary metabolites.

6.3. Individual aims:

In the first chapter, we investigated interactive effect of drought and arbuscular mycorrhizal fungi (AMF) on benzoxazinoids modulation and its consequences for the herbivore performance by *Spodoptera exigua* larvae.

In the second chapter, we investigated the role of benzoxazinoids in forming symbiotic association with the AMF, through MBOA complementation of *bx1* mutant plants. We also investigated the impact of kinetic drought on the colonization efficiency

In the third chapter, we investigated the UDP-glucosyltransferases (UGTs) in the families UGT79, UGT91, and UGT94 that are responsible for the production of DIMBOA-2Glc from DIMBOA-Glc under drought stress.

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Chapter I.

Arbuscular Mycorrhizal Fungi Mitigate Drought-Enhanced Herbivore Performance in Maize

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ABSTRACT

Drought events are becoming increasingly frequent and intense, posing major challenges to crop productivity. Beyond direct water stress, drought can indirectly affect plants by enhancing herbivore performance. While Arbuscular Mycorrhizal Fungi (AMF) have been proposed to alleviate drought stress and to enhance plant resistance to herbivory, their role in mediating plant responses to the two combined pressures remains poorly understood. Here, we examined the individual and interactive effects of drought, AMF colonization, and herbivory on maize (*Zea mays*) performance. We combined a semi-field experiment with two growth chamber assays to assess growth, metabolism, and herbivore responses. Drought reduced maize biomass and chlorophyll content, while AMF improved reproductive traits, independently of soil moisture levels. Both drought and AMF colonization led to a reconfiguration of the plant primary and secondary metabolism. Interestingly, drought transiently decreased DIMBOA-Glc levels in maize leaves, an effect that was exacerbated under AMF colonization. Consistently, drought increased leaf herbivore performance. However, AMF colonization limited the drought-mediated increase in herbivore performance, despite similar leaf damage. Overall, AMF enhanced maize yield and herbivore resistance under drought conditions. This study highlights the need to consider multi-stressor interactions to understand and harness AMF benefits in agriculture under increasing drought pressure.

Keywords:

Drought, Arbuscular Mycorrhizal Fungi (AMF), Herbivory, Maize.

INTRODUCTION

Drought is becoming increasingly frequent and intense across many regions due to shifting climate patterns, posing a serious threat to global food security (Farooq, et al., 2023; Rezaei et al., 2023, IPCC, 2023). While drought directly impairs plant growth and yield by limiting water and nutrient uptake, it can also increase herbivore pressure, either by weakening plant defenses or by improving plant tissue nutritional value. Arbuscular mycorrhizal fungi (AMF) can improve plant drought resilience (Abdalla et al., 2023; Zou et al., 2020) and plant tolerance and/or resistance to herbivory (Dowarah et al., 2022). However, the role of AMF in mediating plant responses under combined drought and herbivory remains poorly understood. Addressing this knowledge gap is essential for developing sustainable strategies to improve plant resilience in increasingly variable environments.

The increasing frequency and severity of drought events threaten agricultural productivity worldwide, especially in regions already vulnerable to water scarcity (IPCC, 2023; Rosenzweig et al., 2014; Yuan et al., 2024). Among climate-related stressors, drought is one of the most damaging, with the potential to reduce crop yields by over 50% on arable land by 2050 (Vinocur and Altman, 2005). The three major cereal crops, maize, wheat, and rice, which together provide over half of the global caloric intake, are particularly sensitive to water stress (Farooq et al., 2023; Deribe, 2024; Kheyruri et al., 2024; Mohammadi, 2024; Sheoran et al., 2022). Drought leads to impaired photosynthesis, stunted growth, disrupted nutrient uptake, early senescence, and reduced yield (Gupta et al., 2020; Qiao et al., 2024). A meta-analysis showed that a 40% water reduction caused yield declines of up to 21% in wheat and 40% in maize in the field (Daryanto et al., 2016). As drought episodes intensify, safeguarding these staple crops is essential to ensure food security for a growing population.

Beyond its direct effects on plant growth and yield, drought can also indirectly exacerbate plant stress by increasing herbivore pressure (Chávez-Arias et al., 2021). Water limitation can trigger increased tissue concentrations of sugars and amino acids due to osmotic adjustment and weaken or delay activation of defense pathways (Ruan, 2014). In crops, drought has been shown to impair the jasmonic acid (JA)- and salicylic acid (SA)-mediated defense responses that normally deter herbivory (Margay at al., 2024). As a result, stressed plants can become more susceptible to insect pests, particularly during early developmental stages. For instance, drought downregulated JA biosynthetic genes such as ZmOPR2 and ZmLOX10 in maize, leading to increased susceptibility to *Spodoptera frugiperda* larvae (Huang et al., 2023). Similarly, in Arabidopsis, drought suppressed SA defense against herbivores by

845 downregulating ICS1 expression via NAC transcription factors (Zhao et al., 2025). These
846 findings underscore the importance of considering biotic and abiotic stress interactions, as
847 drought not only reduces maize vigor but also increases vulnerability to herbivory, further
848 threatening productivity under climate stress.
849 AMF form symbiotic associations with the roots of most terrestrial plants and play a key role
850 in improving plant resilience to drought (Bhupenchandra et al., 2024; Martin & vam der
851 Heijden, 2024; Wang et al.; 2024). AMF penetrates cortical cells of the roots and produce
852 arbuscles where an exchange of nutrients between the two partners takes place. This symbiotic
853 association helps the plant to acquire nutrients, resistance against pathogens, enhanced growth
854 under abiotic stresses (Bhupenchandra et al., 2024; Kumar et al., 2024). The fungi, in return
855 receive carbohydrates and lipids from the plant (Balestrini et al., 2020; Salmeron-Santiago et
856 al., 2021). By extending their hyphal networks into the soil, AMF enhance water uptake beyond
857 the root depletion zone, thereby improving plant hydration under limited water availability
858 (Abrar et al., 2024). Furthermore, AMF increase the acquisition of essential nutrients such as
859 phosphorus, potassium, and micronutrients, which are often less mobile in dry soils
860 (Bhupenchandra et al., 2024; Balestrini et al.; 2020). In addition to improving resource uptake,
861 AMF modulate plant physiological responses to drought by promoting osmotic adjustment
862 through the accumulation of solutes like proline and soluble sugars, enhancing antioxidant
863 enzyme activity, and stabilizing photosynthetic processes (Begum et al., 2019). These effects
864 help maintain cell turgor, delay senescence, and support root hydraulic conductivity under
865 water-limiting conditions (Abdalla et al., 2023). Studies across various species have
866 demonstrated that AMF-inoculated plants maintain higher biomass, chlorophyll content, and
867 stomatal conductance under drought stress compared to non-mycorrhizal plants (Tang et al.,
868 2022). For instance, mycorrhizal symbiosis can increase the uptake nutrients such as nitrogen,
869 phosphorus and iron as demonstrated in a study in *Pelargonium graveolens* under drought
870 stress (Amiri et al., 2017). Similarly. AMF-inoculated pistachio plants revealed high levels of
871 phosphorus, potassium, zinc and manganese (Bagheri et al. 2012). Several studies have
872 indicated that the association of AMF with plants led to an increase in biomass, rise in net CO_2
873 assimilation and stomatal conductance (Ran et al., 2024; Kakabouki et al., 2023). The
874 photosynthetic activity indicated by higher levels of photosynthetic pigments and chlorophyll
875 fluorescence parameters were also observed (Bagheri et al., 2019). Under drought stress, AMF
876 can stabilize water relations, improving plant resilience through mechanisms such as increased
877 root hydraulic conductivity when plants are subjected to drought stress (Erice et al., 2024).
878 Through these multifaceted mechanisms, AMF contribute significantly to plant drought

879 tolerance and represent a promising tool for improving crop resilience in water-scarce
880 environments.
881 AMF can further enhance plant defenses against herbivory under ambient conditions (Meier &
882 Hunter, 2018). AMF can increase plant vigor and support the synthesis of defensive secondary
883 metabolites by improving nutrient acquisition, particularly of phosphorus and nitrogen (Amani
884 et al., 2022, Orine et al., 2025). Additionally, AMF colonization has been shown to prime or
885 amplify defense signaling pathways, notably those mediated by JA (Rivero et al., 2021). In
886 particular, AMF have been shown to alter the production of secondary metabolites including
887 the phenolic compounds quercetin, vanillic acid, rutin, coumaric acid, kaempferol, and
888 tetraterpenoids carotenoids in quinoa (Benaffari et al., 2024), and benzenes and sulphur
889 containing compounds in *Solanum nigrum* (Rashidi et al., 2024). In tomato plants, AMF
890 enhanced tolerance to *S. littoralis* even in JA-deficient genotypes, suggesting that mycorrhizae
891 can even compensate for impaired defense signaling (Formenti & Rasmann, 2019). In
892 *Asclepias* species, AMF inoculation increased foliar phosphorus levels and conferred greater
893 resistance to monarch butterfly larvae (Tao et al., 2015). Similarly, AMF associations reduced
894 aphid performance on *Ageratina adenophora* by lowering nymph survival and supporting
895 stronger plant growth (Du et al., 2022). These examples demonstrate that AMF can bolster
896 plant defenses and mitigate herbivore damage under normal environmental conditions,
897 highlighting their potential as a natural strategy for pest management in agriculture.
898 While AMF have been shown to improve plant tolerance to both drought and herbivory when
899 studied separately, their role under simultaneous exposure to these stressors remains poorly
900 understood. In real-world agricultural settings, plants often face multiple, interacting stresses
901 rather than isolated ones. It is therefore critical to understand whether AMF can continue to
902 support plant performance and defense when both stressors co-occur. Some studies suggest that
903 AMF can prime plant defense pathways even under abiotic stress, potentially maintaining
904 resistance to herbivores during drought. For instance, *Medicago truncatula* inoculated with
905 *Rhizophagus irregularis* showed increased expression of JA-responsive genes and elevated
906 flavonoid levels under combined drought and insect stress, suggesting that mycorrhizal
907 colonization can help sustain chemical defenses even when plants face water limitation
908 (Adolfsson et al., 2017). However, the benefits of AMF may be highly context-dependent,
909 varying with the timing, severity, and combination of stresses, as well as the plant and AMF
910 genotypes involved. Investigating AMF-plant-herbivore interactions under realistic, multi-
911 stress conditions will enable us to better predict and harness their potential for sustainable crop
912 protection and climate-resilient agriculture.

The present study investigated the individual and combined effects of drought stress, AMF colonization, and herbivory on maize. We first assessed how AMF colonization by *Rhizophagus irregularis* influenced maize growth, yield, and natural herbivory under ambient and drought conditions in a semi-field experiment. We then conducted a controlled growth chamber assay to evaluate how AMF modulated plant responses to drought and herbivory by *S. exigua* larvae. By integrating physiological, metabolic, and herbivore performance data, our goal was to determine whether AMF can enhance maize resilience under simultaneous abiotic and biotic stress, and to identify potential mechanisms underlying these effects.

METHODS

Biological resources

B73 maize seeds were obtained from Maize GDB germplasm (MGCSC, Urbana, USA) and multiplied by Delley Semences et Plantes (DSP, Delley-Portalban, Switzerland). Inoculum containing sand, soil, roots, and spores of the AMF *Rhizophagus irregularis* (SAF22) as well as a mock inoculum without AMF was produced in the greenhouse, as previously described by Lutz et al. (2023), and were kindly provided by the Swiss Collection of Arbuscular Mycorrhizal Fungi (SAF, Zurich, Switzerland). Eggs of *S. exigua* were bought from Frontiers Agricultural Sciences, Newark, NJ, USA and larvae were reared on artificial diet (Lepidoptera diet; Frontier Agricultural Sciences, Newark, NJ, USA). Second-instar larvae were used.

Maize growth and yield in the field

The individual and interactive effects of drought and AMF on maize growth and yield were evaluated by conducting a semi-field assay. The experiment was carried out in Ostermundigen (46°57'59.8"N 7°29'13.1"E), Switzerland between June and October 2024. Weather data was provided by MeteoSwiss (Federal Office of Meteorology and Climatology, Zürich, Switzerland) and are presented in Supplementary Table 1. Maize seeds (var. B73) were surface sterilized using 15% (v/v) bleach (Potz, Migros, Zurich, Switzerland) in distilled water for 15 min. The seeds were then rinsed with distilled water and pregerminated by placing them on damped filter papers (90mm; Cytiva, Marlborough, MA, USA) in a plastic box (Semadeni, Bern, Switzerland) in the dark for three days. Ten-liter pots (Hortima, Hausen, Switzerland) were covered at the bottom using fabric sheath (Neeser, Reiden, Switzerland) and filled with approximately 11.4 kg of soil (Landerde, Ricoter, Aarberg, Switzerland), what corresponds to 95% of the pot volume. The soil chemical profile was analyzed by the laboratory Labor für

Boden- und Umweltanalytik (LBU, Steffisburg, Switzerland) (Supplementary Table 2). Approximately 500 g of the AMF inoculum were added to half of the pots (AMF+, n= 27) and mixed with the soil. The same amount of mock inoculum was added and mixed with the soil of control pots (AMF-, n=27). Three pregerminated seedlings were placed 3 cm deep into the soil in individual pots. After seven days, maize growth was assessed and one seedling (the most central) per pot was kept by manually removing additional seedlings. All plants were watered daily for two weeks. After this period, only control plants received water daily (AMF+: n=9, AMF-: n=9), while drought-exposed plants were left unwatered until drought symptoms appeared (leaf wilting score of 4, Sudhakar et al., 2016). Afterwards, all plants were watered once to twice weekly and received either 2.3 L (ambient), 1.9 L (RCP2.6) or 1.66 L (RCP8.5) (n=9 per treatment). The volume of water to add was based on the calculated soil moisture of the current ambient conditions and predicted future climate scenarios RCP2.6 and RCP8.5 with a water content of 23%, 19%, and 16.6% (v/v) respectively (Guyer et al., 2018). All plants received 1% NK fertilizer (NK Flüssigdünger; Biorga, Grossaffoltern, Switzerland) during the eighth and ninth week of the experiment. All pots were covered with 35 L plastic bags (Quick Bag, Galaxus, Zürich, Switzerland) during rain episodes. The 54 pots (2 AMF treatments x 3 drought levels x 9 replicates) were randomly placed in the beds to avoid positional bias.

Plant phenotypic parameters were measured after 60, 85 and 100 days. Relative chlorophyll content of the youngest leaf was measured using Soil and Plant Analysis Development SPAD502 plus (Konica Minolta, München, Germany) around 12 pm for all the plants. The duration of the measurements lasted from 30 min to one hour. Plant height was measured by using a ruler from the tip of the youngest leaf down to the soil surface. Herbivory damage was measured visually using a score of 1-3, one as the lowest scoring (1. Herbivory of < 5% leaf tissue, 2. Herbivory of 5-15% leaf tissue, 3. Herbivory of > 15% leaf tissue.). Maize yield was approximated by measuring tassel and cob development after 85 and 100 days of planting the pregerminated seedlings. When two cobs were present, the length of the oldest cob was taken into account for further analyses. The experiment was unexpectedly interrupted when an individual entered the field and collected most maize cobs, resulting in the premature termination of the experiment on day 118. As a result, and while fresh shoot and root biomass were measured at the termination of the experiment, no final cob parameters are available. Maize youngest leaves were collected on days 60 and 120 and flash frozen in liquid nitrogen for sugars, hormones and benzoxazinoid analysis. Maize roots were collected on day 120 for benzoxazinoid analysis and AMF colonization evaluation.

Herbivore performance under growth chamber conditions

The impact of AMF and drought on maize resistance to herbivory was investigated by measuring herbivore damage and herbivore performance under laboratory conditions. Maize seeds were sterilized and pregerminated as described above. Germinating seedlings were placed in 3 L pots (Hortima, Hausen, Switzerland) covered at the bottom using fabric sheath (Neeser, Reiden, Switzerland). The pots were filled with either 3.4 kg soil (95% of pot volume; Landerde; Ricoter, Aarberg, Switzerland) mixed with 150 g AMF inoculum (AMF+, n= 36) or with 3.4 kg soil (95% of pot volume; Landerde; Ricoter, Aarberg, Switzerland) mixed with 150 g of autoclaved control inoculum (AMF-, n=36). Maize plants were grown in a growth chamber at 23±1°C and 18±1°C with 14/10 hours of light and darkness respectively to simulate natural conditions and 60% (v/v) relative humidity. All plants were watered daily for two weeks. Because no difference was observed between the two drought levels in the field, only one drought treatment (RCP8.5) was used in this experiment. Half of the AMF+ and AMF- plants were further well-watered on a daily basis. The second half of the plants were watered with 500 mL only upon leaf wilting (score 4) symptoms (RCP8.5). After 60 days, five pre-weighed *S. exigua* larvae were placed in the middle of the shoot tip. Control plants did not receive any insects. All plants were covered with a fleece (cover fleece 1.6 × 20 m; Florada, Hannover, Germany) to prevent larvae from escaping. The pots were randomly placed in the growth chamber. Five days later, *S. exigua* larvae were collected and weighed. Infested plants where no larvae were collected were excluded from the analysis. The leaves of infested plants were photographed to analyze the leaf damage area with ImageJ (Rasband, 2018). The youngest leaves and crown roots were collected and flash-frozen in liquid nitrogen to analyze the benzoxazinoid contents. Maize roots were collected and stored at minus 20ºC for AMF colonization assessment by microscopy. The experiment was repeated twice to ensure a sufficient number of replicates per herbivore treatment (n=7-8).

AMF colonization rates

Roots were stained following a previously established procedure (Vierheilig et al., 1998). Maize thin roots (diameter 0.5 - 1 mm) were cut into small segments of approximately 1.5 cm in length and preserved in 50% EtOH (Alcosuisse, Rüti b. Büren, Switzerland). The ethanol was rinsed off using distilled water and the samples were then cleared with 10% w/v KOH (Sigma-Aldrich, Steinheim, Germany) at 80°C in a dry bath (Digital Dry Bath; Labnet, Edison, NJ, USA) for a duration of 30 min. After incubation, the roots were rinsed using distilled water and stained with ink (Pelikan, Hannover, Switzerland) -vinegar solution (5% acetic acid;

MBudget, Migros, Zurich, Switzerland) and incubated at 80°C for 30 min. After a final rinse with distilled water, the samples were stored in 50% glycerol (Dr. Bähler Dropa AG, Bern, Switzerland). The root samples were placed on a microscopic slide, mounted with 50% glycerol, and covered with the help of a cover slip. The samples were observed under a Fluorescence epi microscope with camera (Leica DMC6200; Leica Microsystems, Heerbrugg, Switzerland) at the magnification of 200X (magnifying lens * ocular lens). The average number of root segments analyzed for each plant in the field assay was 100, while the average of 60 root segments for each plant was analyzed for the herbivory assay. To exclude contamination in controls, on average 85 root segments were analyzed in the field assay. The colonization rate in percentage was measured as the proportion of root segments colonized by AMF compared to the total number of root segments (McGonigle et al., 1990).

Soluble sugar quantification

The quantification of soluble sugars was performed using Ultra High Performance Liquid Chromatography (UHPLC) coupled with Mass Spectrometry (MS) following a protocol adapted from (Barzen-Hanson et al., 2018; Yang & Rainville, 2019; Zhu et al., 2015). Maize roots and leaves samples were ground to a fine powder in liquid nitrogen using a mortar and a pestle. Aliquots of 100 ± 1 mg were extracted by adding 500 µL of 50% (v/v) aq. EtOH in 2 mL tubes microtubes (Sarstedt AG & Co. KG, Nümbrecht, Germany). The samples were incubated for 15 min at 78 °C in a dry bath, vortexed, and centrifuged at 14'000 rpm at 4°C for 10 min, and the supernatant was transferred to a new tube. This extraction was repeated twice, adding the supernatants of the same sample to the same tube. The samples were diluted 100 times and stored at -20 °C until analysis. Fructose, glucose, and sucrose profiling were performed with an Acquity UPLC I-Class system coupled to a single quadrupole mass spectrometer (QDa) equipped with an electrospray source (Waters, Milford, MA, USA). Gradient elution was performed on an Acquity BEH Amide (1.7 µm, 2.1 × 150 mm i.d.; Waters, Milford, MA, USA) column maintained at 85 °C, using normal phase chromatography in negative ion mode. The elution conditions were as follows: solvent A consisted of isopropanol (IPA) and aq. 10 mM ammonium formate (50:50 v/v), while solvent B consisted of acetonitrile (ACN), IPA, and aq. 10 mM ammonium formate (90:5:5 v/v). The flow rate was set to 0.7 mL/min. The gradient program was: 100% solvent B from 0.00-2.00 min; a linear gradient from 100% to 60% solvent B from 2.00 to 6.00 min; 60% solvent B from 6.00 to 8.00 min; a rapid linear gradient from 60% to 100% solvent B from 8.00 to 8.10 min; and finally, 100% solvent B from 8.10 to 10.00 min. MassLynx v4.1 SCN923 (Waters, Milford, MA, USA) was

used to control the instrument and for data processing. Absolute quantities were determined using standard curves of the corresponding pure compounds. Glucose, fructose, and sucrose standards were bought from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland).

Phytohormone analyses

Salicylic acid (SA), oxophytodienoic acid (OPDA), jasmonic acid (JA), jasmonic acid-isoleucine (JA-Ile), and abscicic acid (ABA) concentrations were quantified by UHPLC-MS/MS as described by Glauser et al. (2014) with minor adjustments (Gfeller et al., 2023). Aliquots of 85 ± 5 mg ground plant material were extracted by adding 990 µL of extraction solvent, consisting of ethyl acetate (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and formic acid (FA; Thermo Fisher Scientific, Waltham, MA, USA; 99.5:0.5 v/v), and 10 µL internal standard solution (isotopically labeled hormones at 100 ng/mL in water; d_6-SA from Sigma-Aldrich Chemie GmBH, Buchs, Switzerland; d_6-ABA and d_5-OPDA from OlChemIm, Olomouc, Czech Republic; d_5-JA from CDN Isotopes, Quebec, Canada; $^{13}C_6$-Ja-Ile produced in the laboratory of the Neuchatel Platform of Analytical Chemistry). The solution was vortexed (Vortex-Genie 2; Genie, Bohemia, NY, USA) for 10 s before adding 5 to 10 glass beads for mixing in a mixer mill (MM300; Retsch, Haan, Germany) at 30 Hz for 3 min and subsequently centrifuged at 14'000 rpm for 3 min (Centrifuge 5427 R; Eppendorf, Hamburg, Germany). The supernatants were transferred to 2 mL tubes microtubes. The pellet was re-extracted in 500 µL of extraction solvent and centrifuged as described above. The two supernatants were combined. The solvent was evaporated using a centrifugal evaporator (CentriVap; Labconco, Kansas City, MO, USA) and resuspended in 200 µL of aq. MeOH (50:50 v/v; Thermo Fisher Scientific, Waltham, MA, USA) using vortex and ultrasounds (Ultrasonic bath XUBA1; Grant Instruments Ltd, Royston, UK). The supernatant was filtered through a polytetrafluoroethylene hydrophilic syringe filter (0.22 µm × 13 mm i.d.; BGB, Boeckten, Switzerland) and collected in a clean Eppendorf tube (Microtube CapLock; Nolato, Torekov, Sweden). Hormone profiling was conducted using an Acquity UPLC I-Class (Waters AG, Baden-Dättwil, Switzerland) coupled to a QTRAP 6500+ mass spectrometer (Sciex, Framingham, MA, USA) operated in multiple reaction monitoring (MRM) mode with negative ionization. Chromatographic separation was performed on an Acquity BEH C18 column (1.7 µm, 2.1 × 50 mm i.d.; Waters, Milford, MA, USA) coupled to a guard column of identical phase chemistry. UHPLC gradient conditions were as follows: solvent A consisted of H_2O and FA (99.95:0.05 v/v), and solvent B consisted of ACN and FA (99.95:0.05 v/v). The flow rate was set to 0.4 mL/min. The injection volume was 1 µL and the column temperature was maintained

at 35°C. The gradient program was: a linear gradient from 5 to 50% solvent B from 0.00 to 5.00 min; a linear gradient from 60 to 100% solvent B from 5.00 to 8.00 min, 100 % solvent B from 8.00 to 12.00 min; and re-equilibration at 5% solvent B from 12.00 to 16.00 min. Analyst v.1.7.1 (Sciex, Framingham, MA, USA) was used to control the instrument and for data processing.

Benzoxazinoid profiling

Benzoxazinoid contents were characterized using an Acquity UPLC I-Class system coupled to a single quadrupole mass spectrometer (QDa) equipped with an electrospray source (Waters, Milford, MA, USA) as previously described (Hu et al., 2018). The plant metabolites were extracted from 100 ± 1 mg by adding 1 mL MeOH:H_2O:FA (70:30 v/v, 0.1% FA) and thoroughly vortexed for 10 s. The samples were then centrifuged for 20 min at 13'00 rpm at 10°C and the supernatant was collected for analysis. Compounds were separated on an Acquity BEH C18 column (1.7 µm, 2.1 × 100 mm i.d.; Waters, Milford, MA, USA). The flow rate of the mobile phase was maintained at 0.4 mL/min. The injection volume was 1 µL and the temperature of the column was maintained at 40ºC. The MS was operated in negative mode, and data were acquired in the scan range (*m/z* 150–650) using a cone voltage of 10 V. All other MS parameters were left at their default values. The elution conditions were as follows: solvent A consisted of H_2O and FA (99.9:0.1 v/v), while solvent B consisted of ACN and FA (99.9:0.1 v/v). The gradient program was: 2% solvent B from 0.00 to 1.00 min; a linear gradient from 2 to 40% solvent B from 1.00 to 4.00 min; a linear gradient to 100% solvent B from 4.00 to 6.00 min.; 100% solvent B from 6.00 to 8.50 min; a gradient from 100 to 2% solvent B from 8.50 to 8.51 min; and 2% solvent B from 8.51 to 10 min. MassLynx v4.1 SCN923 was used to control the instrument and for data processing. The absolute quantities of HMBOA, DIMBOA, DIMBOA-Glc, DIMBOA-2Glc, HDMBOA-Glc, and MBOA were determined using standard curves of the corresponding pure compounds. MBOA was purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). DIMBOA-Glc DIMBOA-2Glc, and HDMBOA-Glc were isolated from maize plants in our laboratory as previously described (Sutour et al., 2024; Thoenen et al., 2023). DIMBOA and HMBOA were synthesized in our laboratory following published protocols (Macías et al., 2006). HMBOA-Glc, HMBOA-2Glc, HM_2BOA-Glc, DIMBOA-3Glc, DIM_2BOA-Glc, and HDM_2BOA-Glc for which no analytical standards were available, were quantified by comparison with the standard curve of their closest parent compounds, HMBOA, DIMBOA-Glc, and HDMBOA-Glc. Full names and chemical formulas of measured benzoxazinoids can be found in Supplementary Table 3.

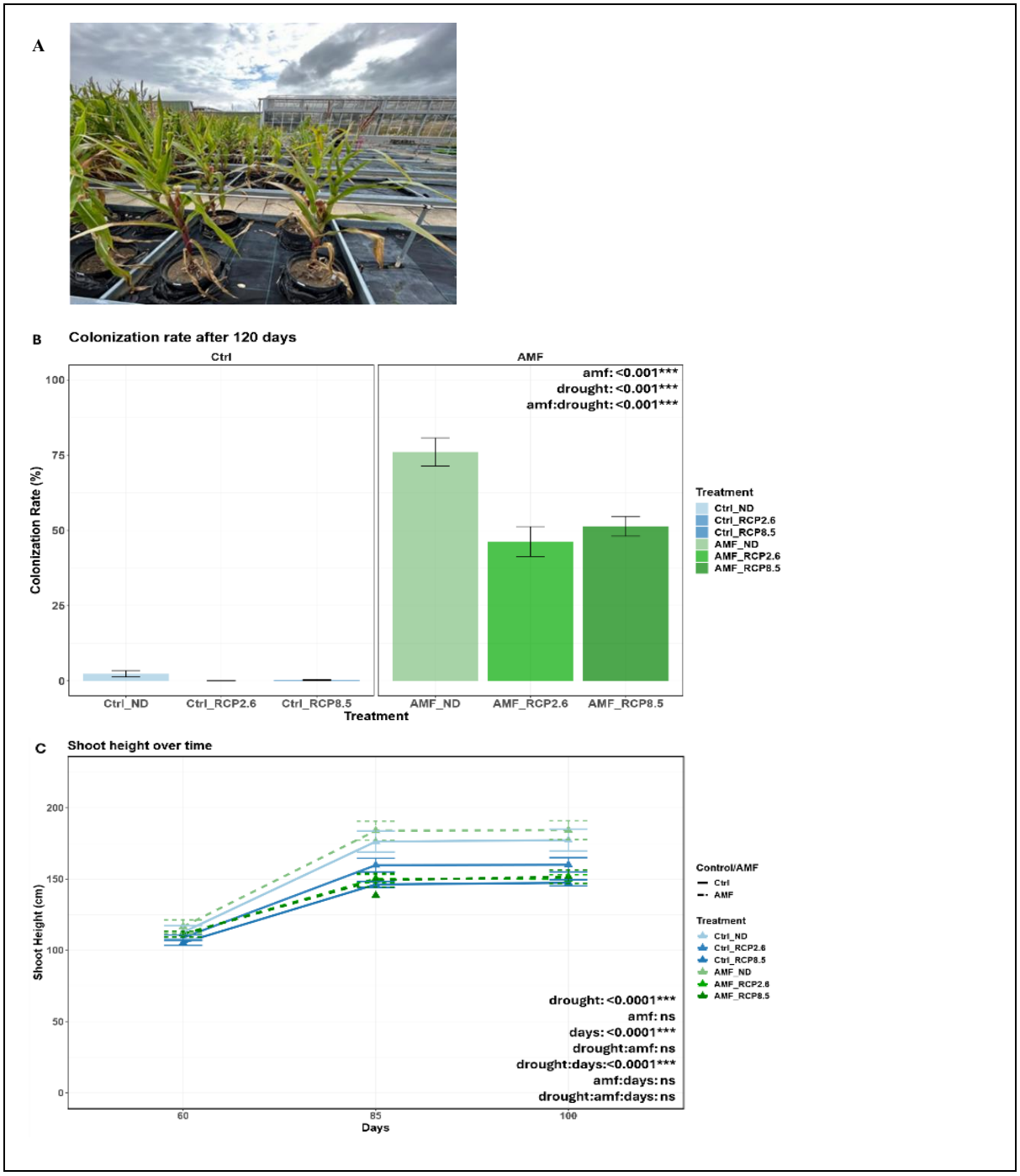
Statistical analyses

Statistical analyses and data visualization were done with R (version 4.4.2; R core team, 2018) using R studio (version 2024.12.0.467; Posit team, 2024). The data was read in with the package readxl (version 1.4.3). For organizing and structuring the data the package dplyr (version 1.1.4) was used. The semi-field assay and the herbivory assay followed a fully multifactorial design and the response variables were analyzed by using simple linear models. Explanatory variables were AMF presence or absence, water regimes, and for the herbivore assay presence or absence of herbivores. Homoscedasticity and normality of distribution of residuals were confirmed visually with the diagnostic plots of base R. If the model fit was not satisfactory, the tested variables were rank transformed prior to analysis. Two- and three-Way ANOVAs were used to detect the effects of response variables, depending on the number of variables in the experiment. For the insect performance data no effect of the experimental repetition could be observed, and thus the data of both experiments were combined for analysis. Plots were made using the package ggplot2 (version 3.5.1) and ggpattern (version 1.1.1).

RESULTS

Drought decreased maize growth, but AMF improved plant growth and reproductive success independently of soil moisture levels

A semi-field experiment was carried out to assess the effects of drought, AMF, and naturally occurring herbivores in conditions relatable to agriculture (Figure 1a). The addition of AMF increased colonization from 2.34% to 76.1% in ambient conditions and from 0% to 46.3% and 0.25% to 51.4% under RCP2.6 and RCP8.5 conditions respectively (Figure 1b). Drought further decreased shoot height already at day 60 and the effect intensified after 85 days but not further after 110 days (Figure 1c). Shoot biomass was also decreased under RCP2.6 and RCP8.5 drought conditions (Figure 1c, d). Drought further decreased leaf chlorophyll contents (Supplementary Figure 1). Conversely, AMF presence increased shoot biomass, cob length, and cob number (Figure 1d-f). No interactions between drought and AMF were observed on any of the measured growth and reproductive parameters under semi-field settings (Figure 1). Root biomass was not affected by treatments (Supplementary Figure 2). Field damage by herbivores was low and did not show a treatment effect (Supplementary Figure 3).


A
B
Colonization rate after 120 days
Ctrl
AMF
amf: <0.001***
drought: <0.001***
amf:drought: <0.001***
Colonization Rate (%)
100
75
50
25
0
Ctrl_ND
Ctrl_RCP2.6
Ctrl_RCP8.5
AMF_ND
AMF_RCP2.6
AMF_RCP8.5
Treatment
Treatment
Ctrl_ND
Ctrl_RCP2.6
Ctrl_RCP8.5
AMF_ND
AMF_RCP2.6
AMF_RCP8.5
C
Shoot height over time
Shoot Height (cm)
200
150
100
50
0
60
85
100
Days
drought: <0.0001***
amf: ns
days: <0.0001***
drought:amf: ns
drought:days:<0.0001***
amf:days: ns
drought:amf:days: ns
Control/AMF
Ctrl
AMF
Treatment
Ctrl_ND
Ctrl_RCP2.6
Ctrl_RCP8.5
AMF_ND
AMF_RCP2.6
AMF_RCP8.5

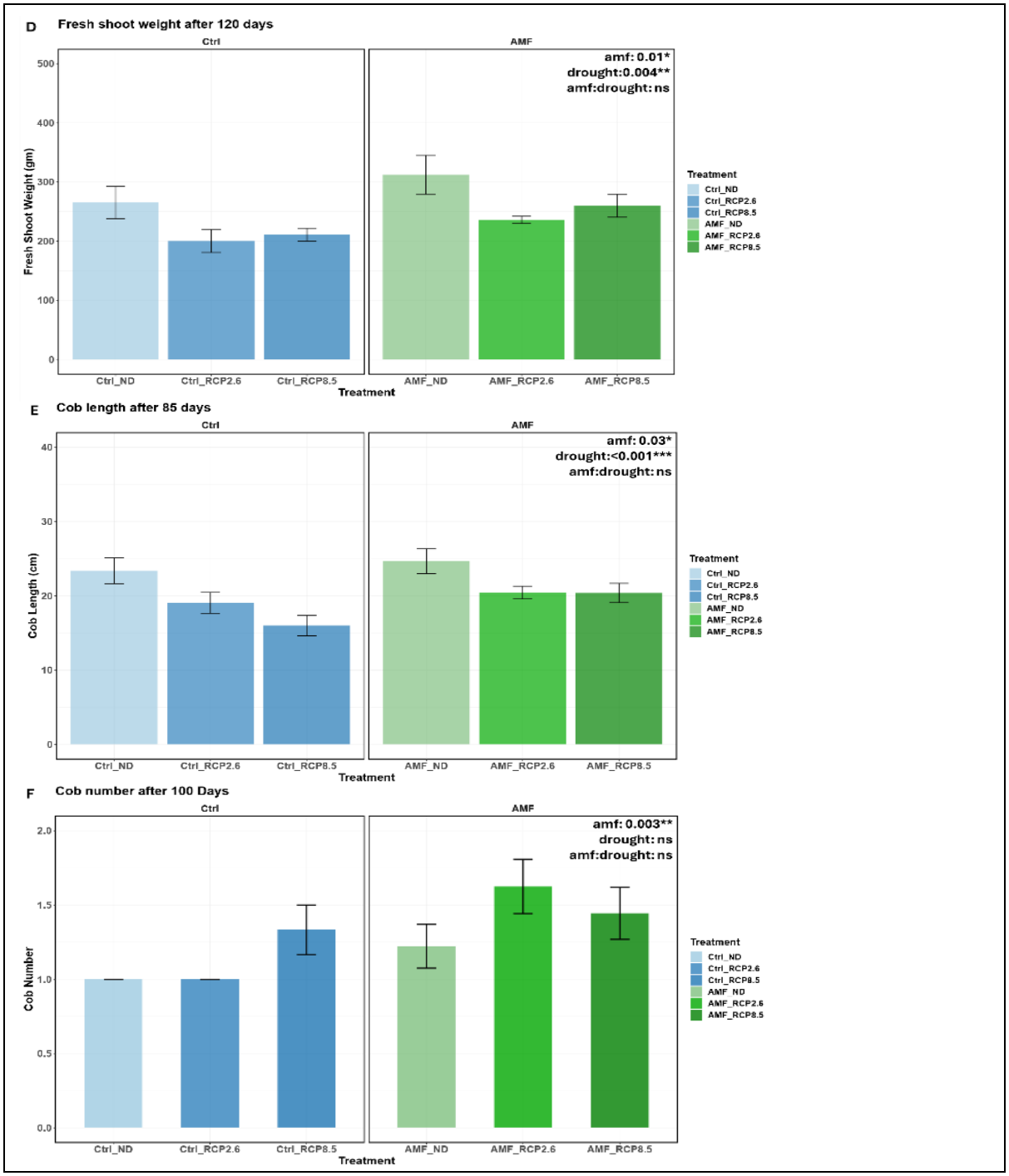


Figure 1. AMF colonization promotes shoot biomass and cob length independently of the moisture conditions. A) A photograph of the semi-field experiment, B) AMF colonization in inoculated plants after 120 days, C) mean shoot height over time, D) mean fresh shoot biomass after 120 days, E) mean cob length after 100 days, F) number of cobs after 100 days. Mean ± standard errors are shown (n = 9 per treatment) (n = 9). ND: Ambient soil moisture: 23% (v/v); Drought soil moisture: RCP2.6 and RCP8.5: 19% and 16.6% (v/v) respectively. AMF = Arbuscular mycorrhizal fungi, *Rhizophagus irregularis* (SAF22). ANOVA tests were run to analyze differences among treatments: ns: not significant; = 0.05<p<0.10, * = p<0.05, ** = p<0.01, *** = p<0.0001. Different letters indicate significant differences between treatments when interactions between AMF and drought were observed. Data on drought and AMF colonization effects on leaf chlorophyll contents, root biomass, and field damage are provided in Supplementary Figures 1-3.

AMF and drought modulated maize metabolism in semi-field conditions

In leaves, drought triggered transient changes in benzoxazinoids at day 60, reflected by a decrease in DIMBOA-Glc levels and an increase in DIM_2BOA-Glc leaf concentrations (Figure 2a; Supplementary Figure 4). The AMF-induced decrease in DIMBOA-Glc was stronger under ambient than drought conditions (Figure 2a; Supplementary Figure 4). AMF colonization was positively correlated with leaf sucrose and ABA concentrations (Supplementary Figure 5). At day 60, AMF colonization was negatively correlated with DIM_2BOA-Glc (Supplementary Figure 6). After 120-day, drought stress decreased sucrose and, albeit not significantly, glucose concentrations, but did not affect fructose levels in leaves (Figure 2b; Supplementary Figure 7). Drought did not affect leaf hormonal levels (Figure 2b; Supplementary Figure 7).

In roots, the prolonged drought increased fructose, JA, OPDA, SA, HMBOA-2Glc, and HM_2BOA-Glc levels (Figure 2c; Supplementary Figure 8). AMF presence increased fructose, glucose, and sucrose concentrations, and decreased OPDA and total benzoxazinoid levels, particularly through lowered concentrations of HMBOA-Glc and DIMBOA-Glc (Figure 2c; Supplementary Figure 8). Drought and AMF presence showed an interactive effect on fructose, as AMF-induced increase in fructose levels was stronger in the RCP2.6 drought scenario (Figure 2c; Supplementary Figure 8). A negative correlation between AMF colonization and HM_2BOA-Glc and DIMBOA-2Glc was observed (Supplementary Figure 9).

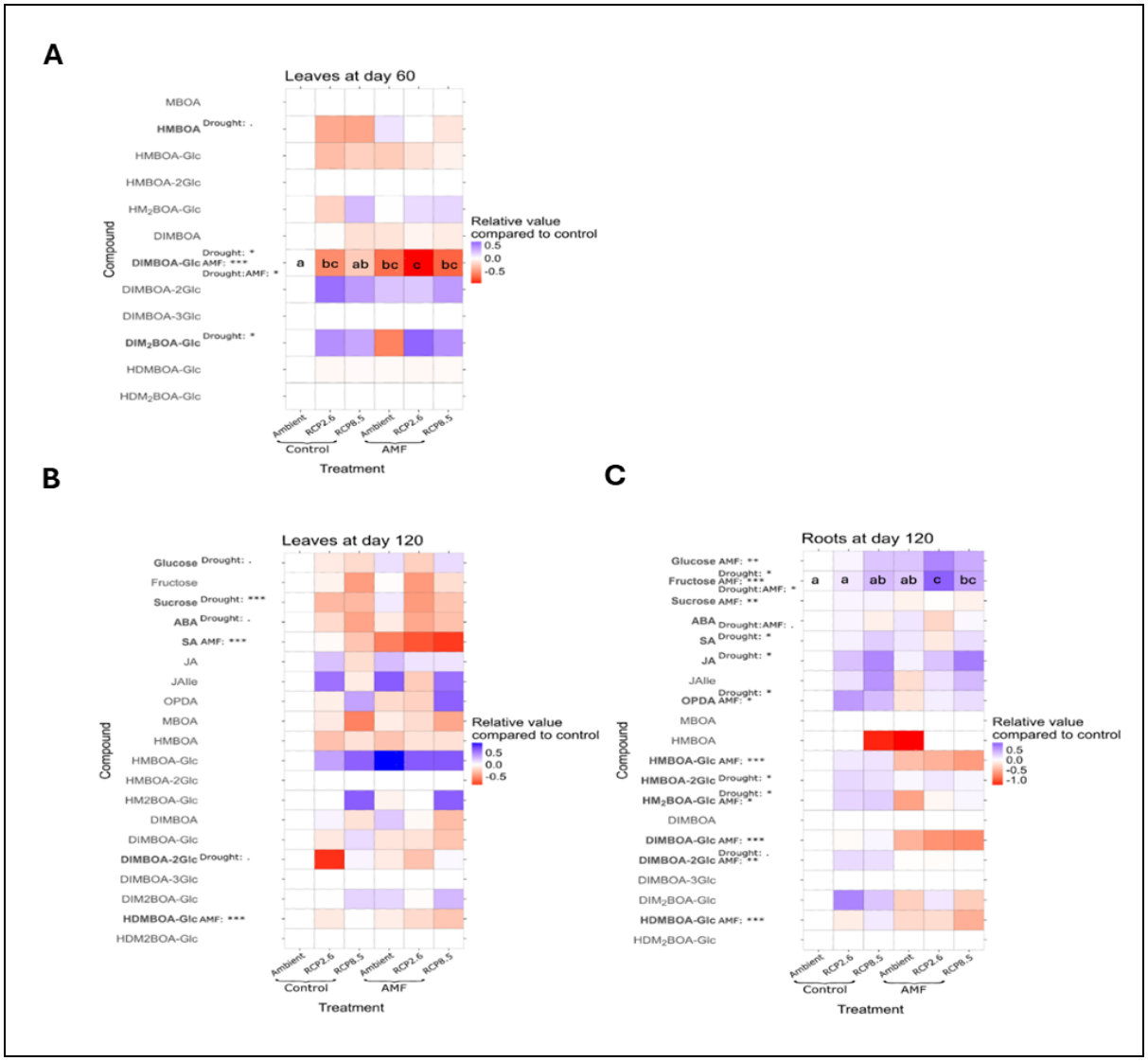


Figure 2. Drought and AMF modulate the maize metabolism. A. Heatmap of leaf metabolite concentrations – relative to concentrations in control plants under ambient conditions after 60 days, B. Heatmap of leaf metabolite concentrations relative to concentrations in control plants under ambient conditions after 120 days, C. Heatmap of root metabolite concentrations relative to concentrations in control plants under ambient conditions after 120 days. Ambient soil moisture: 23% (v/v); Drought soil moisture: RCP2.6 and RCP8.5: 19% and 16.6% (v/v) respectively. AMF = Arbuscular mycorrhizal fungi, *Rhizophagus irregularis* (SAF22). Data were log-transformed (n=9 per treatment). Compounds highlighted in bold showed significant differences. Stars indicate significant differences (linear model for each compound): *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$, = $0.05 < p < 0.1$. Different letters indicate significant differences between treatments when interactions between AMF and drought were observed. ND: Ambient soil moisture: 23% (v/v); Drought soil moisture: RCP2.6 and RCP8.5: 19% and 16.6% (v/v) respectively. AMF = Arbuscular mycorrhizal fungi, *Rhizophagus irregularis* (SAF22). Histograms for individual compound graphs are shown in Supplementary Figures 4, 5, and 8. Correlations between AMF colonization and ABA and between AMF colonization benzoxazinoids are shown in Supplementary Figures 6, 7, and 9.

AMF colonization limited drought-induced increase in insect performance

Drought and AMF showed individual and interactive effects on maize leaf benzoxazinoids in the field (Figure 2). Because the natural herbivore pressure in the field was low (Supplementary Figure 3), the potential effects of drought and AMF-mediated changes in benzoxazinoids on herbivore performance were assessed under controlled conditions. As in the semi-field assay, drought reduced AMF colonization, plant height, and shoot biomass (Supplementary Figure 10). Drought and AMF showed interactive effects on chlorophyll contents, as AMF-induced decrease in chlorophyll content was pronounced only under ambient conditions (Supplementary Figure 10).

After 2 months, plants were subjected to feeding of 5 *S. exigua* larvae for 5 days. The relative growth of the leaf herbivore *S. exigua* was not affected by AMF presence in soil but was slightly increased on plants that were subjected to drought than on plants that grew in ambient conditions (Figure 3a). While the herbivore performed better under drought conditions in the absence of AMF, the effect disappeared in the presence of AMF (Figure 3a). The leaf damage area was not affected by drought nor AMF (Figure 3b), but a significant correlation between the absolute mass gain of larvae and the leaf damage area was observed (Supplementary Figure 11).

In the leaves, levels of HMBOA-Glc and DIM_2BOA-Glc increased and HDM_2BOA-Glc decreased under drought conditions (Supplementary Figure 12). AMF alone showed no effect, but AMF presence induced an increase in DIMBOA-2Glc under drought, but not ambient, conditions (Supplementary Figure 12). Herbivory did not affect benzoxazinoid levels in leaves (Supplementary Figure 12).

In roots, drought increased the concentration of HMBOA-Glc, HMBOA-2Glc, HM2BOA-Glc, DIMBOA-2Glc, DIM_2BOA-Glc, and MBOA, while only HMBOA showed a decrease. AMF treatment affected DIMBOA-3Glc through elevated concentrations in AMF+ plants. Interactive effects between drought and AMF were observed for DIMBOA and DIMBOA-2Glc, yet following opposite trends. While DIMBOA levels were lower in AMF+ plants under drought treatment, DIMBOA-2Glc levels were increased in the same conditions. HMBOA-Glc and DIMBOA-Glc were increased under drought conditions when subjected to herbivory (Supplementary Figure 13).

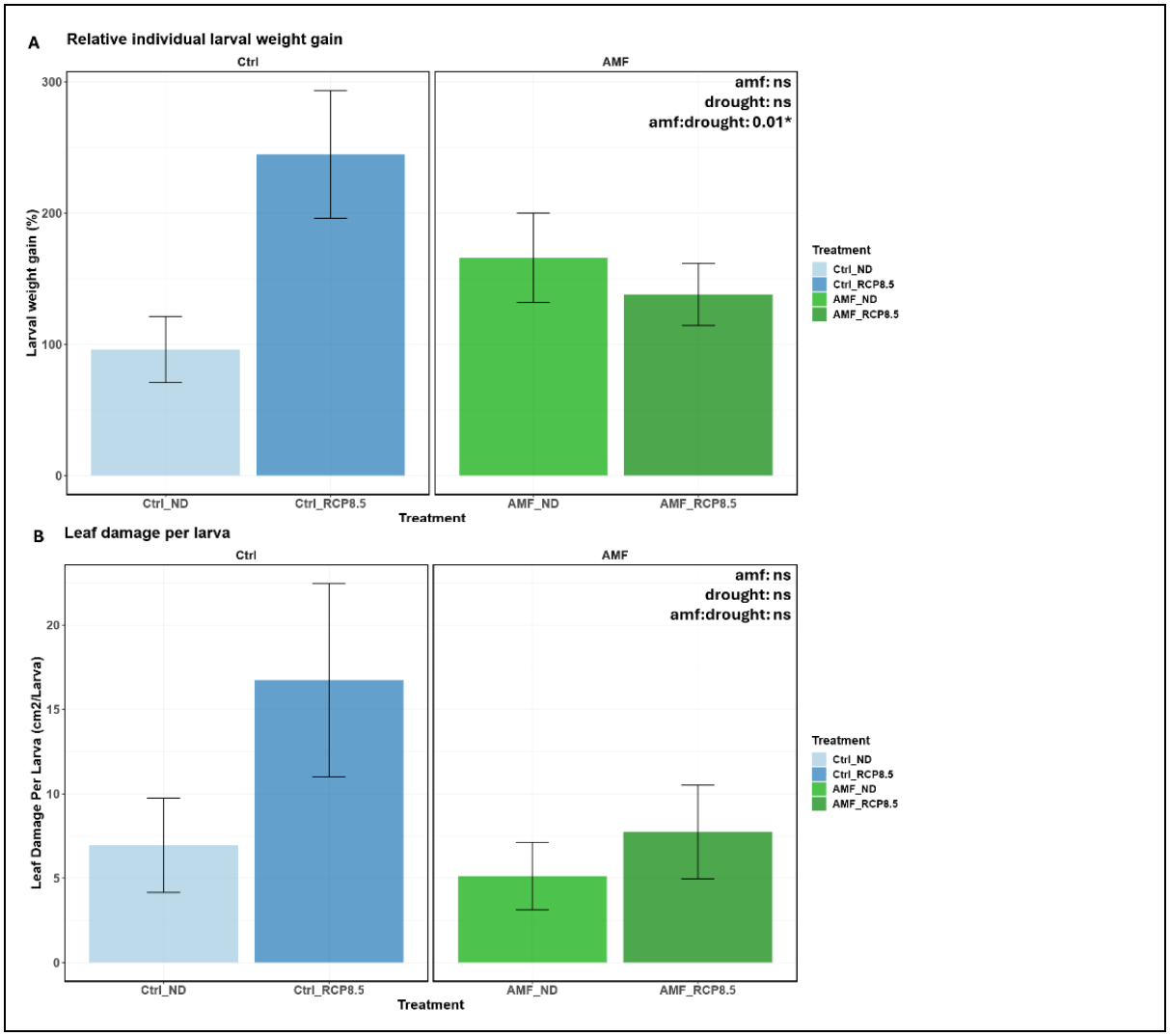


Figure 3. AMF alleviates the drought-mediated increase in insect performance. A. Relative individual weight gain. B. Leaf damage per larval mass gain. Mean ± standard errors are shown (n = 7-8 per treatment). ANOVA tests were run to analyze differences among treatments: ns: not significant; * = $p<0.05$. Different letters indicate significant differences between treatments when interactions between AMF and drought were observed. The effect of drought on AMF colonization under controlled conditions is shown in Supplementary Figure 10. The correlation between the larval mass gain and leaf damage area is shown in Supplementary Figure 11. Benzoxazinoid levels in maize leaves and roots are shown in Supplementary Figures 12 and 13. ND: Ambient soil moisture: 23% (v/v); Drought soil moisture (RCP8.5): 16.6% (v/v). AMF = Arbuscular mycorrhizal fungi, *Rhizophagus irregularis* (SAF22).

DISCUSSION

Our study revealed that drought significantly reduced maize vegetative growth, while AMF colonization improved plant growth and reproductive success independently of soil moisture levels. Under controlled conditions, drought increased herbivore performance, yet this effect was neutralized in AMF-colonized plants, suggesting that AMF may reduce drought-enhanced susceptibility to herbivory. Together, these findings highlight the potential of AMF to support maize reproductive performance and buffer biotic stress under drought.

Drought stress alone had clear effects on maize growth and metabolism, as well as on herbivore performance. Drought led to significant reductions in maize shoot height, biomass, and chlorophyll content, reflecting impaired photosynthetic capacity and overall plant vigor. These observations are consistent with previous studies showing that drought reduces maize performance (Deribe, 2024), although the extent of these effects can vary depending on genotype, developmental stage, and nutrient availability (Blein-Nicolas et ak., 2020; Liu et al., 2021). In roots, prolonged drought increased fructose and glucose concentrations, consistent with the known role of soluble sugars in osmotic adjustment and stress tolerance (Sepulva et al., 2022; Anjum et al., 2017). However, in leaves, drought reduced sucrose and tended to decrease fructose concentrations, while glucose levels remained unchanged. This partially contrasts with studies reporting whole-plant sugar accumulation under drought (Du et al., 2020; Mohammadkhani & Heidari, 2008), possibly due to differences in sampling time, tissue type, or drought severity (Sharma et al., 2019; Gurrieri et al., 2020). Regarding phytohormones, and despite clear wilting symptoms, drought did not affect ABA levels in roots or leaves, which was surprising given its well-established role in stomatal closure and drought signaling (Kim et al., 2010; Aslam et al., 2022). However, drought increased levels of JA, its precursor OPDA, and of SA, reflecting activation of general stress responses. Drought further led to increased concentrations of several benzoxazinoids in roots, including HMBOA-2Glc and HM_2BOA-Glc, and transiently altered DIMBOA-Glc and DIM_2BOA-Glc levels in leaves at day 60. These changes are consistent with the reported induction of benzoxazinoids under abiotic stress as part of plant defense and stress adaptation (Sutour et al., 2024; Robert & Mateo, 2022). Finally, in the herbivory assays, drought increased the performance of *S. exigua* larvae, suggesting that drought-induced changes in primary metabolites or reduced resistance mechanisms may have outweighed the effects of plant defenses. This aligns with previous studies showing that drought can increase herbivore growth by altering plant nutritional quality (Duell et al., 2024; Ximénez-Embún et al., 2017; Carvajal-Acosta et al., 2022).

Under ambient conditions, AMF colonization alone had significant effects on maize growth, yield, and defenses. AMF-inoculated plants showed increased shoot biomass, cob length, and cob number, consistent with the well-established role of AMF in promoting plant growth through improved nutrient acquisition and hormonal modulation (Bhupenchandra al., 2024). Root fructose and glucose concentrations increased under AMF treatment, suggesting enhanced carbon sink strength and possibly greater metabolic activity in roots, a pattern also reported in peach and tomato plants colonized by AMF (Mo et al., 2016; Ge et al., 2008). Interestingly, AMF colonization led to a reduction in root sucrose levels, possibly due to

increased sucrose cleavage or altered sugar transport dynamics, as seen in other studies where AMF modulated sugar transporter expression (Ge et al., 2008; Tang et al., 2022). In terms of hormonal signaling, AMF colonization decreased OPDA in roots and SA in leaves, contrasting with several reports that suggest AMF increase phytohormone levels under stress (Tang et al., 2022). This may indicate a shift toward resource allocation for growth rather than defense when stress levels are low. In secondary metabolism, AMF suppressed root benzoxazinoid levels, including HMBOA-Glc and DIMBOA-Glc, possibly reflecting a trade-off in which improved nutrient status and physiological condition reduce the need for costly chemical defenses. The reduction in constitutive defense compounds under ambient conditions could also imply that AMF-colonized plants rely more on induced defenses or tolerance strategies. However, AMF colonization can also lead to enhanced accumulation of defense metabolites such as DIMBOA under pathogen attack, suggesting a complex context-dependent regulation (Song et al., 2011). In the controlled assay, AMF colonization did not alter benzoxazinoid levels in leaves under ambient conditions. This difference could reflect environmental or developmental factors, as the semi-field experiment involved a longer growth period and greater exposure to fluctuating conditions, possibly inducing stronger AMF-mediated reprogramming of defense metabolism. Consistently, AMF colonization alone did not reduce *S. exigua* growth in the herbivore assays. This aligns with earlier findings indicating that AMF-mediated resistance is often context-dependent and may require either a co-occurring stress or stronger defense priming signals to translate into reduced herbivore performance. For instance, AMF boosted resistance to *S. littoralis* in JA-deficient tomatoes, an effect that was only pronounced when defense pathways were compromised (Formenti & Rasmann, 2019). Overall, these findings highlight the multifaceted role of AMF in modulating maize metabolism, supporting both growth and fine-tuned defense regulation even in the absence of external stressors.

Interactive effects between AMF and drought on maize physiology and metabolism were limited in the semi-field assay but became more apparent under controlled conditions. Consistently with previous studies, drought reduced AMF colonization (Orine et al.; 2022). In the field, AMF and drought affected maize metabolism largely independently with observed interactive effects being limited to root fructose levels and leaf DIMBOA-Glc contents. AMF increased root fructose and the effect that was more pronounced under drought conditions. Such context-dependent enhancement of sugar accumulation may indicate that AMF contribute to osmotic adjustment under moderate water stress as suggested in previous studies (Bahadur et al., 2019; Chandrasekaran & Paramasivan, 2022). At day 60, AMF reduced DIMBOA-Glc concentrations in leaves more strongly under ambient than drought conditions, suggesting

drought constrained the AMF effect. Interestingly, while AMF alone had no effect on herbivore performance, their presence cancelled the drought-induced increase in *S. exigua* growth observed in non-mycorrhizal plants. This suggests that AMF conferred drought-associated protection, possibly through improved nutritional balance or defense priming. The AMF-mediated dampening of drought-induced increases in herbivore performance highlights their potential as a valuable biological tool for promoting crop resilience and reducing reliance on chemical pest control in sustainable agricultural systems.

This study demonstrates that AMF can enhance maize reproductive success and modulate plant metabolism under both well-watered and drought conditions, with additional benefits under combined abiotic and biotic stress. While drought reduced plant growth and increased herbivore performance, AMF colonization improved yield-related traits and mitigated drought-induced susceptibility to herbivory. The context-dependency of AMF effects, particularly their modulation of benzoxazinoids and defense signaling under variable environmental conditions, emphasizes the need for integrated, multi-factorial studies to understand plant responses in realistic scenarios. From a practical perspective, the ability of AMF to buffer drought-enhanced herbivore pressure offers promising opportunities for sustainable agriculture, reducing the need for external inputs while supporting crop resilience. Future research should aim to elucidate the mechanistic basis of these interactions across diverse plant and AMF genotypes, and under fluctuating field conditions, to better harness the full potential of AMF for climate-smart crop management.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY

All data will be provided as supplementary material upon acceptance of the manuscript.

FIGURE LEGENDS

Figure 1. **AMF colonization promotes shoot biomass and cob length independently of the moisture conditions**. A) A photograph of the semi-field experiment, B) AMF colonization in inoculated plants after 120 days, C) mean shoot height over time, D) mean fresh shoot biomass after 120 days, E) mean cob length after 100 days, F) number of cobs after 100 days. Mean ± standard errors are shown (n = 9 per treatment) (n = 9). ND: Ambient soil moisture: 23% (v/v); Drought soil moisture: RCP2.6 and RCP8.5: 19% and 16.6% (v/v) respectively. AMF = Arbuscular mycorrhizal fungi, *Rhizophagus irregularis* (SAF22). ANOVA tests were run to analyze differences among treatments: ns: not significant; = 0.05<p<0.10, * = p<0.05, ** = p<0.01, *** = p<0.0001. Different letters indicate significant differences between treatments when interactions between AMF and drought were observed. Data on drought and AMF colonization effects on leaf chlorophyll contents, root biomass, and field damage are provided in Supplementary Figures 1-3.

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Supplementary Table 1. Meteorological data during the semi-field assay

Calendar week	Mean soil temperature at 5 cm depth [°C]	Mean air temperature 2 m above ground [°C]	Total precipitation [mm]	Mean daily sunshine duration [h]
22	15.00	12.80	7.60	0.70
23	17.87	17.20	27.20	4.87
24	18.39	14.94	26.70	5.10
25	20.30	18.54	36.80	6.11
26	20.67	20.10	5.50	6.16
27	20.46	17.41	35.00	4.10
28	21.33	19.49	27.50	7.21
29	22.27	20.90	17.00	9.31
30	23.01	21.14	2.20	8.51
31	23.26	22.26	5.80	8.83
32	22.64	21.47	19.40	10.71
33	23.27	22.50	64.40	9.17
34	20.89	18.56	2.70	8.09
35	20.76	19.87	2.80	7.81
36	21.31	19.46	15.10	6.37
37	16.66	11.86	18.90	2.64
38	14.11	11.77	1.90	5.67
39	15.10	13.07	60.50	2.66
40	13.17	10.73	37.30	3.07
41	12.55	11.00	1.40	2.15

Supplementary Table 2. Soil profile analysis

Soil Characteristics

Parameter	Unit	Result	Method	Interpretation/Category
Humus	% G/G	2.0	Texture Test (FP)	Low in Humus
Clay	% G/G	11.0	Texture Test (FP)	Very sandy loam
Silt	% G/G	31.0	Texture Test (FP)	
pH Value		7.5	pH (1:2.5 H_2O)	Slightly alkaline

1.1. Available Nutrients (H_2O10)

Nutrient	Unit	Result	Correction Factor	Supply Level
Nitrate	mg/kg	351.4		Enriched
Phosphorus	mg/kg	5.0	1.2	Moderate
Potassium	mg/kg	394.8	0.0	Enriched
Calcium	mg/kg	325.6		Stock
Magnesium	mg/kg	42.6	0.4	Stock

1.2. Reserve Nutrients (AAE10)

Nutrient	Unit	Result	Correction Factor	Supply Level
Phosphorus	mg/kg	276.8	0.0	Enriched
Potassium	mg/kg	1031.5		Enriched
Calcium	mg/kg	325.6	0.4	Poor
Magnesium	mg/kg	373.0	0.2	Stock

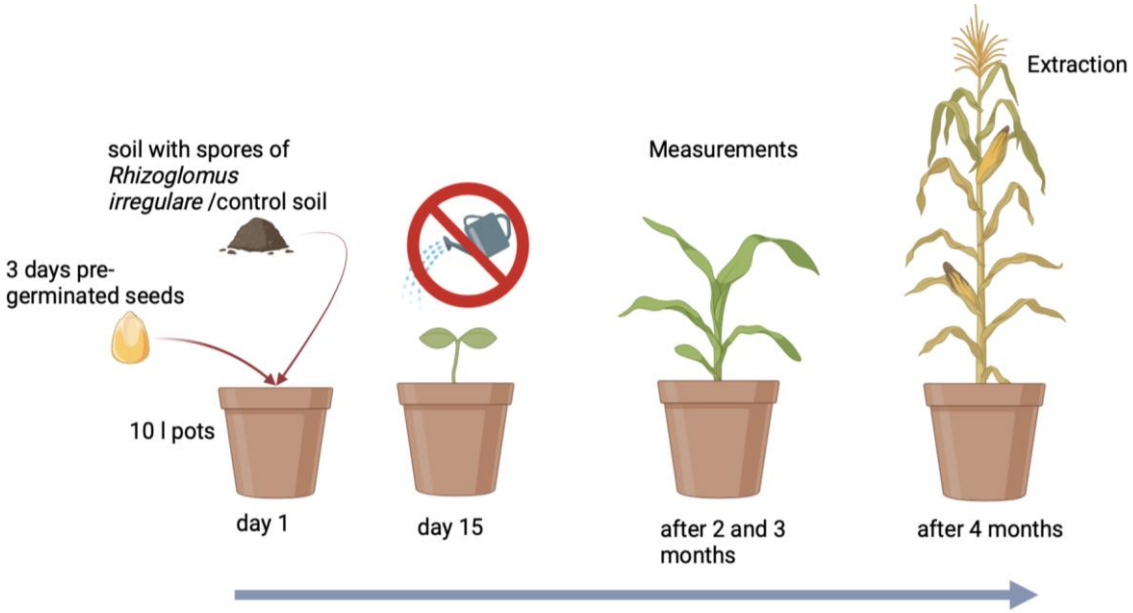
1.3. Trace Elements

Nutrient	Unit	Result	Correction Factor	Supply Level
Boron	mg/kg	1.4		Enough
Manganese	mg/kg	315		Stock
Copper	mg/kg	14.1		Stock
Iron	mg/kg	938		Enriched

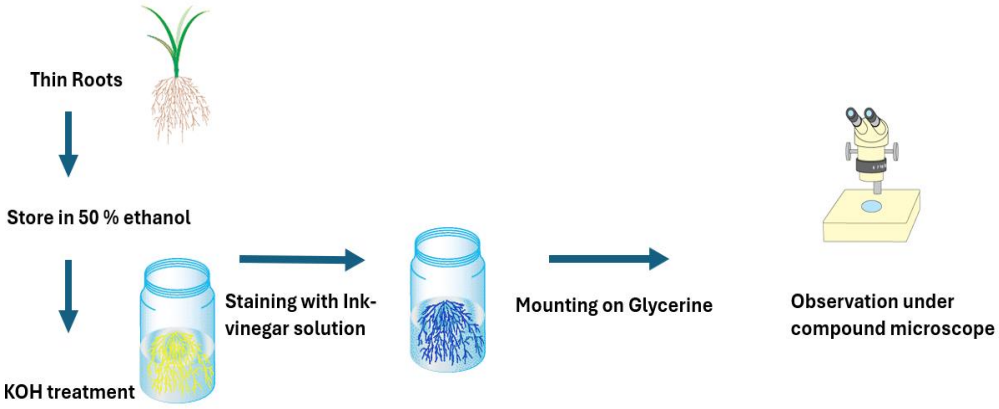
Supplementary Table 3. Benzoxazinoid names and chemical formulas

Name	Chemical name	Chemical formula
HMBOA	2-Hydroxy-7-methoxy-1,4-benzoxazin-3-one	$C_9H_9NO_4$
HMBOA-Glc	2-Hydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside	$C_{15}H_{19}NO_9$
HMBOA-2Glc	2-Hydroxy-7-methoxy-1,4-benzoxazin-3-one diglucoside	$C_{21}H_{29}NO_{14}$
HM_2BOA-Glc	2-Hydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one glucoside	$C_{16}H_{21}NO_{10}$
DIMBOA	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one	$C_9H_9NO_5$
DIMBOA-Glc	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside	$C_{15}H_{19}NO_{10}$
DIMBOA-2Glc	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one diglucoside	$C_{21}H_{29}NO_{15}$
DIMBOA-3Glc	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one triglucoside	$C_{27}H_{39}NO_{20}$
DIM2BOA-Glc	2,4-Dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one glucoside	$C_{16}H_{21}NO_{11}$
HDMBOA-Glc	2-Hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside	$C_{16}H_{21}NO_8$
HDM2BOA-Glc	2-Hydroxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one glucoside	$C_{17}H_{23}NO_{11}$
MBOA	6-Methoxybenzoxazolin-2-one	$C_8H_7NO_3$

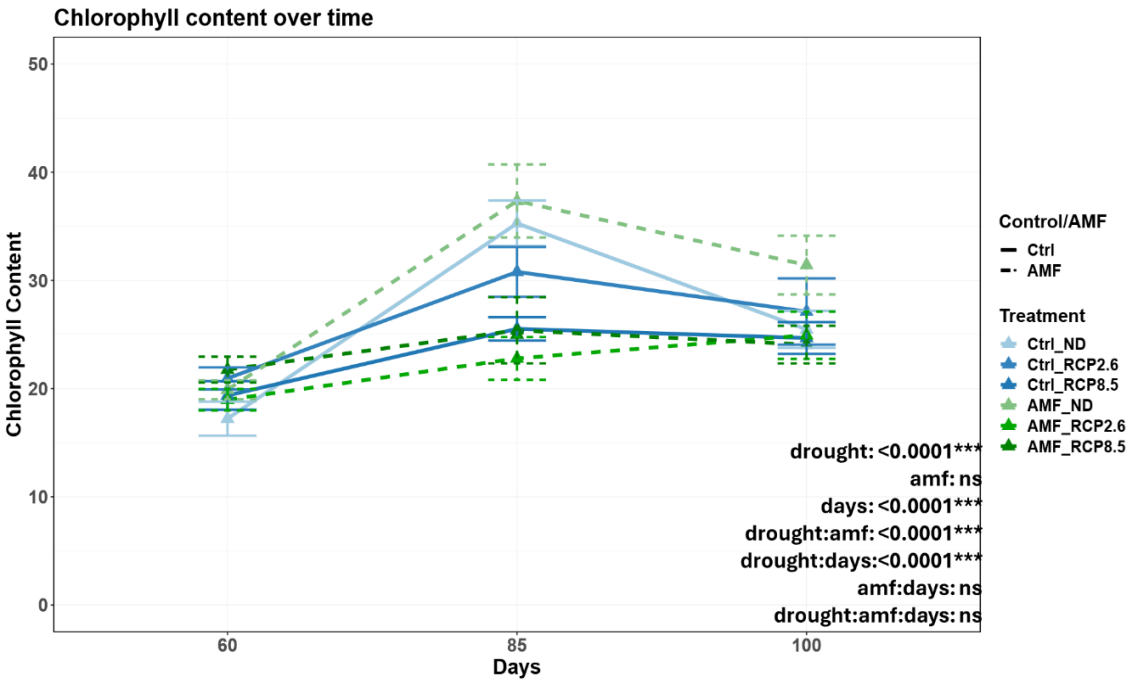
Supplementary Figure 1. Experimental design for the semi-field experiment in Ostermundigen in Summer 2024.



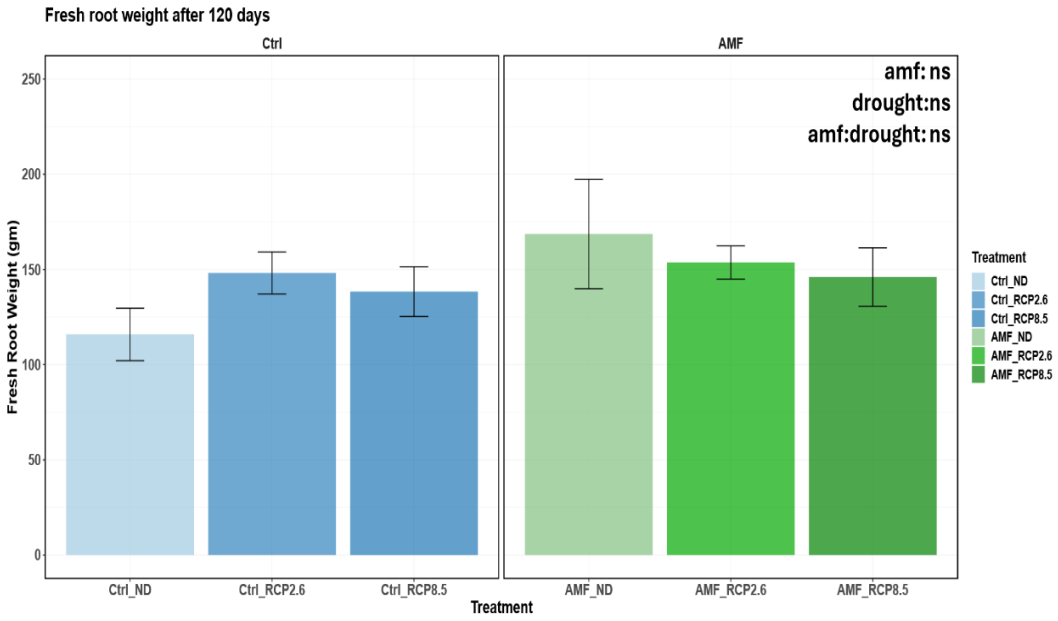
Supplementary Figure 2. Preparation of roots for the microscopic analysis (Mark Brundrett, 2008).



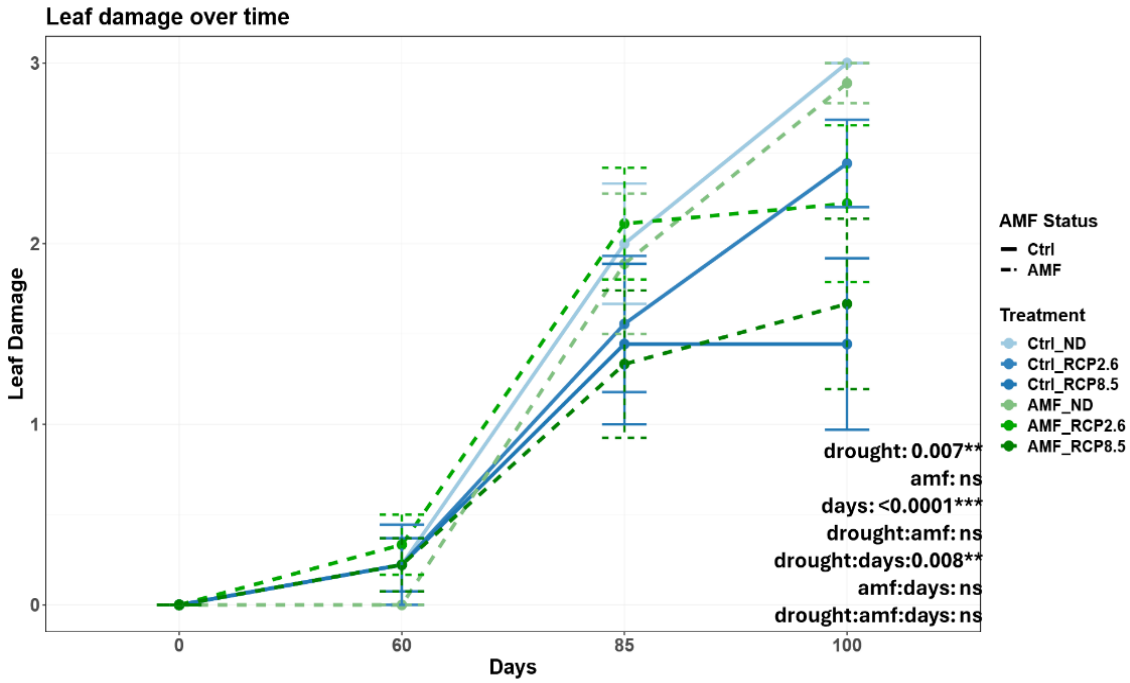
Supplementary Figure 3. Maize leaf chlorophyll contents development over time in the field



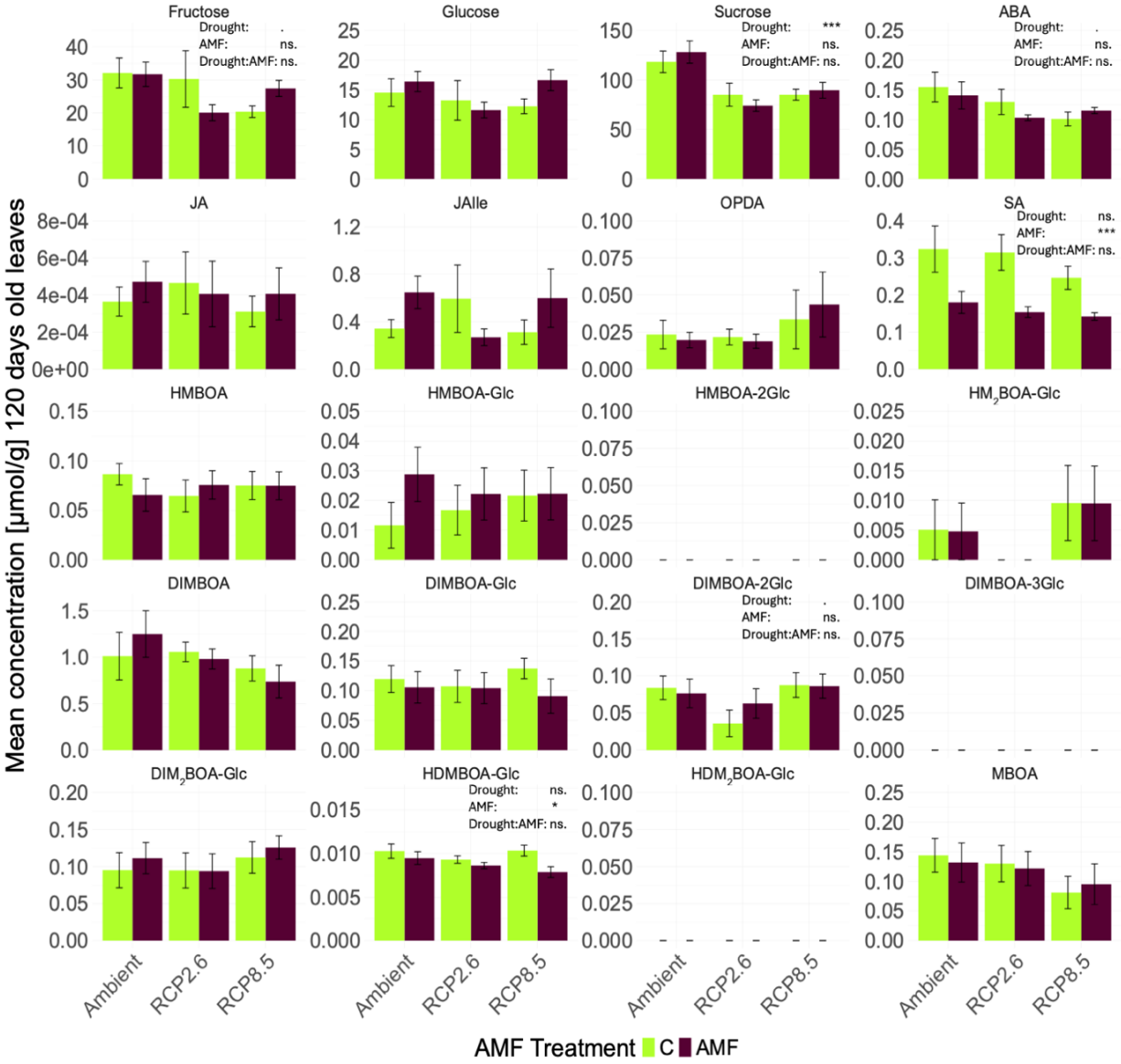
Supplementary Figure 4. Maize root biomass in the field at day 120



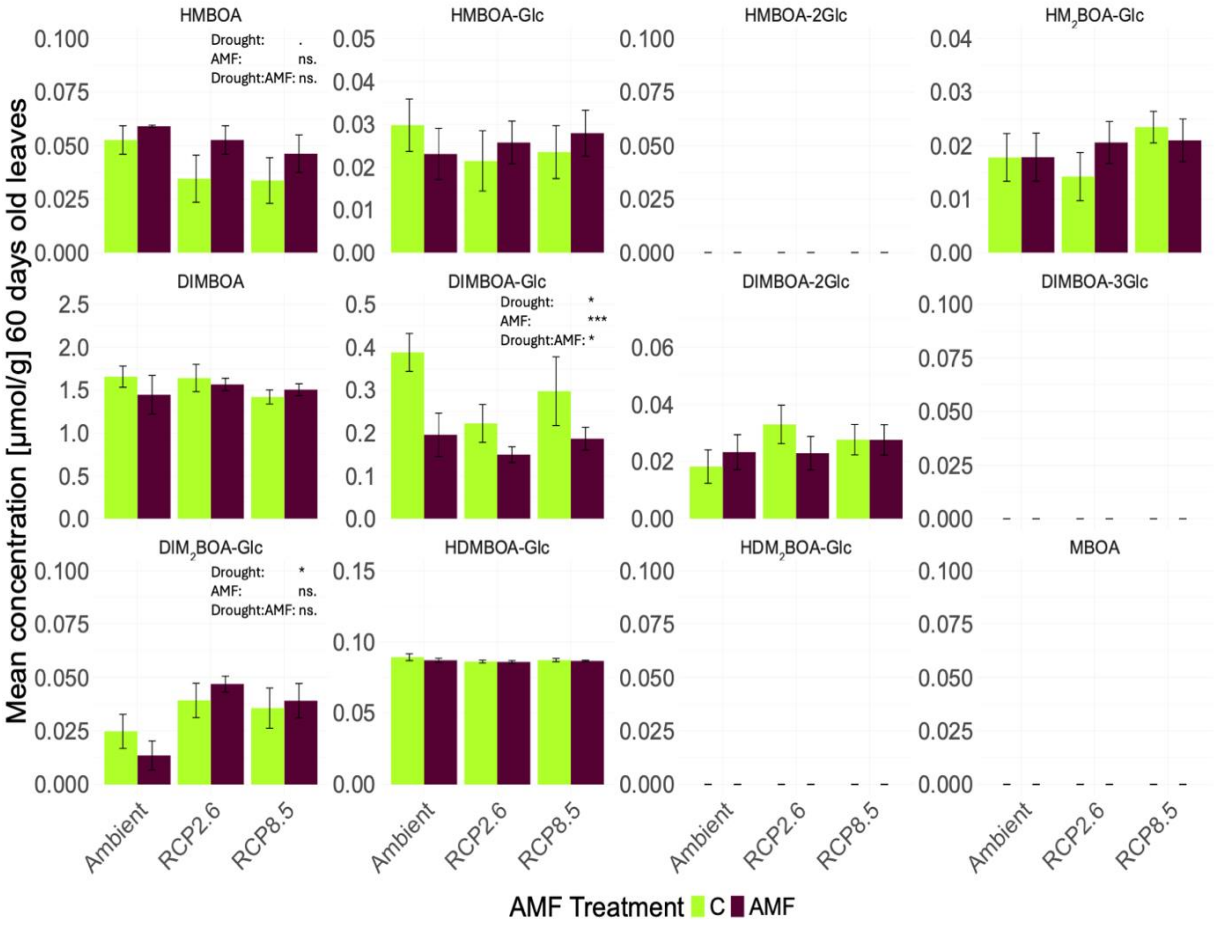
Supplementary Figure 5. Herbivore damage development over time in the field



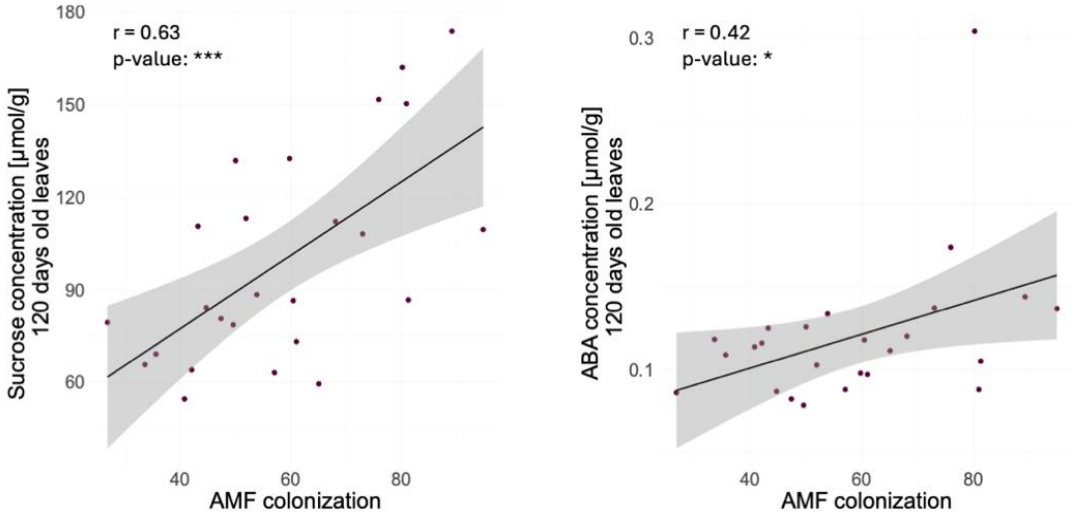
Supplementary Figure 6. Drought had effect on Sucrose and AMF on SA and HDMBOA-Glc in maize leaves in the field at day 120



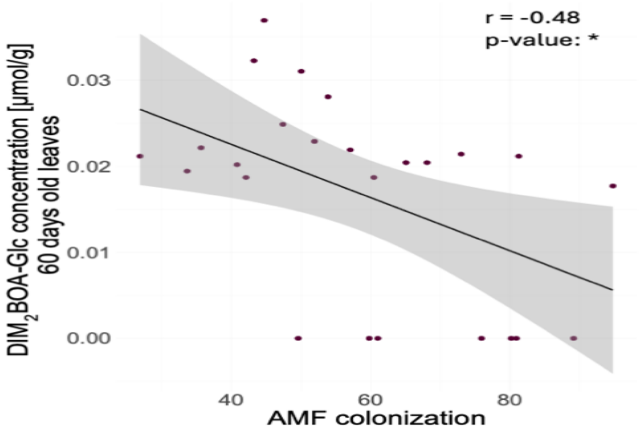
Supplementary Figure 7. Drought and AMF have interactive effects on DIMBOA-Glc in maize leaves in the field at day 60



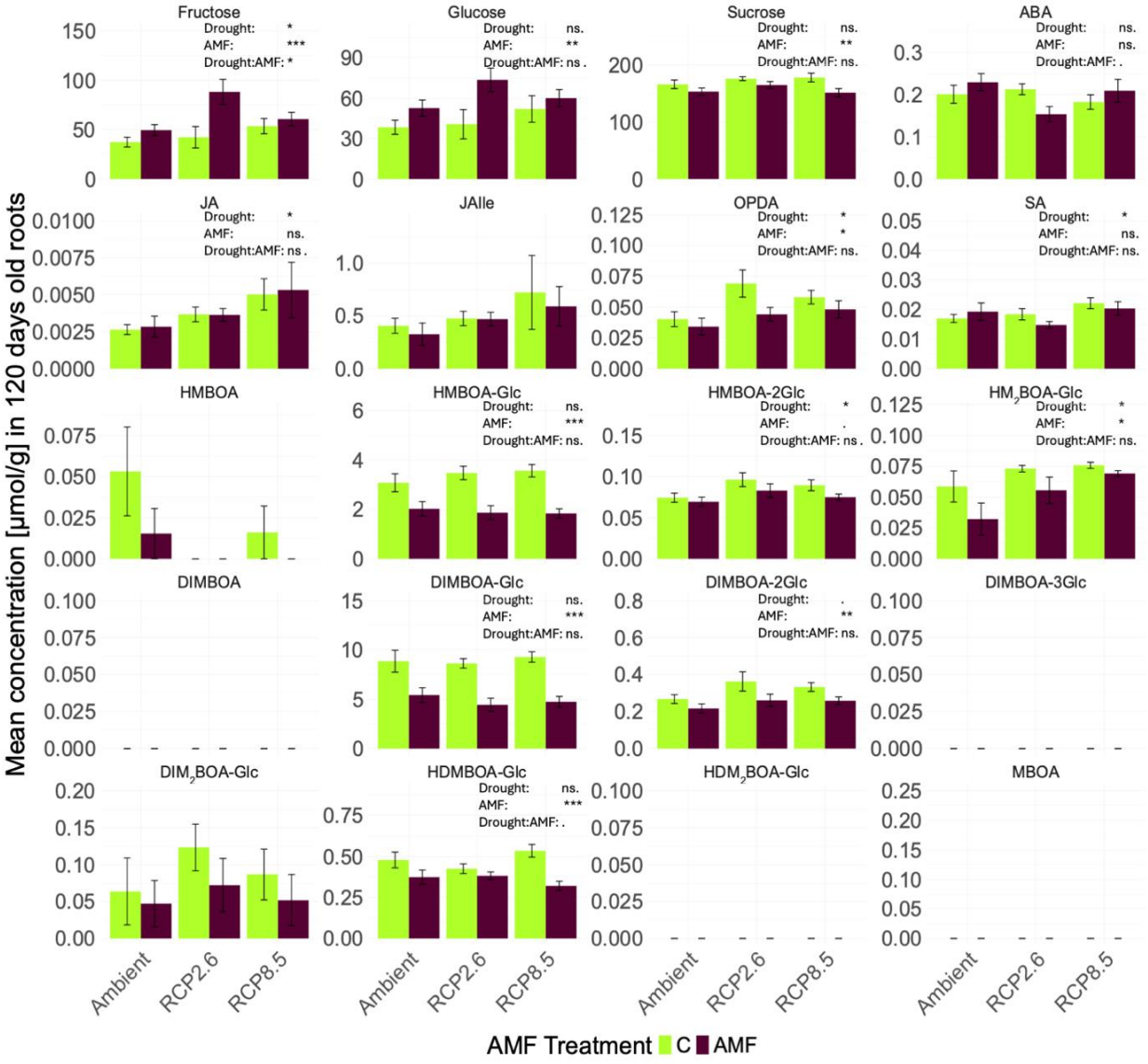
Supplementary Figure 8. AMF colonization had effect on sucrose and ABA levels in maize leaves in the field at day 120



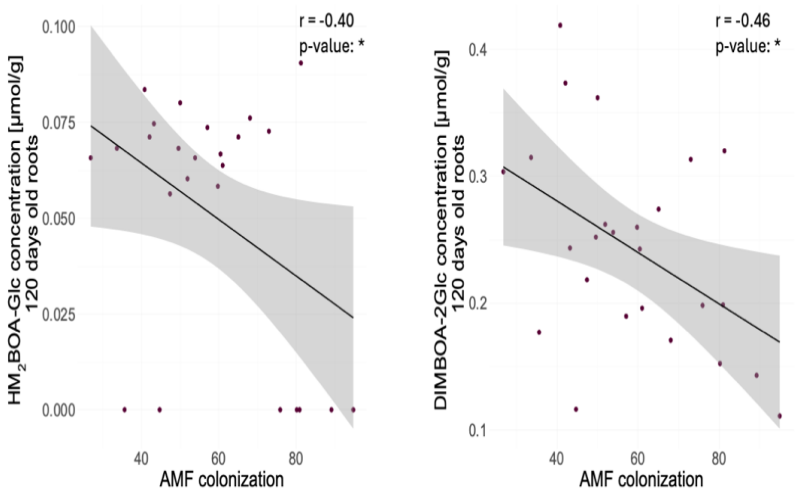
Supplementary Figure 9. AMF colonization had effect on DIM2BOA-Glc levels in maize leaves in the field at day 60



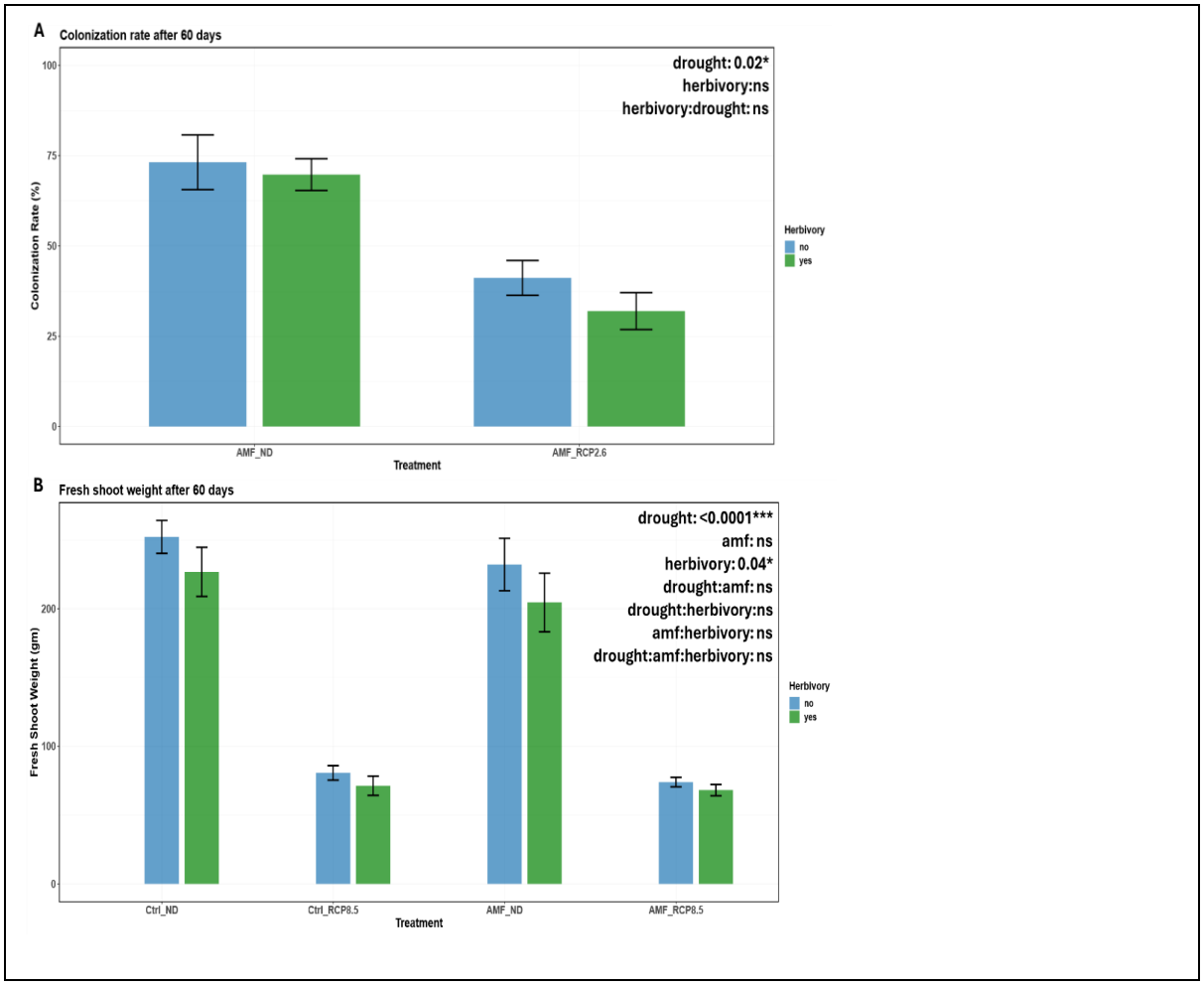
Supplementary Figure 10. AMF affected soluble sugar levels in maize roots in the field at day 120

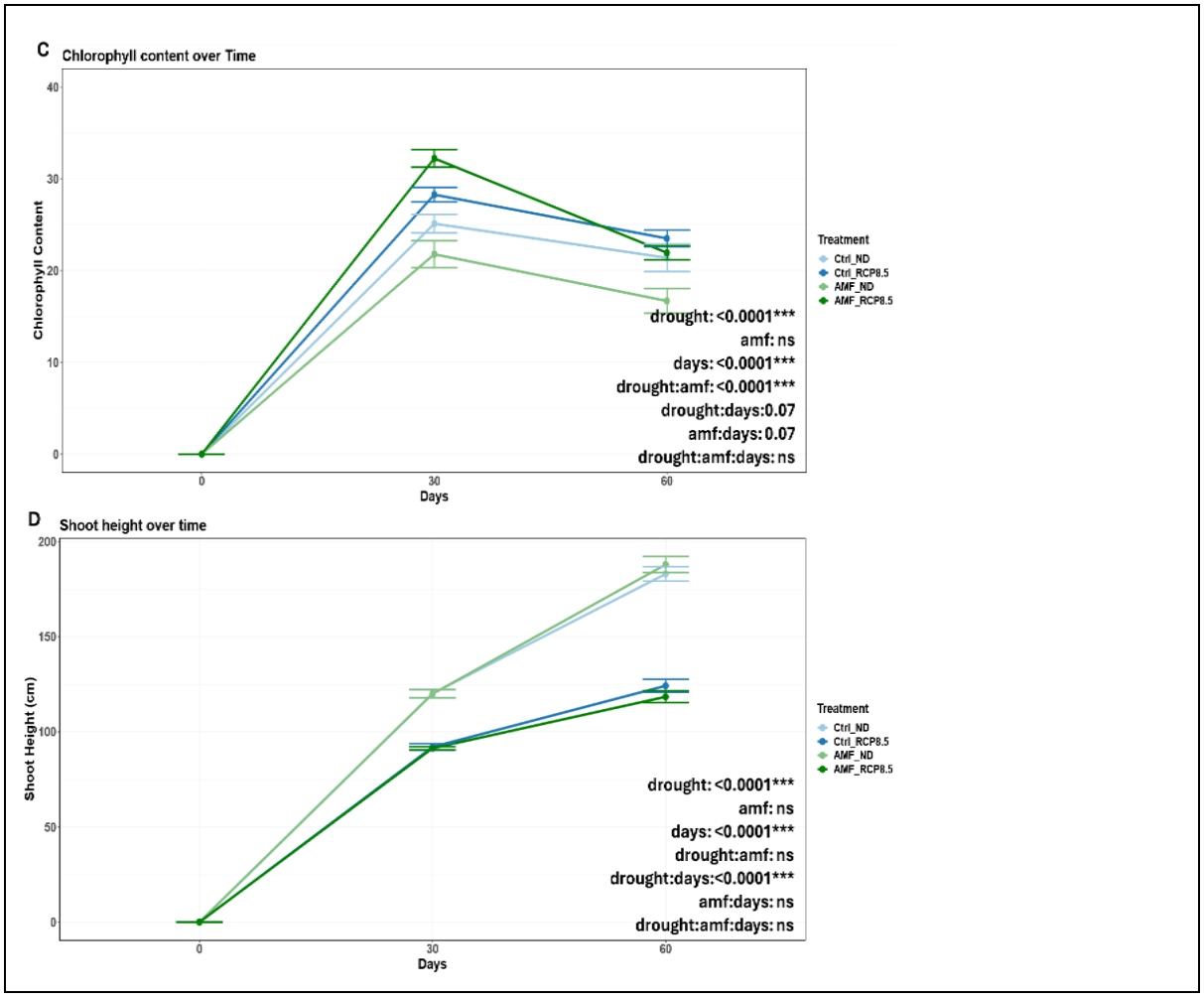


Supplementary Figure 11. AMF colonization had effect on HM2BOA-Glc and DIMBOA-2Glc levels in maize roots in the field



Supplementary Figure 12. Drought reduced AMF colonization under controlled conditions in herbivory assay 2

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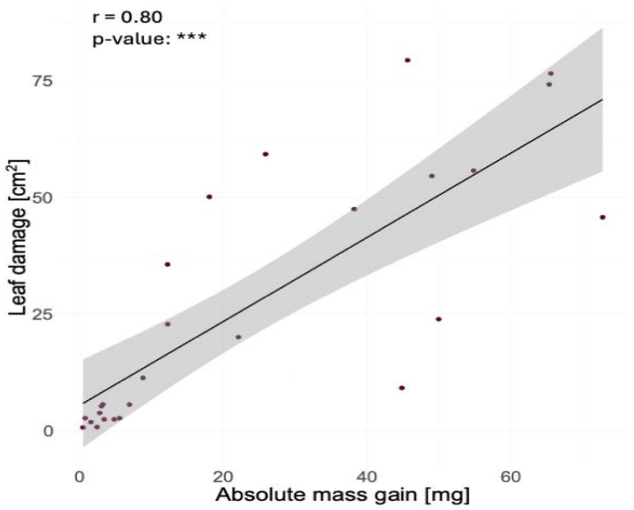
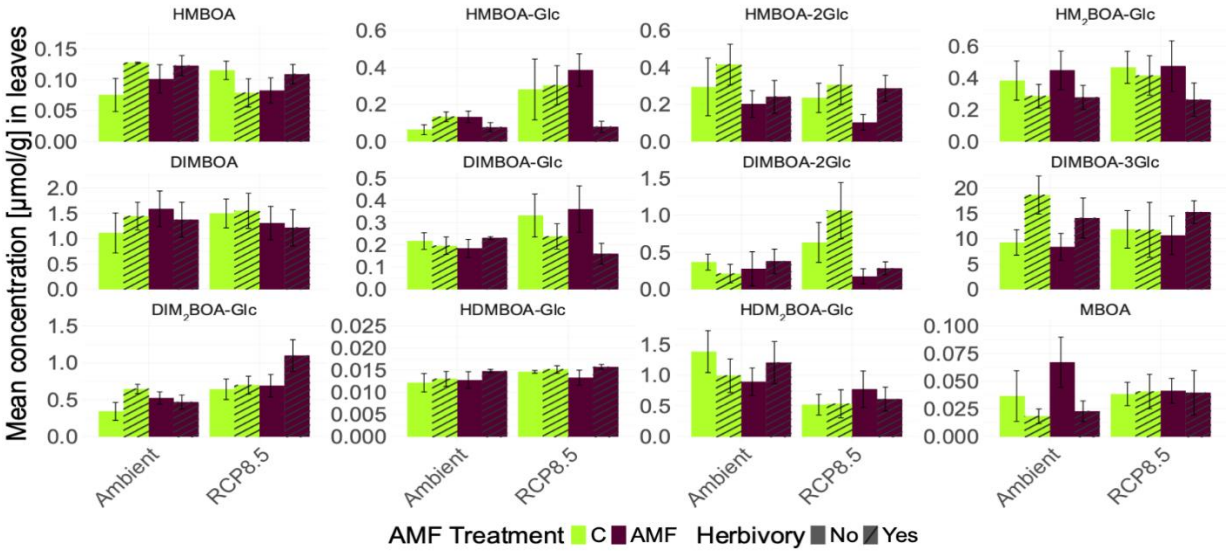
Supplementary Figure 13. Absolute larvae mass gain and leaf damage area are positively correlated in herbivory assays



Supplementary Figure 14. Drought increase HMBOA-Glc and DIM2BOA-Glc and decreased HDM2BOA-Glc levels in maize leaves in herbivory assay (greenhouse)



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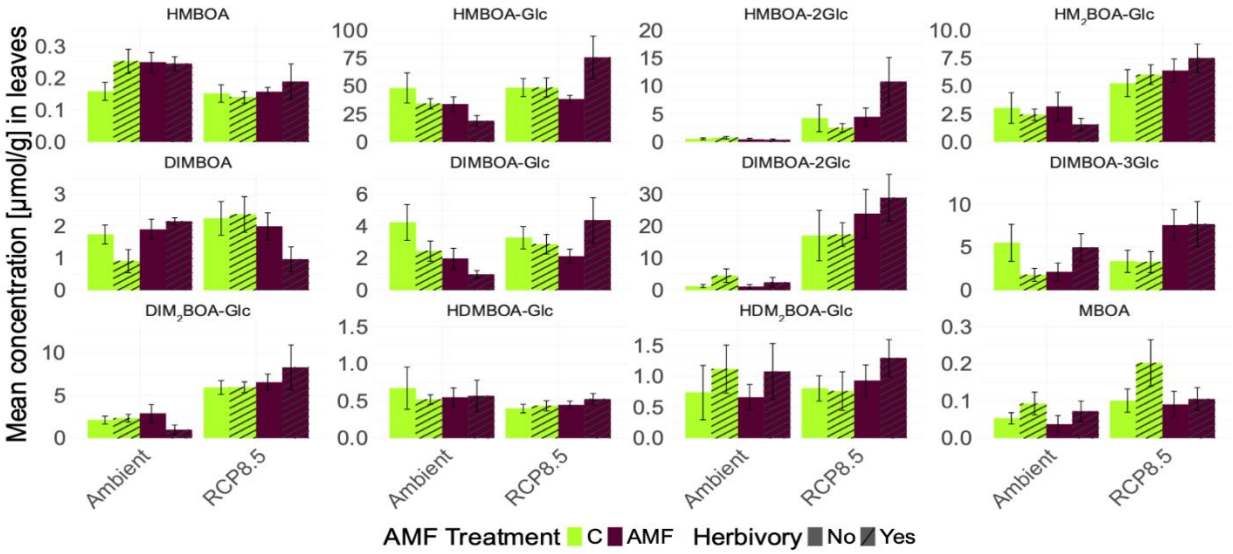
Supplementary Figure 15. Drought increased HMBOA-Glc, HMBOA-2Glc, HM2BOA-Glc, DIMBOA-2Glc, DIM2BOA-Glc and MBOA levels in maize roots in herbivory assay 2



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Chapter II.

The Benzoxazinoid-Derivative MBOA Improves Arbuscular Mycorrhizal Fungi Colonization in Maize

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ABSTRACT

Drought is one of the most devastating environmental stressors impacting crop productivity in agricultural ecosystems. Arbuscular mycorrhizal fungi (AMF) forms association with plants providing them with increased water and nutrient uptake, thereby enhancing plant resilience against drought. Benzoxazinoids (BXDs) are secondary metabolites actively involved in plants defences against drought, though their role in forming these symbiotic associations remains unclear. In this study, we evaluated the role of BXDs in facilitating the establishment of symbiotic associations with the arbuscular mycorrhizal fungi *Rhizophagus irregularis*. We combined a semi-field experiment with greenhouse assay involving *bx1* mutants complemented with MBOA to assess growth, metabolism and AMF colonization. Additionally, we also investigated the impact of kinetic drought on the rate of colonization. In the semi field assay, drought increased DIMBOA, DIMBOA-Glc, DIM_2BOA-Glc and DIMBOA-2Glc concentration in maize roots while AMF decreased DIMBOA, DIMBOA-Glc, DIM_2BOA-Glc concentration after 60 days. In the *bx1* mutant assay, AMF increased fresh shoot weight while MBOA complementation increased colonization rate in *bx1* mutant plants after 20 days. Kinetic drought had no impact on the rate of colonization of AMF with the maize plants. Overall, drought enhanced production of maize secondary metabolites, an effect which was minimized in the presence of AMF. In *bx1* mutant plants, MBOA addition increased the colonization rate highlighting their potential role in signalling and symbiotic formation. These findings highlight the need to better understand the BXDs role in plant defences and symbiotic interactions to develop better strategies for crops experiencing drought stress.

Keywords:

Arbuscular Mycorrhizal Fungi (AMF), Drought, Benzoxazinoids, MBOA, Maize

INTRODUCTION

Plants produce a wide range of low molecular weight organic compounds (Seregin et al., 2024, which can be divided into three categories based on their functions: primary metabolites are needed for plant growth (Salam et al., 2023), secondary or specialized metabolites are required for plant-environmental interactions including attraction, repelling and defense reactions, and hormones, which are involved in regulation of organismal processes and metabolism (Erb and Kliebenstein, 2020). Approximately 200,000 secondary metabolites are produced across the plant kingdom (Dixon, 2003). Some secondary metabolites are toxic owing to their instability and capacity to react with other compounds (Akbar et al., 2024); they play a key role in plants defense by influencing interactions with the environment and ultimately shaping plant fitness and survival.

Benzoxazinoids (BXDs) are well recognized plant specialized metabolites found in wheat (*Triticum* spp.) (Gfeller et al., 2023), rye (*Secale cereale*), maize (*Zea mays*) and other poaceae members (Kukobo et al., 2017). Benzoxazinoids are also found in some dicot species belonging to the Anthaceae, Lamiaceae and Scrophulariaceae (Schullehner et al., 2008). The pathway for BXDs starts with the formation of indole catalysed by the enzyme indole 3-glycerol phosphate lyase (IGL) (*ZmBX1*). These compounds can be glycosylated by UGTs into double and triple hexoses which biologically inactivates them, preventing autotoxicity within the producing plant (Robert & Mateo, 2022, Florean et al., 2023).

Maize plants produce multihexose BXDs when subjected to drought conditions. Drought enhanced production of DIMBOA-2Glc, DIMBOA-3Glc, HMBOA-2Glc, HMBOA-3Glc, and HDMBOA-2Glc in roots and leaf tissues of seven days old maize seedlings, an effect observed across various maize lines (Sutour et al., 2024). A study investigating the genomic basis of maize adaptation to drought stress revealed enhanced expression of *ZmBX12* gene involved in the production of DIMBOA-Glc, underscoring its potential role in plants defense under drought conditions (Zhang et al., 2021). The altered composition of BXDs under drought conditions highlights the plant ability to better cope with changing environmental conditions.

Plants secrete bioactive molecules into the rhizosphere that can modify their growth environment and soil microbiota (Hu et al., 2018). Root exudates consist of both primary metabolites including sugars, amino acids and carboxylic acids and a wide array of secondary metabolites (Hartmann et al., 2009). Root exudates not only serve as the carbon and nitrogen source for the microbial growth but also act as signalling molecules, attractants and stimulants or can have inhibitory repellent effects (Baetz & Martinoia, 2014). The host plant therefore

controls the composition of the exudates and eventually shapes rhizosphere microbial communities (Bulgarelli et al., 2013). Different plant species exude a broad range of bioactive molecules; in maize, root exudates in the rhizosphere are particularly rich in BXDs (Pétriacq et al., 2017; Hu et al., 2018). Extensive studies have shown that BXDs are involved in providing resistance against herbivores and pathogens through root exudation, although their potential role in forming symbiosis has not been explored.

Drought is one of the most significant abiotic stresses affecting global agriculture (Nehra et al., 2024), but AMF has emerged as key allies in enhancing plant resilience under challenging conditions. For example, AMF can enhance plant tolerance to drought stress and reduce its negative effects on plant growth (Li et al., 2019). Maize B73 plants when inoculated with AMF *Funneliformis mosseae* resulted in improved seedling growth, plant biomass, soil nutrient availability and microbial biomass. The AM fungi *Rhizoglomus intraradices* promoted the uptake of copper, iron, manganese and zinc durum wheat (*T. durum* L.) plants when they were subjected to drought conditions (Goicoechea et al., 2016). Similarly, *R. intraradices* also promoted the uptake of potassium, phosphorus, calcium, magnesium, sodium, and iron in Rose-scented geranium (*Pelargonium graveolens*) under drought conditions induced by laser light (Okla et al., 2022). Interestingly, a shift in the microbial community was also observed under association with AMF under drought and well-watered conditions (Li et al., 2025). Drought hinders plant cell metabolism and induces production of reactive oxygen species (ROS). AMF can help alleviate the effect of ROS as it significantly reduces hydrogen peroxide, malondialdehyde and electrolyte leakage (Chandrasekaran et al., 2022). Under changing climatic conditions, AMF is a promising tool for sustainable agriculture by increasing plant tolerance to drought stress.

Nevertheless, environmental factors can limit AMF colonization under drought stress. Moderate temperatures and adequate soil moisture are ideal for enhanced colonization, as drought can lead to reduced spore germination and hyphal growth (Auge, 2001). Mycorrhizal colonization frequency was declined in barley plants under drought condition. Additionally, the abundance of arbuscules and vesicles was also reduced by 58% and 64% respectively while ambient conditions had no effect on all indicators of AMF performance (Sendek et al., 2019). Arbuscule abundance was also decreased with the increasing drought conditions in *Poncirus trifoliata* (L.) plants inoculated with *Rhizophagus irregularis* (Zhang et al., 2024). The impact of drought on AMF colonization needs further investigation, as it is context-dependent and varies with drought intensity, plant species and soil conditions.

Drought can significantly alter the patter of root exudates forcing the plants to adjust the biochemical composition of exuded compounds because of limited water availability. The modified composition includes enhanced release of specific sugars, amino acids and secondary metabolites (Canarini et al., 2019) which are involved in signalling and act as cues for the soil microbes (Canarini et al., 2019). Plant-soil communication under reduced water conditions is reshaped by modulating the soil rhizosphere chemical composition. This modulation can critically impact the arbuscular mycorrhizal fungi (AMF) as it relies on plant-derived carbon which can become inadequate due to limitations in photosynthesis resulting in lowering spore abundance and colonization rates (Augé, 2001; Jayne & Quigley, 2014). Furthermore, modified exuded profiles can impact AMF recruitment and symbiotic efficiency under drought conditions through altered chemical cues in the soil (Santos-Medellín et al., 2017). For example, tomato plants enhance strigolactones exudation under phosphate starvation, but this effect is not always maintained under drought stress highlighting complex regulation (López-Ráez et al., 2010)

Extensive research has been carried out on BXDs and AMF individually under abiotic stress but their direct interaction especially in the context of colonization remains unexplored. This study aims to address key knowledge gaps regarding the interaction between BXDs and AMF in maize under drought conditions through a semi field assay. The study examines whether AMF presence can modulate BXDs biosynthesis, potentially shaping plant growth and defensive strategies in drought-affected environments. Specifically, it also investigates whether BXDs, particularly MBOA (6-methoxy-benzoxazolin-2(3 H)- one), a breakdown product of DIMBOA-Glc in soil, can influence AMF colonization. To explore this, we used both wild type and *bx1* mutant maize lines to assess AMF colonization levels and root BXDs profiles. Furthermore, it evaluates potential trade-offs between BXDs mediated defense and AMF benefits, such as plant growth, chlorophyll content and fresh shoot weight. In addition, the effect of drought on AMF colonization was also evaluated under different drought regimes.

METHODS

Semi-Field Experiment (Ostermundigen; 2023)

Biological resources

B73 and W22 maize seeds were obtained from Maize GDB germplasm (USDA/ARS, University of Illinois, Urbana,) and multiplied by Delley Semences et Plantes (DSP, Delley-Portalban, Switzerland). AMF *Rhizophagus irregularis* (SAF22) inoculum containing sand,

soil, roots, and spores as well as a mock inoculum without AMF was produced in the greenhouse, as previously described by Lutz et al. (2023), and were kindly provided by the Swiss Collection of Arbuscular Mycorrhizal Fungi (SAF, Zurich, Switzerland).

Maize performance in the field

The individual and interactive effects of drought and AMF on maize growth and yield were evaluated through a semi-field experiment (Figure S1). The experiment was carried out in Ostermundigen (46°57'59.8"N 7°29'13.1"E), Switzerland between May and July 2023. Four-liter pots (Hortima, Hausen, Switzerland) were covered at the bottom using fabric sheath (Neeser, Reiden, Switzerland) and filled with approximately 4.4 kg of soil (Landerde, Ricoter, Aarberg, Switzerland). The soil chemical profile was analyzed by the laboratory Labor für Boden- und Umweltanalytik (LBU, Steffisburg, Switzerland) (Supplementary Table 1). Approximately 200 g of the AMF inoculum were added to half of the pots (AMF+, n= 36) and mixed with the soil. The same amount of mock inoculum was added and mixed with the soil of control pots (AMF-, n=9). Three maize B73 seeds were placed 3 cm deep into the soil in individual pots. After ten days, maize growth was assessed and one seedling (the most central) per pot was kept by manually removing additional seedlings. All plants were watered daily for two weeks. After this period, only control plants received water daily (AMF+: n=12, AMF-: n=3), while drought-exposed plants were left unwatered until drought symptoms appeared (leaf wilting score of 4, Sudhakar et al. 2016). Drought treatments were defined based on the calculated soil moisture of the predicted future climate scenarios RCP2.6 and RCP8.5 with a water content of 19% (v/v) and 16.6% (v/v) respectively (Guyer et al., 2021; van Doan et al., 2021; IPCC, 2014). All plants were watered once daily. Drought-exposed plants received either 1.9 L (19% of pot volume; AMF+: n = 12, AMF-: n = 3) or 1.66 L (16.6% of pot volume; AMF+: n = 12, AMF-: n = 3) water. Leaf wilting symptoms were observed at similar frequency and intensity in both drought treatments. According to the manufacturer's instructions, the plants received 1% NK fertilizer solution (NK Flüssigdünger; Biorga, Grossaffoltern, Switzerland) with one liter volume applied per plant during the fourth week of the experiment. All pots were covered with 35 L plastic bags (Quick Bag, Galaxus, Zürich, Switzerland) during rain episodes. The 45 pots (2 AMF treatments x 3 drought levels x 12 replicates) were randomly placed in the beds to avoid positional bias.

Plant phenotypic parameters were measured after 15, 30, 45 and 60 days. Relative chlorophyll content of the youngest leaf was measured using Soil and Plant Analysis Development SPAD502 plus (Konica Minolta, München, Germany) around 12 pm for all the plants. The

overall duration of the measurements lasted from 30 min to one hour. Plant height was measured by using a ruler from the tip of the youngest leaf down to the soil surface. Maize youngest leaves were collected on day 60 and flash frozen in liquid nitrogen for benzoxazinoid analysis. Maize roots were collected on day 60 for benzoxazinoid analysis and AMF colonization evaluation

Effect of kinetic drought on AMF colonization (Greenhouse Ostermundigen; Summer 2024)

The impact of drought on maize AMF colonization was investigated by establishing drought at different time points under greenhouse conditions (Supplementary Figure 3). Maize seeds (var. B73) were surface sterilized as described above. Germinating seedlings were placed in 3 L pots (Hortima, Hausen, Switzerland) covered at the bottom using fabric sheath (Neeser, Reiden, Switzerland). The pots were filled with 3.4 kg soil (95% of pot volume; Landerde; Ricoter, Aarberg, Switzerland). Maize plants were grown in a greenhouse at 23±1°C and 18±1°C with 14/10 hours of light and darkness respectively to simulate natural conditions and 60% (v/v) relative humidity. The plants were subjected to four watering conditions i.e., CC, CD, DC and DD, where C stands for control watering and D for drought treatment according to RCP 8.5 (16.6% soil moisture). For the first fourteen days, CC and CD treated plants received ambient watering while DC and DD treated plants were subjected to drought watering conditions.

After this period, watering was continued for CC and DC while drought was applied to CD and DD. All the plants mixed with 150 g AMF inoculum (AMF+, n= 32). The pots were randomly placed in the greenhouse (Figure S2).

Plant phenotypic parameters were measured after 20, 40 and 60 days. Relative chlorophyll content of the youngest leaf was measured using Soil and Plant Analysis Development SPAD502 plus (Konica Minolta, München, Germany) around 12 pm for all the plants. The duration of the measurements lasted from 30 min to one hour. Plant height was measured by using a ruler from the tip of the youngest leaf down to the soil surface. Fresh shoot biomass root length was also measured at the termination of the experiment. Maize youngest leaves and roots were collected on days 20, 40 and 60 and flash frozen in liquid nitrogen for benzoxazinoid analysis. Maize thin roots were collected on day 60 and stored at -20ºC for AMF colonization evaluation

Maize W22 and *bx1* mutant association with AMF and complementation with MBOA (Ostermundigen; Summer 2024)

The impact of benzoxazinoids on maize AMF colonization was investigated by using W22 type and *bx1* mutant plants under greenhouse conditions (Supplementary Figure 3). Maize seeds were surface sterilized using 15% (v/v) bleach (Potz, Migros, Zurich, Switzerland) in distilled water for 15 min. The seeds were then rinsed with distilled water and pregerminated by placing them on damped filter papers (90mm; Cytiva, Marlborough, MA, USA) in a plastic box (Semadeni, Bern, Switzerland) in the dark for three days. Germinating seedlings were placed in 3 L pots (Hortima, Hausen, Switzerland) covered at the bottom using fabric sheath (Neeser, Reiden, Switzerland). The pots were filled with either 3.4 kg soil (Landerde; Ricoter, Aarberg, Switzerland) mixed with 150 g AMF inoculum to each WT and bx1 mutant plants (AMF+, n= 14) or with 3.4 kg soil (Landerde; Ricoter, Aarberg, Switzerland) mixed with 150 g of autoclaved control inoculum (AMF-, n=14). 8 mg of MBOA was purchased from Sigma-Aldrich Chemie GmbH (Buchs; Switzerland) and was added to bx1 mutant plants (AMF+, n= 7, AMF-, n= 7). Maize plants were grown in a growth chamber at 23±1°C and 18±1°C with 14/10 hours of light and darkness respectively to simulate natural conditions and 60% (v/v) relative humidity. All plants were watered daily for eight weeks. The pots were randomly placed in the growth chamber to avoid positional bias.

Plant phenotypic parameters were measured after 20, 40 and 60 days. Relative chlorophyll content of the youngest leaf was measured using Soil and Plant Analysis Development SPAD502 plus (Konica Minolta, München, Germany) around 12 pm for all the plants. The duration of the measurements lasted from 30 min to one hour. Plant height was measured by using a ruler from the tip of the youngest leaf down to the soil surface. Fresh shoot biomass, root length, tassel and cob development were also measured at the termination of the experiment. Maize youngest leaves and roots were collected on days 20, 40 and 60 and flash frozen in liquid nitrogen for benzoxazinoid analysis. Maize thin roots were collected on day 60 and stored at - 20ºC for AMF colonization evaluation

AMF colonization rates

Roots were stained following a previously established procedure (Vierheilig et al., 1998). Maize thin roots (diameter 0.5 - 1 mm) were cut into small segments of approximately 1.5 cm in length and preserved in 50% EtOH (Alcosuisse, Rüti bei Büren, Switzerland). The ethanol was rinsed off using distilled water and the samples were then cleared with 10% w/v KOH (Sigma-Aldrich, Steinheim, Germany) at 80°C in a dry bath (Digital Dry Bath; Labnet, Edison,

NJ, USA) for a duration of 30 min. After incubation, the roots were rinsed using distilled water and stained with 5% ink (Pelikan, Hannover, Switzerland) -vinegar solution (5% acetic acid; MBudget, Migros, Zurich, Switzerland) and incubated at 80°C for 30 min. After a final rinse with distilled water, the samples were stored in 50% glycerol (Dr. Bähler Dropa AG, Bern, Switzerland). The root samples were placed on a microscopic slide, mounted with 50% glycerol, and covered with the help of a cover slip. The samples were observed under a Fluorescence epi microscope with camera (Leica DMC6200; Leica Microsystems, Heerbrugg, Switzerland) at the magnification of 200X (magnifying lens * ocular lens). The colonization rate in percentage was measured as the proportion of root segments colonized by AMF compared to the total number of root segments (McGonigle et al., 1990). The number of root segments per plant in average was 50, 40 and 40 for semi-field, drought kinetic and *bx1* mutant assays respectively.

Benzoxazinoids profiling

Benzoxazinoid contents were characterized using an acquity i-Class UHPLC system coupled to a single quadrupole mass spectrometer (QDa) equipped with an electrospray source (Waters, Milford, MA, USA) as previously described (Hu et al., 2018). The plant metabolites were extracted from 100 ± 1 mg by adding 1 mL MeOH: H2O:FA (70:30 v/v, 0.1% FA) and thoroughly vortexed for 10 s. The samples were then centrifuged for 20 min at 13'00 rpm at 10°C and the supernatant was collected for analysis. Compounds were separated on an Acquity BEH C18 column (1.7 µm, 2.1 × 100 mm i.d.; Waters, Milford, MA, USA). The flow rate of the mobile phase was maintained at 0.4 mL/min. The injection volume was 1 µL and the temperature of the column was maintained at 40ºC. The MS was operated in negative mode, and data were acquired in the scan range (*m/z* 150–650) using a cone voltage of 10 V. All other MS parameters were left at their default values. The elution conditions were as follows: solvent A consisted of H_2O and FA (99.9:0.1 v/v), while solvent B consisted of ACN and FA (99.9:0.1 v/v). The gradient program was: 2% solvent B from 0.00 to 1.00 min; a linear gradient from 2 to 40% solvent B from 1.00 to 4.00 min; a linear gradient to 100% solvent B from 4.00 to 6.00 min.; 100% solvent B from 6.00 to 8.50 min; a gradient from 100 to 2% solvent B from 8.50 to 8.51 min; and 2% solvent B from 8.51 to 10 min. MassLynx v4.1 SCN923 was used to control the instrument and for data processing. To detect and identify BXDs, targeted mass spectrometry in negative ionization mode was used. The absolute quantities of HMBOA, DIMBOA, DIMBOA-Glc, DIMBOA-2Glc, HDMBOA-Glc, and MBOA were determined using standard curves of the corresponding pure compounds. MBOA was purchased from

Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). DIMBOA-Glc DIMBOA-2Glc, and HDMBOA-Glc were isolated from maize plants in our laboratory as previously described (Thoenen et al., 2023). DIMBOA and HMBOA were synthesized in our laboratory directly from or adapting published protocols (Macías et al., 2006). HMBOA-Glc, HMBOA-2Glc, HM_2BOA-Glc, DIMBOA-3Glc, DIM_2BOA-Glc, and HDM_2BOA-Glc for which no analytical standards were available, were quantified by comparison with the standard curve of their closest parent compounds, HMBOA, DIMBOA-Glc, and HDMBOA-Glc. Full names and chemical formulas of measured benzoxazinoids can be found in Supplementary Table 3.

Statistical analyses

Statistical analyses and data visualization were done with R (version 4.4.2; R core team, 2018) using R studio (version 2024.12.0.467; Posit team, 2024). The data was read in with the package readxl (version 1.4.3; Wickham and Bryan, 2023). For organizing and structuring the data the package dplyr (version 1.1.4; Wickham et al., 2023) was used. The semi-field assay and the herbivory assay followed a fully multifactorial design, and the response variables were analysed by using linear models or ANOVA. Explanatory variables were AMF presence or absence, water regimes, and for the mutant assay presence or absence of *bx1* gene. Homoscedasticity and normality of distribution of residuals were confirmed visually with the diagnostic plots of base R. I applied aligned rank transform (Art) ANOVA using ARTool package (version 0.11.1; Kay et al., 2021) if the model fit was not satisfactory. Depending on the number of variables in the experiment, two-way or three-way ANOVA was used to detect the effects of response variables. P-values below 0.05 were considered significant. Plots were made using the package ggplot2 (version 3.5.1; Wickham, 2016) and ggpattern (version 1.1.1; Wickham and Davis, 2024).

RESULTS

Drought decreased AMF colonization enhanced root benzoxazinoid levels in the field

We conducted a semi-field assay to examine the interactions between drought and AMF *Rhizophagus irregularis* (SAF22) on maize plants (var. B73) growth and defense compounds, specifically benzoxazinoids (Supplementary Figure 1).

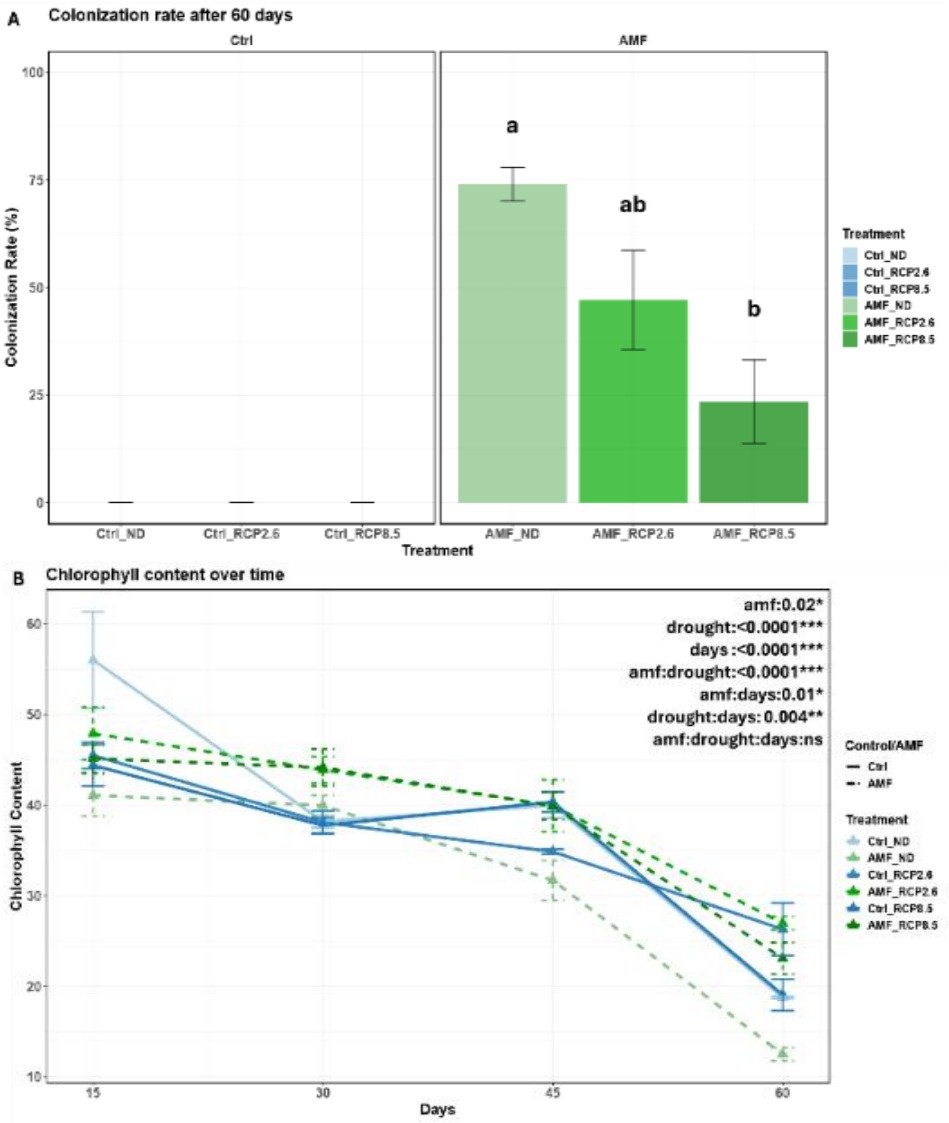
We quantified colonization to make sure that the AMF association was established in the roots. Time required by the AMF to fully colonize plant roots depend on plant genotype, fungal species and the soil conditions. Our preliminary findings suggests that maize reached maximum

colonization with *R. irregulare* after around eight weeks. Our findings revealed that drought drastically reduced AMF colonization in the maize roots after 60 days while ambiently watered plants had the highest colonization rate (Figure 1A).

We measured chlorophyll content to analyse the effect of drought on photosynthesis and whether AMF can help plants in improving chlorophyll content under drought stress. Time series of the chlorophyll content in maize plants is shown in Figure 1B. After 15 days, there was a significant effect of drought and AMF on maize chlorophyll content (Supplementary Figure 4A). After 30 days, AMF inoculation of maize plants improved chlorophyll content (Supplementary Figure 4B). After 45 days, no effect of either drought or AMF was found for chlorophyll content (Supplementary Figure 4C). After 60 days, AMF reduced chlorophyll content of plants that were watered ambiently while no effect on drought treated plants was found (Supplementary Figure 4D).

We analysed how drought can impact the production of benzoxazinoids in maize plants under drought as they are primarily involved in plant defences against environmental stresses. Additionally, whether benzoxazinoids metabolic profiles were positively or negatively modulated under the AMF association was also determined. We found that drought increased root and leaf benzoxazinoid levels while AMF reduced benzoxazinoids content in the roots (Figure 1C-D). In roots, drought increased the production of DIMBOA, DIMBOA-Glc, DIM2BOA-Glc and DIMBOA-2Glc while AMF lowered the DIMBOA, DIMBOA-Glc, DIM2BOA-Glc content. AMF enhanced the production of HDMBOA-Glc in roots while interactive effect of AMF and drought were found for DIMBOA-Glc, DIM2BOA-Glc and HMBOA-2Glc (Supplementary Figure 6). In leaves, drought enhanced the levels of HMBOA, HMBOA-Glc, DIMBOA-Glc, while interactive effect of AMF and drought was found for DIM2BOA-Glc. (Supplementary Figure 7).

Interestingly, in roots, correlations were found between BXDs and colonization rate. DIMBOA, DIMBOA-Glc, DIM2BOA-Glc, DIMBOA-2Glc were positively correlated in roots under low colonization. Alternatively, HDMBOA was negatively correlated in plants roots with higher colonization (Supplementary Figure 8).


A
Colonization rate after 60 days
Ctrl
AMF
Colonization Rate (%)
100
75
50
25
0
a
ab
b
Ctrl_ND
Ctrl_RCP2.6
Ctrl_RCP8.5
AMF_ND
AMF_RCP2.6
AMF_RCP8.5
Treatment
Treatment
Ctrl_ND
Ctrl_RCP2.6
Ctrl_RCP8.5
AMF_ND
AMF_RCP2.6
AMF_RCP8.5
B
Chlorophyll content over time
amf:0.02*
drought:<0.0001***
days :<0.0001***
amf:drought:<0.0001***
amf:days:0.01*
drought:days: 0.004**
amf:drought:days:ns
Chlorophyll Content
60
50
40
30
20
10
15
30
45
60
Days
Control/AMF
Ctrl
AMF
Treatment
Ctrl_ND
AMF_ND
Ctrl_RCP2.6
AMF_RCP2.6
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AMF_RCP8.5

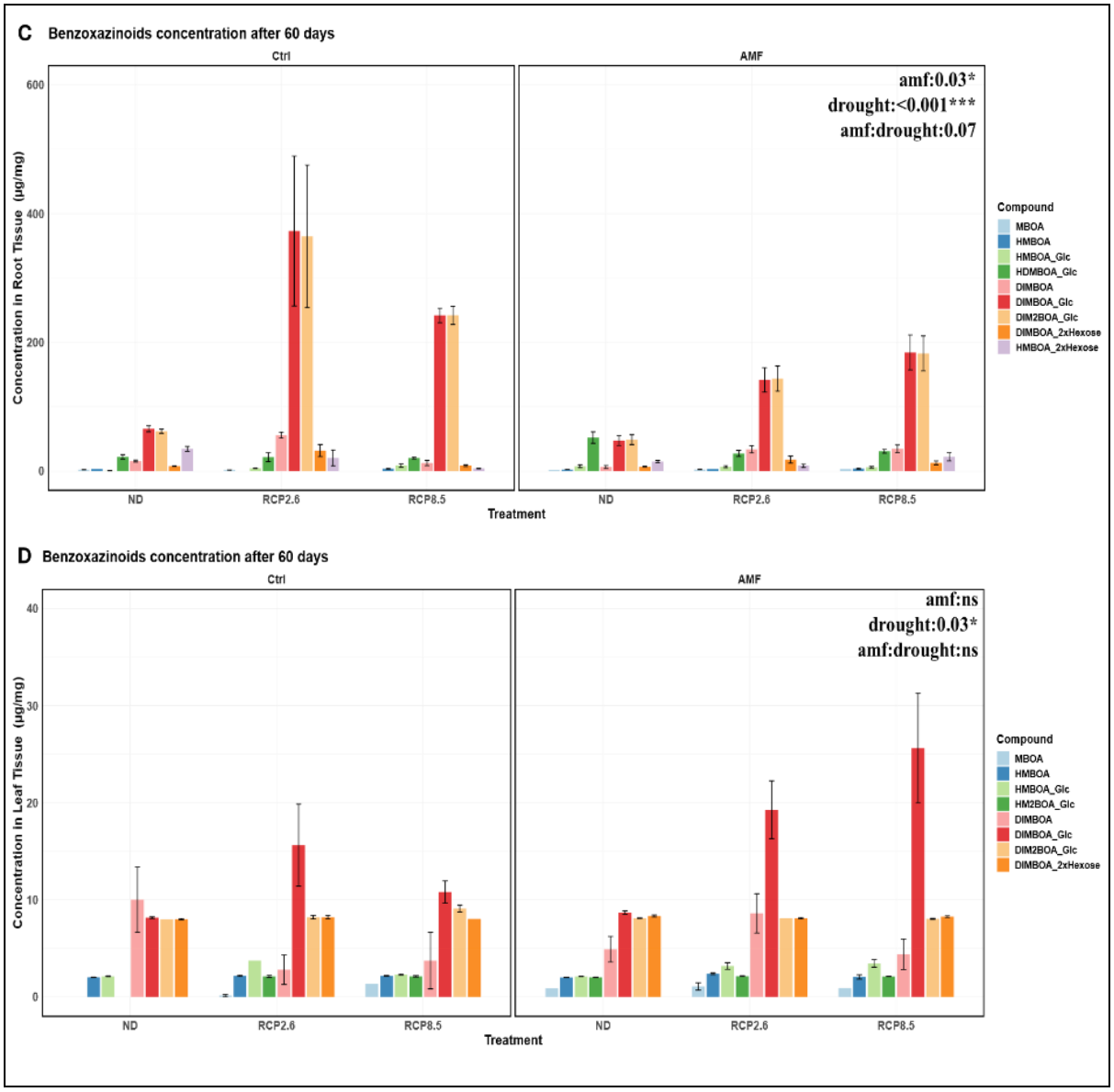


Figure 1. The colonization rate of arbuscular mycorrhizal fungi (AMF) with maize B73 plants declined with increasing drought conditions in the field. Benzoxazinoids levels in maize B73 roots and leaves were induced under drought stress while AMF presence limited this effect in the roots A. Colonization rate of *Rhizophagus irregularis* with maize B73 plants drastically declined under increasing drought conditions after 60 days. B. Time series of the chlorophyll content in maize plants. C-D. Profile of different benzoxazinoids in the root and leaf tissues after 60 days are shown. Mean ± standard errors are shown (Ctrl_ND, Ctrl_RCP2.6, Ctrl_RCP8.5 n=3; AMF_ND, n=7; AMF_RCP2.6, AMF_RCP8.5, n=9). Three-way anova test was used run to analyze differences among treatments. For total BXDs, PERMANOVA test was run to analyze differences among treatments. Stars and letters indicate significant differences, **: $p<0.001$, *: $p<0.05$, $0.05<p<0.10$. Ctrl = Control, AMF = Arbuscular mycorrhizal fungi, *Rhizophagus irregularis* (SAF22). ND: Ambient, soil moisture: 23% (v/v); Drought soil moisture: RCP2.6 and RCP8.5: 19% and 16.6% (v/v) respectively.

Prolonged drought increased chlorophyll and benzoxazinoids content in maize plants, while kinetic drought had no effect on AMF colonization in the greenhouse.

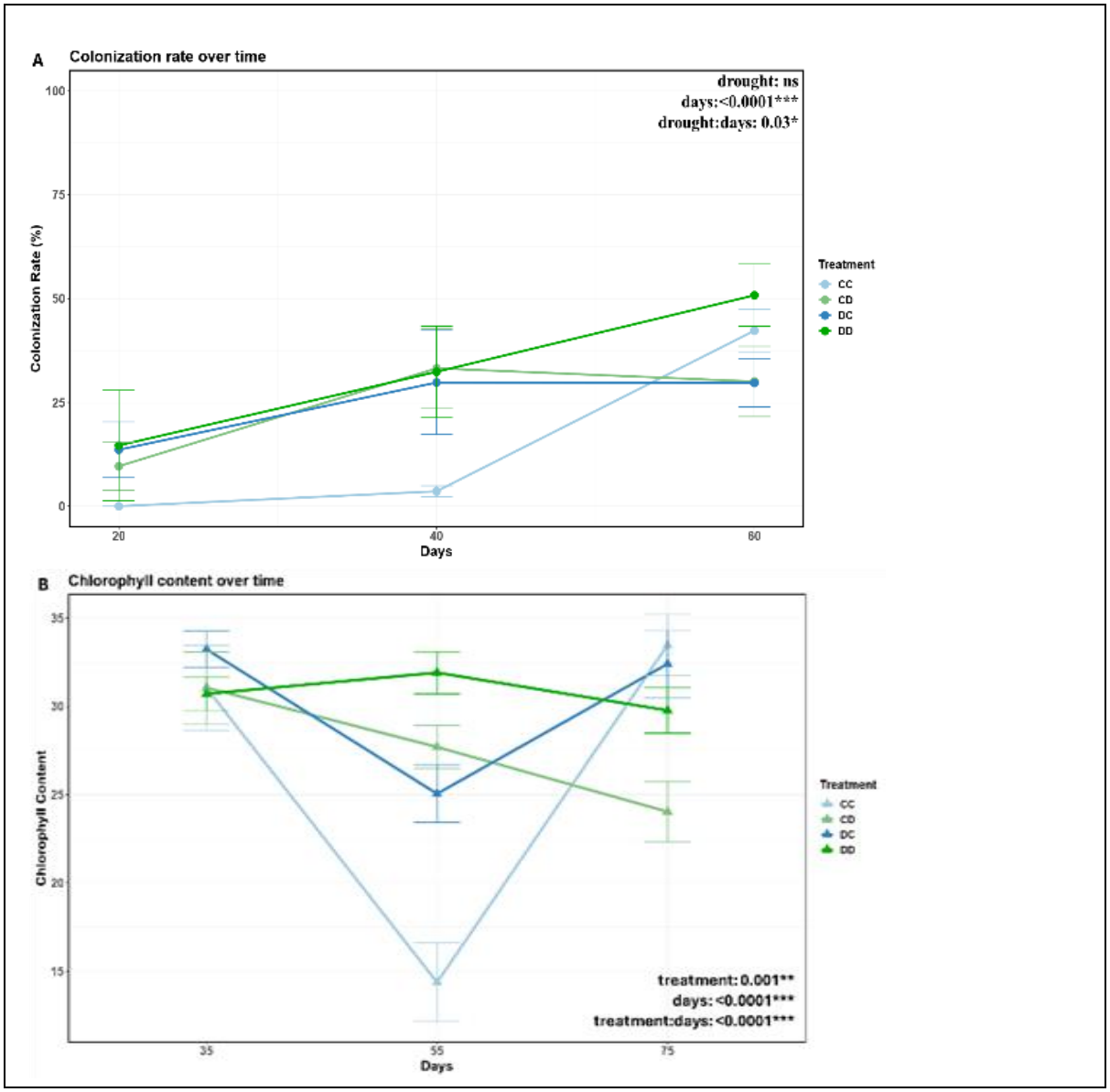
In the first experiment, drought was established after 15 days of ambient watering (Supplementary Figure 2). We, therefore, thought that the timing of drought could explain the Plant-AMF interactions as previous studies with earlier drought establishment also did not report decreased AMF colonization. Additionally, the effect of BXDs seem to occur at early stages of maize and AMF interactions. We established four watering regimes i.e., CC, CD, DC and DD for fourteen days before the addition of AMF inoculum and assessed plant growth, colonization rate and BXDs content. Here, CC refers to ambient watering, CD as late drought, DC refers to early drought, and DD as prolonged drought. All the plants were inoculated with the AMF.

Time series of colonization rate after 35, 55 and 75 days is presented in Figure 2A. After 35, 55 and 75 days, there was no effect of early and late drought on the colonization rate of *Rhizophagus irregularis* (SAF22) with maize B73 plants (Supplementary Figure 12).

Time series of chlorophyll content of maize B73 plants after 35, 55 and 75 days is presented as Figure 2B. After 35 days, there was no effect of drought on the chlorophyll content (Supplementary Figure 9A), while after 55 days late and prolonged drought treatment increased the chlorophyll content in maize B73 plants (Supplementary Figure 9B).

Time series of fresh shoot weight of maize B73 plants after 35, 55 and 75 days is presented as Figure 2C. After 55 days prolonged drought treatment decreased fresh shoot weight of maize B73 plants (Figure 11B). The plants with early drought treatment recovered after 75 days and had the highest fresh shoot weight (Supplementary Figure 11C)

Benzoxazinoids profile in the roots after 75 days is presented as Figure 2D. After 35 days, prolonged drought increased DIM2BOA-Glc (Supplementary Information 13). while after 55 days prolonged drought increased HMBOA and DIMBOA concentrations in the roots (Supplementary Information14). After 75 days, HDMBOA-Glc and DIMBOA-Glc concentrations increased under prolonged drought while there was no effect of early, late and prolonged drought on other benzoxazinoids (Supplementary Information 15).


A
Colonization rate over time
drought: ns
days:<0.0001***
drought:days: 0.03*
Colonization Rate (%)
100
75
50
25
0
20
40
60
Days
Treatment
CC
CD
DC
DD
B
Chlorophyll content over time
Chlorophyll Content
35
30
25
20
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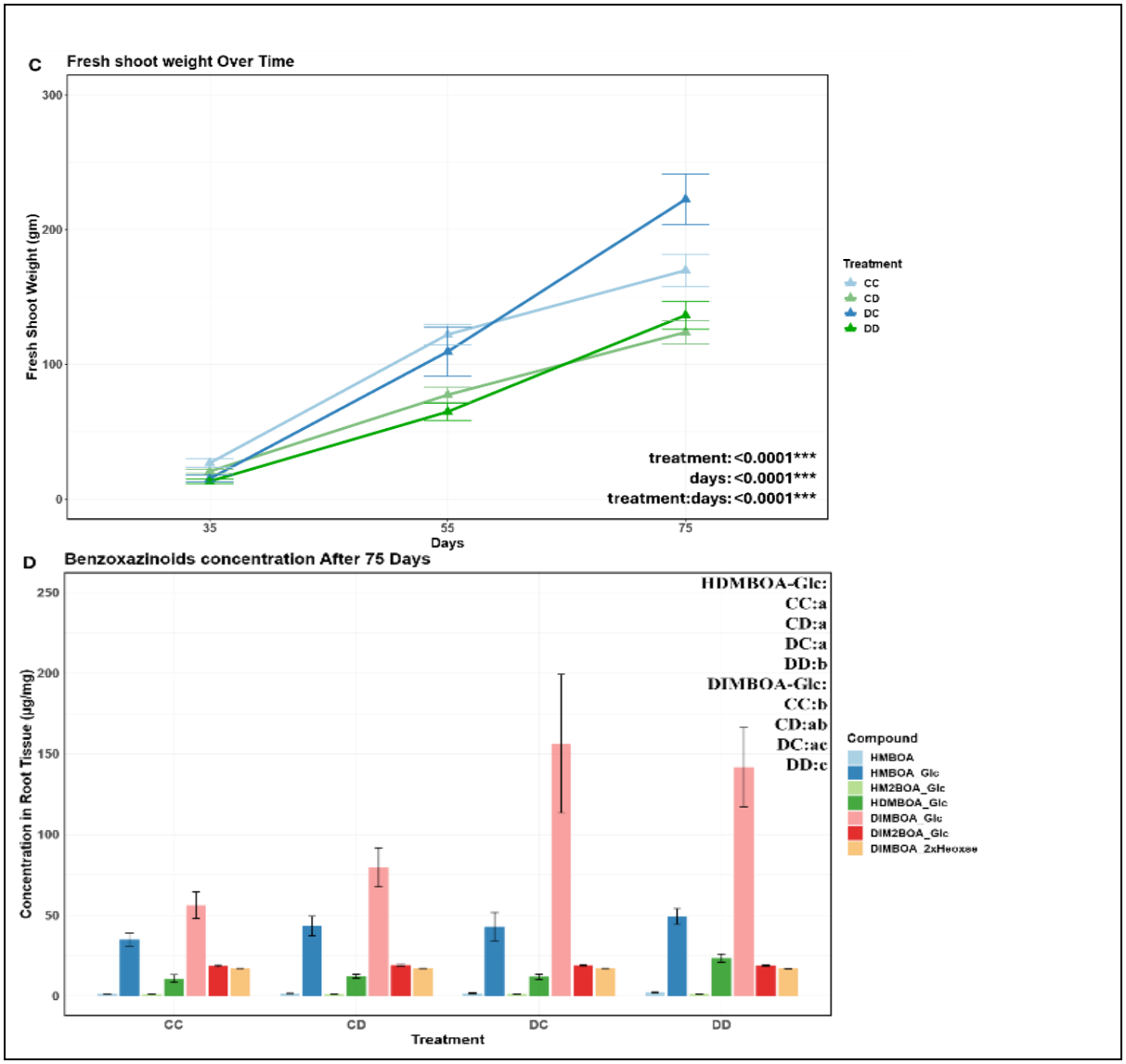

Figure 2. Kinetic drought had no effect on colonization rate of maize plants inoculated with AMF *Rhizophagus irregularis* (SAF22) while prolonged drought increased chlorophyll and decreased fresh shoot weight. A. Colonization rate of maize plants inoculated with AMF after 35, 55 and 75 days. B. Chlorophyll content over time for maize plants inoculated with AMF under different drought treatments. C. Mean fresh shoot weight over time for maize plants inoculated with AMF under different drought treatments. D. Benzoxazinoids profile in the root tissues under different drought conditions and inoculation with AMF after 75 days. Mean ± standard errors are shown (n=8 except for CD; n =7). Anova was performed to analyze differences among treatments. Letters and stars indicate significant differences, ***: $p<0.0001$, **: $p<0.01$, $0.05<p<0.10$. CC here stands for ambient watering, CD is late drought, DC is early drought, and DD is prolonged drought treatment according to RCP 8.5 (16.6% soil moisture). For the first fourteen days, CC and CD treated plants received ambient watering while DC and DD treated plants were subjected to drought watering conditions.

Soil complementation with MBOA increased AMF colonization

In the first experiment, we found that Increasing drought led to increased levels of BXDs and decreased levels of AMF colonization We therefore tested whether BXDs could explain the lower AMF rates. We used *bx1* mutants and complemented the soil with MBOA (Supplementary Figure 3). MBOA is a breakdown product of DIMBOA, it is bioactive and is involved in signalling, defense responses and effects soil microbes. We used ~ 2.5 mg MBOA/kg of soil as it represents the amount in the plant tissues and is consistent with previous studies (Fomsgaard et al., 2006).

For W22 plants, we measured colonization rate after 20 40 and 60 days to visualize the AMF *R. irregulare* association with the genotype. *R. irregulare* successfully colonized W22 plants and had close to maximum colonization rate after 20 days as opposed to B73 maize plants in the field assay. The *bx1* mutants were less colonized by the AMF, but the complementation of *bx1* mutants with MBOA increased the colonization rate significantly after 20 days (Figure 3A) The stimulating effect of MBOA disappeared in *bx1* mutant after 40 and 60 days where all plants displayed similar AMF colonization rate (Supplementary information 20).

We measured the effect of MBOA on plant growth such as the fresh shoot weight considering whether higher colonization rate facilitates in improved plant performance. We found that the addition of MBOA did not affect the fresh shoot weight, while fresh shoot weight of W22 and *bx1* mutant plants was influenced by AMF after 20 (Figure 3A) and 60 days (Supplementary Figure 19B). The fresh shoot weight after 40 and 60 days is presented in Supplementary Figure 19A-B.

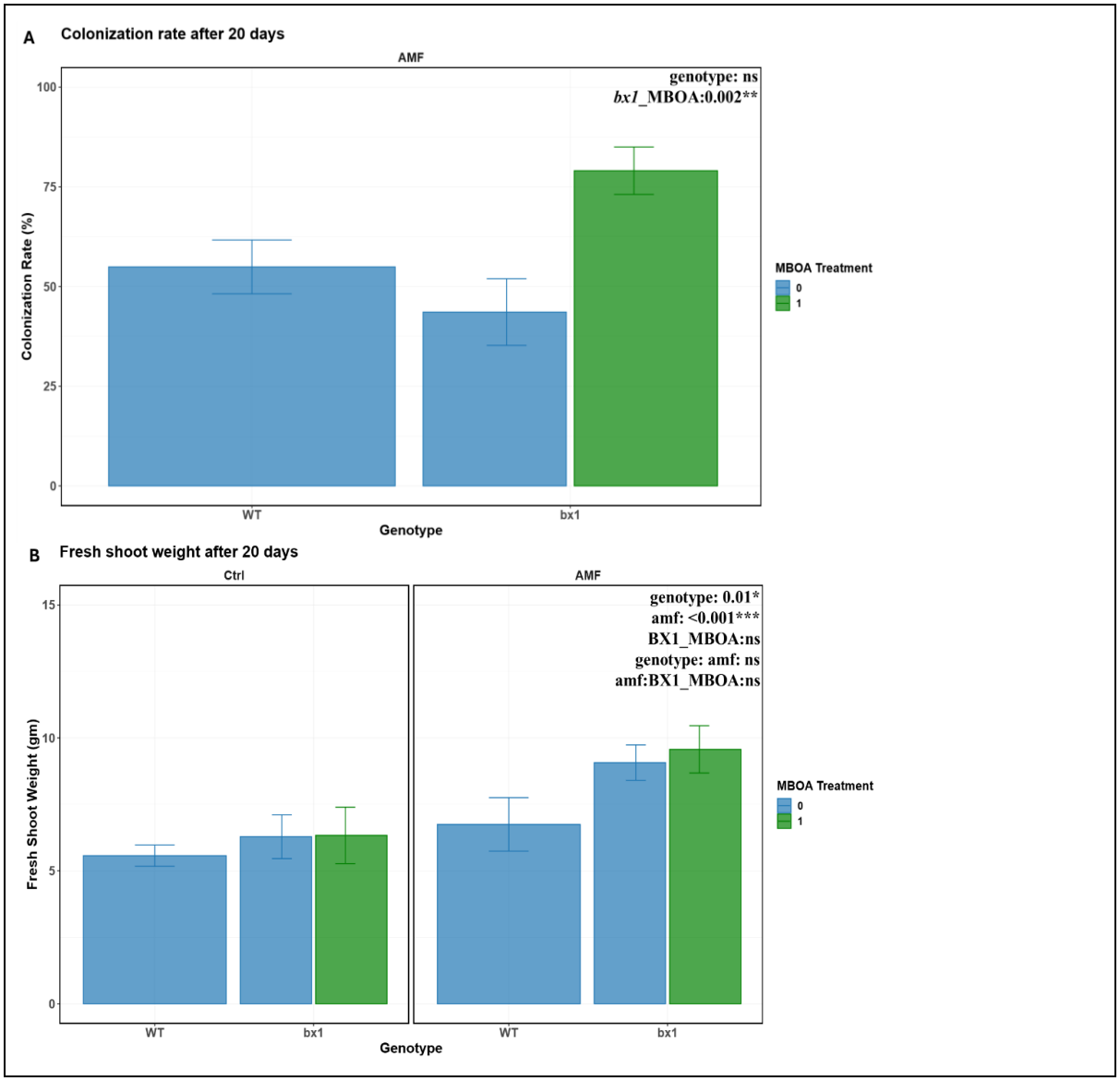

Figure 3. MBOA complementation increased AMF colonization in maize plants after 20 days. A. Colonization rate of *Rhizophagus irregularis* (SAF22) with maize W22, mutant *bx1* and mutant *bx1* plants complemented with MBOA. B. Fresh shoot weight of maize W22, *bx1* mutant plants complemented with MBOA after 20, 40 and 60 days. Mean ± standard errors are shown (n=7; except for BX1_MBOA_Ctrl, n=6). Two-way anova test was run to analyze differences among treatments. Stars indicate significant differences, ***: p<0.0001, **: p<0.01, *: p<0.05, 0.05<p<0.10. Ctrl= Control, AMF = Arbuscular mycorrhizal fungi, *Rhizophagus irregularis* (SAF22). MBOA0 = No MBOA, MBOA1 = MBOA addition.

DISCUSSION

The present study reports contradicting results of the effect of drought on AMF, as drought decreased AMF colonization in the semi-field assay while increased it in the kinetic assay under greenhouse conditions. AMF had an increasing effect on chlorophyll content in maize plants after 30 days but decreased it in well-watered plants after 60 days. Interestingly, whereas drought increased benzoxazinoids content in the roots and leaves, AMF reduced BXDs concentrations in the roots. While first evidence suggests that some BXDs, namely MBOA,

may play a role in stimulating AMF colonization at early stage, more investigations are required to best characterize the role of BXDs in maize-AMF interactions as well as the effect of drought on AMF association.

In the field assay, one of the most significant observations is the decreased AMF colonization under drought conditions. Under standard conditions, AMF form mutualistic associations with plant roots, facilitating nutrient and water uptake in exchange for carbohydrates. Previous studies have reported increased AMF colonization under drought conditions (Chareesri et al., 2020; Orine et al., 2022). AMF can help plants enhance their drought tolerance through different mechanisms: for instance by extending their hyphal networks deeper into the soil, beyond the root zone, they can access water and nutrients that would otherwise be unavailable to the plant (Bhupenchandra et al., 2024). Increased colonization under drought has been attributed to the plant's need for more efficient nutrient and water acquisition during stress, as well as AMF's ability to stabilize soil structure and improve water retention (Aminzadeh et al., 2025). Our field data contrasts with these studies as we found that drought conditions reduced AMF colonization rates. Previous work showed that the relationship between drought and AMF colonization can also vary depending on factors such as the severity and duration of the drought, plant species, and soil conditions. For example, while moderate drought may stimulate AMF colonization as part of a plant's adaptive response, more severe or prolonged drought may lead to resource allocation trade-offs, where plants prioritize survival over maintaining symbiotic relationships. In these cases, colonization rates may decrease, especially if the plant reduces carbon allocation to the roots or if soil moisture levels drop too low to support fungal growth. Interestingly in the greenhouse experiment, establishing different watering regimes including, early, late and prolonged drought before the addition of the AMF inoculum mitigated the effect of drought on AMF colonization. A few studies indicate that drought can result in reduced AMF colonization due to reduced carbon allocation and root growth under drought stress (Augé, 2001). However, pre-establishment of drought conditions can result in root remodelling or stress priming that can support effective fungal colonization. Under water deficient systems, the timing of AMF inoculation can be a key strategy to enhance symbiotic associations, but more investigations are needed to clearly characterize interaction between drought and AMF.

In this study, drought triggered an increase in root BXDs contents, an effect which is consistent with former work in maize (Sutour et al., 2024). BXDs known for their role in plant defense against herbivores and pathogens (Neal et al, 2012) also facilitate the colonization of the rhizosphere by the plant growth promoting rhizobacteria *Pseudomonas putida* (Nael et al.,

2017). Yet, the involvement of BXDs in modulating AMF colonization under abiotic stress such as drought has not been widely explored. An interesting finding of this study is that AMF colonization resulted in reduced BXDs levels in the roots. For successful AMF colonization, plants may suppress their immune responses including downregulation of defense metabolites. This modification of the metabolic processes is expected to result in reduced accumulation of BXDs such as DIMBOA-Glc. At the hormonal level, salicylic acid and jasmonic acid pathways regulate the BXDs biosynthesis genes, *ZMBX1-ZMBX14* (Ahmad et al 2011; Hu et al., 2018; Setotaw et al., 2024). Increased levels of salicylic acid can therefore delay AMF colonization in tobacco plants (Blilou et al., 2000; Medina et al., 2003). Additionally, this symbiotic association requires exchange of energy and reduces the need for investing in high levels of chemical defenses and conserves growth resources.

Drought stress often triggers changes in plant physiology, such as altered root exudation patterns, reduced carbon allocation to the roots, and shifts in hormone signalling, all of which can negatively affect AMF colonization. BXDs act as signalling molecules and can regulate plant-biotic interactions; their exudation in the rhizosphere can impact microbial communities and shape plant interactions with the soil microbiota (Hu et al., 2018). BXDs are involved in altering the root-associated microbiota as marked differences were observed in community composition of bacteria and fungi in WT and *bx1* mutant plants (Cadot et al., 2021). Benzoxazinoids exudation from the maize roots also altered the root microbial community in the field conditions with more pronounced effects observed for the root fungi. BXDs exudation consistently depleted *Flavobacteriaceae* and *Comamonadaceae* across the different environments (Cotton et al., 2019; Cadot et al., 2021).

The use of *bx1* mutants and the addition of MBOA, provided further insights into the role of BXDs in AMF interactions. The lack of difference in AMF colonization rates between wild-type (WT) and *bx1* mutants suggests that BXDs production may not directly inhibit AMF colonization. However, when MBOA was introduced into the soil, AMF colonization rates temporarily increased, which suggests that specific BXDs derivatives may have a stimulatory effect on AMF under certain conditions. This stimulatory effect of MBOA was transient, disappearing after 40 days, indicating a time-sensitive dynamic in the plant-fungi relationship possibly influenced by other environmental or biological factors. Several hypotheses could explain these observations. First, the transient increase in AMF colonization in response to MBOA could be due to an initial enhancement of fungal activity triggered by low levels of BXDs-derived compounds. However, over time, this effect may be counteracted by feedback mechanisms in the plant or fungi, leading to a normalization of colonization rates. Another

possibility is that AMF colonization is influenced by a complex interplay of multiple factors, including plant hormonal responses, root architecture, or changes in the soil microbiome under drought conditions, which may override any potential BXDs-mediated effects. MBOA has demonstrated antimicrobial properties against pathogenic fungi and specific soil bacteria, it can therefore contribute to selective microbial recruitment (Niemeyer, 2009; Hu et al., 2018). Furthermore, beneficial microbes utilize MBOA for metabolic adaptation or niche colonization adaptation (Cotton et al., 2019; Kudjordjie et al., 2019). Dual role of MBOA as carbon substrate and in deterring or favouring specific microbiota depends on the functionality. Together, MBOA impact appears to be context-dependent and current study is consistent that it can act either as a nutrient or stimulator reflecting shifts in microbial profiles. Temporary increase in AMF colonization in response to MBOA indicates that BXDs might serve as signalling molecules that affect symbiotic dynamics without necessarily altering overall plant performance in the short term.

In conclusion, this study highlights the relationship between drought stress, benzoxazinoid levels, and AMF colonization. While drought reduces AMF colonization and increases BXDs production, the timing of drought establishment and AMF inoculation minimized that effect respectively. The exact role of BXDs in mediating these effects also remains unclear. The reducing effect of AMF colonization on the root benzoxazinoids level points towards modulation in plant defense chemistry, but further investigation is needed to confirm the underlying processes. Although MBOA temporarily stimulated AMF colonization, the long-term dynamics of this interaction require further investigation. Future work should focus on validating these findings and exploring the underlying mechanisms driving these interactions over extended periods and in varying environmental conditions. Understanding these processes could help in developing strategies to mitigate the impacts of drought and drought timing on plant-microbe symbioses and improve plant resilience to stress.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY

All data will be provided as supplementary material upon acceptance of the manuscript.

AUTHOR CONTRIBUTION

Sheharyar Khan: Conceptualization, experimental setup, methodology, data collection, formal analysis, writing manuscript

Marcel van der Heijden: Provision of the AMF strain *Rhizophagus irregularis* (SAF22)

Pierre Matteo: Benzoxazinoids analysis

Natacha Bodenhausen: Experimental design, supervision, reviewing and editing, statistical analysis

Christelle Robert: Conceptualization, Experimental design, supervision, reviewing and editing, statistical analysis

FIGURE LEGENDS

Figure 1. The colonization rate of arbuscular mycorrhizal fungi (AMF) with maize B73 plants declined with increasing drought conditions in the field. **Benzoxazinoids levels in maize B73 roots and leaves were induced under drought stress while AMF presence limited this effect in the roots** A. Colonization rate of *Rhizophagus irregularis* with maize B73 plants drastically declined under increasing drought conditions after 60 days. B. Time series of the chlorophyll content in maize plants. C-D. Profile of different benzoxazinoids in the root and leaf tissues after 60 days are shown. Mean ± standard errors are shown (Ctrl_ND, Ctrl_RCP2.6, Ctrl_RCP8.5 n=3; AMF_ND, n=7; AMF_RCP2.6, AMF_RCP8.5, n=9). Three-way anova test was used run to analyze differences among treatments. For total BXDs, PERMANOVA test was run to analyze differences among treatments. Stars and letters indicate significant differences, **: $p<0.001$, *: $p<0.05$, $0.05<p<0.10$. Ctrl = Control, AMF =

Arbuscular mycorrhizal fungi, *Rhizophagus irregularis* (SAF22). ND: Ambient, soil moisture: 23% (v/v); Drought soil moisture: RCP2.6 and RCP8.5: 19% and 16.6% (v/v) respectively.

Figure 2. Kinetic drought had no effect on colonization rate of maize plants inoculated with AMF *Rhizophagus irregularis* (SAF22) while prolonged drought increased chlorophyll and decreased fresh shoot weight. A. Colonization rate of maize plants inoculated with AMF after 35, 55 and 75 days. B. Chlorophyll content over time for maize plants inoculated with AMF under different drought treatments. C. Mean fresh shoot weight over time for maize plants inoculated with AMF under different drought treatments. D. Benzoxazinoids profile in the root tissues under different drought conditions and inoculation with AMF after 75 days. Mean ± standard errors are shown (n=8 except for CD; n =7). Anova was performed to analyze differences among treatments. Letters and stars indicate significant differences, ***: $p<0.0001$, **: $p<0.01$, $0.05<p<0.10$. CC here stands for ambient watering, CD is late drought, DC is early drought, and DD is prolonged drought treatment according to RCP 8.5 (16.6% soil moisture). For the first fourteen days, CC and CD treated plants received ambient watering while DC and DD treated plants were subjected to drought watering conditions.

Figure 3. MBOA complementation increased AMF colonization in maize plants after 20 days. A. Colonization rate of *Rhizophagus irregularis* (SAF22) with maize W22, mutant *bx1* and mutant *bx1* plants complemented with MBOA. B. Fresh shoot weight of maize W22, *bx1* mutant plants complemented with MBOA after 20, 40 and 60 days. Mean ± standard errors are shown (n=7; except for BX1_MBOA_Ctrl, n=6). Two-way anova test was run to analyze differences among treatments. Stars indicate significant differences, ***: $p<0.0001$, **: $p<0.01$, *: $p<0.05$, $0.05<p<0.10$. Ctrl= Control, AMF = Arbuscular mycorrhizal fungi, *Rhizophagus irregularis* (SAF22). MBOA0 = No MBOA, MBOA1 = MBOA addition.

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LIST OF SUPPLEMENTARY INFORMATION

Supplementary Table 1. Soil chemical profile.

Supplementary Table 2. Benzoxazinoids names and chemical formulas

Supplementary Figure 1. Experimental design for the semi-field assay to visualize the individual and interactive effects of drought and AMF on colonization and maize secondary metabolism.

Supplementary Figure 2: Experimental design for the greenhouse experiment to visualize kinetic effect of drought on colonization.

Supplementary Figure 3. Experimental design for the greenhouse experiment to visualize the role of Benzoxazinoids in AMF colonization with W22 and mutant *bx1* plants complemented with MBOA.

Supplementary Figure 4: Interactive effect of drought and AMF *Rhizophagus irregularis* on chlorophyll content after 15, 45 and 60 days. The chlorophyll content was reduced under the effect of AMF in normally watered plants after 60 days.

Supplementary Figure 5: Interactive effect of drought and AMF *Rhizophagus irregularis* on shoot height after 15, 30, 45 and 60 days.

Supplementary Figure 6: Interactive effect of drought and AMF *Rhizophagus irregularis* on root benzoxazinoids content after 60 days.

Supplementary Figure 7: Interactive effect of drought and AMF *Rhizophagus irregularis* on leaf benzoxazinoids content after 60 days.

Supplementary Figure 8: Correlation between colonization rate and root benzoxazinoids content after 60 days.

Supplementary Figure 9. Kinetic drought effect on chlorophyll content in inoculated maize B73 plants after 35 and 75 days.

Supplementary Figure 10. Kinetic drought effect on shoot height in inoculated maize B73 plants after 35, 55 and 75 days.

Supplementary Figure 11. Kinetic drought effect on fresh shoot weight in inoculated maize B73 plants after 35 and 75 days.

Supplementary Figure 12. Kinetic drought effect on colonization rate of *Rhizophagus irregularis* with maize B73 plants after 35, 55 and 75 days.

Supplementary Figure S13. Kinetic drought effect on root benzoxazinoids content after 35 days.

Supplementary Table 1. According to the Labor für Boden- und Umweltanalytik (German for Laboratory for Soil and Environmental Analysis) soil profile analysis, the table represents nutrient profiles in the soil sample. Correction factor is applied to the raw data as a numerical adjustment for variations that affect measurement accuracy, it is not applied in the nutrient values given in the table above.

Parameter	Unit	Result	Method	Interpretation/Category
Humus	% G/G	3.0	Texture Test (FP)	Low in Humus
Clay	% G/G	21.0	Texture Test (FP)	Clay
Silt	% G/G	31.0	Texture Test (FP)	
pH Value		7.5	pH (1:2.5 H_2O)	Weakly alkaline

Available Nutrient (H_2O10)

Nutrient	Unit	Result	Correction Factor	Supply Level
Nitrate	mg/kg	877.1		Enriched
Phosphorus	mg/kg	2.8	1.4	Moderate
Potassium	mg/kg	431.8	0.0	Enriched
Calcium	mg/kg	968.5		Enriched
Magnesium	mg/kg	76.1	0.0	Stock

Reserve Nutrients (AAE10)

Nutrient	Unit	Result	Correction Factor	Supply Level
Phosphorus	mg/kg	296.8	0.0	Enriched
Potassium	mg/kg	662.4	0.0	Enriched
Calcium	mg/kg	26,970		Stock
Magnesium	mg/kg	369	0.2	Stock

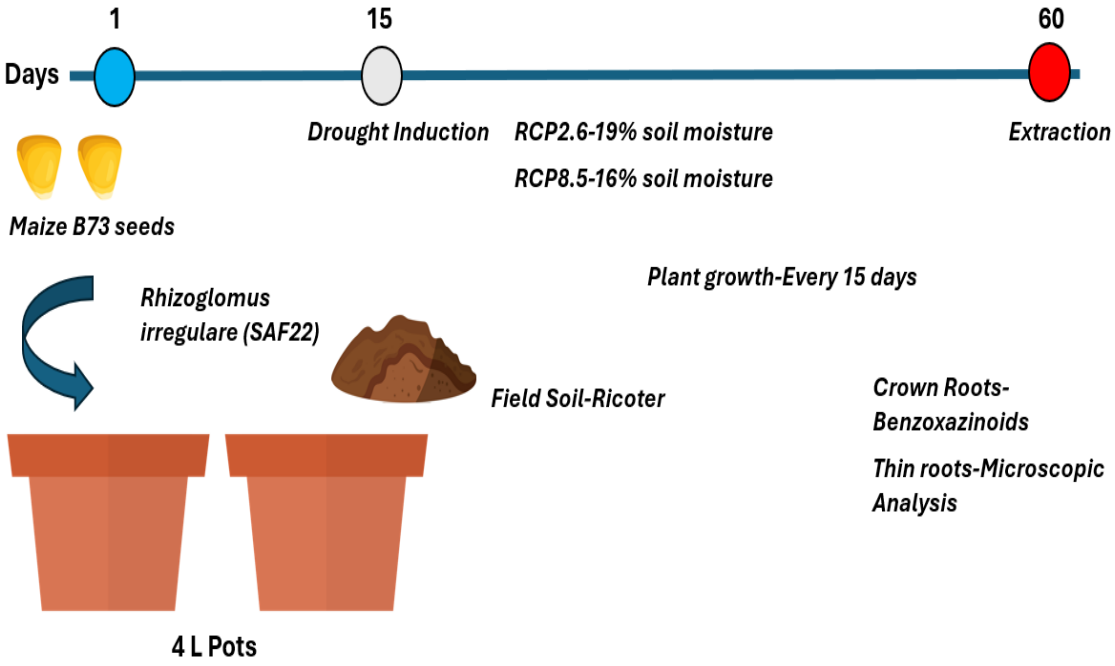
Trace Elements

Nutrient	Unit	Result	Correction Factor	Supply Level
Boron	mg/kg	1.7		Stock
Manganese	mg/kg	360		Stock
Copper	mg/kg	12.9		Stock
Iron	mg/kg	906		Enriched

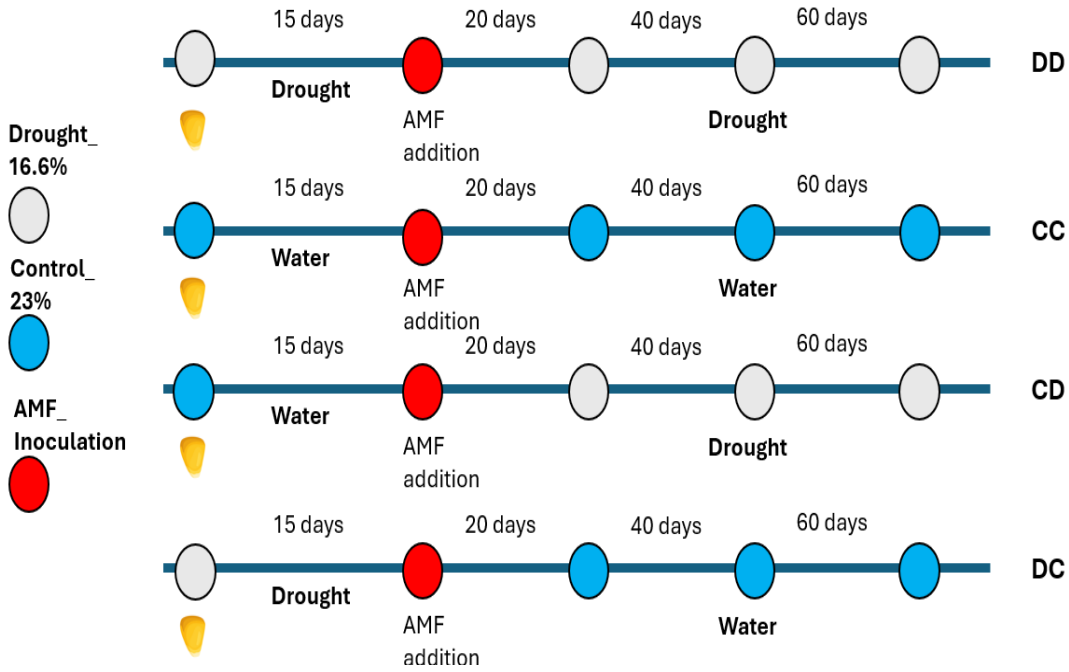
Supplementary Table 2. Benzoxazinoid names and chemical formulas

Name	Chemical name	Chemical formula
HMBOA	2-Hydroxy-7-methoxy-1,4-benzoxazin-3-one	$C_9H_9NO_4$
HMBOA-Glc	2-Hydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside	$C_{15}H_{19}NO_9$
HMBOA-2Glc	2-Hydroxy-7-methoxy-1,4-benzoxazin-3-one diglucoside	$C_{21}H_{29}NO_{14}$
HM_2BOA-Glc	2-Hydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one glucoside	$C_{16}H_{21}NO_{10}$
DIMBOA	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one	$C_9H_9NO_5$
DIMBOA-Glc	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside	$C_{15}H_{19}NO_{10}$
DIMBOA-2Glc	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one diglucoside	$C_{21}H_{29}NO_{15}$
DIMBOA-3Glc	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one triglucoside	$C_{27}H_{39}NO_{20}$
DIM2BOA-Glc	2,4-Dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one glucoside	$C_{16}H_{21}NO_{11}$
HDMBOA-Glc	2-Hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside	$C_{16}H_{21}NO_8$
HDM2BOA-Glc	2-Hydroxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one glucoside	$C_{17}H_{23}NO_{11}$
MBOA	6-Methoxybenzoxazolin-2-one	$C_8H_7NO_3$

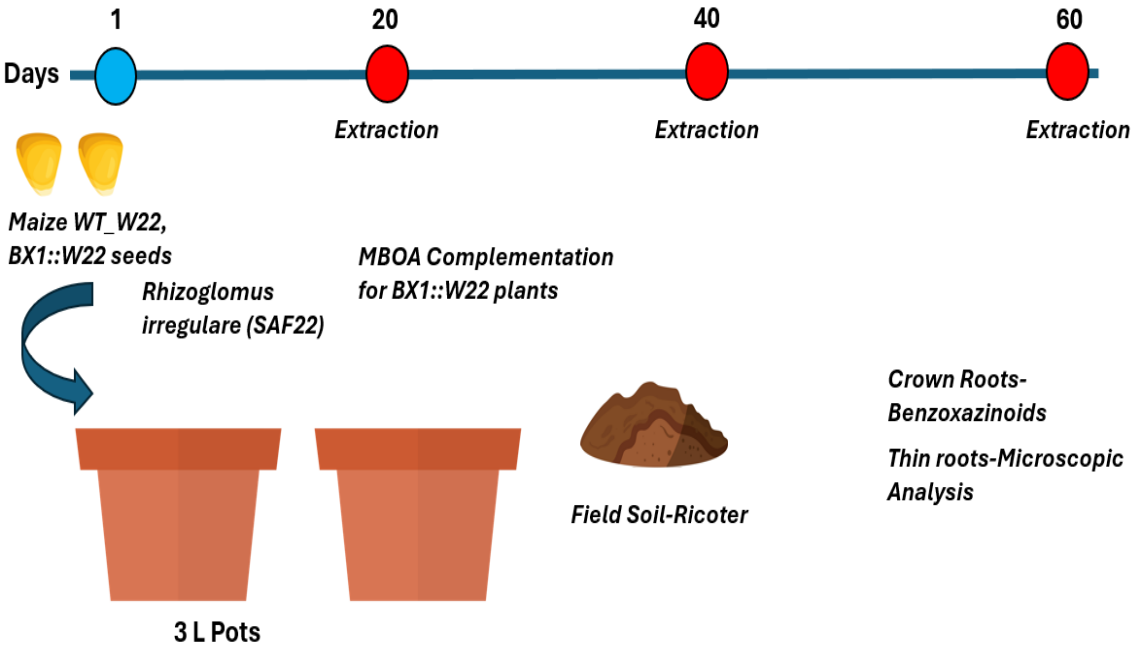
Supplementary Figure 1. Experimental design for the semi-field assay to visualize the individual and interactive effects of drought and AMF on colonization and maize secondary metabolism



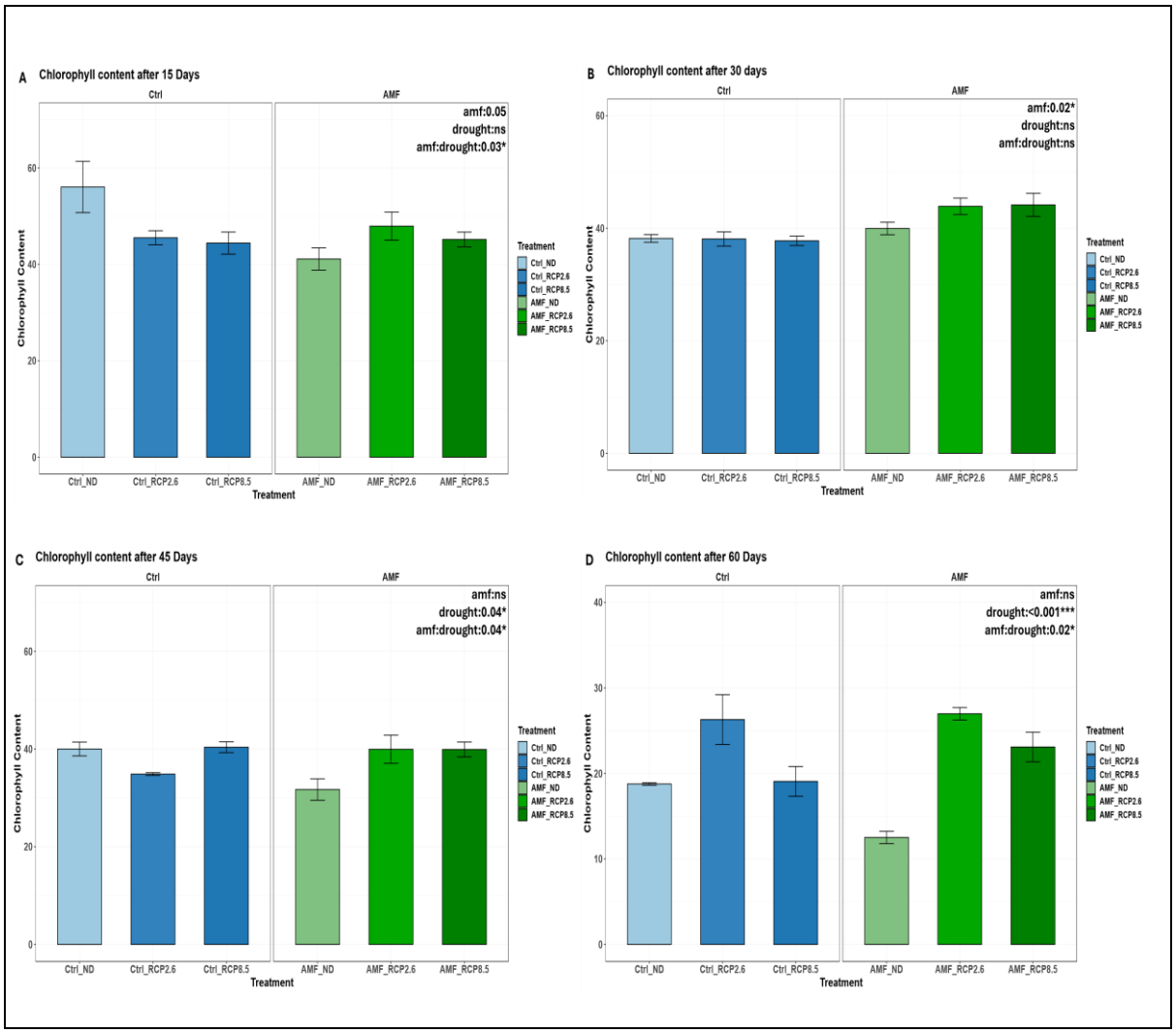
Supplementary Figure 2: Experimental design for the greenhouse experiment to visualize kinetic effect of drought on colonization



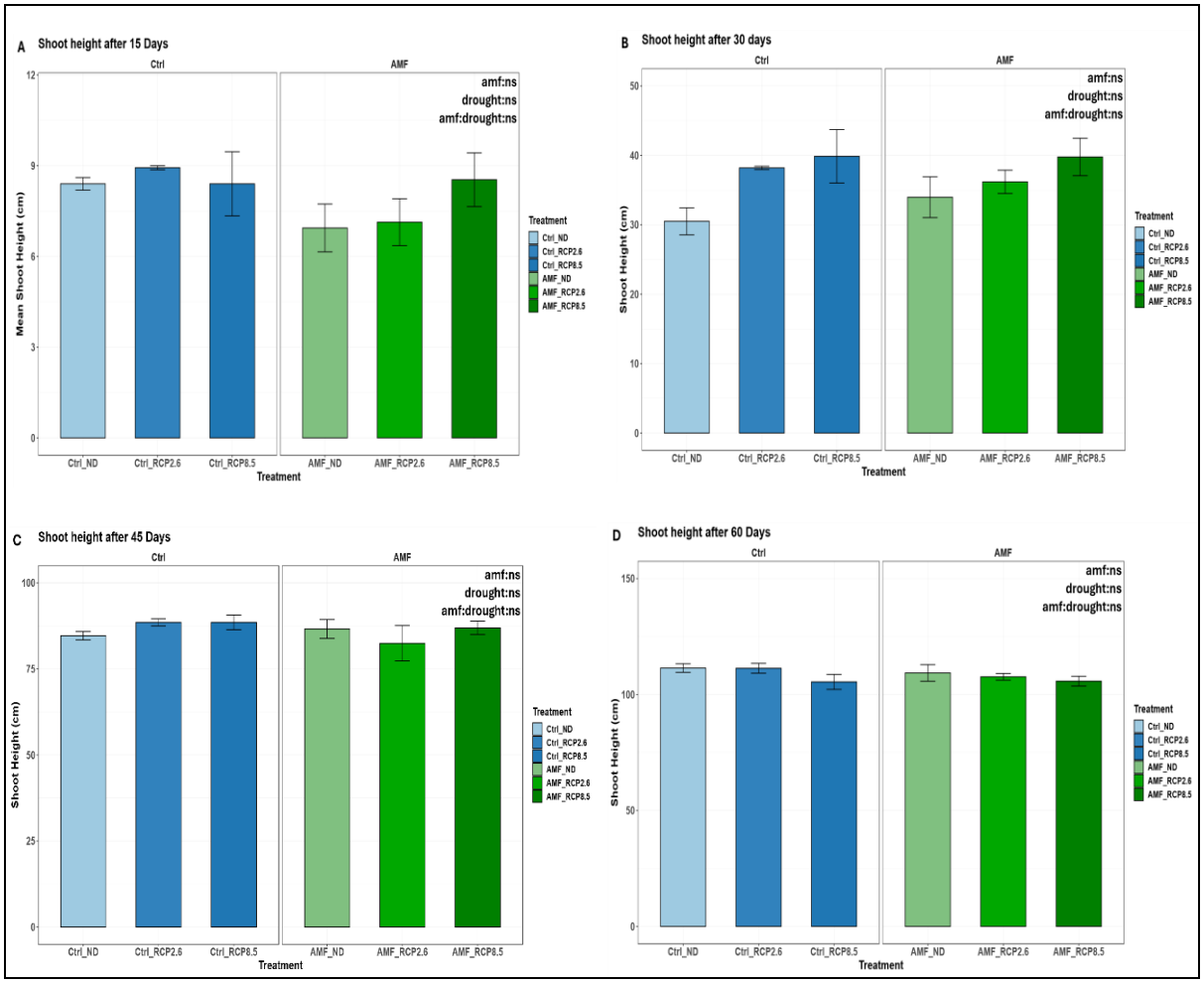
Supplementary Figure 3. Experimental design for the greenhouse experiment to visualize the role of Benzoxazinoids in AMF colonization with W22 and mutant *bx1* plants complemented with MBOA



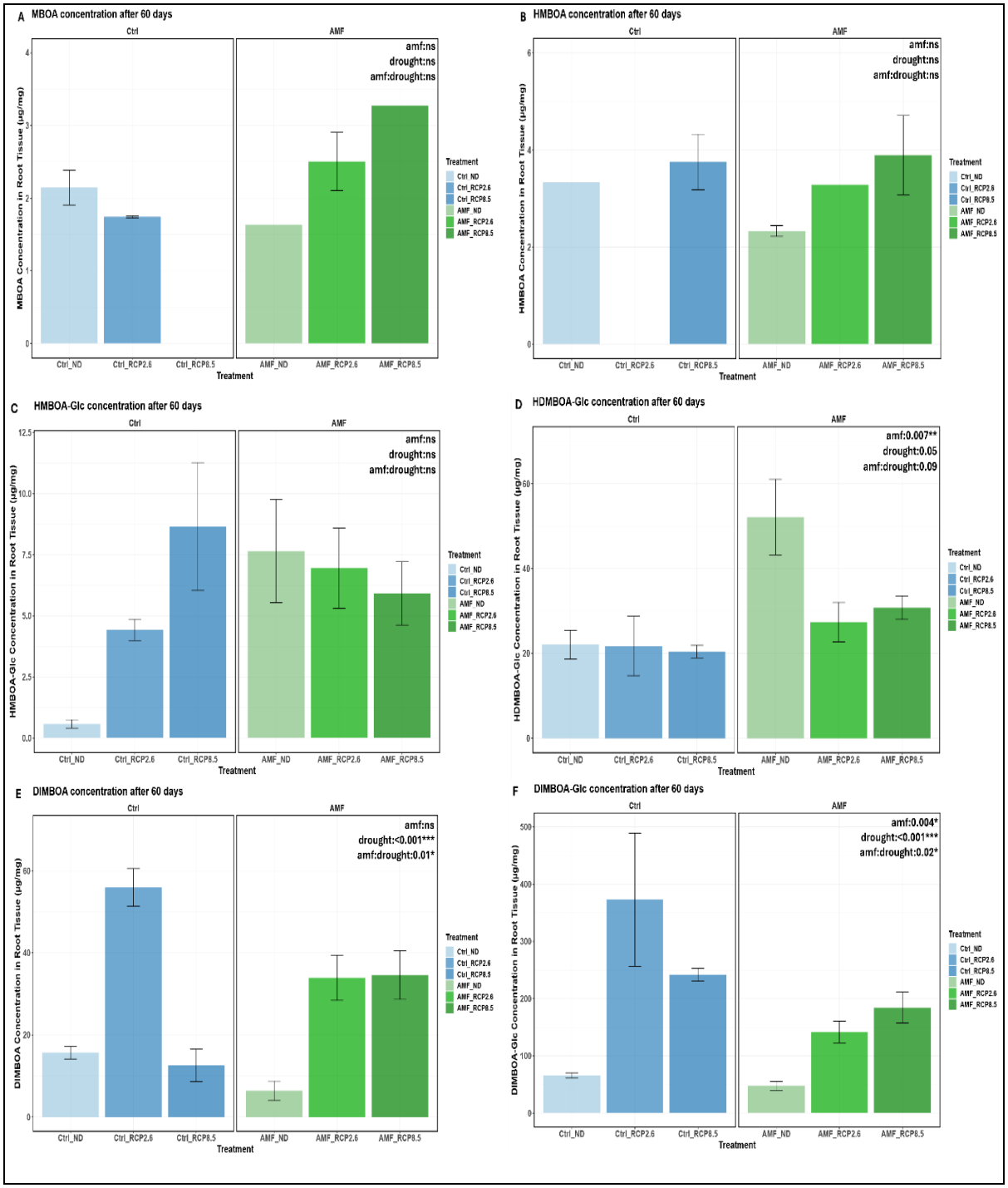
Supplementary Figure 4: Interactive effect of drought and AMF *Rhizophagus irregularis* on chlorophyll content after 15, 45 and 60 days. The chlorophyll content was reduced under the effect of AMF in normally watered plants after 60 days

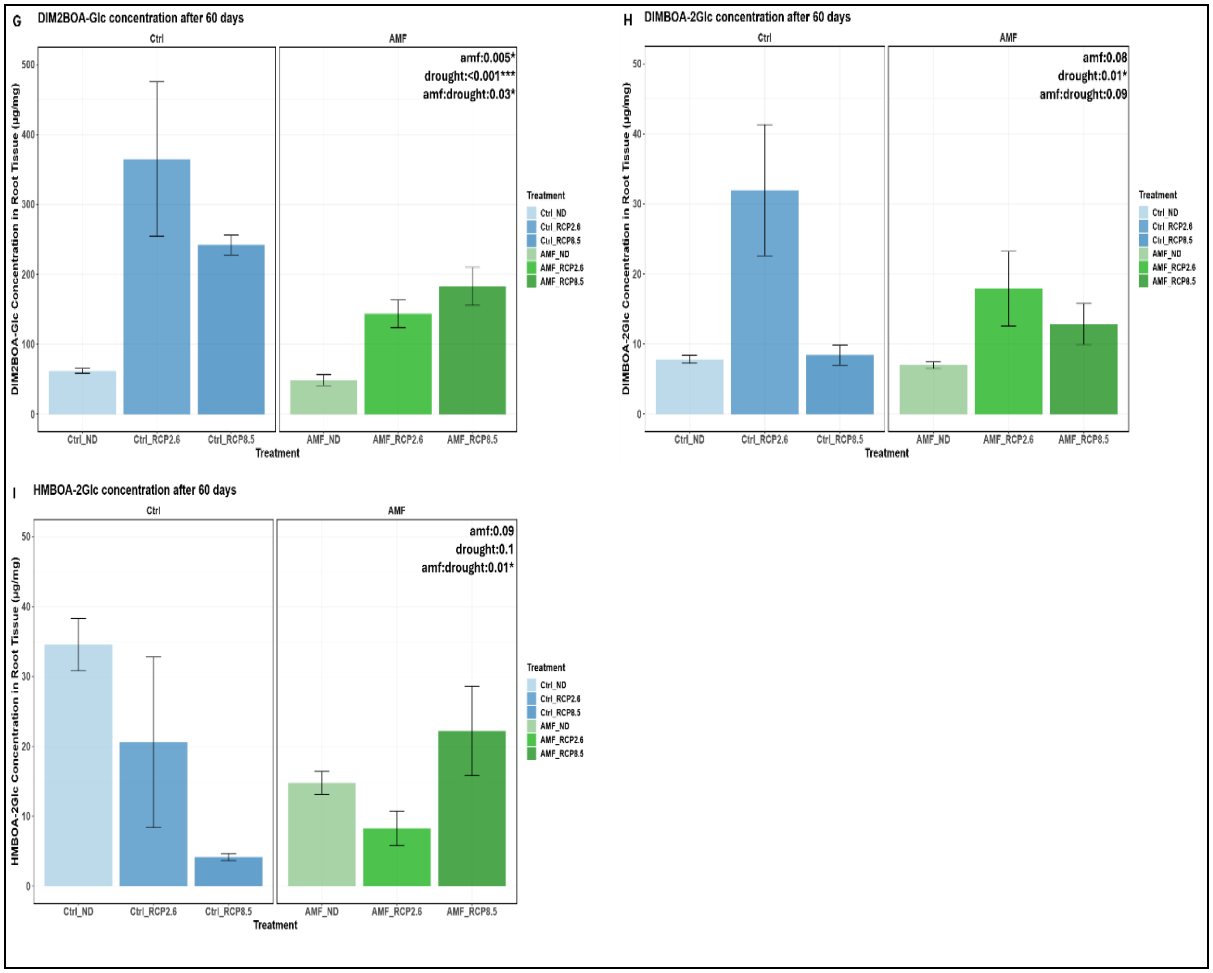


Supplementary Figure 5: Interactive effect of drought and AMF *Rhizophagus irregularis* on shoot height after 15, 30, 45 and 60 days

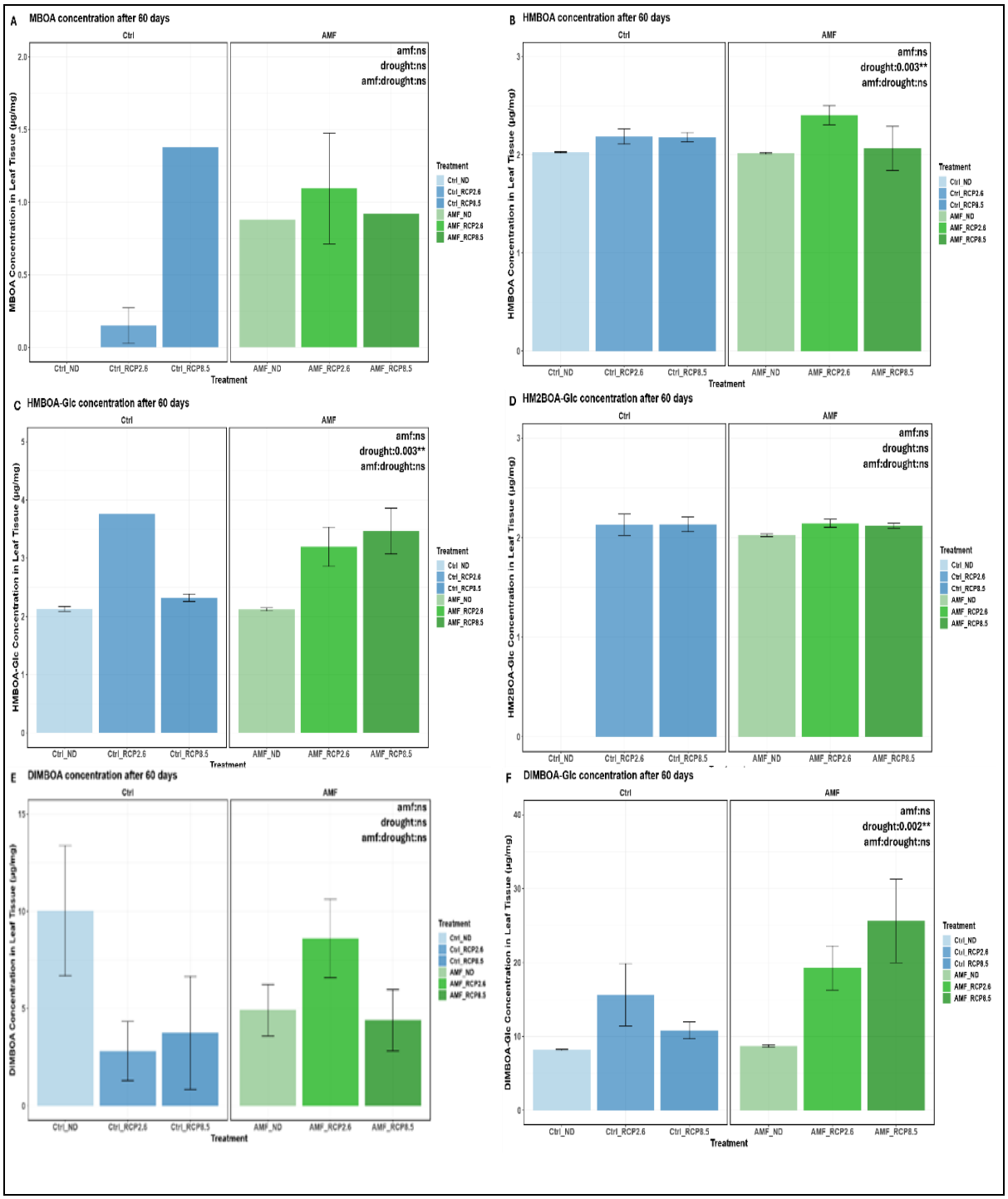


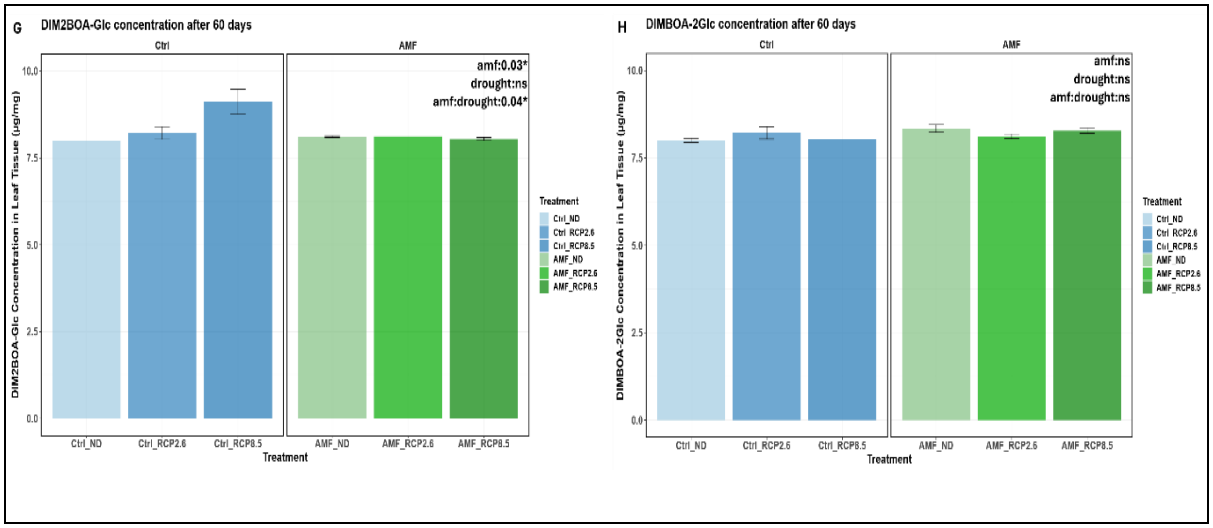
Supplementary Figure 6: Interactive effect of drought and AMF *Rhizophagus irregularis* on root benzoxazinoids content after 60 days



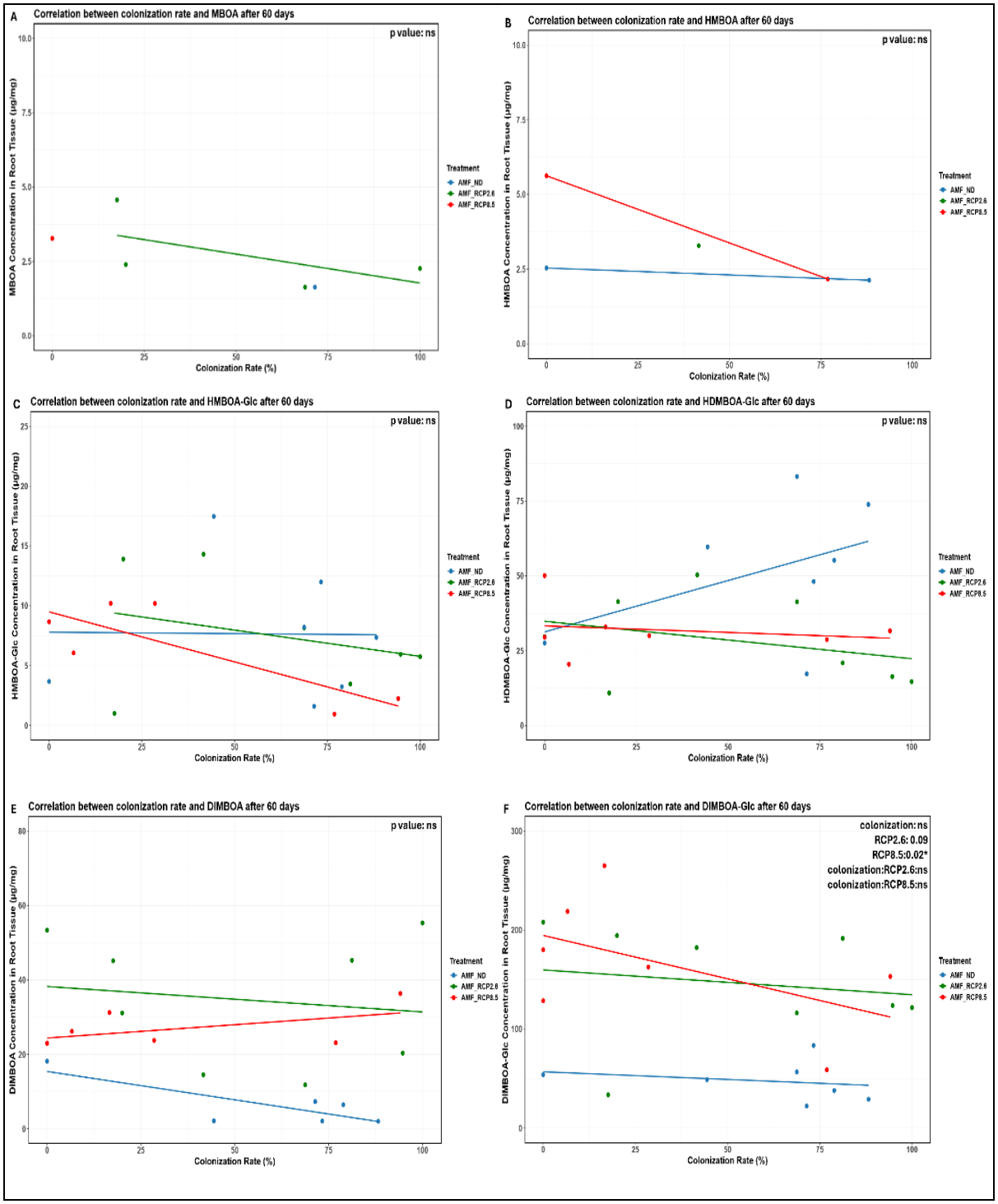

G
DIM2BOA-Glc concentration after 60 days
Ctrl
AMF
amf:0.005*
drought:<0.001***
amf:drought:0.03*
DIM2BOA-Glc Concentration in Root Tissue (µg/mg)
Treatment
Ctrl_ND
Ctrl_RCP2.6
Ctrl_RCP8.5
AMF_ND
AMF_RCP2.6
AMF_RCP8.5
H
DIMBOA-2Glc concentration after 60 days
amf:0.08
drought:0.01*
amf:drought:0.09
DIMBOA-2Glc Concentration in Root Tissue (µg/mg)
I
HMBOA-2Glc concentration after 60 days
amf:0.09
drought:0.1
amf:drought:0.01*
HMBOA-2Glc Concentration in Root Tissue (µg/mg)

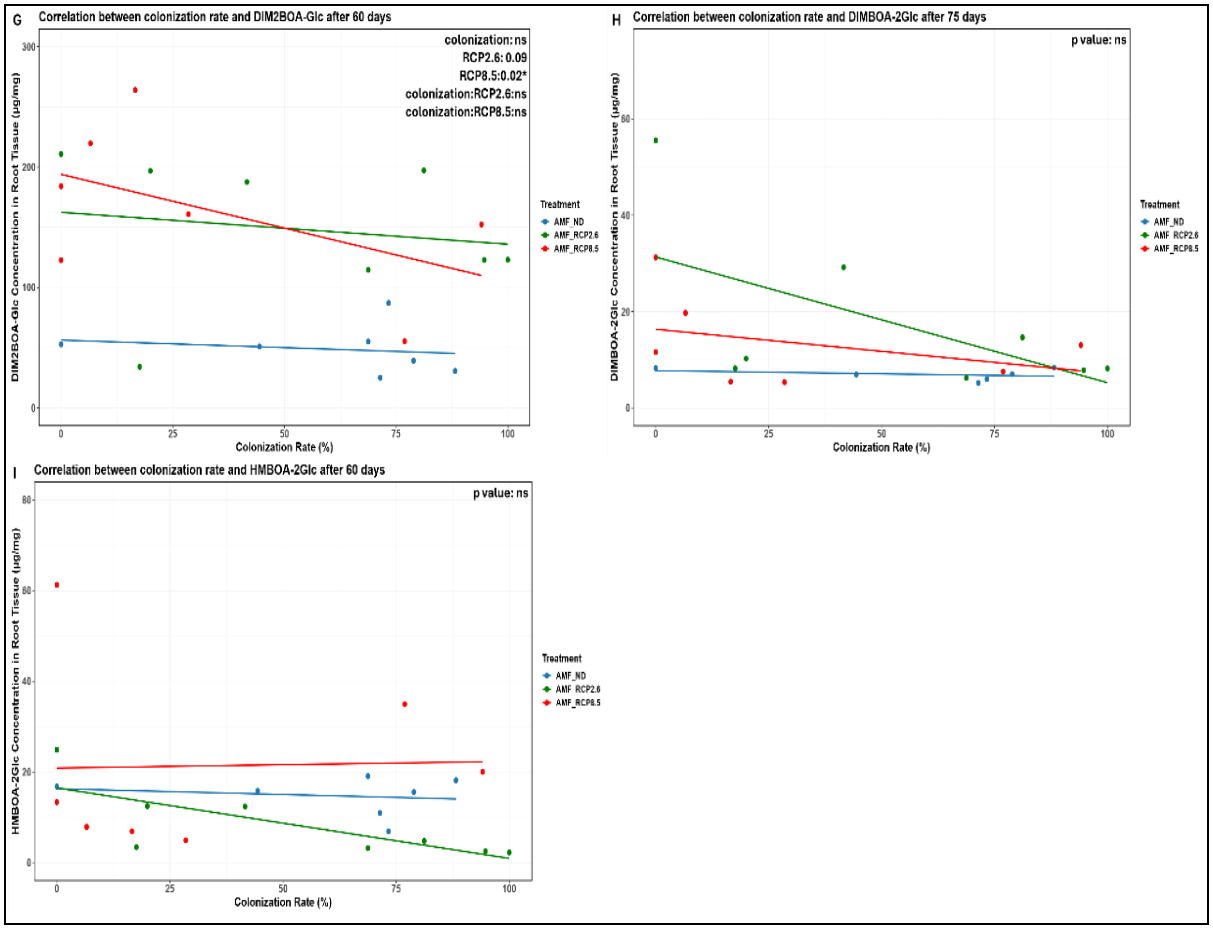
Supplementary Figure 7: Interactive effect of drought and AMF *Rhizophagus irregularis* on leaf benzoxazinoids content after 60 days



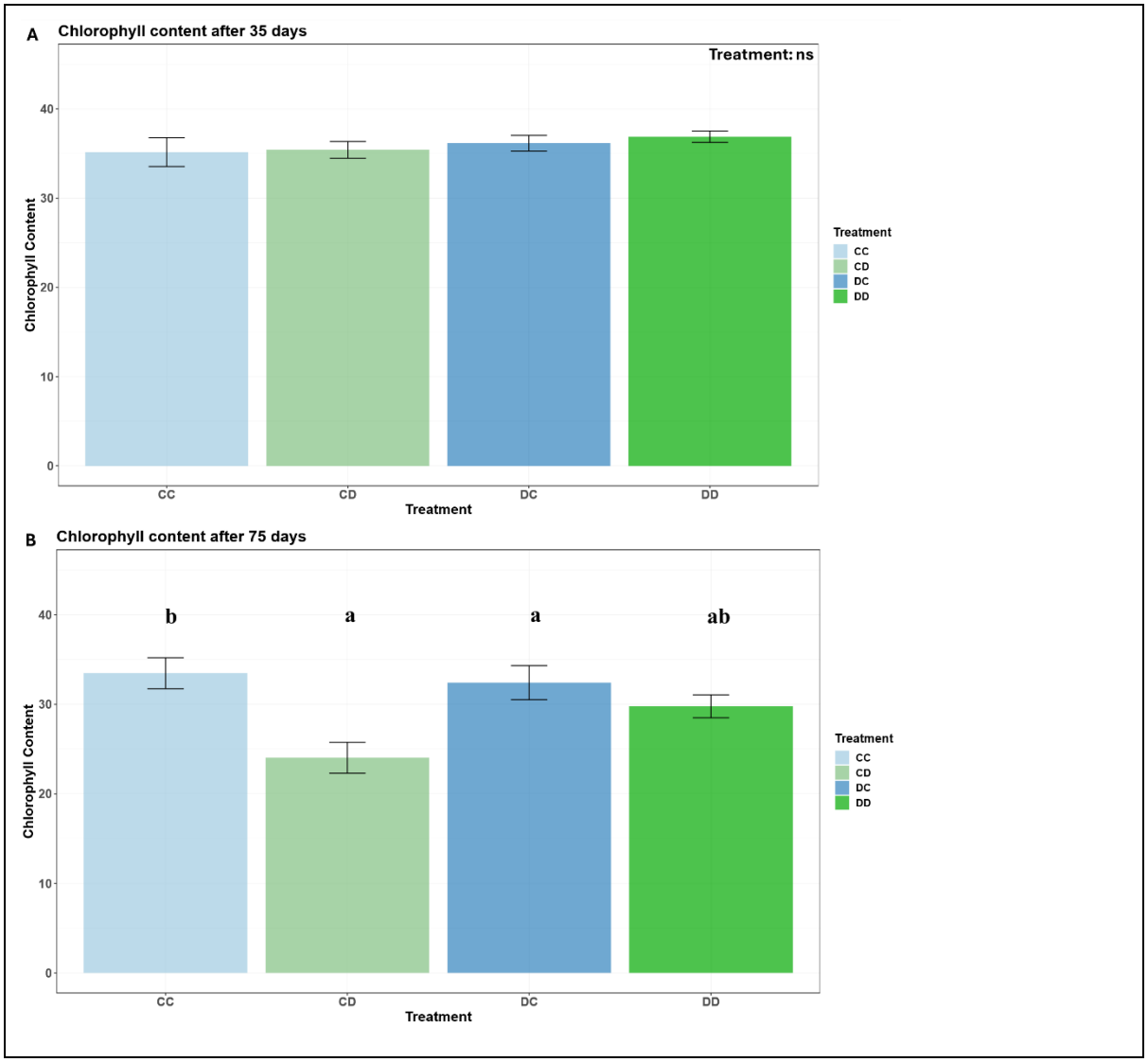

G
DIM2BOA-Glc concentration after 60 days
Ctrl
AMF
amf:0.03*
drought:ns
amf:drought:0.04*
DIM2BOA-Glc Concentration in Leaf Tissue (µg/mg)
10.0
7.5
5.0
2.5
0.0
Ctrl_ND
Ctrl_RCP2.6
Ctrl_RCP8.5
AMF_ND
AMF_RCP2.6
AMF_RCP8.5
Treatment
Treatment
Ctrl_ND
Ctrl_RCP2.6
Ctrl_RCP8.5
AMF_ND
AMF_RCP2.6
AMF_RCP8.5
H
DIMBOA-2Glc concentration after 60 days
Ctrl
AMF
amf:ns
drought:ns
amf:drought:ns
DIMBOA-2Glc Concentration in Leaf Tissue (µg/mg)
10.0
7.5
5.0
2.5
0.0
Ctrl_ND
Ctrl_RCP2.6
Ctrl_RCP8.5
AMF_ND
AMF_RCP2.6
AMF_RCP8.5
Treatment
Treatment
Ctrl_ND
Ctrl_RCP2.6
Ctrl_RCP8.5
AMF_ND
AMF_RCP2.6
AMF_RCP8.5

Supplementary Figure 8: Correlation between colonization rate and root benzoxazinoids content after 60 days

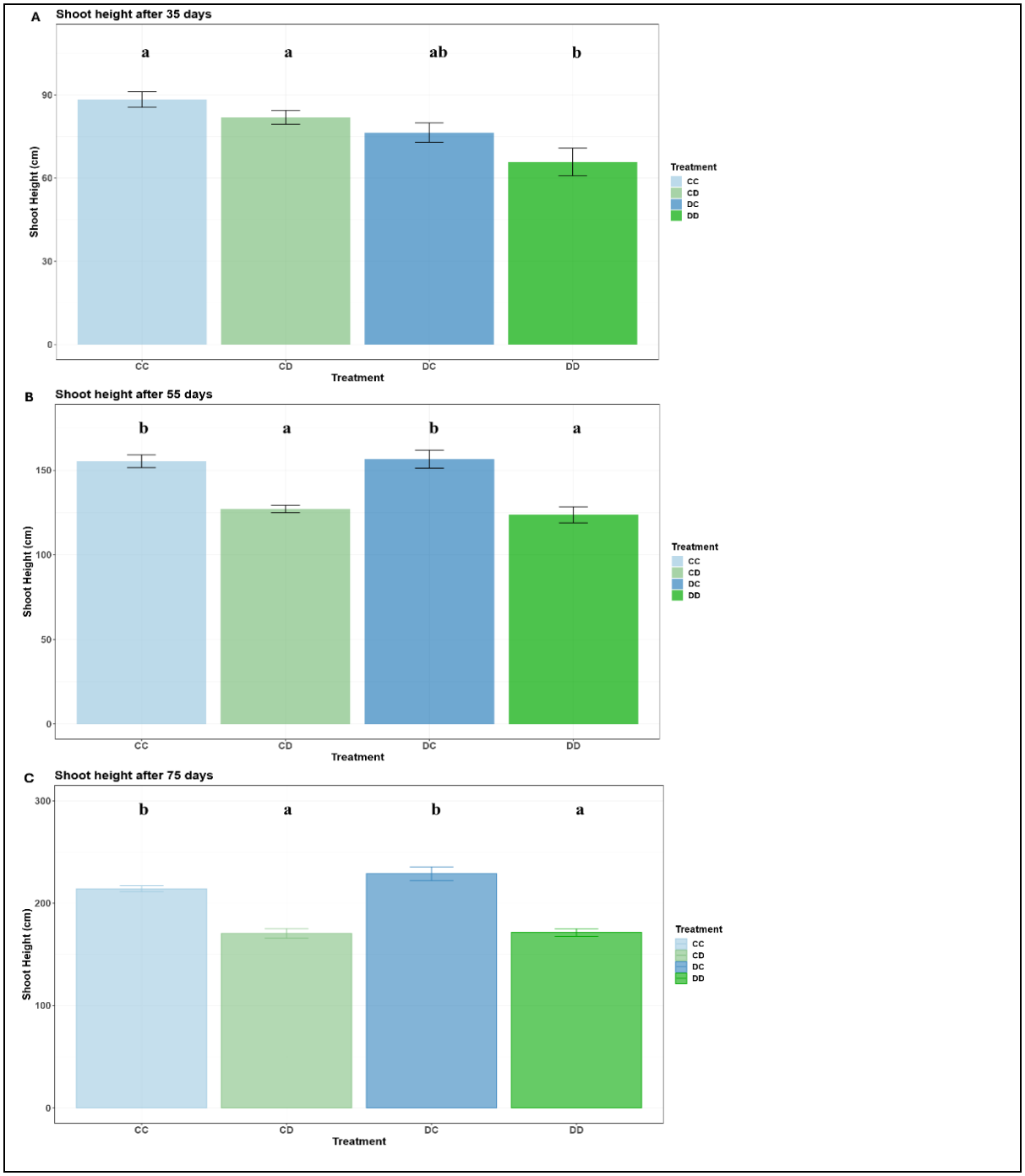



G
Correlation between colonization rate and DIM2BOA-Glc after 60 days
colonization: ns
RCP2.6: 0.09
RCP8.5:0.02*
colonization:RCP2.6:ns
colonization:RCP8.5:ns
DIM2BOA-Glc Concentration in Root Tissue (µg/mg)
Colonization Rate (%)
Treatment
AMF_ND
AMF_RCP2.6
AMF_RCP8.5
H
Correlation between colonization rate and DIMBOA-2Glc after 75 days
p value: ns
DIMBOA-2Glc Concentration in Root Tissue (µg/mg)
Colonization Rate (%)
Treatment
AMF_ND
AMF_RCP2.6
AMF_RCP8.5
I
Correlation between colonization rate and HMBOA-2Glc after 60 days
p value: ns
HMBOA-2Glc Concentration in Root Tissue (µg/mg)
Colonization Rate (%)
Treatment
AMF_ND
AMF_RCP2.6
AMF_RCP8.5

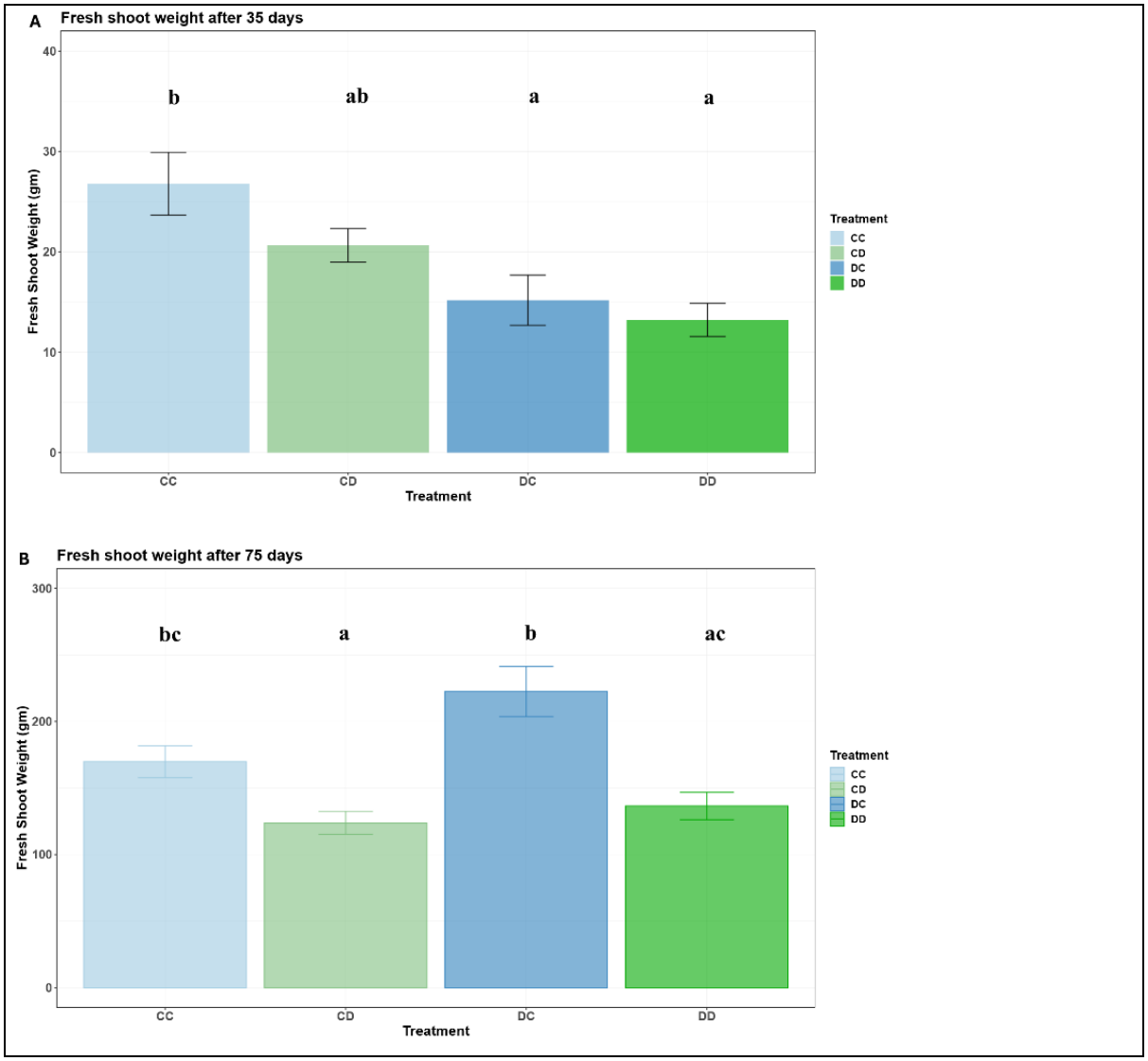
Supplementary Figure 9. Kinetic drought effect on chlorophyll content in inoculated maize B73 plants after 35, and 75 days



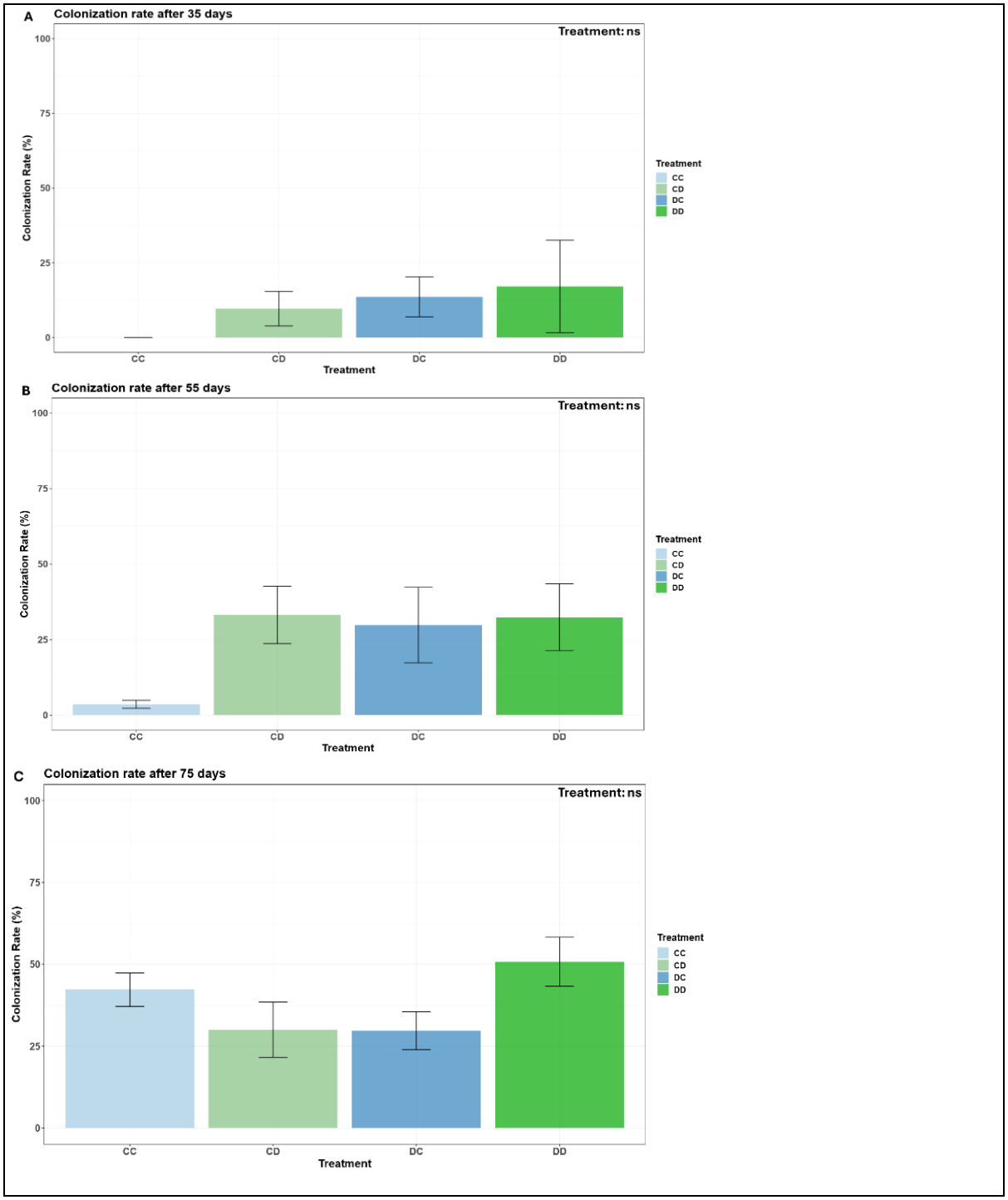
2486 Supplementary Figure 10. Kinetic drought effect on shoot height in
2487 inoculated maize B73 plants after 35, 55 and 75 days



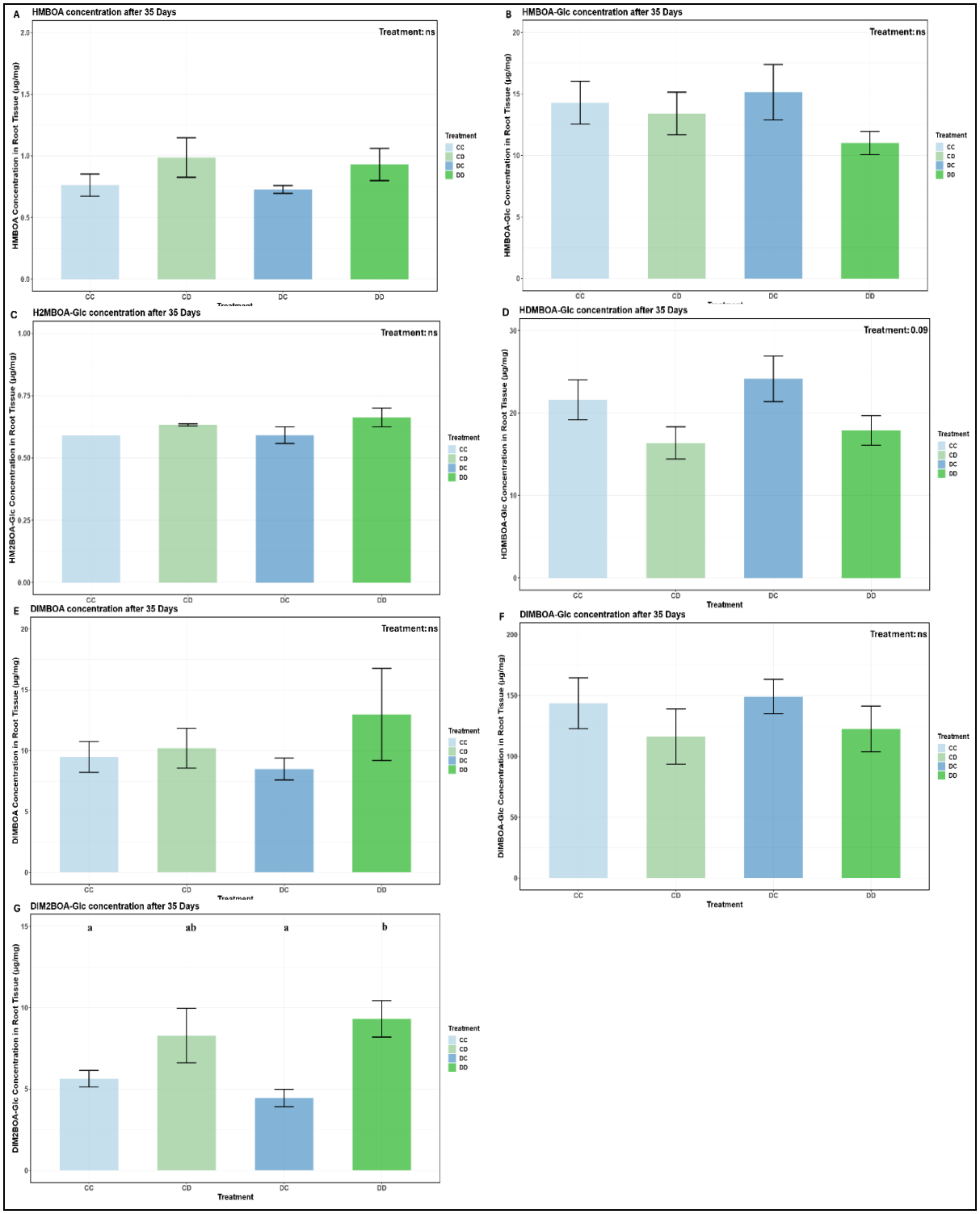
Supplementary Figure 11. Kinetic drought effect on fresh shoot weight in inoculated maize B73 plants after 35 and 75 days.



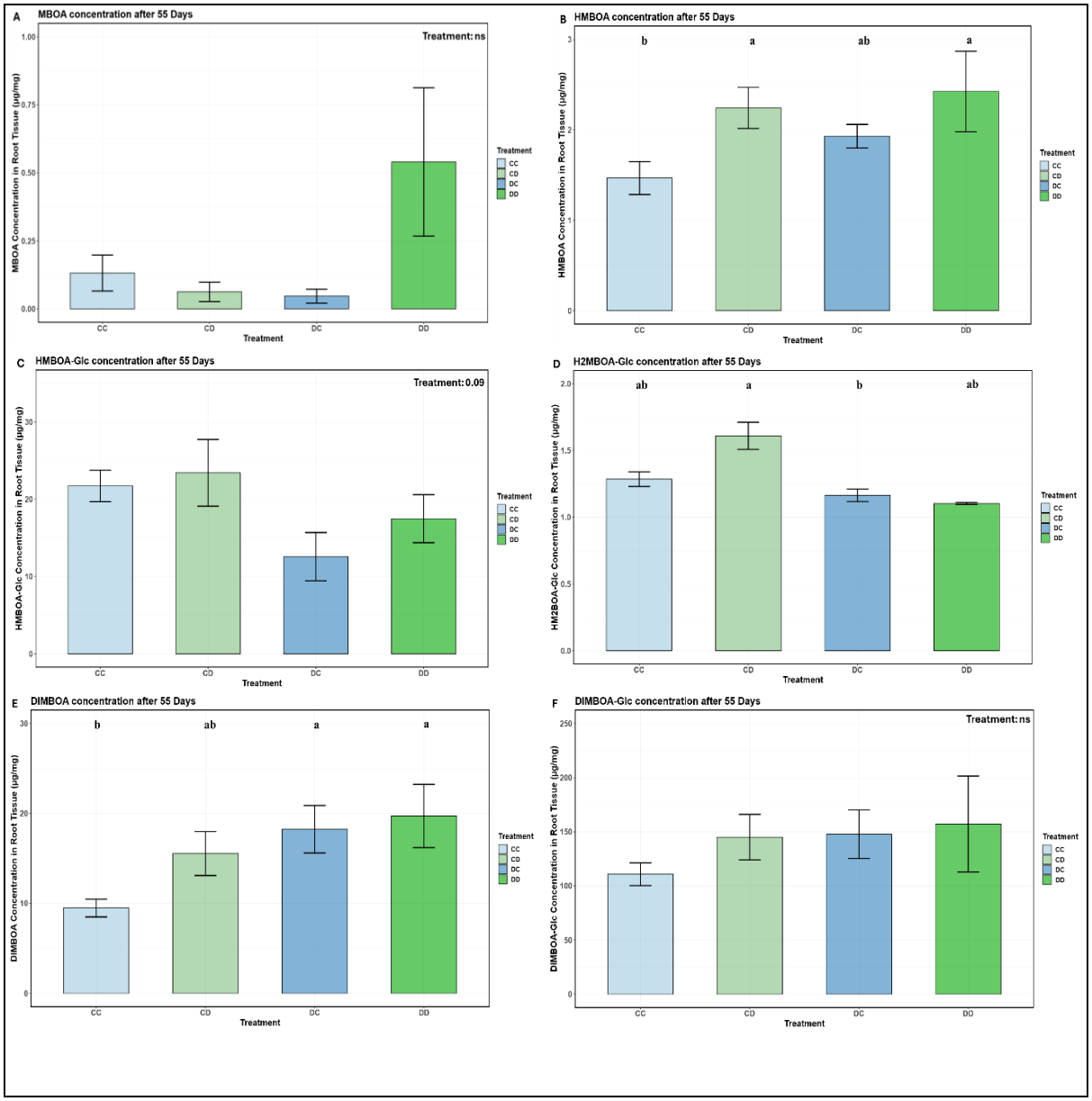
Supplementary Figure 12. Kinetic drought effect on colonization rate of *Rhizophagus irregularis* with maize B73 plants after 35, 55 and 75 days

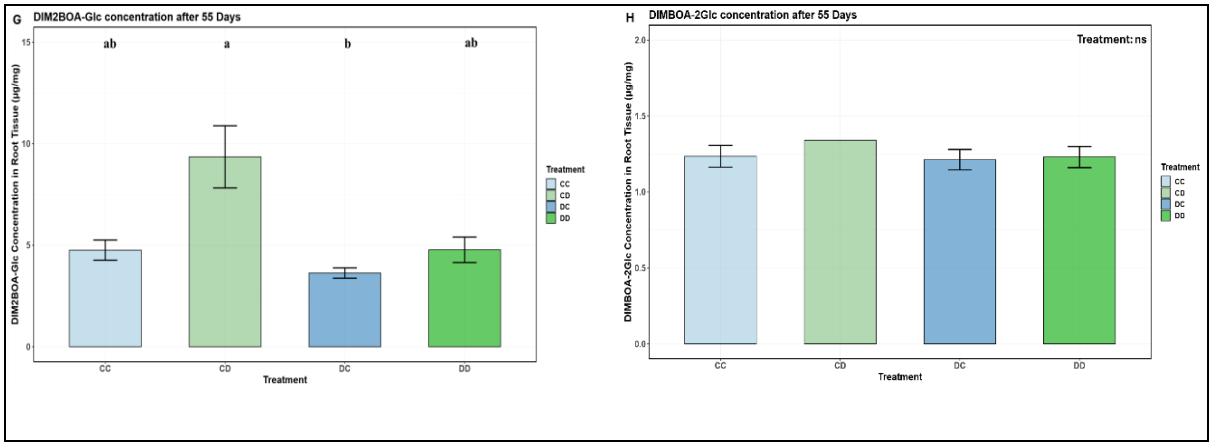


Supplementary Figure S13. Kinetic drought effect on root benzoxazinoids content after 35 days

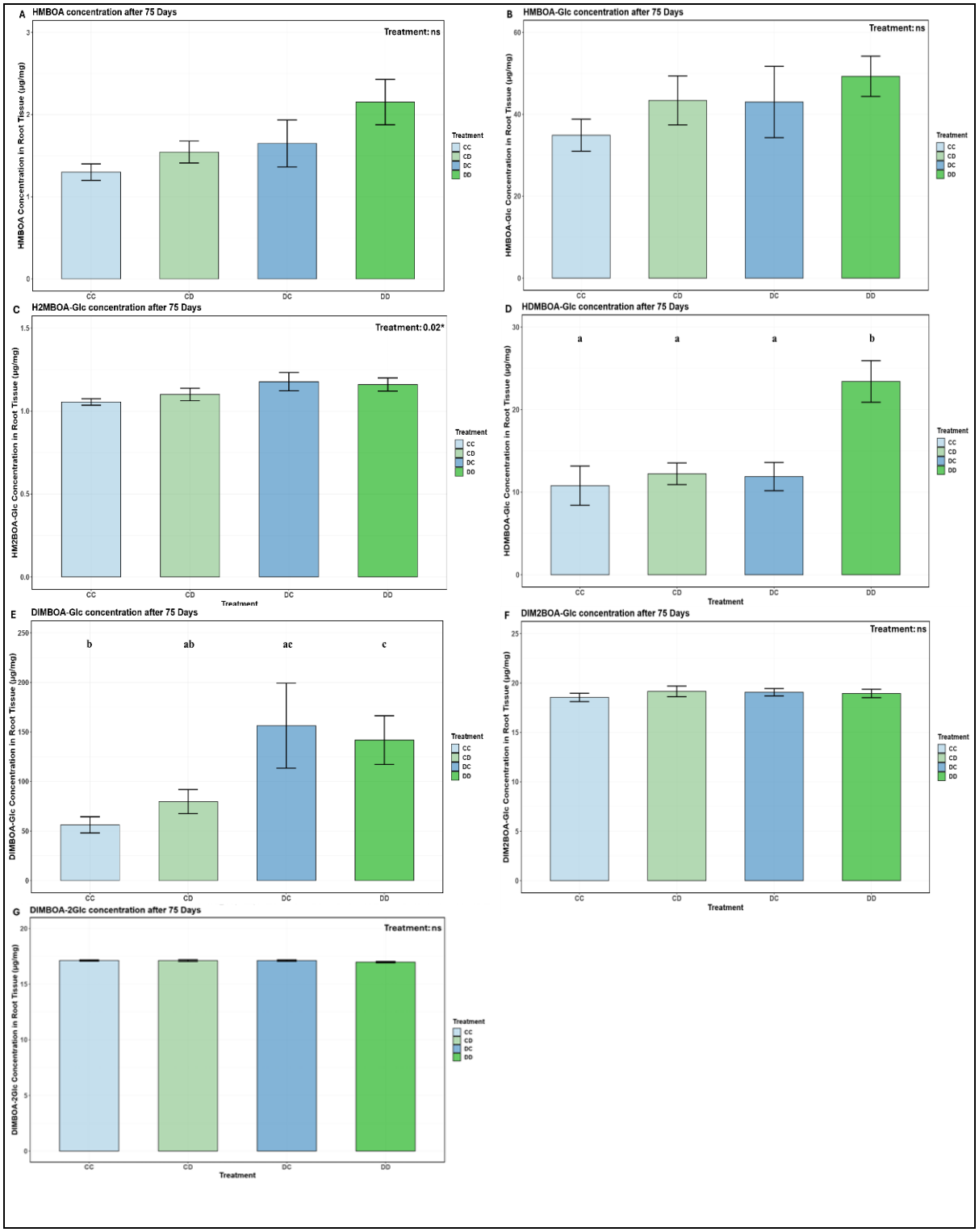


Supplementary Figure S14. Kinetic drought effect on root benzoxazinoids content after 55 days

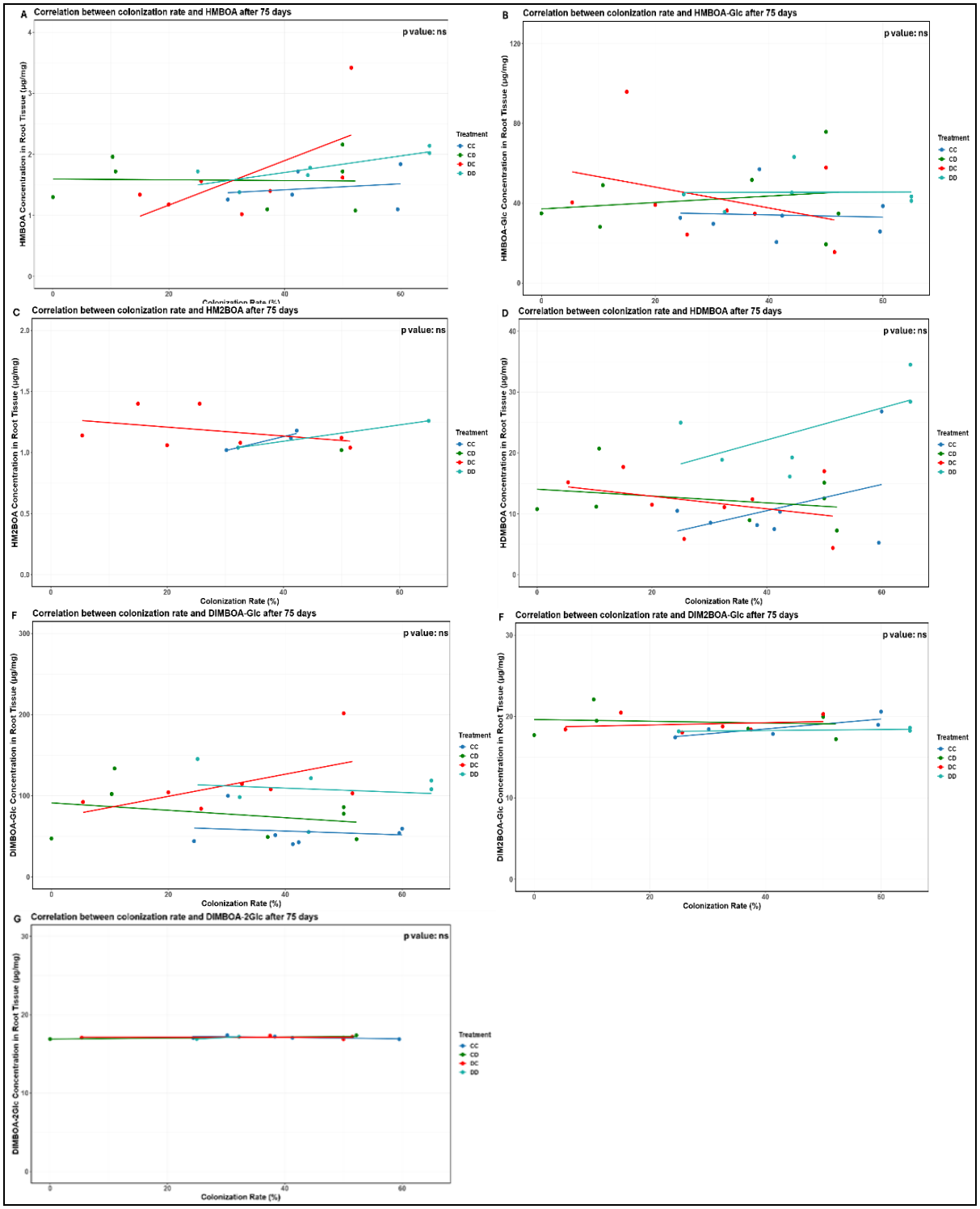



G
DIM2BOA-Glc concentration after 55 Days
ab
a
b
ab
DIM2BOA-Glc Concentration in Root Tissue (µg/mg)
Treatment
CC
CD
DC
DD
H
DIMBOA-2Glc concentration after 55 Days
Treatment: ns
DIMBOA-2Glc Concentration in Root Tissue (µg/mg)

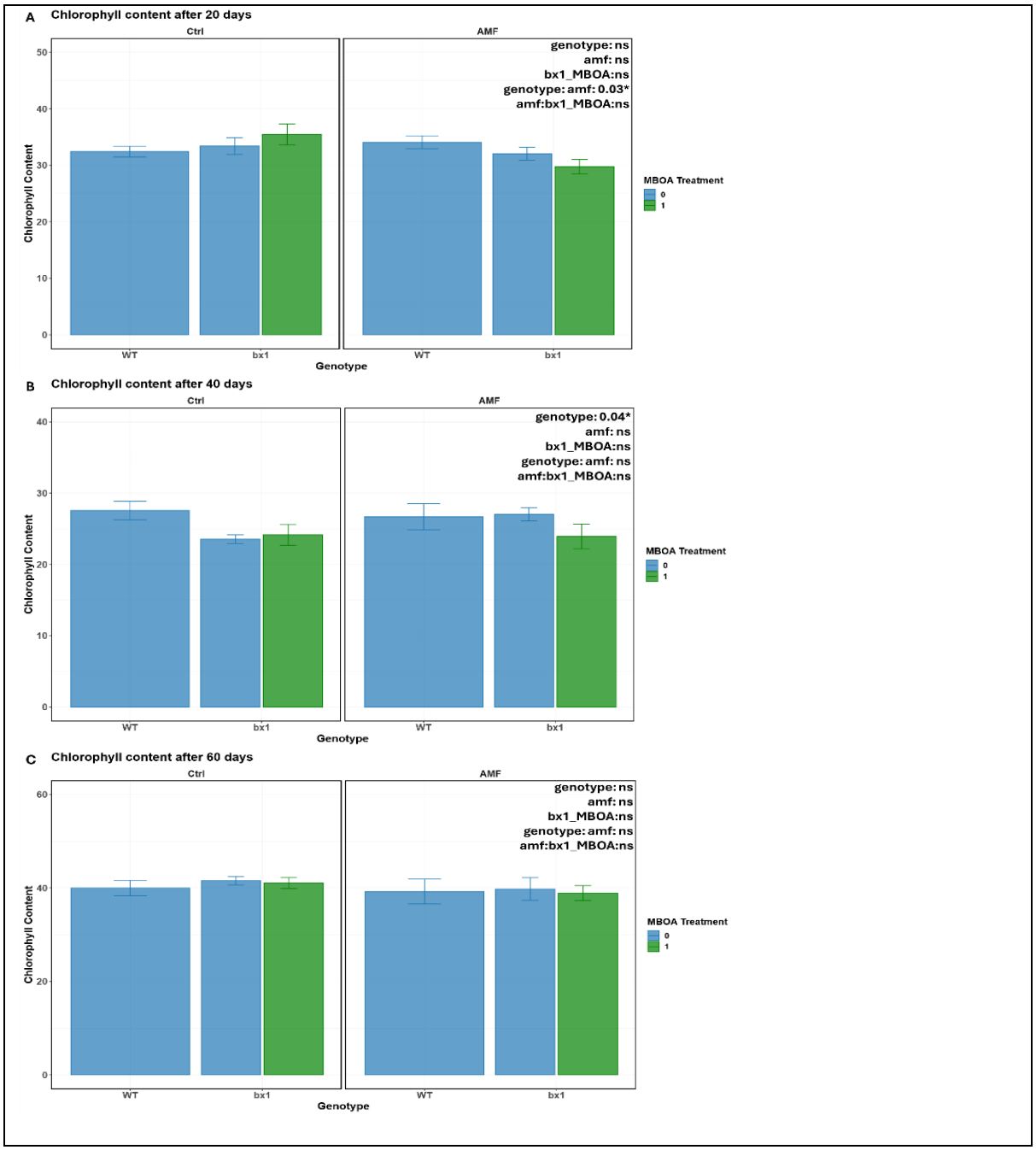
Supplementary Figure 15. Kinetic drought effect on root benzoxazinoids content after 75 days



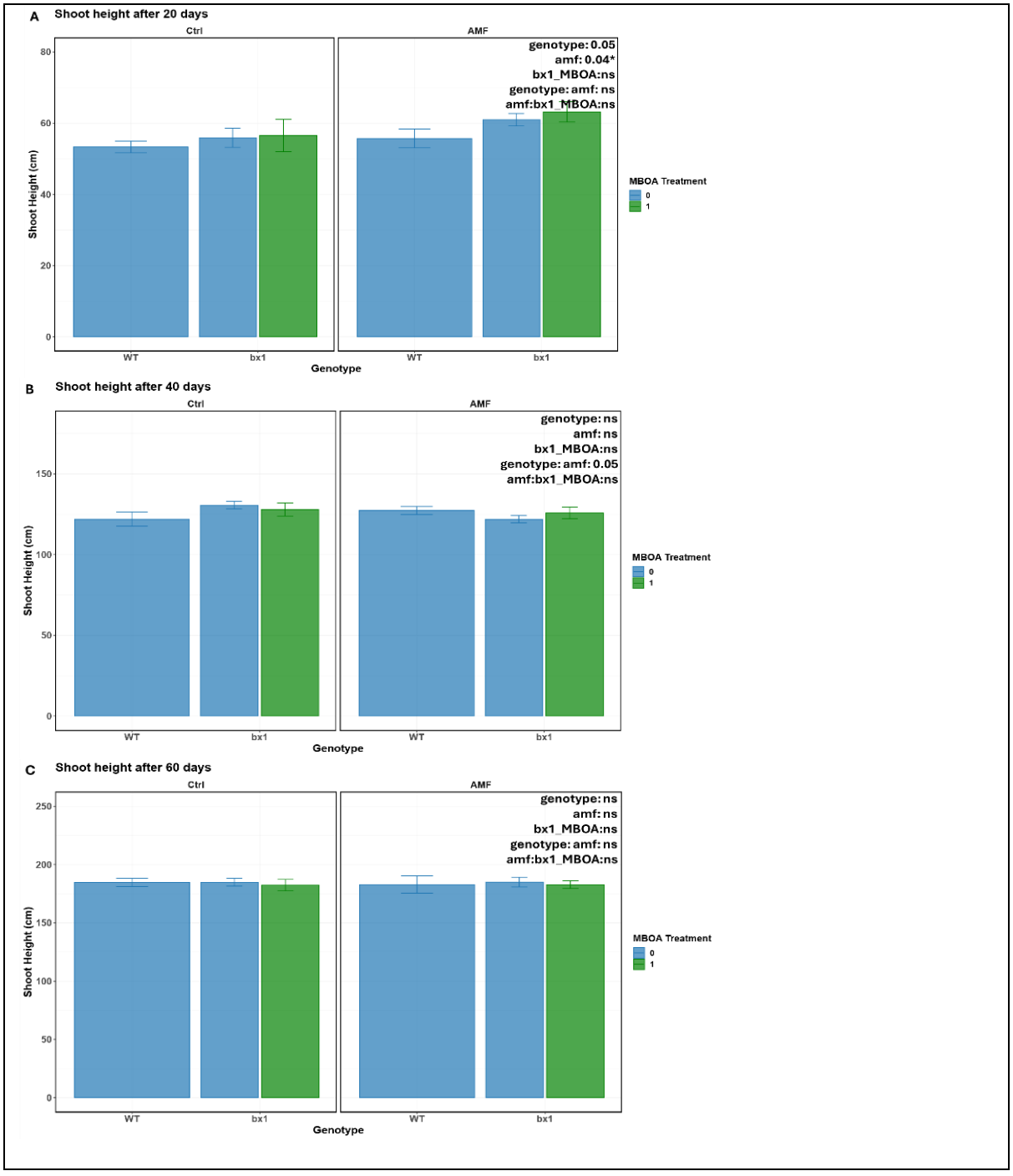
Supplementary Figure 16. Correlation between root benzoxazinoids content and colonization rate after 75 days



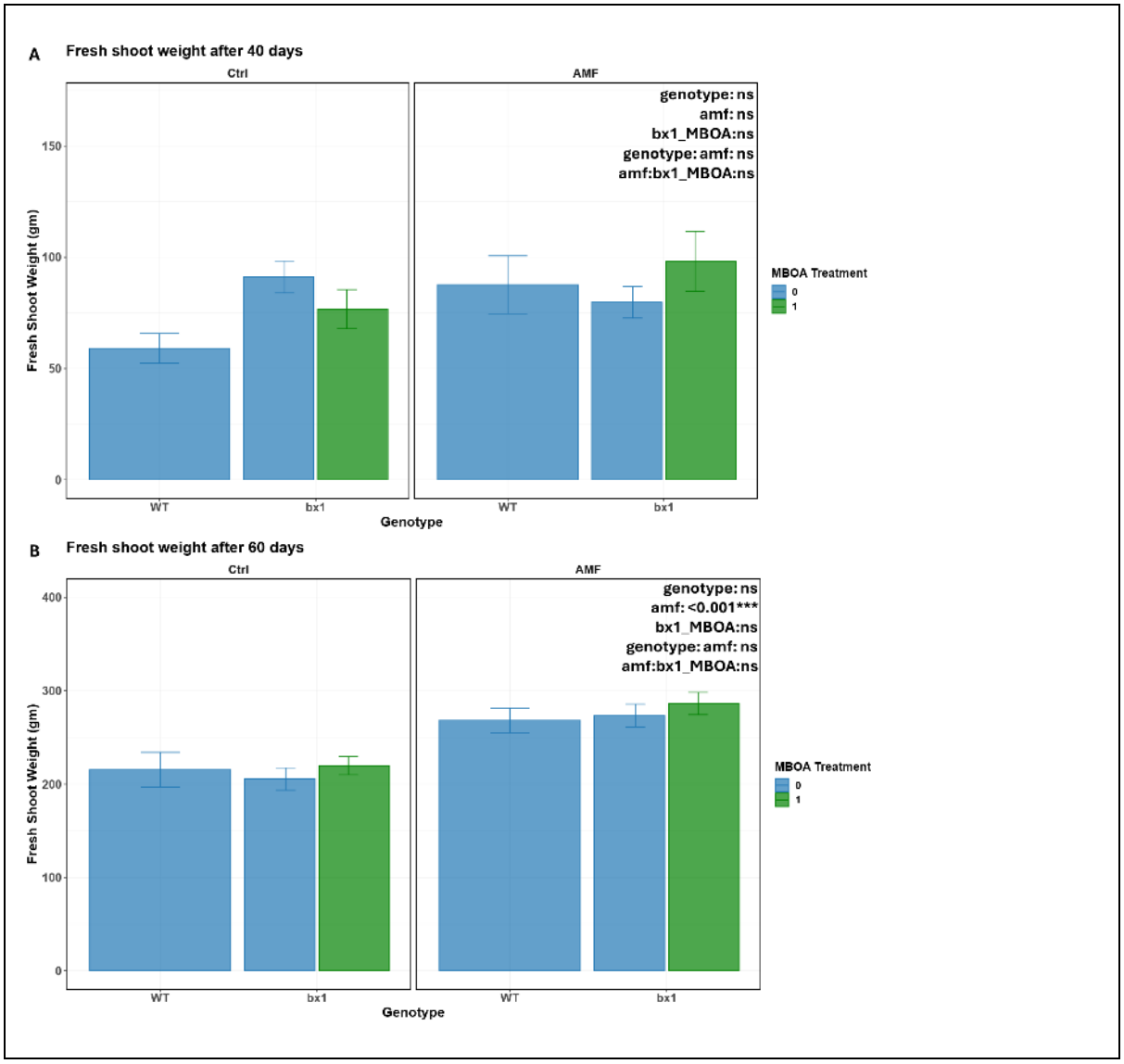
Supplementary Figure 17. Chlorophyll content of inoculated maize W22 and mutant *bx1* plants complemented with MBOA after 20, 40 and 60 days



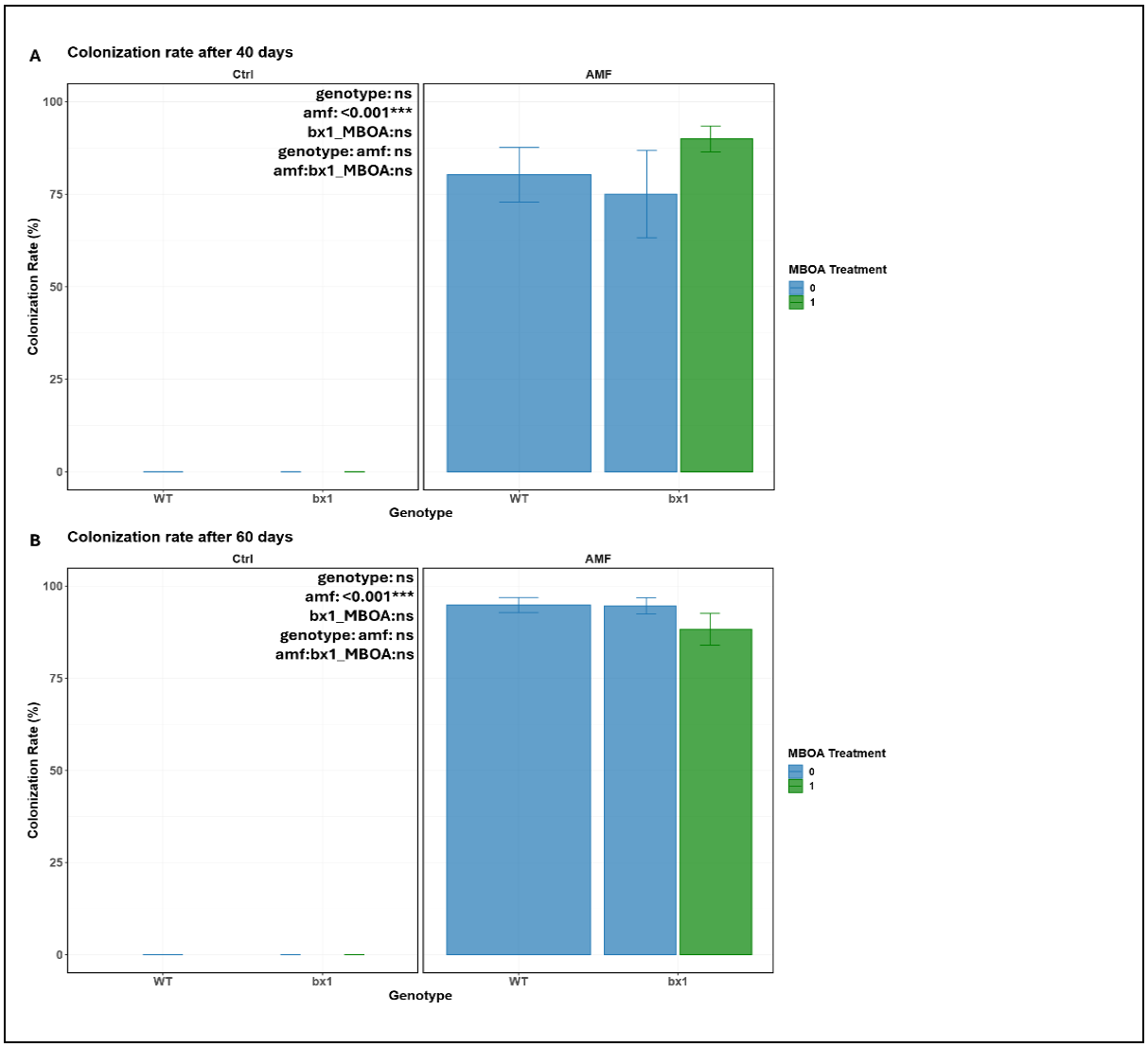
Supplementary Figure 18. Shoot height of inoculated maize W22 and mutant *bx1* plants complemented with MBOA after 20, 40 and 60 days



Supplementary Figure 19. Fresh shoot weight of inoculated maize W22 and mutant *bx1* plants complemented with MBOA after 40 and 60 days



Supplementary Figure 20. Colonization rate of AMF *Rhizophagus irregularis* with W22 and *bx1* mutant plants complemented with MBOA after 40 and 60 days



Chapter III.

Multihexose Benzoxazinoid Synthesis in Maize

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ABSTRACT

Benzoxazinoids are key defense metabolites in maize, and their activity and stability can be modulated through glycosylation. Here, we report a drought-induced biosynthetic pathway for multihexose benzoxazinoids in maize (*Zea mays*). Under drought, the concentrations of DIMBOA-2Glc, DIMBOA-3Glc, and HMBOA-2Glc increased up to 40-fold in roots. Transcriptome mining and phylogenetic analysis identified nine candidate UDP-glycosyltransferases (UGTs) in the UGT79, UGT91, and UGT94 families, of which two, UGT94A1 and UGT94A2, were strongly upregulated by drought. Recombinant expression in *E. coli* demonstrated that both enzymes can convert DIMBOA-Glc to DIMBOA-2Glc. Site-directed mutagenesis of UGT94A1 abolished this activity, confirming the functional role of the target residues. Additionally, CRISPR/Cas9 mutants for UGT94A1 and UGT94A2 were generated in KN5585 inbred line by Weimi Biotechnology Company. To date, a single homozygous ugt94a1 mutant has been isolated, containing a 368 bp deletion between the UGT94A1-1 and UGT94A1-2 target sites. Multiple other mutant lines for both loci are currently still segregating. Our findings uncover key enzymes in a previously uncharacterized multihexose benzoxazinoid biosynthesis pathway and highlight their inducibility by drought, offering new insights into the chemical adaptation of maize to environmental stress.

Keywords:

Multihexoses Benzoxazinoids, UDP-Glucosyltransferases, Recombinant Expression, Maize

INTRODUCTION

Many regions of the world are projected to suffer frequent and severe drought, significantly impacting crop yield (IPCC, 2022; Farooq et al., 2023; Karanth et al., 2023). As compared to 2010, global total food demand is expected to increase by 30-62% by 2050, therefore efforts aimed at increasing crop yields in many regions across the world will face serious challenges (Lobell et al., 2011; van Dijk et al., 2021). Plants adapt to drought through complex biochemical processes by regulating metabolic adjustments and production of defence compounds (Kaya et al., 2023). It is thereby necessary to understand these plant responses to drought to ensure global food security (Janni et al., 2024). This chapter focuses on the UDP-glycosyltransferases (UGTs) that are involved in modulating secondary metabolic profile in maize plants under drought stress.

Maize has played an increasingly diverse role since its domestication some 9000 years ago. As a staple crop, maize provides proteins, calories and essential nutrients for millions of people (FAO, 2022). Maize is the leading cereal in terms of production volume (Asfawa et al., 2024) with production over one billion tons over the last decade and will overtake wheat as the most grown and traded crop in the coming decade. By the year 2050, maize is also expected to provide more than 50% of the cereal demands. The world is currently witnessing a surge in maize production owing to demand and a combination of area expansion, technical advances and yield increase (Erenstein et al., 2022). Global maize production highlights its importance in the agriculture sector and therefore it is needed to ensure resilience against environmental stressors such as drought.

Although maize yield has increased significantly over the past few decades, its susceptibility to drought has also increased in parallel (Lobell, 2014). Drought can lead to significant yield losses by reducing the water use efficiency of maize during critical stages of growth (Hatfield and Dold, 2019). Drought stress is a major barrier in the production of maize as it decreases yield components, leaf photosynthesis and transpiration rate (Li et al., 2018). Maize is the most vulnerable to drought stress during the silking, vegetative and ear stages resulting in yield reduction of upto 25%, 50% and 21% respectively (Sah et al., 2020). Drought can lead to fewer kernels per cob and smaller kernel size by disrupting the plants reproductive process (Farooq et al., 2009). Drought stress during the vegetative growth stage can also lead to reduced growth rate, extension of the vegetative growth stage and redirection of the roots in maize plants (Wang et al., 2019). It is therefore critical to understand the physiological mechanisms that underlie maize tolerance to drought to facilitate its resilience to changing climatic conditions.

Chapter III

Benzoxazinoids (BXDs) are specialized plant secondary metabolites that are produced in the family poaceae including wheat, rye and maize (Niemeyer, 2009; Robert and Mateo, 2022). They are derived from indole and comprise of benzoxazinones (1,4-benzoxazin-3-one skeleton) and benzoxazolinones (1,3-benzoxazol-2-one core structure) and play role in modulating important activities in plants including reproduction, development, nutrition and defenses (Robert and Mateo, 2022). The predominant benzoxazinoid in maize is DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) which is synthesized from the amino acid tryptophan through a series of enzymatic steps (Frey et al., 1997). In response to both biotic and abiotic stresses such as pathogen attack and drought respectively, BXDs such as DIMBOA and its derivatives have been shown to accumulate in maize plants (Meihls et al., 2013; Erb et al., 2015). The role of these BXDs is to enhance plant´s defense strategies. Sutour et al., 2024 identified that the maize plant under drought conditions produces di, tri and tetra BXDs in the leaves and roots of maize plants. Multihexose BXDs that are specifically induced in the drought stress are DIMBOA-2Glc, DIMBOA-3Glc, HMBOA-2Glc, HMBOA-3Glc, HDMBOA-2Glc, highlighting plant metabolism modulation and ability to cope drought stress.

UDP-glycosyltransferases (UGTs) are a family of enzymes that catalyse the glycosylation of various plant secondary metabolites such as alkaloids, flavonoids, and terpenoids (Liu et al., 2025). The physicochemical properties of metabolites including solubility, stability, and reactivity are altered because of glycosylation thereby modulating the availability and bioreactivity of metabolic compounds in the plant tissues (Hou et al., 2004). Several studies in recent years have highlighted the importance of UGTs in plants response to drought stress. For example, UGT87A2 is overexpressed in *Arabidopsis thaliana* under drought conditions, and confers resistance by promoting germination, root growth, and reduced accumulation of reactive oxygen species (ROS) (Li et al., 2017). Similarly, overexpression of UGT79B2 and UGT79B3 under drought stress increased flavonoid accumulation improving plant resistance against drought and cold stress (Li et al., 2017). AtUGT79B2/B3 in *Arabidopsis* leads to enhanced glycosylation of anthocyanins resulting in increased plant tolerance to drought via ROS scavenging (Liu et al., 2017). In rice plants, UGT85E1 glycosylates abscisic acid and enhances tolerance to drought, this involves strengthening the stomatal closing under drought stress (Liu et al., 2021). Similarly, in Solanum plants, formation of flavonoid diglycosides resulted in antipyretic, anti-inflammatory and analgesic properties (Nassar et al., 2013). To enhance absorption and antioxidant capacity as compared to monoglycosides, flavonoid C-glycosides including isovitexin, vitexin and orientin are also multi-glycosylated in plants

(Xiao et al., 2016). This evidence strongly suggests broader the role of UGTS in mediating plant responses to drought and other environmental stresses across various species.

The detrimental effects of climate change are escalating on agriculture (Prajapati et al., 2024); it is therefore necessary to understand mechanism of plant responses to tackle these challenges. This chapter aims to identify the role of UGTs in maize plants that are involved in modulating secondary metabolites during stress conditions. Specifically, we aim to identify the UGTs that are involved in the formation of DIMBOA-2Glc in maize under conditions of drought. We expect that the UGTs involved in the glycosylation activity have a polar end to stabilize the second glucose molecule to already present glucose moiety, which in the current case is due to the amino acid threonine. We also aim to observe whether substitution of threonine with non-polar amino acid isoleucine by site directed mutagenesis can result in the loss of glycosylation activity. This will involve heterologous expression of genes in bacterial cells, purification of protein, performing enzymatic assays and HPLC-MS analysis. These insights can contribute to the better development of crops with enhanced tolerance to stress marking a key step forward in sustainable agricultural productivity.

METHODS

Biological material

Plants

Maize seeds (*Zea mays* L.) of the variety B73 were provided by Delley Semences et Plantes SA (Delley, CHE).

Climatic conditions

Current and predicted climatic conditions were calculated using climatic data from the Swiss Central Plateau (Average of summer conditions from 2004 to 2016, Oensingen, 47°17'11.1" N / 7°44'01.5" E, Switzerland), data were supported by MeteoSwiss (Federal Office of Meteorology and Climatology, Zürich, Switzerland), and predictions from the Representative Concentration Pathway 8.5 (RCP 8.5, Intergovernmental Panel on Climate Change (IPCC) report (IPCC 2014). RCP 8.5 corresponds to an extreme scenario in which CO_2 emissions continue to rise throughout the 21st century. Consequently, current and RCP 8.5 atmospheric CO_2 concentrations were of 450 ppm (± 50ppm), and 850 ppm (± 50 ppm) respectively. Current and RCP 8.5 of soil temperatures were 19.6 °C and 23 °C respectively. Because daily temperature variation can affect insect performance and predator-prey interactions (Stoks et al 2017), current and RCP 8.5 soil temperatures followed a diurnal variation of 3.5 °C (minimal

temperature at 6 am and maximal temperature at 4 pm) and reached a maximum temperature of 21.4 °C and 24.8 °C respectively (Figure S1). Current and RCP 8.5 soil volumetric moisture levels were adjusted to 23% and 16.6% (corresponding to 28 % less precipitation) (Figure S2).

Microcosm systems

To manipulate CO_2 levels, temperature, and moisture, we used a microcosm system using dry-bath cyclers and a custom-made CO2-dosage system. Falcon tubes (50 mL, Falcon, Greiner Bio-One, Frickenhausen, Germany) were filled with 10 g dry (80 °C for 48 hrs), sieved (2 cm mesh) soil (40% sand, 35% silt, 25% clay; Landerde, Ricoter, Aarberg, Switzerland). The natural soil microbiota was re-implemented to the soil as previously described (Hu et al., 2018). All falcon tubes were placed in dry bath cyclers (Digital Heating Cooling Drybath, Thermo Scientific, Fisher Scientific AG, Reinach, Switzerland) equipped with heating blocks that can accommodated up to nine falcon tubes. A CO_2 mixing and distribution system was designed to continuously mix CO_2 ambient air, measure the CO_2 concentration of the mixture, and distribute it to different channels. Mixing CO_2 and air was achieved using an air compressor (Prematic AG, Affeltrangen, Switzerland) coupled to two mass-flow-controllers (for CO_2: Bronkhorst El-Flow Select F-200CV (0.6 mL.min-1), Ruurlo, Netherlands; and for air: CKD FCM-0010AI (0-10 L.min-1), CKD Corporation, Aichi, 485-8551, Japan). Ambient air from outside the building was used for mixing, therefore no CO_2 was added to mimic current conditions (=450 ppm ± 50 ppm). A concentration of 400 ppm CO_2 (purity 100%, 54.6 L bottle, and pressure of output at 0.8 bars, Gümligen, Switzerland) was added + 400 ppm to ambient air (=850 ppm ± 50 ppm) to reach expected RCP 8.5 scenarios. The resulting CO_2: air mix was pushed through a filter of activated carbon (Camozzi, Warwickshire, United Kingdom) and split through valves (Needle Valve 2839-⅛, CKD, Aichi, 485-8551, Japan) into seven individual channels in a series. The first channel, referred thereafter as "CO_2 measuring channel", was connected to a CO_2 sensor (Rotronic AG, Bassersdorf, Switzerland). The air flow circulated alternatively between the CO_2 measuring channel (for 2 min) and experimental channels (for 2 min). The two minutes duration between experimental channels was sufficient to reach stable expected CO_2 concentrations. In all assays, four experimental channels were used, alternating between ambient (channels 2 and 4) and CO_2 enriched (channels 3 and 5) air. Therefore, the ambient or CO_2-enriched air was distributed through all channels within 16 min. This cycle was repeated every 30 min (16 min air distribution followed by 14 min pause) over the course of the experiment. Each of the experimental channels had 12 outlets (One-Touch fittings-male Straight, Sang-A Pneumatic Co., Daegu, Korea). Polyurethane tubing (outer/inner

diameter: 4/2.5 mm, length: 2 m, Sang-A Pneumatic Co., Daegu, Korea) was connected to the outlets and distributed the air to the Falcon tubes. The tubing was attached to the lids of the Falcon tubes using One-Touch fittings-male Elbow (Sang-A Pneumatic Co., Daegu, Korea). The flow rate sent through individual Falcon tubes was adjusted to 1 L.min-1. The outflow of the Falcon tubes was connected to a collection system, itself connected to the CO_2 sensor to verify CO_2 levels. The collected air was then released in the environment.

The temperature in the Falcon tubes was controlled through the dry-bath cyclers and followed a diurnal variation of 3.5 °C. Soil temperatures used to mimic current conditions were of 17.8 °C at 6 am, and gradually increased to reach 21.4 °C at 4 pm (Figure S1), as reported for the Swiss Plateau over the past two decades (MeteoSwiss, Federal Office of Meteorology and Climatology, Zürich, Switzerland). The temperatures mimicking the RCP 8.5 scenario were set to 21.2 °C at 6 am and progressively increased to reach 24.8 °C at 4 pm (Figure S1).

The moisture present in the tubes was controlled by adding the soil leachates to the tubes once at the beginning of the experiment. The volume of water to add in the tubes was calculated based on the soil density of 1.2 $g.cm^{-3}$. Current moisture levels (23% soil moisture) were achieved by adding 16.6% (v/v) microbiota extracts contained in tap water and 6.4% (v/v) additional tap water. Predicted moisture levels (RCP 8.5, 28% less precipitation, Figure S2) were achieved by adding 16.6% (v/v) microbiota extracts contained in tap water only. The temperatures were adjusted to the different scenarios over a six-hour adaptation period (Figure S1).

Benzoxazinoids analyses

The leaf and root samples were grinded using liquid nitrogen in the pestle and mortar. The plant metabolites were quantified using 100 mg of grinded material which was extracted using extraction buffer MeOH:H_20: (70:30 v/v, 0.1% Formic acid) and thoroughly mixed for 10 seconds on the vortex. The samples were centrifuged for 20 min at 13,000 rpm at 10 °C and supernatant was collected and stored in glass vials. The supernatant was analyzed with an acquity UHPLC-MS system equipped with an electrospray source (Waters i-Class UHPLC-QDA, USA). Gradient elution was performed on an Acquity BEH C18 column (2.1 × 50 mm i.d., 1.7 µm particle size) at 99–72.5% A over 3.5 min, 100% B over 2 min, holding at 99% A for 1 min, where A = 0.1% formic acid/water and B = 0.1% formic acid/acetonitrile and the flow rate of mobile phase was maintained at 0.4 mL/min. The injection volume was 1 ul and the temperature of the column was maintained at 40ºC. The MS was operated in negative mode, and data were acquired in scan range (*m/z* 150–650) using a cone voltage of 10 V. All other MS

parameters were left at their default values as suggested by the manufacturer (Adapted from Robert et al., 2017; Steinauer, 2021).

Cloning and heterologous expression in *E. coli* cells

To identify candidate genes involved in glycosylation activity belonging to families, UGT79, UGT91, and UGT94, phylogenetic analysis combined with transcriptome mining were employed. The genes of interest, Zm0001eb111430 (UGT94A1) Zm0001eb111270 (UGT94A2) and were amplified using a specific set of primers.

Primer's sequence:

UGT94A1-F, 5'- ggtgccgcgcggcagccataTGGCGCAGATGGAGCGCGAG-3';

UGT94A1-R, 5'- acggagctcgaattcggatcTCAGTTGGGCACGGCCACTC-3';

UGT94A2-F, ggtgccgcgcggcagccatATGGCGCAGGCGGAGCGCGA-3';

UGT94A2-R, acggagctcgaattcggatcTCAGTTGGGCACGGCCACAC-3';

Extensions for Gibson Assembly are in lower case. Zm0001eb111430 (UGT94A1) and Zm0001eb111270 (UGT94A2) were cloned into the NdeI and BamHI restriction sites of the pET28b vector (Novagen, Madison, WI) in-frame with an N-terminal hexahistidine tag. Briefly, full-length coding sequences were amplified using gene-specific primers with 20-bp extensions at their 5′ ends homologous to the termini of the linearized vector.

Amplified gene fragments (2 µL) were individually inserted into 1 ul the pET28b plasmid (Novagen, Madison, WI) using 5 ul of Gibson Assembly Master Mix (New England Biolabs; NEB). The solution was incubated at 50°C for 30 minutes to allow annealing of each fragment at the insertion site of the plasmid. The process involved chewing 5′ends of the pET28b plasmid by the exonuclease, creating overhangs at the 3′ ends complementary to each of the gene of interest. The polymerase then extended the 3′ ends by filling the gaps, and the nicks were sealed by the ligase. The Gibson assembly reaction products containing the pET28b plasmid and each gene of interest were mixed with *Escherichia coli* (DH5α cells) separately. To facilitate transformation, cells were given heat shock treatment at 42°C for 45 seconds and then placed on ice for 5 minutes. The transformed cells were diluted in 1 mL of Super Optimal Broth with catabolite repression media (SOC) as it allows for the recovery of *E. coli* cells. The cells were plated on agar plates containing 50 ug/mL of kanamycin to screen for the cells that are successfully transformed as our plasmid contains gene for the kanamycin resistance. The successfully plated colonies were picked to perform colony PCR to ensure the incorporation of the plasmids into the cells based on the fragment size (1.8 kb). The colonies with the correct fragment size were then subjected to mini preparation (mini prep) to isolate plasmids which

were also sequenced to confirm the correct assembling of each gene fragment. These plasmids for each gene were then transformed into expression cells of *E.coli* BL21 (DE3) using the protocol described above. BL21 (DE3) cells contain the antibiotic resistance gene for chloramphenicol. The transformed cells were plated on agar plates containing both kanamycin and chloramphenicol to screen for the transformed cells. Colony PCR was again performed to verify the correct incorporation of the plasmids. The correct fragment length was selected for each gene from the stab plate and grown into an overnight liquid culture in LB media containing both antibiotics at 37°C. Two Erlenmeyer flasks were filled with 250 mL of liquid LB media, 50 µg per mL of kanamycin and 35 µg per mL of chloramphenicol. The media was inoculated with 250 uL of overnight cultures of each gene. The culture was grown at 37 °C with shaking until it reached an OD_{600} of 0.6–0.8, at which point IPTG was added to a final concentration of 1 mM. The induced cells were then incubated at 16 °C with shaking for 18 hours and harvested by centrifugation. After harvesting, the cell pellet was resuspended in one-tenth the original culture volume of buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM PMSF, and 1 mg/mL lysozyme), and the cells were lysed by three rounds of freeze-thaw. After the incubating with DNase, the lysate was clarified by centrifugation, and the His-tagged recombinant protein was purified from the supernatant using HisPur Cobalt Resin.

The purified protein was used for enzymatic assay, the reaction was performed in 200 µL of Tris-HCl buffer (pH 7.5) containing 200 µM substrate and 2 µg of affinity-purified recombinant protein. The mixture was incubated at 30 °C for 1 hour, after which an equal volume of methanol was added to stop the reaction. The sample was then centrifuged at 12,000 rpm for 10 minutes and filtered through a 0.2 µm filter before analysis.

Site directed mutagenesis

A single nucleotide substitution was introduced to change the gene of interest (GOI), Zm0001eb111430 (UGT94A1). Specifically, cytosine (C) was replaced with thymine (T) at position 143 of the gene sequence resulting in codon alteration from ACC to ATC. The new codon ATC encodes the amino acid isoleucine instead of threonine encoded by ACC in the original gene sequence. Modified primers

UGT94A1-T143I-F, GCACCTCAGCATCTTCAGCGCCG;

UGT94A1-T143I-R, CGGCGCTGAAGATGCTGAGGTGC

were used to amplify two gene fragments both containing the desired mutation. The two fragments were then assembled using the overlap extension PCR and the final product was then

cloned into the pET28b expression construct as described previously. Sequencing was performed to confirm the insertion of mutation at the desired position.

Mutant generation

CRISPR/Cas9 knockout lines targeting the UGT94A1 and UGT94A2 loci were generated in the KN5585 inbred line by Weimi Biotechnology Company using the following sgRNAs:
UGT94A1-1, GACCCCTCGGATCCGCTTCGCGG;
UGT94A1-2, GGCGCAGTACATCCTCCGCGAGG;
UGT94A2-1, CCTCGGGGTTCGTGGCCATCAAG; and
UGT94A2-2, CCGCGTCACGCGGTGGCTCGACC.
To date, a single homozygous ugt94a1 mutant has been isolated, containing a 368 bp deletion between the UGT94A1-1 and UGT94A1-2 target sites. Multiple other mutant lines for both loci are currently still segregating.

Statistical analyses

Statistical analyses were conducted using R (version 3.5.3, https://www.r-project.org) and online tools (http://quantpsy.org; https://www.graphpad.com). Normality and heteroscedasticity of error variance were assessed using Levene's and Shapiro-Wilk tests, as well as by visualizing quantile-quantile plots and model residuals versus fitted values. ANOVA analysis was used to analyze effects of response variables. Comparisons of means were performed using Tukey's HSD tests ($p \leq 0.05$). The heat map of BX profiles was expressed in log fold change value compared to ambient BXDs by using the functions foldchange () in package gtools and heat.map2() in the package of gplots().

RESULTS

Drought induces the production of multihexose benzoxazinoids

Climatic components have specific effects on BXDs profiles contents in shoot, kernel, and roots (Figure 1). Drought strongly increased the concentrations of DIMBOA-2Glc, HMBOA-2Glc, and DIMBOA-3Glc in a tissue specific manner. Low precipitation increased the concentrations of DIMBOA double and triple glycosides about 40 times in the roots (Figure 1). Elevated temperature increased the concentrations of HMBOA, HMBOA-Glc, DIMBOA-Glc, DIM_2BOA-Glc, HDMBOA-Glc, and HDM_2BOA-Glc in kernels and roots. Elevated CO_2 alone changed total glucoside BXDs profiles in shoot. Combined drought and elevated temperature synergistically increased BXDs in roots and shoots. Combined drought or elevated

CO_2 with elevated temperature affected on glucosides BXDs contents. Combined elevated CO_2 and elevated temperature increased total glucoside contents in the shoot of maize seedlings.

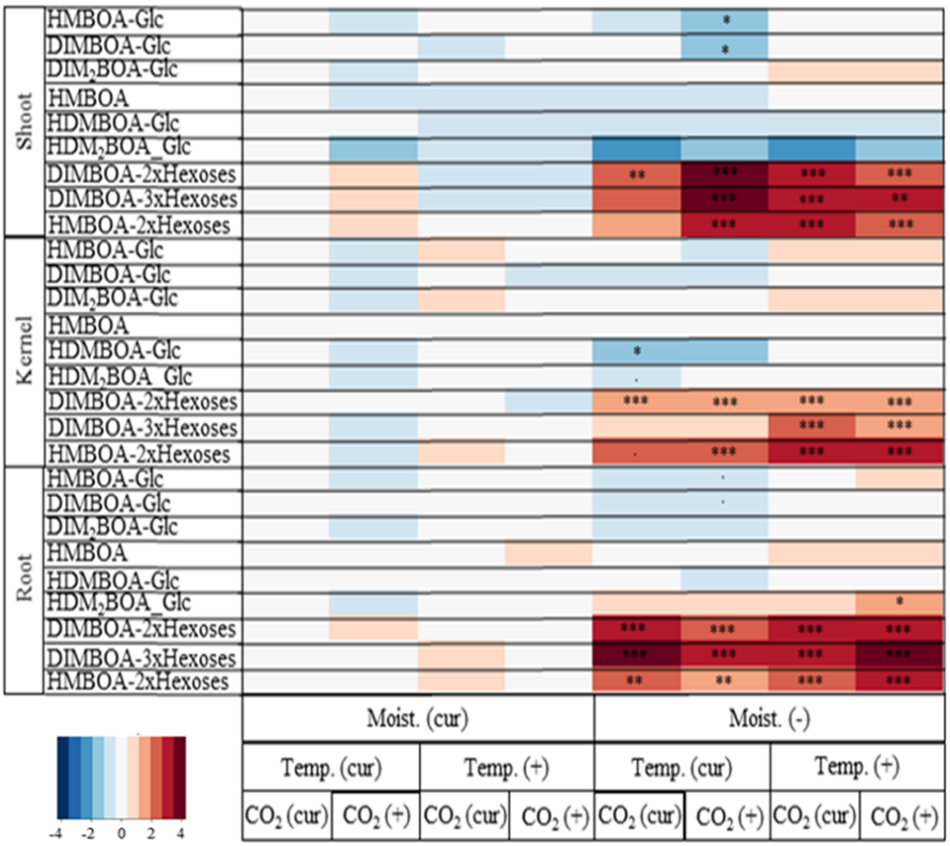

Figure 1. Climatic variables increase maize benzoxazinoid contents in maize. BXDs concentration were log 10 transformed and expressed as fold changes compared to current conditions. Blue indicates lower concentrations compared to current conditions. Red indicates higher concentrations than in current conditions. Stars indicate significant differences to current condition: ***: $p \leq 0.001$; **: $p \leq 0.01$; *: $p \leq 0.05$; $p \leq 0.1$. Moist: soil moisture, Temp: temperature, CO2: CO2 levels, cur: current conditions, +/-: elevated or decreased levels of soil moisture, temperature or CO2 as predicted by the RCP 8.5 scenario IPCC, 2014.

Figure reproduced with permission from Van Cong Doan (2020), *Interactive effects of elevated temperature, drought and elevated CO_2 on tritrophic interactions in maize*. Doctoral dissertation, University of Bern.

The glucosyltransferases Zm00001eb330430 (UGT94A1) Zm00001eb111270 (UGT94A2) are induced by drought

In the roots of maize plants, Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) and were highly induced under drought stress as indicated by the drought induced expression data analysis. The maize UGT Zm00001eb330430 (UGT94A1), located on the chromosome 7 at the genomic locus Zm00001d022467 encodes a protein containing a conserved UGT domain in addition to plasmodesma, plasma membrane activity and biosynthesis of anthocyanin-related compounds. The full-length cDNA sequence spans 1,883 bp, translating into a 476-amino-acid protein (Woodhouse et al., 2021). The maize UGT Zm00001eb111270 (UGT94A2), located on the chromosome 2 at the genomic locus LOC103647933 encodes a protein containing a conserved UGT domain in addition to localization in plasmodesmata and plasma membrane. The full-length cDNA sequence spans 1,708 bp, translating into a 475-amino-acid protein (Woodhouse et al., 2021).

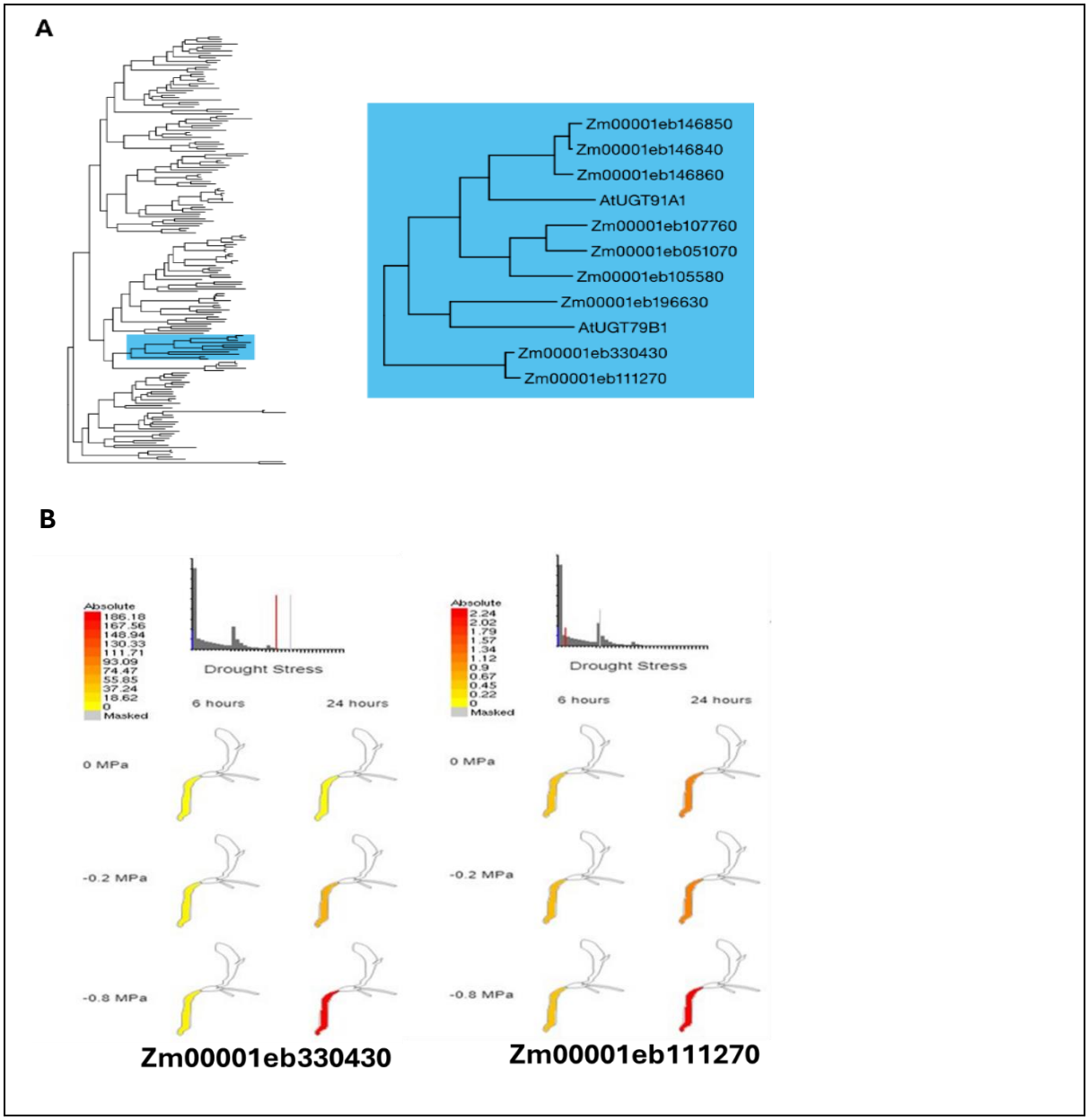

Figure 2. Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) are glucosyltransferases induced by drought in roots of maize plants. A. Phylogenetic tree of maize and *Arabidopsis thaliana* UDP-glucosyltransferases (UGTs). Multiple sequence alignment was performed, and the tree was generated using HMMER indicating evolutionary relationships among the selected UGT genes. B. The concentration of Zm00001eb330430 (UGT94A1) produced under drought in roots of maize B73 plants is 83 folds higher than the Zm00001eb111270 (UGT94A2) (Reproduced from Opitz et al. (2014), *BMC Plant Biology*, licensed under CC BY 4.0.).

Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) produce DIMBOA-2Glc from DIMBOA-Glc

Both Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) were able to successfully glycosylate DIMBOA-Glc to DIMBOA-2Glc.

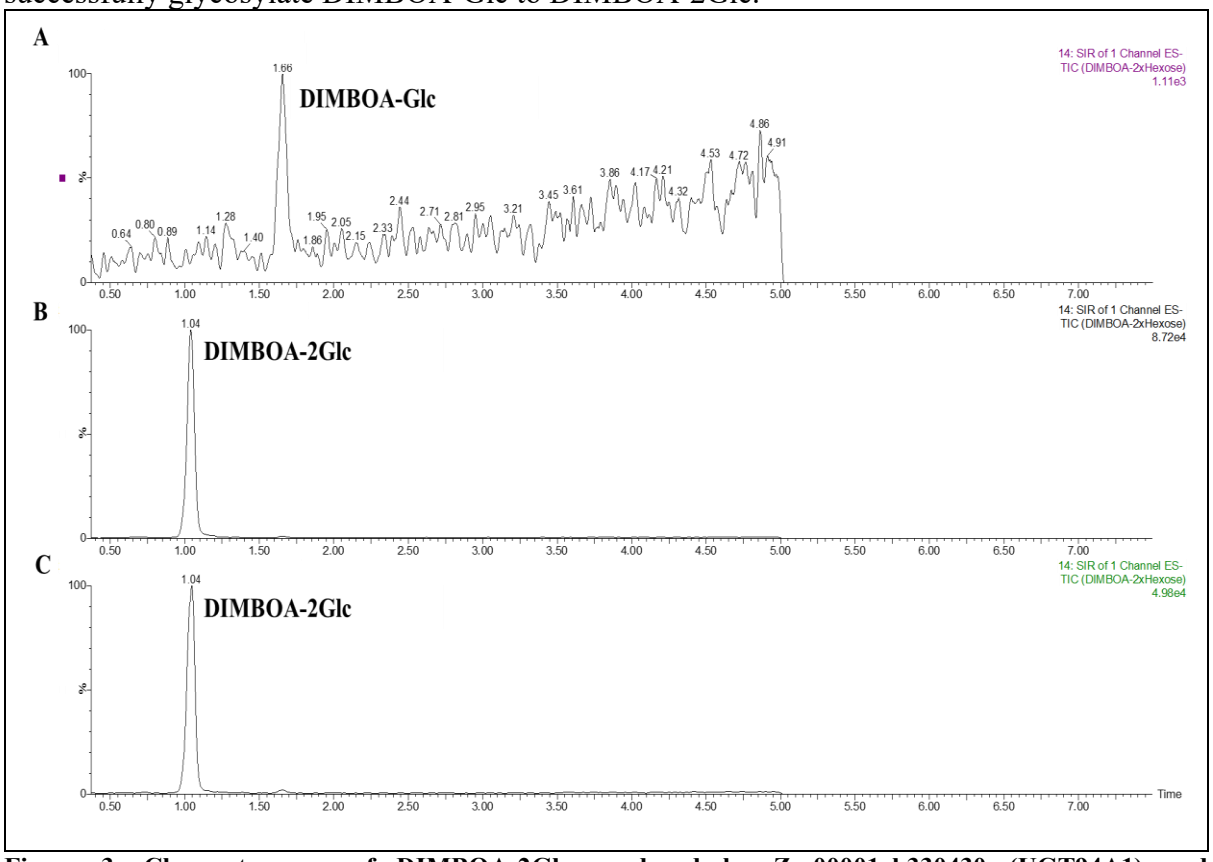

Figure 3. Chromatograms of DIMBOA-2Glc produced by Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2). A. Chromatogram showing DIMBOA-Glc at a retention time of 1.66 minute, observed in the control reaction. B. Chromatogram of DIMBOA-2Glc at a retention time of 1.04 minute, produced by Zm00001eb330430 (UGT94A1) when supplemented with 2 mM UDP-glucose after 60 minutes at 30ºC. Chromatogram of DIMBOA-2Glc at a retention time of 1.04 minute, produced by Zm00001eb111270 (UGT94A2) when supplemented with 2 mM UDP-glucose after 60 minutes at 30ºC. We were not able to detect traces of DIMBOA-3Glc.

Site-directed mutagenesis of Zm00001eb330430 (UGT94A1) abolished glycosylation of DIMBOA-Glc to DIMBOA-2Glc

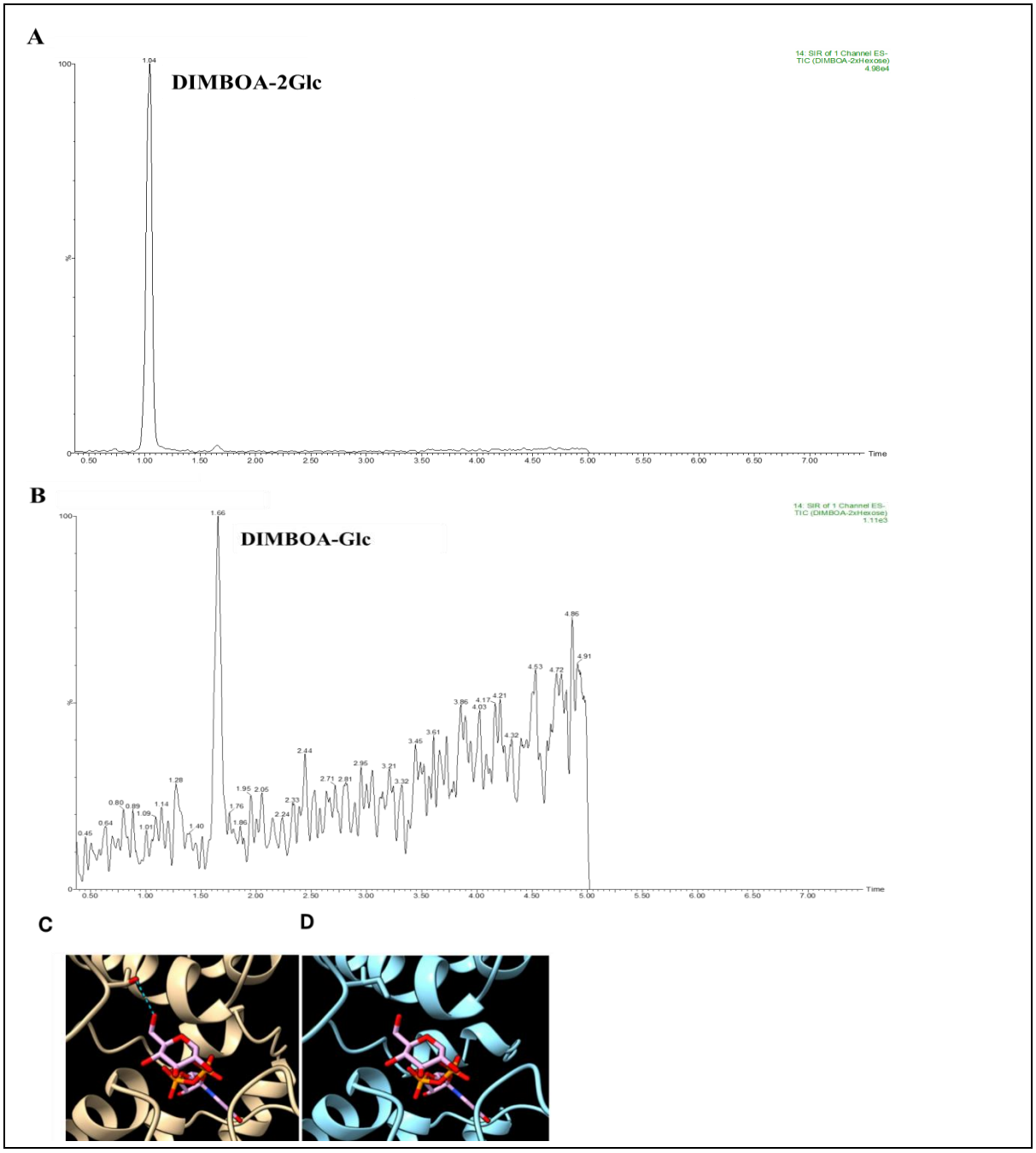

Figure 4. **The mutated gene lost the ability to glycosylate DIMBOA-Glc to DIMBOA-2Glc**. A. Chromatogram of DIMBOA-2Glc produced by the Zm00001eb330430 (UGT94A1) at a retention time of 1.04 minute, when supplemented with 2 mM UDP-glucose after 120 minutes at 30ºC. B. Chromatogram illustrating DIMBOA-Glc as the mutated protein lost its ability to glycosylate DIMBOA-Glc. C. The wild type ESMFold Protein Structure of Zm00001eb330430 (UGT94A1) (Modified from Meta AI, Lin et al., 2022, Licensed under under a CC-BY-NC-ND 4.0). D. Mutated ESMFold Protein Structure of Zm00001eb330430 (UGT94A1) (Modified from Meta AI, Lin et al., 2022, Licensed under under a CC-BY-NC-ND 4.0).

DISCUSSION

In the current study, we detected glycosylated BXDs, DIMBOA-2Glc, DIMBOA-3Glc, and HMBOA-2Glc in maize plants under drought conditions highlighting underexplored mechanism of stress adaptation. We identified nine candidate UDP-glycosyltransferases (UGTs) in the UGT79, UGT91, and UGT94 families using transcriptome mining and phylogenetic analysis. Out of nine, Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) were strongly upregulated by drought. Recombinant expression analysis in *E. coli* demonstrated that both these enzymes are involved in the production of DIMBOA-2Glc from DIMBOA-Glc. Site-directed mutagenesis in Zm00001eb330430 (UGT94A1) impaired its ability to produce DIMBOA-2Glc from DIMBOA-Glc.

In the study, interesting finding demonstrate that the concentrations of DIMBOA-2Glc, DIMBOA-3Glc, and HMBOA-2Glc are increased up to 40-fold in the roots. BXDs are defence related compounds primarily involved in providing resistance against herbivores and pathogens (Robert and Matteo, 2022). Currently, there is increasing evidence they also play role in acquiring resistance to abiotic stress such as drought, although the functions remain largely unidentified (Frey et al., 2009). Glycosylated BXDs such as DIMBOA-Glc are inactive, non-toxic, storage forms of BXDs under drought stress conditions when metabolism is suppressed (Niculaes et al., 2018). This modification can help plants conserve energy which is critical when metabolic activity is suppressed under water limiting conditions. Glycosylation can also help plants in priming against biotic stresses by rapidly activating BXDs from non-toxic compounds (Niculaes et al., 2018; Israni et al., 2020). Furthermore, glycosylated compounds are more water soluble, increasing their movement within the plant tissues or exudation through root tissues. Glycosylation of benzoxazinoids (BXDs) can be a crucial mechanism adopted by plants to regulate their bioactivity and distribution.

In the current study, based on preliminary analysis and expression profiles, we identified two out of nine UGTs that are involved in BXDs glycosylation. These two UGTs Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) were highly induced in the roots under drought stress pointing towards tissue specific role in adaptation to drought stress. UGTs are enzymes involved in plant metabolism and catalyse the addition of sugar molecules to a wide range of acceptor molecules such as secondary metabolites and hormones. As a result of this glycosylation, solubility, stability, and bioactivity of these compounds is increased thereby modulating their roles in plant growth and development under stress conditions (Gharabli et al., 2023). The specific roles of glycosylated BXDs are currently

unknown, though various hypotheses can be provided based on functions of glycosylated BXDs in different systems. For example, formation of glycosylated flavonoids can help release the stress of reactive oxygen species (ROS) owing to their antioxidant properties (Pourcel et al., 2007). BXDs are not antioxidant in nature, their glycosylation can result in indirect redox homeostasis or alternatively prevent their breakdown into harmful degradation products. For instance, BXDs such as DIMBOA can be cytotoxic at higher concentrations, their glycosylation can prevent plants cells during drought stress where cell damage can result in membrane permeability or metabolite leakage (Ahmad et al., 2011). Glycosylation also enables the sequestration of metabolites in vacuolar compartments and serves as a reservoir that can be rapidly utilized under stress condition (Jones at el., 2003). Under drought stress, BXDs glycosylation can serve as reservoir pool that can be hydrolysed when needed. Initial evidence is provided through mutant analysis supporting their involvement, we await the availability of CRISPR-generated knockout lines to thoroughly confirm this.

Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) are also actively involved in cellular functions owing to their localization in plasma membrane and plasmodesmata. Additionally, Zm00001eb330430 (UGT94A1) is involved in the biosynthesis of anthocyanin containing compounds, suggesting its role in biological processes (Woodhouse et al., 2021). Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) are largely conserved and have orthologs in Sorghum bicolor (Sorghum), *Setaria italica* (Foxtail millet), *Oryza sativa japonica* (Rice), *Brachypodium distachyon* (Brachypodium) (Woodhouse et al., 2021). The Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) share a stretch of conserved 40 amino acids with other UGTs in *Arabidopsis* and tea plants that are involved in glycosylation activities including AtUGT71B1, AtUGT74C1, AtUGT79B1, AtUGT79B6, CsUGT94P1B1. Moreover, they all possess a conserved Adenine in their sequence along with our two UGTs (Ohgami et al., 2015).

Under drought, glycosylation of BXDs represents a novel mechanism adopted by plants to manage drought stress. Although, the functional aspects of this biochemical adaptation are not completely understood, glycosylation of BXDs under water stress is likely aimed to modulate their stability, storage and bioactivity. A novel biosynthetic pathway is elucidated in this study involving Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) genes that modify plant metabolic responses to drought. Future work will be carried out to perform qPCR analysis of the identified UGTs and their relevance in mutant plants for drought resilience. Furthermore, more investigations are needed to properly characterize the function of these compounds in maize plants under water limiting conditions.

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AUTHOR CONTRIBUTION

Tristan Cofer: Conceptualization, experimental setup, methodology, data collection, formal analysis for phylogenetic and transcriptomic mining, heterologous expression, enzymatic activity, mutant homozygous lines.

Sheharyar Khan: Conceptualization, experimental setup, methodology, data collection, formal analysis, writing manuscript for heterologous expression and enzymatic activity.

Cong van Doan: Conceptualization, experimental setup, methodology, data collection, formal analysis, writing manuscript for benzoxazinoids under drought

Pierre Mateo: Benzoxazinoids analysis

Natacha Bodenhausen: Supervision, reviewing and editing, statistical analysis

Christelle Robert: Conceptualization, experimental design, methodology, supervision, reviewing and editing, statistical analysis

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY

All data will be provided as supplementary material upon acceptance of the manuscript.

FIGURE LEGENDS

Figure 1. Climatic variables increase maize benzoxazinoid contents in maize. BXDs concentration were log 10 transformed and expressed as fold changes compared to current conditions. Blue indicates lower concentrations compared to current conditions. Red indicates higher concentrations than in current conditions. Stars indicate significant differences to current condition: ***: $p \leq 0.001$; **: $p \leq 0.01$; *: $p \leq 0.05$; $p \leq 0.1$. Moist: soil moisture, Temp: temperature, CO2: CO2 levels, cur: current conditions, +/-: elevated or decreased levels of soil moisture, temperature or CO2 as predicted by the RCP 8.5 scenario IPCC, 2014.

Figure 2. Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) are glucosyltransferases induced by drought in roots of maize plants. A. Phylogenetic tree of maize and *Arabidopsis thaliana* UDP-glucosyltransferases (UGTs). Multiple sequence alignment was performed, and the tree was generated using HMMER indicating evolutionary relationships among the selected UGT genes. B. The concentration of Zm00001eb330430 (UGT94A1) produced under drought in roots of maize B73 plants is 83 folds higher than the Zm00001eb111270 (UGT94A2) (Reproduced from Opitz et al. (2014), *BMC Plant Biology*, licensed under CC BY 4.0.).

Figure 3. **Chromatograms of DIMBOA-2Glc produced by Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2). A.** Chromatogram showing DIMBOA-Glc at a retention time of 1.66 minute, observed in the control reaction. B. Chromatogram of DIMBOA-2Glc at a retention time of 1.04 minute, produced by Zm00001eb330430 (UGT94A1) when supplemented with 2 mM UDP-glucose after 60 minutes at 30ºC. Chromatogram of DIMBOA-2Glc at a retention time of 1.04 minute, produced by Zm00001eb111270 (UGT94A2) when supplemented with 2 mM UDP-glucose after 60 minutes at 30ºC. We were not able to detect traces of DIMBOA-3Glc.

Figure 4. **The mutated gene lost the ability to glycosylate DIMBOA-Glc to DIMBOA-2Glc**. A. Chromatogram of DIMBOA-2Glc produced by the Zm00001eb330430 (UGT94A1) at a retention time of 1.04 minute, when supplemented with 2 mM UDP-glucose after 120 minutes at 30ºC. B. Chromatogram illustrating DIMBOA-Glc as the mutated protein lost its ability to glycosylate DIMBOA-Glc. C. The wild type ESMFold Protein Structure of Zm00001eb330430 (UGT94A1) (Modified, Meta AI). D. Mutated ESMFold Protein Structure of Zm00001eb330430 (UGT94A1) (Modified, Meta AI).

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LIST OF SUPPLEMENTARY INFORMATION

Supplementary Figure 1. Microcosm temperature regimes. To mimic natural conditions, the two temperature conditions were set to reach a maximum at 4 pm and progressively reduced by 3.5 °C to reach a minimum around 6 am. Adaptation stage was set 6 hours before three day-night cycles.

Supplementary Figure 2. Correlation between precipitation and soil moisture. Linear correlation between average June precipitation sum (in mm) and average June soil moisture (v/v) at a soil depth of 15 cm between 2004 and 2016 (years 2005, 2006, 2012 and 2013 were not recorded in the field) in the Swiss Central Plateau (47°17'11.1" N / 7°44'01.5" E), Switzerland.

Supplementary Figure 3. Nine candidate genes in maize reveals a conserved glutamic acid residue that is a hallmark for sugar acceptor recognition

Supplementary Figure 4. Genes in *Arabidopsis thaliana* and *Camellia sinensis* involved in adding glucose to another glucose molecule have a conserved threonine, including two Zm00001eb330430 and Zm00001eb111270 of the nine candidate genes in maize

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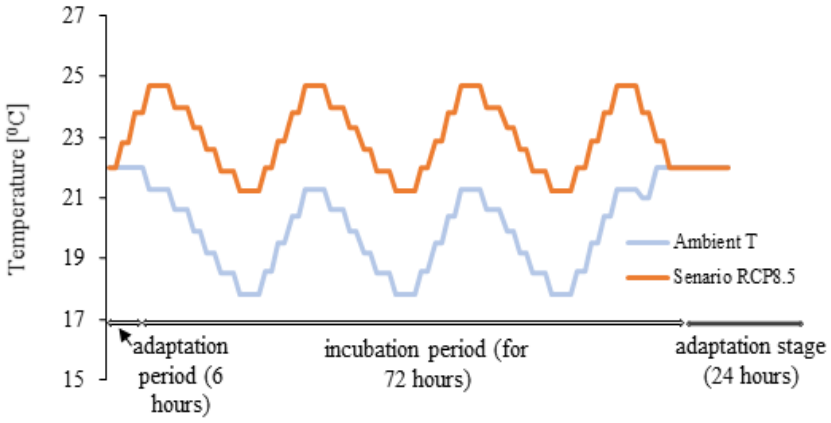

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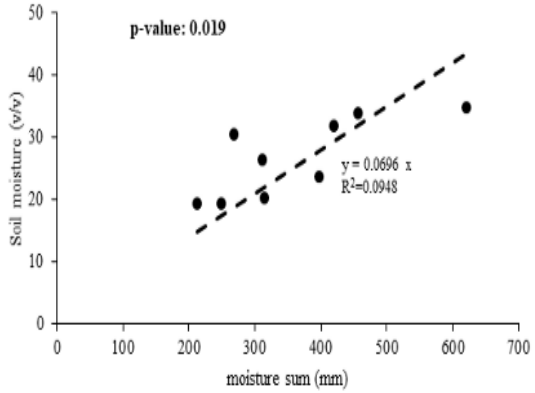

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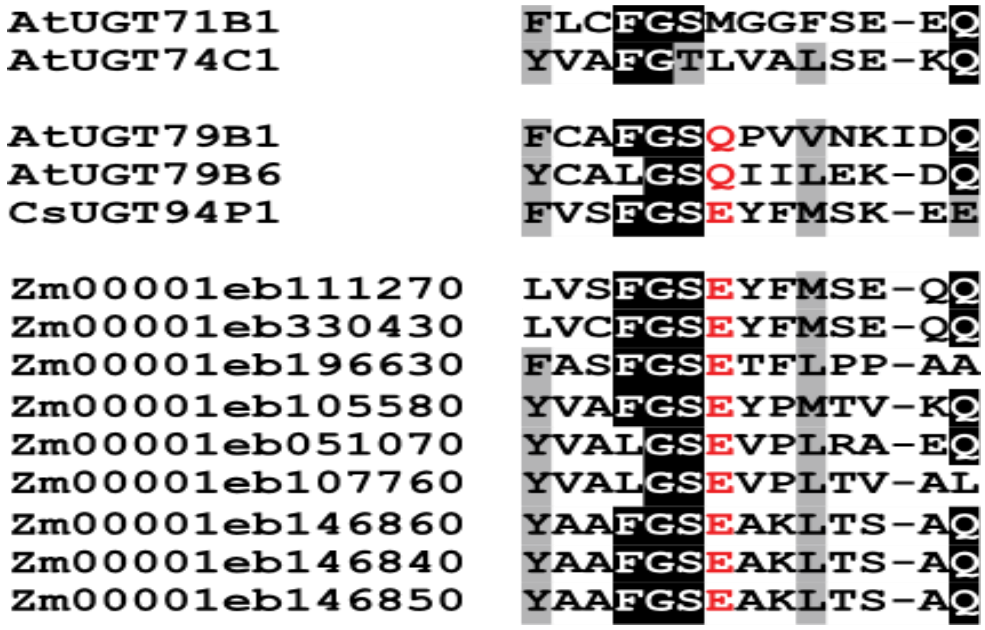

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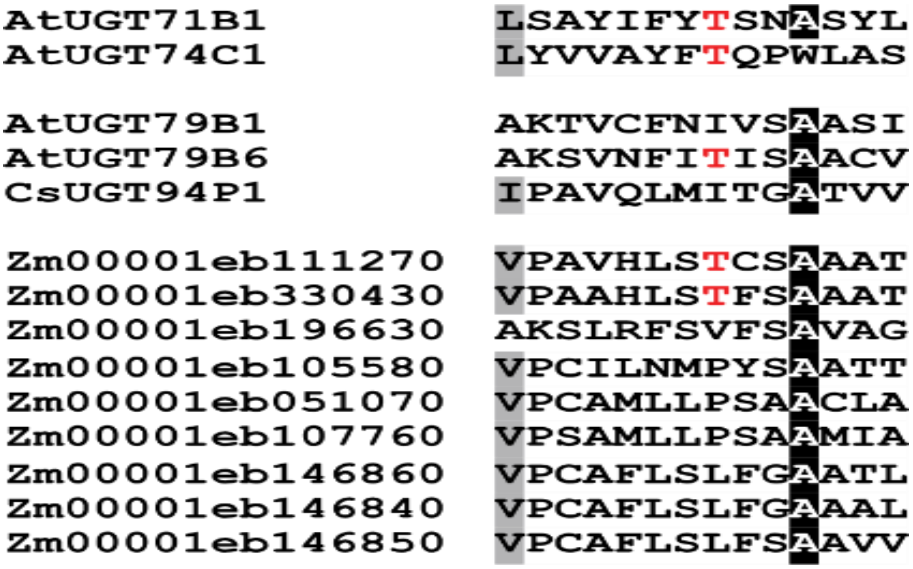

GENERAL DISCUSSION

My PhD work was focused to fill knowledge gaps about the modulation of maize secondary metabolism when subjected to drought stress. Firstly, the interactive effects of drought and AMF on BXDs modulation and its effect on the herbivore performance was analysed. Under drought stress, maize biomass and chlorophyll content was reduced while AMF on the other hand increased reproductive traits and altered metabolic profiles. Under drought and AMF treatments, metabolic changes in sugars, phytohormones and BXDs were also observed. Interestingly, drought increased the performance of the leaf herbivore *Spodoptera exigua*, an effect limited in the presence of the AMF. (Chapter I).

Secondly, the role of BXDs in facilitating the establishment of symbiotic associations with the arbuscular mycorrhizal fungi *Rhizophagus irregularis* was evaluated. Furthermore, how colonization efficiency is affected by kinetic drought was also investigated. In semi-field assay, drought increased DIMBOA, DIMBOA-Glc, DIM_2BOA-Glc and DIMBOA-2Glc concentration in maize roots while AMF decreased DIMBOA, DIMBOA-Glc, DIM_2BOA-Glc concentration after 60 days. Kinetic drought had no impact on the rate of colonization in maize plants with the AMF. MBOA complementation increased colonization rate in *bx1* mutant plants after 20 days while AMF increased fresh shoot weight (Chapter II).

Finally, we identified UGTs Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) that are specifically involved in the formation of double hexose DIMBOA-2Glc from DIMBOA-Glc in maize plants under drought condition. These UGTs were identified by employing phylogenetic analysis combined with transcriptome mining of UGT79, UGT91, and UGT94, families. The enzymatic function of these two UGTs was confirmed by recombinant expression in *E. coli* cells. Site-directed mutagenesis of UGT94A1 abolished this activity, confirming the functional role of the target residues (Chapter III). Below I discuss future possibilities that arise from these findings.

Plant growth under drought and AMF

Drought is one of the most limiting factors for plant growth and results in reduced biomass and altering of key physiological processes involving photosynthesis, stomatal regulation and nutrient uptake (Ahmad et al., 2018; Liu et al., 2024; Zhao et al., 2024). AMF has emerged as a key ally that can help boost plant resilience by promoting the uptake of water and nutrients (Ansari et al., 2025; El Malahi et al., 2025; Priyadarshani et al., 2025). In line with previous studies, our findings demonstrated the negative effect of drought on maize growth and how

AMF can help mitigate these effects by improving plant development and reproductive success under reduced soil moisture levels (Duan et al., 2025; Nader et al., 2025; Yang et al., 2025). The findings highlighted the negative impact of drought on plant growth resulting in significantly lower fresh shoot biomass, plant height, cob length, and cob number, AMF colonization on the other hand facilitated in alleviating these impacts. Plant stress resilience under AMF association can be attributed to the extensive hyphal networks that enhances plants access to water and nutrient uptake, thus sustaining metabolic activity during drought (Akter et al., 2024; Ahmed et al., 2025).

Secondary metabolism under drought and AMF

Climate change is global phenomena with drought being as one of the most critical stressor predicted to increase in severity and frequency (IPCC, 2023; Savari et al., 2024). AMF forms mutualistic symbiotic association with plants and can help modify physiological and metabolic processes under stress conditions (Begum et al., 2019; Sonbol et al., 2025; Deng et al., 2025). The interactive effect of drought and AMF elicit plant responses that were significantly different from the responses triggered by individual's stressors alone (Hussain et al., 2019). In the current study, both drought and AMF were able to induce changes in maize secondary metabolism although the effects were antagonistic. Drought enhanced the production of root BXDs including DIMBOA, DIMBOA-Glc, DIM_2BOA-Glc DIMBOA-2Glc (Sutour et al., 2024) while AMF on the other hand decreased their levels of DIMBOA, DIMBOA-Glc and DIM_2BOA-Glc.

These findings suggest that a regulatory crosstalk exists between symbiosis and secondary metabolism. Drought stress upregulates maize chemical defences leading to the increased accumulation levels of BXDs. This effect can be explained by several hypotheses, firstly BXDs can maintain cell turgor pressure and osmotic balance by acting as osmoprotectants. Secondly, hydrogen bond can be formed in the sugar moieties leading to reduced water loss linked with transpiration. Thirdly, multihexose compounds can protect damage due to reactive oxygen species better than their precursors. Fourth, sugars may be stored as an energy source in the form of multihexoses as a mechanism to tolerate drought and reduced photosynthetic rates. Fifth, BXDs are involved in modulating plant interactions with the herbivores, multihexose BXDs may play role in protecting plant from biotic stress under drought conditions. These sugars compounds can also be exuded in the rhizosphere to incorporate beneficial microbiota. AMF on the hand, can likely dampen stress signalling pathways including jasmonic acid and salicylic acid signalling, thereby reducing the induction of defensive compounds (Pozo &

Azcón-Aguilar, 2007). Likely, AMF through enhanced water and nutrient uptake during drought stress can limit the needs for the stress-induced metabolic compounds (Smith & Read, 2008; Khoza et al., 2025). Another plausible explanation is to supress antifungal compounds to better facilitate the colonization with the maize plant. There also exists an energy trade off to spend on the symbiosis rather than on the defensive chemistry.

Drought and AMF interactions

My study also highlighted the impact of drought on AMF colonization. In the two semi-field experiments, drought significantly reduced AMF colonization. Plants form association with AMF to increase drought tolerance and previous studies suggests more colonization success under drought conditions (Chareesri et al., 2020). Relationship between drought and AMF colonization can vary depending on factors such as the drought severity and duration, plant species, and soil conditions. Plants can stimulate colonization to adapt to drought stress, but severe or prolonged drought can result in resource allocation trade-offs, where plants prioritize survival over maintaining symbiotic relationships. The decreased AMF colonization can be explained by the fact that reduced soil moisture had a negative effect on fungal growth, spore germination and hyphal proliferation (Augé, 2001; Trouvelot et al., 2015). The establishment and maintenance of fungal structures is also limited as fungal hyphae require adequate soil moisture to explore and transfer nutrients (Smith & Read, 2008). Root exudation patterns are altered under drought stress resulting in modified chemical signals that are involved in regulating AMF colonization (Badri & Vivanco, 2009). Conversely in the greenhouse experiment where drought was established prior to the inoculum induction, we did not observe the effect of drought on colonization success. Interestingly, plants with prolonged drought exhibited the highest levels of colonization success. This suggested that prior drought induction can prime the root architecture or exudation patterns that eventually favors the AMF establishment. This finding aligns with the research that root exudates or strigolactones can be enhanced under mild stress and they are key signals involved in AMF association (Besserer et al., 2006; Ruiz-Lozano et al., 2016). These findings underscore the fact that not only the drought intensity, but its chronology is also crucial in determining the colonization efficiency of the AMF, however more investigations are required to best characterize the effect of drought on AMF association.

Drought and AMF differently affect spodoptera feeding

The current study gave emphasis to the herbivore performance under drought and AMF. Herbivore performance of *Spodoptera exigua* was increased under drought conditions when

there was more accumulation of secondary metabolites. Conversely, AMF association reduced the performance of *S. exigua* larvae under drought conditions, highlighting role of AMF in deterring herbivores through possible stabilization of the metabolite profiles beyond BXDs. Secondary metabolites deter herbivores, but the certain metabolites are not that effective against all herbivores. Drought stress also increases sugar content (Jahan et al., 2024; Xiao et al., 2024) due to increased accumulation of certain secondary metabolites; this provides a rich energy source for herbivores and eventually increases their performance (Züst & Agrawal, 2017). AMF association can prime plant defences preparing them more rapidly in case of herbivore attack. Although the overall levels of secondary metabolites are reduced, certain anti-herbivore compounds are primed resulting in lower herbivore performance as compared to non-mycorrhizal plants. For example, colonization of tomato plants by the AMF resulted in higher mortality rates of the herbivore *Spodoptera exigua*. Although the overall metabolome of the leaf was not impacted by mycorrhizal association, but accumulation of alkaloids and fatty acid derived compounds was exhibited resulting in priming of plant defence responses (Rivero et al., 2021). In conclusion, the study depicted contrasting effects of AMF and drought on herbivory. This supports the hypothesis that AMF can mitigate both biotic and abiotic stresses by modulating chemical defences of the plant. However, this balance is delicate as BXDs accumulation was also limited raising questions about metabolic pathways that are involved in reducing insect herbivory.

MBOA increases AMF colonization

There has also been a growing interest in how secondary metabolites can shape AMF symbiotic association including phenolics, terpenoids (Pozo et al., 2015) and BXDs. Although these metabolites are tightly regulated, they can be modulated by the AMF to prime plant defences. This means that in case of herbivory, plants respond more effectively even without high constitutive levels of defense compounds (Pozo et al., 2015). My study illustrates the possible role BXDs can have in AMF colonization. The *bx1* mutant line had lower rates of colonization with the AMF *Rhizophagus irregularis* but the complementation of bx1 mutant plants with MBOA resulted in higher colonization rates after 20 days. The AMF colonization reduction observed in *bx1* mutant plants suggests that specific metabolic pathways or genetic factors are required for maintaining symbiosis. This also aligns with previous studies that highlight the role of root exudates and specialized metabolites in symbiotic efficiency (McLaughlin et al., 2022; Chen & Liu, 2024; Cui et al., 2024; Robert et al., 2025). Interestingly, our finding that MBOA complementation can enhance AMF colonization, indicates an either direct or indirect

role of these compounds in facilitation of AMF symbiosis. Benzoxazinoids have documented role in not only plant defense against pathogens and herbivores but also in altering the rhizosphere community and signalling (Hu et al., 2018; Cotton et al., 2019). It is, therefore, reasonable to suggest that BXDs have role in modifying root exudate profiles enabling fungal recognition and growth, albeit further investigations are needed.

UGTs and multihexose BXDs

Out of nine candidate genes, Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2 were identified in carrying out glycosylation activity and forming DIMBOA-2Glc from DIMBOA-Glc. Under drought stress, these two UGTs are highly expressed in the maize roots (Opitz et al., 2014). Glycosylation is characterized mechanism by which plants modify, store or detoxify toxic metabolites (Gharabli et al., 2023), the formation of multihexose compounds suggests another layer of metabolic adaptation by the maize plants (Barreda et al., 2024). Specific enzymes for forming multihexoses signifies that a degree of functional specialization exists within the UGT family and that these compounds can have distinct biological roles, including enhanced solubility, autotoxicity reduction, transport and storage. For example, UGTs in Arabidopsis and tea plants that are involved in glycosylation activities include AtUGT71B1, AtUGT74C1, AtUGT79B1, AtUGT79B6, CsUGT94P1B1 (Ohgami et al., 2015), these UGTs share a stretch of conserved 40 amino acids. These UGTS glycosylate particularly, flavonoids and phenylpropanoids (Yonekura-Sakakibar 2014; Dai et al., 2018; Liu et al., 2018). The compounds produced because of glycosylation are actively involved in storage, solubility, bioactivity, regulating antioxidant activity and defence responses such as SA levels (Yang et al., 2024).

DIMBOA-2Glc can be characterized as a safe metabolite reservoir and can be remobilized or activated under stress condition of drought and herbivory. As some UGT expression overlap with stress treatment, DIMBOA-2Glc can have role in possible root exudation or microbial signalling. This finding expands our understanding that plants do not utilize glycosylation as merely a detoxification step but as a regulatory mechanism to fine tune metabolic profiles in a context-dependent manner.

Impact

This thesis advances our understanding that how maize plant responds to drought stress at both physiological and metabolic levels, particularly under association with the arbuscular mycorrhizal fungi, *Rhizophagus irregularis*. Although, drought had significant impact on plant growth, AMF symbiosis helps in alleviating these impacts. Drought had a contrasting effect on

colonization rate in semi-field and greenhouse experiments, drought establishment prior to inoculum addition and duration can possibly minimize the effect on colonization rate. These findings shed light on the complex interactions that takes place between plants and AMF highlighting that chronology in addition to water availability is also crucial. Notable work also includes the upregulation of maize defensive metabolites including DIMBOA-Glc, DIM_2BOA-Glc, DIMBOA-2Glc and HMBOA-2Glc under drought stress, while AMF association attenuates this chemical upregulation by improving growth and suppressing herbivore performance at the same time. These key findings demonstrated that AMF not only buffers physiological drought impacts but also effects higher trophic level by modulating secondary metabolism. Additionally, role of MBOA in increasing AMF colonization elucidates the potential role of metabolites in the regulatory feedback and establishment of symbiosis. Finally, the identification of UGTs Zm00001eb330430 (UGT94A1) and Zm00001eb111270 (UGT94A2) that are involved in glycosylation of DIMBOA-Glc to DIMBOA-2Glc opens new avenues for biochemical and functional studies focused on bioengineering and crop improvement strategies.

Perspectives

In the future, there is a need to understand the signalling pathways through which interaction of drought and AMF modulates the BXDs biosynthesis. This can involve transcriptomic analysis of the regulatory genes that could be differentially expressed under interactive effect of drought and AMF. As AMF successfully supressed BXDs accumulation and herbivore performance, field-based trails can be conducted where AMF inoculants can be integrated with pest management strategies to develop solutions for sustainable agriculture in regions that are affected by drought. To unravel the mechanisms behind reduced herbivory, targeting feeding assays can be carried out alone with BXDs and compounds induced under AMF association. Role of other metabolite classes such as flavonoids and terpenoids must be investigated in conferring deterrence against herbivory. The novel finding of MBOA increasing AMF colonization can help to promote its use or synthetic analogs to boost symbiosis in poor soils. Lastly, one of the key priorities will be to functionally characterize the role of multihexose compounds produced under drought stress.

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Declaration of consent

on the basis of Article 18 of the PromR Phil.-nat. 19

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Study program: PhD in Ecology and Evolution

Bachelor ☐ Master ☐ Dissertation ☑

Title of the thesis. Arbuscular mycorrhizal fungi-mediated modulation of maize secondary metabolism under drought conditions.

Supervisor: Prof. Dr. Christelle Robert
Dr. Natacha Bodenhausen

I declare herewith that this thesis is my own work and that I have not used any sources other than those stated. I have indicated the adoption of quotations as well as thoughts taken from other authors as such in the thesis. I am aware that the Senate pursuant to Article 36 paragraph 1 litera r of the University Act of September 5th, 1996 and Article 69 of the University Statute of June 7th, 2011, is authorized to revoke the doctoral degree awarded on the basis of this thesis.

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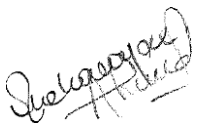

Signature

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