Micronutrients, Silicon and Biostimulants as Cold Stress Protectants in Maize

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Low temperature (**LT**)
Reactive oxygen species (**ROS**)
Zinc (**Zn**)
Indole acetic acid (**IAA**)
Dry weight (**DW**)
Superoxide dismutase (**SOD**)
Manganese (**Mn**)
Iron (**Fe**)
Silicon (**Si**)
Plant growth promoting microorganism (**PGPM**)
Plant growth promoting fungus (**PGPF**)
Plant growth promoting bacteria (**PGPB**)
1-aminocyclopropane-1-carboxylate (**ACC**)
Eukaryotic initiation factors (**EIFs**)
Transcription factors (**TFs**)
Late embryogenesis-abundant (**LEA**)
Dehydration responsive element binding protein/C-repeat binding factor (**DREB/CBF**)
No apical meristem (**NAC**)
Arabidopsis thaliana activating factor1 (**ATAF1**)
Cup-shaped cotyledon (**CUC2**)
Zinc-finger homeodomain (**ZF-HD**)
ABA-responsive element binding protein/ABRE binding factor (**AREB/ABF**)
Myelocytomatosis/myeloblastosis (**MYC/ MYB**)
Zinc-finger proteins (**ZFPs**)
Arabidopsis zinc finger proteins (**AZF**)
Salt-tolerance zinc finger (**STZ**)
Abscisic acid (**ABA**)
Basic leucine zipper (**bZIP**)
ABA-responsive element (**ABRE**)
Enhanced embryogenesis level (**EEL**)
Linker histone H1–3 (**HIS1–3**)
ATPases associated with diverse cellular activities (**AAA**)
Open stomata1/ sucrose non-fermenting related protein kinases 2E (**OST1/SRK2E**)
Cold regulated proteins/cold responsive genes (**COR/COR**)
Brassinosteroids (**BA**)
Brassinazoleresistant 1 (**BZR1**)
Brassinosteroidinsensitive 2 (**BIN**)
Cold shock domain protein 2 (**CSP2**)
Dehydrins (**DHN**)
Cold induced (**KIN**)
Heptahelical transmembrane protein (**HHP**)
C-repeat binding factor (**CBF**)
Calcium ion (**Ca^{2+}**)
Inositol 1,4,5-triphosphate (**IP3**)
Phospholipase C (**PLC**)
Calcium dependent protein kinases (**CPKs**)
Calcineurin B-like proteins (**CBLs**)
CBL-interacting protein kinases (**CIPKs**)

C_repeat/dehydration responsive element (**CRT/DRE**)
Ras-mitogen-activated protein kinase (**MAPK**)
Phosphoryl group (**Pi**)
Jasmonic acid (**JA**)
Gibberellic acid (**GA**)
Cytokinin (**CTK**)
Salicylic acid (SA
Arabidopsis cytokinin receptor histidine kinases (**AHK**)
DELLA protein (**DELLA**)
Arabidopsis histidine kinase (**AHK**)
Arabidopsis histidine phosphor-transferase (**AHP**)
Arabidopsis response regulator (**ARR**)
Ethylene insensitive3 (**EIN3**)
Jasmonate-zim-domain protein (**JAZ**)
MYC-related transcriptional activator2 (**MYC2**)
Calmodulin-binding transcriptional activator (**CAMTA**)
not SA-deficient mutant (**EDS1**)
Transcript-derived fragments (**TDFs**)
Quantitative trait loci (**QTL**)
CBF expression 1 (**ICE1**)
Low-temperature induced (**LTI**)
Responsive to dehydration (**RD**)
High expression of osmotically responsive gene 1 (**HOS1)**
Late elongated hypocotyl (**LHY**)
LUX ARRYHTHMO (**LUX**)
Circadian and clockassociated 1 (**CCA1**)
Brassinazoleresistant 1 (**BZR1**)
Suppressor of overexpression of constans 1 (**SOC1**)
Phosphorylation (**P**)
Small ubiquitin-related modifier (**SUMO**)
Ubiquitin (**U**)
Plant growth-promoting potential (**PGPRs**)
Low root zone temperature (**RZT**)
Root zone temperatures (**RZT**)
Days after sowing (**DAS**)
Catalases (**CAT**)
Peroxidases (**POD**)
Ascorbate peroxidase (**APX**)
water holding capacity (**WHC**)
Dry matter (**DM**)
1,1-diphenyl-2-picrylhydrazyl radical (**DPPH •**)
Trichoderma haruzianum OMG16 and mixture of *Bacillus* spp with Zn and Mn **CombiA^{+}**
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Biological fertilizer OD (**BFOD**)
Fresh weight (**FW**)
Cytokinin (**CK**)
Auxin response factor 12 (**ZmAFR12**)
Abscisic acid responsive element-binding factor 2 (**ZmABF2**)
Elongation factor-1alpha (**EF1α**)
Root length (**RL**)

SMALL AUXIN UP RNAs (**SAURs**)
Supplementary (**Suppl.**)

1. Summary

Mitigation of abiotic stress in crops is a feature attributed to various so-called biostimulants based on plant growth-promoting microorganisms (PGPMs) plant-, compost- and seaweed extracts, protein hydrolylates, chitosan derivatives etc. but also to mineral nutrients with protective functions, such as zinc (Zn), manganese (Mn), boron (B), calcium (Ca) and silicon (Si), recommended as stress protectants in commercial formulations. This study focussed on the effects of selected biostimulants on cold stress mitigation during early growth in maize, as a major stress factor for cultivation of tropical and subtropical crops in temperate climates.

Chilling stress and micronutrient supplementation

Chilling stress, induced by moderately low soil temperatures (8-14°C) in a controlled root cooling system, was associated with inhibition of shoot growth, oxidative leaf damage (chlorosis, necrosis accumulation of stress anthocyanins) and a massive decline in root length (Chapter 4 and 5). Due to inhibition of root growth, nutrient acquisition in general was impaired. However, nutrient deficiencies were recorded particularly for the micronutrients zinc (Zn) and manganese (Mn). The impaired Zn and Mn status was obviously related with the observed limitations in plant performance, which were reverted by exogenous Zn and Mn supplementation (0.5 mg plant-1), finally leading to restored nutrient acquisition and improved plant recovery after termination of the cold stress period. Zinc and manganese deficiency was mainly related with impaired uptake of the micronutrients, since the cold stress-induced deficiency symptoms persisted even in hydroponic culture when all nutrients were freely available. Beneficial effects of Zn/Mn supplementation were only detectable when the micronutrients were supplied prior to the onset of the stress period via seed soaking, seed dressing or fertigation, when uptake and internal translocation was still possible. A transcriptome analysis of the shoot tissue (Chapter 5) revealed 1400 differentially expressed transcripts (DETs) after 7-days exposure of maize seedlings to chilling stress of 12°C, mostly associated with down-regulation of selected functional categories (BINs), related with photosynthesis, synthesis of amino acids, lipids and cell wall precursors, transport of mineral nutrients (N, P, K,), metal handling and synthesis of growth hormones (auxins, gibberellic acid) but also of jasmonic (JA) and salicylic acids (SA) involved in stress adaptations. In accordance with the impaired micronutrient status and oxidative leaf damage in response to the cold stress treatments, downregulation was also recorded for transcripts related with oxidative stress defence (superoxide dismutases SOD, catalase, peroxidases POD, synthesis of phenylpropanoids and lignification), particularly dependent on the supply of micronutrients as co-factors. Upregulation was recorded for BINs related with degradation of lipids, of cell wall precursors, synthesis of waxes and certain flavonoids and of stress hormones, such as abscisic acid (ABA) and ethylene but degradation of growth-promoting cytokinins (CK). Accordingly, supplementation of Zn and Mn increased the accumulation of anthocyanins and antioxidants, the activities of superoxide dismutase and peroxidases, associated with reduced ROS accumulation (H_2O_2), mitigation of oxidative leaf damage and improved plant recovery at the end of the cold stress period (Chapter 5 and 6).

Effects of seaweed extracts

Cold-protective properties similar to Zn/Mn supplementation, associated with an improved Zn/Mn-nutritional status and reduced oxidative damage, were recorded also after fertigation with seaweed extracts prior to the onset of the stress treatments (Chapter 4). However, this effect was detectable only with seaweed extract formulations rich in Zn/Mn (Algavyt+Zn/Mn; Algafect; 6-70 mg kg DM^{-1}) but not with a more highly purified formulation (Superfifty) without detectable micronutrient contents. This finding suggests that the cold-protective effect by soil application of seaweed extracts is based on an improved micronutrient supply and not to an elicitor effect, frequently reported in the literature for stress-protective functions after foliar application of seaweed extracts.

Silicon fertilization

Similar to seaweed extracts, also silicon (Si), applied by seed soaking or fertigation with silicic acid, mimicked the cold-protective effects of Zn/Mn supplementation in maize seedlings (Chapter

5). The Zn/Mn status of the Si-treated plants was improved although, in this case no additional micronutrient supply was involved. However, Si application significantly reduced leaching losses of Zn/and Mn by 50-70%, as a consequence of cold stress-induced membrane damage in germinating maize seeds and favoured the root to shoot translocation of Zn. This was associated with a restoration of gene expression, similar to the profiles recorded for unstressed control plants. However, the expression of genes related with synthesis and signal transduction of ABA, as central regulator of adaptive cold stress responses in plants, was even more strongly upregulated than in the cold-stressed controls. Accordingly, expression of cold stress adaptations involved in oxidative stress defence (SOD, peroxidases, phenolics, antioxidants) and the reduction of oxidative leaf damage and improved plant recovery were similar to the plants with Zn/Mn supplementation.

Plant growth promoting microorganisms

Cold-protective functions were recorded also for selected microbial inoculants (Chapter 6). However, out of five tested inoculant formulations, based on strains of *Pseudomonas* sp., DSMZ13134, *Bacillus amyloliquefaciens* FZB42, *Bacillus atrophaeus* ABI05, *Penicillium* sp. PK112 (BFOD) and a consortium of *Trichoderma harzianum* OMG16 and five *Bacillus* strains (Combi-A), a significant protective effect was detectable only for *Penicillium* sp. and particularly for CombiA. The CombiA consortium significantly increased root length and reduced oxidative leaf damage of cold-stressed plants, associated with increased SOD and POD activities and accumulation of phenolics and antioxidants. Root growth stimulation was related with increased IAA (indole acetic acid) tissue contents and increased expression of genes involved in IAA biosynthesis (ZmTSA) transport (ZmPIN1A) and perception (ZmAFR12). The tissue concentrations of ABA were not affected by the microbial inoculants, but the shoot concentrations of JA and SA increased, suggesting an effect by induced systemic resistance (ISR). Moreover, root concentrations of cytokinins (CKs) as ABA antagonists and expression of IPT genes involved in CK biosynthesis declined, leading to an increased ABA/cytokinin ratio and accordingly to increased expression of ABA responsive genes (ZmABF2). These findings suggest that CombiA mainly acted via improvement of root growth and nutrient acquisition by activation of the plant auxin metabolism and activation of cold protective metabolic responses by induction of ISR via JA/SA signalling and ABA-mediated responses, due to inhibition of CK biosynthesis.

Synergistic interactions

While the different cold-stress protectants investigated in this study induced similar protective plant responses, synergistic effects were obtained by combined applications (Chapter 6). The combination of CombiA inoculation with Zn/Mn supplementation further increased the plant micronutrient status and the cold-protective effects of CombiA. For all treatments, generally the expression of cold-protective effects was further improved by use of DMPP-stabilized ammonium fertilizers instead of nitrate fertilization. Ammonium fertilization promoted micronutrient acquisition via root-induced rhizosphere acidification, increased the ABA shoot concentrations with a moderate activation of metabolic cold stress responses and stimulated root colonization of *Trichoderma harzianum* OMG16 (CombiA).

Field performance

A comparative evaluation of the various cold protectants under field conditions with stabilized ammonium starter fertilization, revealed a severely reduced seedling emergence at six weeks after sowing (44%) due to extremely cold and wet soil conditions by the end of April in 2016, associated with a low Zn-nutritional status (32 mg kg^{-1} shoot DM). Significant improvements were recorded particularly for starter treatments including Zn/Mn seed dressing (emergence 56%) or seed priming with K_2SiO_4 (emergence 72%) and also by inoculation with the fungal PGPM strain *Penicillium* sp. BFOD (emergence 49%) associated with a doubling of the Zn tissue concentrations. Even after re-sowing, a significant yield increase for silo maize was recorded exclusively for the K_2SiO_4 treatment (Chapter 5). Taken together, the findings suggest that exploitation of synergistic interactions by combined starter applications of protective nutrients with selected biostimulants, could offer a cost-effective option for cold-stress prophylaxis in sensitive crops.

2. Zusammenfassung

Der Einsatz sogenannter Biostimulanzien auf Basis pflanzenwachstums-stimulierender Mikroorganismen, verschiedener Pflanzen-, Kompost- oder Algenextrakten, Protein-hydrolysaten oder Chitosanderivaten etc., aber auch Mineralstoffe mit Schutzfunktionen, wie z.B. Zink (Zn), Mangan (Mn), Bor (B), Calcium (Ca) oder Silizium (Si) wird in zahlreichen kommerziellen Formulierungen zur Erhöhung der pflanzlichen Stresstoleranz empfohlen. Die vorliegende Arbeit befasst sich daher exemplarisch mit der Charakterisierung der Wirkungen ausgewählter Biostimulanzien zur Erhöhung der Kältetoleranz in der Jugendentwicklung von Mais, als einer der Hauptstressfaktoren beim Anbau tropischer und subtropischer Kulturpflanzen in gemäßigten Klimazonen.

Kältestress und Mikronährstoff-Supplementierung

Kältestress, der durch moderat erniedrigte Bodentemperaturen von 8-14°C in einem kontrollierten Wurzelraumkühlungssystem induziert wurde, führte zu einer Hemmung des Sprosswachstums, oxidativen Blattschäden (Chlorosen, Nekrosen, Bildung von Stress-anthocyanen) und einem drastischen Rückgang des Wurzelwachstums (Kapitel 4 und 5). Durch die Wurzelwachstumshemmung wurde die Nährstoffaneignung generell beeinträchtigt. Jedoch wurde physiologischer Nährstoffmangel explizit für Zn und Mn diagnostiziert, der offensichtlich wesentlich für die beobachteten Beeinträchtigungen der Pflanzenentwicklung verantwortlich war, und durch Zn und Mn Supplementierung behoben werden konnte, verbunden mit einer allgemein verbesserten Nährstoffaufnahme und einer effizienteren Erholung nach Beendigung der Kälteperiode. Zink und Manganmangel beeinträchtigten hauptsächlich die Mikronährstoffaufnahme, denn die Mangelsymptome traten sogar in Nährlösungskultur auf, wenn alle Nährstoffe frei verfügbar waren. Die Schutzwirkung einer Zn/Mn Supplementierung war nur nachweisbar, wenn die Mikronährstoffe vor Beginn der Stressperiode über Einquellen des Saatgutes mit Mikronährstofflösung, Saatgutbeizung oder Fertigation appliziert wurden, solange eine Aufnahme und Verlagerung innerhalb der Pflanze noch möglich war. Eine Transkriptomanalyse im Sprossgewebe (Kapitel 7) ergab 1400 differenziell exprimierte Transkripte (DETs) nach 7-tägiger Kältebehandlung von Maiskeimlingen bei 12°C, die hauptsächlich im Zusammenhang mit verminderter Expression bestimmter funktioneller Kategorien (BINs) standen, in Verbindung mit der Photosynthese, Synthese von Aminosäuren, Lipiden und Zellwandvorstufen, Transport von Mineralstoffen (N,P,K), Interaktionen mit metallischen Kationen, der Synthese von Wachstumshormonen (Auxine, Gibberellinsäure) aber auch von Jasmon-, (JA) und Salicylsäure (SA) mit Beteiligung an der Regulation von Stressanpassungen. In Übereinstimmung mit dem beeinträchtigten Mikronährstoffstatus und der Ausbildung oxidativer Blattschäden als Folge der Kältebehandlung, wurde auch eine verminderte Expression von Transkripten in Verbindung mit der oxidativen Stressabwehr beobachtet (Superoxiddismutasen SOD, Katalase, Peroxidasen POD, Synthese von Phenylpropanoiden und Lignifizierung), die besonders auf eine ausreichende Versorgung mit Mikronährstoffen als Co-Faktoren angewiesen ist. Erhöhte Expression wurde für BINs in Verbindung mit dem Abbau von Lipiden und Zellwandvorstufen, der Synthese von Wachsen und bestimmter Flavonoide sowie von Stresshormonen wie Abscisinsäure (ABA) und Ethylen und dem Abbau wachstumsfördernder Cytokinine (CK) beobachtet. Entsprechend erhöhte die Supplementierung von Zn und Mn die Bildung von Anthocyanen und Antioxidanzien, die Aktivitäten von Superoxiddismutase und Katalase, verbunden mit verminderter Akkumulation freier Radikale (H_2O_2), einer Verminderung oxidativer Blattschäden und einer verbesserten Erholung nach Ende der Kältestressperiode (Kapitel 5 und 6).

Wirkung von Algenextrakten Die Fertigation mit Algenextrakten vor Beginn der Kältebehandlung zeigte ähnliche Kälteschutzwirkungen wie die Zn/Mn Supplementierung (Kapitel 4). Jedoch war diese Wirkung nur bei Algenextraktformulierungen mit hohem Zn/Mn Konzentrationen nachweisbar (Algavyt+Zn/Mn; Algafect; 6-70 mg kg TM^{-1}), jedoch nicht bei einer stärker aufereinigten Formulierung (Superfifty) ohne nachweisbaren Mikronährstoffgehalt. Dies weist auf eine Mikronährstoffwirkung der Algenextrakte bei Bodenapplikation hin und nicht auf Elicitoreffekte, die in der Literatur häufig für Blattapplikationen beschrieben werden.

Silizium Düngung Ähnlich wie die Algenextrakte zeigte auch Siliziumapplikation über Saatgutbehandlung oder Fertigation mit Kieselsäure die typischen Kälteschutzeffekte einer Zn/Mn-Supplementierung bei Maiskeimlingen (Kapitel 5). Der Zn/Mn-Status Si-behandelter Pflanzen wurde

signifikant erhöht, obwohl keine zusätzliche Mikronährstoffdüngung erfolgt war. Allerdings verminderte die Si Behandlung die durch Membranschäden induzierten Mikronährstoffverluste keimender Maissamen unter Kältestress signifikant um 50-70% und förderte die Zinkverlagerung in den Spross. Dabei traten Genexpressionsprofile vergleichbar mit ungestressten Kontrollpflanzen auf. Allerdings wurde die Expression von Genen in Verbindung mit der Synthese und Signaltransduktion von ABA, als zentraler Regulator pflanzlicher Anpassungen an Kältestress, sogar noch stärker erhöht als bei kältegestressten Kontrollpflanzen. Entsprechend wurde die Expression von Kältestressanpassungen im Zusammenhang mit der oxidativen Stressabwehr (SOD, POD, Phenole, Antioxidanzien) in ähnlicher Weise erhöht wie nach Zn/Mn Supplementierung und oxidative Blattschäden entsprechend vermindert.

Pflanzenwachstums-stimulierende Mikroorganismen Für einige mikrobielle Inokulanzien auf Basis von Pilz-, und Bakterienstämmen konnten Kälteschutzfunktionen nachgewiesen werden (Kapitel 6). Jedoch traten signifikante Schutzwirkungen nur bei zwei von fünf getesteten Formulierungen auf (*Pseudomonas* sp., DSMZ13134, *Bacillus amyloliquefaciens* FZB42, *Bacillus atrophaeus* ABI05, *Penicillium* sp. PK112 (BFOD) und ein Konsortium aus *Trichoderma harzianum* OMG16 und 5 *Bacillus* Stämme (Combi-A) mit signifikanten Effekten für BFOD und besonders für CombiA. Das CombiA-Konsortium erhöhte die Wurzellänge signifikant und verminderte oxidative Blattschäden in Verbindung mit erhöhter Aktivität von SOD und POD, sowie Akkumulation von Phenolen und Antioxidanzien, Die Förderung des Wurzelwachstums war mit erhöhten Gewebekonzentrationen von Indolessigsäure (IAA) und erhöhter Expression von Genen der IAA-Synthese (ZmTSA), IAA-Transport (ZmPIN1A) und der IAA Signalperzeption (ZmAFR12) verbunden. Die Konzentrationen von ABA wurden durch die Inokulanzien nicht beeinflusst aber die Sprosskonzentrationen von JA und SA stiegen an, was auf eine induzierte systemische Resistenzreaktion (ISR) hindeutet. Darüber hinaus wurden die Konzentrationen von Cytokininen (CKs) als ABA-Antagonisten verringert was zu einem erhöhten ABA/CK Verhältnis und einer erhöhten Expression ABA-responsiver Gene (ZmABF2) führte. Diese Beobachtungen weisen auf eine wurzelwachstumsfördernde Wirkung von CombiA über Interaktionen mit dem pflanzlichen Auxinstoffwechsel hin, sowie auf eine Aktivierung von Kältestressanpassungsreaktionen durch ISR-Induktion über JA/SA-vermittelte Signaltrans-duktion und ABA-abhängige Stressantworten, in Folge der gehemmten CK Synthese.

Synergistische Interaktionen Während die verschiedenen, in dieser Arbeit getesteten Kälteschutzapplikationen ähnliche Schutzwirkungen induzierten, konnten durch kombinierte Anwendungen auch synergistische Effekte induziert werden. Die kombinierte Inokulation von CombiA mit Zn/Mn Supplementierung führte zu einem erhöhten Zn/Mn Ernährungsstatus, der die Schutzwirkung von CombiA noch weiter verbesserte. Bei allen Behandlungen konnte die Kälteschutzwirkung durch die Verwendung DMPP-stabilisierter Ammoniumdünger an Stelle von Nitratdüngung weiter erhöht werden. Ammonium Düngung verbesserte die Mikronährstoffaneignung durch wurzelinduzierte pH-Absenkung in der Rhizosphäre, erhöhte die Sprosskonzentrationen von ABA verbunden mit einer moderaten Aktivierung der Kälteschutzanpassungen und verbesserte die Wurzelbesiedelung mit *Trichoderma harzianum* OMG16 (CombiA).

Anwendung unter Feldbedingungen, Eine vergleichende Untersuchung der Wirkung der verschiedenen Kälteschutzanwendungen mit stabilisierter Ammonium Starterdüngung im Feldversuch, ergab einen drastisch verminderten Feldaufgang sechs Wochen nach Aussaat (44%) als Folge von extrem hoher Bodenfeuchte und niedriger Bodentemperaturen nach Aussaat Ende April 2016, verbunden mit einem niedrigen Zink-Ernährungsstatus (32 mg kg^{-1} Spross TM). Eine signifikante Verbesserung des Feldaufgangs wurde besonders durch Zn/Mn Saatgutbeizung (56%) Saatgutbehandlung mit K_2SiO_4 (72%) aber auch durch Inokulation mit dem Penicillium-Stamm BFOD (49%) erreicht. Dabei wurden die Zn-Sprosskonzentrationen verdoppelt. Selbst nach Lückennachsaat in Folge der massiven Kälteschäden, zeigte die K_2SiO_4 Behandlung noch eine signifikante Ertragserhöhung im Vergleich zur unbehandelten Kontrolle (Kapitel 5). Zusammenfassend weisen die Ergebnisse darauf hin, dass besonders durch synergistische Wirkungen kombinierter Applikationen von Mineraldüngern mit Schutzfunktionen und ausgewählten Biostimulanzien, kostengünstige Anwendungen für die Kältestressprophylaxe bei kälteempfindlichen Kulturpflanzen entwickelt werden könnten.

Chapter 1. General Introduction

1.1 Maize and cold stress

Many crop species from subtropical or tropical climates, such as *Zea mays* (maize), *Glycine max* (Soybean), Sorghum, or *Solanum Lycopersicum* (tomato) are particularly sensitive to cold stress (chilling (0–15 ◦C) or freezing (≤0 ◦C); Ruelland et al., 2009). Maize (*Zea Mays* L.) belongs to Poaceae family and is one of the most important annual cereal crops worldwide. Maize is the staple food in many parts of the world and is the third most-produced crop after wheat and rice. Although maize is mainly used as a food/feed crop, it has also vast industrial potentialities (Rouf Shah et al., 2016). Despite its tropical origin and its high sensitivity to low temperatures, maize is cultivated in a wider range of climates than any other cereal crop (Frederiks et al., 2015, Wang et al., 2018).

The optimum temperature for maize growth ranges from 21 to 27 °C and suboptimal temperatures (10–20 °C as chilling stress) lead to growth retardation and declined yield, while temperatures below 10 °C cause irreversible damage and result in plant death (Mao et al., 2017, Zhu et al. 2007). Early sowing of maize can improve yield as the result of a longer growing season (Carneiro et al., 2017), and is an important strategy for avoiding the effect of summer drought (Hund et al., 2004), however, it potentially exposes seedlings to cold stress in the early spring in temperate regions. In the early sowing of cold spring, maize development and physiology is impaired throughout two stages at the early development. The first stage covers from germination to the three-leaf stage where growth relies mainly on seed reserves, while the second stage initiates with the development of a functional photosynthetic apparatus (Rodrı´guez et al., 2014). In the latter regard, cold stress inhibits CO_2 assimilation, and induces the overproduction of potentially dangerous reactive oxygen species (ROS), leading to the destruction of cellular structures and disrupt metabolism.

Reduction in nutrient and water uptake is also induced by cold stress due to impaired root development (Foyer et al., 2002). Accordingly, maize is a cold-intolerant plant and there is a need for improving its tolerance against cold stress for having a maximum possible yield in temperate zones like central Europe (Zhu et al. 2007, Riva-Roveda et al., 2016). One approach to cope with this situation is based on adapted fertilization with critical nutrients or application of stress-protective biostimulants, discussed as mitigation strategies (Bradacova et al., 2016; Gómez-Muñoz et al., 2018; Moradtalab et al., 2018). However, to optimize the exploitation of the genetic yield potential of maize under suboptimal environmental conditions, a better understanding of the effects of cold stress on its growth and the physiological, genetic, and molecular base of potential mitigation strategies is required.

1.2 Significance of micronutrients

Stress is defined as an external biotic or abiotic factor that causes a deleterious effect on plants by limiting their development and survivorship. The concept of stress is closely related to stress tolerance, which is the plant's ability to face an unfavorable environment (Rehem et al., 2012). Low temperature (LT) or cold is one of the major environmental stresses lands leads to multiple physiological and metabolic disturbances in plant growth and development (Li et al., 2018, Foyer et al., 2002). One of the major negative effects of cold stress is the loss of membrane integrity and a nutrient limitation in response to impaired root growth and activity (Awasthi et al., 2015). Cold stress increases the levels of ROS and typically plants respond to this by increasing the expression and activity of ROS-scavenging enzymes and increasing the production of antioxidants to reduce the risk of oxidative damage of membranes and other cellular components including detrimental effects on hormone homeostasis (Xie et al., 2019). Micronutrient deficiency can limit these

defense reactions since most of the ROS-scavenging enzymes and the biosynthesis of phenolic antioxidants are dependent on micronutrients such as Zn, Mn, Fe, and Cu as cofactors (Cakmak, 2000; Datnoff et al., 2007), thus, their role in improving cold stress tolerance is critical.

1.2.1 Zinc

Zinc (Zn) has several physiological roles in plants include catalytic, co-catalytic, and structural involvement in more than 300 enzymes such as carbonic anhydrase, carboxypeptidase, alcohol dehydrogenase, Cu/Zn superoxide dismutase (SOD), alkaline phosphatase (microorganisms), phospholipase, RNA polymerase, Zn-PPiase (tonoplast), fructose 1,6 bis-phosphatase, aldolase, participating in membrane integrity, Zn-finger motif class of transcription factors, the integrity of ribosomes, protein, RNA, DNA and carbohydrates, reactive oxygen species and indole acetic acid (IAA) metabolism (Marschner, 1995; Hajiboland, 2012; Ahangar et al., 2016). In many parts of the world, Zn deficiency is an important cause of nutritional disorders in plants (Marschner, 1995). In plants, shoot Zn concentrations generally below a 15–20 mg kg^{-1} dry weight (DW) is the threshold level for deficiency symptoms, detectable in young leaves due to low phloem mobility. Under stress conditions, such as Zinc deficiency, the activity of Cu/Zn superoxide dismutase (Cu/Zn-SOD) as the first detoxifying barrier of plants response to stress conditions is frequently not sufficiently expressed. Limited ROS detoxification leads to damage to membranes, proteins, chlorophyll, and enzymes, resulting in leaf chlorosis and the inhibition of photosynthesis and growth (Cakmak 2000). Excessive production of ROS can promote the oxidative degradation of IAA. Therefore, auxin deficiency was considered another important factor for growth limitation in Zn deficient plants causing growth depression of plants under Zn deficiency (Cakmak et al., 1989).

1.2.2 Manganese

Manganese (Mn) is involved in the activation of various enzymes of the tricarboxylic acid cycle and shikimic acid pathway leading to the biosynthetic pathway of aromatic amino acids aromatic secondary metabolites and particularly the synthesis of phenolic compounds (datnoff et al., 2007) Manganese is also involved in the photosynthetic system related to photosystem II, ATP synthesis, RuBP carboxylase reactions and the biosynthesis of fatty acids, acyl lipids and proteins. PSII has several transition metal cofactors such as haem, non-haem iron, and an oxygen-evolving manganese cluster containing four manganese ions (Ahangar et al., 2016). Mn deficiency on thylakoid structure and chlorophyll degradation (Papadakis et al. 2007), leading to the development of characteristic interveinal leaf chlorosis, whereas rates of respiration and transpiration remain unaffected. In dicotyledonous plants, interveinal chlorosis of the younger leaves is the most distinct symptom of Mn deficiency, whereas, in cereals, greenish-gray spots on the more basal leaves (gray speck) are the major visual symptoms (Husted et al. 2005).

Manganese has an important role in keeping chlorophyll concentration and superoxide dismutase activity well balanced (Upadhyaya et al., 2012). MnSOD is more sensitive to UV light than Cu/ZnSOD suggesting the importance of the manganese cofactor in the photoinhibition of MnSOD (Hakala et al., 2006). Manganese can act as a scavenger of superoxide (O_2) and hydrogen peroxide (H_2O_2) radicals (Millaleo et al., 2010). Oxidative stress caused by manganese deficiency indirectly causes chlorophyll losses in leaves (Hajiboland, 2012).

1.2.3 Iron

Iron (Fe) is involved in the production of chlorophyll. It is a component of many enzymes associated with energy transfer, nitrogen reduction, and fixation, and lignin formation. In

association with sulfur, iron forms compounds that catalyze other reactions in plants (Marschner 1995). Drought-induced deficiency of iron causes chlorosis of leaves due to low levels of chlorophyll. Leaf chlorosis first appears on the younger upper leaves in interveinal tissues. Severe iron deficiencies cause leaves to turn completely yellow or almost white leading to their death (Ahangar et al., 2016). Uptake of iron decreases with increased soil pH and high levels of available phosphorus; manganese and zinc in soils also have adverse effects on iron uptake (Waraich et al., 2011).

Because of the low solubility of iron-bearing minerals, plants use two strategies to absorb sufficient iron. Strategy I plants (dicotyledonous and non-graminaceous monocotyledonous plants) can respond to the lack of iron in the soil by increasing the capacity of root tissues to reduce apoplastic Fe, by the acidification of the rhizosphere and accumulation and release of organic acids (mainly citrate) to increase iron solubility in soil. By the advent of these events, the iron uptake activities in rhizodermal root cells and mobility within plants are increased. In Strategy II plants, members of the mugineic acid family of phytosiderophores are secreted into the rhizosphere, which helps to solubilize Fe^{3+} by chelation to form the Fe^{3+}-mugineic acid complex. The Fe^{3+}- mugineic acid complex is then taken up by root cells through the action of the Yellow Stripe 1 (YS1) protein (Curie et al., 2001). Abadia et al. (1999) demonstrated that iron-deficiency-induced leaf chlorosis is due to a decrease in the leaf concentrations of photosynthetic pigments (chlorophylls and carotenoids). Iron-deficient leaves showed decreases in the actual PSII efficiency at steady-state photosynthesis, due to the reduction of photochemical quenching and intrinsic PSII efficiency (Abadia et al., 1999). Iron nutrition has a critical role in the protection of plants against oxidative stress by affecting the activity POD, APX, and Cu/ZnSOD enzymes. Iron deficiency reduces the activity of CAT and PODs, the ubiquitous haem-containing

enzymes. High levels of H_2O_2 in iron-deficient plants (Marschner, 1995) indicate the reduced capacity of iron-deficient plants for detoxification of peroxide radicals and therefore increasing oxidative damage (Hajiboland, 2012).

1.2.4 Boron

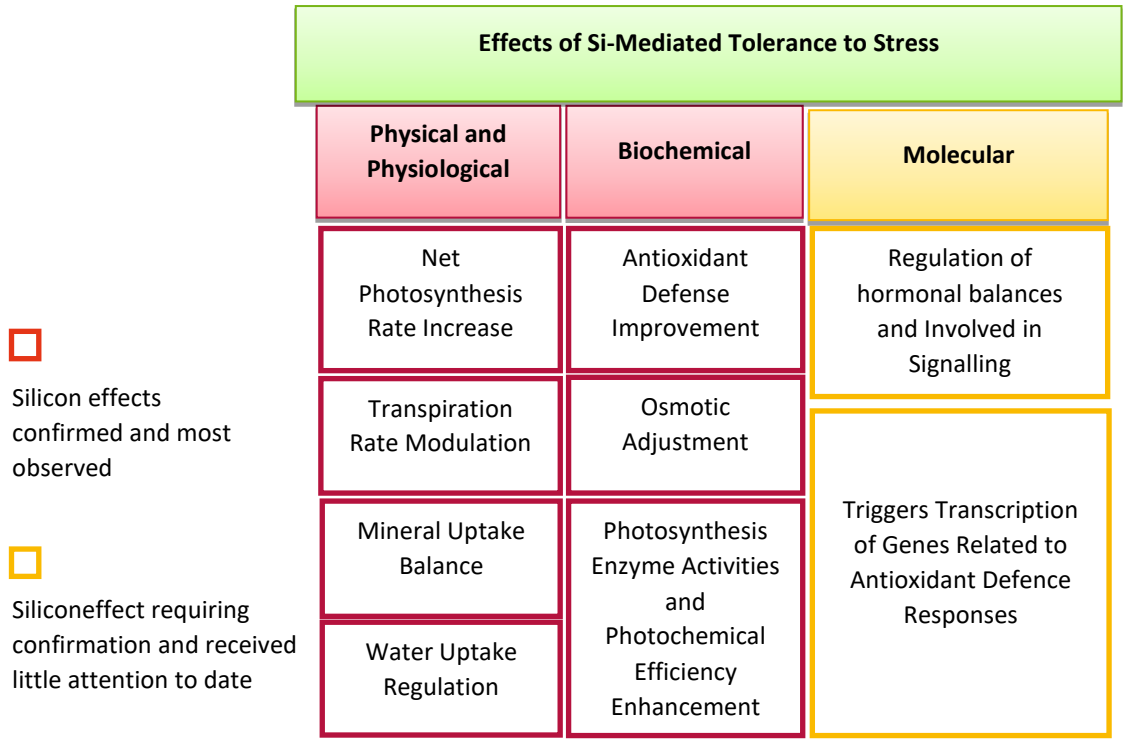
Boron is a constituent of the cell wall in cross-links of rhamnogalacturonan II, involved in cell wall synthesis and cell extension, improves regulation of lignin biosynthesis, and xylem differentiation. Moreover, it enhances the photosynthetic rate and integrity of membranes, improves sugar transport and IAA synthesis, maintains carbohydrate, protein, and RNA metabolism, improves seed and pollen germination and pollen tube growth as well. It affects the metabolism of phenolics as antioxidants and hence reduces the production of ROS (Waraich et al., 2011; Hajiboland, 2012).

1.2.5 Copper

Copper (Cu) plays key roles in photosynthetic and respiratory electron transport chains, ethylene sensing, lignification, cell wall metabolism, and protection from oxidative stress (Yruela, 2005). Copper is also involved in pollen formation and has an important role in maintaining its viability, mediates pollination, biosynthesis of lignin, quinones, and carotenoids (Hajiboland, 2012). Several enzymes bear copper ion as a cofactor, for example, Cu/ZnSOD, cytochrome c oxidase, ascorbate oxidase, amino oxidase, laccase, plastocyanin, and polyphenol oxidase. At the cellular level, copper plays an essential role in cell wall metabolism, signaling, protein trafficking machinery, oxidative phosphorylation, iron mobilization and the biogenesis of molybdenum cofactor. Thus, an appropriate concentration of copper is essential for normal growth and development, and its deficiency develops specific symptoms in plants (Ahangar et al., 2016).

1.3 Significance of silicon

Although silicon (Si) has not been yet considered to be an essential element for higher plants (Epstein, 1999), its beneficial effects have been demonstrated for many plants, especially when they are exposed to biotic or abiotic stresses (Ma and Yamaji, 2006; Liang et al., 2007). It is an essential nutrient for species from Equisetaceae and wetland Poaceae. It prevents toxicity of P, manganese, and iron and reduces heavy metal stress, causes the stability of plants, cell wall rigidity, and elasticity, increases leaf erectness and the volume, rigidity of aerenchyma and root oxidizing power of wetland plants, reduces cuticular transpiration and effects of mutual shading and susceptibility to lodging (Hajiboland, 2012). Proposed key mechanisms in Si-mediated alleviation of abiotic stresses in higher plants are: (1) silica deposition inside the plant tissues which provides mechanical strength and erectness, nutrient and water mobility inside the plants, (2) stimulation of antioxidant systems in plants, (3) complexation or co-precipitation of toxic metals with Si both in plant tissues and in soil, (4) modulation of gene expression and signaling through hormones (Savvas and Ntatsi, 2015) Fig. 1.

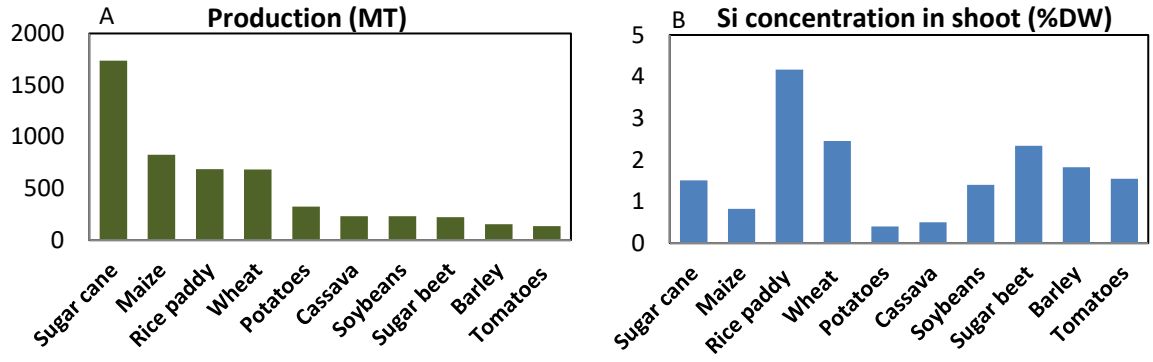


Chapter 1. Fig. 1. Scheme of Si-mediated tolerance to stress in plants (Modified from Zhu and Gong, 2014).

1.3.1 Seven out of the ten most important crops are classified as Si accumulators

Plants take up Si from soil solution where Si is mainly present as H_4SiO_4 (mono-silicic acid) (Dietzel, 2000). The transporters responsible for Si uptake by roots (Lsi1 and Lsi2) were identified in several higher plant species including rice, barley, maize, wheat, and pumpkin. Depending on the extent of plant Si uptake, the process of Si uptake can be classified as active (accumulator), passive (intermediate), or rejective (excluders). Seven out of the ten most important crops are classified as Si accumulators (Guntzer et al., 2012, Fig. 2). The existence of an active metabolic importer of Si to the roots may highlight the importance of Si in metabolism by accumulator crops, which is neglected in many years. Once the solubility of H_4SiO_4 is exceeded (i.e. > 2 mM), SiO_2 polymerization occurs and, for cells, this can be toxic (Exley, 2015); thus, it stands to reason that H_4SiO_4 transported through healthy root cells (via Lsi1 and Lsi2) must maintain a cytosolic concentration of <

2 mM (Coskun et al., 2019).



Chapter 1. Fig. 2. A) Most important crops ranked by production; B) Si concentration in the crops (Guntzer et al., 2012), MT: Million Tones, DW: Dry Weight.

1.4 Significance of plant growth-promoting microorganisms (PGPMs) and seaweed extracts

Soil life is characterized by a high diversity of microorganisms such as archaea, bacteria, fungi, protozoa, and algae. Microbes that interact with the plant roots, stimulate the release of root exudates and rhizodeposits, which can influence soil properties including soil pH and nutrient status, and altering the diversity and activity of plant-associated beneficial microbes and also pathogens (Adak et al., 2016). Various plant beneficial microorganisms have been isolated, characterized and are used as inoculants to stimulate plant growth and to improve nutrient acquisition and stress resistance of their host plants (PGPMs) in commercial formulations. Apart from Rhizobia as symbionts in leguminous plants, diazotrophic bacteria (Azotobacter, Azospirillum) and arbuscular mycorrhizal fungi, members of the genera *Trichoderma*, *Bacillus*, and *Pseudomonas* are among the most widespread PGPMs used as plant inoculants, which have been investigated also in the present study. *Tri*choderma spp. is one of the most popular genera of useful microbes commercially available as a plant growth-promoting fungus (PGPF) and biological control

agent (Keswani et al., 2014). The major modes of *Tri*choderma spp. action that benefits plant growth and health are mycoparasitism (confrontation with myco-pathogens), secretion of cell wall degrading enzymes (subsequent penetration and killing myco-pathogens), antibiosis secretion of secondary metabolites (antimicrobial properties), competition (being decomposers by nature), efficiently mobilize and uptake macro- and micro-nutrients from soil (Keswani et al., 2014). *Tri*choderma spp. induces tolerance against abiotic stresses and improve plant growth. *Trichoderma* colonized plants produce certain compounds (auxins, ethylene, gibberellins, plant enzymes, antioxidants) and phytoalexins and phenols that provide tolerance to abiotic stresses and enhance the branching capacity of the root system (Hidangmayum et al., 2019).

Among the different soil microorganisms, bacteria are by far the most common (95%, Schoenborn, et al., 2004). However, the number and the type of bacteria that are found in different soils are dependent on the soil conditions such as temperature, moisture, presence of salt and other chemicals, the number and types of plants found in those soils, and possible environmental stress (Glick et al., 1999). The bacteria that can promote plant growth are named as plant growth-promoting bacteria (PGPB) that include free-living (making symbiotic relationships with plants such as Rhizobia spp. and Frankia spp.), endophytes (can colonize plant's different tissues), and cyanobacteria (blue algae). PGPB may promote plant growth directly via facilitating resource acquisition and modulating plant hormone levels (the effects are depending on the endogenous pool of plant hormone) or indirectly through decreasing the inhibitory effects of various pathogenic agents on plant growth and development as biocontrol bacteria (Glick, 2012, Table. 1). The application of the predominant and important genera of PGPB (*Pseudomonas* and *Bacillus*) in the rhizosphere alleviate plants stresses due to their unique characteristics, diversity and relationship to

plants. It was reported that *Pseudomonas* and *Bacillus* strains investigated explicitly in this study can increase plant tolerance against abiotic stress along with multiple PGPM traits like ACC deaminase activity, minerals solubilization, hormones production hormonal regulation, biofilm formation, siderophore activity (Kumar et al., 2016).

Seaweed application in plants can increase the content of important nutrients such as nitrogen (N) and sulfur (S) and also chlorophyll, thus, the photosynthesis. As a consequence of the increased photosynthesis, the carbon content (C) is also raised. Therefore, the increase of N, S, and C can be responsible for increased plant growth by seaweed application (Jannin et al., 2013). Seaweed extracts (mainly obtained from *Asophyllum nodosum*) also increase plant tolerance against abiotic and biotic stresses (Calvo et al., 2014). Biochemical components of seaweed that act a role to improve plant tolerance against stress are carbohydrates, minerals, trace constituents, amino acids, betaines, and betaine-like compounds, hormones, hormonal like compounds. These compounds can trigger and balance different response pathways of plants against stress conditions (Khan et al., 2009).

Chapter 1. Table. 1. The mechanisms via that PGPB promote plant growth (modified from Glick 2012).

Effect	Mechanism
Direct	• Facilitating Resource Acquisition: Nitrogen Fixation, Phosphate Solubilization, Sequestering Iron • Modulating Phytohormone Levels: Cytokinins, Gibberellins, Indoleacetic Acid production
Indirect	• Antibiotics and Lytic Enzymes Synthesis • Siderophores production • Competition with pathogens • Regulating the synthesis of Ethylene • Induced Systemic Resistance: priming plant defense mechanisms

1.4.1 PGPB modulate the effects of environmental stress

Plant growth may be affected by different biotic and abiotic stress factors such as nematodes, pathogens including viruses, bacteria, and fungi, drought, cold, high light, toxic metals, and salinity. To overcome these stresses, the plant adjusts its metabolism, thus plant growth may be limited. However, when PGPB are added to plants, they may employ several strategies to mitigate this growth inhibition. Many stress situations growth arrest is part of the adaptation, actively regulated and efficient e.g against excessive water losses during drought or salinity and protective against cold stress. Mitigation of the growth inhibition by PGPMs under these conditions would be rather detrimental and not beneficial. The main function of efficient biostimulants including PGPMs is the protection against irreversible damage during the stress period and supporting recovery. The most proposed mechanism that is applied by bacteria under abiotic stress is summarized in Table 2.

Chapter 1. Table. 2. Mechanisms that PGPB employ to mitigate abiotic stress effects on plant growth (modified from Glick 2012).

Effect	PGPB	Mechanism
Ethylene	Production of ACC deaminase	1. Various biotic and abiotic stresses can stimulate the transcription of the gene for ACC synthase. 2. ACC is the immediate precursor of stress hormone ethylene with inhibitory effects e.g. on root growth at higher concentrations. 3. Some ACC can be taken up by PGPB bound to the plant and degraded to ammonia and α-ketobutyrate. 4. Excessiveethylene accumulation in the rhizosphere is avoided by minimizing detrimental effects on plant growth and root development.
Auxin	Production of IAA	1. The amino acid tryptophan is excluded by plant roots and then taken up by PGPB bound to the roots where it is converted into IAA. 2. Bacterially produced IAA is secreted, taken up by plant cells, and, together with the plant's pool of IAA stimulates an auxin signal transduction pathway, including various auxin response factors.
Cytokinin	Production of Cytokinin or	1. Overproduction of cytokinin in transgenic plants significantly protected the plants from the

	Cytokinin-like compounds	deleterious effects of abiotic stress. 2. Cytokinins promote shoot growth and counteract senescence
Trehalose	Production of α-α-1,1-glucoside	1. Trehalose, a highly stable molecule can form a gel phase as cells dehydrate, replacing water and, as a result, decreasing damage from drought and salt. 2. Trehalose can prevent some of the protein degradation and aggregation that often occurs under both high and low-temperature stresses.
Antifreeze	Secretion of antifreeze proteins	1. Antifreeze proteins secretion into the surrounding medium when the bacteria are grown at low temperature. 2. The possibility of using bacterial antifreeze proteins, to facilitate the functioning of plants at low temperatures has not yet been studied. 3. Bacterial antifreeze proteins may have ice-nucleation activity, to regulate the formation of ice crystals outside of the bacterium. 4. The bacterial cell wall and membrane protect from potentially lethal damage (piercing) from the formation of large ice crystals.
Exopolysaccharides	Secretion of bacterial exopolysaccharides	1. It inhibits the movement of toxic ions and helps to maintain the ionic balance, promotes the movement of water in plant tissues, and inhibits the growth of pathogenic microbes.

Organic Compounds	Production of volatile organic compounds (VOCs) and Quorum-sensing signals	1. VOCs help plants to resist pathogen attack and balance hormonal responses of plants. They may involve in gene expression regulation 2. Quorum sensing signals may involve in phytohormonal balances and trigger host phytohormone and metabolite regulation

1.5 Environmental stress and regulation of gene expression

Stress conditions induce the expression of genes, which protect cells by producing not only functional but also regulatory proteins such as TFs and protein kinases (Shinozaki and Yamaguchi- Shinozaki, 2007). Signal transduction pathways consist of kinases, phosphatases, TFs, and other regulatory factors such as late embryogenesis-abundant (LEA) proteins, chaperones, and enzymes for osmolyte biosynthesis, and ROS detoxification (Moradtalab and Hajiboland, 2016).

Translation of stress signals into changes in gene expression occurs through multiple TF families, including dehydration responsive element binding protein/C-repeat binding factor (DREB/CBF) regulon; the no apical meristem (NAC) regulon, Arabidopsis thaliana activating factor1 (ATAF1), cup-shaped cotyledon (CUC2), and zinc-finger homeodomain (ZF-HD) regulons; the ABA-responsive element-binding protein/ABRE binding factor (AREB/ABF) regulon; and the myelocytomatosis/myeloblastosis (MYC/ MYB) regulon (Lata et al., 2011). Zinc-finger proteins (ZFPs) are TFs in both animals and plants with roles in growth and development. Cys2/His2 (C2H2)-type ZFPs, consist of the EAR transcriptional repressor domain and regulate plant responses to several stress factors. Overexpression of ZFPs causes resistance to low temperature, drought, and salinity at both germination and seedling stages in tobacco. Several ZPT2 (C2H2 type ZFP) related proteins Arabidopsis zinc finger proteins (AZF), AZF1, AZF2, AZF3, and salt-tolerance zinc finger (STZ) function as repressors of other TFs. Overexpression of STZ in transgenic Arabidopsis plants results in growth delay and higher resistance under water stress, indicating that AZF2 and STZ as repressors act in increasing tolerance to stresses through growth cessation (Singh et al., 2010).

1.6 The important role of abscisic acid in plant stress responses

The plant stress hormone, abscisic acid (ABA) is a key regulator of abiotic stress in plants (Kim et al., 2010). ABA induces leaf stomatal closure, reduces water loss via transpiration, and improves plants' water use efficiency (Bray, 2004). The AREB or ABFs are basic leucine zipper (bZIP) TFs and activate ABA-dependent gene expression and bind to the ABA-responsive element (ABRE) motif. ABFs can activate several ABA/stress-responsive genes in Arabidopsis. ABFs operate in different stress response pathways; for example, ABF1 in low temperature; ABF2 in salinity, drought, and high temperature; ABF3 in salinity; ABF4 in low temperature, salinity, and drought stress (Fujita et al., 2005).

AREB1/ABF2, AREB2/ ABF4, and ABF3 are mostly expressed in vegetative tissues, specifically under drought, whereas ABI5 and enhanced embryogenesis level (EEL) are expressed during seed development (Nakashima and Yamaguchi- Shinozaki, 2006). AREB1 is a transcription activator of ABA- and dehydration related genes such as linker histone H1–3 (HIS1–3), ATPases associated with diverse cellular activities (AAA), ATPase, and LEA class genes, which ameliorate water stress (Fujita et al., 2005).

AREB1 and AREB2 act in ABA-inducible expression of rd29B, which is one of the dehydration-responsive genes in Arabidopsis. The N-terminal domain in the AREB proteins are phosphorylated by an ABA-activated protein kinase, for example, open stomata1/ sucrose non-fermenting related protein kinases 2E (OST1/SRK2E) (Umezawa et al., 2004). In Arabidopsis, OST1/SRK2E phosphorylates Ser/Thr residues located in the conserved regions of AREB1 (Furihata et al., 2006). SRK2C as a positive regulator of drought tolerance in Arabidopsis is a central regulator of stomatal closure. Over-expression of SRK2C causes hypersensitivity to ABA and ameliorates drought tolerance by reduction of water loss (Umezawa et al., 2004).

ABA plays a role in cold acclimation by triggering specific cellular and molecular osmotic responses. Continuous application of ABA induces chilling tolerance in chilling-sensitive plant species, such as maize, rice, cucumber, and pepper. Furthermore, exogenous ABA application in temperate plants such as poplar, barley, wheat, and Arabidopsis can partially mimic cold acclimation and enhance freezing tolerance. ABA biosynthesis pathway may be required for the full development of the cold response, as defects in both basal and acquired freezing tolerance have been observed in ABA-deficient mutants. For instance, *ABA1* and *ABA3* are identified as genes encoding enzymes involved in the ABA biosynthetic pathway. Cold induction of the cold-response genes such as cold-induced (*COR*) genes is reduced in the aba3 mutants aba3/los5/frs1.

Cold acclimation was shown to be impaired in an aba1 mutant, which has also reduced the expression of specific *COR* genes. The expression of none of the ABA biosynthesis genes is affected by cold treatment in Arabidopsis. Therefore, ABA biosynthesis is not an early event in response to cold stress. Consistent with this, foliar application of ABA to a wide range of plant species does not induce cold hardness or freezing tolerance. Cold stress specifically activates the expression of ABA biosynthesis genes in reproductive organs, such as the inflorescence meristem, with only slightly increased expression in the leaves and vegetable organs. thus, ABA function in stimulating and hastening plant harvests under adverse environmental conditions. Taken together, ABA may function late in the development of cold-induced metabolic changes and is required for determining the maximum levels of cold tolerance in plants and under cold stress, the expression of the *COR* genes is regulated by both ABA-dependent and ABA independent pathways (Shi and Yang, 2014).

1.6.1 ABA-dependent pathway

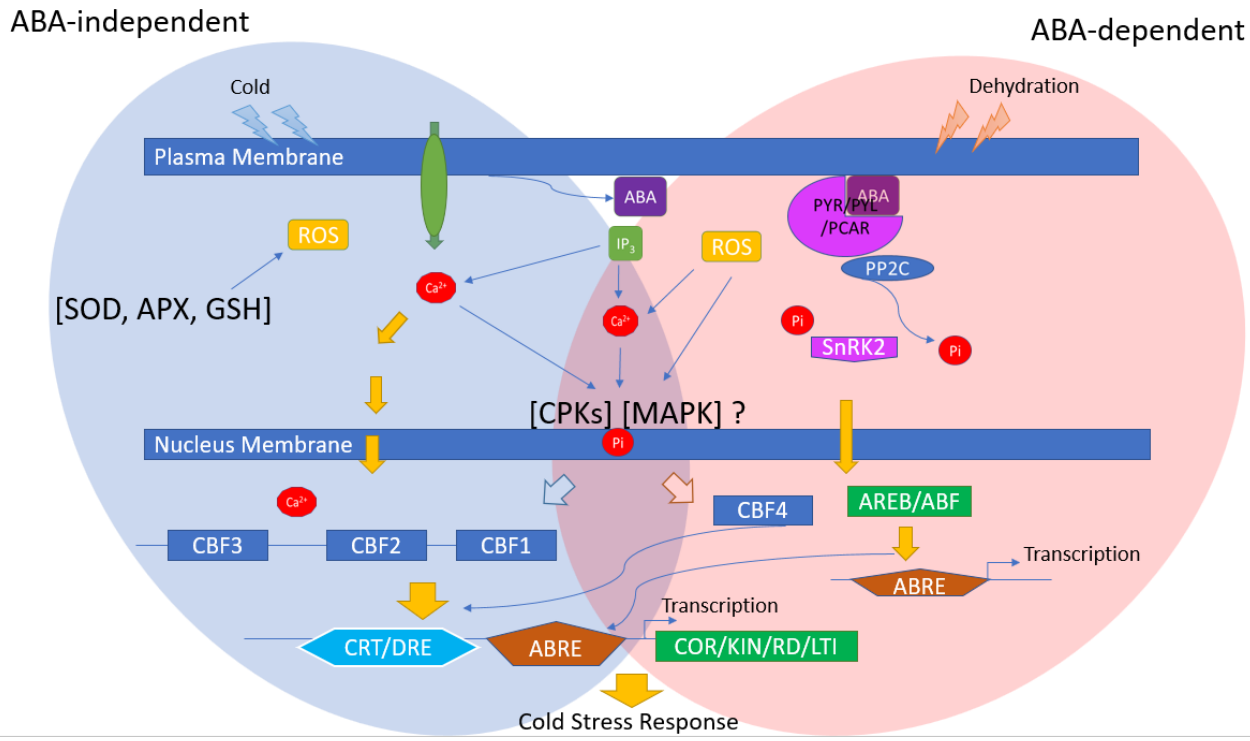
ABA-dependent signaling pathway plays an essential role in stress-responsive gene expression during cold-induced osmotic stress. Cold induction of some ABA-responsive genes is regulated by different kinds of transcription factors (Shi and Yang, 2014). ABRE and DRE/CRT are cis-acting elements presented in the promoter region of the genes that are induced by water, salt, and cold stresses. In the ABA-dependent pathway, ABRE is the main ABA-responsive element (Valliyodan and Nguyen, 2006). ABA biosynthesis genes contribute to the enrichment of *COR* genes, such as *RD29A*, *RD22*, *COR15A*, *COR47*, and *P5CS* via the activity of their ABRE *cis*-element. ABF1 expression is significantly induced by cold, but not by osmotic stress (Choi et al. 2000), whereas AREB1/ABF2, AREB2/ABF4, and ABF3 are specifically induced by ABA and drought stress. In addition to AREBs, other bZIP transcription factors belonging to the ABI5 subfamily have been characterized as ABRE-binding proteins and were shown to affect seed germination as well as the cold stress response (Zhang, 2014). Several MYC and MYB family genes have been reported to be important regulators of ABA-responsive gene expression under cold stress. For instance, MYB96 is induced by ABA and drought, and it enhances ABA-mediated drought and freezing tolerance (Zhang, 2014). NAC domain family members are involved in stress responses (Shao et al. 2015). Expression of a rice NAC gene, OsNAC5, is induced by osmotic stress and ABA, and overexpression of OsNAC5 increases stress-induced proline and soluble-sugar levels and enhances tolerance to cold, salt, and drought stresses (Hong et al., 2016).

1.6.2 ABA-independent pathway

In ABA-independent pathways, overexpression of the C-repeat binding factor (CBF) family of transcription factors/DREB1 genes in transgenic plants improves tolerance to

cold, drought, and salt stresses. Another component of the ABA-independent pathway, MYB family genes. The rice *Osmyb4* gene codes MYB4 TF, which is expressed at low temperature and its expression in Arabidopsis promotes its resistance to low and freezing temperatures. Overexpression of rice MYB4 improves drought tolerance by increasing the cellular concentrations of osmolytes such as glycine betaine, sinapoyl malate, glucose, fructose, sucrose, and proline under non-stress and stressful conditions (Mattana et al., 2005). Both cold stress and ABA treatment induce the production and accumulation of inositol 1,4,5-triphosphate (IP3), calcium ion (Ca^{2+}), and ROS inside the cell. These molecules act as second messengers in signaling networks to amplify and transduce signals through activating protein kinases or TF cascades. Increased IP3 levels, which are catalyzed by phospholipase C (PLC) under cold stress, are known to release Ca^{2+} from vacuoles into the cytosol. In plants, Ca^{2+} is a widely used and important signaling molecule during early responses to abiotic stresses, as it functions by activating protein kinase cascades, which in turn can activate transcription factors and stress-responsive genes. More specifically, a transient influx of cytoplasmic Ca^{2+} occurs in response to cold shock, and it has been suggested that low-temperature-induced changes in membrane fluidity are the primary thermo-sensor signal that activates the Ca^{2+} influx in plants. Furthermore, calcium is required for the full expression of the COR genes in Arabidopsis. Ca^{2+} may function through calmodulin or Ca^{2+} sensors to modulate the activation of downstream calcium-dependent protein kinases (CPKs). Calcineurin B-like proteins (CBLs), one kind of calcium sensors, was also shown to regulate expression of the CBF genes, based on the observations that overexpression of CBL1 resulted in reduced freezing tolerance, Several CBL-interacting protein kinases (CIPKs) and CPKs have been implicated in the responses to ABA, cold, and high-salt stress. In Arabidopsis, the expression of CIPK3 is strongly

induced by cold stress and ABA treatment, but not by drought stress. It has also been suggested that CIPK1 is a convergence point for ABA-dependent and ABA-independent stress responses (Shi and Yang, 2014, Fig. 3).



Chapter 1. Fig. 3. Schematic illustration of the cold and dehydration response regulatory networks through ABA-dependent and ABA-independent pathways. In Arabidopsis IP3, Ca2+, and ROS act as second messengers in signaling networks to transduce signals through protein kinases or transcription-factor cascades. CBFs and AREB/ABFs transcription factors are responsible for the regulation of COR genes containing CRT/DRE (CCGAC) and ABRE (ACGT) motifs in their promoters, respectively. In Arabidopsis, approximately 10 % of the cold-induced transcriptome contains both CRT and ABRE motifs in their promoter regions, which are upregulated by both cold and dehydration stresses. IP3 inositol 1,4,5-triphosphate, ROS reactive oxygen species, CPK calcium-dependent protein kinase, MAPK Ras-mitogen-activated protein kinase, Pi phosphoryl group (Modified from Shi and Yang, 2014).

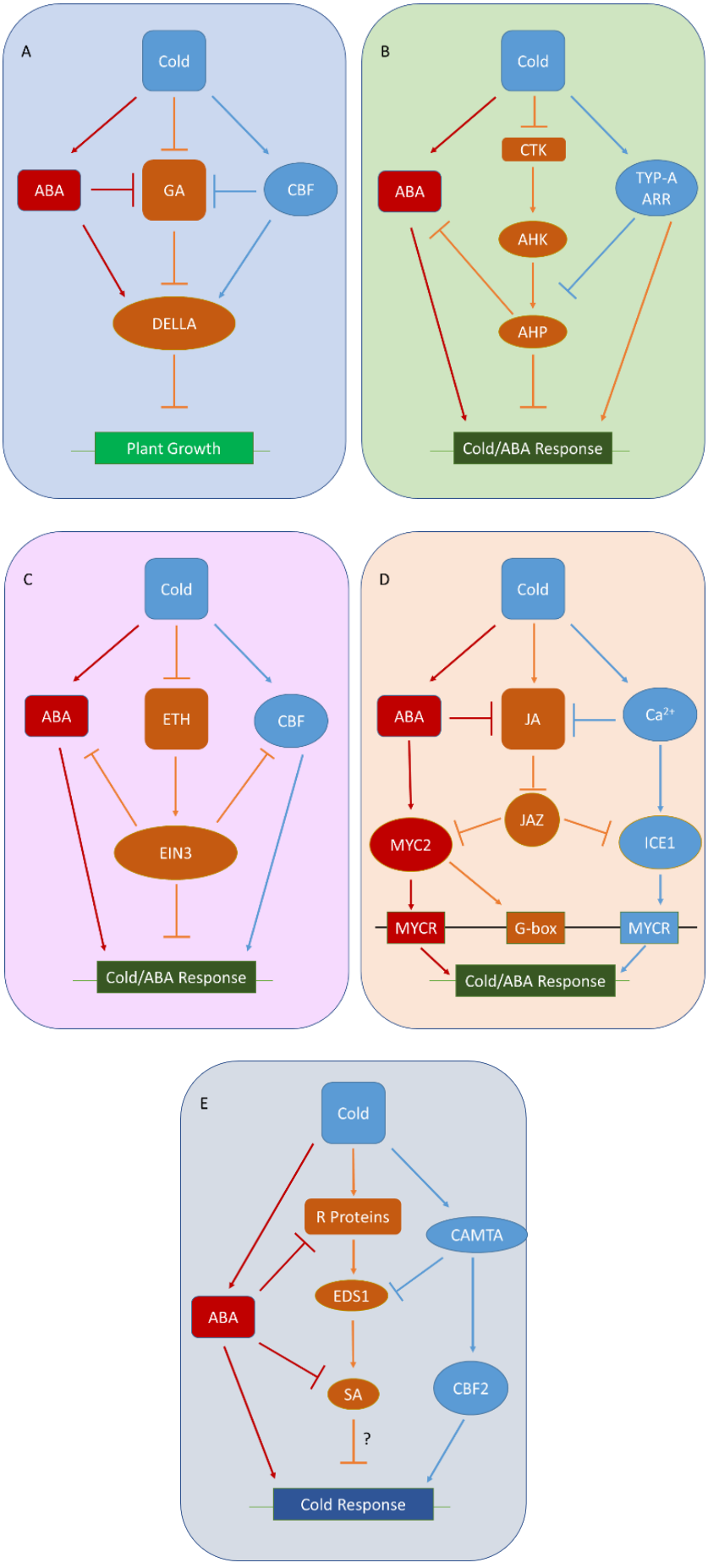
1.5 Interactions among plant hormones in cold stress responses

Cold acclimation is affected by changes in the homeostasis of various hormones. A complex interplay between ABA and other phytohormone-signaling pathways is crucial for the regulation of plant response to cold stress. When plants are challenging by stress

conditions, in general, the growth inhibition occurs that decreases the capacity for energy utilization, which in turn, results in cold acclimation processes. Stress hormones ABA and jasmonic acid (JA) inhibit plant growth by modulating the actions of auxin, gibberellic acid (GA), and cytokinin (CTK) (Peleg and Blumwald, 2011). DELLA proteins acting as GA-signaling repressors, are ABA promotes the accumulation of DELLA proteins, which induces growth repression. Consistently, overexpression of CBFs represses plant growth via the accumulation of DELLA proteins (Achard et al., 2008, Fig. 4A).

Cytokinin acts as a negative regulator to modulate abiotic stress signaling (Nishiyama et al. 2011). The cytokinin receptor histidine kinases AHK2, AHK3, and CRE1 in Arabidopsis play important roles in the regulation of plant abiotic responses in both ABA-dependent and ABA-independent signaling pathways (Tran et al. 2007). It is suggested that cytokinin has a negative role for signaling in cold- and ABA-mediated abiotic stress. For example, ahk2 ahk3 double mutants are highly tolerant of cold, drought, and salt stress and show strong expression of ABA-responsive genes (Jeon et al. 2010). Type-A ARR genes that are induced by cytokinin as well as by cold stress, are negative response regulators in the cytokinin signaling pathway (Fig. 4B). However, whether cytokinin receptors can perceive stress signals is still unclear.

The role of ethylene in plant responses to cold stress is complex and species-dependent. Enhanced chilling and freezing tolerance were observed with an increase of ethylene biosynthesis in several plant species, including tomato, cucumber, and tobacco. In contrast, the suppression of ethylene biosynthesis in mung bean and Arabidopsis increased cold tolerance.


A
Cold
ABA
GA
CBF
DELLA
Plant Growth
B
Cold
CTK
ABA
TYP-A ARR
AHK
AHP
Cold/ABA Response
C
Cold
ABA
ETH
CBF
EIN3
Cold/ABA Response
D
Cold
ABA
JA
Ca2+
JAZ
MYC2
ICE1
MYCR
G-box
MYCR
Cold/ABA Response
E
Cold
R Proteins
CAMTA
EDS1
ABA
SA
CBF2
?
Cold Response

Chapter 1. Fig. 4. Models of multiple interactions among plant hormones in cold stress response. There are multiple points of interaction between ABA and other plant hormones including GA (A), cytokinin (B), ethylene (C), JA (D), and SA (E) in the regulation of plant response to cold stress. CTK cytokinin, ETH ethylene, DELLA DELLA protein, AHK Arabidopsis histidine kinase, AHP Arabidopsis histidine phosphor-transferase, ARR Arabidopsis response regulator, EIN3 Ethylene insensitive3, JAZ jasmonate-zim-domain protein, MYC2 MYC-related transcriptional activator2 (Modified from Shi and Yang, 2014).

In Arabidopsis, the application of the ethylene precursor ACC decreases freezing tolerance, whereas the application of the ethylene biosynthesis inhibitor promotes freezing tolerance (Shi and Yang, 2014). Although, the complex cross-talk between ethylene, ABA, and cold signaling is still unclear but genetic and biochemical analyses revealed that ethylene negatively regulates cold signaling, at least partially, through direct transcriptional control of the COR, CBFs, and type-A ARR genes via EIN3 (Shi et al. 2012, Fig. 4C).

The exogenous application of JA significantly improves freezing tolerance in *Arabidopsis*. primarily through an ABA independent pathway. JAZ1 and JAZ4 proteins physically interact with the ICE1-proteins to repress their transcriptional activity, thereby repressing the expression of CBFs (Hu et al. 2013). However, there is an antagonistic interaction between ABA and JA signaling pathway. in this regard, MYC2 acts as a node for the integration of ABA and JA signaling. MYC2 directly represses JAZ-mediated signaling and acts as a positive regulator of the ABA-dependent signaling pathway (Fernandez-Calvo et al. 2011, Fig. 4D). In Arabidopsis, mutants that are defected in R/R-like protein that overproduce SA, are chilling sensitive phenotypes. The calmodulin-binding transcriptional activator (CAMTA3) in signaling pathway interacting with Ca^{2+} is involved in the regulation of freezing tolerance and positively regulate *CBF2* expression during cold stress, but it also interacts with the EDS1 promoter (in not SA-deficient mutants) to repress SA-dependent disease resistance (Kim et al., 2013). However, the exact role of SA in cold tolerance is still unclear (Fig. 4E).

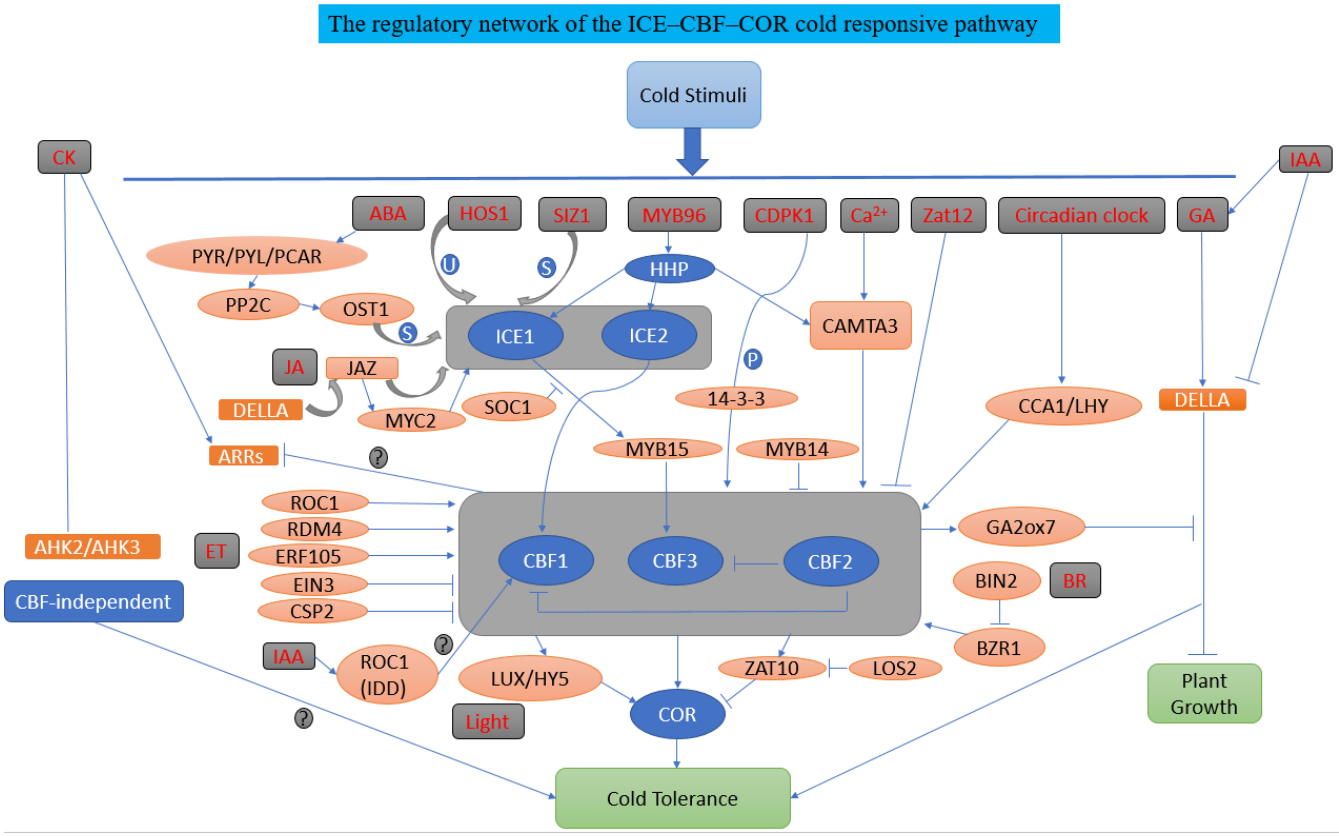
1.6 Regulatory network of the ICE–CBF–COR cold-responsive pathway in plants

Based on the sequence analysis, the transcript-derived fragments (TDFs) of known functions in maize plants undergo a complex responsive process to cold stress via changes in metabolism, photosynthesis, signal transduction, and defense responses (Yang et al., 2011). Many quantitative trait loci (QTL) were identified for cold tolerance in maize (Rodríguez et al., 2014). RNA-Seq technology is an approach used to identify large numbers of genes associated with specific traits and to discover novel stress response pathways. For cold responses, the most reported QTL were associated with photosynthesis (Mao et al., 2017). Several genes also involved in photosynthesis, sugar metabolism, signal transduction, circadian regulation, and cell wall function have been identified as candidate genes associated with cold response and cold acclimation (Yang et al., 2011).

Cold temperatures trigger the expression of the C-repeat binding factor (CBF) family of transcription factors, which in turn activate many downstream genes that confer chilling and freezing tolerance to plants. The inducer of CBF expression 1 (ICE1), is an upstream transcription factor that regulates the transcription of CBF genes in the cold. An *Arabidopsis* ice1 mutation blocks the expression of CBF3 and decreases the expression of many genes downstream of CBFs, which leads to a significant reduction in plant chilling and freezing tolerance. ICE1 encodes an MYC-like bHLH transcriptional activator. ICE1 binds specifically to the MYC recognition sequences in the CBF3 promoter. ICE1 is expressed constitutively, and its overexpression in wild-type plants enhances the expression of the CBF regulon in the cold and improves freezing tolerance of the transgenic plants (Chinnusamy et al., 2003, see Fig. 5).

CBFs, is also known as dehydration-responsive element-binding factor proteins (DREBs), which function upstream transcription factors that bind to the CRT/DRE element and

activate the transcription of the cold-response genes such as COR, low-temperature induced (LTI), response to dehydration (RD) (Yang et al., 2011, see Fig. 5). The transgenic expression of maize DREBs known as ZmDREB1A in *Arabidopsis* enhanced drought and freezing tolerance (Wang and Dong et al., 2009). The regulatory network of the ICE–CBF–COR cold-responsive pathway is illustrated in Fig. 5.



Chapter 1. Fig. 5. Regulatory network of the ICE–CBF–COR cold-responsive pathway. MYB96 regulates HHP genes by binding directly to promoters of ICE1, ICE2, and CAMTA3, respectively. Transcription factor ICE1 is regulated through sumoylation of HOS1 and phosphorylation of SIZ1. PP2C negatively regulates OST1/SnRK2, which interacts with ICE1, stabilizes ICE1, and promotes its transcriptional activity. CE1 functions upstream from the CBF/DREB1 regulon. JAZ proteins interact with ICE to inhibit the activation of CBFs; DELLAs competitively bind to JAZs to release MYC2. MYC2 interacts with ICE1 to enhance CBF gene transcription. SOC1 inhibits ICE1 from binding to the CBF3 promoter. ICE1 represses the expression of MYB15. The expression of CBFs is negatively regulated by MYB14, MYB15, ZAT12, and EIN3. ROC1, RDM4, CSP2, ERF105, and CAMTAs positively regulate the expression of CBFs. Plasma membrane CRPK1 phosphorylates 14_3_3 proteins to promote destabilization of CBF proteins. CBFs reduce the bioactive levels of GA and promote the accumulation of DELLAs; DELLAs restrain plant growth. GA2ox7 is a CBF3 regulon, which is activated by CBF3 to decrease the bioactive GA level and promote the accumulation of DELLAs; DELLAs restrain plant growth. BIN2 is a negative regulator of BZR1, and BZR1 positively modulates the expression of CBF gene transcripts. CBFs regulate the expression of COR genes that contribute to cold tolerance. CBFs induce the expression of ZAT10, which negatively regulates the expression of COR genes. Cold upregulated LOS2 represses the transcription of ZAT10. Circadian regulation of CCA1 and LHY positively regulates the level of CBF genes. Light regulation of HY5 mediates the induction of COR genes under low temperatures. P, phosphorylation; S, SUMO (small ubiquitin-related modifier); U, ubiquitin, Modified from Wang et al., 2017.

1.7 Research focus

Maize seeds are planted at spring with cold events in the temperate zones like central Europe for having a maximum possible yield even though this crop largely lacks the capacity for cold acclimation. Thus, to maintain the highest genetic yield potential of maize under cold stress via early sowing in spring, a better understanding of the effects of cold on its growth and development is very important. To boost maize performance under cold stress in this study, we have focused on developing strategies to improve its cold tolerance using stress protectants including:

1. Fertigation and seed treatments with nutrients (Zn, Mn, and Si) important for oxidative stress defense
2. Fertigation with seaweed extracts (mainly obtained from *Asophyllum nodosum*) with antioxidative and membrane-protective properties
3. Inoculation with plant growth-promoting microorganisms (PGPMs) including cold-tolerant bacterial strains with the potential ability to maintain growth, metabolic activity, phytohormone production, and nutrient mobilization even at lower soil temperatures

1.7.1 Research questions

1. What are the most efficient cold stress protectants (Zn/Mn, Si, seaweed extracts)?

2. What is the suitable application techniques?

3. What is the mode of action at the physiological and molecular levels?

4. Is it possible to translate stress-protective effects into field performance and yield responses?

5. What is the cost-benefit level of each cold stress protectant at the field scale?

1.7.2 Aims and objectives

The aims and objectives of this study are to:

1. investigate the biochemical changes in root and shoot tissues during the early growth of maize seedlings affected by cold stress to identify possible cellular metabolites and compounds involved in stress-related tolerance such as enzymatic and non-enzymatic antioxidants, growth, and stress-related hormones.

2. understand the impacts of the cold stress protectants on cold-responsive gene expression.

3. to characterize the molecular mechanisms of tolerance to cold stress in maize plants via seed treatments with protective nutrients (Zn, Mn, and Si) and PGPMs.

4. to find the most suitable application technique and cost-effective of cold stress protectants.

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Chapter 2. Micronutrients (Zn/Mn), Seaweed Extracts, and Plant Growth Promoting Bacteria as Cold-Stress Protectants in Maize

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Authors' contributions

KB, NFW, and NMT equally contributed to the setup and evaluation of the soil culture experiments under controlled RZT and prepared the manuscript. MW was involved in setup of the cooling system and adaptation of the SOD determination. MA and MI were responsible for the nutrient seed priming experiment in hydroponic culture. GN designed the experiments and was involved in proof reading and final editing of the manuscript. All authors read and approved the final manuscript.

2.1 Abstract

Background: Low soil temperature in spring is a major constraint for cultivation of tropical crops in temperate climates, associated with impaired seedling development, inhibition of root growth and root activity. In this study, potential cold-stress protectants, such as supplemented micronutrients (Zn, Mn), seaweed extracts, and rhizobacteria with plant growth-promoting potential (PGPRs) were tested in order to improve the tolerance of maize to low root zone temperatures (RZT) during early growth.

Methods: Maize (v. Colisee) was cultivated in a root cooling system for adjustment of the RZT. In three independent experiments, after germination at 20 °C, the cold-stress phase (12–14 °C) started at 14 days after sowing to simulate a cold period in spring. Micronutrients, seaweed extracts, and PGPRs were supplied by fertigation (experiment 1), fertigation and seed dressing (experiment 2), and nutrient seed priming (experiment 3). At the end of the experiments, scoring of oxidative leaf damage, biomass production, chlorophyll status (SPAD), root length density, superoxide dismutase activities in leaf and root tissues, and the shoot mineral-nutritional status were determined.

Results: Positive effects on plant growth and particularly on root development at low RZT were detected exclusively for seaweed extracts with high Zn/Mn contents and similar growth promotions were induced by Zn and Mn application in comparable amounts. This finding suggests that the selected seaweed extracts were mainly acting via improved Zn and Mn supply to the plants. It was essential that the cold-stress protectants were present during seed imbibition. The beneficial effect of Zn/Mn treatments and sea weed extracts was associated with increased superoxide dismutase activity in the root and leaf tissue, with key functions in antioxidative stress defense, depending on Zn, Mn, Cu, and Fe as enzymatic co-factors. Accordingly, leaf damage, shoot and root growth inhibition in

cold-stressed plants was associated with a low Zn-nutritional status, mitigated by application of the cold-stress protectants.

Conclusions: Since micronutrients are effective already at low concentrations, starter applications of Zn/Mn or the respective seaweed extracts may offer an economic option for cold-stress prophylaxis in crops.

Keywords: Maize, Seaweed extract, Micronutrients, Cold-stress tolerance, Oxidative stress, Antioxidative stress defense, Superoxide dismutase, Plant growth-promoting rhizobacteria

2.2 Background

Cultivation of tropical and subtropical crops, such as maize, soybean, or sorghum in temperate climates continuously increases as a consequence of global warming. However, for plant species like maize with optimum temperatures of 25–30 °C for germination and plant growth [1, 2], even moderately low soil temperatures <15 °C are already detrimental to root development, fine root branching, and root elongation [3, 4]. Apart from root growth, also root activity in terms of nutrient uptake and nutrient translocation, adaptive root exudation for nutrient mobilization [5], and hormonal balances are impaired by low root zone temperatures (RZT), [6, 7]. Particularly in maize, plant growth is not only affected by limitations of the root system, since also the shoot meristem is located close to the soil surface and remains below- ground even until the V6 stage [8]. Oxidative stress plays a significant role for the induction of chilling injury in low temperature-sensitive plants [9, 10], indicated visually by formation of oxidative leaf-damage symptoms, such as chloroses, anthocyanin formation, and leaf de-colouration, later resulting in formation of necrotic areas (see Fig. 3). As an additional stress factor, also soil nutrient availability

decreases with declining soil temperatures, as a consequence of lower solubility of mineral nutrients in cold soils, while the viscosity of water increases and reduces the speed of transport processes of mineral nutrients from soil to the root surface [11, 12]. Therefore, plant availability of sparingly soluble nutrients, such as P, NH_4^+, K, Fe, Zn, Mn, and Cu is particularly affected by low soil temperatures. Under favorable conditions, short cold periods can be tolerated and later compensated during plant development until final harvest. However, longer stress periods can easily induce irreversible dam- age already in the seedling stage [13].

Various strategies have been proposed as practical measures to counteract low temperature stress in crops:

1. Fertilizer placement close to the seedling roots is currently the most widely employed approach to support root uptake of limiting nutrients, such as P and N, in some cases also including Zn, Mn, and Fe as micro- nutrients. Imran et al. [14] demonstrated that even soaking seeds in (Zn, Mn, Fe) micronutrient solutions (seed nutrient priming) could partially restore root growth and nutrient uptake of maize seedlings exposed to low RZT of 12 °C, associated with a final yield increase of 10 % in two independent field experiments.
2. Application of seaweed extracts (mainly obtained from *Asophyllum nodosum)* with antioxidative and membrane-protective properties (reviewed by Sangha et al. [15]).
3. Improving root growth and plant nutrient acquisition by inoculation with plant growth-promoting microorganisms (PGPRs), particularly using cold-tolerant bacterial strains (psychro-tolerant bacteria) with the ability to maintain phytohormone production, nutrient mobilization (siderophores), or degradation of excessive ethylene levels even at low soil temperatures (reviewed by Subramanian

et al. [16]).

This study was designed as a comparative evaluation of approaches to mitigate cold-stress during early growth of maize under controlled root zone temperatures. The selected mitigation strategies comprised (1) fertigation and seed treatments with micronutrients (Zn, Mn) important for oxidative stress defense [14, 17]; (2) fertigation with seaweed extracts of different origins; and (3) inoculation with plant growth-promoting and psychro-tolerant bacteria.

2.3 Methods

2.3.1 Plant cultivation

Zea mays L cv. Colisee was used as test plant. Soil material (silty-loam, pH 6.9) was derived from the Ap horizon of a maize cultivation field site at the Hohenheim University experimental station Ihinger Hof, Renningen, Germany. After sieving with 2 mm mesh size, fertilization was performed with Ca $(NO_3)_2$, 100 mg N kg^{-1} DM; $Ca(H_2PO_4)_2$, 80 mg P kg^{-1} DM; K_2SO_4, 150 mg K kg^{-1}DM and $MgSO_4$, 50 mg Mg kg^{-1} DM. For improvement of the soil structure, the fertilized soil was mixed with quartz sand (ratio 2:1). Plastic pots with a volume of 1275 cm3 were filled with the soil substrate and inserted into a cooling system, designed to control the root zone temperature of plants. An immersion water bath circulator (Thermomix 1480/Frigomix 1497, Braun, Melsungen, Germany) was connected to the cooling system containing a closed pipe system, installed in moist peat culture substrate to circulate the refrigerating fluid through the moist peat layer. The plants were regularly watered to 70 % of substrate water holding capacity (WHC) with distilled water (Additional file 1: Figure S1). Hydroponic culture with the nutrient solution containing 2 mM Ca $(NO_3)_2$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.5 mM $MgSO_4$, 0.1 mM KH_2PO_4, 10 µM

H_3BO_3, 0.5 µM $MnSO_4$, 0.2 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$, 0.5 µM ZnSO4, and 20 µM Fe–EDTA. pH 6.0–6.5 was performed as described by Imran et al. [14].

2.3.2 Application of plant growth-promoting rhizobacteria, micronutrients, and seaweed extracts

Commercial products and micronutrients ($ZnSO_4$, $MnSO_4$) were applied (1) as fertigation treatments with a pipette close to the plant, directly on top of the soil substrate in three weekly intervals, starting with the sowing date (experiment 1); (2) single starter fertigation vs three weekly applications for seaweed extracts, and Zn/ Mn seed dressing with Lebosol® Mn^{500} SC and Lebosol® Zn^{700} SC (Lebosol® Dünger GmbH, Elmstein, Germany), according to the manufacturer instructions (experiment 2); (3) micronutrient seed priming (experiment 3) according to Imran et al. [14].

4.3.2.1 Plant growth-promoting rhizobacteria

- Proradix® WP (Sourcon Padena, Tübingen, Germany); active ingredient: *Pseudomonas* sp. DSMZ 13134. Dosage per application: 1*109 CFU kg^{-1} dry soil substrate.
- RhizoVital® FZB42 (ABiTEP, Berlin, Germany); active ingredient: *Bacillus amyloliquefaciens* subsp. *plantarum.* Dosage per application: 1*109 CFU kg^{-1} dry soil substrate.
- R41 (ABiTEP, Berlin, Germany); active ingredient: cold-resistant *Bacillus simplex* strain R41. Dosage per application: 1*109 CFU kg^{-1} dry soil substrate.

4.3.2.2 Sea weed extracts

- Super Fifty® (BioAtlantis, Tralee, Ireland); active ingredient: *Ascophyllum nodosum* extract. Dosage per application: 17 mg kg^{-1} dry soil substrate.

- Algavyt Zn/Mn (Agriges, San Salvatore Telesino, Italy); active ingredient: extracts from *Ascophyllum nodosum*, *Fucus* spp., *Laminaria* spp., +Zn/Mn. Dosage per application: 16 mg kg^{-1} dry soil substrate.
- Algafect (Agriges, San Salvatore Telesino, Italy); active ingredient: extracts from *Ascophyllum nodosum*, *Fucus* spp., *Laminaria* spp. Dosage per application: 16 mg kg^{-1} dry soil substrate.

4.3.2.3 Micronutrients

- $ZnSO_4$ fertigation. Dosage per application: 0.5 mg kg^{-1} dry soil substrate.
- $MnSO_4$ fertigation. Dosage per application: 0.5 mg kg^{-1} dry soil substrate.
- Zn seed dressing. Lebosol® Zn^{700} SC: 2 mL 4000 $seeds^{-1}$
- Mn seed dressing. Lebosol® Mn^{500} SC: 4 mL 4000 $seeds^{-1}$
- Zn/Mn seed priming. 4 mM Zn + 2.5 mM Mn solution as $ZnSO_4 \cdot H_2O$ and as $MnSO_4 \cdot 7H_2O$. Sixty seeds were soaked in 200 mL of priming solutions (distilled water as control) in the dark for 24 h. Thereafter, seeds were taken out, rinsed with running distilled water for 1 min to remove excess priming solution. Subsequently, seeds were air dried at room temperature for a minimum time period of 1 h [14].

23.3 Plant analysis

Visual scoring of leaf chloroses and necroses was performed for experiment 1 and replaced by reflectometric leaf chlorophyll measurements (SPAD) for experiment 2. Plant height, root and shoot dry matter after 60 °C oven drying, and root length measurements were performed for all experiments (WinRHIZO root analysis software, Regent Instruments Inc., Quebec, Canada).

2.3.4 Analysis of mineral nutrients

One hundred milligrams of dried shoot material were ashed for 5 h in a muffle furnace at 500 °C. After cooling, the samples were extracted twice with 1 mL of 3.4 M HNO_3 and evaporated until dryness to precipitate SiO_2. The ash was dissolved in 1 mL of 4 M HCl, subsequently diluted ten times with hot deionized water, and boiled for 2 min to convert meta- and pyrophosphates to orthophosphate. After addition of 0.1 mL Cs/La buffer to 4.9 mL ash solution, Fe, Mn, and Zn concentrations were measured using atomic absorption spectrometry (ATI Unicam Solaar 939, Thermo Electron, Waltham, USA). Spectrophotometrical determination of orthophosphate was conducted after addition of molybdate-vanadate color reagent (Hitachi U-3300 spectrophotometer, Hitachi LtD. Corporation Japan) according to the method of Gericke and Kurmies [18]. Determination of Mg was conducted by atomic absorption spectrometry, while K and Ca were measured by flame emission photometry (ELEX 6361, Eppendorf, Hamburg, Germany).

2.3.5 Superoxide dismutase assay

The superoxide dismutase (SOD) assay was optimized for root and shoot tissues of maize with reference to the method described by Beauchamp and Friedovich [19] and modifications suggested by Giannopolitis and Ries [20] and Hajiboland and Hasani [21]. One hundred milligrams of fresh plant material, frozen in liquid nitrogen and stored at −80 °C, were ground with a pre-cooled mortar and pestle, and homogenized in 1.5 mL extraction buffer containing

25 mM HEPES pH 7.8 and 0.1 mM EDTA. After centrifugation at 10000×g (4 °C for 10 min), aliquots of the supernatant were transferred into 2 mL reaction tubes and kept on ice. For preparation of the reaction mixture, 1 mL cuvettes, covered with aluminum foil for

light protection, were filled with 300 µL 62.5 mM HEPES, 75 µL 1.0 mM EDTA, 75 µL 120 mM Na2CO3, 75 µL 120 mM l-methionine, 150 µL 750 µM nitro-blue tetrazolium (NBT), and 100 µL of plant extract. Finally, 225 µL 10 µM riboflavin were added. The light reaction was started by removing the aluminum foil, exposing the samples to light (8000 Lux) for 25 min. During the light phase, NBT is reduced to a dark blue formazan, measured spectrophotometrically (U-3300, Hitachi, Tokyo, Japan) at a wavelength of 650 nm. The final SOD activity, which inhibits the NBT reduction, was calculated as difference between absorbance of the sample and a control without plant extract, divided by 50 % absorbance of the control. The SOD activity was expressed as SOD units per g fresh weight (FW).

2.3.6 Statistical analyses

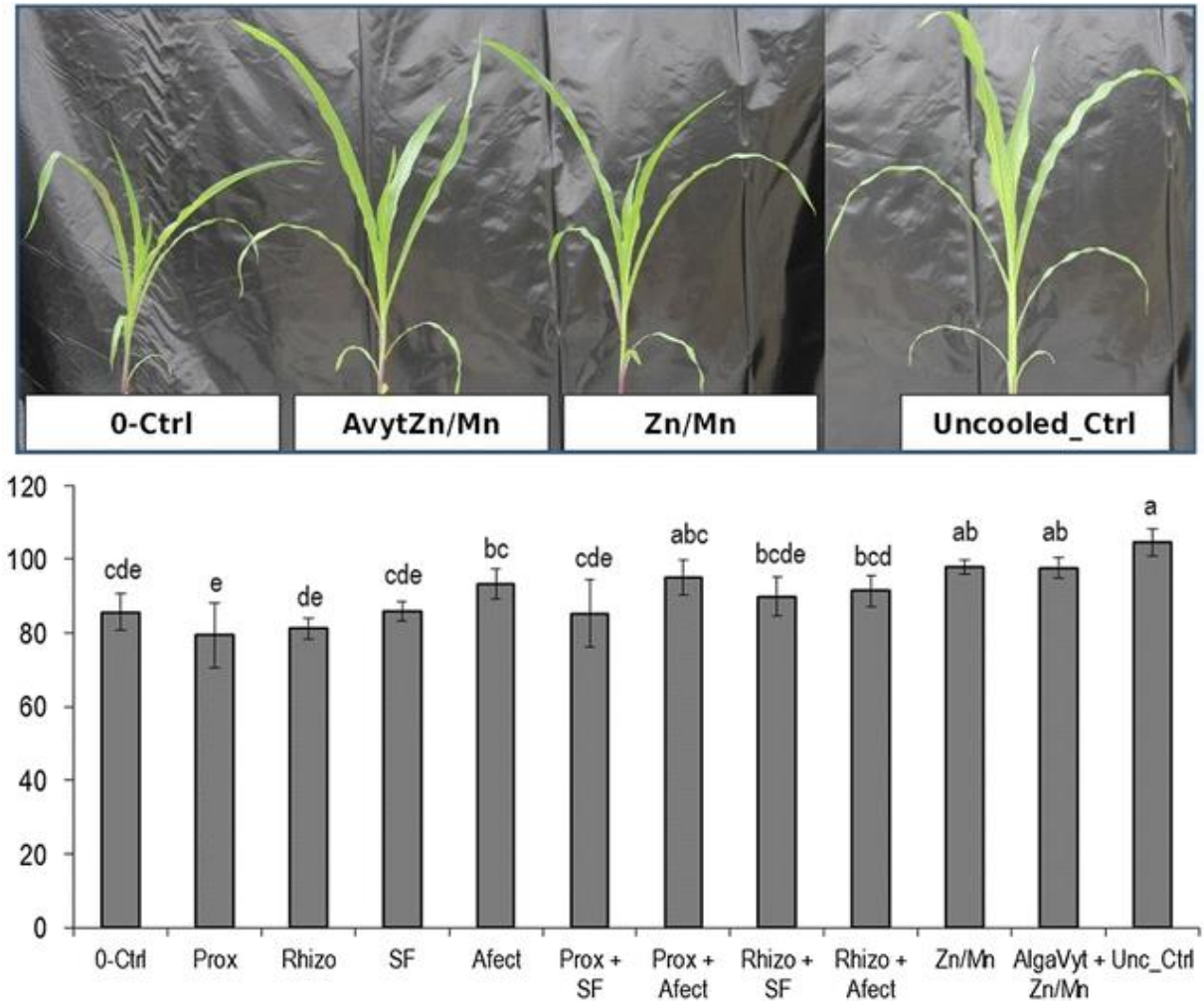
The study was carried out in a completely randomized design. Data are presented as mean ± SE. For statistical analysis of significant differences between treatment groups, a one-way ANOVA followed by a Tukey test ($p < 0.05$ significance level) were performed using the SAS/STAT software package of SAS® 9.3.

2.4 Results

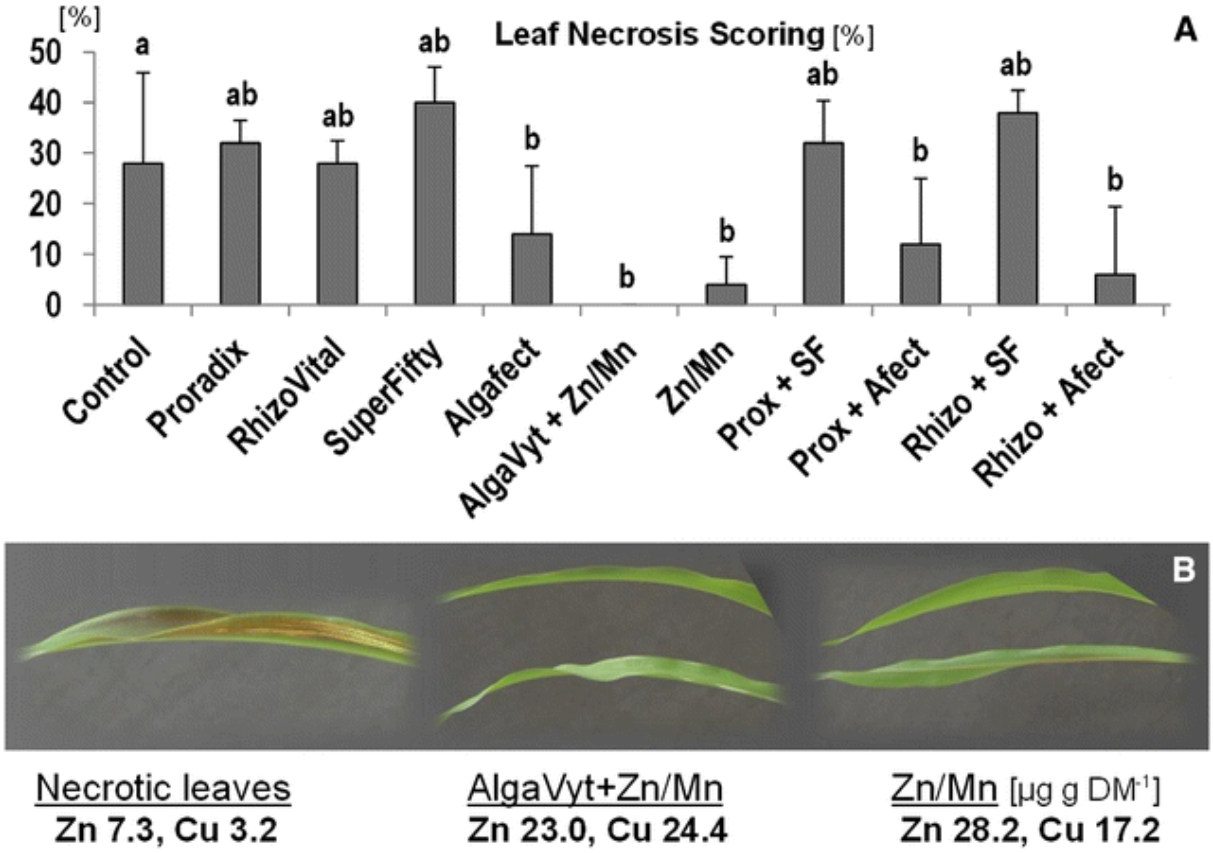
2.4.1 Experiment 1

Maize seedlings, germinated for 2 weeks on a silty-loam soil pH 6.9 (taken from a maize field site) at a root zone temperature (RZT) of 20–22 °C, followed by a two-week low RZT treatment with 12–14 °C and a recovery period of 10 days, exhibited significantly reduced shoot growth as compared with the unstressed control (Fig. 1). During the cold-stress period, plants developed leaf chlorosis, subsequently turning into necrotic spots, associated with anthocyanin formation, which finally affected 30–40 % of the total leaf area (Fig. 2).

A significant reduction of leaf damage was observed after Zn/Mn fertigation (5 % affected leaf area) and in the treatments with the seaweed extract combination products Algafect and Algavyt+Zn/Mn (*Ascophyllum nodosum*, *Fucus* spp., *Laminaria* spp.; 0–15 % affected leaf area) but not for the pure *Ascophyllum nodosum* extract Super Fifty (40 % affected leaf area, Fig. 2). Accordingly, shoot growth inhibition, induced by low RZT was reverted particularly in maize plants with Zn/Mn and Algavyt+Zn/Mn fertigation (Fig. 2). By contrast, the treatments with plant growth promoting rhizobacteria had no protective effects on formation of leaf necrosis (Fig. 2) and inhibition of shoot growth (Fig. 1) under low RZT. Combinations of the PGPRs with the seaweed extract Algafect resulted in a reduction of necrotic leaf area but the effect was not bigger than the treatment with the seaweed extract alone (Fig. 2).

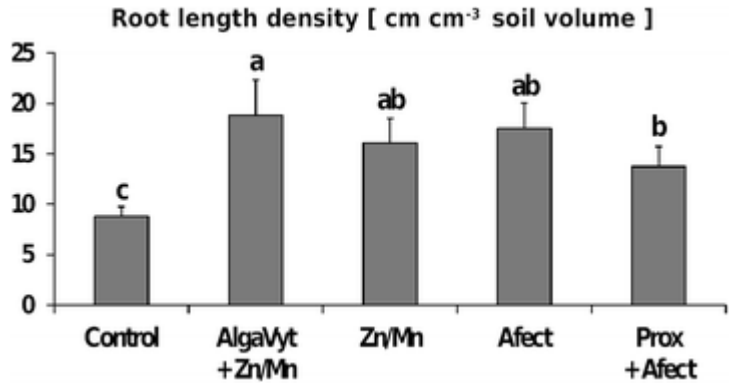


Chapter 2. Fig. 1. Shoot length of maize plants exposed to a root zone temperature of 12–14 °C for 2 weeks in a cooling system. 0-Ctrl untreated control, Prox Proradix, SF Super Fifty, Rhizo Bacillus amyloliquefaciens FZB42 + Bacillus simplex R41, Afect Algafect; Unc. Ctrl uncooled control at 20–22 °C. Data represent means and SE of five independent replicates for each treatment. Significant differences (Tukey test, $\alpha < 5$ %) are marked with different letters.



Chapter 2. Fig. 2. Leaf damage of maize plants exposed to a root zone temperature of 12–14 °C for 2 weeks in a cooling system. A Scoring of leaf necrosis (% affected leaf area). B Appearance and micronutrient status (Zn, Cu µg g^{-1} DM] of damaged ad corresponding undamaged leaves treated with cold-stress protectants. Data represent means and SE of five independent replicates for each treatment. Significant differences (Tukey test, $\alpha < 5$ %) are marked with different letters. Prox Proradix, SF Super Fifty, Rhizo Bacillus amyloliquefaciens FZB42 + Bacillus simplex R41, Afect Algafect.

The beneficial effects of fertigation with Zn/Mn, Algafect, and Algavyt+Zn/Mn on cold-stress-induced leaf necrosis and inhibition of shoot growth were associated with a doubling of root length density (Fig. 3), indicating strong effects on root elongation and fine root production with outstanding importance for nutrient acquisition [22]. A closer look on the nutritional status of the damaged leaf tissue in cold-stressed maize plants [23] revealed deficiencies of Zn (7.3 mg kg^{-1} DM, critical threshold 20 mg kg^{-1} DM) and Cu (3.2 mg kg^{-1} DM; critical threshold 5 mg kg^{-1} DM), not detectable in the corresponding leaves after fertigation with Zn/Mn and Algavyt+Zn/Mn (Fig. 3).



Chapter 2. Fig. 3. Root length density of maize plants exposed to a root zone temperature of 12–14 °C for two weeks in a cooling system. 0-Ctrl untreated control, Prox Proradix, Afect Algafect. Data represent means and SE of five independent replicates for each treatment. Significant differences (Tukey test, $\alpha < 5$ %) are marked with different letters.

The Mn concentrations were sufficient in all treatments (40–50 mg kg^{-1} DM), while the P status of 0.25–0.3 % was low but not critical (data not shown). Analysis of critical nutrients supplemented with the applied cold protectants revealed high levels of Zn and Mn as common micronutrients present in the seaweed extracts Algafect, Algavyt+Zn/Mn (6–70 mg kg^{-1} DM), and in the Zn/Mn fertigation solution, exerting beneficial effects on cold-stressed plants but not in the pure *Ascophyllum nodosum* extract Super Fifty and in the microbial inoculants (≤0.06 mg kg^{-1} DM), lacking protective activity (Table 1).

Chapter 2. Table. 1. Mineral nutrients [mg kg−1 DM] in bacterial inoculants and seaweed extracts used in the experiments.

Product	P	K	Ca	Mg	Zn	Fe	Cu	Mn
Proradix®	9.10	13.20	9.90	1.09	0.03	b.d.	b.d.	b.d.
RhizoVital®	1.42	0.56	1.03	0.44	b.d.	b.d.	b.d.	0.02
Bacillus simplex	3.63	0.71	1.78	0.87	0.03	b.d.	b.d.	0.06
Super fifty®	b.d.	70.00	0.36	2.48	b.d.	0.06	b.d.	b.d.
Algafect	0.23	47.10	2.33	1.02	20.80	0.09	b.d.	6.64
Algavyt+ZnMn	b.d.	4.91	0.15	0.31	71.90	b.d.	b.d.	59.60

b.d. below detection limit

2.4.2 Experiment 2

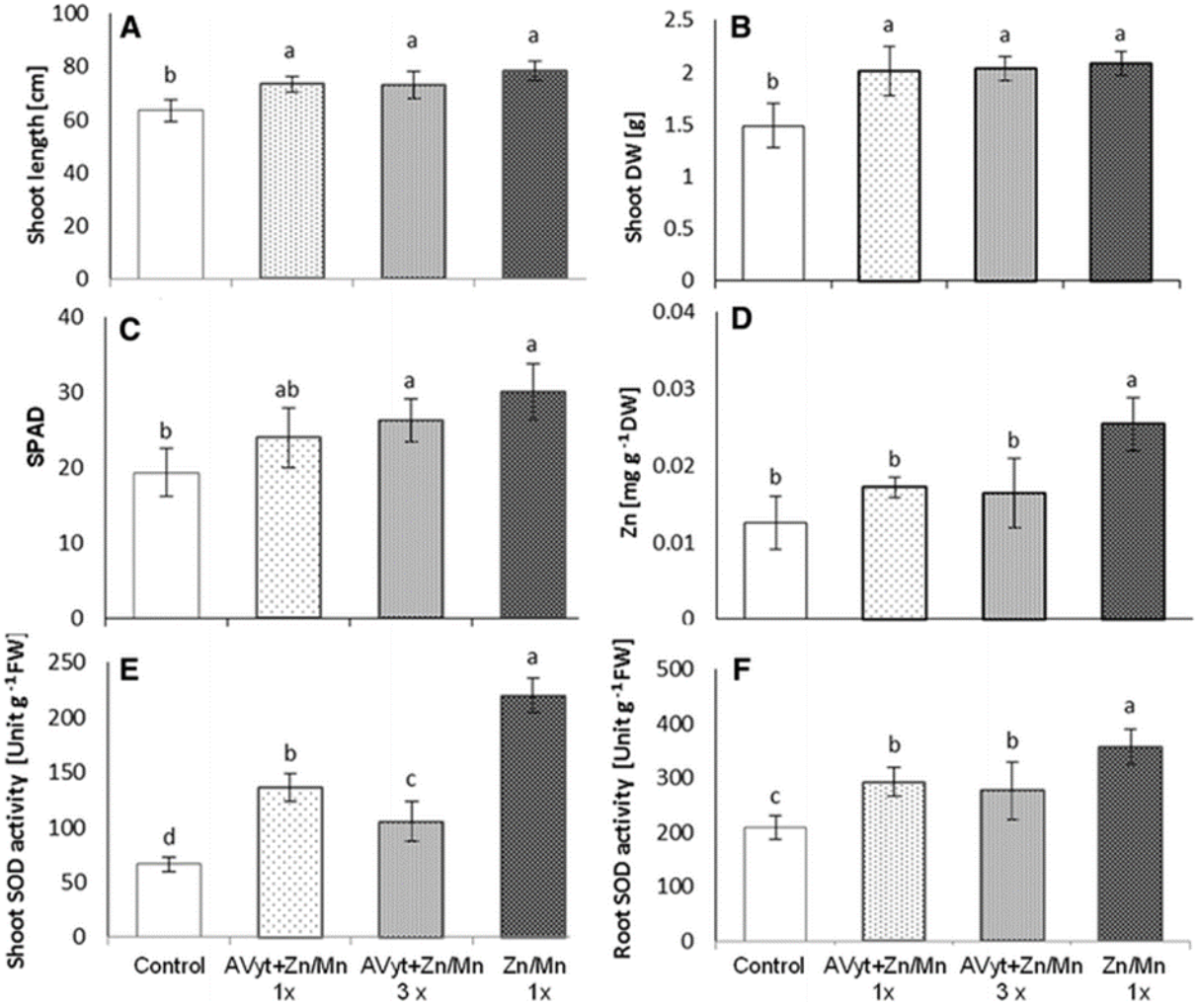
In face of the obvious indications for a role of micronutrients, such as Zn in cold-stress mitigation by the applied cold-protectants, a second experiment was conducted to investigate a putative link with SOD as key enzymes in oxidative stress protection, with Zn, Mn, Cu, and Fe as co-factors [17]. Moreover, in the first experiment, fertigation with cold-protectants was performed in weakly intervals during the first 3 weeks after sowing, difficult to perform under real practice conditions. Therefore, in the second experiment also the more realistic option of a single starter application of promising cold-protectants at the time of sowing was tested: Algavyt+Zn/Mn was supplied as single fertigation treatment directly after sowing, as compared with three weekly applications. Zn/Mn was supplied once as a starter application, using a commercial seed dressing formulation (Lebosol Dünger GmbH, Elmstein, Germany). At 6 weeks after sowing, shoot length and shoot biomass production of maize plants exposed to low RZT of 12–14° C was increased by approx. 20 and 30 %, respectively, in the treatments with cold-protectants without differences between single and triple applications of Algavyt+Zn/Mn (Fig. 5a, b). Cold-stress induced leaf chlorosis (SPAD values) declined in the order Zn/Mn < Algavyt+Zn/Mn (three applications) = Algavyt+Zn/Mn (one application) < untreated control (Fig. 4C).

Declining leaf chlorosis was associated with increasing Zn leaf concentrations (Fig. 4D) and increased activity of superoxide dismutase in the leaf and root tissue (Fig. 4D, E). Mineral nutrient analysis [23] revealed a low but not critical status for P (0.23–0.3 %) and Mg (0.17–0.19 %), and Ca and K concentrations in the sufficiency range, without significant treatment differences (Table 2). The micronutrient status of the untreated control was deficient for Zn (12 mg kg^{-1} DM) and Mn (24 mg kg^{-1} DM), increased after application of cold-stress protectants and reached the sufficiency range in the Zn/Mn seed-dressing treatment. The Fe and Cu status was low but not critical without significant differences within the treatments (Table 2). For all investigated nutrients, total shoot contents increased in response to the treatments with cold-stress protectants (Table 3), demonstrating an improved nutrient acquisition in general.

Chapter 2. Table 2. Shoot concentration of mineral nutrients in maize plants exposed during four weeks to a root zone temperature of 12–14 °C with and without Zn/Mn seed dressing and single or triple seaweed extract applications of Algavyt+Zn/Mn.

Treatment	Ca (mg g^{-1} DW)	Mg (mg g^{-1} DW)	K (mg g^{-1} DW)	P (mg g^{-1} DW)
Untreated control	4.46 ± 0.35^{a}	1.65 ± 0.17^{a}	3.42 ± 0.56^{a}	2.53 ± 0.32^{ab}
Algavyt single appl.	4.33 ± 0.34^{a}	1.70 ± 0.23^{a}	3.49 ± 0.90^{a}	2.30 ± 0.24^{b}
Algavyt triple appl.	4.68 ± 0.82^{a}	1.89 ± 0.30^{a}	4.44 ± 1.02^{a}	3.02 ± 0.50^{a}
Zn/Mn seed dressing	4.72 ± 0.55^{a}	1.67 ± 0.20^{a}	3.72 ± 0.60^{a}	2.36 ± 0.25^{ab}
Treatment	**Zn (mg g^{-1}DW)**	**Mn (mg g^{-1}DW)**	**Fe (mg g^{-1}DW)**	**Cu (mg g^{-1}DW)**
Untreated control	0.012 ± 0.003^{b}	0.024 ± 0.004^{b}	0.054 ± 0.017^{a}	0.005 ± 0.001^{a}
Algavyt single appl.	0.017 ± 0.001^{b}	0.028 ± 0.004^{b}	0.081 ± 0.023^{a}	0.005 ± 0.001^{a}
Algavyt triple appl.	0.016 ± 0.004^{ab}	0.027 ± 0.009^{b}	0.068 ± 0.012^{a}	0.006 ± 0.0007^{a}
Zn/Mn seed dressing	0.025 ± 0.003^{a}	0.048 ± 0.013^{a}	0.090 ± 0.024^{a}	0.006 ± 0.001^{a}

Data represent means and SE of five independent replicates for each treatment. Significant differences (Tukey test, $\alpha < 5$ %) are marked with different letters (a, b).



Chapter 2. Fig. 4. A: Shoot length, B: Shoot biomass (DM), C: SPAD values (Chlorophyll), D: Zinc shoot concentration, E: Superoxide dismutase (SOD) leaf activity, and F: SOD root activity of maize plants exposed to a root zone temperature of 12–14 °C for two weeks in a cooling system. 0-Ctrl untreated control, Prox Proradix, Afect Algafect. Data represent means and SE of five independent replicates for each treatment. Significant differences (Tukey test, $\alpha < 5$ %) are marked with different letters.

2.4.3 Experiment 3

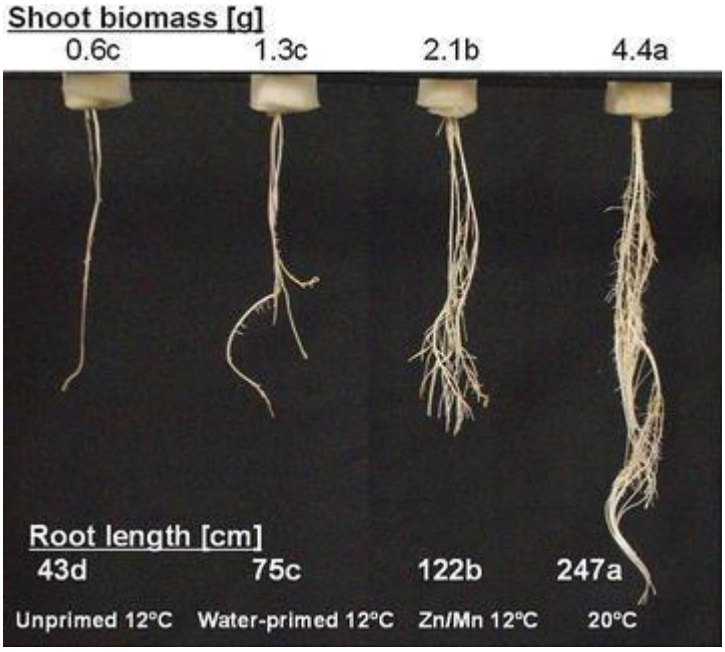
Generally, effects of single Algavyt+Zn/Mn treatments were not smaller than the effects of triple applications and were most pronounced in Zn/Mn seed-dressing treatments (Fig. 4; Tables 2, 3), demonstrating the outstanding importance of a starter application at the time of sowing. This was further confirmed in a nutrient solution experiment with seeds, pre-soaked in Zn/Mn nutrient solutions and water, respectively, subsequently germinated between filter paper and transferred for 5 weeks to a full-strength nutrient solution

containing all essential mineral nutrients. During 4 weeks of the culture period, the nutrient solutions were cooled to 12 °C. Analysis of plant growth revealed a cold-protective effect of Zn/Mn treatments only when the micronutrients were applied as seed treatments prior to the onset of the cold stress period. By contrast, even continuous supply of all essential nutrients in unlimited amounts via the nutrient solution during the cold-stress period directly to the roots of the maize seedlings, had no protective effects, as demonstrated by the control variant without Zn/Mn seed treatment (Fig. 5).

Chapter 2. Table 3. Shoot accumulation of mineral nutrients in maize plants exposed during four weeks to a root zone temperature of 12–14 °C with and without Zn/Mn seed dressing and single or triple seaweed extract applications of Algavyt+Zn/Mn.

Treatment	**Ca (mg plant^{-1})**	**Mg (mg plant^{-1})**	**K (mg plant^{-1})**	**P (mg plant $^{-1}$)**
Untreated control	6.66 ± 1.21^{b}	2.47 ± 0.51^{b}	5.06 ± 0.84^{b}	3.78 ± 0.83^{b}
Algavyt single appl.	8.73 ± 1.18^{ab}	3.40 ± 0.45^{ab}	6.92 ± 1.34^{ab}	4.61 ± 0.55^{ab}
Algavyt triple appl.	9.53 ± 1.66^{ab}	3.84 ± 0.0.60^{a}	9.00 ± 1.82^{a}	6.13 ± 0.73^{a}
Zn/Mn seed treatment	9.81 ± 0.89^{a}	3.50 ± 0.54^{a}	7.45 ± 1.90^{ab}	6.35 ± 1.05^{a}
Treatment	**Zn (mg plant^{-1})**	**Mn (mg plant^{-1})**	**Fe (mg plant^{-1})**	**Cu (mg plant^{-1})**
Untreated control	0.019 ± 0.008^{b}	0.037 ± 0.012^{c}	0.080 ± 0.021^{b}	0.007 ± 0.002^{b}
Algavyt single appl	0.035 ± 0.006^{ab}	0.056 ± 0.008^{b}	0.160 ± 0.034^{a}	0.009 ± 0.002^{ab}
Algavyt triple appl.	0.034 ± 0.010^{ab}	0.055 ± 0.0.022^{a}	0.140 ± 0.045^{ab}	0.012 ± 0.001^{a}
Zn/Mn seed treatment	0.053 ± 0.008^{a}	0.101 ± 0.032^{a}	0.190 ± 0.058^{a}	0.0012 ± 0.002^{a}

Data represent means and SE of five independent replicates for each treatment. Significant differences (Tukey test, $\alpha < 5$ %) are marked with different letters (a, b, c).



Chapter 2. Fig. 5. Root development and shoot biomass of maize plants grown in hydroponics and exposed to a root zone temperature of 12 °C during four weeks in a cooling system. Effects of seed priming with water and Zn/Mn [14] as compared to an untreated control grown under ambient temperature at 20 °C. Data represent means of four independent replicates for each treatment. Significant differences (Tukey test, $\alpha < 5$ %) are marked with different letters.

2.5 Discussion

This study was designed to compare the efficiency of different mitigation strategies against low root zone temperatures during early growth as a major constraint for maize cultivation in temperate climates [1–4], including supplementation of critical micronutrients (Zn, Mn) [14], application of seaweed extracts [15], and inoculation with plant growth-promoting and cold- tolerant bacteria [16]. Surprisingly, despite a proven plant growth-promoting potential [24, 25] even in combination with the tested maize cultivar [26, 27], the investigated microbial inoculants, including a psychro-tolerant strain of *Bacillus simplex*, failed to show any beneficial effects on growth of maize seedlings exposed to moderately low RZT of 12–14 °C. It remains to be established whether other cold-resistant microbial inoculants, directly isolated from the maize rhizosphere, such as *Acinetobacter rhizosphaerae* BIHB727, *Pseudomonas putida* B0, or *Mycoplana bullata* MpB46 [16],

could be more effective in this context. In accordance with the observations of Imran et al. [14], supplementation of critical micronutrients, particularly Zn, was effective in mitigating growth depressions (Fig. 1) and oxidative leaf damage in maize seedlings exposed to low RZT (Fig. 2). Oxidative stress may represent the physiological link between micronutrient supply and chilling tolerance, and has been characterized as an important stress factor in cold-stressed plants [28]. Reduced shoot and root growth of maize plants exposed to low temperatures has been attributed to severe oxidative damage induced by cold stress [13, 29]. The different isoforms of SO as key enzyme for detoxification of free radicals, strongly depend on Zn, Mn, Cu, and Fe as cofactors and have been implicated in chilling tolerance of higher plants [29]. Accordingly, our study revealed increased in vitro activity of SOD in leaves by supplementation of mineral Zn/Mn fertilizers (Fig. 4). In addition to the mitigation of oxidative leaf damage and shoot growth inhibition, as one of the most striking effects, fertigation with Zn/Mn solutions or Zn/Mn-rich seaweed extracts (Algafect, Algavyt+Zn/Mn) doubled the root length density of cold-stressed maize plants (Fig. 4), similarly reported also by Imran et al. [14]. Apart from lipid peroxidation, an increased oxidative degradation of auxins as a consequence of Cu/ZnSOD limitation has been discussed as a mechanism for reduction of plant growth in response to Zn deficiency [6, 30]. Accordingly, external Zn/Mn supply to cold-stressed plants inducing an increased SOD activity (Fig. 4), may exert a protective effect against oxidative auxin degradation, thereby maintaining auxin levels sufficiently high to trigger root and shoot elongation even at low RZT. Recent studies also suggest that particularly auxin transport rather than auxin synthesis is affected by cold stress [7]. Stimulation of root growth may explain the increased accumulation also of other mineral nutrients in the shoot tissue of the Zn/Mn-treated maize plants (Table 3) as a consequence of improved spatial nutrient acquisition.

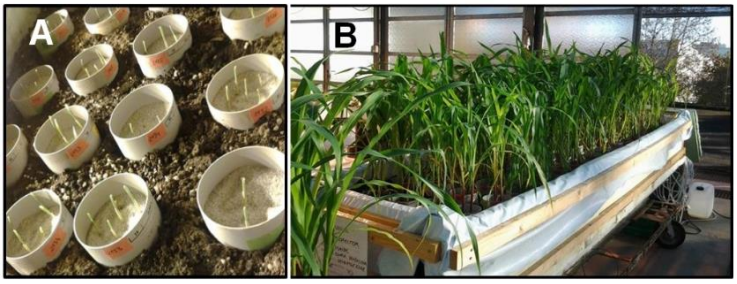
However, apart from auxins, cold stress affects hormonal balances via positive and negative interactions with a wide range of phytohormones and signal compounds, including cytokinins, gibberellins, jasmonic acid, ABA, salicylic acid, and there is no information on putative effects of micronutrient availability on these processes [7]. Surprisingly, improved Zn/Mn supply seems to be also the mechanism behind cold-stress mitigation mediated by application of seaweed extracts, since only seaweed extracts rich in Zn/Mn (Algafect 20.8/6.6 mg kg^{-1} DM; Algavyt+Zn/Mn 71.9/59.6 mg kg^{-1} DM) exerted protective effects against low RZT, while the highly concentrated and purified *Ascophyllum nodosum* extract "Super Fifty" without detectable Zn/Mn contents was completely ineffective (Fig. 2). This result was unexpected since in the available literature, protective effects of seaweed extracts against abiotic stresses have been mainly related with the organic fraction [15, 32]. However, in many cases these applications refer to foliar treatments [15] and to extracts obtained with lipophilic solvents supplied in agar media [31]. It is not clear whether the same compounds are active in the fertigation treatments applied in the present study, since adsorption and microbial degradation processes may occur in soils, as a source of interference not present in case of foliar applications. More detailed chemical fractionation experiments are required for further testing the hypothesis that micronutrients and particularly Zn are the major active ingredients of seaweed extracts used for soil applications as antioxidative cold-stress protectants. Another interesting result was the finding that the application timing of the cold-stress protectants was obviously more important than the number of applications, and single starter applications of Zn/Mn or Zn/Mn-rich seaweed extracts were equally effective than weekly applications during the first 21 DAS (Fig. 4). The nutrient solution experiment even demonstrated that Zn/Mn treatments could only exert a protective effect against cold stress when the micronutrients

were present during seed imbibition prior to the onset of the cold stress period. This effect could not be replaced by later applications of micronutrients to the germinated seedling roots, even with unlimited supply of all essential nutrients in a full nutrient solution (Fig. 5). Since the micronutrients are taken up by the seeds during imbibition [14], this may indicate that only the micronutrient fraction already present in the plant tissue can exert a protective function after onset of the cold-stress period, while root uptake from the external medium is largely inhibited under these conditions. This is in line with observations of Engels et al. [32, 33], demonstrating that Zn and Mn shoot accumulation in maize exposed to low RZT was particularly dependent on cold-stress effects affecting root activity, while the uptake of other nutrients was more strongly determined by the shoot demand. The efficiency of starter treatments with micronutrients or seaweed extracts could also be a big advantage from the practical point of view, since it facilitates integration into common sowing techniques, such as seed dressings or underfoot placement of fertilizers, provided that suitable formulations are available. By contrast, repeated applications would largely increase the application costs due to a higher dosage and additional workload. Since micronutrients are effective already at low application doses, starter applications of Zn/Mn or the respective seaweed extracts may offer a highly economic option for cold-stress prophylaxis in crops. Assuming a field planting density of six maize plants m^{-2} (60.000 plants ha^{-1}), one single application of Zn/Mn-rich seaweed extract would translate into a dose of approx.1 kg ha^{-1}. In case of Zn/Mn sulfate fertigation, already 30 g ha^{-1} would be sufficient. Recommended dosages for underfoot placement are in the range of 300 g ha^{-1}. In case of micronutrient seed priming [14], the dose would be even lower (2–3 g ha^{-1}) and translated into a maize grain yield increase of approx. 10 % in two independent field experiments [14].

2.6 Outlook and concluding remarks

The beneficial effects of starter treatments with micronutrients (particularly Zn) or seaweed extracts acting via the antioxidative stress defense system on cold-stress tolerance of the target plants are obvious, but the detailed mode of action is still not fully understood. Also, field performance requires further confirmation on different soils. The various successful application modes and formulations, such as seed priming, seed dressing, fertigation, supplementation in form of pure mineral nutrients or as seaweed extracts may offer high flexibility for different sowing techniques, underfoot fertilization, organic vs conventional farming etc., but still requires optimization of the application dosage. Apart from improved cold-stress tolerance, both, micronutrient fertilization and seaweed extracts have documented effects on plant growth promotion and tolerance against other biotic and abiotic stress factors [16, 17, 34]. Therefore, additional beneficial effects may be expected under stress conditions not addressed in the present study.

2.7 Additional file



Chapter 2. Suppl. Fig. 1. Cooling system for adjustment of low root zone temperature based on moist peat culture substrate with cooling tubes. A) Pots with germinating maize seedlings inserted into the cooling system. B) Overview at final harvest.

2.8 References

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Chapter 3. Silicon improves chilling tolerance during early growth of maize by effects on micronutrient homeostasis and hormonal balances

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Authors' contributions

NM, GN, and MW conceived and designed the experiments. NM conducted the experiments, collected the data and wrote the manuscript with GN and UL. FW, BH, and NM developed and performed the UHPLC–MS analysis of phytohormones. All authors approved the final manuscript.

3.1 Abstract

Low soil temperature in spring is a major constraint for the cultivation of tropical and subtropical crops in temperate climates, associated with inhibition of root growth and activity, affecting early growth and frequently plant performance and final yield. This study was initiated to investigate the physiological base of cold-protective effects induced by supplementation with silicon (Si), widely recommended as a stress-protective mineral nutrient. Maize was used as a cold-sensitive model plant, exposed to chilling stress and low root-zone temperature (RZT) during early growth in a lab to field approach. In a pot experiment, 2–weeks exposure of maize seedlings to low RZT of 12–14◦C, induced leaf chlorosis and necrosis, inhibition of shoot and root growth and micronutrient limitation (particularly Zn and Mn). These phenotypes were mitigated by seed treatments with the respective micronutrients, but surprisingly, also by Si application. Both, silicon and micronutrient treatments were associated with increased activity of superoxide dismutase in shoot and roots (as a key enzyme for detoxification of reactive oxygen species, depending on Zn and Mn as cofactors), increased tissue concentrations of phenolics, proline, and antioxidants, but reduced levels of H_2O_2. These findings suggest that mitigation of oxidative stress is a major effect of Zn, Mn, and Si applied as cold stress protectants. In a soil–free culture system without external nutrient supply, Si significantly reduced large leaching losses of Zn and Mn from germinating seeds exposed to low-temperature stress. Silicon also increased the translocation of micronutrient seed reserves to the growing seedling, especially the Zn shoot translocation. In later stages of seedling development (10 days after sowing), cold stress reduced the root and shoot contents of important hormonal growth regulators (indole acetic acid, gibberellic acid, zeatin). Silicon restored the hormonal balances to a level comparable with non-stressed plants and

stimulated the production of hormones involved in stress adaptation (abscisic, salicylic, and jasmonic acids). Beneficial effects of Si seed treatments on seedling establishment and the nutritional status of Zn and Mn were also measured for a field-grown silage maize, exposed to chilling stress by early sowing. This translated into increased final biomass yield.

Keywords: silicon, micronutrients, germination, chilling stress, maize, oxidative stress

3.2 INTRODUCTION

In the context of global warming, there is an increasing trend for the cultivation of crops with tropical and subtropical origins, such as maize, soybean, Miscanthus or Sorghum also in temperate climates, e.g., in Central Europe. Under these conditions, early sowing is required for efficient use of the comparatively shorter vegetation periods and to escape from detrimental effects of summer drought (Hund et al., 2004). However, low temperature and cold and wet soils during early spring represent major constraints for the cultivation of tropical crops bearing the risk of poor germination, impaired seedling establishment, and reduced nutrient acquisition due to limited root growth and activity. This is frequently associated with poor vegetative plant development, reduced stress resistance and finally reduced crop yield (Duncan and Hesketh, 1968; Muldoon et al., 1984; Imran et al., 2013), although under favorable conditions short cold periods can be tolerated and later compensated until final harvest (Saeidnejad et al., 2012). In plant species, such as maize, with optimum temperatures of 25–30°C for germination and plant growth, even moderately low soil temperatures <15°C are already detrimental (Cutforth et al., 1986; Kasper and Bland, 1992). Due to root growth limitation and slow diffusion rates, plant availability, and acquisition of sparingly soluble nutrients, such as P, NH_4^+, K, Fe, Zn, Mn, and Cu, is particularly affected by low soil temperatures (Duncan and Hesketh, 1968; Kramer and

Boyer, 1995; Wan et al., 2001). Therefore, placement of these nutrients close to the seedling roots or as seed treatments are among the most widespread practical measures to counteract detrimental effects of low soil temperatures on seedling establishment and early growth of maize (Imran et al., 2013; Bradáˇcová et al., 2016; Nkebiwe et al., 2016). Meanwhile, starter applications of ammonium phosphates by shallow subsurface placement below the seeds (Nkebiwe et al., 2016) belong to the standard fertilization strategies employed for maize production systems in temperate climates. Micronutrients with stress-protective functions, such as Zn and Mn, are frequently applied as foliar sprays. However, this is not possible in the seedling stage and usually, the formulations are more expensive than soil fertilizers. More cost-effective placement strategies including seed dressings are increasingly employed to promote stress resistance, early growth, and crop establishment (Farooq et al., 2012; Bradáˇcová et al., 2016). Similar stress-protective effects have been recorded also for amendments with Si (Liang et al., 2015).

When plants are exposed to environmental stress factors, such as chilling, drought or salinity, an imbalance between production and detoxification of reactive oxygen species (ROS) promotes accumulation of ROS, which induces oxidative damage to cellular components (Gong et al., 2005). Many studies have highlighted a role of Si in the suppression of oxidative damage in various plant species under a wide range of stress conditions. Protective effects have been reported against drought stress in wheat (Gong et al., 2005, 2008; Pei et al., 2010), salinity and boron toxicity in tomato, spinach (Al-aghabary et al., 2005; Gunes et al., 2007), cotton (Gossett et al., 1994), and barley (Liang et al., 2003; Inal et al., 2009), or low temperature stress in wheat (Liang et al., 2008) and cucumber (Liu et al., 2009). Mitigation of oxidative stress by Si treatments has been related to increased expression of enzymatic ROS detoxification systems, such as superoxide

dismutases, catalases, peroxidases, ascorbate peroxidase, and increased accumulation of antioxidants (phenolics, proline, ascorbic acid), similar to the effects mediated by stress-protective micronutrients, such as Zn, Mn, Fe, and Cu (Cakmak, 2000; Datnoff et al., 2007). However, the links between Si application and induction of the protective mechanisms against oxidative stress are still largely unknown. Therefore, this study was designed to investigate more in detail the physiological background of Si effects on cold stress mitigation during early growth of maize. We hypothesized that the protective role of silicon is related to the homeostasis of cold stress-protective micronutrients, such as Zn and Mn (Imran et al., 2013; Bradáˇcová et al., 2016). In a comparative investigation, Zn, Mn, and Si were applied as seed treatments or by starter fertigation to maize plants, subsequently exposed to low temperatures of 12–14°C on a silty loam soil taken from a field site of maize cultivation and also in a soil free culture system. Plant growth, symptoms of oxidative leaf damage and the mineral nutritional status in different plant organs were documented in relation with the expression of various physiological stress indicators (production of H_2O_2, activities of superoxide dismutase and peroxidase, accumulation of antioxidants, phenolics, and proline) and with changes in hormonal balances. Finally, a preliminary field experiment was conducted on the same soil, where low-temperature stress was provoked due to early sowing at the mid of April. This allowed evaluating the expression of micronutrient and Si effects and their impact on final yield under practical conditions.

3.3 MATERIALS AND METHODS

3.3.1 Plant Cultivation

3.3.1.1 Soil Culture Experiment

Zea mays L cv. Colisee was used as test plant. Soil material (silty loam, pH 6.9) was derived from the Ap horizon of a maize cultivation field site at the Hohenheim University

experimental station Ihinger Hof, Renningen, Germany (Supplementary Table 1). After sieving with 2mm mesh size, fertilization was performed with Ca $(NO_3)_2$, 100mg N kg^{-1} DM; Ca $(H_2PO_4)_2$, 80mg P kg^{-1} DM; K_2SO_4, 150mg K kg^{-1}DM and $MgSO_4$, 50mg Mg kg^{-1} DM. For improvement of the soil structure, the fertilized soil was mixed with quartz sand (ratio 2:1). Plastic pots were filled with 1,800ml of this soil substrate and inserted into a cooling system, designed to control the root zone temperature of plants. An immersion water bath circulator (Thermomix 1480/Frigomix 1497, Braun, Melsungen, Germany) was connected to the cooling system, equipped with a closed pipe system which was installed into moist peat substrate to circulate the refrigerating fluid through the moist peat layer surrounding the culture vessels (Bradáˇcová et al., 2016). The plants were regularly watered to 70% of substrate water holding capacity (WHC) with deionized water and cultivated for 2 weeks at a root zone temperature of 20–22◦C, 2 weeks at low root zone temperature (12–14◦C), followed by a 2 weeks' recovery phase at 20–22◦C.

Silicon was supplied as silicic acid (H_4SiO_4) prepared by passing K_2SiO_3 through a column filled with a cation–exchange resin (Amberlite IR−120, H^+ form, Sigma Aldrich, Germany) according to Maksimovic et al. (2007) and Pavlovic et al. (2013). Silicon fertigation was performed at a dosage of 40mg H_4SiO_4 kg^{-1} soil DM applied with a pipette close to the plant, directly on top of the soil substrate in four weekly intervals, starting at the sowing date. Seed dressing with Zn and Mn was performed with commercial formulations: Lebosol® Mn^{500} SC and Lebosol® Zn^{700} SC (Lebosol® Dünger GmbH, Ermstein, Germany) according to the manufacturer instructions. Lebosol® Zn^{700} SC: 2 ml 4,000 $seeds^{-1}$, Lebosol® Mn^{500} SC: 4ml 4,000 $seeds^{-1}$.

3.3.1.2 Soil–Free Culture Experiment

Germination of maize seeds (Z. mays cv. Colisee), surface–sterilized by 1min soaking in ethanol (99% v/v), was performed in pre-sterilized Petri dishes (9 cm diameter) on moist filter paper (10 seeds per petri dish). The seeds were soaked with 3mL deionized water (–Si control) or 3mL freshly prepared H_4SiO_4 [1.0mM Si] (Maksimovic et al., 2007; Pavlovic et al., 2013), respectively with four replicates per treatment. The optimum level of Si seed application was determined in a pilot experiment with different levels of Si supply (0–3.0mM Si, Supplementary Figure 1). Covered Petri dishes were placed into a laboratory incubator (AtmoCONTROL, ICP, 750 Memmert GmbH, Schwabach, Germany) for 3 days in the dark at 18◦C in thin unsealed plastic bags to minimize evaporation. For further seedling development, the germinated seeds were placed on the upper edge of rolled filter papers (10∗60 cm; 2 seeds per roll), moistened with 100ml distilled water or H_4SiO_4 solution [1mM Si] at 3 and 5 days after sowing (DAS). The filter rolls were transferred into the laboratory incubator and incubated for 7 days at 12°C (16/8 h light/dark period and relative humidity of 60%, with a light intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ or at 18◦C outside the incubator.

3.3.1.3 Field Experiment

In 2016, a field experiment was established for silo maize production (*Z. mays* cv. Rolandinio) at the experimental station “Ihinger Hof” University of Hohenheim, Renningen, Germany (soil properties are specified in Supplementary Table 1). To increase the probability of low temperature stress in spring, sowing was conducted on April 22nd. Accordingly, a mean air temperature of 13.6◦C during April and May was recorded associated with heavy rainfall (469mm) leading to cold and wet soil conditions during germination and emergence. Fertilization was conducted prior to sowing by broadcast

application with subsequent soil incorporation of stabilized ammonium sulfate NovaTec R solub 21: 161 kg N ha^{-1} (Compo Expert, Münster, Germany) and by underfoot placement of di-ammonium phosphate (29 kg N, 32 kg P ha^{-1}). Seed treatments were performed as seed dressings with Zn and Mn (Lebosol® Mn^{500} SC and Lebosol® Zn^{700} SC (Lebosol R Dünger GmbH, Ermstein, Germany) as described in experiment (1), and as seed priming with 1mM potassium silicate (KSi, PottaSol, BioFa, Münsingen Germany) with a 24 h seed soaking period and re-drying during 24 h at 28 ± 2°C. A seed water priming treatment without Si was included as a control. Sowing was performed on April 22nd with a sowing density of 9 seeds m^{-2}, a row distance of 75 cm and a sowing depth of 6–7 cm. Foliar Si application (Vitanica R Si, Compo, Münster, Germany 16 L ha^{-1} + 100ml ha^{-1} Greemax R Stallen Bio Agro AG, Basel, Switzerland) was conducted with a backpack sprayer (Solo R , Sindelfingen–Maichingen, Germany) at 69, and 75 DAS. Due to the extremely cold and wet soil conditions by the end of April, seedling emergence was severely biased, particularly in the untreated control variants. Therefore, after recording of emergence rates at 41 days after sowing (DAS), re–sowing was performed in the heavily affected plots to maintain a comparable level of inter-plant competition for light, water, and nutrients within the rows during the rest of the culture period. Final harvest was conducted at 214 DAS.

3.3.2 Plant Analysis

Visual scorings of leaf chlorosis, necrosis, and anthocyanin formation and determination of shoot height was performed for all experiments. Reflectometric leaf chlorophyll measurements were performed with a SPAD meter (Konica Minolta INC, Osaka, Japan). Estimates of damaged leaf area were obtained by the equation, leaf area (cm^2) = x/y, where x is the weight (g) of the area covered by leaf drawings on a transparent millimeter graph paper, and y is the weight of 1 cm^2 of the same graph paper (Pandey and Singh, 2011).

Based on these data, the percentage of the necrotic area was calculated. After final harvest, root and shoot dry matter was determined after 60°C oven-drying. Root length measurements were performed by digital image analysis using the WinRHIZO root analysis software package (Regent Instruments Inc., Quebec, Canada).

3.3.3 Analysis of Mineral Nutrients

One hundred milligrams of dried, milled shoot material were ashed for 5 h in a muffle furnace at 500◦C. After cooling, the samples were digested twice with 1mL of 3.4M HNO_3 and evaporated until dryness to precipitate SiO_2. The ash was dissolved in 1mL of 4M HCl, subsequently diluted 10 times with hot deionized water, and boiled for 2 min to convert meta-, and pyro-phosphates to orthophosphate. After addition of 0.1mL Cs/La buffer to 4.9mL ash solution, Mg, Fe, Mn, and Zn concentrations were measured by atomic absorption spectrometry (ATI Unicam Solaar 939, Thermo Electron, Waltham, USA). Spectrophotometrical determination (Hitachi U−3300 spectrophotometer, Hitachi LtD. Corporation Japan) of orthophosphate was conducted after addition of molybdate-vanadate color reagent according to the method of Gericke and Kurmis (1952). K and Ca were measured by flame emission photometry (ELEX 6361, Eppendorf, Hamburg, Germany). Silicon was analyzed by ICP–OES (Vista–PRO, Varian Inc., Palo Alto, USA). For the digestion, 0.250 g of sample DM was suspended in 1mL of H_2O and 2.5mL of conc. HNO_3. The digestion is carried out by means of microwave–heated pressure digestion with HNO_3 and HF at 220°C in a digestion system Ultra clave II (MLS GmbH, Leutkirch, Germany). The digestion took place over 20min, the entire digestion program with heating and cooling phases comprised 2 h. After digestion, 0.5ml HF solution (1% v/v) was added to dissolve sparingly soluble silicates. The solutions were adjusted to 10mL with distilled H_2O (VDLUFA Method book VII, 2011) and used for ICP–OES analysis.

3.3.4 Superoxide Dismutase Assay

The superoxide dismutase (SOD, EC 1.15.1.1) assay was optimized for root and shoot tissues of maize according to the method described by Beauchamp and Fridovich (1971) and modifications suggested by Giannopolitis and Ries (1977) and Hajiboland and Hasani (2007). One hundred milligrams of fresh plant material, frozen in liquid nitrogen and stored at −80◦C, were ground with a pre-cooled mortar and pestle, and homogenized in 1.5ml extraction buffer containing 25mM HEPES pH 7.8 and 0.1mM EDTA. After centrifugation at 10,000 × g (4◦C for 10min), aliquots of supernatant were transferred into 2ml reaction tubes and kept on ice. For preparation of the reaction mixture, 1ml cuvettes covered with aluminum foil for light protection, were filled with 300 µl 62.5mM HEPES, 75 µl 1.0mM EDTA, 75 µl 120mM Na_2CO_3, 75 µl 120mM Lmethionine, 150 µl 750µM nitro-blue tetrazolium (NBT), and 100 µl of plant extract. Finally, 225 µl of 10µM riboflavin was added. The light reaction was started by removing the aluminum foil, exposing the samples to a light source (8000 Lux) for 25min. During the light phase, NBT is reduced to a dark blue formazan, measured spectrophotometrically (Spectrophotometer U−3300, Hitachi, Tokyo, Japan) at a wavelength of 650 nm. The final SOD activity, which inhibits the NBT reduction, was calculated as the difference between the absorbance of the sample and a control without plant extract, divided by 50% absorbance of the control. The specific SOD activity was expressed as SOD units per mg total protein. Total, protein content was determined according to Bradford (1976).

3.3.5 Peroxidase Assay

Peroxidase (POD, EC1.11.1.7) activity was determined using the guaiacol test (Chance and Maehly, 1955; Hajiboland and Hasani, 2007). The tetra-guaiacol formed during the reaction is measured photometrically at 470 nm. The enzyme was extracted from fresh leaf

material (100mg) by 10mM phosphate buffer (pH 7.0) and centrifuged 1,000 g for 10min. The test mixture (1mL) contained 10mM phosphate buffer (300 µL, pH 7.0), 5mM H_2O_2 (300 µL), and 4mM guaiacol (300 µL). The reaction was started by addition of the enzyme extract (100 µL) at 25°C. The formation of tetraguaiacol was recorded over a reaction period of 5min and the specific enzyme activity was expressed in µmoles tetraguiacol formation mg^{-1} total protein.

3.3.6 Determination of H_2O_2

Hydrogen peroxide levels were determined as described by Harinasut et al. (2003). Leaf tissues (100mg fresh weight) were homogenized in an ice bath with 5ml 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12,000 g for 15min. 0.5ml of the supernatant was added to 0.5ml of 10mM potassium phosphate buffer (pH 7.0) and 1ml of 1M KI. The absorbance of the supernatant was recorded at 390 nm. The concentration of hydrogen peroxide was determined using a standard curve ranging from 0 to 120µM of H_2O_2.

3.3.7 Determination of Total Soluble Sugars, Phenolics, and Proline

For determination of soluble sugars, leaf and root samples were homogenized in 100mMphosphate buffer (pH 7.5) at 4°C. After centrifugation at 12,000 g for 15min, the supernatant was used for determination of total soluble sugars (Yemm and Willis, 1954). An aliquot of the supernatant was mixed with anthronesulfuric acid reagent (Yemm and Willis, 1954) and incubated for 10min at 100◦C. After cooling, the absorbance was recorded at 625 nm. A calibration curve was created using glucose as external standard (Merck, Darmstadt, Germany). Total phenolics concentration was determined spectrophotometrically at 750 nm, using the Folin method (Hajiboland et al., 2017). For determination of proline, samples were homogenized with 3 % (v/v) sulfosalicylic acid and

the homogenate was centrifuged at 3,000 g for 20min. The supernatant was treated with acetic acid and acid ninhydrin, boiled for 1 h, and then the absorbance was determined at 520 nm. Proline (Sigma-Aldrich, Munich, Germany) was used for the production of a standard curve (Bates et al., 1973).

3.3.8 Determination of Total Soluble Anthocyanins and Flavonoids

Determination of anthocyanins was conducted spectrophotometrically at 510 nm according to Plessi et al. (2007). One hundred milligrams of fresh shoot material were extracted with 2ml methanol/HCl conc. (98:2 v/v). After centrifugation at 12,000 × g for 10min, each 0.5mL of the supernatant was used for spectrophotometric determination by using a pH differential method at pH 1 and pH 4.5 adjusted with 4.5ml of MES buffer After 5 h incubation at 4°C the absorbance was read at 510 nm from each group. The results were calculated as cyanidine-3-glycoside equivalents, using the formula $1A \times MW \times DF \times V \times 100/\varepsilon \times Wt$, where 1A is Abs (pH 1) – Abs (pH 4.5), MW = molecular weight of cyanidine-3-glycoside (484.83 g mol^{-1}), DF = dilution factor, V = final volume of the supernatant (0.5ml), ε = molar absorbance factor of cyanidine-3-glycoside (26,900Mm^{-1} cm^{-1}), L = diameter of the light path [cm], and Wt = sample fresh weight (0.1 g). Total leaf flavonoids were determined in methanolic extracts according to Hajiboland et al. (2014). One hundred milligrams of fresh leaf material were extracted in AlCl3-methanol (2%, w/v) and after centrifugation at 12,000 × g for 10min, the supernatant was used for determination at 415 nm with quercetin (Sigma, Munich, Germany) as an external standard.

3.3.9 Determination of Total Antioxidants

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH •) has been used to evaluate the free radical scavenging activity of antioxidants (Panico et al., 2009). The DPPH solution was

prepared by adding 2.37mg DPPH (Sigma-Aldrich, Munich, Germany) in 2ml 99% ethanol. One hundred milligrams of fresh leaf samples were grinded in 1ml of extraction solution (1:1 Ethanol:Water). After centrifugation at 12,000 g for 10min at 4◦C, 50 µl supernatant was used in a reaction cell which contained 50 µl freshly prepared 3mM DPPH solution and 900 µl ethanol (99%). After incubation for 10min in a dark room at 25◦C, the absorbance was determined at 515 nm. A reference solution contained 50 µl DPPH solution and 950 µl of ethanol (99%). The decline in absorbance at 515 nm was recorded for each sample and the quenching percentage of the DPPH radical was calculated based on the observed decrease in absorbance using the formula:

% Inhibition = $[(A_0–A_1)/A_0] \times 100$, where A_0 is the absorbance value of the DPPH blank solution and A_1 is the absorbance value of the sample solution.

3.3.10 Determination of Phytohormones by UHPLC-MS Analysis

Frozen maize tissue samples (shoot, roots) of 1 g of were ground to a fine powder with liquid nitrogen and extracted twice with 2.5ml of 80% methanol in falcon tubes. Thereafter, the samples were further homogenized by ultrasonication (Micra D-9 homogenizer, Art, Müllheim Germany) for 1min and 15 s at 10,000 rpm. Two milliliters of the methanol extracts were transferred to microtubes and centrifuged at 5,645 × g for 5min. Thereafter, 350 µl of the supernatant was mixed with 700 µl ultra-pure water and centrifuged at 5,645 × g for 5min. The supernatant was cleaned bymembrane filtration (ChromafilR O-20/15MS) and transferred to HPLC vials. UHPLC-MS analysis was carried out on a Velos LTQSystem (Thermo Fisher Scientific, Waltham, Massachusetts, USA) fitted with a Synergi Polar column, 4µ, 150 × 3.0mm, (Phenomenex, Torrance, California, USA). The injection volume was 3 µL and the flow rate was adjusted to 0.5ml min^{-1} for gradient elution with mobile phase (A): water and 5% acetonitrile; mobile phase (B): acetonitrile

and a gradient profile of: 0–1min, 95% A, 5% B, 11–13min, 10% A, 90% B, 13.1min, 95% A, 5% B, 16min 95% A, 5% B). All standards were purchased from Sigma Aldrich, (Sigma Aldrich, St. Louis, Missouri, USA) including (+/–)-jasmonic acid; 3-indoleacetic-acid, gibberellic acid, (+/–) abscisic acid; trans-zeatin; salicylic acid.

3.3.11 Statistical Analyses

The study was carried out in a completely randomized design for pot experiments and a randomized block design for the field experiment. Data are presented as means ± SE. For statistical analysis of significant differences between treatment groups, a one-way ANOVA followed by a Tukey–test ($p<0.05$ significance level) were performed using the Sigma-Plot software 10.0 (Systat Software GmbH, Erkrath, Germany). For the statically evaluation of yield data from the field experiment, t-grouping instead of the Tukey test was applied as recommended by Mudra (1958).

3.4 RESULTS

3.4.1 Soil Culture Experiment

In the first experiment, soil-grown maize seedlings were exposed to 14 days reduced root zone temperature of 12–14°C in a root cooling system. Cooling of the roots started at 2 weeks after sowing, followed by a 14-d recovery period. Seed treatments were performed with a commercial Zn/Mn seed dressing formulation. Silicon was applied as silicic acid (40mg kg^{-1} dry soil) by fertigation in four weekly intervals. The optimal dosage for silicic acid application has been determined in a pilot experiment (Fig. 1).

3.4.1.1 Plant Growth and Development

Confirming the results of our earlier studies (Bradáˇcová et al., 2016), 2 weeks exposure of maize plants to low RZT of 12–14°C was associated with induction of leaf chlorosis (Fig. 2A, C) necrosis, formation of stress anthocyanins (Fig. 2A) limited shoot and root growth

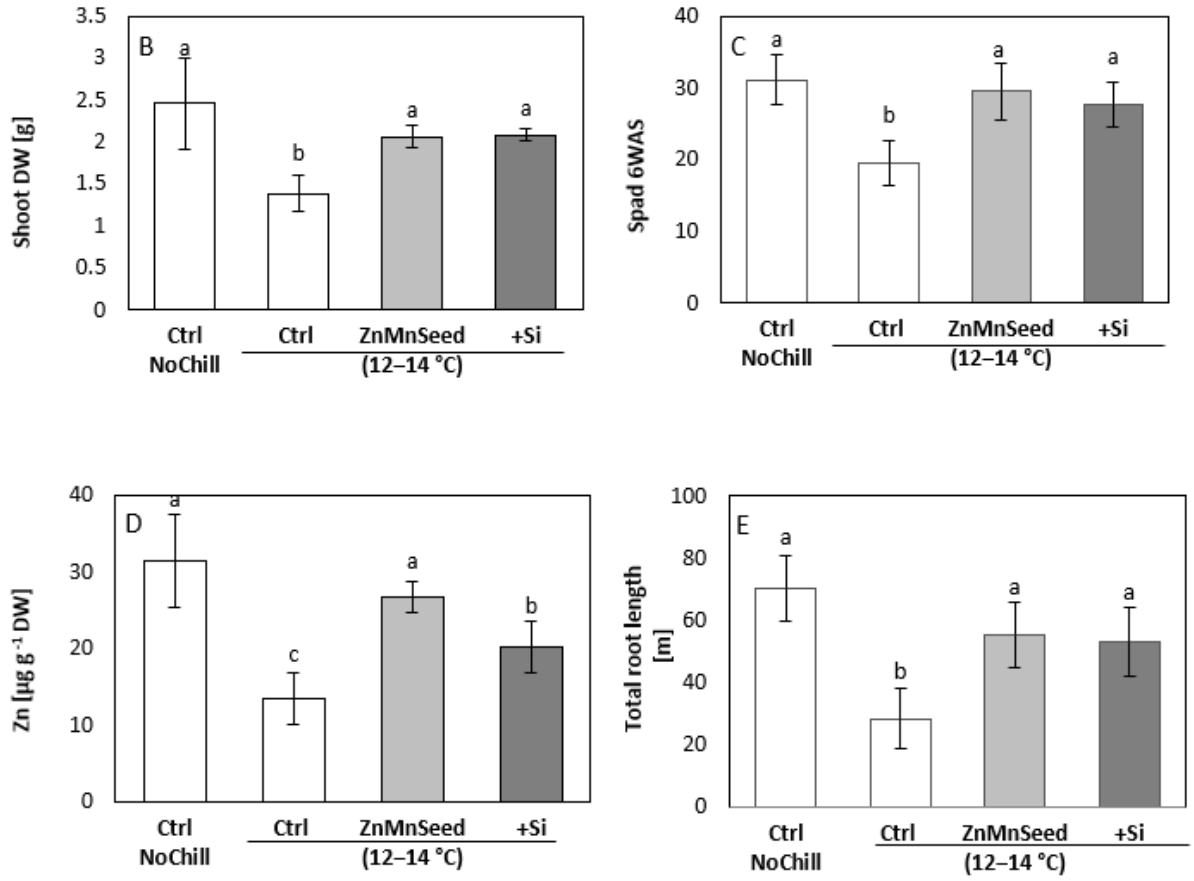
(Figs. 2 B,E) and an impaired micronutrient status (particularly Zn and Mn) below the deficiency thresholds (Figs. 2D, 3A; Bergmann, 1988). The stress responses and micronutrient deficiencies were mitigated by seed treatments with the respective micronutrients, but surprisingly, also by Si application (Fig. 2). Shoot biomass and chlorophyll contents determined by SPAD measurements were increased by ~50% (Figs. 2B, C) and total root length even by 90% (Fig. 2E).

Chapter 3. Fig. 1. (A) Shoot and (B) root development and biomass of maize plants including untreated control (–Si), and different levels (L) of Si (silicic acid) fertigation (L1: 25, L2: 85, L3:1,000 and L4: 10,000mg Kg^{-1} soil DM). Culture period: 8 weeks under greenhouse conditions with 7 days minimum temperature of 12–14 °C. Biomass data represent mean values ± SE of four replicates. Significant differences ($P < 0.05$) are indicated by different letters.

3.4.1.2 Mineral Nutritional Status

Macronutrient analysis of the shoot tissue revealed no significant differences for the P, Ca, and K concentrations (Fig. 3A). While the Ca and K status was in the sufficiency range,

low concentrations were recorded for P and Mg. Seed dressing with Zn/Mn or Si fertigation significantly increased particularly the Mg status of the plants (Bergmann, 1988). The shoot micronutrient concentrations of the untreated control were 0.013mg g^{-1} DM for Zn and 0.025mg g^{-1} DM for Mn (Fig. 3B) which is below the reported deficiency thresholds (Bergmann, 1988). The concentrations increased to the sufficiency range in response to the Zn/Mn seed dressing treatment, but interestingly also after Si fertigation (Fig. 3A). The Fe and Cu status was low but not critical, without significant differences between the treatments (Fig. 3A). Shoot Si was significantly increased only by Si fertigation (Table. 1). In contrast to the nutrient concentrations, as indicators for the plant nutritional status, total shoot contents of all investigated nutrients were significantly increased by the treatments with the chilling stress protectants Si and Zn/Mn (Figs. 3C, D). This demonstrates that the Si and Zn/Mn applications generally improved nutrient acquisition and any surplus of mineral nutrients was readily transformed into biomass production (Fig. 2B).



Chapter 3. Fig. 2. (A) Leaf chlorosis, necrosis, and anthocyanin formation, (B) shoot dry weight (DW), (C) SPAD values, (D) shoot Zn concentration and (E) total root length of maize plants exposed to a 2-weeks period of reduced root zone temperature (RZT, 12–14°C**)** on a silty loam soil, pH 6.9. Un-cooled control: (Ctrl NoChill) and low RZT variants including untreated control (Ctrl); Zn Mn seed dressing (ZnMnSeed), and silicon (H_4SiO_4) fertigation (+Si). Means of three replicates. Significant differences ($P < 0.05$) are indicated by different characters.

3.4.1.3 Oxidative Stress Indicators

In the root tissue directly exposed to low-temperature stress, Zn/Mn and Si applications increased the activities of superoxide dismutase (SOD, Table. 1) and peroxidase (POD, Table. 1) by 45– 46 and 71–84%, respectively. These enzymes are involved in oxidative stress defense and accordingly, H_2O_2 concentrations declined by 54–63% (Table. 1). In the shoot, SOD activities even increased by 179 183% (Table. 1). Shoot concentrations of total proteins, antioxidants, total phenolics, total flavonoids, and proline concentrations were

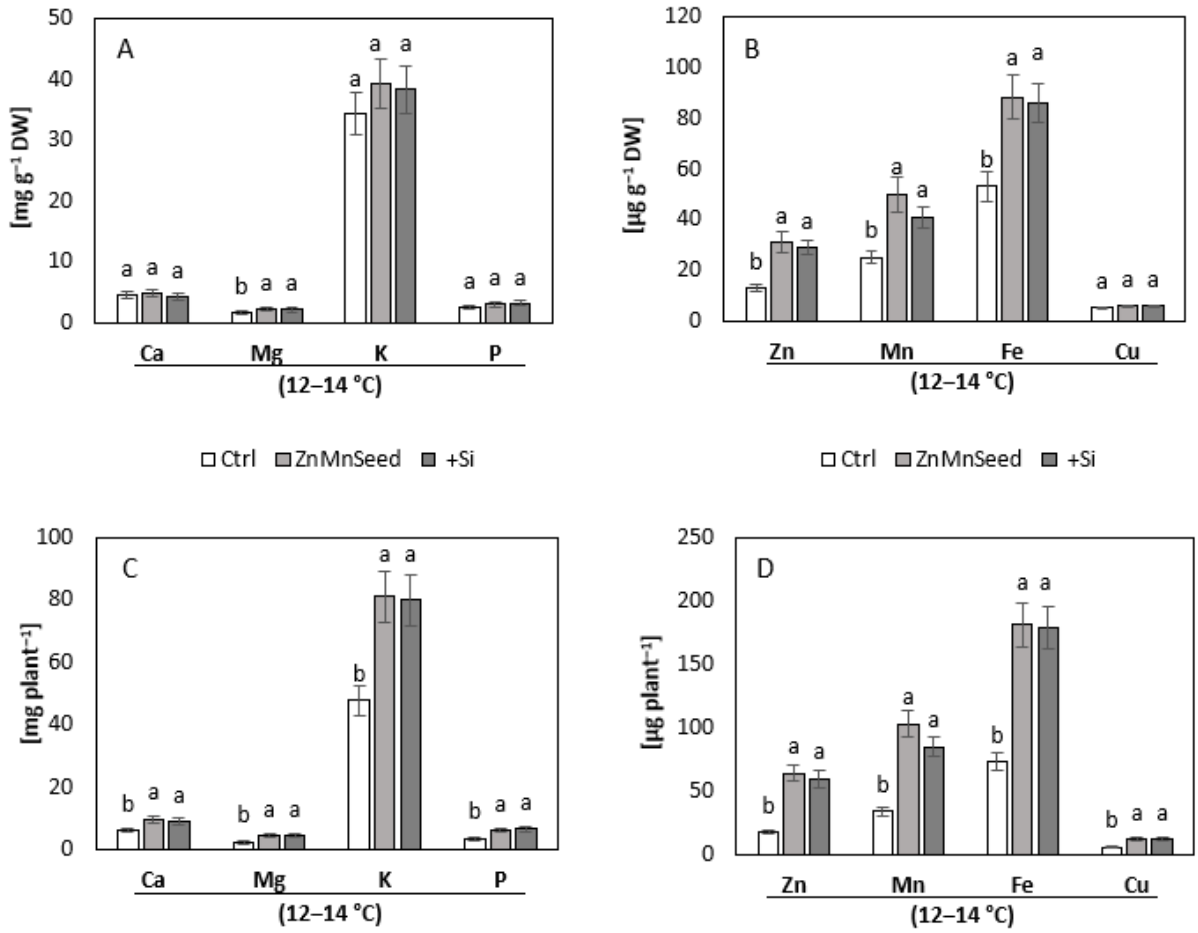
also strongly increased (Table. 1) by both the Zn/Mn and Si treatments, while leaf anthocyanins, significantly declined (Table. 1). In addition, the concentration of total soluble sugars of shoot tissue was significantly enhanced by Si treatment (Table. 1).

Chapter 3. Table. 1. Superoxide dismutase activity (SOD), peroxidase activity (POD), and H_2O_2 concentrations in root tissue and SOD, total protein, total antioxidant capacity, total soluble phenolics, total soluble flavonoids, proline, anthocyanin, total soluble sugars, and Si concentrations in the shoot tissue of maize plants (cv Colisee) exposed to a 2-weeks period at reduced root zone temperature (12–14°C) on a silty loam soil, pH 6.9. Untreated control: (Ctrl); Zn Mn seed dressing: (ZnMnSeed), and silicon (H_4SiO_4) fertigation: (+Si). Means of three replicates. Significant differences ($P < 0.05$) are indicated by different characters.

Tissue	Determination	Ctrl	ZnMnSeed	+Si
Root	**SOD [U mg $^{-1}$ protein]**	85.94±8.68 b	124.52±12.44 a	125.29±12.62 a
	POD [µmol tetra guaicol mg $^{-1}$ protein]	54.19±5.51 b	99.76±10.07 a	92.51±9.34 a
	H_2O_2 [µmol g^{-1} FW]	85.70±8.66 a	39.81±4.07 b	32.50±3.34 b
Shoot	**SOD [U mg $^{-1}$ protein]**	10.06±1.10 b	26.51±2.74 a	28.02±2.89 a
	Total protein [mg g^{-1} FW]	6.58±0.75 b	8.39±0.93 a	8.19±0.91 a
	Total Antioxidants [%]	69.11±7.00 b	91.20±9.21 a	88.13±8.90 a
	Phenolics [mg gallic acid equivalents g $^{-1}$ FW]	50.31±5.12 b	79.15±8.01 a	78.19±7.91 a
	Flavonoids [mg g $^{-1}$ FW]	2.25±0.32 b	3.41±0.43 a	3.35±0.43 a
	Proline [µmol g $^{-1}$ FW]	0.92±0.18 b	1.68±0.26 a	1.89±0.28 a
	Anthocyanin [µmol cyanidine–3–glucoside equivalents g^{-1} FW]	9.88±1.08 a	6.40±0.73 b	6.16±0.71 b
	Sugar [mg g $^{-1}$ FW]	19.23±2.01 b	21.18±2.21 ab	24.15±2.51 a
	Si [mg g $^{-1}$ DW]	2.70±0.36 b	2.88±0.38 b	4.15±0.51 a

3.4.2 Soil–Free Culture Experiment

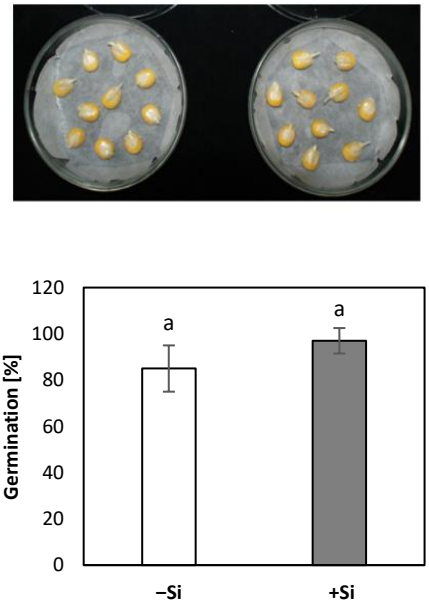
Although Si induced a recovery of maize seedling growth after exposure to low RZT, which was associated with improved root development and mitigation of Zn and Mn deficiency (Fig. 3, Table. 1) we hypothesized that Si may exert also effects on improvement of the micronutrient status independent of root uptake. Thus, a second experiment was conducted to separate the root–mediated nutrient acquisition from internal redistribution of Zn and Mn from the seed to the establishing seedling.



Chapter 3. Fig. 3. (A, B) Concentrations and (C, D) contents of macro-, and micro-nutrients in shoots of maize plants (cv Colisee) exposed to a 2-weeks period at reduced root zone temperature (12–14˚C**)** on a silty loam soil, pH 6.9. Untreated control: (Ctrl); Zn Mn seed dressing: (ZnMnseed), and silicon (H_4SiO_4) fertigation (Si). Means of three replicates. Significant differences ($P < 0.05$) are indicated by different characters.

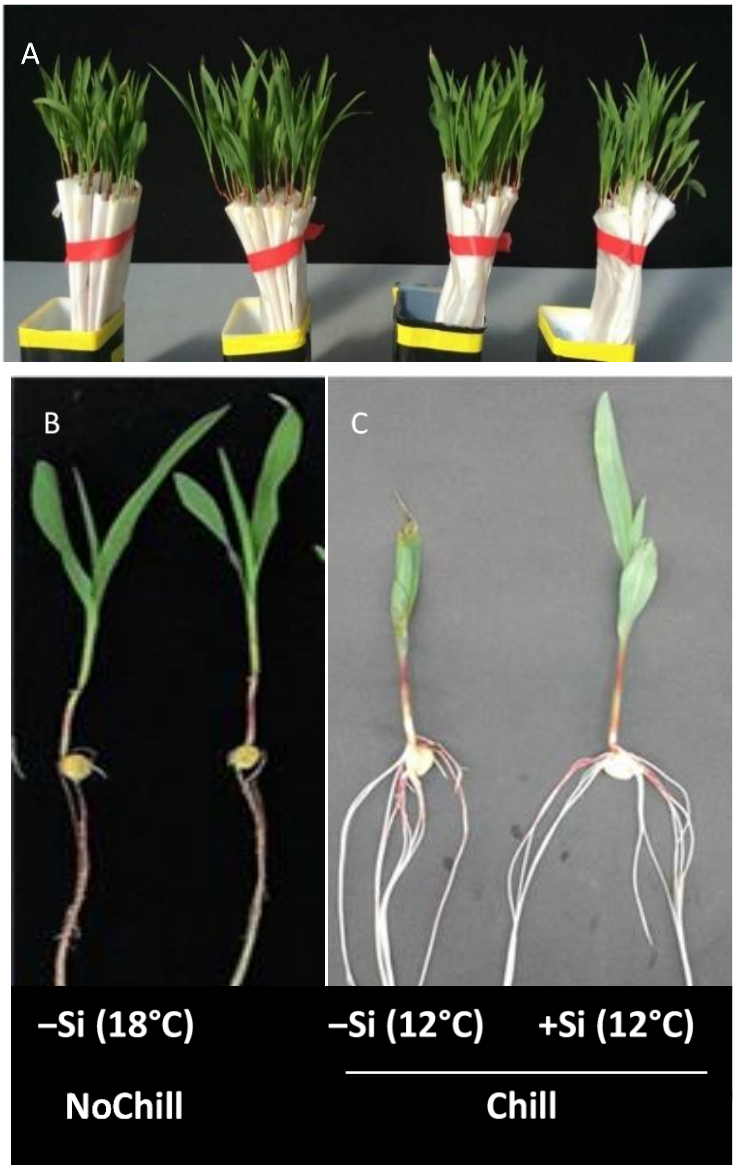
This was achieved by exposing maize seedlings to low temperature stress (7 d, 12˚C) in a soil–free culture system with and without Si application via seed soaking (3 d during germination at 18˚C), where the seedlings were exclusively dependent on their nutrient seed reserves and root uptake was excluded. During the first 3 days of cultivation at 18˚C, no significant treatment differences were recorded for germination rates (Fig. 4). However, the subsequent 7 d–cold stress periods at 12˚C induced a retardation in seedling growth, associated with a reduction in shoot and root biomass production by 53 and 60%, respectively, and intense necrotic and chlorotic leaf damage (Table. 2) similar to the

symptoms observed in soil culture (Figs. 2A and 5). Silicon treatments reduced the necrotic leaf area by more than 90%. This was associated with increased levels of total antioxidants (+27%) and soluble sugars (+117%) in the leaf tissue. The nutritional status of Zn and Mn was significantly increased by the Si treatment and a trend for increased root and shoot biomass production was detectable after the cold period of 7 d (Table. 2). During the 7-days cold stress period, shoot and root concentrations of all-important hormonal growth regulators (indole acetic acid—IAA, zeatin, gibberellic acid—GA), as well as stress–related hormones, such as abscisic (ABA), jasmonic and salicylic acids, declined significantly. However, after Si seed soaking, hormonal concentrations were restored comparable to those of unstressed plants or even further increased (shoot concentrations of IAA, gibberellic acid, and ABA, Table. 3).



Chapter 3. Fig. 4. Germination rate [%] radicle emergence at 3 DAS; 18°C) of maize seedlings (cv Colisee) on moist filter paper with (+Si) or without (–Si) 3-days seed soaking treatments with 0.1mM Si (H_4SiO_4).

Since plant cultivation in this experiment was conducted in a nutrient–free culture system, lacking any additional Zn or Mn (Si treatment solution pre-purified by cation exchange chromatography) (Maksimovic et al., 2007; Pavlovic et al., 2013), it was possible to monitor the redistribution of seed– stored Zn and Mn during seedling development.



Chapter 3. Fig. 5. (A–C) Maize seedlings germinated in filter rolls for 7 d at 18°C (NoChill) or at 12°C (Chill) without (−Si) or with (+Si) after 3 d seed soaking in 0.1mM Si (H_4SiO_4).

Chapter 3. Table. 2. Effect of Si seed soaking on biomass production, oxidative leaf damage [% necrotic leaf area], tissue concentrations of Zn, Mn, Si, soluble sugars, and total antioxidants in maize seedlings (cv Colisee) exposed to 7 days chilling stress at 12°C in a soil-free filter roll culture system. Means of five replicates. Significant differences (P< 0.05) are indicated by different characters.

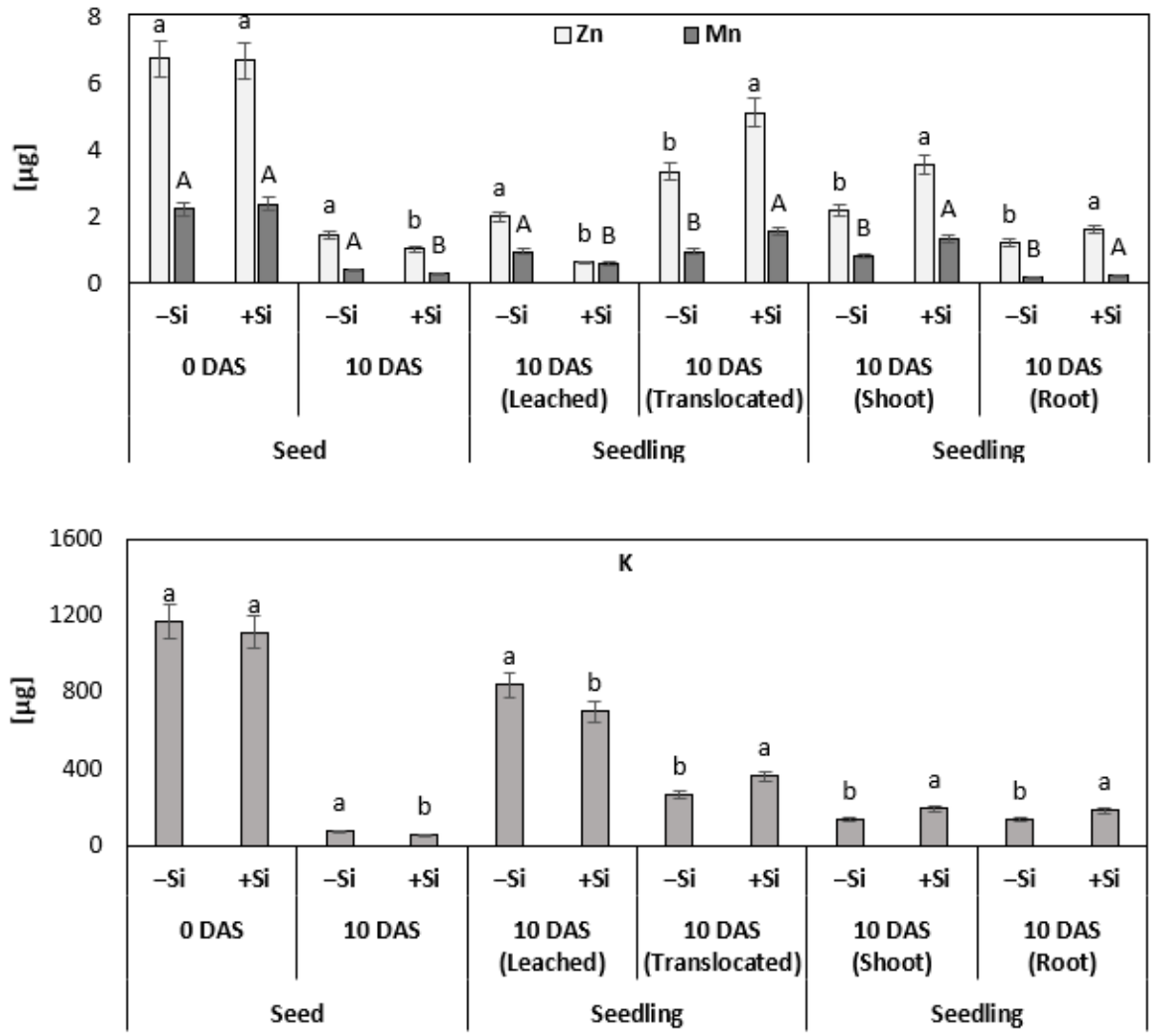
Determination	**–Si (18°C)**	**–Si (12°C)**	**+Si (12°C)**
Dry weight (Shoot) [g]	0.98±0.11 a	0.46±0.06 b	0.63±0.27 ab
Dry weight (Root) [g]	0.85±0.10 a	0.34±0.04 a	0.41±0.05 b
Zn (Shoot) [µg g^{-1} DW]	16.25±1.63 a	11.42±1.15 c	13.73±1.38 b
Zn (Root) [µg g^{-1} DW]	11.85±1.20 a	9.08±0.92 b	10.80±1.09 a
Mn (Shoot) [µg g^{-1}DW]	3.23±0.33 a	1.56±0.17 b	2.63±0.27 a
Mn (Root) [µg g^{-1} DW]	2.12±0.22a	0.99±0.11 b	1.43±0.69 ab
Total Antioxidants (Shoot) [%]	62.25±6.24 b	67.30±6.74 b	85.21±8.53 a
Necrotic leaf area [%]	0.50±0.06 c	25.25±2.54 a	2.35±0.25 b
Soluble Sugars (Shoot) [mg g^{-1} FW]	28.15±2.83 a	12.12±1.22 b	26.23±2.63 a
Si (Shoot) [mg g^{-1} DW]	0.86±0.20 b	0.63±0.07 c	1.32±0.14 a

Chapter 3. Table. 3. Endogenous concentrations of phytohormones in maize seedlings (cv Colisee) grown for 7 days at 18 or 12°C, in a soil-free filter roll culture system with (+Si) or without (–Si) silicic acid seed soaking (–Si) during a 3-day pre-germination period at 18°C. IAA, indole acetic acid; ABA, abcsisic acid; GA, gibberellic acid; JA, jasmonic acid; SA, salicylic acid. Means of three replicates. Significant differences (P < 0.05) are indicated by different characters. ND, Not Detectable.

Tissue	**Shoot**			**Root**		
Temperature	**18°C**	**12°C**	**12°C**	**18°C**	**12°C**	**12°C**
Treatment	–Si	–Si	+Si	–Si	–Si	+Si
Phytohormones	[ng g^{-1} fresh weight]					
IAA	76.77±9.90 a	28.14±4.65 b	102.12±16.54 a	31.45±8.81 a	12.76±0.37 b	40.10±13.43 a
Zeatin	3.24±0.84 a	1.10±0.22 b	3.62±0.65 a	1.29±0.49 a	0.40±0.04 b	1.21±0.31 a
ABA	50.63±4.13 b	31.02±4.04 c	109.56±13.69 a	46.56±8.65 a	14.97±0.91 b	48.78±14.19 a
GA	55.60±10.29 b	18.26±3.14 c	67.20±9.77 a	25.07±6.27 a	9.51±0.70 b	30.12±10.49 a
JA	0.44±0.07 a	0.28±0.03 b	0.42±0.09 a	ND	ND	ND
SA	44.89±6.79 a	16.78±3.17 b	56.27±15.26 a	21.96±4.71 a	9.53±0.79 b	28.94±12.00 a

Thus, any additional nutrient uptake via the roots was excluded. During the 10 days culture period, 50% of the seed Zn contents and 42% of seed Mn were translocated to the developing control seedlings. Interestingly, the translocation of micronutrient seed reserves was stimulated by the Si treatments, resulting in significantly higher total Zn (+67%) and Mn (+62%) contents at the end of the cold stress period, as compared with the untreated controls (Fig. 6). By comparison with the remaining nutrient contents in the seeds, it was

also possible to calculate the nutrient leaching losses. In the untreated control plants exposed to chilling stress, leaching accounted for 29% of the original seed Zn contents and for 41% of Mn. However, silicon treatment significantly reduced the large leaching losses of Zn and Mn by 70 and 48%, respectively (Fig. 6).



Chapter 3. Fig. 6. Fate and distribution of Zn, Mn, and K [µg seed−1 or µg seedling−1] during germination and seedling growth of maize (cv Colisee) exposed to chilling stress (12°C; 3–10 DAS) in a soil-free filter roll culture system with (+Si) or without (–Si) silicic acid seed soaking (–Si) during a 3-day pre-germination period at 18°C. Means of five replicates. Significant differences ($P < 0.05$) are indicated by different characters.

Apart from the micronutrients, also the re-distribution of K was investigated, because of its

well-established role as an indicator for nutrient leaching in response to impairment of tissue and membrane integrity (Cakmak and Marschner, 1987) and its function in cold stress protection (Wang et al., 2013). At the end of the cold stress period, untreated control seedlings had lost 72% of their K seed reserves by leaching and only 22% had been translocated to the developing seedling. Silicon treatments reduced these large leaching losses by 17% and increased the K contents of the seedling by 38% (Fig. 6). For other tested macronutrients, such as P, Mg, and Ca, no significant treatment effects were recorded (data not shown). However, Si not only reduced the leaching losses and improved the translocation of seed reserves of mineral nutrients to growing tissues. Additional selective effects were detectable for the mineral nutrient ratio between shoots and roots. While Si pre-treatments increased the Mn and K contents in shoots and roots to a similar extent (Mn: +68–77%; K: +38–46%), Si preferentially increased the Zn shoot contents by 64%, but less, only by 34%, in the root tissue (Fig. 6).

3.4.3 Field Experiment

To investigate the benefit of Zn/Mn and Si starter treatments on maize performance under on-farm conditions, a field experiment was established. Early sowing on April 22nd resulted in cold stress during germination and early growth. Due to low temperatures (13.6°C) and high precipitation (469 mm) during April and May, emergence and seedling growth were heavily affected by cold stress, but also by oxygen limitation and *Aspergillus niger* infections on the cold and wet silty-loam soil. By the mid of May, at 41 DAS (BBCH 15, stage 1), an emergence rate of only 44% was recorded in the untreated control variant (Table. 1). However, emergence was significantly increased by the Zn/Mn seed dressing (+12%) and particularly by seed soaking with KSi (+28%). To account for a potential water priming effect of the seed soaking treatment (Lutts et al., 2016), also a water-soaked control

was included, which increased emergence by 8% (Table. 4). At 49 DAS (BBCH 17, stage 1), amineral nutrient analysis was conducted for the youngest fully developed leaves. In the control variant, the Zn–nutritional status was low (Table. 4) as expected, and K and P were even below the deficiency thresholds. Mg, Cu, and Mn supply, by contrast, was sufficient (Supplementary Table 2). Similar to the pot experiment, both, Zn/Mn and Si treatments significantly increased the Zn concentrations to a sufficient level of 65 and 59 μg g−1 dry matter (Bergmann, 1988). A trend for an improved status was recorded also for the remaining nutrients (Supplementary Table 2). The protective effects of Zn/Mn and Si starter treatments were finally reflected in substantially higher biomass yield of the heavily cold-affected plots by 56 and 82%, respectively, when calculated according to the plant density determined at 41 DAS. In the Si treatments, 25% of the yield increase could be attributed to a water priming effect. Foliar Si application had no significant impact on final biomass yield (Table. 4). However, due to the extremely low emergence (44%), particularly in the untreated plots, re-sowing was performed in the most heavily affected parts, by the mid of May after the end of the stress period, to provide comparable inter-plant competition for light, water, and nutrients within the rows during the rest of the culture period. But even including the non-stressed plants after re-sowing into the yield calculation, a significant yield increase (+10.6%) was measured only for the Si seed priming variant (Table. 4).

Chapter 3. Table. 4. Emergence, Zn/Mn status (DAS), and final biomass yield of field-grown silo maize (Rolandinio) on a silty loam soil pH 6.9 at the experimental station "Ihinger Hof" University of Hohenheim with underfoot placement of di-ammonium phosphate (29 kg N, 32 kg P ha^{-1}) and stabilized ammonium sulfate fertilization (161 kg N ha^{-1}) with or without Zn/Mn seed dressing (Lebosol Mn500 SC, Zn700 SC), potassium silicate [1mM] seed priming, water priming of foliar Si application of Si as (16 L Si [1mM] + 100ml Greemax® ha−1). Yield determinations with (in brackets) and without re-sowing by the end of May 2016. Means of five replicates per treatment. Significant differences ($P < 0.05$) are indicated by different characters.

Seed treatment	Application mode	Emergence 41 DAS [%]	Yield [t ha^{-1}]	Zn status (45DAS) [µg g DW^{-1}]	Mn status (45 DAS) [µg g DW^{-1}]
Untreated	**–**	44.00±6.00 d	7.14±0.46 d (16.10±1.0 b)	32.00±2.60 b	48.00±4.00 a
Zn/Mn	**Seed–dressing**	56.00±7.00 b	11.07±0.79 b (16.40±1.2 b)	65.00±5.40 a	56.00±4.50 a
Water	**Priming**	52.00±9.00 c	8.10±0.81 c (17.2±1.60 ab)	49.00±4.08 a	57.00±4.75 a
K_2SiO_4	**Priming**	72.00±15.00 a	12.89±0.74 a (17.8±1.0 a)	59.00±4.90 a	61.00±5.08a
untreated	**Si foliar**	46.00±7.00 d	7.60±0.37 d (16.6±3.40 ab)	36.00±3.00 b	51.00±4.25 a

3.5 DISCUSSION

3.5.1 Si Mimics Cold Stress-Protective Effects of Zn/Mn Starter Applications

Similar to earlier reports (Imran et al., 2013; Bradáˇcová et al., 2016), starter treatments by seed dressing or seed priming with Zn and Mn significantly increased cold tolerance of maize in the pot experiment with controlled root-zone temperature (Fig. 2). The 14-d chilling treatments with 12◦C root zone temperature induced Zn and Mn deficiencies in the soil-grown maize seedlings (Fig. 3). This seems to be not primarily related to Zn/Mn availability in the soil, since Imran et al. (2013) demonstrated that Zn and Mn accumulation in the shoot tissue was suppressed during the cold stress period, even with freely available nutrient supply in a hydroponic culture system. Only Zn and Mn uptake before the onset of the stress period via seed priming, seed dressing or fertigation, could increase shoot micronutrient accumulation above the deficiency thresholds, detectable even after the end of the 2-weeks cold stress period (Fig. 3). This is in line with findings of Engels and

Marschner (1992, 1996), demonstrating that limited Zn and Mn shoot accumulation in maize exposed to low root zone temperatures was particularly dependent on cold-stress effects affecting nutrient uptake and root activity. In accordance with the low Zn/Mn status, the cold-stressed control plants exhibited various symptoms characteristic for Zn and Mn limitation (Cakmak, 2000), such as chlorosis and oxidative leaf damage, stunted shoot, and root growth (Fig. 2) reduced activity of enzymes involved in ROS detoxification with micronutrients as co-factors (SODs, PODs), and impaired biosynthesis of phenolic antioxidants (Table. 1) which depends on Cu and Mn cofactors (Datnoff et al., 2007). Consequently, excessive accumulation of ROS (Table. 1) resulted in oxidative damage of plant tissues (Fig. 2) considered as one of the major constraints for cold-stressed plants (Baek and Skinner, 2012; Saeidnejad et al., 2012). Supplementation of Zn and Mn via seed priming (Imran et al., 2013) or seed dressing were able to overcome Zn/Mn deficiency (Fig. 3) and largely mitigated the related stress symptoms described above. Surprisingly, also starter fertigation or seed soaking with Si completely mimicked all cold-stress-protective effects of Zn/Mn starter applications (Fig. 2, Table. 1). Silicon increased the Zn/Mn status, activities of ROS detoxification enzymes, and accumulation of antioxidants to a comparable level as the Zn/Mn treatments (Fig. 3, Table 1) although Si was applied as free silicic acid (H_4SiO_4), pre-purified via cation exchange chromatography, and no Zn or Mn was detectable in the application solution. Also, in the field experiment, seed treatments with Si and Zn Mn improved emergence and the micronutrient status (particularly Zn) of maize seedlings, exposed to sub-optimal germination temperatures and cold and wet soil conditions by early sowing at the mid of April (Table. 4). A certain protective effect was recorded also for the control treatment with water-primed seeds but less expressed as compared with Si seed priming. This is in line with the well-documented beneficial effects

of water priming on seed germination by metabolic pre-activation (Lutts et al., 2016). In accordance with earlier observations (Imran et al., 2013; Bradáˇcová et al., 2016), application of cold stress protectants was ineffective after the onset of the stress period, i.e., when Si was supplied by the foliar application (Table. 4). By contrast, silicon seed priming was the most effective treatment and increased emergence by 64% as compared with the untreated control, which translated into a significant increase in final yield in both scenarios of yield determination (with and without re-sowing after the end of the cold stress period; Table. 4). Similar effects have been reported also in two additional field experiments conducted under comparable climatic conditions when the micronutrient status of the maize seedlings was increased by direct supplementation of Zn, Mn, and Fe via seed priming. In these field trials, marketable grain yields increased by 13–15% (Imran et al., 2013). The surprisingly long-lasting effects of the starter treatments with cold stress protectants may be related to the intense stimulation of root growth (Fig. 2D) which has an advantage for plant performance not only during the stress period but also during the recovery phase and under more favorable growth conditions.

3.5.2 Silicon Reduces Leaching Losses and Promotes Utilization of Zn/Mn Seed Reserves

The striking similarity of Zn/Mn and Si effects on cold-stressed maize seedlings raises the question whether Si exerts its protective effects via improvement of the plant micronutrient (Zn/Mn) status. Restoration of cold stress-induced root growth inhibition was among the most apparent effects of Si or Zn/Mn applications (Fig. 2E). Of course, root growth stimulation can contribute to improved nutrient acquisition in general, as demonstrated for increased shoot accumulation of P, K, Mg, Ca, Fe, Zn, Mn, and Cu recorded after the cold stress period in Si-treated maize plants (Fig. 3). However, Si was able to improve

selectively the Zn/Mn status of maize seedlings, already during the first week of the cold stress period, before a marked stimulation of root growth was detectable (Table. 2). Moreover, this effect was observed in a soil–free culture system excluding the option for further Zn/Mn root uptake from the external medium (Fig. 5). This implicates that the improved micronutrient status of the Si treated maize seedlings exposed to low-temperature stress cannot be exclusively attributed to Si-induced root growth stimulation but involves also Si effects counteracting nutrient leaching and promoting internal distribution of Zn and Mn. Imran et al. (2015) demonstrated that seed reserves can cover the Zn and Mn demand of maize seedlings for about 2–3 weeks. However, cold stress is a well-documented stress factor leading to electrolyte leakage via oxidative membrane damage (Bewley and Black, 1994), which can limit the seed reserves of mineral nutrients. Accordingly, our study revealed large leaching losses of 30– 40% for the Zn/Mn seed reserves and even 70% for K, as another mineral nutrient with cold-protective functions (Wang et al., 2013), during the first 10 days of seedling development after a 12◦C cold stress period of 7 days (Fig. 6). Seed soaking with Si dramatically reduced the leaching losses by 70% (Zn), 50% (Mn), and 15% (K), leading to an improved nutrient supply to the developing seedling. This effect may be attributed to the well-documented protective functions of Si against oxidative membrane damage (He et al., 2010). However, Si seed soaking increased the root and shoot contents of Mn by 77 and 68%, respectively, while the root contents of Zn were increased only by 34%, but by 64% in the shoot tissue (Fig. 6). This indicates a selective effect of Si on the root/shoot distribution of Zn. A similar improved Zn status by Si treatment has been reported for Zn-deficient soybean plants (Pascual et al.,

2016). Different mechanisms have been proposed for effects of Si, improving internal Zn

availability in Zn-deficient plants. Increased production of phenolics with metal chelating properties induced by Si treatments (Pavlovic et al., 2013), as observed also in the present study (Table. 1) may increase internal mobility and transport of Zn within the plant. This may be related to the improved Mn status of Si treated plants (Fig. 3) as an important enzymatic cofactor for the biosynthesis of phenolics (Datnoff et al., 2007). In later stages of plant development, the same mechanism may be responsible also for the remobilization of Zn sequestered in the apoplast together with iron plaques (Chen et al., 1980), as similarly demonstrated for apoplastic Fe remobilization in cucumber (Pavlovic et al., 2013). Bityutskii et al. (2014) could not confirm this interaction, but in that study, plants were grown in nutrient solution without Zn supply, which may have prevented the accumulation of apoplastic Zn pools. Other studies suggest direct Si-metal interactions counteracting apoplastic metal immobilization and supporting metal transport in plants (Pavlovic et al., 2013; Hernandez– Apaolaza, 2014; Stevic et al., 2016). Effects of Si on the expression of metal acquisition and transport genes have been reported by Pavlovic et al. (2013, 2016), but the underlying mechanisms are unknown.

3.5.3 Si Restores the Levels of Hormonal Growth Regulators in Cold-Stress-Affected Maize Seedlings

Due to the impairment of Zn/Mn-dependent ROS detoxification systems, induced by limited Zn/Mn availability in cold stressed plants (Figs. 2, 3) excessive ROS accumulation causes oxidative damage, leading to leaf chlorosis and necrosis (Fig. 2). This is associated with an impairment of photosynthesis, resulting in a reduced root allocation of assimilates required for root development, as previously reported also by Sowinski et al. (1998). Moreover, excessive production of ROS can promote oxidative degradation of indole acetic acid and result in a 50% reduction of IAA contents in Zn-deficient Phaseolus vulgaris,

which could be restored by Zn fertilization. Therefore, auxin deficiency was considered as another important factor for growth limitation in Zn deficient plants (Cakmak et al., 1989). Similarly, in our study cold stress induced, both, Zn/Mn limitation (Table. 1) and a 60% reduction of IAA accumulation in the shoot and root tissue (Table. 3) associated with inhibition of shoot and root growth and all symptoms were reverted by exogenous Si application (Fig. 2). More recent studies suggest that cold stress additionally affects root growth via inhibition of PIN2 and PIN3-mediated basipetal auxin transport within the roots (Shibasaki et al., 2009). Shoot growth in cold-stressed plants seems to be also affected by a reduction of bioactive growth-promoting gibberellic acid (GA) levels, leading to an increased abundance of nuclear DELLA-protein growth repressors via a signaling pathway involving CBF/DREB1 transcription factors (Miura and Furumoto, 2013; Eremina et al., 2016). Accordingly, in our study GA levels in shoot and roots of cold-stressed maize seedlings declined by ~60–70%. This effect was completely reverted by Si seed soaking (Table. 3). In line with this observation, in Arabidopsis thaliana it was demonstrated that cold stress stimulates GA degradation by upregulation of the GA 2-oxidase gene and simultaneously impairs GA biosynthesis by repressing the GA 20-oxidase gene (Eremina et al., 2016). For Zn-deficient plants, reduced GA concentrations have been recorded (Suge et al., 1986; Sekimoto et al., 1997) similar to the reduction in IAA levels reported by Cakmak et al. (1989). More recently, it was shown that various steps of GA biosynthesis depend on the presence of IAA (Ross et al., 2001). Therefore, the observed reduction of GA levels in cold-stressed maize seedlings (may be a consequence of auxin deficiency, resulting from the limited Zn supply caused by low temperature stress. Interestingly, it was shown that exogenous application of IAA and GA could increase the accumulation of flavonoids and other phenolics in buckwheat (Fagopyrum esculentum) seedlings (Park et

al., 2017). This observation is in line with the increased production of phenolics and antioxidants, induced by Si application in cold stressed maize seedlings (Table. 1) that may be triggered by the increased IAA and GA levels in the respective plants (Table. 3). Similar to auxin and GA, also the levels of the metabolically active cytokinin form "zeatin," with functions in stimulation of cell division and cell expansion, declined in cold-stressed maize seedlings (Table. 3). A similar decline had been previously reported for Arabidopsis, rice and, wheat (Eremina et al., 2016). In rice, this was associated with a significant downregulation of gene expression related to cytokinin biosynthesis (Maruyama et al., 2014). Accordingly, external application of cytokinins increased cold tolerance in Arabidopsis (Jeon et al., 2010; Shi et al., 2012). In our study, cold-stress protection by Si application was also associated with increased zeatin concentrations in the root and shoot tissue (Table. 3). Taken together, the results demonstrated that cold stress significantly reduced the internal concentrations of the most important hormonal regulators of plant growth. Furthermore, the cold-protective effect of Si application was related to a restoration of hormonal levels comparable to those of unstressed plants (Table. 3).

3.5.4 Si Increases the Levels of Hormonal Stress Regulators in Cold Stress-Affected Maize Seedlings

The Si treatments influenced also the levels of abscisic (ABA) salicylic (SA) and jasmonic acids (JA), known as hormones more directly involved in the regulation of abiotic and biotic stress responses (Table 3). These hormones have been also implicated in cold stress signaling (Miura and Furumoto, 2013; Eremina et al., 2016). Improved cold acclimation and increased cold tolerance by exogenous applications of ABA, SA, and JA have been reported for various plant species (Horváth et al., 2007; Kumar et al., 2008; Eremina et al., 2016; Hu et al., 2017). Mutants affected in ABA, SA, and JA metabolism show altered

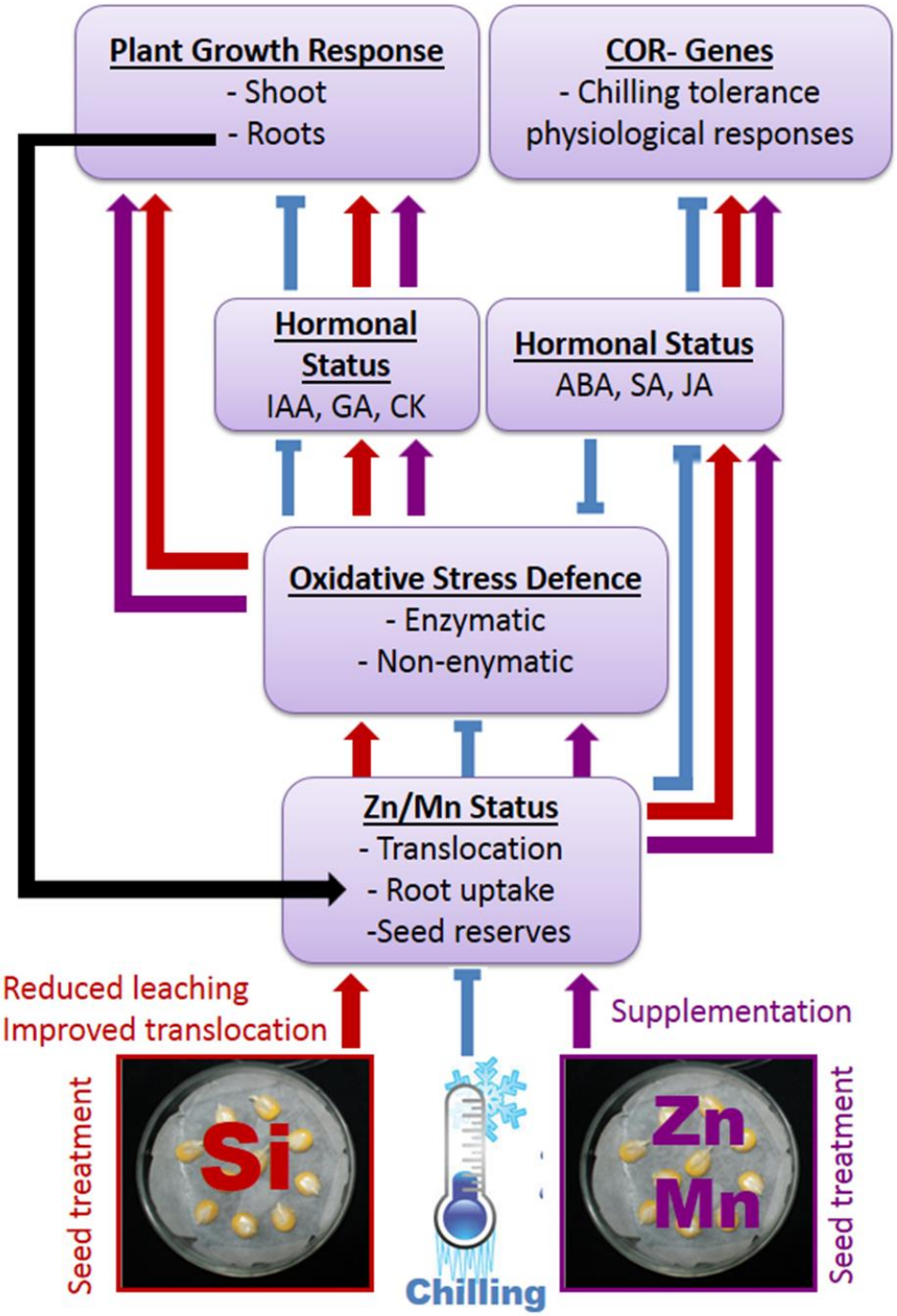
responsiveness to cold stress (Eremina et al., 2016). Accordingly, in our study, exposure of maize, as cold-sensitive plant species to a 7-days cold period, significantly decreased the levels of ABA, SA, and JA. The cold-protective effect of Si starter treatments was associated with an increase of ABA and to a smaller extent also of SA and JA, particularly in the shoot (Table. 3). Abscisic acid is considered as a central regulator of cold stress responses in plants and seems to regulate the adaptive expression of cold related genes with cold-protective functions via CBF-dependent and independent pathways, in cross-talks involving also SA and JA (Szalai et al., 2011; Eremina et al., 2016). Direct links between ABA and induction of oxidative stress defense enzymes, such as SOD in cold-stressed plants similar to our study (Fig. 3) have been reported by Kumar et al. (2008), Szalai et al. (2011), and Li and Zhang (2012). Moreover, the cold stress-induced accumulation of cryo-protectants, such as proline (Fig. 3) has been linked with a reduction of leaching losses via ABA-induced protection against oxidative membrane damage (Chen and Li, 2002). Proline biosynthesis at least partially depends on ABA signaling (Szabados and Savouré, 2010). Increased ABA levels in the Si-treated plants may be also related to the improved Zn nutritional status induced by the Si starter treatments (Fig. 3). Accordingly, Cakmak et al. (1989) and more recently Wang et al. (2012) reported that Zn deficiency reduced the ABA levels in Phaseolus vulgaris and in apple rootstocks. The surprisingly distinct effects of the Si treatments on the hormonal balances (Table. 3) and the timing of hormonal changes just at the beginning of detectable growth responses (Table. 2) are clear indicators for the proposed interactions of the Si amendments with hormonal cold stress signaling. Nevertheless, the obvious interactions of Si with hormonal balances require further investigations considering quantitative changes during plant development and a higher spatial resolution, since it is known that local changes in hormone concentrations at the

cellular level control adaptive growth and development (Eremina et al., 2016). A conceptual model of the proposed interactions between Si, micronutrients, and hormones mediating chilling tolerance in maize seedlings and its relation to plant growth is presented in Figure 7 and summarizes our findings.

3.6 CONCLUDING REMARKS

The findings of the present study suggest that induced deficiency of Zn and Mn as a consequence of leaching during early development and limited root growth and activity is a major factor determining the sensitivity of young maize plants to chilling stress, with options for mitigation by supplementing germinating seeds with Zn, Mn, or Si. In this context, the protective effect of Si treatments is related to an improved Zn and Mn status, starting already during germination with a protective effect of Si against leaching losses of micronutrient seed reserves and improved translocation to the developing seedling. The improved micronutrient status can at least partially explain the ability of the plants to maintain a balanced hormonal (IAA, GA, cytokinin) status that restores plant growth and particularly root development, which allows further nutrient uptake. The improved micronutrient status also helps to increase the expression of enzymatic (SOD, POD) and non-enzymatic (phenolic antioxidants) defense systems against cold-induced oxidative stress. Finally, it stimulates the accumulation of stress hormones (ABA, SA, JA), potentially priming various physiological adaptations to chilling stress via expression of cold response genes. It remains to be established whether this applies also to protective effects of Si against other abiotic stresses, such as drought or salinity, which are also associated with oxidative stress. Remarkably, the improved micronutrient status by Si treatment under low temperature stress is not only restricted to controlled lab conditions. The preliminary field experiment demonstrated that the observed protective effects of Si

starter treatments on seedling performance can translate into improved yields in agricultural practice and requires further investigation considering different soil types and maize varieties.



Chapter 3. Fig. 7. Proposed interactions between Si or Zn/Mn seed treatments and expression of chilling tolerance in maize. Arrows indicate stimulation, t–shaped lines inhibition of the respective processes.

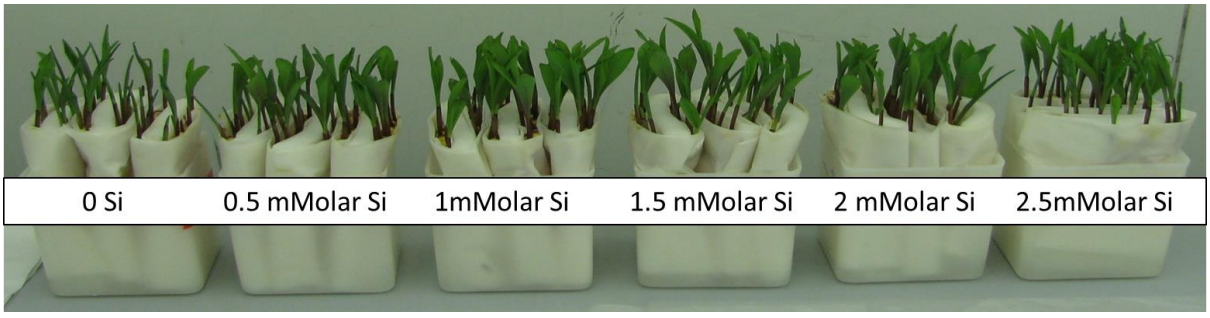
3.7 SUPPLEMENTARY MATERIAL

Chapter 3. Suppl. Table. 1. Chemical and physical properties of the soil material from IHO.

pH-Value ($CaCl_2$-solution)	6.5
Humus	2.12 %
C_{org}	1.23 %
Carbonate-C	< 0.20 %
C_{total}	1.27 %
N	0.18 %
P (CAL-extract VDLUFA)	83 mg P kg^{-1}
K (CAL-extract VDLUFA)	149 mg K kg^{-1}
Mg ($CaCl_2$-extract VDLUFA)	200 mg Mg kg^{-1}
Sand (63 -2000 µm)	2 %
Silt (2 – 63 µm)	71 %
Clay (< 2 µm)	27 %

Chapter 3. Suppl. Table. 2. Mineral nutrient analysis at 49 days after sowing (BBCH (Code 17) stage 1), of the youngest fully developed leaves.

Treatment	Zn (µg g^{-1} DM)	Mn (µg g^{-1} DM)	Cu ((µg g^{-1} DM)	K (µg g^{-1} DM)	Mg (µg g^{-1} DM)	P (µg g^{-1} DM)
No treatment	32 B	48 A	8 A	15.5 A	2.6 A	1.13 B
Zn/Mn seed dressing	65 A	56 A	10 A	16.0 A	3.3 A	1.22 A
KSi seed soaking	59 A	61 A	11 A	15.6 A	2.7 A	1.20 A



Chapter 3. Suppl. Fig. 1. A pilot experiment was done to determine the optimum Si concentration for priming seed experiment.

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Chapter 4. Synergisms of microbial consortia, N forms, and micronutrients alleviate oxidative damage and stimulate hormonal cold stress adaptations in maize

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Author Contributions

NM and GN conceived and designed the experiments. NM and AA conducted the experiments, performed the analyses, and collected the data. GN, UL, FW, BH, and JG provided the facilities for analyses. GN, UL, JG, andFWread and edited the manuscript. All authors approved the final manuscript. NM and AA equally contributed to manuscript writing.

4.1 Abstract

Aims: Low soil temperature in spring is a major constraint for the cultivation of tropical crops in temperate climates. This study aims at the exploitation of synergistic interactions of micronutrients, consortia of plant growth-promoting microorganisms and N forms as cold-stress protectants.

Methods: Maize seedlings were exposed for two weeks to low root zone temperatures at 8–14°C under controlled conditions on a silty clay-loam soil (pH 6.9) collected from a maize field cultivation site. A pre-selection trial with fungal and bacterial PGPM strains revealed superior cold-protective performance for a microbial consortium of *Trichoderma harzianum* OMG16 and *Bacillus spp.* with Zn/Mn supplementation (CombiA^{+}), particularly in combination with N-ammonium as a starting point for the characterization of the underlying physiological and molecular mechanisms.

Results: In nitrate-treated plants, the cold stress treatment increased oxidative leaf damage by 133% and reduced the shoot biomass by 25%, related with reduced acquisition of phosphate (P), zinc (Zn) and manganese (Mn). The supplying of N as ammonium improved the Zn and Mn nutritional status and increased the ABA shoot concentration by 33%, as well as moderately increased detoxification of reactive oxygen species (ROS). Moreover, use of N as ammonium also increased the root auxin (IAA) concentration (+76%), with increased expression of auxin-responsive genes, involved in IAA synthesis (ZmTSA), transport (ZmPIN1a), and perception (ZmARF12). Additional inoculation with the microbial consortium promoted root colonization with the inoculant strain *T. harzianum* OMG16 in combination with ammonium fertilization (+140%). An increased ABA/cytokinin ratio and increased concentrations of jasmonic (JA) and salicylic acids (SA) were related to a further increase in enzymatic and non-enzymatic ROS

detoxification. Additional supplementation with Zn and Mn further increased shoot IAA, root length and total antioxidants, resulting in the highest shoot biomass production and the lowest leaf damage by oxidative chemical species.

Conclusion: Our results suggest the mitigation of cold stress and reduction of stress priming effects on maize plants due to improved ROS detoxification and induction of hormonal stress adaptations relying on the strategic combination of stress-protective nutrients with selected microbial inoculants.

Keywords: synergisms, plant growth-promoting microorganisms, ammonium, colding, phytohormones, ROS detoxification

4.2 INTRODUCTION

The cultivation of tropical and subtropical crops in agricultural production systems under temperate climates continuously increases and is further promoted by global warming. Under these conditions, short vegetation periods due to low temperatures in early spring remain a major challenge for crops, such as maize, tolerating soil temperatures not much lower than 15°C for normal germination and early growth (Cutforth et al., 1986; Kaspar and Bland, 1992). This is further complicated by the more widespread adoption of no-tillage or conservation tillage, leading to a slower seedbed warming in spring (Hayhoe et al., 1996).

As a consequence of cold stress (5–15°C), poor field establishment due to inhibition of root development, impaired uptake and translocation of water and nutrients can translate into poor vegetative growth, low-stress resistance and finally reduction of yield (Duncan and Hesketh, 1968; Muldoon et al., 1984; Imran et al., 2013). Besides, the maize shoot meristem is directly affected since it remains belowground even until the V6 stage (Stone et al., 1999). Impairment of root growth particularly limits the acquisition of phosphate (P)

and micronutrients, such as zinc (Zn), manganese (Mn) and iron (Fe) due to low soil mobility, leading to induced nutrient deficiencies (Engels and Marschner, 1996; Bradacova et al., 2016; Moradtalab et al., 2018). Due to the importance of micronutrients as co-factors for enzymatic and non-enzymatic detoxification of reactive oxygen species (ROS), oxidative stress appears in consequence of ROS overproduction, which causes a severe damage to membranes, organelles, and cell functions (Cakmak, 2000; Gong et al., 2005). Impairment of photosynthesis due to oxidative leaf damage and impaired auxin production related to zinc limitation are factors further contributing to inhibition of root growth, impaired nutrient acquisition and limited plant regrowth (Moradtalab et al., 2018).

Natural cold stress adaptations are weakly expressed in tropical and subtropical-originated plant species. Most breeding programs toward improved cold tolerance use Flint maize inbred lines as a source of adaptation, originally based on the Northern Flint race adapted to cold temperate regions of Northeastern America (Riva-Roveda et al., 2016). Temporary growth and rapid recovery from limitations due to low photosynthesis rates have been described as major adaptation traits of these genotypes (Riva-Roveda et al., 2016). As a complementary approach, adapted fertilization for supplementation of critical nutrients, such as phosphate (P) and micronutrients or application of stress-protective biostimulant are discussed as mitigation strategies to cope with cold-stress (Bradacova et al., 2016; Gómez-Muñozet al., 2018; Moradtalab et al., 2018):

(i) Placement of P starter fertilizers as ammonium phosphates close to the seed is meanwhile regarded as a standard measure for maize cultivation in temperate climates (Nkebiwe et al., 2016). Both, P and ammonium N are applied close to the seedling root, where rhizosphere acidification, induced by preferential ammonium uptake (Marschner and Römheld, 1983) can increase the solubility of critical nutrients, such as P, Zn, Mn, Fe,

and Cu, with particular importanc on soils with neutral to alkaline pH (Neumann and Römheld, 2002; Jing et al., 2010).

(ii) The application of stress-protective nutrients, such as Zn, Mn, Fe, B, Cu, or Si by seed treatments or starter fertigation to promote oxidative stress defense, is another strategy with proven beneficial effects on cold tolerance in maize (Imran et al., 2013; Bradacova et al., 2016; Moradtalab et al., 2018).

(iii) Also, inoculation with plant growth-promoting microorganisms (PGPMs) is discussed as a potential measure to promote early growth and field establishment of sensitive crops under challenging environmental conditions (Kumar and Verma, 2018). In the context of cold tolerance, the selection of so-called psychrotolerant PGPM strains with the ability to propagate also at soil temperatures below 15°C and sometimes even close to the freezing point, may provide a significant advantage (Selvakumar et al., 2008a,b; Subramanian, 2011). The same holds true for the use of microbial consortia as plant inoculants, combining different PGPM strains with complementary properties and differing stress tolerance (Nuti and Giovannetti, 2015; Woo and Pepe, 2018). A common feature of the described mitigation strategies is the mode of application of the aforementioned agricultural inputs. This may offer opportunities for the development of multifunctional products, combining beneficial properties and exploiting potential synergisms.

Based on this hypothesis, the aim of this study was to explore the synergistic interactions and the combined application of PGPMs, micronutrients (Zn and Mn), and the use of N asammonium and nitrate on the recovery and early growth of maize after two weeks exposure to low root zone temperatures at 8–14°C on a silty clay-loam field soil, collected from a maize cultivation site.

A pre-selection trial was conducted with a range fungal and bacterial PGPM strains based

on *Penicillium* sp. with cold-protective properties (Gómez-Muñoz et al., 2018), a cold-tolerant strain of *Bacillus atrophaeus* (ABI02) and a microbial consortium product (MCP), based on a combined formulation of *Trichoderma harzianum* OMG16 and *Bacillus spp.* with Zn/Mn supplementation (CombiA). The CombiA consortium was selected according to the MCP concept, by combining different as microbial strains with complementary properties as discussed an approach to increase the efficiency and the flexibility of PGPM-based production strategies under variable environmental conditions (Woo and Pepe, 2018). *Trichoderma-Bacillus* combinations are among the well-documented examples in this context. Although co-cultivation of *Trichoderna* and *Bacillus* strains on artificia growth media was frequently characterized by antagonisms (Gyu Kim et al., 2008), in many plant species, including *Oryza sativa* (Ali and Nadarajah, 2014), *Triticum aestivum* (Karuppiah et al., 2019), *Cicer arietinum* (Zaim et al., 2018), *Solanum melongena* and *Capsicum annuum* (Abeysinghe, 2009), synergistic beneficial effects were reported after co-inoculation. This included stimulation of germination and growth promotion, as well as biocontrol effects against fungal pathogens, such as *Rhizoctonia*, *Fusarium* and *Pythium*, known as important damping-off diseases in cold and wet soils with potential relevance also for the present study. Testing seven different fungal and bacterial inoculants, Mpanga et al. (2019b) found superior root growth-promoting properties and improved nutrient acquisition after CombiA inoculation in maize under P limitation. Similarly, superior cold-protective performance in terms of biomass production and reduction of oxidative leaf damage was reported for the CombiA consortium in the previous selection trial conducted in this study (Supplementary Table S1).

To evaluate the mode of action of the selected cold stress mitigation strategies, we hypothesized that (i) ammonium-dominated N supplying will increase the availability of

critical nutrients, such as P, Zn, Mn, Fe via rhizosphere acidification on the investigated soil with neutral pH and additionally stimulate rhizosphere interactions with the selected inoculants as previously reported by Mpanga et al. (2019a), (ii) The starter application of micronutrients will additionally provide co-factors (Zn, Mn) for the systems of ROS detoxification already before the onset of the cold stress treatments. The improved micronutrient status will support the expression of adaptive responses to cold stress by mitigation of oxidative damage, as similarly reported by Bradacova et al. (2016) and Moradtalab et al. (2018), (iii) The selected microbial inoculants will interact with plant hormonal homeostasis, supporting nutrient acquisition by stimulation of root growth, induction of stress priming effects and provide protection against pathogen attack.

To dissect the investigated mitigation strategies to low temperatures, the monitoring of physiological stress indicators, hormonal homeostasis and expression of related genes was conducted for the single and combined application of the selected cold-stress protectants.

4.3 MATERIALS AND METHODS

4.3.1 Plant Cultivation

For all experiments, maize (*Zea mays* L. cv. Rolandinio) plants were grown for six weeks under greenhouse conditions. For the preparation of the growth substrate, a silty clay loam field soil pH 6.9 (see Table 1 for soil properties) was freshly collected from the Ap horizon of a maize cultivation site at the Hohenheim University experimental station Ihinger Hof (48°45’ N, 8°56’ E, Renningen, Germany), air-dried and sieved using 2 mm mesh size. The soil substrate was mixed with quartz sand (30% w/w) to improve the soil structure. Fertilization was performed with $Ca(H_2PO_4)_2$: 80 mg kg^{-1} dry matter (DM) P; K_2SO_4: 150 mg kg^{-1} DM K and $MgSO_4$: 50 mg kg^{-1} DM Mg. Nitrogen was applied at a rate of 100 mg kg^{-1} DM N, either as a commercial Ca-nitrate formulation, (YaraLiva®, CALCINIT® ,

YARA GmbH & Co., KG, Germany) or as stabilized ammonium sulfate (NovaTec® Solub 21, COMPO EXPERT GmbH, Germany).

Chapter 4, TABLE 1 | Chemical and physicochemical properties of the soil sampled at the Hohenheim University experimental station Ihinger Hof Renningen, Germany), 2016.

Determination	Unit	Value
pH-Value ($CaCl_2$)		6.9
Available P CAL-Extract VDLUFA)	mg/kg	82.9
Availabke K CAL-Extract VDLUFA)	mg/kg	141.1
Mg ($CaCl_2$)	mg/kg	190
Humus	mg/kg	22000
Carbon $_{total}$	mg/kg	12800
N	%	0.177
S	%	0.054
Sand (63-2000 µm)	%	2.9
Silt (2-63 µm)	%	66.8
Clay (<2 µm)	%	30.3
Carbonate (Scheibler)	mg/kg	1.1
Fe (CAT-Extract)	mg/kg	126
Cu (CAT-Extract)	mg/kg	4.22
Mg (CAT-Extract)	mg/kg	215
Mn (CAT-Extract)	mg/kg	404
Zn (CAT-Extract)	mg/kg	2.92
K (CAT-Extract)	mg/kg	97.2
P (Olsen)	mg/kg	49.2
B (ICP-OES KW)	mg/kg	17.8
Ca ((ICP-OES KW)	mg/kg	4.600
Fe (ICP-OES KW)	mg/kg	26.912
K (ICP-OES KW)	mg/kg	4.407
Cu (ICP-OES KW)	mg/kg	23.1
Mg (ICP-OES KW)	mg/kg	5.630
Mn (ICP-OES KW)	mg/kg	991
P (ICP-OES KW)	mg/kg	990
Zn (ICP-OES KW)	mg/kg	62.6
Base saturation (Co-hexamine VDLUFA)	%	80
Calcium (Ca) exchangeable (CoHexamin)	cmol(c)/kg	13.7
KAK pot (co-hexamine VDLUFA)	cmol(c)/kg	20.6
Potassium (K) exchangeable (CoHexamin)	cmol(c)/kg	0.42
Magnesium (Mg) exchangeable (CoHexamin)	cmol(c)/kg	2.37
Sodium (Na) exchangeable (CoHexamin)	cmol(c)/kg	0

Thereafter, 2 L plastic pots were filled with 1.8 kg of the substrate prior to sowing, which was conducted with five seeds per pot and reduction to one seedling after germination. During the first two weeks, plants were grown under ambient greenhouse temperature

conditions (ranging from 8–25°C) and as soon as the plants were established, they were exposed to low root zone temperature (RZT in a thermostatic root-cooling device, described by Bradacova et al. (2016). The root-cooling system is based on an immersion water bath circulator (Thermomix 1480, Frigomix 1497, Braun, Melsungen, Germany) connected to a pipe system, installed in moist peat culture substrate to circulate the cooling fluid through the moist peat layer. For adjustment of the reduced RZT regime (two weeks-cold stress a 8–25°C), the culture vessels were immersed into the cooled peat bed followed by 2-weeks recovery at ambient greenhouse temperature. Soil and air temperatures were constantly recorded by a LogTag device (Supplementary Figure S1). Every second day, soil moisture was adjusted gravimetrically to 70% of the substrate water-holding capacity (18.3% w/w).

4.3.2 Microbial Inoculants

To select the most effective cold stress-protectants, five different treatments were tested in a pre-screening experiment: (i) Seed dressing with a commercial Zn/Mn formulation (0.2. ml Lebosol® Mn500 SC and 0.1 ml Lebosol® Zn700 SC 100 g^{-1} seeds, Lebosol® Dünger GmbH, Ermstein, Germany); (ii) fertigation with 10^9 spores Kg^{-1} DM ABI02, a cold-tolerant *Bacillus atrophaeus* strain (ABITEP, Berlin, Germany) combined with Zn/Mn seed dressing. (iii) fertigation with 10^8 spores Kg^{-1} DM Biological fertilizer OD (BFOD), a *Penicillium* sp. formulation (Bayer Crop Science Biologicals GmbH, Malchow, Germany) with documented cold–stress protecting effects combined with Zn/Mn seed dressing. (iv) Fertigation with 2.5×10^7 cfu Kg^{-1} DM Combi A^+, a microbial consortium formulation with Zn (13% w/w) + Mn (9% w/w) + *Trichoderma harzianum* OMG16 (9 $\times10^9$ spores g^{-1}) + Vitabac (1 $\times10^{11}$ cfu g^{-1}, as a mixture of *Bacillus licheniformis*, *B. megaterium*, *B. polymyxa*, *B. pumilis* and *B. subtilis*, Bactvita GmbH, Straelen, Germany).

Untreated variants exposed to cold stress and ambient greenhouse temperature were included as controls. In the follow-up experiments, only CombiA with ($CombiA^{+}$) and without Zn/Mn supplementation ($CombiA^{-}$) were used as inoculants.

The first inoculation was performed one week after sowing (WAS), followed by a 2nd application one day prior to exposure to low RZT 13 days after sowing (DAS) and a final application at the start of recovery phase (29 DAS). All microbial inoculants were applied through soil drenching with a dispenser pipette into the top-soil close to the seedlings roots.

4.3.3 Assessment of leaf damage, biomass, root length, and rhizosphere pH

At the final harvest (6 WAS), cold stress-induced oxidative leaf damage (chlorosis/necrosis, anthocyanin formation) was quantified by counting the number of damaged leaves $plant^{-1}$. Root systems were excavated, and rhizosphere soil was collected by shaking-off root adhering soil and mixed in a plastic bag. Thereafter, pH was determined according to VDLUFA (1991). Fresh weight (FW) of the shoot and root tissue was measured and finally dry weight (DW) was recorded after oven-drying at 65 °C. For root length determination, washed roots previously-stored in 30% ethanol, were separated, submerged in a water film on transparent Perspex trays, and subsequently digitalized using a flat-bed scanner (Epson Expression 1000 XL, Tokyo, Japan). The root length of the digitalized samples was measured by the use of the WinRHIZO root analysis system (Reagent Instruments, Quebec, QC, Canada).

4.3.4 Analysis of shoot nutrient contents

To assess the plant mineral nutrient status, dried shoot material was homogenized using a grinding mill (Labor Scheibenschwingmühle TS-100A, Sieb Technik GmbH, Mühlheim-Ruhr, Germany). After grinding, 250 mg of plant material was ashed in a muffle furnace for five hours at 500 °C. After cooling to room temperature, the samples were extracted as

described by Moradtalab et al., (2018). Spectrophotometrical determination (Hitachi U−3300, Hitachi LtD. Corporation Japan) of orthophosphate was conducted by molybdo-vandate method of Gericke and Kurmis (1952). Potassium and Ca were determined by flame emission photometry (ELEX 6361, Eppendorf, Hamburg, Germany). Magnesium, Fe, Mn, Zn, and Cu concentrations were measured by atomic absorption spectrometry (ATI Unicam Solaar 939, Thermo Electron, Waltham, USA).

4.3.5 Superoxide dismutase and peroxidase activity

Extraction and determination of superoxide dismutase (SOD, EC 1.15.1.1) and peroxidase (POD, EC1.11.1.7) activities were optimized for root and shoot tissues of maize according to the method described (Moradtalab et al. 2018). Spectrophotometrical determination (Spectrophotometer U−3300, Hitachi, Tokyo, Japan) of SOD activity was performed based on the nitro-blue tetrazolium (NBT) method at a wavelength of 650 nm (Beauchamp and Friedovich, 1971). The activity of POD was determined at 470 nm using the tetra-guaiacol assay described by Hajiboland and Hasani (2007).

4.3.6 Analysis of metabolites and antioxidants

Details for metabolite determinations have been described previously by Moradtalab et al. (2018). For the determination of soluble sugars, leaf and root samples were homogenized in 100 mM phosphate buffer (pH 7.5) at 4 °C. After centrifugation at 12000 g for 15 min, the supernatant was used for the determination of total soluble sugars by the anthrone-sulfuric acid method of Yemm and Willis, (1954). Total phenolics concentration was determined spectrophotometrically at 750 nm, using the Folin method (Hajiboland et al., 2017). For proline analysis, samples were homogenized with 3 % (v/v) sulfosalicylic acid and the homogenate was centrifuged at 3,000 g for 20 min. Proline was determined spectrophotometrically at 520 nm after acetic acid and acid ninhydrin derivatization (Bates

et al., 1973). The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) method was used to evaluate the free radical scavenging activity of antioxidants in the plant tissue (Panico et al., 2009).

4.3.7 UHPLC-MS analysis of phytohormones

Frozen maize tissue samples (shoot, roots) of 1 g of were ground to a fine powder with liquid nitrogen and extracted twice with 2.5ml of 80% methanol in falcon tubes. Thereafter, the samples were further homogenized by ultrasonication (Micra D-9 homogenizer, Art, Müllheim Germany) for 1min and 15 s at 10,000 rpm. Two milliliters of the methanol extracts were transferred to microtubes and centrifuged at 5,645 × g for 5min. Thereafter, 350 µl of the supernatant was mixed with 700 µl ultra-pure water and centrifuged at 5,645 × g for 5min. The supernatant was cleaned through a filtration membrane (Chromafil R O-20/15MS) and transferred to HPLC vials. UHPLC-MS analysis was carried out on a Velos LTQSystem (Thermo Fisher Scientific, Waltham, Massachusetts, USA) fitted with a Synergi Polar column, 4µ, 150 ∗ 3.0mm, (Phenomenex, Torrance, California, USA). The injection volume was 3 µL and the flow rate was adjusted to 0.5ml min^{-1} for gradient elution with mobile phase (A): water and 5% acetonitrile; mobile phase (B): acetonitrile and a gradient profile of 0–1min, 95% A, 5% B, 11–13min, 10% A, 90% B, 13.1min, 95% A, 5% B, 16min 95% A, 5% B). All standards were purchased from Sigma Aldrich, (Sigma Aldrich, St. Louis, Missouri, USA) including (+/–)-jasmonic acid; 3-indoleacetic-acid, gibberellic acid, (+/–) abscisic acid; trans-zeatin; salicylic acid (Moradtalab et al., 2018).

4.3.8 Expression of hormonal target genes

The expression of selected target genes was analyzed to evaluate potential interactions of applied cold stress protectants with hormonal signaling. Relative expression of the IAA efflux transporter *ZmPIN1a (PINFORMED)*, the auxin response factor 12 (*ZmAFR12*),

tryptophan synthase (*ZmTSA*), the auxin early response gene *ZmAuxIAA*, the abscisic acid responsive element-binding factor 2 (*ZmABF2*) and the isopentenyl transferases (*ZmIPT4* and *ZmIPT5*) were determined. Elongation factor-1alpha (*EF1α*) and *tubulin* were selected as reference genes. The results are expressed according to *EF1α* with particularly stable expression under cold stress (Tang et al., 2017). Isolation of mRNA and RT-qPCR quantification of relative transcript abundances was performed from frozen root material by GenXPro GmbH, Frankfurt am Main, Germany.

4.3.9 Rhizosphere tracing of *Trichoderma harzianum* OMG16

For the quantification of the *T. harzianum* strain OMG16 (DSMZ accession no.: 32722) in the maize root endosphere, roots were thoroughly cleaned with a soft brush and water to remove residual soil particles, shortly dried between paper towels and cut into small pieces. Approximately 80 mg fine roots were placed in 2 mL tubes containing 1.0 mm silica spheres including one single 0.25-inch ceramic bead (MP Biomedicals, France) and 400 µL peqGOLD lysis buffer (VWR Peqlab, Germany). Root tissue was homogenized for 3x 30 s at a speed of 6 m/s in a FastPrep 24 bead-beating system (MP Biomedicals). After each cycle samples were cooled on ice for 1 min. DNA was subsequently extracted utilizing the peqGOLD Fungal DNA Kit (VWR Peqlab), following the manufacturer's instructions. DNA was eluted in a TE buffer (pH 8.0) and checked on 0.8% TAE agarose gels. DNA concentrations were determined using a Qubit® 3.0 Fluorometer and the Qubit dsDNA HS Assay Kit according to the instructions of the manufacturer (Thermo Fisher Scientific, Germany). A *T. harzianum* OMG16-specific primer pair, designed from OMG16 genomic DNA sequences were used for qPCR quantification of *T. harzianum* OMG16 DNA in the DNA samples according to the method described by Mpanga et al., (2019).

4.4 Statistical analysis

The study was carried out in a completely randomized design. Data are presented as means ±SD. For statistical analysis of significant differences between treatment groups, a one-way ANOVA followed by a Tukey-test ($p < .05$) were performed using the SAS software 9.4 (SAS Institute Inc., Cary, NC, USA). Pairwise comparisons (t-Test, $p < .05$) were conducted with the Sigma Plot 13 software package (SYSTAT Software Inc., Erkrath, Germany).

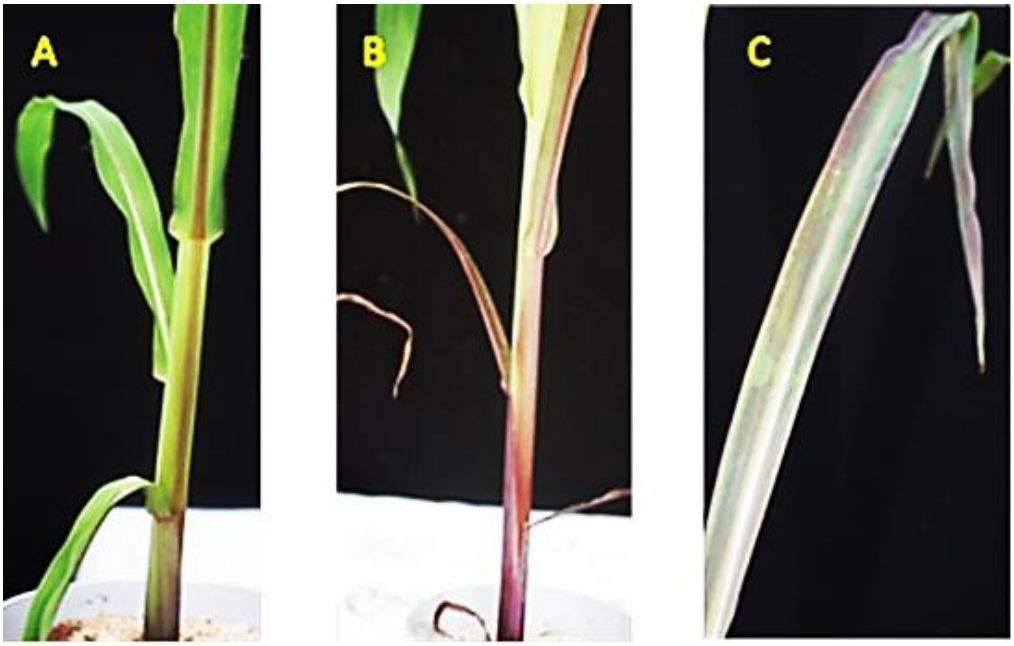
4.5. Results

4.5.1 Cold-protective effects of the CombiA+ consortium as related to N forms

The first experiment addressed the impact of ammonium fertilization versus nitrate supply on the cold-protective performance of the CombiA$^+$ consortium. The form of N had no significant effect on the shoot biomass of non-stressed control plants (Table 2). No macro- or micronutrient deficiencies were recorded, irrespective of the N fertilization regime (Table S2), while shoot P and Zn accumulation were significantly increased in the ammonium variant (Table S2), associated with a decline in rhizosphere pH by 0.6 units as compared with nitrate fertilization (Table 2). The two weeks cold-stress period decreased shoot biomass production of the plants with nitrate supply by 25 % (Table 2), associated with a 133 % increase in oxidative leaf damage, indicated by chlorosis, necrosis, and stress-induced anthocyanin formation (Figs.1 and 2).

Chapter 4. Table. 2. Shoot dry weight (DW), oxidative leaf damage (number of damaged leaves plant-1), Zn and Mn shoot concentrations and rhizosphere pH of maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14 °C) with (CombiA$^+$) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. Data represent the means and SD of five replicates. In each row, different letters indicate significant differences (Tukey-Test, $p < 0.05$).

N-Form	Stress Factor	Treatment	Shoot DW [g]	Oxidative leaf damage [Leaf number]	Zn [µg g $^{-1}$DW]	Mn [µg g $^{-1}$DW]	pH Mean value ($CaCl_2$)	pH Δ (Rhizo-Bulk soil)
Nitrate	No-Cold	Ctrl	6.0±0.6 a	2.40±0.54 c	46.0±0.8 b	50.5±1.9 b	7.3±0.02 a	+0.4
	8–14 °C	Ctrl	4.5±0.5 b	5.6±0.89 a	24.4±3.9 c	34.8±5.9 c	7.2±0.03 b	+0.3
		Combi A^+	5.8±0.7 a	3.4±0.554 b	47.8±5.6 b	48.0±1.3 b	7.0±0.05 c	+0.1
Ammonium	No-Cold	Ctrl	6.6±0.2 a	1.6±0.53 c	59.0±2.0 a	51.0±2.6 b	6.7±0.02 d	-0.2
	8–14 °C	Ctrl	6.2±0.4 a	3.6±0.52 c	59.4±3.8 a	53.7±1.3 ab	6.7±0.02 d	-0.2
		Combi A^+	6.9±1.1 a	2.4±0.53 bc	57.6±5.9 a	52.9±3.4 ab	6.6±0.03 e	-0.3

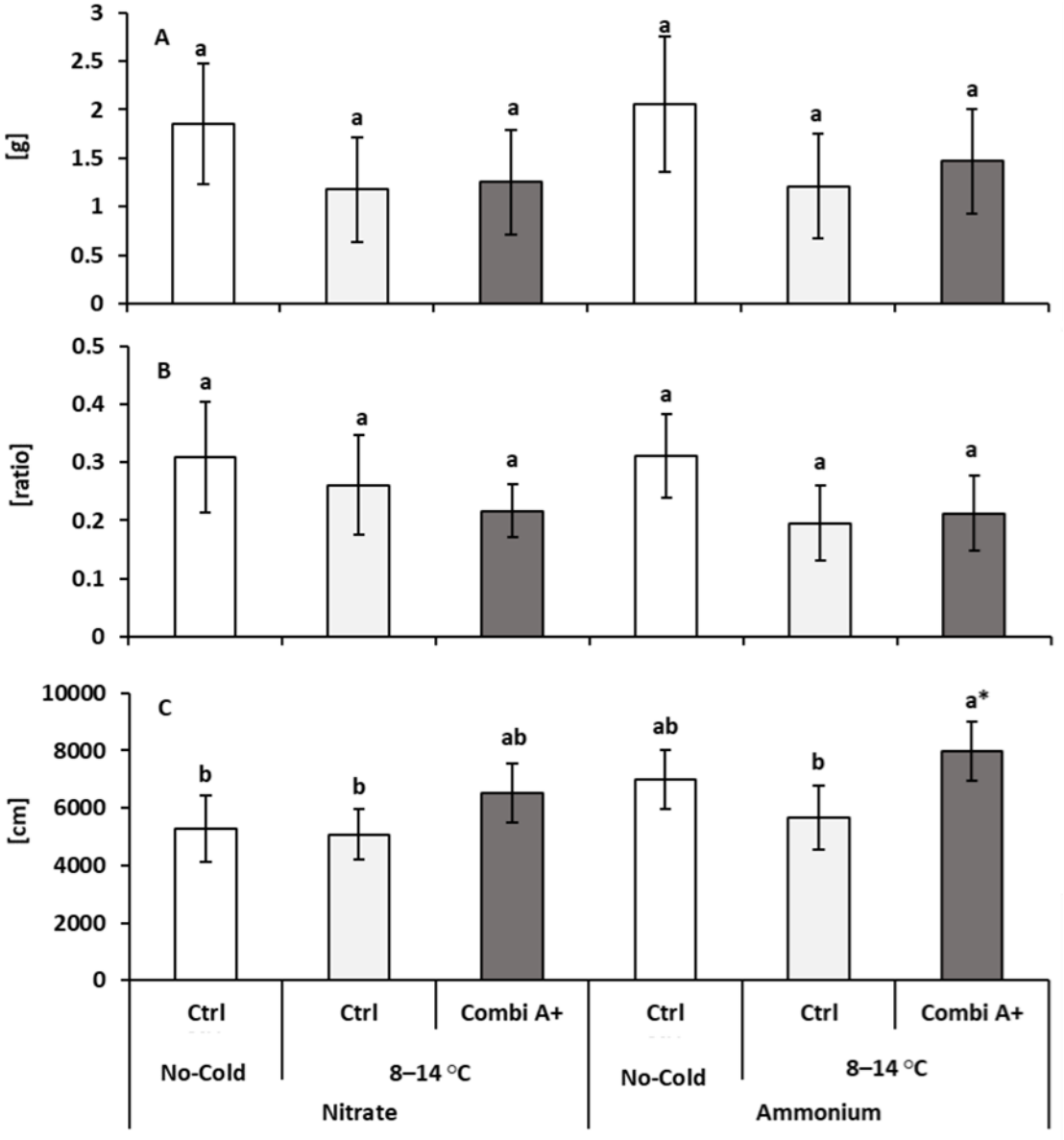


Chapter 4. Fig. 1. A) Undamaged leaves of non-stressed maize plants grown for four weeks under ambient greenhouse temperature (18–25 °C), B) and C) Oxidative leaf damage and symptoms of P limitation (chlorosis, necrosis, stress-anthocyanins) of cold stressed- plants exposed to two weeks of reduced root zone temperature (8–14 ° C).

Compared with nitrate fertilization, oxidative leaf damage was significantly lower in the ammonium variant and there was no significant decline in shoot biomass (Table 3). The Zn and Mn-nutritional status in cold-stressed plants with nitrate supply dropped close to the deficiency threshold but remained in the sufficiency range in combination with ammonium fertilization (Table 2; Table S2), associated with a lower rhizosphere pH. Nevertheless, ammonium-induced rhizosphere acidification had no effect on the P-

nutritional status, which declined below the deficiency threshold in the cold-stressed plants (Table S2).

The application of the microbial consortium product CombiA$^+$ significantly increased shoot biomass production in the cold-stressed nitrate variant with the same trend in combination with ammonium supply (Table 2), which additionally increased total root length (Fig. 2). Under both N form regimes, CombiA$^+$ application significantly reduced cold stress-induced oxidative leaf damage but only in the ammonium variant, the level of leaf damage was not significantly different from the non-stressed control (Table. 2) and the P status reached the sufficiency range (Table S2). No significant differences were recorded for root biomass and the root/shoot biomass ratio (Fig. 2)



Chapter 4. Fig. 2. Root dry weight (A), root/shoot biomass ratio (B) and total root length (C) of maize plants exposed to a 2-weeks period of reduced root zone temperature (RZT, 8–14 °C) on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14 °C) with (CombiA^{+}) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. Data represent the means and SD of five replicates. Different letters indicate significant differences (Tukey-Test, $p < 0.05$).

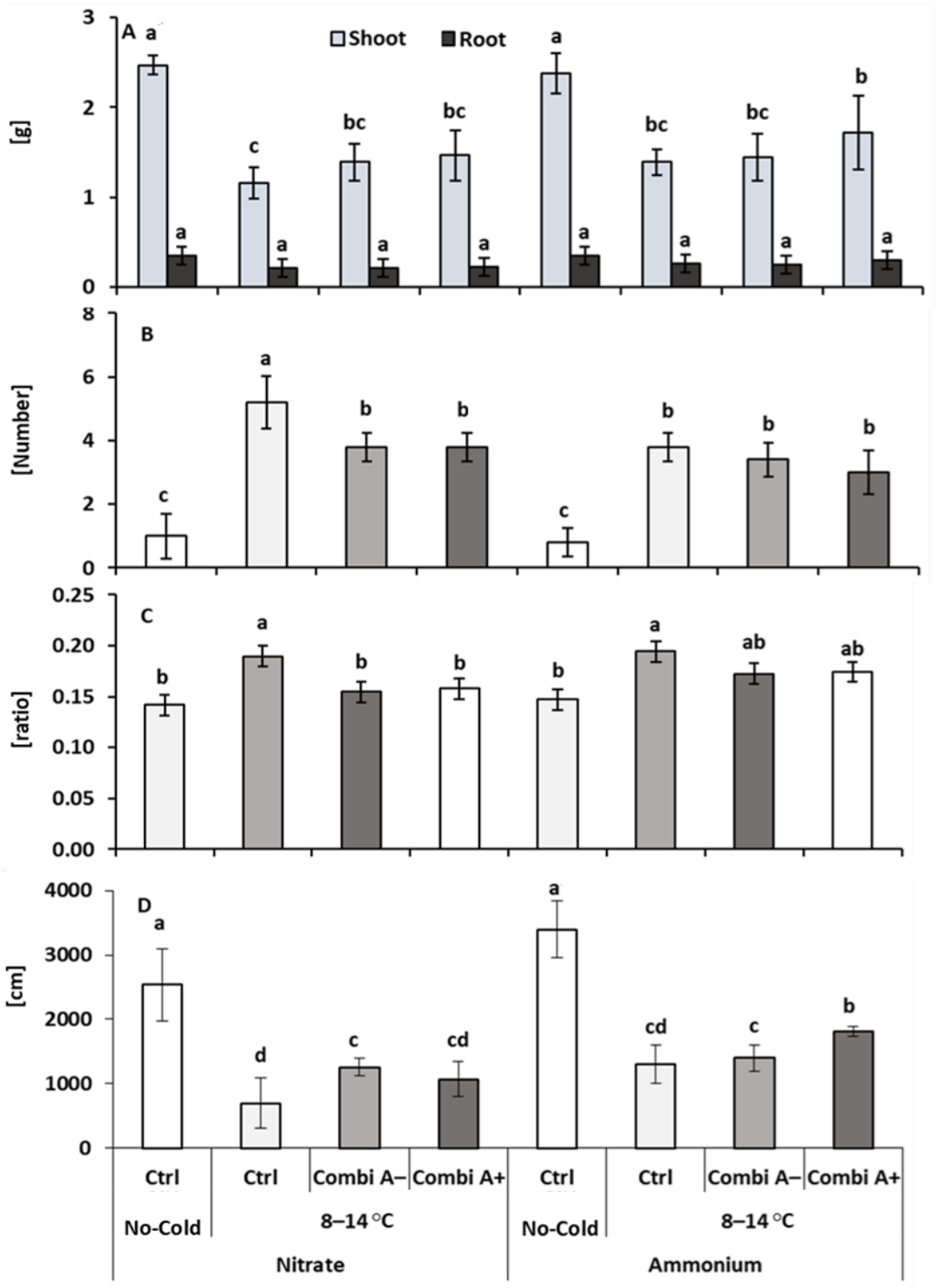
4.5.2 Synergistic effects of N-form supply, micronutrient supplementation and PGPM inoculants adaptive cold-stress responses in maize

A second experiment was conducted to dissect the individual contributions of ammonium fertilization, Zn/Mn supplementation and CombiA application to the cold-protective effect at the physiological and molecular level. Micronutrient effects were identified by

comparison of MCP inoculant formulations with (CombiA^{+}) and without additions of Zn/Mn (CombiA^{-}).

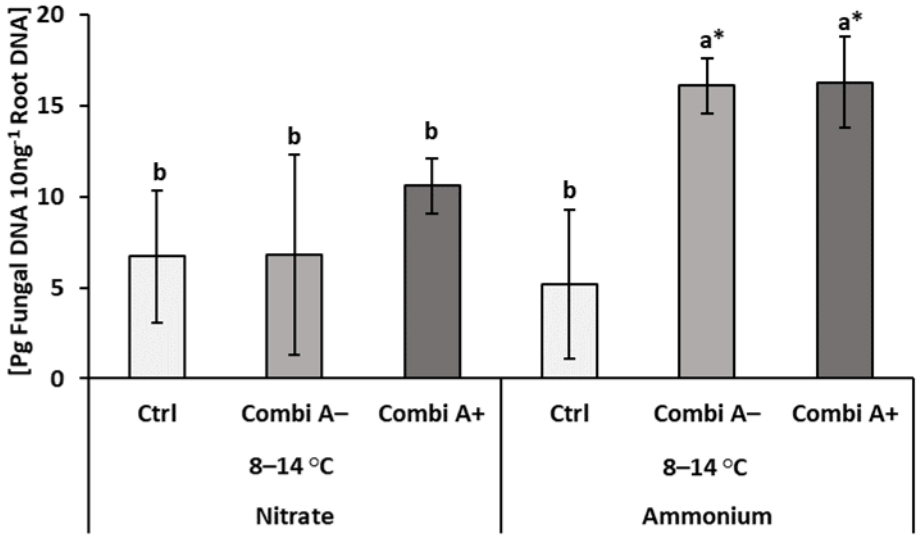
4.5.2.1 Plant growth and MCP root colonization

Superior cold-protective performance by combined application of stabilized ammonium, Zn/Mn supply, and the PGPM consortium as compared with nitrate fertilization (described under 5.1) was confirmed also in the second experiment. This was reflected in the highest shoot biomass production (+48%), increased root length (+161%) and the lowest level of cold-stress induced oxidative leaf damage (-42%). Ammonium fertilization and particularly CombiA^{+} application reverted cold-stress induced Zn limitation of the host plants, while root biomass remained unaffected (Fig. 3).



Chapter 4. Fig. 3. Shoot and root dry weight (A), oxidative leaf damage (B) root/shoot biomass ratio (C) and root length (D) of maize plants exposed to a 2-weeks period of reduced root zone temperature (RZT, 8–14 ○C) on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants including untreated control (Ctrl), Combi A^- (without Zn/Mn) and Combi A^+ (containing Zn/Mn) under nitrate or ammonium fertilization. Means and SD of five replicates. Different letters: significant differences (Tukey-Test, $p < .05$).

A strain-specific primer was available for *Trichoderma harzianum* OMG16 in the CombiA formulation. This enabled rhizosphere tracing to evaluate the root colonization efficiency of the inoculant. Traces of *T. harzianum* OMG16 DNA were detectable also in the root samples of non-inoculated controls. A significant increase in OMG16 root colonization was recorded exclusively in CombiA-inoculated roots of maize plants with ammonium fertilization and was not affected by additional Zn/Mn supplementation (Fig. 4).



Chapter 4. Fig. 4. Root colonization with Trichoderma harzianum OMG16 of maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14 °C) with ($CombiA^{-}$; $CombiA^{+}$) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. $CombiA^{-}$ formulation without Zn/Mn; $CombiA^{+}$ formulation with Zn/Mn. Data represent the means and SD of five replicates. Different letters indicate significant differences (Tukey-Test, $p < 0.05$).

4.5.2.2 Accumulation of antioxidants and cryoprotective solutes

Cold stress increased the shoot concentration of proline with cryo-protective an anti-oxidative functions (Szabados, Savouré 2010) by 67 % under nitrate supply and by 200 % in the ammonium variant. A significant increase in soluble sugar concentrations was recorded only in combination with nitrate fertilization. Finally, the highest shoot concentrations of proline and soluble sugars accumulated in cold-stressed maize plants with

CombiA inoculation and ammonium supply. This could be attributed to the presence of the MCP inoculant since additional Zn/Mn supplementation had no additional effects (Table 3).

Total phenolics and antioxidants increased in the shoot tissue of cold stressed maize plants particularly in combination with ammonium fertilization, while antioxidants in roots rather declined. Again, CombiA inoculation combined with ammonium supply resulted in the highest accumulation of phenolics and total antioxidants, both, in shoot and root tissues. Additional Zn/Mn supplementation further increased the root concentrations of antioxidants (Table 3).

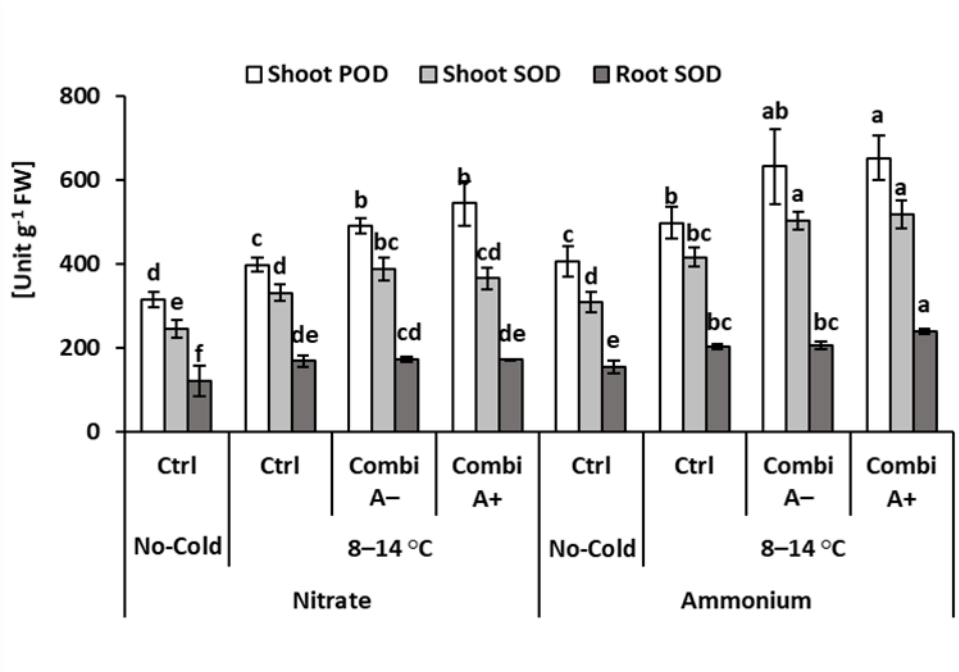
Chapter 4. Table. 3. Tissue concentrations of sugars, proline, total phenolics and total antioxidants in maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14 °C) with (CombiA^{-}; CombiA^{+}) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. CombiA^{-} formulation without Zn/Mn; CombiA^{+} formulation with Zn/Mn. Data represent the means and SD of five replicates. In each row, different letters indicate significant differences (Tukey-Test, $p < 0.05$). *: significant in pairwise comparisons with RTZ untreated Ctrl (t-Test, $p < 0.05$).

			Shoot				Root
N-Form	Stress Factor	Treatment	Sugar [mg g^{-1} FW]	Proline [mg g^{-1} FW]	Phenolics [mg g^{-1} FW]	Total antioxidants [%]	Total antioxidants [%]
Nitrate	No-Cold	Ctrl	1.7±0.2 d*	0.3±0.04 d*	3.1±0.1 d*	52.5±0.94 d*	39.0±2.5 c*
	8–14 °C	Ctrl	2.3±0.2 c	0.5±0.05 c	3.9±0.1 c	67.1±1.89 c	25.7±3.6 d
		Combi A^{-}	3.2±0.4 b*	0.7±0.04 b*	4.2±0.4 c	91.4±1.40 b*	62.3±4.7 b*
		Combi A^{+}	2.9±0.6 c	0.7±0.03 b*	4.6±0.2 bc*	85.7±6.93 b*	69.6±5.4 ab*
Ammonium	No-Cold	Ctrl	2.3±0.2 c	0.2±0.03 d*	3.9±0.1 c*	58.0±3.80 d*	39.4±2.7 c
	8–14 °C	Ctrl	2.5±0.2 c	0.6±0.03 c	4.9±0.1 b	77.1±3.63 bc	29.0±4.0 cd
		Combi A^{-}	3.8±0.1 a*	1.0±0.04 a*	5.5±0.5 ab	97.1±1.97 a*	71.5±5.3 b*
		Combi A^{+}	3.6±0.2 a*	0.9±0.04 a*	5.6±0.4 a*	98.9±5.92 ab*	87.1±5.2 a*

4.5.2.3 Enzymatic ROS detoxification

Activities of superoxide dismutase and peroxidase were determined as key enzymes involved in the detoxification of cold-stress-induced production of reactive oxygen species. Accordingly, the lowest SOD and POD activities were recorded in the non-stressed controls

but with higher values in the ammonium variants. Cold stress further increased SOD and POD in root and shoot tissues with higher levels in plants with ammonium fertilization as compared with nitrate supply. After all, the highest activities were found after CombiA inoculation in combination with ammonium supply. This effect could be mainly attributed to the presence of the MCP inoculant with a small additional impact of Zn/Mn supplementation. (Fig. 5).



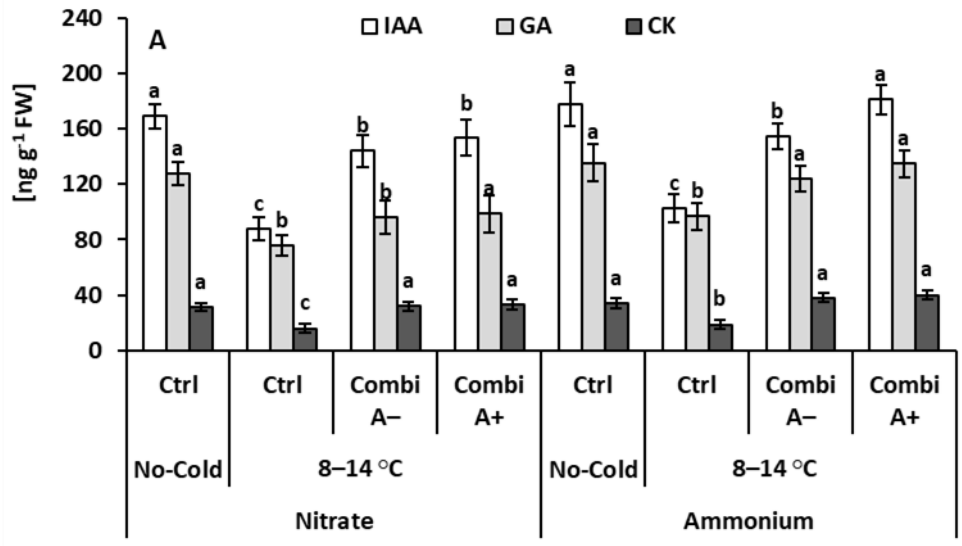
Chapter 4. Fig. 5. Activities of peroxidase (POD) and superoxide dismutase (SOD) in maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14 °C) with (CombiA^{-}; CombiA^{+}) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. CombiA^{-} formulation without Zn/Mn; CombiA^{+} formulation with Zn/Mn. Bars represent the means and SD of five replicates. For each enzyme, different letters indicate significant differences (Tukey-Test, $p < 0.05$).

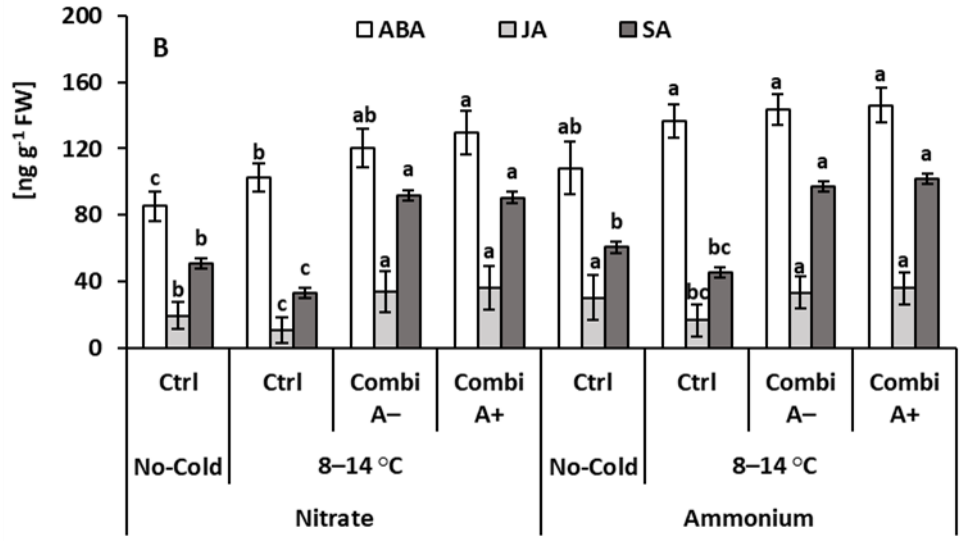
4.5.2.4 Interactions with hormonal homeostasis

The form of N supply had no effects on the shoot concentrations of the growth hormones indole acetic acid (IAA), gibberellic acid (GA) and zeatin (CK) in non-stressed controlled plants, while the concentrations declined in the cold stress variants without significant

differences between plants with nitrate or ammonium supply. The negative cold stress effect on shoot concentrations of IAA, GA and CK was reverted by CombiA inoculation and more strongly expressed for the GA concentrations in plants with ammonium fertilization as compared with nitrate supply. Additional Zn/Mn supplementation further increased the IAA concentrations in the ammonium variants (Fig. 6).

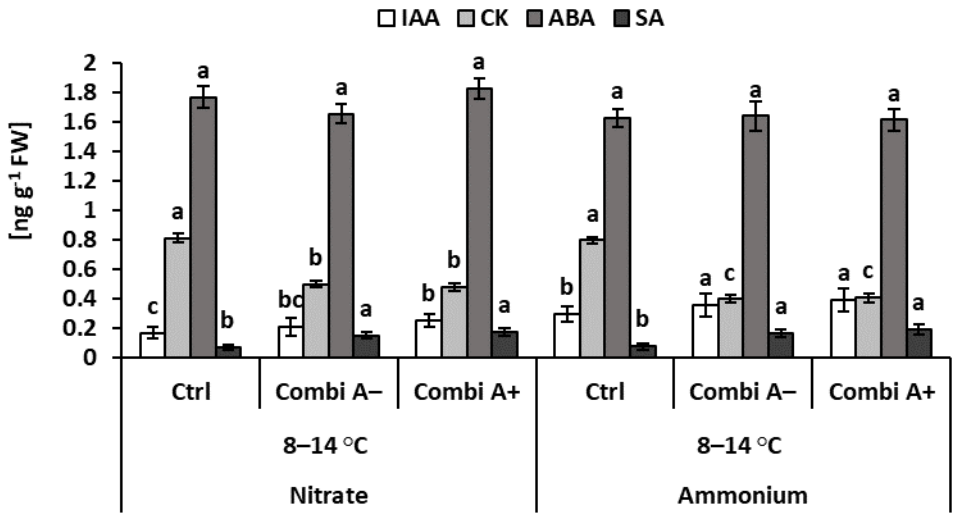
Ammonium fertilization increased the concentrations of the stress hormones abscisic acid (ABA) and jasmonic acid (JA) even in the shoot tissue of non-stressed control plants. Cold stress further increased the ABA levels particularly in the ammonium variant, while the concentrations of JA and salicylic acid (SA) declined. By contrast, JA and SA concentrations increased after CombiA inoculation while ABA increased only in the nitrate variant but not with ammonium supply. Additional effects of Zn/Mn supplementation in the CombiA^{+} variants were not detectable (Fig. 6).





Chapter 4. Fig. 6. Endogenous concentrations of growth (A), stress (B) phytohormones in the shoot tissue of maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14 °C) with (CombiA^{-}; CombiA^{+}) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. CombiA^{-} formulation without Zn/Mn; CombiA^{+} formulation with Zn/Mn. IAA= indole acetic acid: GA= gibberellic acid; CK = cytokinins (zeatin); ABA = abscisic acid; JA = jasmonic acid; SA = salicylic acid. Bars represent the means and SD of five replicates. For each hormone, different letters indicate significant differences (Tukey-Test, $p < 0.05$).

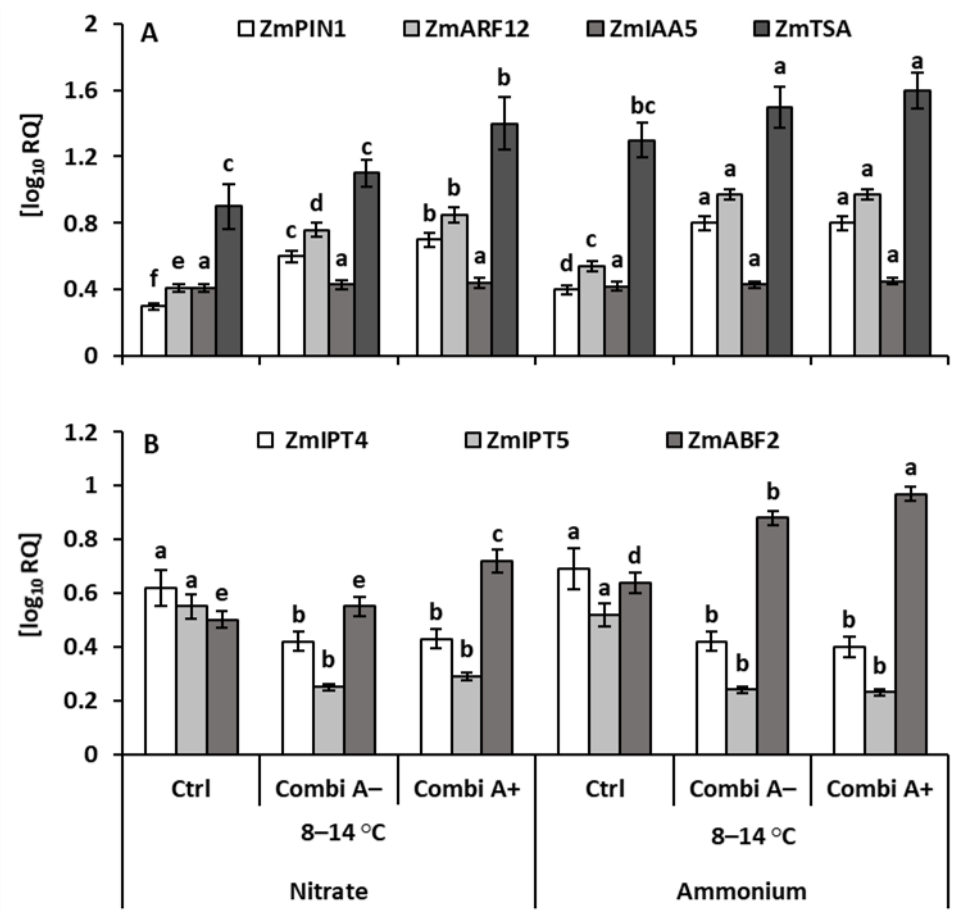
In the roots of cold stressed plants, ammonium supply and particularly the combination with CombiA inoculation increased the IAA tissue concentrations by 75 % and 131%, respectively (Fig. 7), as similarly recorded also for the shoot tissue (Fig. 6). Ammonium fertilization had no effect on the level of root cytokinins (zeatin) but root CKconcentrations declined in response to CombiA application, contrary to the opposite effect, recorded in the shoot tissue (Fig. 6). The decline in CK concentrations was particularly expressed in the variants with ammonium supply (-50%). CombiA also increased SA concentrations in the root tissue without additional effects by Zn/Mn supplementation (Fig. 7). No treatment differences were detectable for root ABA concentrations. Jasmonic acid (JA) ranged below the detection limit.



Chapter 4. Fig. 7. Endogenous concentrations of phytohormones in the root tissue of maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9. Low RZT variants (8–14 °C) with (CombiA^{-}; CombiA^{+}) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. CombiA^{-} formulation without Zn/Mn; CombiA^{+} formulation with Zn/Mn. IAA= indole acetic acid; CK = cytokinins (zeatin); ABA = abscisic acid; SA = salicylic acid. Data represent the means and SD of five replicates. For each hormone, different letters indicate significant differences (Tukey-Test, $p < 0.05$).

In accordance with the increased IAA concentrations in the root tissue (Fig. 7), expression of the tryptophan synthase gene (*ZmTSA*), involved in the biosynthesis of IAA and other indole compounds (Mano and Nemoto, 2012), was enhanced in response to ammonium fertilization and further increased in combination with CombiA inoculation. This was associated with a correspondingly increased expression of genes encoding for the auxin transporter *ZmPIN1* (Li et al., 2018) and the auxin response factor 12 (*ZmAFR12*) involved in IAA perception (Fig. 8A). The *ZmAuxIAA5* gene was selected as a well-studied member of the auxin early response genes, found to be rapidly up-regulated by external auxin supply (Park and Hasenstein, 2015), to test responses to potential IAA production of the inoculants but in this case, no significant treatment differences were detectable (Fig. 8A). Declining cytokinin concentrations recorded in the root tissue of CombiA-inoculated plants (Fig. 7) were reflected in decreased expression of the genes encoding the isopentenyl transferases4,5 (*ZmIPT4,5*) involved in cytokinin biosynthesis. (Fig. 8B). Ammonium

fertilization and particularly the combination with CombiA$^+$ inoculation increased gene expression of the ABA-responsive ABA-binding factor2; ABF2 (Fig. 8B).



Chapter 4. Fig. 8. Transcript abundances of root tissue in maize plants exposed to a 2-weeks period of reduced root zone temperature (RZT, 8–14 °C) on silty loam soil, pH 6.9. Low RZT variants including untreated control (Ctrl), Combi A$^-$ (formulation without Zn/Mn) and Combi A$^+$ (formulation with Zn/Mn) under nitrate or ammonium fertilization. Means and SD of five replicates. For each gene, different letters: significant differences (Tukey-Test, $p < .05$). PIN1: PINFORMED1, ARF12: Auxin response factor12, IAA5: Aux/IAA-transcription factor5, TSA: tryptophan synthase, IPT4,5: Isopentenyl transferases 4 and 5, ABF2: Abscisic acid-binding factor2.

4.6. Discussion

4.6.1 Cold protective effects induced by the form of N supply

In both experiments, a certain cold protective effect of stabilized ammonium supply compared with nitrate fertilization was indicated by a 27-36% decline of oxidative leaf damage (chlorosis, necrosis; Fig. 1), detectable at the end of the 2-weeks cold stress period

(Table 2 and Fig. 3). Ammonium fertilization counteracted cold-stress induced zinc and manganese deficiencies, which dropped to critical levels (Campbell and Plank 2013) in the cold-stressed plants with nitrate supply (Table 2; S2). Hence, micronutrient deficiencies (Zn, Mn, Fe) have been characterized as growth-limiting factors for cold-stressed maize plants also in previous studies, reverted by micronutrient supplementation via seed priming (Imran et al., 2013), seed dressing or fertigation (Bradacova et al., 2016; Moradtalab et al., 2018) prior to the onset of the stress period. Under ammonium fertilization, increased shoot concentrations of Zn and Mn were related to the well-documented ammonium-induced rhizosphere acidification (Römheld and Marschner, 1983; Neumann and Römheld 2002) by 0.6 pH units compared with nitrate supply (Table 2), which apparently increased Zn and Mn solubility in the rhizosphere. However, the rhizosphere acidification effect was obviously not sufficient to mobilize significant amounts of P, since the P status remained in the deficiency range (Table S2).

Components of both, enzymatic and non-enzymatic ROS detoxification are particularly dependent on sufficient micronutrient supplying (Cakmak, 2000; Datnoff et al., 2007), providing enzymatic co-factors for superoxide dismutases (Zn, Mn, Fe, Cu), peroxidases (Fe) and enzymes involved in biosynthesis of phenolics with antioxidative potential (Mn, Cu). Consequently, Zn/Mn supplementation by seed dressing increased superoxide dismutase (SOD) activity, accumulation of phenolics and antioxidants associated with a decline in ROS accumulation and reduced oxidative leaf damage in cold-stressed maize plants (Bradacova et al., 2016; Moradtalab et al. 2018). Similarly, in this study, an improved Zn/Mn status in response to ammonium-induced rhizosphere acidification (Table 2) may be related with increased activities of superoxide dismutases (+25%) and peroxidases (+25 %; Fig, 5) and increased Mn-dependent shoot accumulation of phenolics

(+26%; Table 3), while oxidative leaf damage declined by 27% (Fig. 3). This was associated with increased shoot concentrations of abscisic acid (ABA, Fig. 6) as a central regulator of cold stress adaptations in plants (Szalai et al., 2011; Eremina et al., 2016). Direct links between ABA and enzymatic ROS detoxification in cold-stressed plants have been reported by Kumar et al. (2008), Szalai et al. (2011), Li and Zhang (2012) and Moradtalab et al., (2018), while Peuke et al. (1994) reported ammonium-induced stimulation in root to shoot translocation of ABA in *Ricinus* seedlings Accordingly, the cold-protective effect of ammonium fertilization observed in this study may be related with a stimulatory effect on ABA accumulation in the shoot tissue, which promoted the expression of enzymatic (SOD, POD, Fig. 6) and non-enzymatic (phenolics, Table 3) ROS detoxification. Similarly, ammonium-induced induction of ABA accumulation and a relationship with improved oxidative stress defence was reported also in response to other abiotic stress factors such as drought and salinity (Hessini et al., 2013; Ding et al., 2016).

In the root tissue of cold stressed plants, ammonium fertilization significantly increased the IAA concentration by 41 % as compared with nitrate supply (Fig. 7), with a similar trend for shoot IAA, which was not detectable in the absence of cold stress. In our study, this was related to a significantly increased expression of the *ZmPIN1a* gene (Fig. 6A), encoding an auxin efflux carrier with functions in the lateral root formation in maize (Li et al., 2018). Increased gene expression was recorded also for the auxin response factor 12 (*ZmAFR12*) involved in IAA perception (Fig. 6A) and upregulated in cold-stressed maize plants (Sobkiwiak et al., 2014). Thus, a trend for increased root length development in the ammonium-treated plants was recorded in both experiments, although this difference was not statistically significant (Fig. 3). Excessive production of ROS can promote the oxidative degradation of IAA. and resulted in a 50% reduction of IAA contents in Zn-deficient

Phaseolus vulgaris, which was suppressed due to Zn fertilization (Cakmak et al., 1989), promoting enzymatic ROS detoxification (Cakmak, 2000). Similarly, a ROS-protective effect of higher SOD activities recorded in the root tissue of ammonium-treated plants (Fig. 5) may counteract oxidative IAA degradation and provide an explanation for greater root IAA levels in cold-affected maize over nitrate-treated plants (Fig. 7).

As an additional beneficial effect of ammonium fertilization, root colonization by the CombiA-PGPM strain *Trichoderma harzianum* OMG16 was increased in comparison with plants supplied with nitrate fertilizer (Fig. 4). The reasons for this preference are not entirely clear but recently Mpanga et al. (2019b) found ammonium-induced promotion of root hair development in P-deficient maize plants, also identified as limiting nutrients in this study (Table S2). This may provide additional infection sites, since preferential colonization of root hairs has been reported for various strains of *Trichoderma harzianum*, including T22 and OMG16 (Harman 2000; Mpanga et al. 2019b). Additionally, the various cold stress-protective effects, induced by ammonium fertilization as described above, may improve the rhizosphere establishment of the inoculants by strengthening the host plant. Similarly, improved performance of a wide range of bacterial and fungal PGPMs, including single strain inoculants and microbial consortia in combination with stabilized ammonium fertilization, has been documented in various pot and field experiments under conditions of P limitation (Mpanga et al., 2019 a,b; Bradacova et al., 2019 a,b).

4.6.2 Cold-protective effects of the CombiA inoculation as related to Zn and Mn supplementation

For both forms of nitrogen fertilization, the inoculation with CombiA induced cold-protective effects in maize plants, which were still detectable after a two-weeks recovery period at soil temperatures ≥ 20°C. This may indicate not only direct stress mitigation, as

indicated e.g. by reduced oxidative leaf damage recorded at the end of the 2-weeks cold stress period (Table 2; Fig.3) but also due to the induction of longer-lasting stress priming effects.

The most intense expression of cold protection in terms of increased shoot biomass production, reduced oxidative leaf damage, and stimulation of root growth, was recorded for the ammonium-CombiA^{+} combination (Table 2; Figs. 2 and 3). The effects were detectable in both experiments conducted under controlled root zone temperatures (RZT), although shoot biomass production was different, probably due to differences in ambient air temperature at the greenhouse (Fig. S3).

The two-weeks cold stress treatments with 8–14°C RZT reduced the shoot dry matter production by 25-52 % when nitrate was the N source (Table 2; Fig. 3). This was associated with a significant decline in the shoot concentrations of the growth hormones IAA, GA, and CK (Zeatin) by 48, 41 and 49 %, respectively (Fig. 6), as previously reported also by Moradtalab et al. (2018). Reduction of shoot growth is regarded as a component of cold stress adaptations, which is actively regulated by a reduction of bioactive growth-promoting gibberellic acid (GA) levels, leading to an increased abundance of nuclear DELLA-protein growth repressors via a signaling pathway involving CBF/DREB1 transcription factors (Miura and Furumoto, 2013; Eremina et al., 2016). However, the decline of GA and IAA in cold stressed plants have been also related to cold-induced Zn-limitation (Moradtalab et al., 2018) since reduced GA and IAA levels are characteristic for Zn-deficient plants (Suge et al., 1986; Sekimoto et al., 1997; Cakmak et al. 1989). More recently, it was shown that various steps of GA biosynthesis depend on the presence of IAA (Ross et al., 2000) and Zn limitation promotes oxidative IAA degradation and impairs auxin transport (Cakmak et al., 1989; Shibasaki et al., 2009).

Interestingly, two weeks after recovery from the cold stress treatment, CombiA inoculation particularly in combination with ammonium fertilization, restored the concentrations of IAA, GA, and CK to the levels characteristic for non-stressed plants (Table 4). This was associated with the lowest level of oxidative leaf damage, increased shoot biomass production (Table 2, Figs. 2 and 3), increased enzymatic (POD, SOD) and non-enzymatic (total antioxidants, phenolics, proline) ROS defence and accumulation of cryoprotectants (Table 4 and Fig. 5), indicating an improved recovery from the cold stress treatment. Strengthening of ROS detoxification in the shoot tissue was not related to Zn and Mn supplementation by the CombiA^{+} treatment since the same effect was observed also for CombiA^{-} inoculation without additional micronutrient supply (Table 4 and 5). In this case, the improved micronutrient supply, induced by ammonium fertilization (Table 2), was already sufficient to cover the requirements of the systems for ROS detoxification.

Also increased ABA production with the potential to trigger ROS defence was not detectable in CombiA treatments but was characteristic for sole ammonium supply (Fig. 6). By contrast, CombiA inoculation was related to increased shoot accumulation of JA and SA (Fig. 6). This points to induction of induced systemic resistance (ISR) via JA and SA signaling pathways, which is well documented for various *Trichoderma* and *Bacillus* strains, with stimulatory effects e.g. on the accumulation of phenolics and POD activity (García-Gutiérrez, 2013; Martınez-Medina et al. 2013; 2014, Shahzad et al. 2017). Unfortunately with the currently available data set, it is not possible to unfold the individual contributions of the selected *Trichoderma* and *Bacillus* inoculants to the ISR effect. This would require additional experiments with single-strain inoculations. Although abscisic acid is considered as a central regulator of cold stress responses in plants, it seems to regulate the adaptive expression of cold- related genes in cross talks involving also SA and

JA (Szalai et al., 2011; Eremina et al., 2016). This may also explain the improved cold acclimation by CombiA inoculation via ISR-induced production of JA and SA (Table 5). The only superior cold-protective feature related to the increased Zn/Mn supply provided by CombiA^{+} in combination with ammonium fertilization recorded in this study was the increased accumulation of antioxidants in the root tissue (Table 3), which promoted root elongation (Figs. 2 and 3). Since at the same time, root biomass production was not significantly affected, obviously fine root production was stimulated, characterized by a higher root length with the same root biomass after CombiA^{+} application as compared to the CombiA^{-} variant, (Figs 2 and 3). This may indicate a protective effect against oxidative auxin degradation leading to root growth inhibition (Cakmak et al., 1989., Moradtalab et al., 2018). Generally, CombiA inoculation increased IAA concentrations not only in the shoot but also in the root tissue, associated with a decline in root CK (Fig. 5). This was related to increased expression of auxin-responsive genes involved in IAA biosynthesis (*ZmTSA*), transport (*ZmPIN1A*) and IAA signal perception (*ZmARF12*), while the expression of genes involved in CK biosynthesis (*ZmIPT4* and *5*) declined (Fig. 6A). By contrast, the expression of the *AuxIAA5* gene (*ZmIAA5*), reported to be rapidly activated by exogenous IAA supply (Park and Hasenstein, 2016), was not changed by CombiA inoculation. This finding suggests that CombiA rather acted via signals interacting with internal IAA homeostasis of the host plant and not via microbial IAA production. Accordingly, Garnica-Vergara (2015) found an auxin-independent activation of *PIN* genes (*PIN1, PIN2, PIN3, PIN7*), associated with increased lateral root formation in *Arabidopsis thaliana* by 6-pentyl-2H-pyran-2-one (6-PP), a major bioactive volatile organic compound (VOC) with potential cross-kingdom signaling functions, emitted by *Trichoderma* spp. Interestingly, increased levels of IAA and declining CK concentrations in the root tissue in

response to *Trichoderma* inoculation detected in our study, have been observed also in earlier reports with melon seedlings and cherry rootstocks (Sofo et al., 2011; Martínez-Medina et al., 2014). This strongly suggests that the corresponding hormonal changes induced by CombiA inoculation (Figs. 5 and 6) are likely caused by the *T. harzianum* OMG16 strain in the inoculum, associated with preferential root colonization in combination with ammonium fertilization (Fig. 4). Similar effects on root growth and plant IAA homeostasis have been reported also for certain N-acyl homoserine lactones secreted by various rhizosphere bacteria for intercellular communication (quorum sensing; Hartmann et al., 2014).

AA is considered as a central feature of *Bacillus* strains leading to root growth promotion via external supplementation of IAA by the inoculant (Borriss, 2015). Hence, IAA production has been reported for many *Bacillus* species, such as *B. subtilis* (Hashem et al., 2019), *B. megaterium* (Marulanda et al., 2009), *B. licheniformis* (Singh and Jha, 2016) and *B. velezensis* (Mpanga et al., 2019b), where IAA production was even stimulated in the presence of ammonium fertilizers. On the other hand, inactivation of genes responsible for bacterial tryptophan synthesis inhibited IAA formation and plant growth promotion (Idris et al., 2007). However in our study, no upregulation of the *ZmIAA5* gene, activated by external IAA supplying was detectable (Fig. 8A), suggesting that external IAA supplementation by the bacterial inoculants was not involved in the observed root growth increase, at least under the investigated cold stress conditions.

Declining CK concentrations induced by CombiA inoculation also had important consequences for the hormonal homeostasis in the root tissue, known to be even more important for the hormonal regulation of physiological processes than the absolute concentrations of individual phytohormones (Mueller and Leyser 2011; Nordström et al.,

2004). The decline in root CK increased the IAA/CK ratio by factor 3 and doubled the ABA/CK ratio in plants with CombiA inoculation and supplying of N as ammonium (Table 4).

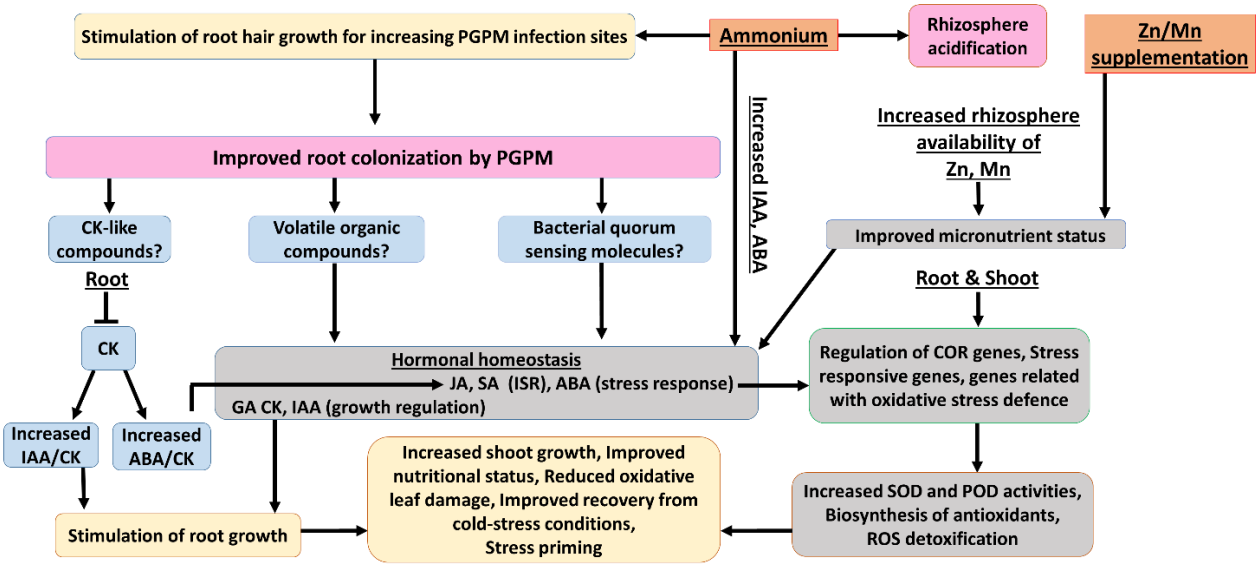
Chapter 4. Table. 4. The ratio of abscisic acid/cytokinin (ABA/CK) and auxin/cytokinin (IAA/CK) concentrations in the root of maize plants exposed to a 2-weeks period of reduced root zone temperature (RZT, 8–14 °C) on silty clay loam soil, pH 6.9. Low RZT variants including untreated control (Ctrl), Combi A^- (without Zn/Mn) and Combi A^+ (containing Zn/Mn) under nitrate or ammonium fertilization. Means of five replicates. In each row different letters: significant differences (Tukey-Test, $p < 0.05$).

	8–14 °C					
	Nitrate			Ammonium		
Ratio	Ctrl	Combi A^-	Combi A^+	Ctrl	Combi A^-	Combi A^+
ABA/CK	2.18 c	3.33 b	3.81 b	2.04 c	4.14 a	3.96 a
IAA/CK	0.21 c	0.42 b	0.53 b	0.37 c	0.91 a	0.96 a

Since CKs are acting as potent hormonal antagonists of IAA, e.g by inhibiting polar IAA transport mediated by PIN transporters (Fukaki and Tasaka, 2009), the increased IAA/CK ratio in CombiA treated plants (Table 4), may result in an improved shoot to root allocation of IAA, increased IAA activity with subsequent stimulation of root growth (Figs. 2 and 3). Similarly, the antagonistic effects of CK on ABA-mediated responses have been reported (Wilkinson et al., 2012; Pavlů et al., 2018). Therefore, the increased ABA/CK ratio may promote ABA-induced induction of cold-adaptations in the root tissue, although endogenous ABA concentrations were not changed (Fig. 8), as similarly reported also for cold acclimation in durum wheat (Veselova et al. 2005). This is in line with increased gene expression of the ABA response factor *ZmABF2* particularly in the root tissue of CombiA^+-inoculated plants with ammonium supply (Fig. 6B), known to be upregulated in cold-stressed maize plants (Sobowiak et al., 2014).

4.6.3 Contribution of N forms, Zn, Mn, and microbial inoculants to the cold-protective maize response

In summary, the results of the present study indicate a differential activation and stimulation of adaptive cold-stress responses, induced by the selected fertilization strategies. The various complementary and synergistic effects of ammonium fertilization, CombiA inoculation and ZnMn supplementation on cold stress adaptations in maize are schematically summarized in Fig. 9, while Table 4 provides an overview of the relative importance of the selected mitigation strategies (ammonium fertilization, PGPM inoculation, Zn/Mn supplementation) for the expression of cold-protective effects.



Chapter 4. Fig. 9. Proposed interactions of stabilized ammonium fertilization, PGPM (CombiA) inoculation and Zn/Mn supplementation contributing to increased cold tolerance during the early growth of maize (for description of details see 4.6.2 and 4.6.3).

Effects of ammonium fertilization: In accordance with the hypothesis (i), ammonium-dominated fertilization stimulated rhizosphere acidification, which improved the availability and the nutritional status of critical nutrients such as Zn and Mn on the investigated soil with neutral pH (Fig. 9), although the effect on P availability was

marginal. The improved Zn and Mn-nutritional status, with important functions in oxidative stress defence, moderately increased the enzymatic and non-enzymatic ROS detoxification, counteracted oxidative IAA degradation and oxidative leaf damage (Table 5). Ammonium fertilization was also the major factor contributing to increased ABA concentrations in the shoot tissue (Table 5), as a central regulator of adaptive cold stress responses and stimulated root colonization with the PGPM inoculant CombiA (Fig. 9).

Effects of PGPM (CombiA) inoculation: Root growth promotion by stimulation of IAA biosynthesis and reduction of antagonistic cytokinins in the root tissue of the host plant, was a major feature induced by PGPM inoculation with CombiA. Additionally, PGPM inoculation was associated with typical responses of ISR signaling via induction of jasmonic and salicylic acid accumulation even in the shoot tissue and an increase in the ABA/cytokinin ratio in roots. This was related with a further increase in enzymatic (SOD, POD) and non-enzymatic (antioxidants, phenolics, proline) ROS detoxification expressed mainly in the shoot tissue, and consequently a further decline of oxidative leaf damage. The observed effects are in line with the assumptions of the initial hypothesis (iii).

Effects of Zn/Mn supplementation: Partially in line with the hypothesis (ii), the additional supplementation with Zn and Mn mainly contributed to an additional increase of antioxidants and SOD activity in the root tissue. This was associated with increased IAA accumulation, reflecting a reduction of oxidative IAA degradation, which is typically induced under Zn deficiency related with high soil pH and impairment of root activity under cold stress. In consequence, further stimulation of root growth contributed to improved nutrient (P) acquisition, a generally improved plant nutritional status, improved plant performance and induced longer-lasting stress priming effects, still detectable two weeks after recovery from the cold stress treatments.

Chapter 4. Table. 5 Relative changes (%) of phenotypic and physiological responses in maize plants induced by stabilized ammonium fertilization (Ctrl ammonium), ammonium fertilization +PGPM inoculation (Ammonium + CombiA^{-}) and ammonium fertilization + PGPM inoculation + Zn/Mn supplementation (Ammonium+CombiA^{+}) after recovery (14 d) from two weeks exposure to low root zone temperatures (8-14°C) over plants supplied with N in the nitrate form.

Tissue	Factor	Ctrl (Ammonium)	Ammonium + Combi A^{-}	Ammonium + Combi A^{+}
Root	Length	n.s	+101	+161
	PGPM Colonization	n.s	+140	+143
	ROS Defence			
	Antioxidants	n.s	+178	+239
	SOD	+20	+22	+42
	Hormonal Effects			
	IAA	+75	+112	+131
	CK	n.s	-51	-50
	ABA	n.s	n.s	n.s
	SA	n.s	+123	+162
	ZmPIN1	+33	+167	+167
	ZmARF12	+32	+137	+137
	ZmIAA5	n.s	n.s	n.s
	ZmTSA	n.s	+67	+78
	ZmIPT4	n.s	-32	-35
	ZmIPT5	n.s	-56	-58
	ZmABF2	+28	+76	+94
Shoot	Biomass	n.s	n.s	+48
	Oxidative leaf damage	-27	-35	-42
	ROS Defence			
	SOD	+25	+52	+56
	POD	+25	+59	+64
	Phenolics	+26	+41	+44
	Antioxidants	n.s	+45	+47
	Cryoprotectants			
	Proline	n.s	+100	+80
	Sugar	n.s	+65	+57
	Hormonal Effects			
	IAA	n.s	+76	+106
	GA	n.s	+63	+78
	CK	n.s	+141	+153
	ABA	+33	+40	+43
	JA	+55	+208	+231
	SA	+38	+195	+211

SOD: Superoxide dismutase, POD: Peroxidase, IAA: Auxin, CK: Cytokinin, GA: Gibberellic acid, ABA: Abscisic acid, JA: Jasmonic acid, SA: Salicylic acid, PIN1: PINFORMED1, ARF12: Auxin response factor12, IAA5: Aux/IAA-transcription factor5, TSA: tryptophane synthase, IPT4,5: Isopentenyl transferases4,5, ABF2: Abscisic acid-binding factor2, n.s: not significant.

4.6.4 Conclusions

The combined use of N as ammonium, Mn, Zn and the *Trichoderma/Bacillus* inoculant is a suitable strategy to improve the tolerance of maize plants in the early growth stage to cold-stress conditions. This approach could be easily integrated into existing strategies for starter fertilization of maize production systems, such as seedbed fertilization with stabilized ammonium phosphates and micronutrient supplementation in combination with granulated spore formulations of the *Trichoderma/Bacillus* inoculant. Field performance of the agronomic practice proposed needs further evaluation in field trials, mirroring the already demonstrated effectiveness of single applications of micronutrients and silicon to improve the growth of maize plants (Imran et al., 2013; Moradtalab et al. 2018). Due to overlapping, adaptive plant responses to several abiotic stress factors, and additional biocontrol properties of the inoculants, even a wider spectrum of stress-protective effects might be expected.

4.7. Author Contributions

NM and GN conceived and designed the experiments. NM and AA conducted the experiments, performed the analyses, and collected the data. GN, UL, FW, BH, JG provided the facilities for analyses. GN, UL, JG, and FW read and edited the manuscript. NM and AA equally contributed to manuscript writing. All authors approved the final manuscript.

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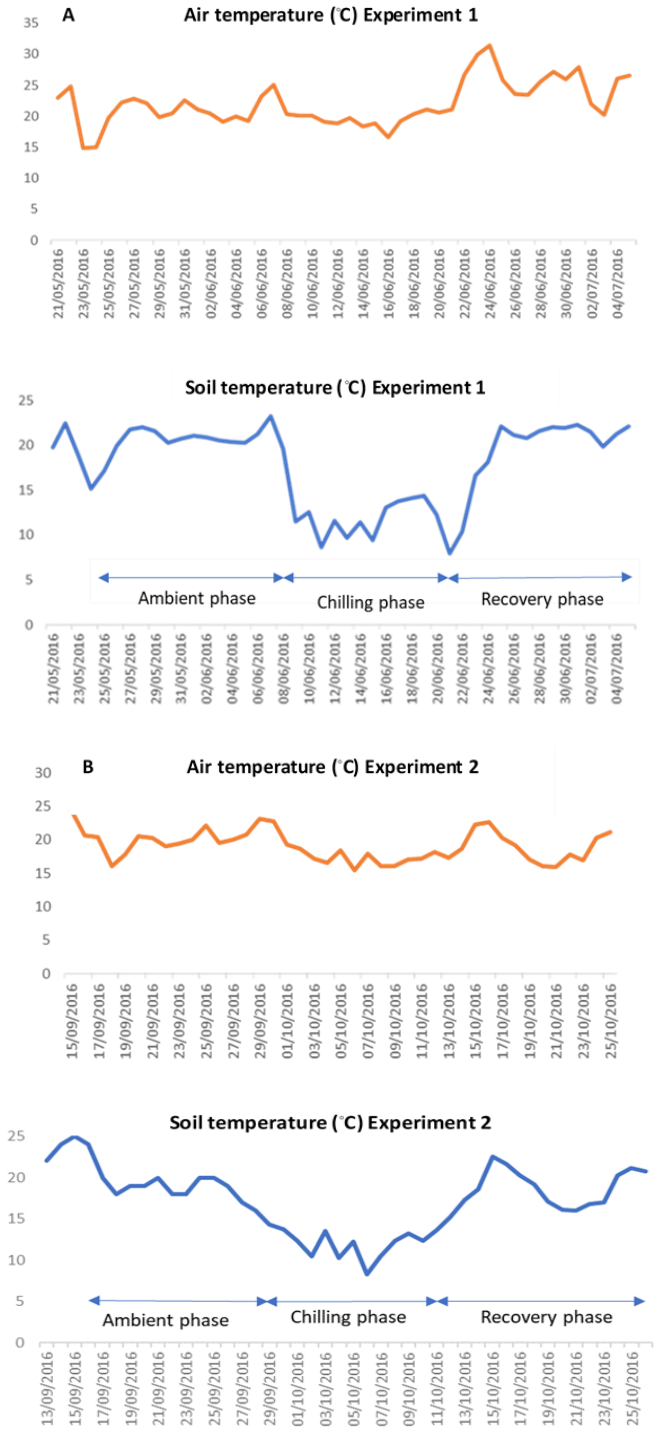
4.9. Supplementary Material



Chapter 4. Fig. S1. Time courses of ambient air and soil temperature during A) Experiment 1 and B) Experiment 2.

Chapter 4. Table. S1. Shoot and root dry weight (DW) and cold-stress induced oxidative leaf damage of maize plants exposed to a 2-weeks period of reduced root zone temperature (RZT, 8–14 °C) on a silty clay loam field soil, pH 6.9 with nitrate fertilization. Uncooled positive control (No-

Cold Ctrl) and low RZT variants including (i) a negative untreated control (Ctrl), (ii) Zn/Mn: seed dressing with Zn/Mn, (ii) Zn/Mn + ABI02: *Bacillus atrophaeus* ABI02 combined with Zn/Mn seed dressing, (iii) Zn/Mn +BFOD: *Penecillium* sp. BFOD combined with Zn/Mn seed dressing, and (iv) Combi A^+: *Trichoderma harzianum OMG16* + Vitabac. Supplemented with Zn/Mn Data represent means and SD of five replicates. Different letters indicate significant differences (Tukey-Test, $p < 0.05$); *: significant in pairwise comparisons with 8-14°Ctrl. (t-Test, $p < 0.05$).

N-Form	Stress	Treatment	Shoot DW [g]	Root DW [g]	Oxidative leaf damage [leaves plant^{-1}]
Nitrate	No-Cold	Ctrl	6.0±0.6 a*	1.9±0.2 a*	2.4±0.5 c*
	8–14 °C	Ctrl	4.5±0.5 c	1.2±0.2 b	5.6±0.9 a
		Zn/Mn	5.1±0.5 bc	1.1±0.2 b	4.4±1.1 a
		Zn/Mn +ABI02	5.2±0.3 b	1.2±0.2 b	4.6±0.5 a
		Zn/Mn +BFOD	5.8±0.2 ab*	1.2±0.3 b	4.4±0.5 ab*
		Combi A^+	5.8±0.7 ab*	1.3±0.2 b	3.4±0.5 b*

(i) Seed dressing with Zn/Mn Lebosol® Dünger GmbH, Ermstein, Germany), (ii) Zn/Mn seed dressing + fertigation with 10^9 spores Kg^{-1} soil DM ABI02, a cold-tolerant *Bacillus atrophaeus* strain (ABITEP, Berlin, Germany), (iii) Zn/Mn seed dressing + fertigation with 10^8 spores Kg^{-1} soil DM biological fertilizer OD (BFOD), a *Penicillium* sp. formulation (Bayer Crop Science Biologicals GmbH, Malchow, Germany), (iv) Fertigation with 2.5×10^7 cfu Kg^{-1} soil DM Combi A^+, a combination product of Zn (13%w(w) + Mn (9%w/w) + *Trichoderma harzianum OMG16* (9×10^9 spores g^{-1}) + Vitabac (1×10^{11} cfu g^{-1}, mixture of *Bacillus licheniformis, B. megaterium, B. polymyxa, B. pumilis and B. subtilis,* Bactvita GmbH, Straelen, Germany).

Chapter 4. Table. S2. A) Mineral concentrations in shoot dry matter (DM) with published deficiency thresholds, and B) total shoot contents of minerals in maize plants exposed to a 2-weeks period of reduced root zone temperature on silty clay loam soil, pH 6.9. Un-cooled control: (No-Cold Ctrl) and low RZT variants (8–14 °C) with (CombiA+) and without (Ctrl) PGPM inoculation under nitrate or stabilized ammonium fertilization. Data represent the means and SD of five replicates. Different letters indicate significant differences (Tukey-Test, $p < 0.05$).

A

N-Form	Stress Factor	Treatment	Ca [mg g^{-1} DM]	Mg [mg g^{-1} DM]	P [mg g^{-1} DM]
Nitrate	No-Cold	Ctrl	4.85 a	2.24 a	3.76 a
	12–14 °C	Ctrl	3.79 b	1.85 a	1.11 c
		Combi A^+	5.10 a	2.28 a	3.01 b
Ammonium	No-Cold	Ctrl	4.69 a	2.14 a	3.79 a
	12–14 °C	Ctrl	3.13 b	1.44 a	1.43 c
		Combi A^+	4.31 ab	1.96 a	4.14 a
Deficiency threshold*			2.5	1.5	3
N-Form	Stress Factor	Treatment	Zn [µg g^{-1} Dm]	Mn [µg g^{-1} DM]	Cu [µg g^{-1} DM]
Nitrate	No-Cold	Ctrl	46.00 a	50.51 a	4.82 a
	12–14 °C	Ctrl	24.40 b	34.82 b	4.03 a
		Combi A^+	47.81 a	48.01 a	4.16 a
Ammonium	No-Cold	Ctrl	59.01 a	51.04 a	4.30 a
	12–14 °C	Ctrl	59.42	53.72 a	4.06 a
		Combi A^+	57.63	52.93 a	4.88 a
Deficiency threshold*			20	20	5

B

N-Form	Stress	Treatment	Ca [mg Plant^{-1}]	Mg [mg Plant^{-1}]	P [mg Plant^{-1}]

	Factor				
Nitrate	No-Cold	Ctrl	29.10 a	13.44 a	22.56 a
	12–14 °C	Ctrl	17.06 b	8.33 b	5.00 c
		Combi A^+	29.58 a	13.22 a	17.46 b
Ammonium	No-Cold		30.95 a	14.12 a	25.01 a
	12–14 °C		19.41 b	8.93 b	8.87 b
			29.74 a	13.52 a	28.57 a
N-Form	Stress Factor	Treatment	Zn [µg $Plant^{-1}$]	Mn [µg $Plant^{-1}$]	Cu [µg $Plant^{-1}$]
Nitrate	No-Cold	Ctrl	276.00 a	300.06 a	28.92 a
	12–14 °C	Ctrl	109.80 b	156.69 b	18.14 b
		Combi A^+	277.24 a	278 .46 a	24.13a
Ammonium	No-Cold	Ctrl	389.40 a	336.86 a	28.38 a
	12–14 °C	Ctrl	368.40 a	333.06 a	25.17 a
		Combi A+	397.65 a	365.22 a	33.67 a

* Campbell C. R. and Plank C. O. (2013). Chapter: Reference Sufficiency Ranges — Field Crops/Corn at Early Growth. Campbell C. R. (Ed.). In Reference sufficiency ranges for plant analysis in the southern region of the united states (3rd ed.), southern cooperative series Bulletin #394. p. 122.

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Chapter 5. Transcriptomic Profiling of Silicon-affected Maize (*Zea mays* L.) Seedlings under Cold Stress

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NM, UL, and GN conceived and designed the experiments. NM conducted the experiments, performed the analyses, and collected the data. GN and UL provided the facilities for analyses. NM wrote the manuscript. GN and UL read and edited the manuscript. All authors approved the final manuscript.

5.1 Abstract

The most important abiotic stresses for Europe, are too high and too low temperatures. In central Europe, cold stress strongly affects maize growth and yield due to the early sowing. Silicon (Si) has a beneficial effect on limiting the adverse effects of cold stress through physiological and metabolic responses. However, the molecular regulatory mechanisms by Si application in maize seedlings in response to cold stress remain poorly understood. In this study, using Massive Analysis of cDNA Ends (MACE), we investigated the transcriptome profiles of 10-day-old *Zea mays* L (cv. Colisee) shoot with pre-germination by three days at 20 °C in filter paper rolls following by seven days at 20 °C or 12 °C without silicon or with silicon as 0.1 mM H_4SiO_4. By two-sided comparisons, a total of 553 differentially expressed transcripts (DETs) were identified between cold- and Si-affected maize shoot. Bioinformatic analysis revealed that cold stress downregulated most transcripts involved in primary and secondary metabolism. While transcripts related to hormonal stress regulators (abscisic acid, ethylene) were upregulated. At 12 °C, Si restored cold stress affected expression of transcripts in primary and secondary metabolism to a level similar to non-stressed plants at 20 °C. However, At 12 °C Si-induced transcripts expression even higher than non-stressed controls (at 20 °C) pointed to Si effects in cycloartenol synthase, mevalonate pathway that has functions in cold and drought resistance, membrane re-modeling under cold stress, antioxidative terpenoids, and cuticular wax layers biosynthesis. Si also regulated the expression of transcripts related to metal handling, cell wall modifications, and antioxidants defense at the molecular level. The consequence of these effects caused oxidative damage decline and increased membrane stability. This study extends the understanding of the molecular events via Si application of maize seedlings exposed to cold stress and will be useful for identifying major candidate

genes and molecular markers for improving resistance to cold stress in maize plants.

Keywords: Maize (*Zea mays* L.), cold stress, Massive Analysis of cDNA Ends (MACE), Silicon, hormonal homeostasis, cycloartenol synthase, mevalonate pathway, metal handling, cell wall modification, and stability

5.2 Introduction

Maize (*Zea Mays* L.) is one of the most important annual cereal crops worldwide, but despite its tropical origin and its high sensitivity to cold, maize is now cultivated in a varied range of climates (Rouf Shah et al., 2016). The optimum temperature for maize growth ranges from 21 to 27 °C and suboptimal temperatures (10–20 °C) lead to growth and yield decline and temperatures below 10 °C cause severe oxidative damage and result in plant death (Mao et al., 2017). Although, maize is a cold-intolerant plant (Zhu et al., 2007, Mammadov et al., 2018), however, the current climate change encourages early planting that potentially increases yield and avoids water deficit in drought events of summer. Early harvesting is also preferred to prevent fungal growth in grain and reduce drying costs (Riva-Roveda et al., 2016). However, earlier sowing dates increase the risk of exposure of the plants to cold stress. In maize plants improved tolerance to cold stress is the most cost-effective management approach (Mahajan and Tuteja, 2005), thereby developing approaches for improving maize cold tolerance is very important to boost maize production. Cold stress induces physiological, molecular, and biochemical changes that disturb various cellular processes, which negatively influence the growth of maize plants. Cold stress impacts are cell membranes damage, osmotic stress, and reactive oxygen species (ROS) overproduction resulting in impaired photosynthesis and growth decline (Riva-Roveda and Périlleux, 2015, Krasensky and Jonak, 2012). To counter these negative effects of cold stress, plant responses at all different levels including cellular responses,

metabolic changes, and transcriptional regulation of gene expression. At the cellular level, plants can adjust membrane systems and modify the cell wall architecture. Several compatible solutes (e.g., proline) can be produced to help stabilize proteins and cellular structures (Yadav, 2010). Hormone cross-talk and lipid signaling play a vital role in response to cold stress at metabolites and molecular levels (Huang, et al., 2012). These responses are controlled at the molecular level by regulating the expression of genes involved in osmolyte biosynthesis, ROS detoxification, transporters, and of genes encoding regulatory proteins such as protein kinases, phosphatases, transcription factors (TFs), late embryogenesis-abundant (LEA) protein, and, chaperones (Moradtalab and Hajiboland, 2016). Cold stress induces some abscisic acid (ABA)-responsive genes regulating by different kinds of TFs (Shi and Yang, 2014) belonging to AP2/EREBP, MYB, WRKY, NAC, bZIP families (Shan et al., 2013) with different regulons such as dehydration responsive element binding protein/C-repeat binding factor (DREB/CBF) regulon; the no apical meristem (NAC) regulon, zinc-finger homeodomain (ZF-HD) regulons, the ABA-responsive element-binding protein/ABRE binding factor (AREB/ABF) regulon, and the myelocytomatosis/myeloblastosis (MYC/MYB) regulon (Kimotho et al., 2019).

Although silicon (Si) has not considered being an essential element for higher plants (Epstein, 1999), its beneficial effects have been demonstrated for many plants, especially when they are exposed to biotic or abiotic stresses (Ma and Yamaji, 2006; Liang et al., 2007). In maize, as an active Si-accumulator crop, Si concentration is 21 mg g^{-1} dry weight (Marschner, 2012). In our previous study, we have observed that cold stress-induced deficiency of Zn and Mn and limited maize shoot and root growth but seed and seedlings supplementation with Zn, Mn, or Si protect young maize plants against cold stress (Moradtalab et al., 2018). Effects of Si treatment were related to an improved Zn and Mn

status, a restored and balanced hormonal (auxin (IAA), gibberellic acid (GA), cytokinin (CK)) status, increased activity of enzymatic (superoxide dismutase (SOD) and peroxidase (POD)) and non-enzymatic (phenolic antioxidants) defense systems, primed various physiological adaptations to cold stress via an accumulation of stress hormones (abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA; Moradtalab et al., 2018). However, all of the above analyses remain fragmentary, as these focus on specific cellular or tissue functions. To obtain a complete overview of processes that are modified by Si and cold, transcriptomic analyses can give an overview of altered cellular or tissue functions. The molecular mechanisms of tolerance to cold stress in maize are not well understood since resistance to cold stress in maize has a very complex responsive mechanism. Investigations of the molecular mechanisms of maize response to cold stress may facilitate the development of resistant maize varieties and more effective management strategies. RNA-sequencing (RNA-seq) is a powerful technology for whole-genome gene expression profile analysis and is especially useful for studying complex gene regulatory networks (McGettigan, 2013). In this study, we hypothesized that enhanced maize plant tolerance to cold stress via exogenous Si application could be tracked at the transcriptomic level and Si may act via triggering multiple defense pathways. Accordingly, we used a high-resolution and cost-efficient RNA-Seq technology so-called Massive Analysis of cDNA Ends (MACE, GenXPro GmbH, Frankfurt am Main, Germany) to investigate transcriptome expression profile in Si-treated and non-Si-treated maize shoot under cold stress to understand molecular mechanisms involved in the response to cold stress via Si application in the seedling stage of maize. Differentially expressed transcripts (DETs) were identified by comparisons between the control and cold stress in Si-treated and non-Si-treated seedlings, and these DETs were compared to detect the unique and common transcripts and

pathways responding to cold stress in maize and via Si application. This study may lead to an increase in our knowledge about the molecular mechanisms of tolerance to cold stress in maize plants.

5.3 Material and Methods

5.3.1 Plant Preparation and Cold Treatment

Germination of maize seeds (*Zea mays* cv. Colisee), surface–sterilized by 1 min soaking in ethanol (99% v/v), was performed in pre-sterilized Petri dishes (9 cm diameter) on moist filter paper (10 seeds per petri dish). The seeds were soaked with 3 mL deionized water (–Si control) or 3 mL freshly prepared H_4SiO_4 (1.0 mM Si) (Maksimovic et al., 2007; Pavlovic et al., 2013), respectively with ten replicates per treatment. The optimum level of Si seed application was determined in a pilot experiment with different levels of Si supply (Moradtalab et al., 2018). Covered Petri dishes were placed into a laboratory incubator (AtmoCONTROL, ICP, 750 Memmert GmbH, Schwabach, Germany) for three days in the dark at 20 °C in thin unsealed plastic bags to minimize evaporation. For further seedling development, the germinated seeds were placed on the upper edge of rolled filter papers (10∗60 cm; 2 seeds per roll), moistened with 100 ml distilled water or H_4SiO_4 solution (1 mM Si as Si treatment) at three and five days after sowing (DAS). The filter rolls were transferred into the laboratory incubator and incubated for seven days at 12 °C (as cold treatment) or 20 °C (as an ambient control condition). The incubator set up was 16/8 h and relative humidity of 60%, with a light intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Thereafter, the treatments as three libraries for transcriptomic analysis were seedlings grown for 7 days at 20 °C without Si application (negative control: NC), and at 12 °C without Si application (positive control: PC) or with Si application (Si-treated: Si). Harvested shoot material was immediately frozen in liquid nitrogen and stored at -80 ◦C until further use.

5.3.2 RNA Extraction, Quantification, and Quality Assessment

For total RNA isolation, frozen leaves were ground to a fine powder in liquid nitrogen using a mortar and pestle. RNA isolation method was obtained from GenXPro GmbH and done as described by Parreira et al., 2018.

5.3.3 Massive Analysis of cDNA Ends (MACE)

MACE libraries were generated using GenXPro's MACE kit (GenXPro GmbH, Frankfurt, Germany) as described in Hradilová et al., (2017). Briefly, cDNA from 5 μg of total RNA was randomly fragmented and biotinylated 3′ ends were captured after binding to a streptavidin matrix. A library ready for high-throughput sequencing was prepared using TrueQuant adapters included in the kit. The library consisted of 50–700 bp-long fragments derived from the 3′-end of the cDNAs. The 3′-ends of the libraries were sequenced on a HiSeq 2000 machine (Illumina) with 100 cycles to generate the MACE tags, each tag representing one single transcript molecule. The three libraries (each one pooled with ten seedlings) with three biological replicates produced in total 9 cDNA for sequencing. The raw data processing was performed with the MACE analysis tool of GenXPro. First Illumina adapter sequences were removed, this step is called "clipping", followed by TrueQuant correction, that was performed to eliminate PCR bias and PCR introduced duplicates and artifacts. Also, the TrueQuant adapters are "clipped". Also, a normalization concerning the sequencing depth according to Anders and Huber (2010) was performed. The average of the raw counts of each gene in a library was divided by the geometric mean of the total counts in one sample. For each library, a median of these quotients was calculated, and the raw counts were divided through the library-specific median value. The displayed number represents the average of the normalized values of all samples (GenXPro GmbH, Frankfurt, Germany).

5.3.4 Bioinformatics

Differential gene expression was quantified as the log2 ratio of the normalized values among three libraries (log2 FC). To reduce the noise and false discovery rate (FDR) of low expressed samples, only transcripts with mean counts averaged over all treatments larger than 10 were included in the analysis. Lists of DETs for comparisons of libraries (NC, PC, and Si) were made based on a combination of pairwise comparisons of the log2 FC ratio of the normalized values (log2 FC ≥ 3, log2 FC ≤ -3) and FDR (FDR $< .01$) among three libraries. The pairwise comparisons are NC vs. PC =cold affected transcripts, PC vs. Si = Si-affected transcripts under cold stress, NC vs. Si = Si-cure of cold). Functional characterization was performed using the MapMan 3.6.0RC1 tool (https://mapman.gabipd.org/mapmanstore). Thus, the BIN names and annotated transcripts descriptions are according to the identified or putative Arabidopsis, rice, and maize transcripts databases. The convert of the old GRMZM annotations of MapMan library (2012, B73v2) to recently published and released of new maize annotations (2018, B73v4) was done via the translation tool in https://www.maizegdb.org according to Portwood et al., 2018.

5.3.5 Real-time PCR analysis

Real-time PCR and primers design were done by GenXPro GmbH, Frankfurt, Germany (Tables S9, S10). The isolation of high-quality total RNA from plant cells was done according to the instruction described for the InviTrap® Spin Plant RNA Mini Kit (STRATEC Molecular GmbH, D-13125 Berlin).

5.3.6 Statistical Analysis

Statistical analysis was performed with SAS 9.4 by analysis of variance (ANOVA) based on a t-test (two-group comparisons between treated samples and control samples). Means

were considered significantly different based on t-test threshold value corresponding to the P-value ($P < .05$ and $P < .0001$). Principal component analysis and heatmaps were performed by RStudio 3.4.1.

5.4 Results

5.4.1 Transcriptome profiles of maize seedlings at 20 °C and 12 °C

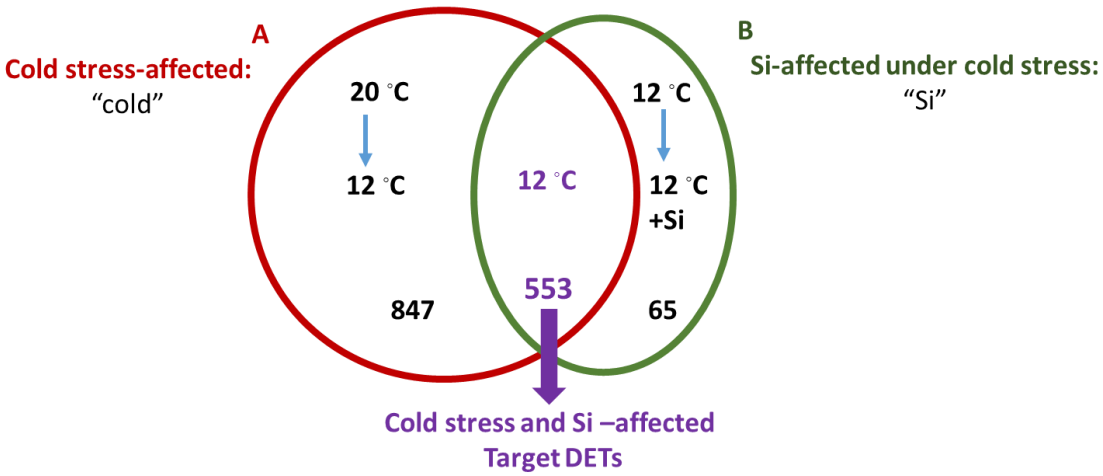
Among the total transcripts (27,186) of seedling of the maize inbred line B73, 23,865 transcripts were expressed in the NC, while 22,794 in PC and 24,208 in Si (Fig. S1). Principal component analysis (PCA) of the top 20 highly expressed transcripts (Supplementary Table. S1) confirmed that the three libraries significantly different by 99.75 % between NC and PC, 99.78% between PC and Si, and 97.25 % between NC and Si. The replicates of each library were not significantly different by 0.17 % for NC and PC and 1.54% for Si (Fig. S2).

Chapter 5. Fig. 1. 10-day-old maize seedlings, pre-germination 3 days at 20 °C in filter paper rolls for 7 days grown at 20 °C without Si application (negative control: NC), and at 12 °C without Si application (positive control: PC) or with Si application (Si-treated: Si) as 0.1 mM H_4SiO_4.

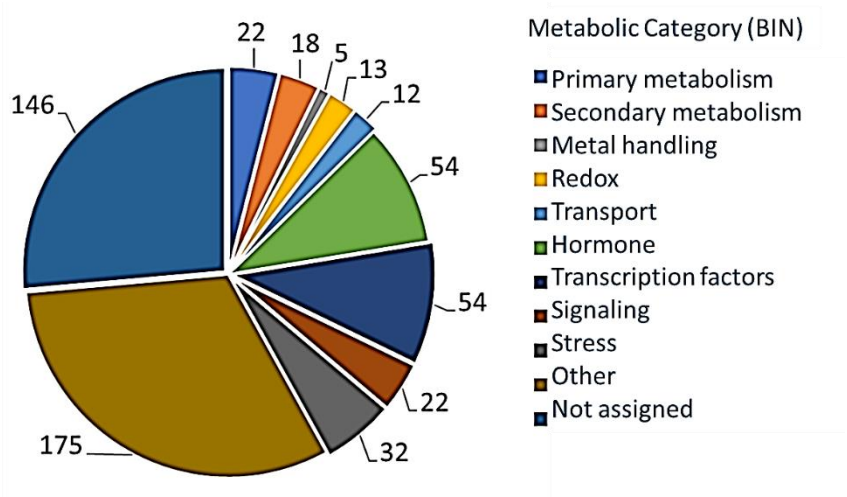
5.4.2 Functional classification of differentially expressed transcripts (DETs)

To reduce unspecific assignments form noise due to low expression and to concentrate on

major differences, our analysis concentrated on substantially expressed transcripts (>10 counts) with log2 FC ≥ 3, log2 FC ≤ −3 threshold by two-sided comparisons. By a reduction of temperature from 20 °C (NC) to 12 °C, a total of 1400 DETs were identified in cold stress-affected plants, while 847 unique DETs of the total transcripts were detected to respond only to cold stress, but 553 of the total overlapped with Si affected transcripts (Fig. 2). Analyzing the unique 847 DETs of cold stress-affected transcripts indicated that there was mostly down-regulation of selected functional categories (BINs) including photosynthesis, transport, and primary, secondary, and hormone metabolism. On the contrary, there was mostly up-regulation of these BINs in Si-treated seedlings. Among 65 unique DETs in Si-affected seedlings that were not responding to cold, a total of 12 unique DETs were identified that belonging to transcription factor families that were members of ABI3, ethylene-responsive element-binding protein, bZIP, bHLH, WRKY, Argonaute, and MYB related TF families (Table. S2). The WRKY family consisting of WRKY46, WRKY5, WRKY25, and WRKY20 was the largest TF family responding exclusively to Si application, without relation to cold stress (Table. S2). However, the massive overlap of 553 transcripts that were affected by both cold and Si suggests that Si promotes growth in cold via simulating higher temperatures. To identify the possible alleviation effects of Si under cold stress at the molecular level (Fig. 2) these 553 target DETs responsive to both “cold” and “Si” were analyzed further and fall into BIN categories including primary and secondary metabolism (totally 40 transcripts), metal handling, redox and transport (totally 30 transcripts), hormone and transcription factors (totally 108 transcripts), other BINs (totally 175 transcripts) and not assigned BINs (totally 146 transcripts; Fig. 3).



Chapter 5. Fig. 2. Differentially expressed transcripts (DETs) (log2 FC ≥ 3, log2 FC ≤ −3) in cold stress-affected (A), and Si-affected in cold stressed plants (B) from10 day-old maize seedlings grown in filter paper rolls for 7 days at 20 °C or 12 °C without silicon or with silicon as 0.1 mM H_4SiO_4.

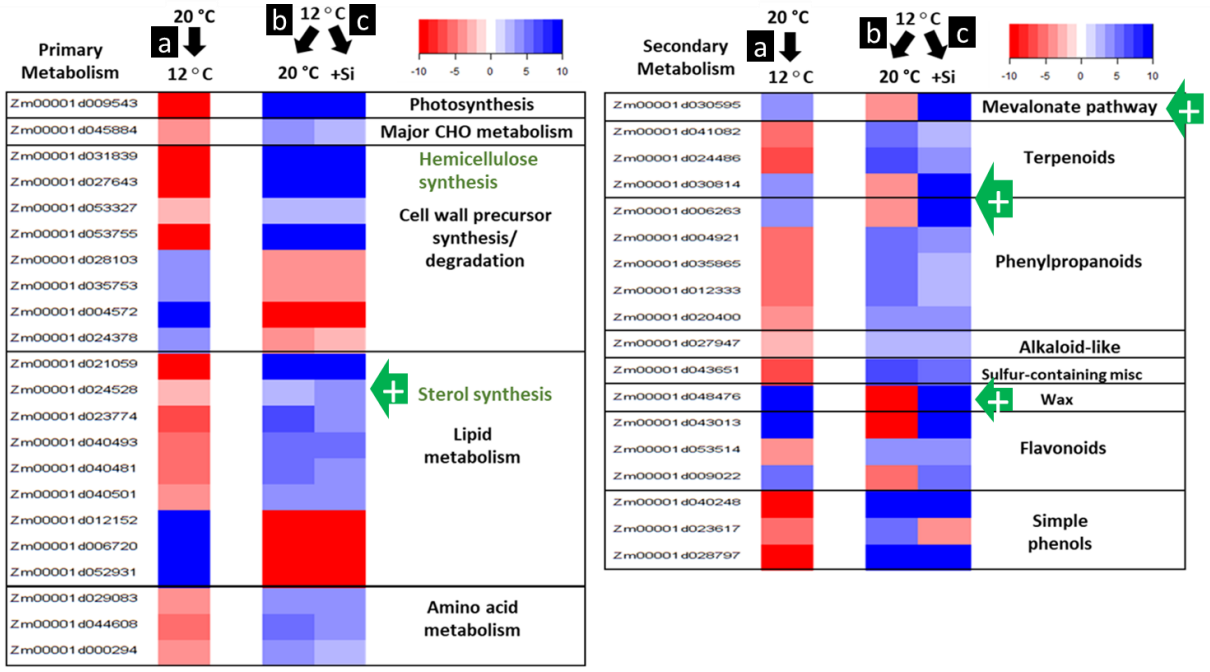


Chapter 5. Fig. 3. 553 Target transcript counts of differentially expressed transcripts (DETs) responsive to both "cold" and "Si" (threshold:log2 FC ≥ 3, log2 FC ≤ −3) of selected metabolic categories (BINs) from 10-day-old maize seedlings grown in filter paper rolls for seven days at 12 °C as cold stress.

5.5 Primary and secondary metabolisms responses of targeted transcripts in cold- and Si -affected maize seedlings at 12 °C and 20 °C

By the reduction of temperature from 20 °C to 12 °C as"cold"-affected transcripts, among a total of 22 DETs involved in primary metabolism pathways, 15 transcripts were down-regulated and seven were up-regulated by "cold" (Fig. 4, Table S3) while their expression

pattern was opposite in comparison to the plants exposed to “Si” or at 20 °C (Fig. 4, Table S3). Among these DETs, transcripts involving in cell wall precursor synthesis were down-regulated by “cold” (Fig. 4) while up-regulated by “Si” or at 20 °C (Fig. 4, Table S3). Transcripts involving in cell wall degradation were found up-regulated by “cold” (Fig. 4) while oppositely down-regulated in “Si” or at 20 °C (Fig. 4). Transcripts involved in phospholipid and desaturation pathways in lipid metabolism were down-regulated by “cold” (Fig. 4) while up-regulated by “Si” or at 20 °C (Fig. 4). However, transcripts including in lipid degradation were up-regulated by “cold” (Fig. 4) but down-regulated by “Si” or at 20 °C (Fig. 4, Table. S3). Analyzing DETs encoding secondary metabolism indicated that most of the identified secondary metabolism transcripts were involved in terpenoids and phenolic pathways (Table. S4). Among these DETs of secondary metabolism, transcripts involving mevalonate pathway, terpenoids and phenylpropanoids, and flavonoids biosynthesis enzymes and simple phenols metabolism (related to L-ascorbate oxidase precursor) were down-regulated by “cold” but up-regulated by “Si” or at 20 °C (Fig. 4, Table S4). Although at 12 °C, as mentioned above, Si restored cold-stress affected expression of transcripts in primary and secondary metabolism to a level similar to non-stressed plants at 20 °C but particularly Si-induced transcripts expression even higher than non-stressed controls and up-regulated the expression of transcripts encoding key enzymes such as acyl-(acyl-carrier-protein) desaturase, hydroxymethylglutaryl-CoA reductase, terpene synthase, cycloartenol synthase, shikimate O-hydroxycinnamoyltransferase, wax synthase-related involved in the biosynthesis of sterols, mevalonate, terpenoids, phenolics, and wax layer (Fig. 4 see green arrows, Tables. S3 and S4).

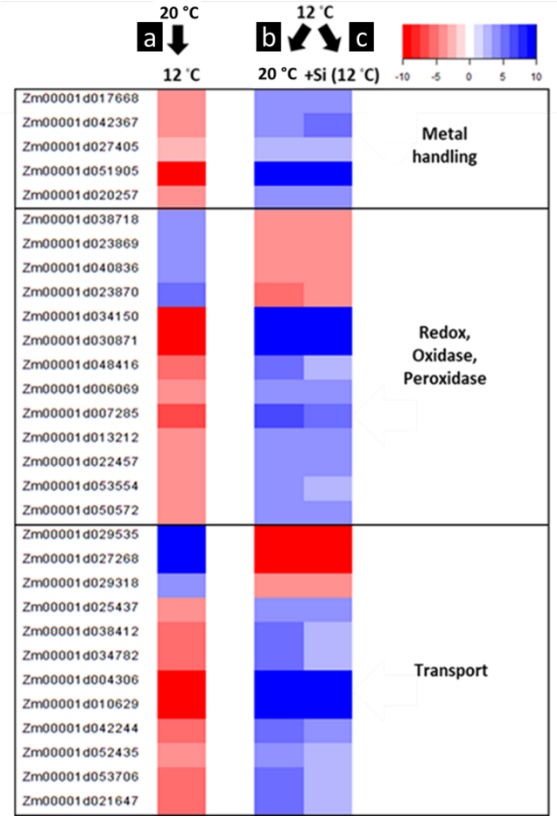


Chapter 5. Fig. 4. Targeted cold- and Si- affected transcripts of maize seedlings involved in primary and secondary metabolisms. Values are presented as log2 FC ≥ 3, log2 FC ≤ −3 of expressed transcripts including (a) cold stress-affected (20 °C to 12 °C = "cold"), (b) ambient condition (12 °C to 20 °C), (c) Si-affected in cold stressed plants (20 °C to 12 °C +Si = "Si"). Red and blue indicate down- and up-regulated transcripts, respectively, (p-value < .05). Green arrows indicate Si-induced transcripts expression (at 12 °C) even higher than non-stressed controls (at 20 °C).

5.6 Metal handling of targeted transcripts in cold- and Si -affected maize seedlings at 12 °C and 20 °C

A total of 30 DETs involved in metal handling, redox, and transport were identified including 23 down-regulated transcripts and seven that were up-regulated in response to "cold" while regulated oppositely by 20 °C and "Si" (Fig. 5, Table. S5). Among these DETs, transcripts involving in metal ion binding were identified, which were down-regulated by "cold" while up-regulated by 20 °C and "Si". Transcripts of redox category encoding related proteins in oxidases activities (chloroplast superoxide dismutase protein, oxidoreductase, monooxygenase, NADP-dependent oxidoreductase, Peroxidase 1 precursor, Peroxidase 2 precursor, peroxidases and transporters of amino acids,

ammonium, phosphate, metal, and potassium) were all down-regulated by "cold" while up-regulated by 20 °C and "Si" (Fig. 5, Table. S5).

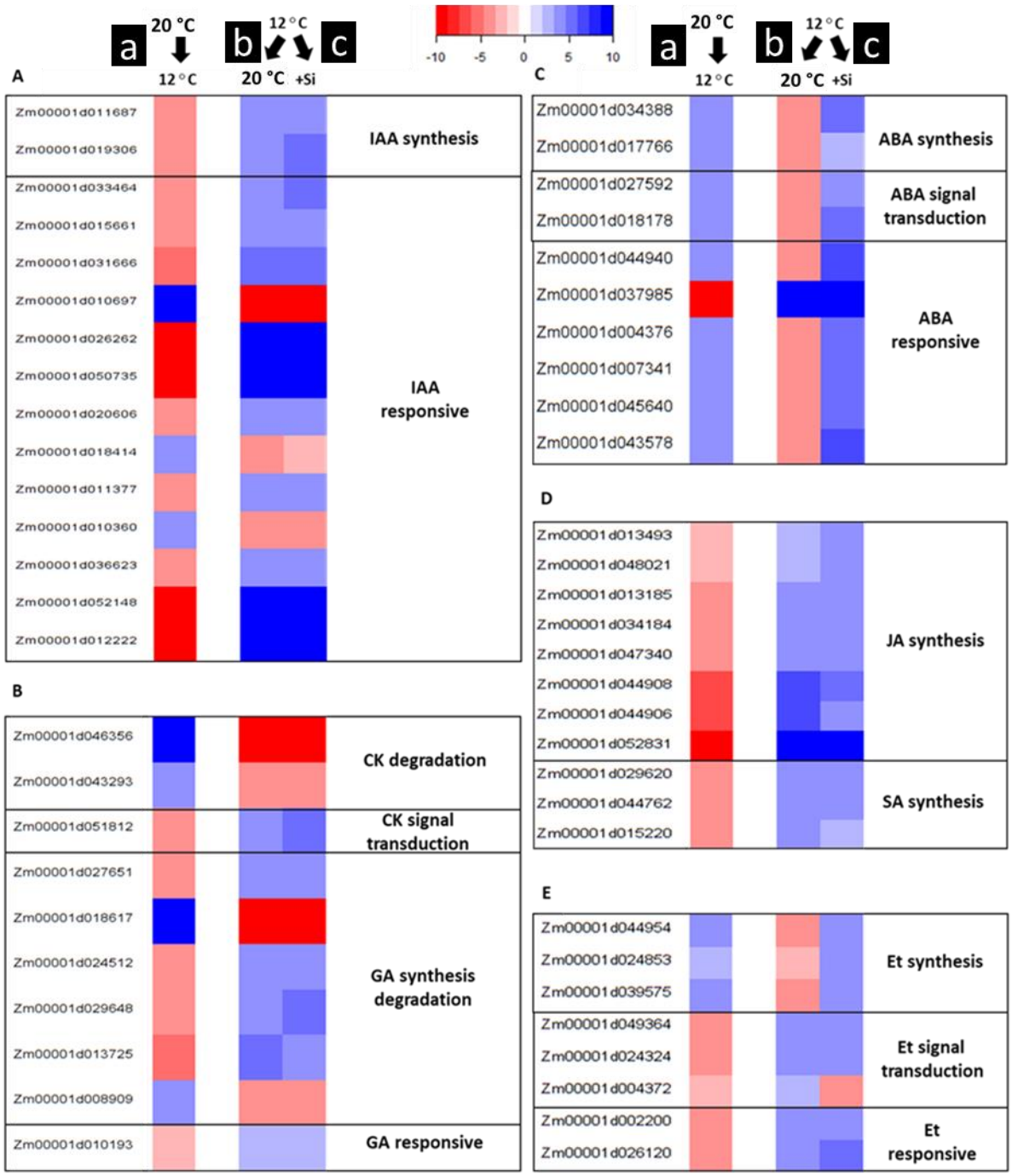


Chapter 5. Fig. 5. Targeted cold- and Si- affected transcripts of maize seedlings involved in metal handling. Values are presented as log2 FC ≥ 3, log2 FC ≤ −3 of expressed transcripts including (a) cold stress-affected (20 °C to 12 °C = "cold"), (b) ambient condition (12 °C to 20 °C), (c) Si-affected in cold stressed plants (20 °C to 12 °C +Si = "Si"). Red and blue indicate down- and up-regulated transcripts, respectively, (p-value < .05).

5.7 Phytohormonal response of targeted transcripts in cold- and Si -affected maize seedlings at 12 °C and 20 °C

A total of 54 DETs involved in hormone biosynthesis, degradation, and signal transduction pathways, such as IAA, CK, GA, ABA, JA, SA, and ethylene (Et), were identified of target DETs in "cold" and "Si" -affected maize seedlings (Fig. 6. Table. S7). We detected 15, three, and seven DETs involved in the IAA, CK, and GA pathways, respectively. Transcripts involved in IAA biosynthesis or IAA responsive genes were all down-regulated by "cold" while up-regulated by 20 °C and "Si" (Fig. 6. Table. S7). Transcripts involving

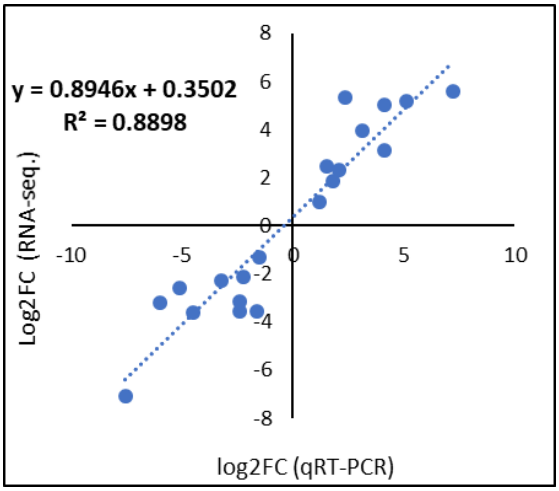
in CK degradation were up-regulated by “cold” while down-regulated by 20 °C “Si”. While transcripts involving CK signal transduction were down-regulated by “cold” while up-regulated by 20 °C and “Si” (Fig. 6. Table. S7). Transcripts in GA biosynthesis were down-regulated by “cold” while up-regulated by the “Si” application (Fig. 6. Table. S7). While GA deactivating enzymes transcripts were up-regulated by “cold” while down-regulated by 20 °C and “Si” (Fig. 6. Table. S7). Among ABA-related DETs, the expression levels of eight ABA biosynthesis enzyme, signal transduction, and responsive genes were all up-regulated in maize seedling leaves by both “cold” and “Si” while down-regulated at 20 °C. However, encoding Late Embryogenesis Abundant transcripts as ABA-responsive gene displayed decreased expression level under “cold” while was up-regulated by 20 °C and “Si” (Fig. 6, Table. S8). The expression levels of JA and SA biosynthesis enzymes transcripts were down-regulated by “cold” and up-regulated at 20 °C and “Si” application (Fig. 6, Table. S8). DETs involved in the Et biosynthesis were all up-regulated in maize seedling leaves under both “cold” and “Si” while down-regulated at 20 °C. Transcripts that negatively regulate Et signal were down-regulated in maize seedling leaves by both “cold” and “Si” while up-regulated by 20 °C. However, transcripts involved in signal transduction and responsive pathways were all down-regulated in response to “cold” but up-regulated by 20 °C and “Si” (Fig. 6, Table. S8). These results indicated that phytohormone related biosynthesis and signal transduction pathways were reprogrammed in response to “cold” with “Si” and in comparison, to ambient condition, Si partially can mitigate cold stress adverse effects.



Chapter 5. Fig. 6. Targeted cold- and Si- affected transcripts of maize seedlings involved in phytohormones homeostasis including auxin (IAA), gibberellic acid (GA), cytokinin (CK), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), and ethylene (Et). Values are presented as log2 FC ≥ 3, log2 FC ≤ −3 of expressed transcripts including (a) cold stress-affected (20 °C to 12 °C = "cold"), (b) ambient condition (12 °C to 20 °C), (c) Si-affected in cold stressed plants (20 °C to 12 °C +Si = "Si"). Red and blue indicate down- and up-regulated transcripts, respectively, (p-value < .05).

5.8 Validation of RNA-Seq analysis by quantitative Real-Time PCR (qRT-PCR)

To validate the reliability of the gene expression data obtained by the RNA-seq analysis (Table S9) in maize seedling leaves, ten DETs were selected for qRT-PCR analysis (Table. S10). The ratio of expression levels found using qRT-PCR was compared to the ratio of expression as measured by RNA-Seq. A significant correlation (r^2= 0.8898, Fig. 7) was observed between the RNA-Seq. and Quantitative real-time PCR (qRT-PCR) data, which confirmed the authenticity of the DETs in this study. Thus, these comparisons of data from qRT-PCR and RNAseq analyses of B73 seedling leaves fully validated the findings from our transcriptome study.



Chapter 5. Fig. 7. Correlation of log2 Fold changes between quantitative real-time PCR and RNA-sequencing data. A significant validation (r^2= 0.8898) of DETs characterized by RNA-sequencing.

5.9 Discussion

Previously, we have observed that cold stress-induced deficiency of Zn and Mn and limited maize shoot and root growth but seed and seedlings supplementation with Zn/Mn, or Si protected maize seedlings against cold stress (Moradtalab et al., 2018). Effects of Si treatment were related to an improved Zn and Mn shoot concentration, a restored and

balanced hormonal (IAA, GA, and CK) status, increased activity of enzymatic (SOD, POD) and non-enzymatic (phenolic antioxidants) defense systems, and primed various physiological adaptations to cold stress via an accumulation of stress hormones (ABA, SA and JA) (Moradtalab et al., 2018). However, despite our observation and numerous scientific reports of positive effects of Si on the growth of plants exposed to a stress condition, molecular events of Si effects under cold stress is not fully studied. Thus, this study would provide insights into the possible molecular basis of Si induced-protection against cold stress that was previously observed at physiological and biochemical levels in previous work by Moradtalab et al., 2018. In this regard, RNA-seq as a useful approach to identify DETs and regulatory mechanisms at the transcriptomic level (Li et al., 2017) was applied in this study. We selected the period of third leaves (10-day old maize seedling, see Fig. 1) because it was suggested that this seedling stage of maize is especially sensitive to cold stress (Peleg and Blumwald, 2011). The number of active transcripts in each treatment sample is comparable and similar to previous studies that examined B73 maize seedling leaves under different abiotic stress conditions (Li et al., 2017, Opitz et al., 2016).

5.10 Key response for resistance to cold stress in maize seedling by Si application is related to membrane stability, cell wall modification, and metal handling

In our study, Si application up-regulated transcripts involving in cell wall precursor synthesis, phospholipid, and desaturation pathways in lipid metabolism (Fig. 4). It was reported that phosphatidic acid, phosphoinositides, sphingolipids, and other lipids are involved in the resistance to abiotic and biotic stresses in plants (Hou et al., 2016). Zinc-binding dehydrogenase (that was up-regulated by Si application in our study) involved in lignin biosynthesis exhibiting defense-related activity and strengthening of the cell wall (Kumar et al., 2016). Transcripts of phenylpropanoids and flavonoids biosynthesis

enzymes were also up-regulated in Si-affected seedlings under cold stress (Fig. 4). In this regard, reports revealed that seed-priming with Si increased phenolics concentration and boosted antioxidative tolerance of maize plants to stress conditions (Latef and Tran, 2016, Moradtalab et al., 2018). Increased phenolic production and their subsequent incorporation into the cell wall either as suberin or lignin are important for the plants to interact with cold stress for adaptation and defense (Ramakrishna and Ravishankar, 2011).

In our study, 32 transcripts among a total of 618 Si-affected-DETs that were directly dependent on metal handling were detected being down-regulated in Zn-deficient maize seedlings under cold stress while up-regulated by Si application (Table. S6). The up-regulated transcripts in our study included metal and Zn ion binding proteins, zinc-binding dehydrogenase, ascorbate peroxidase (AXP) precursor, monooxygenase, SOD and POD proteins, Fe ion transmembrane transporter, ZIFL1 (Zn-induced facilitator like 1) (Table. S6). Similar to our results, Zn deficiency in maize seedlings down-regulated most of the genes encoding enzymes involved in pathways regulating ROS and cell wall modifications, however, upon prolonged Zn deficiency, the high-affinity transporter genes ZmZIP3, 4, 5, 7, and 8 and nicotianamine synthases, primarily ZmNAS5, were up-regulated in maize roots (Mager et al., 2018). In this regard, studies revealed that Zn-deficiency-tolerance in maize lines tolerant to Zn deficiency was associated with the up-regulation of Zn transporter genes and antioxidant activities. The molecular mechanistic basis of Zn deficiency tolerance was (i) up-regulation of Zn transporter genes (ZmZIP1, ZmZIP4, and ZmIRT1) and (ii) increased antioxidant defense (SOD and POD activities) operated in roots (Khatun et al., 2018). The increased expression of the SOD-regulating transcript in Zn-efficient wheat cultivar was also reported compared with Zn-inefficient wheat grown under Zn-limiting conditions (Hacisalihoglu et al. 2003). Selenium-binding proteins (SBP)

transcripts were also up-regulated by Si application under cold stress. The potential function of SBP1 in plants is not fully understood but its involvement in detoxification mechanisms is largely suggested. Overexpression of SBP1 in *Arabidopsis thaliana* showed significantly enhanced tolerance to H_2O_2 (Hugouvieux et al., 2009). It was indicated that Zn-deficiency at the cellular level increased oxidative damage of membrane proteins, phospholipids, chlorophyll, nucleic acids, enzymes, and IAA, and thus caused inhibition of plant growth (Cakmak, 2000). We have observed that Si exerted its protective effects against excess ROS via improvement of the plant micronutrient (Zn/Mn) status. In a soil-free culture medium, Si was able to improve selectively the Zn/Mn status in the shoot of maize seedlings, already during the early growth (10-day-old), even before the stimulation of root growth or further Zn/Mn root uptake from the external medium (Moradtalab et al., 2018). Different mechanisms are proposed for the effects of Si in internal Zn availability improvement in Zn-deficient plants including induced-biosynthesis of phenolics with metal chelating properties (Pavlovic et al., 2013), direct Si-metal interactions counteracting apoplastic metal immobilization, and supporting metal transport in plants (Hernandez–Apaolaza, 2014; Stevic et al., 2016), expression of metal acquisition and transport genes (Pavlovic et al., 2013, 2016). For example, in rice plants as a higher Si accumulator, it was suggested that a hemicellulose-bound form of Si with net negative charges is responsible for the inhibition of Cd uptake in the cells by a mechanism of (Si-hemicellulose matrix) Cd complexation and subsequent co-deposition (Ma et al., 2015).

H_4SiO_4 transport through root cells via Lsi1 and Lsi2 as Si transporters must maintain a cytosolic concentration of < 2 mM (Coskun et al., 2019). Direct cytosolic measurements are currently lacking; thus, it keeps an open question if low cytosolic Si concentration can play a signaling agent role or not. Thus, we cannot exclude an unknown active biological

role for Si (Fig. 8). H_4SiO_4 for silicification interacts with cell wall constituents, such as (Hemi)cellulose, callose, pectin, and lignin (Guerriero et al., 2016). According to the recent apoplastic obstruction hypothesis (Coskun et al., 2019), root-to-shoot translocation of toxicants produced from stress via symplastic and apoplastic routes towards the stele can be blocked by apoplastic Si deposition. Thus, less-toxicant levels in shoots in comparison with NoSi-stressed plants result in less ROS and decreased oxidative stress and thereby increased membrane stability (MS), increased enzyme activities, changed gene expression, increased photosynthesis rate, and ultimately growth (Coskun et al., 2019). The apoplastic obstruction model associated most of Si roles under stress condition with the prevention of the de-regulation inherent to the stress itself, which is indirect, rather than direct effects (Coskun et al., 2019). In this regard, in maize seedlings that indicating Zn-deficiency result from cold stress, interactions between Si-hemicellulose-matrix and metal ions may produce a metal-complex in the apoplast of root and shoot tissue, these metal-complexes can be used by metal handling proteins and benefited plants response to tolerate cold stress. Consequently, elevated ROS detoxification by enhanced metal-dependent antioxidants can balance ROS signaling and hormonal responses. From another point of view, metal-dependent TFs can also regulate cold stress-responsive genes activated by a balanced hormonal signature which will cause cold tolerance. Thus, a Si-hemicellulose-matrix with net negative charges may bound to the metal's ions including Zn^{2+} to produce storage of metal-Si-hemicellulose-matrix-complexation that can be used whenever needed at deficiency condition like cold stress.

At 12 °C, up-regulation of Si-induced secondary metabolism transcripts (cycloartenol synthase, mevalonate pathway, sterol, terpenoids, phenolics, and wax layer biosynthesis, Figs. 4 and 5, Tables. S4 and S5) even higher than non-stressed seedlings at 20 °C, point

to possible direct Si effects. It is particularly important to be discussed as a novel finding of the Si-priming effect. The striking effect of Si application under cold stress was that Si mimicked the transcripts expression profiles of numerous transcripts characteristic for non-stressed control at 20 °C (Figs. 4, 5 and 6). Although, this broad effect is an important result, but it can hardly provide some information on primary Si effects. Since Si treatment had a general rescue effect on cold stressed plants, the transcripts expression profiles may largely reflect the better plant performance and show the indirect consequences of some primary Si effects which cannot be identified with this approach since it is not possible to distinguish between direct and indirect effects. However, much more interesting in this context are those transcripts of Si treated plants that show different and, in most cases, even higher expression than the unstressed control and at the same time, also different expression than the cold stressed controls without Si treatment. These are candidates for the more specific Si effects, e.g. for the secondary metabolites the interesting candidate's transcripts including terpenoids and particularly cycloartenol synthase, which is the key enzyme for phytosterol biosynthesis. Sterols have important functions in membrane re-modeling under cold stress (Rogowska and Szakiel, 2020), are precursors for antioxidative terpenoids (Sharma, et al., 2019, Isah, 2019), brassinosteroids (Sharma, et al., 2017), and for cuticular waxes with functions in cold and drought resistance (Shepherd and Griffiths, 2006). Si interactions are also observed in this context. There are some effects on phenylpropanoids (Fig. 4, Table. S5) and particularly on ABA and ethylene metabolism (Fig. 7, Table. S8), all related to stress adaptations. Thus, this high expression of discussed transcripts under Si treatments may reflect some priming effects of Si application in increasing the membrane stability and reducing metal leaching that consequently may trigger the downstream cold tolerance and adaptive effects.

5.11 Restored growth hormonal balance by Si application was detectable at the molecular Level

Cold tolerance is affected by changes in the homeostasis of various hormones. A complex interplay among phytohormone-signaling pathways is crucial for the regulation of plant response to cold stress. In this regard, previously, we reported that cold stress-induced 60% reduction of IAA accumulation in the shoot and root tissue was associated with inhibition of shoot and root growth while all these symptoms were reverted by exogenous Si application by improving Zn status, inhibited oxidative degradation of IAA resulted from excess ROS production (Moradtalab et al., 2018). It was reported that Zn deficiency blocked the IAA signal with a significant decrease in antioxidant metabolites in root tissue, suggesting that IAA signaling is closely linked with the antioxidant defense underlying Zn deficiency in rice seedlings (Begum et al., 2016). In the current study, Si application altered the expression of IAA genes involving in its biosynthesis and response pathways. Accordingly, the reduction of IAA responses under cold stress was confirmed at the molecular level, since cold stress up-regulated the expression of DFL1, IAA9, and IAA16 that were down-regulated by Si application (Fig. 7, Table. S7). DFL1 is an auxin responsive GH3 gene homolog that negatively regulates shoot cell elongation and lateral root formation, and positively regulates the light response of hypocotyl length (Nakazawa et al., 2001). IAA9 and IAA16 are Aux/IAA proteins as repressors of early auxin response genes at low auxin concentrations. IAA16 reduced responses to IAA and ABA and impedes plant growth (Rinaldi, et al., 2010). Oppositely, cold stress down-regulated the expression of IAR3, ILR1, IAR3, JAR1, and SMALL AUXIN UP RNAs (SAURs) which were up-regulated by Si application (Fig. 7, Table. S7). IAR3 and ILR1 as IAA-amino acid hydrolases regulate the pool of active IAA by releasing free IAA via cleaving IAA-amino

acid conjugates (LeClere et al., 2002).

JAR1 and JAR3 are responsible for the adenylation of JA to form the active JA-amino acid conjugates (Staswick et al., 2002) which regulates JA homeostasis. Although SAURs are the largest family of early auxin response genes, their functions have remained elusive, however, it was proposed that they are key effector outputs of hormonal and environmental signals that regulate plant growth and development (Ren and Gray, 2015). In our previous study, cold-stress protection by Si application was also associated with increased zeatin (CK) concentrations in the root and shoot tissue (Moradtalabe et al., 2018). The results of DETs demonstrated that cold stress significantly increased the transcripts of CKX1, CKX5 (Fig. 7, Table. S7). Cytokinin oxidase/dehydrogenase enzymes (CKX) selectively inactivated CK by oxidative cleavage of its side chain (Vyroubalova et al., 2009). Similar to our results (Fig. 7, Table. S7) under salt and osmotic stresses, most of the genes CKXs were up-regulated in roots of maize plants (Vyroubalova et al., 2009). ZmCKX1 is a key regulator of active CK levels in developing maize roots and is induced by ABA under stress conditions, results in aberrant degradation of CK and impaired normal development (Brugiere et al., 2003). However, up-regulation of CRE1 and down-regulation of CKXs genes by Si application under cold stress indicated that CK level was restored and the signaling pathway of CK responses was active (Fig. 7, Table. S7) since CRE1 is a CK receptor that triggers a cascade of phosphorylation reactions upon a perception of this hormone and regulates root growth. Shoot growth in cold-stressed plants is also affected by a reduction of GA levels, leading to an increased abundance of nuclear DELLA-protein growth repressors via a signaling pathway involving CBF/DREB1 TFs (Miura and Furumoto, 2013; Eremina et al., 2016). Accordingly, in our previous study GA levels in shoot and roots of Zn-deficient maize seedlings declined by ∼60–70% under cold stress

(Moradtalab et al., 2018). It was reported that Zn deficiency in maize markedly reduced the level of GA1, but not GA20, suggesting blockage of 3β hydroxylation (Sekimoto et al., 1997). This cold-induced inactivation of GA is also confirmed by our results due to the up-regulation of two important genes involved in GA deactivation GA2 OX1 and GA2 OX8 (GA 2-oxidase genes, Fig. 7, Table. S7). These two genes are GA dioxygenase, which catalyzes catabolism and inactivation of bioactive GAs or their precursors (Zhao et al., 2007). In *Arabidopsis*, GA 2-oxidase genes impair GA biosynthesis by repressing the GA 20-oxidase gene (Eremina et al., 2016). However, this effect was completely reverted by Si seed soaking since GA 20-oxidase (GA20OX), GA1, and GASA2 transcripts were up-regulated by Si application (Fig. 7, Table. S7). GA20OX and GA1 are involved in GA biosynthesis. Over-expression of GA20OX1 causes high levels of bioactive gibberellins (Oikawa, 2004). Bioactive GA promotes the degradation of DELLA proteins to release their suppression on the GA signaling pathway. Similar to our results (Fig. 7, Table. S7) bioactive GA further induces GASA genes that control shoot elongation and leaf sheath length (Oikawa, 2004 and Eremina et al., 2016).

5.12 Stress-related hormones changes by Si application reprograms cold stress adaptations

When plants are challenging by stress conditions, in general, the growth inhibition occurs that decreases the capacity for energy utilization, which in turn, results in cold acclimation processes. Stress hormone ABA inhibits plant growth by modulating the actions of other hormones (Peleg and Blumwald, 2011). In our previous study, Si treatment in maize seedlings under cold stress influenced the levels of ABA, SA, and JA (Moradtalab et al., 2018). Increased cold tolerance by exogenous applications of ABA, SA, and JA has been reported for various plant species (Horváth et al., 2007; Eremina et al., 2016; Hu et al.,

2017). In the current study, exposure of maize, as cold-sensitive plant species to a 7-days cold period, significantly increased the transcripts of NCED4, NCED5, RTE1, ABF3, and ABF4 in both not-treated and treated plants with Si (Fig. 7, Table. S8). NCED4 and NCED5 (9-cis-epoxycarotenoid dioxygenase) are the key enzymes in ABA biosynthesis (Qin and Zeevaart, 2002). In Arabidopsis, in the ABA-dependent pathway, ABA biosynthesis genes contribute to the enrichment of COR genes, such as RD29A, RD22, COR15A, COR47, and P5CS via the activity of their ABRE cis-element (Shi and Yang, 2014). ABF3 and ABF4 are master TFs cooperatively regulate the ABRE-dependent signaling and improve stress tolerance in Arabidopsis and rice (Yoshida et al., 2010). Accordingly, in our study, in maize seedlings, the ABA-dependent pathway was activated under cold stress and by Si application (Fig. 7, Table. S8). However, in our findings, the different regulated gene in ABA-related transcripts was LEM1, that was down-regulated by cold and up-regulated by Si (Fig. 7, Table. S8) suggesting a possible signaling effect of metal handling in cold stress tolerance to keep osmotic homeostasis of the cell since LEM1 is a late embryogenesis abundant (LEA) protein that protects other proteins from aggregation due to desiccation or Cold-induced osmotic stress (Saucedo et al., 2017).

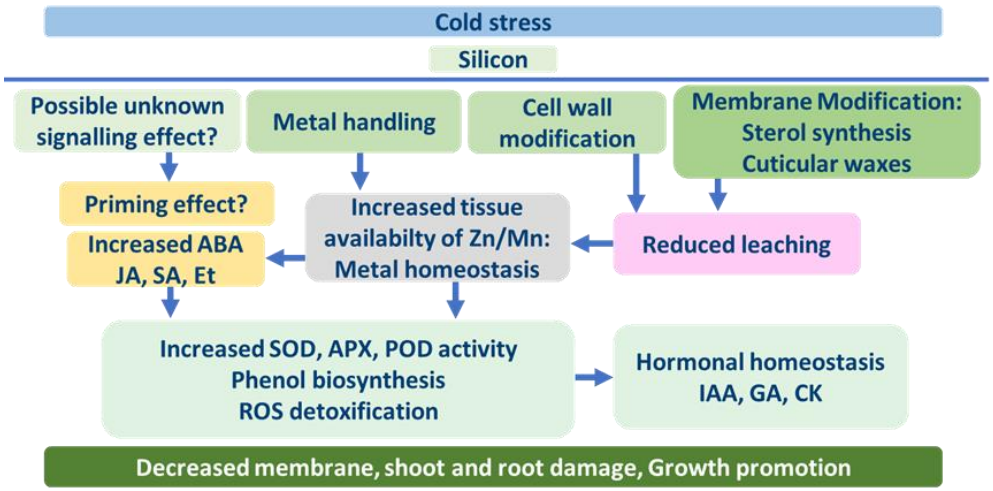
Unlike cold -affected plants, the cold-protective effect of Si starter treatment was also associated with an increase of genes involving in JA and SA biosynthesis including LOX1, AOC3, AOS, OPR1, and BCM respectively (Fig. 7, Table. S8). Our results are in the line with ABA roles in Arabidopsis and rice regulating the adaptive expression of cold-related genes with cold-protective functions via ABA-dependent and independent pathways, in cross-talks with Et, SA, and JA responses (Yang et al., 2015, Szalai et al., 2011; Eremina et al., 2016). Up-regulation of RTE1 in our study indicates that ABA controls Et responses in maize seedlings under cold stress and by Si treatment (Fig. 7, Table. S8) since RTE1 is

a conserved membrane protein that specifically regulates ethylene responses (Yang et al., 2015). In ABA-independent pathways, overexpression of CBF/DREB1 genes improves tolerance to cold, drought, and salt stresses. The components of the ABA-independent pathway are MYB family genes (Mattana et al., 2005, Vannini et al., 2004), calcium signal (required for the full expression of the COR genes in Arabidopsis), the activation of calcium-dependent protein kinases (CPKs) and calcineurin B-like proteins (CBLs) to regulate expression of the CBF genes. In Arabidopsis, the expression of CIPKs is strongly induced by cold stress and ABA treatment. CIPKs are the convergence point for the ABA-dependent and ABA-independent stress responses (Shi and Yang, 2014). In Arabidopsis, the exogenous application of JA significantly improves freezing tolerance through this ABA-independent pathway (Hu et al. 2013). Arabidopsis mutants that are defected in overproducing SA, are chilling sensitive phenotypes. SA responses are activated via the Ca^{2+} signaling pathway in the regulation of freezing tolerance and CBF2 expression during cold stress (Kim et al., 2013). Anyhow, the exact role of SA in cold tolerance is still unclear. However, unlike Et responses in Arabidopsis under cold stress, the Et biosynthesis in maize plants was not repressed in both not-treated or treated plants with Si (Fig. 7, Table. S8). ACC $_{OX}$ that catalyzes the final step of Et biosynthesis (Park et al., 2018) was up-regulated by cold stress while EIN4 transcripts were oppositely down-regulated (Fig. 7, Table. S87). EIN4 (ETHYLENE INSENSITIVE 4) is the Et receptor that negatively regulates Et signaling pathway (Yang et al., 2015). Accordingly, the role of Et in plant responses to cold stress is complex and species-dependent. Although, the complex cross-talk between Et, ABA, and cold signaling is still unclear but studied revealed that Et in Arabidopsis negatively regulates cold signaling, at least partially, through direct transcriptional control of the COR, CBFs via EIN3 (Shi et al. 2012). In contrast, enhanced chilling and freezing

tolerance has been observed with an increase of Et biosynthesis in several plant species, including tomato, cucumber, and tobacco (Shi and Yang, 2014). Thus, in our study, the Et biosynthesis pathway was triggered under cold stress but Et signaling seems to be impaired by cold stress which in turn restored by Si application (Fig. 7, Table. S8).

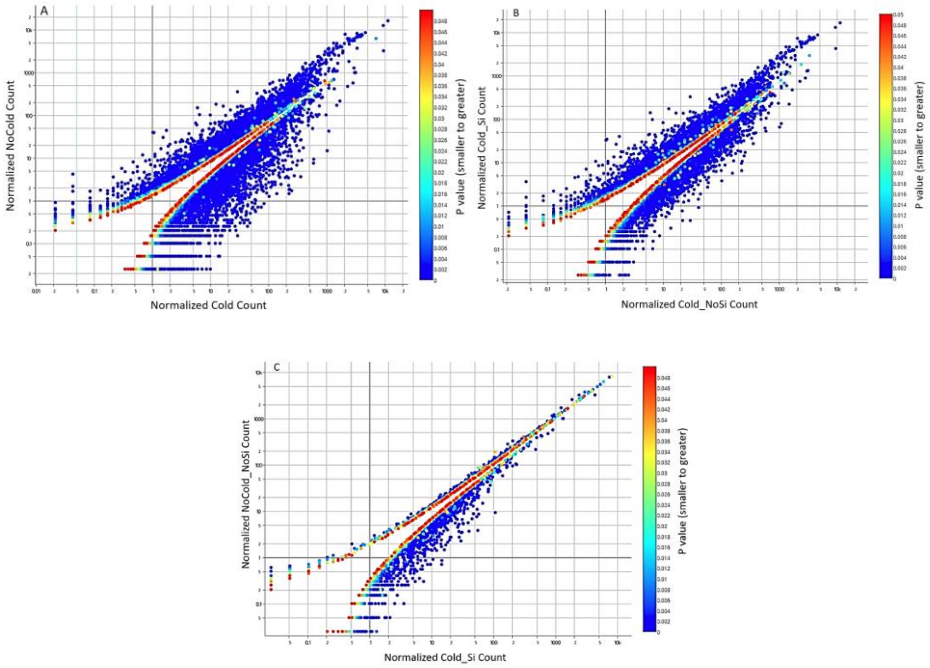
5.13 Conclusion

In the present study, RNA-seq was applied to detect the transcriptional changes in seedling maize leaves in response to cold stress. Our results suggest that Si protects maize seedling from cold stress via increasing membrane stability (upregulating sterol synthesis, terpenoids, and cuticular waxes biosynthesis), metal handling, cell wall modifications, and decreased membrane leaching. The consequence of the reduced metal leaching particularly metal homeostasis causes decreased ROS damage and increased maize seedlings tolerance to cold stress. This study extends the understanding of the molecular events via metal homeostasis of maize responses to cold stress in the seedling stage and will be useful for identifying major candidate genes and molecular markers for improving resistance to cold stress in maize plants. A conceptual model for Si-induced cold stress mitigation mediating cold tolerance in maize seedlings is summarized in Fig. 8.

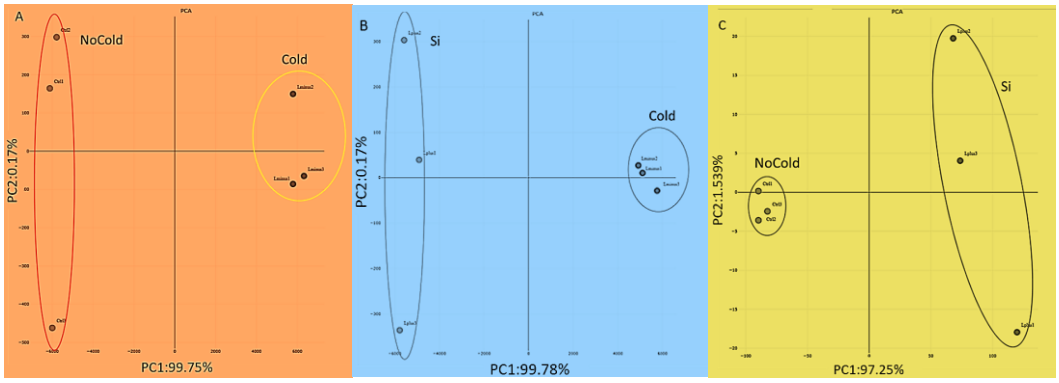


Chapter 5. Fig. 8. Model summarizing Si mediating cold tolerance in maize seedlings

5.14 Supplementary Material



Chapter 5. Fig. S1. Total normalized counts of transcripts in the shoots of 10-day-old maize seedlings in filter paper roll for 7 days at 18 ◦C (NoCold_NoSi) or 12 ◦C without silicon (Cold_NoSi:) or with silicon (Cold_Si) as 0.1 mM H_4SiO_4; The illustrated plots are: (A) 13740 chosen transcripts from Cold vs. NoCold without Si application, (B) 11931 chosen transcripts from Si vs. NoSi under Cold stress, (C) 3450 chosen transcripts from Cold_Si vs. NoCold_NoSi. The counts of transcripts were chosen of total 27186 active genes. P values < .05 are indicated by different colors, as smaller (blue) to greater (red).



Chapter 5. Fig. S2. Principal component analysis (PCA) of top 20 highly expressed transcripts of maize leaves grown in filter paper rolls for 7 days at 18 ◦C (NoCold_NoSi) or at 12 ◦C without silicon (Cold_NoSi) or with silicon (Cold_Si) as 0.1 mM H_4SiO_4; The replicates of each library were not significantly different by 0.17 % for NoCold_NoSi and Cold_NoSi and 1.54% for Cold_Si, (A) NoCold and Cold without Si is significantly different by 99.75 %, (B) NoSi and Si under Cold stress by 99.78%, (C) NoCold_NoSi and Cold_Si by 97.25 %.

Chapter 5. Table. S1. Principal component analysis (PCA) of the top 20 highly expressed transcripts

Gene ID	Description
Zm00001d034705	Putative uncharacterized protein
Zm00001d046170	PEP carboxylase
Zm00001d044099	Putative uncharacterized protein
Zm00001d052165	Ferredoxin/nitrite reductase
Zm00001d030557	Putative uncharacterized protein
Zm00001d045621	Plastocyanin
Zm00001d008706	Photosystem I reaction center subunit XI isoform 1
Zm00001d021620	ATP synthase gamma chain
Zm00001d038579	Phosphoglycerate kinase
Zm00001d053015	Fructose-bisphosphate aldolase
Zm00001d009028	Putative uncharacterized protein
Zm00001d023559	Fructose-bisphosphate aldolase
Zm00001d005996	Photosystem I reaction center subunit V
Zm00001d038984	Photosystem I reaction center subunit VI%2C chloroplast
Zm00001d045621	Putative uncharacterized protein
Zm00001d016134	Cytochrome b6-f complex iron-sulfur subunit
Zm00001d039715	Ultraviolet-B-repressible protein
Zm00001d008681	Ultraviolet-B-repressible protein
Zm00001d035001	Chloroplast ferredoxin 1
Zm00001d027488	Glyceraldehyde-3-phosphate dehydrogenase

Chapter 5. Table. S2. Putative description of selected BINs according to Maize GDB Gene Record (B73v4, Portwood, et al., 2018) of 78 unique up-regulated expressed genes by Si application (0.1 mM H_4SiO_4) in 10-day-old maize leaves grown in filter paper rolls for 7 days at 12 ◦C as Cold stress.

BIN Name	Gene ID	Description
Cell wall. Hemicellulose synthesis	Zm00001d050885	transferring glycosyl groups
Cell wall. Pectin esterases	Zm00001d050082	pectin methylesterase 44 (ATPME44, PME44)
Lipid metabolism	Zm00001d005383	lecithin: cholesterol acyltransferase family protein
Secondary metabolism. PAL	Zm00001d051166	Phenylalanine ammonia-lyase (PAL)
Secondary metabolism. flavonoids. anthocyanins.	Zm00001d024432	transferring acyl groups other than amino-acyl groups
Secondary metabolism. simple phenols	Zm00001d048759	L-ascorbate oxidase precursor
Hormone metabolism. salicylic acid. synthesis	Zm00001d015216	S-adenosyl-L-methionine: benzoic acid carboxyl methyltransferase
Co-factor	Zm00001d002755	Mo-molybdopterin cofactor sulfurase
Stress	Zm00001d005506	osmotin-like protein
Stress	Zm00001d013610	unknown protein
Transcription factor (TF.) ABA response	Zm00001d035903	DNA-binding protein: ABI3
TF. Ethylene responsive	Zm00001d027924	Ethylene-responsive transcription factor 1 (ERF1_ORYSA)
TF. bHLH, Basic Helix-Loop-Helix	Zm00001d030028	MYC2, DNA binding / transcription activator/ transcription factor
TF. Constans-like zinc finger	Zm00001d036214	zinc ion binding
TF. C2H2 zinc finger	Zm00001d038338	MYB 85
TF. Homeobox	Zm00001d044081	homeobox-leucine zipper protein ATHB-4
TF. WRKY	Zm00001d017712	WRKY46
TF. WRKY	Zm00001d044680	WRKY5
TF. WRKY	Zm00001d032265	WRKY25

TF. WRKY	Zm00001d009698	WRKY20
TF. Argonaute	Zm00001d006351	AGO18 a: miRNA binding/protein binding / siRNA binding
TF. ZFHD	Zm00001d009674	ZHD11: DNA binding protein ZF-HD homeobox protein
TF. Unknown	Zm00001d037400	chloroplast nucleoid DNA-binding protein
Transport. metal	Zm00001d046243	iron ion transmembrane transporter activity
Transport. potassium	Zm00001d012717	KCH8: Potassium channel 8

Chapter 5. Table. S3. Transcript description of DETs involved in primary metabolism in pairwise comparisons of Cold_NoSi and Cold_Si treatments of 10-day-old maize leaves grown in filter paper rolls for 7 days at 12 ◦C as Cold stress.

BIN Name	Gene ID	Description
photosynthesis.lightreaction.photosystem	Zm00001d009543	Light-harvesting complex I 11 kDa protein
major CHO metabolism.synthesis.starch.transporter	Zm00001d045884	rotein ADP, ATP carrier protein, mitochondrial precursor
cell wall.precursor synthesis.UXS	Zm00001d031839	NAD-dependent epimerase/dehydratase family protein
cell wall.hemicellulose synthesis	Zm00001d027643	catalytic/ transferase, transferring glycosyl groups
	Zm00001d053327	fucosyltransferase/ transferase, transferring glycosyl groups
cell wall.cell wall proteins.AGPs.AGP	Zm00001d053755	protein fasciclin-like arabinogalactan protein 8 precursor
cell wall.degradation.mannan-xylose-arabinose-fucose	Zm00001d028103	glycosyl hydrolase family 10 protein
cell wall.degradation.pectate lyases and polygalacturonases	Zm00001d035753	protein binding (Polygalacturonase-inhibiting protein) (PGIP-1)
cell wall.modification	Zm00001d004572	hydrolase
	Zm00001d024378	hydrolase, acting on glycosyl bonds/hydrolase
lipid metabolism.FA synthesis and FA elongation.ACP desaturase	Zm00001d021059	acyl-(acyl-carrier-protein) desaturase
lipid metabolism.Phospholipid synthesis.choline-phosphate cytidylyltransferase	Zm00001d024528	catalytic/ choline-phosphate cytidylyltransferase
lipid metabolism."exotics" (steroids, squalene etc)	Zm00001d023774	protein erg28 like protein
	Zm00001d040493	protein erg28 like protein
	Zm00001d040481	protein erg28 like protein
	Zm00001d040501	protein erg28 like protein
lipid metabolism.lipid degradation	Zm00001d012152	triacylglycerol lipase
	Zm00001d006720	protein triacylglycerol lipase
	Zm00001d052931	acyl-CoA oxidase
amino acid metabolism.synthesis.	Zm00001d029083	alanine--glyoxylate aminotransferase
	Zm00001d044608	protein asparagine synthetase
	Zm00001d000294	catalytic/ methionine gamma-lyase

Chapter 5. Table. S4. Transcript description of DETs involved in secondary metabolism in pairwise comparisons of Cold_NoSi and Cold_Si treatments of 10-day-old maize leaves grown in filter paper rolls for 7 days at 12 ◦C as Cold stress.

BIN Name	Gene ID	Description
secondary metabolism.isoprenoids.mevalonate pathway.HMG-CoA reductase	Zm00001d030595	hydroxymethylglutaryl-CoA reductase
secondary metabolism.isoprenoids.terpenoids	Zm00001d041082	GA2 (GA REQUIRING 2); ent-kaurene synthase
	Zm00001d024486	terpene synthase/cyclase family protein
	Zm00001d030814	CAS1 (cycloartenol synthase 1); cycloartenol synthase
secondary metabolism.phenylpropanoids	Zm00001d006263	shikimate O-hydroxycinnamoyltransferase/ transferase

	Zm00001d004921	O-methyltransferase family
	Zm00001d035865	transferase family protein
	Zm00001d012333	transferase family protein
secondary metabolism.phenylpropanoids.lignin biosynthesis.CAD	Zm00001d020400	catalytic/ oxidoreductase/ zinc ion binding
secondary metabolism.N misc.alkaloid-like	Zm00001d027947	tyrosine decarboxylase tyrosine
secondary metabolism.sulfur-containing.misc.alliinase	Zm00001d043651	alliinase family protein
secondary metabolism.wax	Zm00001d048476	membrane bound O-acyl transferase (MBOAT) family protein / wax synthase-related
secondary metabolism.flavonoids.dihydroflavonols	Zm00001d043013	cinnamoyl-CoA reductase family
secondary	Zm00001d053514	UDP-glucosyltransferase/ UDP-glycosyltransferase/ transferase, transferring glycosyl groups
	Zm00001d009022	IFRH_MAIZE Isoflavone reductase homolog IRL (EC 1.3.1.-) - Zea mays (Maize)
secondary metabolism.simple phenols	Zm00001d040248	LAC7 (laccase 7), laccase
	Zm00001d023617	chrL-ascorbate oxidase precursor (EC 1.10.3.3) (Ascorbase), protein copper ion binding protein
	Zm00001d028797	ASO_CUCMA L-ascorbate oxidase precursor (EC 1.10.3.3) (Ascorbase) (ASO)

Chapter 5. Table. S5. Transcript description of DETs involved in metal handling, redox, and transport in pairwise comparisons of Cold_NoSi and Cold_Si treatments of 10-day-old maize leaves grown in filter paper rolls for 7 days at 12 ◦C as Cold stress.

BIN Name	Gene ID	Description
metal handling	Zm00001d017668	SBP1 (selenium-binding protein 1); selenium binding
	Zm00001d042367	SBP1 (selenium-binding protein 1); selenium binding
metal handling.binding, chelation, and storage	Zm00001d027405	metal ion binding protein
	Zm00001d051905	metal ion binding protein
	Zm00001d020257	heavy-metal-associated domain-containing protein/copper chaperone (CCH)-related, metal ion binding protein
redox.heme	Zm00001d038718	HBL1_ORYSA Non-symbiotic hemoglobin 1 (rHb1) (ORYsa GLB1a) - Oryza sativa (Rice)
redox.glutaredoxins	Zm00001d023869	glutaredoxin family protein
	Zm00001d040836	electron carrier/ protein disulfide oxidoreductase
	Zm00001d023870	glutaredoxin family protein
redox.dismutases and catalases	Zm00001d034150	protein superoxide dismutase, chloroplast
misc.oxidases - copper, flavone, etc.	Zm00001d030871	YUCCA5; mono oxygenase disulfide oxidoreductase/ monooxygenase/ oxidoreductase
	Zm00001d048416	monooxygenase, putative (MO2) monooxygenase
	Zm00001d006069	oxidoreductase, zinc-binding dehydrogenase family
	Zm00001d007285	NADP-dependent oxidoreductase
misc.peroxidases	Zm00001d013212	peroxidase, PER1_ORYSA Peroxidase 1 precursor (EC 1.11.1.7) - Oryza sativa (Rice)
	Zm00001d022457	peroxidase, PER2_ORYSA Peroxidase 2 precursor (EC 1.11.1.7) - Oryza sativa (Rice)

	Zm00001d053554	peroxidase, PERP7_BRARA Peroxidase P7 (EC 1.11.1.7) (TP7) - Brassica rapa (Turnip)
	Zm00001d050572	peroxidase, PER1_ARAHY Cationic peroxidase 1 precursor
transport.p- and v-ATPases	Zm00001d029535	methyltransferase/ nucleic acid binding protein
transport.sugars	Zm00001d027268	STP1 (SUGAR TRANSPORTER 1); carbohydrate transmembrane transporter/ sugar:hydrogen symporter
transport.amino acids	Zm00001d029318	amino acid permease family protein
	Zm00001d025437	BAT1 (BIDIRECTIONAL AMINO ACID TRANSPORTER 1); amino acid transmembrane transporter
transport.ammonium	Zm00001d038412	ATAMT2 (AMMONIUM TRANSPORTER 2); ammonium transmembrane transporter/ high affinity secondary active ammonium transmembrane transporter
	Zm00001d034782	ATAMT2 (AMMONIUM TRANSPORTER 2); ammonium transmembrane transporter/ high affinity secondary active ammonium transmembrane transportorter
transport.phosphate	Zm00001d004306	carbohydrate transmembrane transporter/ phosphate transmembrane transporter/ sugar:hydrogen symporter
transport.metal	Zm00001d010629	ATCHX15; monovalent cation:proton antiporter/ sodium:hydrogen antiporter
transport.potassium	Zm00001d042244	HAK5 (HIGH AFFINITY K+ TRANSPORTER 5); potassium ion transmembrane transporter/ potassium:sodium symporter
transport.ABC transporters	Zm00001d052435	ZIFL1 (ZINC INDUCED FACILITATOR-like 1); tetracycline:hydrogen antiporte
	Zm00001d053706	ABC transporter family protein
	Zm00001d021647	PDR11 (PLEIOTROPIC DRUG RESISTANCE 11); ATPase, coupled to transmembrane movement of substances

Chapter 5. Table. S6. Expression values of genes dependent on Zn and metal handling as log2 FC ≥ 3, log2 FC ≤ −3 in pairwise comparisons of Cold_NoSi and Cold_Si treatments (“Si”**)** in 10-day-old maize leaves grown in filter paper rolls for seven days at 12 °C as Cold stress. (A) among common 553 DETs in Cold- and Si- affected shoot and (B) among 65 unique up-regulated expressed genes by Si application under cold stress.

A

BIN Name	**Gene ID**	**Description**	**“cold”**	**“Si”**
secondary metabolism.phenylpropanoids.lignin biosynthesis.CAD	Zm00001d020400	catalytic/ oxidoreductase/ zinc ion binding	-4.17	+3.57
secondary metabolism.simple phenols	Zm00001d023617	chrL-ascorbate oxidase precursor (EC 1.10.3.3) (Ascorbase), protein copper ion binding protein	-5.87	+4.12
	Zm00001d028797	L-ascorbate oxidase precursor (EC 1.10.3.3) (Ascorbase) (ASO-CUCMA)	-10	+10
metal handling	Zm00001d017668	SBP1 (selenium-binding protein 1); selenium binding	-3.62	+3.35
	Zm00001d042367	SBP1 (selenium-binding protein 1); selenium binding	-4.21	+4.89
metal	Zm00001d027405	metal ion binding protein	-3.22	+3.29

handling.binding, chelation and storage	Zm00001d051905	metal ion binding protein	-10	+10
	Zm00001d020257	heavy-metal-associated domain-containing protein / metal ion binding protein	-4.21	+4.67
redox.dismutases and catalases	Zm00001d034150	superoxide dismutase protein, chloroplast	-10	+10
misc.oxidases	Zm00001d030871	mono oxygenase disulfide oxidoreductase/ monooxygenase/ oxidoreductase: YUCCA5;	-10	+10
	Zm00001d048416	monooxygenase, putative	-5.12	+3.01
	Zm00001d006069	oxidoreductase, zinc-binding dehydrogenase	-4.45	+3.60
	Zm00001d007285	NADP-dependent oxidoreductase	-6.46	+5.45
misc.peroxidases	Zm00001d013212	peroxidase, PER1-ORYSA Peroxidase 1 precursor (EC 1.11.1.7) - Oryza sativa (Rice)	-3.55	+3.55
	Zm00001d022457	peroxidase, PER2-ORYSA Peroxidase 2 precursor (EC 1.11.1.7) - Oryza sativa (Rice)	-4.05	+3.72
	Zm00001d053554	peroxidase, PERP7-BRARA Peroxidase P7 (EC 1.11.1.7) (TP7) - Brassica rapa (Turnip)	-4.60	+3.29
	Zm00001d050572	peroxidase, Cationic peroxidase 1 precursor	-4.52	+3.93
transport.metal	Zm00001d010629	monovalent cation: proton antiporter	-10	+10
	Zm00001d046243	iron ion transmembrane transporter activity		
transport.ABC transporters	Zm00001d052435	ZIFL1 (Zn-induced facilitator like 1)	-10	+10
ABA.signal transduction	Zm00001d018178	ABF4 (ABRE BINDING FACTOR 4); DNA binding / protein binding / transcription activator / bZIP transcription factor/ ABI5 family protein	-3.91	+3.04
	Zm00001d044940	ABF3 (ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 3); DNA binding / protein binding / transcription activator / bZIP transcription factor family protein	-4.79	+3.25

B

BIN Name	**Gene ID**	**Description**	**"cold"**	**"Si"**
secondary metabolism. PAL	Zm00001d051166	Phenylalanine ammonia-lyase (PAL)	-2.42	+3.58
transcription factors	Zm00001d036214	zinc ion binding: Constans-like zinc finger	-1.47	+3.06
	Zm00001d038338	MYB 85: C2H2 zinc finger	-1.54	+3.55
	Zm00001d044081	homeobox-leucine zipper protein ATHB-4	-2.20	+3.20
	Zm00001d017712	WRKY46	-2.74	+3.74
	Zm00001d044680	WRKY5	-2.33	+3.33
	Zm00001d009698	WRKY20	-1.66	+4.00
	Zm00001d009674	ZHD11: DNA binding protein	-2.25	+3.43

Chapter 5. Table. S7. Transcript description of DETs involved in growth hormonal biosynthesis and signal transduction in pairwise comparisons of Cold_NoSi and Cold_Si treatments of 10-day-old maize leaves grown in filter paper rolls for 7 days at 12 ◦C as Cold stress.

BIN Name	Gene ID	Description
IAA.synthesis-degradation	Zm00001d011687	IAR3, JR3 | IAR3 (IAA-ALANINE RESISTANT 3); IAA-Ala conjugate hydrolase/ metallopeptidase
	Zm00001d019306	ILR1 (IAA-LEUCINE RESISTANT 1); IAA-Leu conjugate

		hydrolase/ IAA-Phe conjugate hydrolase/ metallopeptidase
IAA.induced-regulated-responsive-activated	Zm00001d033464	auxin-responsive family protein, Auxin-responsive SAUR gene family member
	Zm00001d015661	auxin-responsive SAUR gene family member
	Zm00001d031666	ATB2; oxidoreductase
	Zm00001d010697	GH3.6, DFL1 (DWARF IN LIGHT 1); indole-3-acetic acid amido synthetase, (Auxin-responsive GH3-like protein 4)
	Zm00001d026262	auxin-responsive protein, small auxin up RNA (SAUR_D), Auxin-responsive SAUR gene family member
	Zm00001d050735	auxin-responsive protein-related
	Zm00001d020606	auxin-responsive protein-related
	Zm00001d018414	OsIAA9 - Auxin-responsive Aux/IAA gene family member
	Zm00001d011377	JAR1 (JASMONATE RESISTANT 1); ATP binding / adenylyltransferase/ catalytic/ jasmonate-amino synthetase, Auxin-responsive GH3-like protein 5)
	Zm00001d010360	IAA16_ORYSA Auxin-responsive protein IAA16 (Indoleacetic acid-induced protein 16), Auxin-responsive Aux/IAA gene family member
	Zm00001d036623	OsSAUR25 - Auxin-responsive SAUR gene family member
	Zm00001d052148	OsSAUR12 - Auxin-responsive SAUR gene family member
	Zm00001d012222	OsSAUR24 - Auxin-responsive SAUR gene family member
CK.synthesis-degradation	Zm00001d046356	cytokinin dehydrogenase, CKX1_MAIZE Cytokinin dehydrogenase 1 precursor (EC 1.5.99.12) (ZmCKX1) - Zea mays (Maize)
	Zm00001d043293	CKX5 (CYTOKININ OXIDASE 5); cytokinin dehydrogenase CKX1_MAIZE Cytokinin dehydrogenase 1 precursor (EC 1.5.99.12) (Cytokinin oxidase 1) (CKO 1) (COX 1) (ZmCKX1) - Zea mays (Maize)
CK.signal transduction	Zm00001d051812	CRE1, WOL1, AHK4, ATCRE1 | WOL (WOODEN LEG); cytokinin receptor/ osmosensor/ phosphoprotein phosphatase/ protein histidine kinase
GA.synthesis-degradation	Zm00001d027651	2-oxoglutarate-dependent dioxygenase
	Zm00001d018617	ATGA2OX8, GA2OX8 | GA2OX8 (GIBBERELLIN 2-OXIDASE 8); gibberellin 2-beta-dioxygenase
	Zm00001d024512	GA1 (GA REQUIRING 1); ent-copalyl diphosphate synthase/ magnesium ion binding
	Zm00001d029648	GA1 (GA REQUIRING 1); ent-copalyl diphosphate synthase/ magnesium ion binding
GA.synthesis-degradation.GA20 oxidase	Zm00001d013725	ORYSA Gibberellin 20 oxidase 2 (EC 1.14.11.-) (Gibberellin C-20 oxidase 2) (GA 20-oxidase 2)
GA.synthesis-degradation.GA2 oxidase	Zm00001d008909	ATGA2OX1 (gibberellin 2-oxidase 1); gibberellin 2-beta-dioxygenase (Gibberellin 2-beta-hydroxylase 1), Gibberellin 2-oxidase 1, GA 2-oxidase 1) gibberellin 2-beta-dioxygenase
GA.induced-regulated-responsive-activated	Zm00001d010193	GASA2 | GASA2 (GAST1 PROTEIN HOMOLOG 2), gibberellin-regulated protein 2 precursor

Chapter 5. Table. S8. Transcript description of DETs involved in stress-related hormonal biosynthesis and signal transduction in pairwise comparisons of Cold_NoSi and Cold_Si treatments of 10-day-old maize leaves grown in filter paper rolls for 7 days at 12 ◦C as Cold stress.

BIN Name	Gene ID	Description
ABA.synthesis-degradation	Zm00001d034388	indole-3-acetaldehyde oxidase
	Zm00001d017766	NCED4 | NCED4 (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 4)
	Zm00001d027592	NCED5, ATNCED5 | NCED5 (NINE-CIS-

		EPOXYCAROTENOID DIOXYGENASE 5); 9-cis-epoxycarotenoid dioxygenase
	Zm00001d018178	ABF4, AREB2 | ABF4 (ABRE BINDING FACTOR 4); DNA binding / protein binding / transcription activator/bZIP transcription factor ABI5
ABA.signal transduction	Zm00001d044940	ABF3, DPBF5 | ABF3 (ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 3); DNA binding / protein binding / transcription activator/ bZIP transcription factor family protein
ABA.induced-regulated-responsive-activated	Zm00001d037985	ATEM1, LEM1 (LATE EMBRYOGENESIS ABUNDANT 1) protein embryonic abundant protein 1
	Zm00001d004376	RTE1 (REVERSION-TO-ETHYLENE SENSITIVITY1)
	Zm00001d007341	protein HVA22-like protein
	Zm00001d045640	HVA22F (HVA22-LIKE PROTEIN F), protein TB2/DP1, HVA22 family protein
	Zm00001d043578	protein TB2/DP1, HVA22 family protein
JA.synthesis-degradation	Zm00001d013493	LOX1; lipoxygenase, LOX4_ORYSA Probable lipoxygenase 4 (EC 1.13.11.12) - Oryza sativa (Rice)
	Zm00001d048021	AOS (ALLENE OXIDE SYNTHASE); allene oxide synthase/ hydro-lyase/ oxygen binding
	Zm00001d013185	AOS (ALLENE OXIDE SYNTHASE); allene oxide synthase/ hydro-lyase/ oxygen binding
	Zm00001d034184	AOS (ALLENE OXIDE SYNTHASE); allene oxide synthase/ hydro-lyase/ oxygen binding
	Zm00001d047340	AOC3 (ALLENE OXIDE CYCLASE 3); allene-oxide cyclase
	Zm00001d044908	OPR1; 12-oxophytodienoate reductase
	Zm00001d044906	ATOPR1 | OPR1; 12-oxophytodienoate reductase
	Zm00001d052831	JMT (JASMONIC ACID CARBOXYL METHYLTRANSFERASE); jasmonate O-methyltransferase
SA.synthesis-degradation	Zm00001d029620	Indole-3-acetate beta-glucosyltransferase (EC 2.4.1.121) (IAA-Glu synthetase) ((Uridine 5'-diphosphate-glucose:indol-3-ylacetyl)-beta-D-glucosyl transferase) - Zea mays (Maize)
	Zm00001d044762	S-adenosyl-L-methionine:carboxyl methyltransferase family protein
	Zm00001d015220	Benzoate carboxyl methyltransferase (EC 2.1.1.-) (S-adenosyl-L-methionine:benzoic acid carboxyl methyltransferase) -
Et.synthesis-degradation	Zm00001d044954	2-oxoglutarate-dependent dioxygenase, putative
	Zm00001d024853	1-aminocyclopropane-1-carboxylate oxidase, putative / ACC oxidase, putative
	Zm00001d039575	2-oxoglutarate-dependent dioxygenase
Et.signal transduction	Zm00001d049364	ERF7 (ETHYLINE RESPONSE FACTOR 7); DNA binding / protein binding / transcription factor/ transcription repressor
	Zm00001d024324	ethylene-responsive transcription factor
	Zm00001d004372	EIN4 (ETHYLENE INSENSITIVE 4); ethylene binding / glycogen synthase kinase 3/ protein histidine kinase/ receptor
Et.induced-regulated-responsive-activated	Zm00001d002200	ethylene-responsive protein -related, DNA binding protein
	Zm00001d026120	ethylene-responsive protein -related

Chapter 5. Table. S9. Sequence and primers ID of ten DETs were selected from Cold_NoSi and Cold_Si for qRT-PCR analysis

Primer	Reference Assays
ID	**Sequence 5' > 3'**
ZmActin1fwd	GAGATCACGTCCCTGGCTCCT
ZmActin1rev	CCACATCTGCTGAAAAGTGCTG
ZmZIP1fwd	ATGGTTCTGTTGGTGGCTGGT
ZmZIP1rev	CAACGCAGAGAGGAAATTGAAGAA
ZmZIP8fwd	TCTTGAGCTTGGGATTGTGGTG
ZmZIP8rev	CTTGAACTTGGCCTGAACGATG
ZmIAA5fwd	GGTCTGCATAAATCATCGAGCAA
ZmIAA5rev	AAGATCACCGACAAGCATCCAG
ZmIAA14fwd	GGAGAAGAAGACGACCTACTGGAA
ZmIAA14rev	TCCTTGAACCATCACCTCCATC
ZmARF12fwd	GTCAGTGGTGCAGAGCATGAA
ZmARF12rev	GCCTAGCGATTTTGGCATTTGT
ZmLsi1fwd	CACTGGCACTCGCTCGTCA
ZmLsi1rev	GCGAAGATGGACGTAATGCAAA
ZmLsi2fwd	GTGACGGTGGGCATGGTG
ZmLsi2rev	CAGCGAGTAGGAGACGGTGTTG
ZmPIPfwd	TGATCTTCGCCCTCGTGTACTG
ZmPIPrev	TTTCTTGCCCTCAAAACCCTTC
ZmZnbZIP17fwd	TGGATAACCGAAAGTCCAACGA
ZmZnbZIP17rev	GCTCAAGCCACCAGCTATTTGA
ZmABF2fwd	GCCCAGGACACACATAACCAAG
ZmABF2rev	TCAATGTTTTTGCCACACAGTCC
EF1arev	ACTAACCCACGCTTCAGATCCT
EF1afwd	TGGGCCTACTGGTCTTACTACTGA
b-Tubfwd	CTACCCTCACGGCATCTGCTATGT
b-Tubrev	GTCACACACAC1CGACTTCACG

Chapter 5. Table. S10. RNA quality used of 9 libraries for Massive Analysis of cDNA Ends (MACE)

QUBIT: [c] RNA after DNaseI digest & RIN Labchip					* LabChip before DNaseI digest	
sample	**ng/µl dil.**	**ng/µl dil.**	**Ø ng/µl dil.**	**Ø ~ ng/µl stock**	**ng in ~12 µl**	**RIN***
L 1	38.70	38.80	38.75	775.00	9,300.00	**8.5**
L 2	32.30	32.40	32.35	647.00	7,764.00	**8.8**
L 3	34.30	34.00	34.15	683.00	8,196.00	**8.6**
L 4	41.50	41.20	41.35	827.00	9,924.00	**8.3**
L 5	34.90	35.10	35.00	700.00	8,400.00	**7.4**
L 6	42.80	43.70	43.25	865.00	10,380.00	**8.9**
L 7	31.40	31.50	31.45	629.00	7,548.00	**6.8**
L 8	32.70	32.80	32.75	655.00	7,860.00	**8.1**
L 9	29.20	29.30	29.25	585.00	7,020.00	**6.7**

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Chapter 6. General Discussion

6.1 Challenge for cold protection of sensitive crops in temperate climates

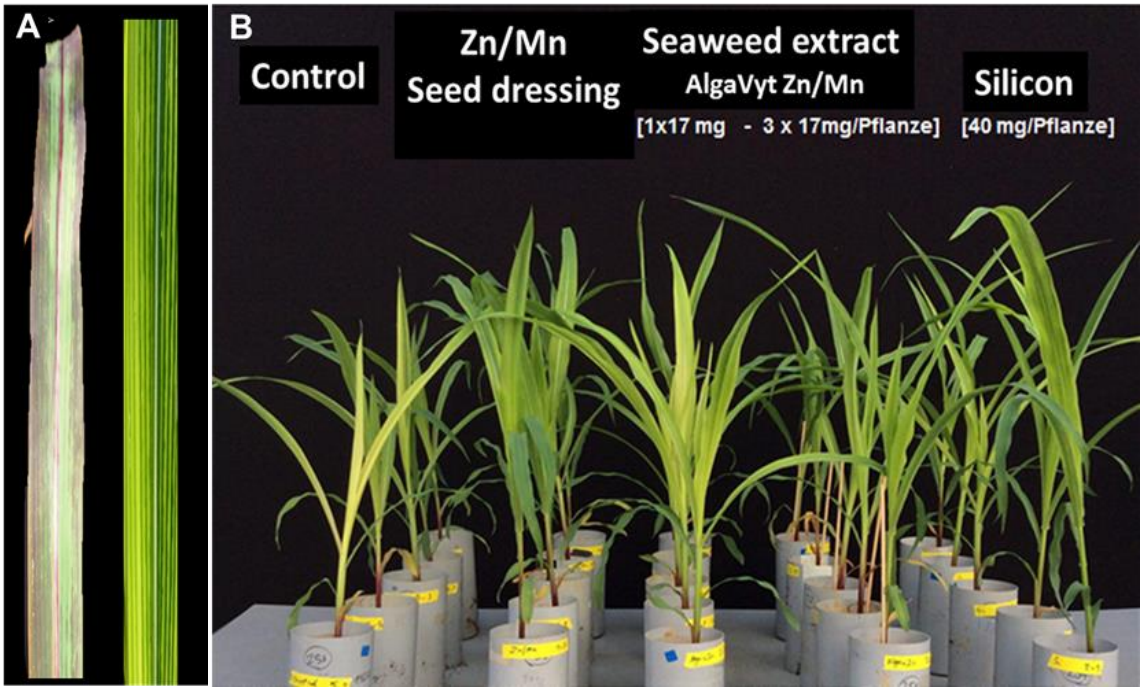
Many crop species from subtropical or tropical climates, such as *Zea mays* (maize), *Glycine max* (Soybean), Sorghum, or *Solanum Lycopersicum* (tomato) particularly sensitive to cold stress (chilling (0–15 ◦C) or freezing (≤0 ◦C); Ruelland et al., 2009) are increasingly cultivated in geographical regions where temperature preferences of these crops are not fully met during the growing season (Frederiks et al., 2015). Thus, developing approaches to cope with late frost or chilling events during spring due to early sowing and improving crop establishment and productivity are challenges to have reliable and high-quality crop yields. In maize as a cold-intolerant crop, cold stress causes inhibition of root growth and activity; induced nutrient deficiencies, limiting ROS detoxification, oxidative damage, disturbance of hormonal balances finally growth repression and reduced yields (Rodrıguez et al., 2014).

Approaches to improve cold tolerance in maize involve breeding programs, mainly based on Flint maize inbred lines used as a source of adaptation to cold in most programs in Northern Europe. The Northern Flint race that adapted to cold temperate regions of Northeastern America was introduced in Northern Europe probably at the beginning of the 16th century (Rebourg et al. 2003). Characterization of more cold-tolerant lines revealed growth inhibition during the chilling treatment and limitation of processes related with photosynthesis but fast recovery upon return to a warm temperature (Riva-Roveda et al. 2016) This finding shows that minimizing irreversible damage of leaf tissue during the chilling period seems to be an important trait of cold-tolerant imbred lines and points to a central role of an effective ROS detoxification as postulated also in previous studies (Prasad et al.,1994, Baek and Skinner, 2012; Saeidnejad et al., 2012). Therefore, approaches to

strengthen physiological ROS detoxification systems under cold stress conditions could provide complementary measures to improve maize seedling performance and establishment under cold stress. This study addressed a range of approaches discussed to improve cold tolerance using stress protectants including supplemented seeds with protective nutrients (Zn, Mn, and Si: chapter 4, 5 and 7), fertigation with seaweed extracts (chapter 4), and application of plant growth-promoting microorganisms including cold-tolerant strains (PGPM, chapter 6) within pot and field trials. Special emphasis was placed on interactions different forms of N fertilization, frequently used in maize cultivation systems, perspectives for the exploitation of synergisms, and the investigation of functional mechanisms

6.2 Overview of promising cold-protective strategies tested in model experiments

As shown in chapters 4, 5, and 6, low soil temperatures (<12-14 °C) could easily induce micronutrient deficiencies (Zn, Mn, Fe) in maize plants even with sufficient nutrient supply due to inhibition of root growth and activity (Chapter 4). Consequences comprised impaired photosynthesis, oxidative leaf damage (chlorosis, necrosis, Fig. 1A), and finally inhibition of shoot-, and root growth while early supplementation of the respective micronutrients (seed treatments, fertigation) before the onset of the stress treatments could mitigate these deficits and interestingly similar protective effects were induced by treatments with silicon or micronutrient-rich seaweed extracts (Fig. 1B, Table 1).



Chapter 6. Fig. 1. A) Oxidative leaf damage (chlorosis, necrosis) by 2 weeks 12-14 °C Soil temperature, B) Maize pot experiment with controlled root zone temperature: 2 weeks 12-14° C soil temperature, silty loam pH 6.9, from experimental station „ Ihinger Hof “.

The protective agents exhibited similar physiological effects: (i) improved micronutrient status (ii) increased levels of antioxidants and enzyme activities involved in detoxification of free radicals, strongly dependent on sufficient micronutrient supply; (iii) lower levels of reactive oxygen species (ROS) (iv) improved shoot or root growth and less oxidative leaf damage as a consequence (Table 1). However two commercial PGPR inoculants (*Bacillus amyloliquefaciens* FZB41; *Psudomonas* sp. DSMZ13134) with a well-characterized potential for growth promotion in maize, both under lab and field conditions (Nkebiwe et al., 2016a; Mpanga et al. 2019 a,b), completely failed concerning induction of cold-protective effects (Chapter 4).

Further investigations revealed that the expression of the cold-protective effects was also N form dependent and further promoted by stabilized ammonium fertilization in comparison with nitrate supply (Chapter 6). Ammonium fertilization already exerted a

certain stress priming effect on cold stressed maize plants to oxidative stress adaptations, which may be related to an improved Zn status (Table 8.1) as a consequence of ammonium-induced rhizosphere acidification (Chapter 6) but also to the induction of ABA accumulation (Chapter 6) as a key regulator of cold stress adaptations in higher plants (Miura and Furumoto, 2013; Eremina et al., 2016). Moreover, further screening of microbial inoculants including cold-tolerant strains revealed cold protective properties of a microbial consortium based on *Trichoderma harzianum* OMG16 in combination with five *Bacillus* strains (Combi A) and for a commercial PGPM product based on *Penicillium sp*. PK112 (BFOD, Bayer Crop Science Biologics) as well (chapter 6). For the investigated cold-protectants, the combination with stabilized ammonium fertilization generally improved the cold-protective performance (Table 1). This may be of particular relevance for maize production systems where ammonium-based fertilization is meanwhile a common approach for starter fertilization in form of underfoot placement or seedbed fertilization close to the developing root system (Nkebiwe et al. 2016). Table 1 summarizes the effects of the various tested cold stress-protectants on plant recovery and related physiological parameters, after a two-weeks cold stress period with reduced root zone temperature (12 °C). The data show that effects on plant growth and reduction of cold-stress-induced leaf damage were most intensively expressed in the combination with ammonium supply and the microbial consortium CombiA with Zn/Mn supplementation, associated with high levels of shoot phenolics and antioxidants, SOD activity, and improved Zn-nutritional status.

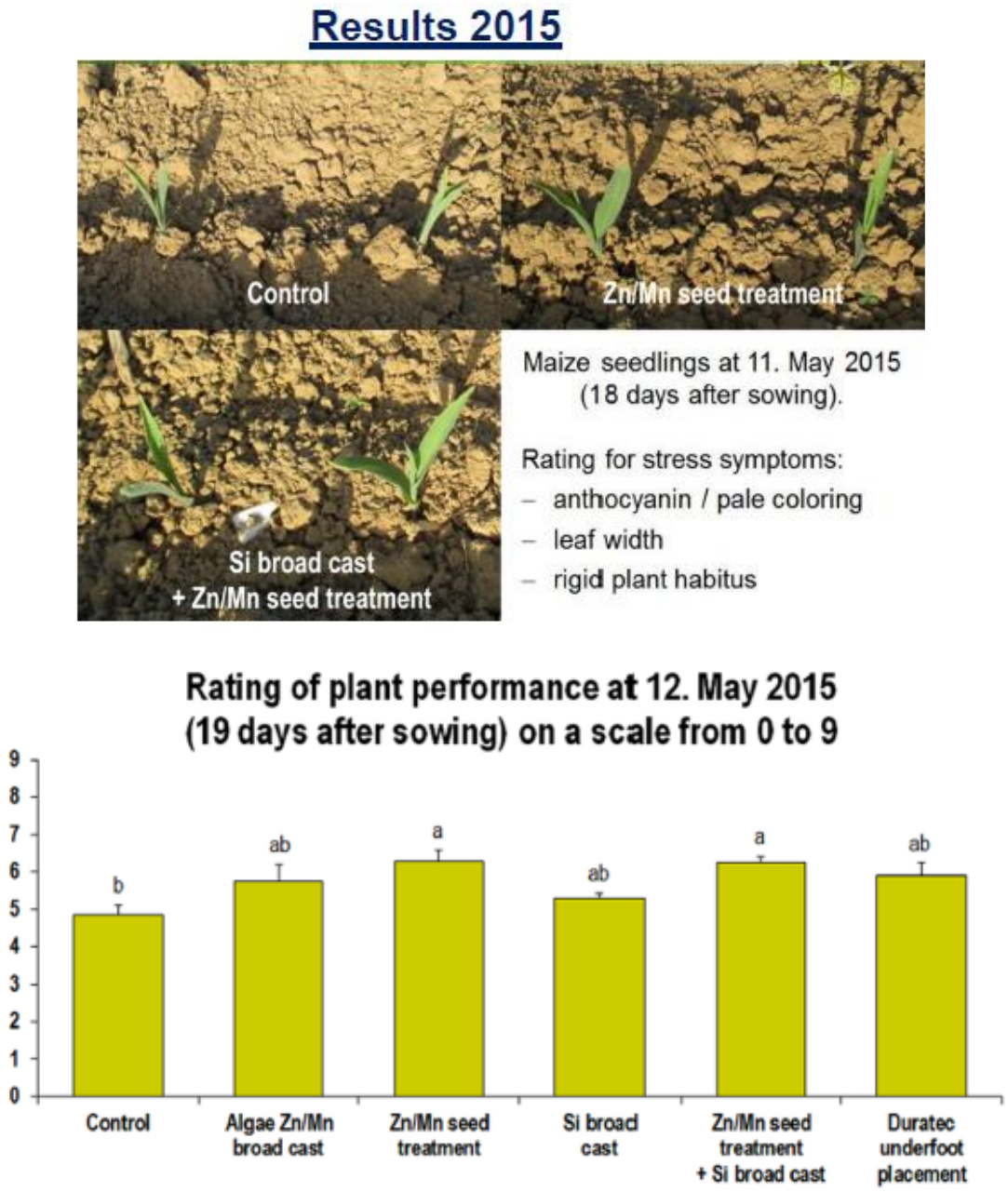
Chapter 6. Table 1. Mitigation effects by cold protective agents identified in pot experiments with controlled root zone temperature (chapters 4, 5, and 6). Data indicate the changes [%] relative to cold-stressed_control plants with nitrate fertilization. Maize plants were grown for six weeks, (two weeks ambient temperature, two weeks root cold stress (12 °C), two weeks recovery phase), Variants include untreated-ammonium controls (NH_4^+ only), Zn/Mn seed dressing (Zn/Mn), Silicic acid fertigation (Si), Combi A (*Trichoderma harzianum* OMG16 + 5 *Bacillus* strains) without or with Zn/Mn in the formulation), Biological fertilizer OD (*Penicillium* sp.PK112 BFOD) and a seaweed extract (Algavyt with Zn/Mn in the formulation) n.s: not significant. n.d = not determined.

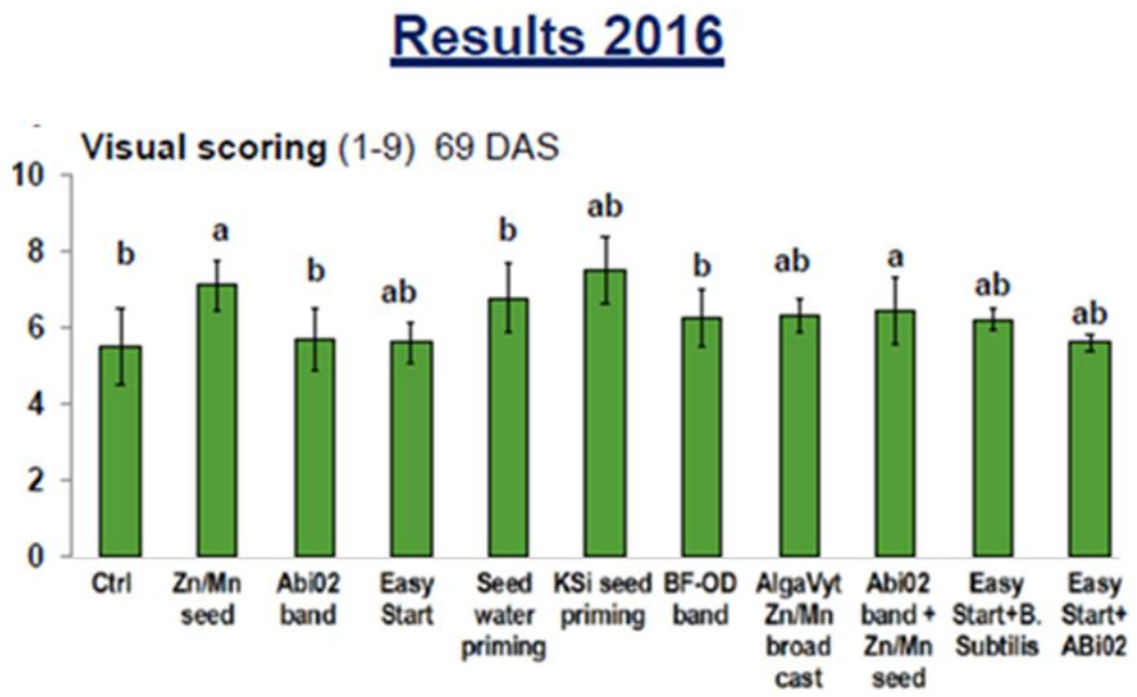
% change relative to NO_3 cintrol	NH_4 Only	Zn/Mn +NH_4	Si +NH_4	CombiA + NH_4	CombiA Zn/Mn + NH_4	BFOD ZnMn + NH_4	Algavyt ZnMn + NO_3
Shoot biomass	n.s	n.s	n.s	n.s	+51	+29	+33
Root length	n.s	+69	+157	+ 86	+108	+n.s	+111
Oxidative stress							
Leaf damage	- 53	- 87	- 62	- 43	- 78	- 83	- 56
SOD	+23	+49	+52	+51	+ 66	+38	+110
POD	+29	n.d	+58	+58	+ 58	n.d	n.d
Antioxidants	+15	+32	+35	+46	+ 46	n.d	n.d
Phenolics	+13	+29	+43	+38	+130	+100	+24
Protective solutes							
Proline	n.s	+83	+104	+104	+102	n.d	n.d.
Sugars	n.s	+10	+ 47	+ 72	+ 34	ns	n.d.
Nutrient status							
Zinc	+72	+130	+106	n.s.	+133	+146	+30

6.3 Most promising applications: from lab to the field

Two field experiments were conducted at the Hohenheim experimental station Ihinger Hof (IHO) in 2015 (chapter 4) and 2016 (chapter 5). To increase the probability of cold stress during early spring, sowing was performed already by the mid of April 2015 and 2016. Micronutrients and Si were applied as seed treatments or as broadcast applications in commercial starter fertilizers, which were also employed for seaweed extracts. Microbial inoculants were applied by fertigation of the experimental plots with or without

combination with the micronutrient treatments. Rating of plant performance of field trials in 2015 and 2016 revealed that particularly Zn/Mn seed treatments andSi+Zn/Mn applications improved emergence and plant performance at 19 days after sowing (DAS) and 69 DAS respectively (Fig. 2). However, in 2015 improved seedling establishment did not translate into a yield increase probably due to compensation effects induced by almost optimal growth conditions in later stages of plant development.





Chapter 6. Fig. 2. Visual plant performance ratings of field trials in 2015 and 2016 at 19 days after sowing (DAS) and 69 DAS respectively. In 2016 treatments include respectively: Ctrl: untreated control, Zn/Mn seed dressing (Lebosol Mn500 SC, Zn700 SC), inoculation with *Bacillus atrophaeus* Abi02 (banded application), EasyStart (finely granulated Zn/Mn/Fe/ammonium phosphate starter fertilizer), seed water priming, KSi: K2SiO4, seed priming, banded application of *Penicillium* sp (BFOD), broadcast application of seaweed extract as Algavyt Zn/Mn, Easy start with inoculation with *Bacillus atrophaeus* Abi02 and *Bacillus subtilis.*

Due to extremely cold and wet soil conditions by the end of April in 2016, seedling emergence was severely biased, particularly in the untreated control variants with significant improvements mainly by starter treatments including additional Zn/Mn or Si supply and also by inoculation with the fungal PGPM strain Penicillium sp.PK112 (BFOD) (Fig. 2; Table 2) The predicted yield decline in consequence of impaired emergence (-10% per 25% emergence decline, Pioneer Agronomy Information, 2016) was significantly reduced in the treatments with Si or Zn/Mn seed treatments, and with the fungal inoculant (BFOD) (Table 2). This is in line with the observations of the pot experiments conducted in this thesis (Table 1) and the results of Gómez-Muñoz et al. (2017). Unfortunately, the most promising consortium CombiA + ZnMn was not yet available in sufficient amounts for field experiments. In face of the extremely reduced field emergence, finally, re-sowing

was performed in the most heavily affected plots by mid of May to maintain a normal level of inter-plant competition for light, water, and nutrients within the rows during the rest of the culture period. The final harvest was conducted at 214 DAS. However, even with re-sowing, a significantly increased yield response was finally recorded with Si seed treatment, (Table 2).

Chapter 6. Table 2. Emergence, Zn/Mn status, and final biomass yield of field-grown silo maize (cv. Rolandinio) on a silty loam soil pH 6.9 at the experimental station "Ihinger Hof" the University of Hohenheim with underfoot placement of di-ammonium phosphate (29 kg N, 32 kg P ha^{-1}) and stabilized ammonium sulfate fertilization (161 kg N ha^{-1}) with or without Zn/Mn seed dressing (Lebosol Mn500 SC, Zn700 SC), potassium silicate [1 mM] seed priming, water priming, foliar Si application (16 L Si + 100 ml Greemax® ha^{-1}), EasyStart (finely granulated Zn/Mn/Fe/ammonium phosphate starter fertilizer, Compo Expert, Münster, Germany) and inoculation with *Bacillus atrophaeus* ABI02 (ABI), *Bacillus subtilis*, *Penicillium* sp (BFOD) or application of Algavyt Zn/Mn. Yield determinations with (in brackets) and without re-sowing by the end of May 2016). Means of five replicates per treatment. Significant differences (P< 0.05) are indicated by different characters. Predicted yield decline according to Pioneer Agronomy information (2016).

Treatments	Application mode	Emergence 41 DAS [%]	P< 0.05	Predicted yield decline [%]	P< 0.05	Yield [t ha^{-1}] after re-sowing	P< 0.05	Zn status	P< 0.05	Mn status	P< 0.05
Untreated Control	-	44	f	22.4	a	16.1	b	32	b	48	a
Zn/Mn	Seed Dressing (SD)	56	b	17.6	b	16.4	b	65	a	56	a
Water	Priming	52	c	19.2	b	17.2	ab	49	a	57	a
K_2SiO_4	Priming	72	a	11.2	c	17.8	a	59	a	61	a
Untreated	Si Foliar	46	e	21.6	ab	16.6	ab	36	b	51	a
ABI02 *Bacillus atrophaeus*	Banding	44	f	22.4	a	16.2	ab	55	a	58	a
ABI02 + Zn/Mn	Banding + SD	52	c	19.2	b	16.9	ab	57	a	55	a
Easy Start	Underfoot	48	e	20.8	ab	16.6	ab	53	a	53	a
Easy Start + *Bacillus Subtilis*	Underfoot	47	e	21.2	a	15.2	b	59	a	56	a
Easy Start + *Bacillus Subtilis* + ABI02	Underfoot + Talcum SD	41	f	23.6	a	15.6	b	49	a	56	a
BFOD (*Penicillium sp*)	Banding	49	d	20.4	ab	16.9	ab	54	a	58	a
BFOD + Zn/Mn	Banding + SD	47	d	21.2	ab	16	ab	63	a	56	a
Algavyt Zn/Mn	Broadcast	45	f	22	ab	16.8	ab	54	a	60	a

Therefore, yield improvement induced by Zn/Mn/Si/Microbial treated variants are most probably associated with better seed germination and seedling establishment during the wet and cold soil conditions after early sowing in spring in comparison with untreated control plants, as similarly reported by Finch-Savage and Bassel (2016). The results are also in line with earlier observations on yield increase observed in maize exposed to low soil temperatures in spring, by seed priming treatments with micronutrients, such as Zn, Mn, and Fe (Imran et al. 2013, Table 3). From the practical point of view, Zn/Mn seed dressings are the most economical approaches with fertilizer requirements of only 1-2 L /t of seeds (Table 4). The same holds for seed priming conducted by seed soaking with potassium silicate (Table 4). However, in contrast to seed dressings compatibility with other seed treatments is problematic in this case and specific formulations for Si seed dressings could be more promising in this context. Also, the repeated inoculations with the PGPM inoculants via soil drenching conducted in the model experiment is difficult to perform under field conditions. However, the results, with BFOD inoculation in the field experiment suggest that already a single starter inoculation may be sufficient. Nkebiwe et al., (2017) found excellent compatibility of inoculants based on *Trichoderma* and *Bacillus* strains with ammonium fertilizers. This may offer the opportunity to develop formulations for combined application with ammonium-based starter fertilizers.

Chapter 6. Table 3. Effects of cold stress protectants on field emergence and yield of grain and silo maize

Sowing Date	Control	Zn/Mn Seed Dressing	Silicon (KSi) Fertigation/ Seed Soaking	Seaweed-Extract Algavyt Fertigation	Location/ Soil
Mid April 2010					Heidfeldhof
Emergence	n.d	n.d	n.d	n.d	Clay-silt
Grain yield [t/ha]	7.9	9.1 (+15%)*	n.d	n.d	pH 6.9
Mid April 2011					
Emergence [Biomass]	3.0	3.7 (+23%)*	n.d	n.d	Heidfeldhof
Grain Yield [t/ha]	11.7	13.4 (+15%)*	n.d	n.d	
Mid April 2015					Ihinger Hof
Emergence [visual rating]	4.9	5.8 (n.s)	5.3 (n.s)	6.3 (+29%)*	Silty loam
DM Yield [t/ha]	18.4	16.5 (n.s)	17.2 (n.s)	17.3 (n.s.)	pH 6.9
Mid April 2016					
Emergence [%]	44	56 (+27%)*	72 (+64%)*	45 (n.s)	Ihinger Hof
DM Yield [t/ha]	12.5	13.9 (+11%)*	15.8 (+26%)*	12.6 (n.s)	
Mid May 2017					Ihinger Hof
Emergence	n.d	n.d	n.d	n.d	
DM Yield [t/ha]	23.4	23.0 (n.s)	22.6 (n.s)	n.d	

*significant differnce, n.d: not determined; n.s. not significant; p<.05, t-test, 2010/2011 Imran et al. 2013; 2015-18 Moradtalab et al.

Chapter 6. Table 4. Cost and benefits of the cold stress protectants on the yield of grain and silo maize

Substracted from related-control yield	Zn/Mn	Silicon (KSi)	Seaweed-Extract Algavyt
Dosage	0.75 [L/ha] Seed dressing	3 [kg/ha] Fertigation 1.2 [kg/ha] Seed soaking	1 [kg/ha] Fertigation
Price	9.7 [€/ha]	717 [€/t]	150 [€/t]
Mid April 2010			
Cost [€/ha]	7.3	n.a	n.a
Benefit [€/ha]	+197*		
Mid April 2011			
Cost [€/ha]	7.3	n.a	n.a
Benefit [€/ha]	+282*		
Mid April 2015			
Cost [€/ha]	7.3	2.2 (Fertigation)	0.15
Benefit [€/ha]	0 (-152 n.s)	0 (-86 n.s)	0 (-84 n.s)
Mid April 2016			
Cost [€/ha]	7.3	0.86 (Seed soaking)	0.15
Benefit [€/ha]	+114*	+303*	0 (-7.5 n.s)
Mid May 2017			
Cost [€/ha]	7.3	2.2	n.a
Benefit [€/ha]	0 (-30 n.s)	0 (-61 n.s)	n.a

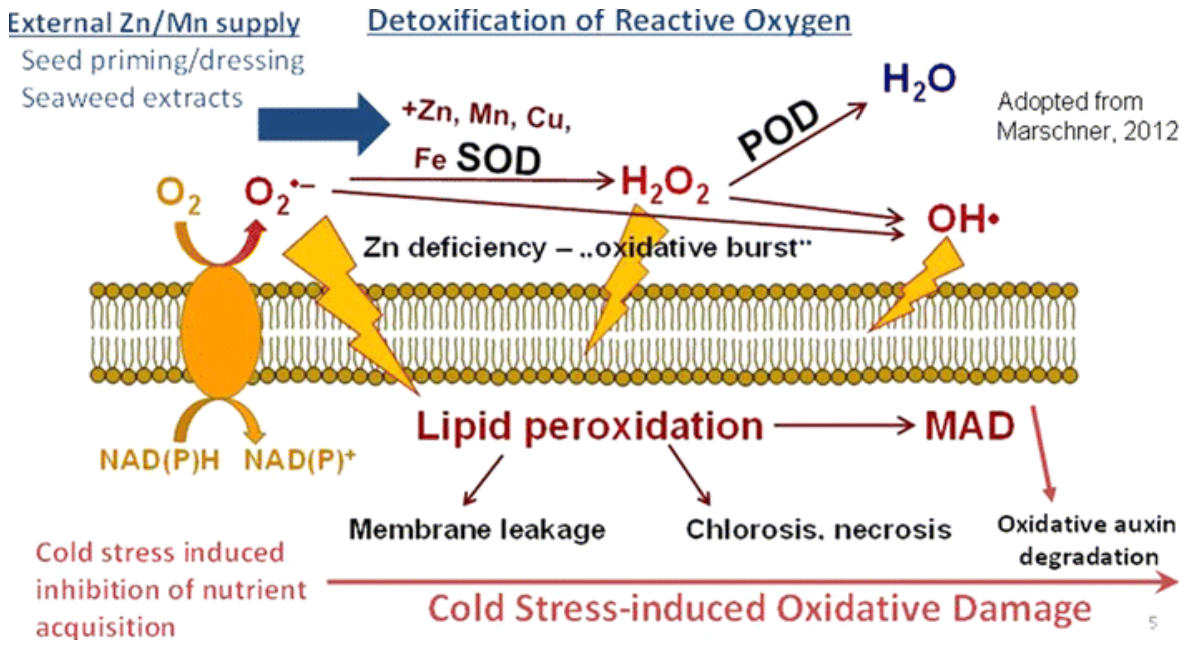
n.a: not applied, *significant yield, n.s: not significant, p<.05, t-test

Taken together the results of all pot and field trials discussed above suggest a synergistic activation of plant defense responses against cold stress-induced by arising the Zn/Mn, Supplementation, and microbial inoculants in combination with ammonium fertilization. The underlying mechanisms are addressed in the following sections

6.4 Cold-stress protectants enhance ROS detoxification, mediating cold stress tolerance during early growth of maize

A key response to cold stress is growth repression leading to re-allocate resources from growth to processes that increase cold stress resistance (Zhou et al., 2018). In addition to morphology alteration, upon cold stress, biochemical and physiological changes occur (Cramer et al., 2011). These changes included an accumulation of reactive oxygen species (ROS) and the formation of cryoprotective metabolites such as soluble sugars and amino acids (Ruelland et al., 2009). In our study, two–weeks exposure of maize seedlings to low root zone temperature (LRZT) of 12–14 °C in a soil culture medium (chapter 4), induced leaf chlorosis and necrosis, inhibition of shoot and root growth, and micronutrient limitation (particularly Zn and Mn). These phenotypes were mitigated by seed treatments with Zn, Mn, and fertigation with seaweed extract (containing Zn/Mn (chapter 4)), but surprisingly, also by Si fertigation (chapter 5 and 7). In line with our findings, studies have reported that micronutrient fertilization and seaweed extract fertigation improved tolerance against biotic and abiotic stress (Subramanian 2011, Huber and Thompson, 2007, Cakmak 2000). In the soil-free culture (chapter 5), Si reduced leaching losses of micronutrients in germinating maize seedlings and promoted root and shoot translocation of micronutrient seed reserves with a preferential effect on Zn shoot translocation. Both, micronutrients and Si mitigation effects were associated with increased activity of superoxide dismutase (SOD) in shoot and roots (as a key enzyme for detoxification of ROS, depending on Zn

and Mn as cofactors), increased tissue concentrations of phenolics (dependent on Mn and Cu for biosynthesis), proline, and total antioxidants, but reduced levels of H_2O_2 leading to reduced oxidative damage and IAA degradation (Fig. 3).



Chapter 6. Fig. 3. Schematic illustration of cold stress effects and the role of micronutrients in ROS detoxification in maize plants exposed to suboptimal soil temperature. Increased activity of superoxide dismutase (SOD) and peroxidase (POD) in shoot and roots (as a key enzyme for detoxification of ROS, depending on Zn and Mn) leading to reduced oxidative damage and IAA degradation, (modified from Marschner, 2012, derived from chapter 4).

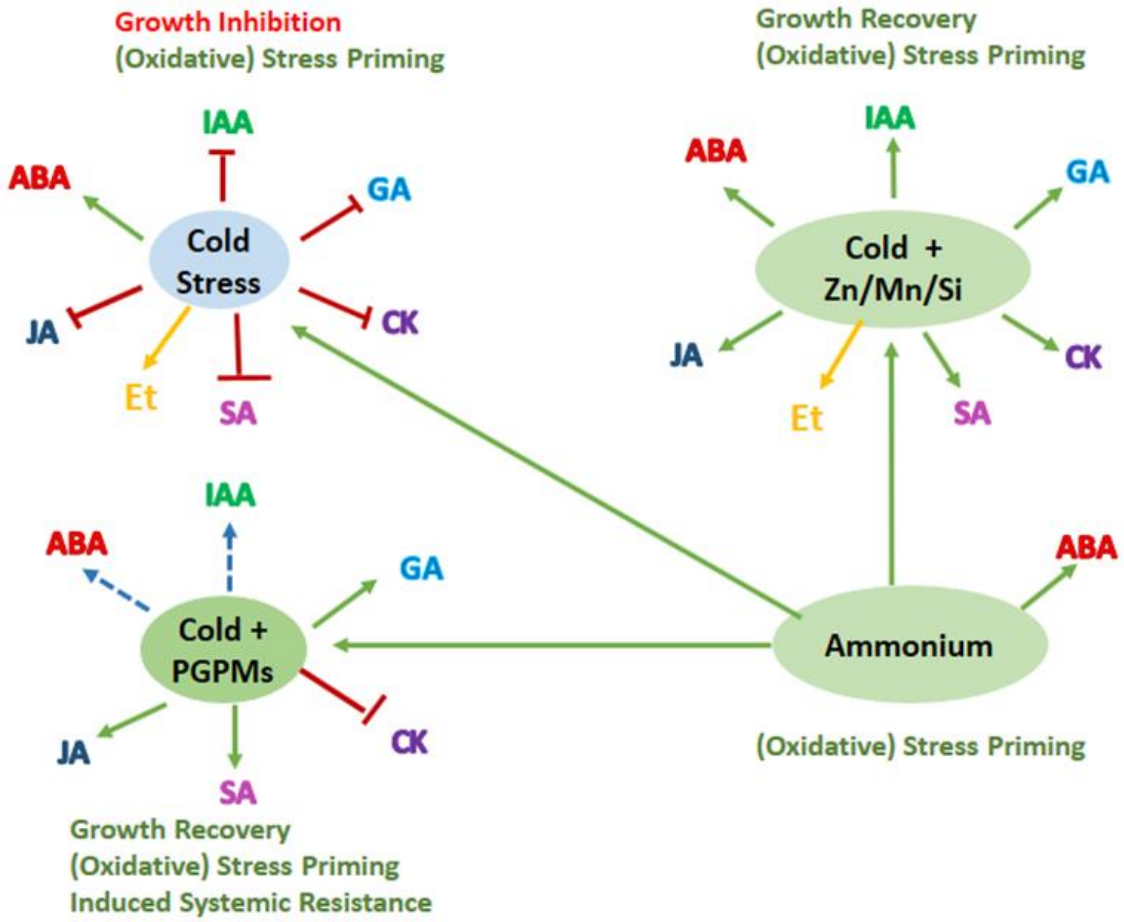
6.5 A Complex network of hormonal interactions in plants

Complex signaling cascades that induce changes in gene expression, enable plants to respond to cold temperature. These signaling cascades are governed by the activity of plant hormones (Santner et al., 2009). Hormonal homeostasis including biosynthesis, catabolism, and transport ultimately integrates and modulates cold responses. Understanding the tuned hormone modes of activity under cold stress responses is complicated because hormonal cross talks can be dependent on plant species, stress conditions targeted tissue, and stage of growth (Achard et al., 2006). Accordingly, different hormones have specific as well as

overlapping functions but at the same time, they also display synergistic or antagonistic effects on outputs of each other that create a complex network of hormonal interactions so-called cross-talk (Santner et al., 2009).

6.6 CombiA (PGPM) and micronutrient supplementation modulate hormonal homeostasis under cold stress at early growth of maize

The complex signaling cascades governed by the activity of plant hormones induce changes in cold-responsive gene expression that enable plants to withstand cold stress (Eremina et al., 2016). Accordingly, in maize seedlings, cold stress reduced the root and shoot contents of important hormonal growth and stress regulators (IAA, GA, CK, JA, and SA) while increased the level of ABA and Et leading to growth retardation and upregulation of stress defense responses (chapters 6 and 7; Fig. 4). The investigated stress protectants, such as stress-protective nutrients (Zn/Mn and Si) and PGPM inoculants restored the levels of growth hormones and hormonal balances (IAA, GA, CK) after stress recovery, leading to stimulation of root and shoot growth and at the same time induced hormonal signatures characteristic for stress priming (increased levels of ABA, JA, and SA) induced systemic resistance. These processes were further promoted by ammonium fertilization (Figs. 4 and 5).



Chapter 6. Fig. 4. Schematic overview of the effects of cold stress and the investigated cold-protectants on hormonal balances in maize seedlings and the related responses in plant performance and physiology. Arrows represent stimulatory effects at the level of gene expression and/or tissue concentrations (upregulation of biosynthesis or down-regulation of genes involved in hormone inactivation.) Blocked arrows represent inhibitory effects (declining concentrations and/or down-regulation of genes involved in hormone biosynthesis or up-regulation of genes involved in the inactivation of a hormone. Dotted arrows indicate increases in the IAA and ABA/CK ratios. Hormones include auxin (IAA), cytokinin (CK), gibberellic acid (GA), abscisic acid (ABA), ethylene (Et), jasmonic acid (JA), and salicylic acid (SA).

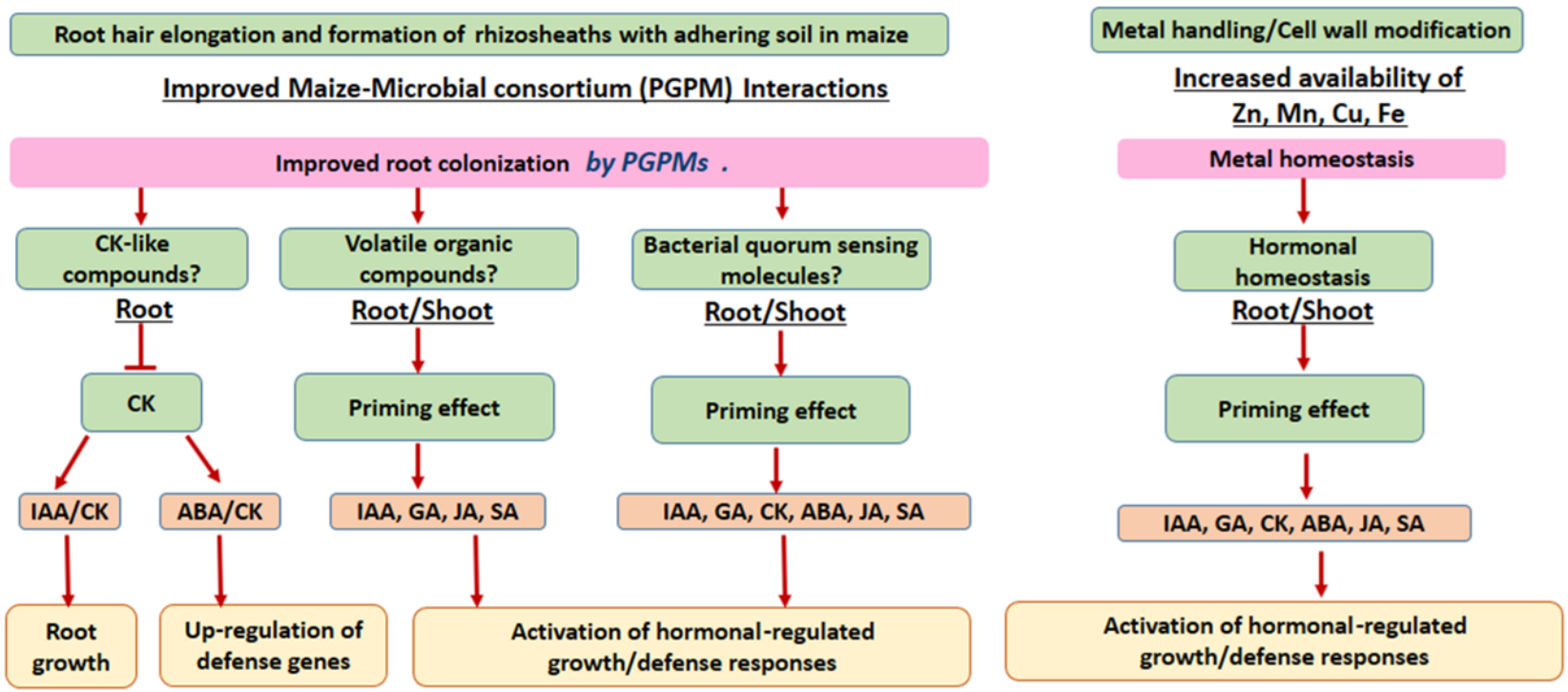
Previous studies indicate that cold stress affects plant IAA level and thus developmental and physiological responses of plants (Kosova et al., 2012, Maruyama et al., 2014). The results of chapters 2 and 3 also showed a decrease in IAA level results from oxidative damage of cold stress. While, Zn supplementation under cold stress, protected IAA from oxidative degradation (chapter 2, 3, and 4), suggesting that IAA signaling is closely linked with the antioxidant defense (Begum et al., 2016). It was shown that IAA transport was

also impaired by cold stress (Shibasaki et al., 2009) similar to this study, our result showed IAA transport-related genes expression was downregulated (chapter 5). The reduction of IAA responses under cold stress was also confirmed at the molecular level (Chapter 5) since cold stress up-regulated the genes responsible for the repression of early auxin responses genes and inactivation of GA and CK. Similar to our findings reports indicated that in Arabidopsis, tobacco, tomato, and rice cold stress-induced a reduction of bioactive IAA, GA, CK and increase ABA, SA, and JA levels (Horváth et al., 2007; Eremina et al., 2016; Hu et al., 2017). In contrast, elevated levels of Zn and Mn in a metal-Si-complex (Chapter 3) restored the hormonal balances to a level comparable with non-stressed plants and stimulated the production of hormones involved in growth regulation (IAA, GA, and CK) and stress adaptations (ABA, SA, and JA). Accordingly, we propose that this metals availability and handling under cold stress give a major role of metal homeostasis in hormonal homeostasis that is also influence the metal transcriptional regulatory network, to coordinate gene expression that expresses enzymatic and non-enzymatic antioxidative defense to decrease ROS damage, regulate cell wall modification and increase membrane stability (Fig. 5). Regarding hormonal homeostasis in our study with fungal and bacterial PGPM strains (chapter 4), we have observed that the best performance of maize seedlings under 12 °C root zone temperature detected for a combined formulation of *Trichoderma haruzianum* OMG16 and *Bacillus* spp with Zn/Mn (Combi A) particularly in combination with stabilized ammonium fertilization. Cold stress caused severe leaf damage and Zn deficiency, associated with reduced root length and impaired IAA accumulation in the shoot tissue of maize plants. In contrast, ammonium and Zn/Mn fertilization increase the cold-protective potential of Combi A in maize by synergistic activation of metabolic defense lines. Our findings showed that ammonium fertilization reduced oxidative leaf

damage by increasing plant availability of micronutrients (Zn/Mn) as cofactors for ROS detoxification systems (SOD, phenolics) via rhizosphere acidification (chapter 4, Fig. 3). Combi A also stimulates root growth and accumulation of cryo-protectants, antioxidants, and phenolics (chapter 4). Combi A + Zn/Mn further improves the micronutrient status with the additional promotion of root growth, SOD activity, accumulation of phenolics, and finally reduced oxidative leaf damage (chapter 4). In this regard, ammonium fertilization stimulates maize root colonization with the Combi A inoculant strain *T. harzianum* OMG16 while there were no effects of Zn/Mn (chapter 4). It was recently reported that ammonium stimulated root hair elongation and formation of rhizosheaths with adhering soil in maize (Mpanga et al., 2019). It was also shown that *Trichoderma* sp. have preferential colonization sites of root hairs e.g. *T. harzianum* OMG16 (Mpanga et al., 2019) and *T. harzianum* T22 (Harman, 2000) both preferred root hairs for colonization and also the bacteria (*Bacillus amyloliquefaciens* FZB42, Fan et al., 2012) preferred root hairs surface for infection sites. This evidence shows that ammonium indirectly by increasing the infection sites increased the plant-microbe interactions (Fig. 5)

Regarding hormonal responses, in the shoot, Combi A restores the accumulation of growth- and stress-related phytohormones in cold-stressed maize plants to the level of the non-stressed controls (Fig. 5) but how the Combi A proceeds this modulating is still not clear. There are some new findings confirmed that the application of *Trichoderma* strains influences the phytohormonal network of their host plant, leading to an improvement of plant growth and stress tolerance (Martínez-Medina et al., 2013, 2014; Sofo et al., 2011). According to literature PGPM can produce IAA that may alter the pool of host hormonal status (Glick, 2012) but our results of expression analyses indicated that ZmIAA5 (as a gene responsive to external IAA level, Park and Hasenstein, 2015) has not changed by

Combi A application (chapter 4). Thus, how Combi A trigger the changes in host hormonal response? Many recent reports show plant-associated endophytic microbes known, which are living within plants can trigger host phytohormone and metabolite regulation. For example, bacterial quorum-sensing molecules like N-acyl homoserine lactones modulates plant responses towards contact with bacteria (Hartman et al., 2014). Regarding *Trichoderma,* there are many species as bio-fungicides and bio-fertilizers reduce plant diseases and promote plant growth and productivity through overlapping modes of action including induced systemic resistance, antibiosis, enhanced nutrient efficiency, and myco-parasitism. *Trichoderma* species produce many metabolites with antifungal, antibacterial, and anticancer properties. Thus, the volatile metabolites of *Trichoderma* can alter plant-microbe interactions and modulate host phytohormone and metabolite production (Lee et al., 2016). Therefore, we can hypothesis that quorum sensing molecules of Combi A bacteria sp. or volatile organic compounds emitted by *Trichoderma* may regulate maize phytohormone and metabolite in our experiment that needs further investigation (Fig. 5).



Chapter 6. Fig. 5. A schematic illustration of potential mechanisms in plant PGPM interactions and metal homeostasis- mediating cold stress tolerance during the early growth of maize plants. (A). Ammonium fertilization stimulates root hair elongation and formation of rhizosheaths with adhering soil in maize result in greater root surface area (Mpanga et al. 2019b), improving plant microbial interactions and also (B) decreases rhizosphere pH causing optimal uptake of Zn leading to reduced oxidative damage and providing better nutrients availability. (A) PGPM may regulate the pool of auxin (IAA), cytokinin (CK), gibberellin (GA), salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA). Volatile organic and CK like-compounds emitted by *Trichoderma* or quorum sensing molecules of Combi A bacteria sp may regulate the phytohormones and metabolic pathways. The possible priming effects of PGPM on hormonal balance may help plants to tolerate cold stress by enhancing the antioxidant system and reducing oxidative damage; improving photosynthetic capacity, stimulating the growth of the root system, and uptake of water and nutrients. (C) Si protects maize seedling from cold stress by increasing metal handling, providing a sufficient level of micronutrients (Zn, Mn, Cu and Fe) resulting in regulation of hormonal balance and transcription factors responses of COR genes, increased phenolic metabolism and superoxide (SOD) and peroxidases (POD) activities, increased detoxification of overproduction of reactive oxygen species (ROS), decreased oxidative damage and increased cell wall and membrane stability.

6.7 Conclusion

This research has shown that cold stress responses in maize seedlings are complex events that alter the biochemical composition of cells with an impact on plant morphology, causing leaf necrosis and chlorosis, and growth repression. The results demonstrated that independent of composition and mode of applications, surprisingly all protective agents tested in this study exerted similar beneficial effects against cold stress in maize seedlings that were improved acquisition of sparingly soluble nutrients such as Zn/Mn, increased root growth, internal metal homeostasis and reprogramming the hormonal responses e.g. by microbial consortia via VOCs/QS or CK like compounds particularly in combination with NH_4^+ nutrition. The field trials confirmed the reproducibility of the pot experiment results that improved Zn/Mn nutritional status via protective agents' applications could not only enhance maize plant performance at the early growth but also increase the yield. The protective effect was associated with increased ROS-detoxification via oxidative stress defense system including superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities, phenolics, proline, and total antioxidants accumulations leading to reduced oxidative damage, balanced endogenous hormonal responses protecting or increasing plant growth.

The concluding remarks are from…

- **Chapters 2, 3 and 4: The identification of the most efficient cold stress protectants (Zn/Mn, Si, seaweed extracts/ microbial consortia):** Mitigation of cold stress-induced inhibition of root/shoot growth and improved Zn/Mn status in all variants except seaweed extracts with low Zn/Mn content showed that the effects mainly act via Zn/Mn supplementation. The combined use of nitrogen fertilization as ammonium, Mn, Zn, and the *Trichoderma/Bacillus* inoculant was also a suitable

strategy to improve the tolerance of maize plants in the early growth stage to cold-stress conditions.

- **Chapters 2, 3, and 4: The identification of suitable application techniques:** Similar efficiency of seed priming, seed dressing, or fertigation were observed when micronutrients were taken up prior to the stress period. The combined use of nitrogen fertilization as ammonium, Mn, Zn, and the *Trichoderma/Bacillus* inoculant could be easily integrated into existing strategies for starter fertilization of maize production systems, such as seedbed fertilization with stabilized ammonium phosphates and micronutrient supplementation in combination with granulated spore formulations of the *Trichoderma/Bacillus* inoculant.

- **Chapters 2, 3, 4 and 5: Mode of action at the physiological and molecular level:** Pleiotropic effects were found via Zn/Mn supplementation on Zn/Mn dependent pathways of enzymatic (SOD) and non-enzymatic (phenolics, antioxidants) ROS defense and hormonal growth- (IAA, GA, CK) and stress regulators (ABA, JA, SA) also at the transcriptional level. Potentially additional beneficial effects of Si were observed by the activation of sterol synthesis (cold-stress adaptive membrane remodeling, antioxidative terpenoids, cold protective cuticular wax layers) and Zn/Mn distribution. Root growth promotion by stimulation of IAA biosynthesis and reduction of antagonistic cytokinins in the root tissue of the host plant was a major feature induced by *Trichoderma/Bacillus* inoculation. Additionally, this inoculation was associated with typical responses of ISR signaling via induction of jasmonic and salicylic acid accumulation even in the shoot tissue and an increase in the ABA/cytokinin ratio in roots. This was related with a further increase in enzymatic and non-enzymatic ROS detoxification expressed mainly in the shoot tissue, and

consequently a further decline of oxidative leaf damage.

- **Chapters 2, 3, and 6: Translation of stress-protective effects into field performance and yield responses:** All tested cold protectants improved emergence and seedling establishment after cold/wet stress events in early spring by 23-64 %. Only Zn/Mn and Si seed treatments translated into significant yield benefits (11-26%). No yield effects in the absence of cold stress events or under compensatory growth conditions after cold stress were found. The *Trichoderma/Bacillus* inoculant was not tested at the field scale in this thesis.

- **Chapter 6. Cost-benefit analysis:** Particularly seed treatments may provide low-cost cold-stress prophylaxis (2-7 €/ha) avoiding significant economic losses even in the absence of yield effects. Yield benefits between 114-303 €/ha were observed in 3 out of 4 cold stress experiments at the field scale. In contrast to Zn/Mn products, there is a limited market availability of suitable Si formulations.

6.8 Outlook

Based on the findings in this thesis, further investigations are open…

- From Chapter 2, 3, and 5:

1. The novel finding that Si activates terpenoid biosynthesis pathways at the transcriptional level (sterols, mevalonate pathway, waxes) requires further investigations at the posttranscriptional and metabolome level to verify a potential contribution to stress resistance.

2. Characterization of potential Si interactions with metal (Zn/Mn) transport identified by transcriptomics.

3. Characterization of Si and Zn/Mn effects on gene expression (direct?

/indirect?)

4. Responses to other stress factors (drought, salinity, biotic stress)?

5. Development of Si application techniques suitable for field conditions (seed treatments?)

6. The longevity of the cold stress priming effects and factors affecting their expression (soil type, fertilization, other stress factors, plant genotype, exploitation of potential synergisms with other stress-protective measures)?

- From Chapter 4:

1. To identify quorum sensing molecules of Combi A bacteria sp., tend to have a larger impact on the regulation of maize phytohormone biosynthesis and metabolic pathways. To determine quorum sensing molecules exposure time in plant responses in different timelines of an experiment to clarify the priming effect of these agents on plant metabolism.

2. To identify volatile organic compounds (VOCs) emitted by *Trichoderma*. To identify VOCs tend to have a larger impact on the regulation of maize phytohormone biosynthesis and metabolic pathways. To determine volatile exposure time in plant responses in different timelines of an experiment to clarify the priming effect of these agents on plant responses.

3. To identify cytokinin like compounds and to determine the transcriptomic outcome of their interaction on gene expression of plant metabolism under cold stress or even other abiotic stress such as drought.

6.9 Reference

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Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic
Micronutrients, Silicon and Biostimulants as Cold Stress Protectants in Maize
is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.
I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Stuttgart, 01.09.2020_____
Place, Date Signature

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Some Talk contributions in conferences and External seminar

1. Foliar application of SuperFifty® as drought stress protectant, The 3rd Biostimulants

World Congress, Miami, November 2017. USA.
2. Silicone, micronutrients and microbial inoculants as cold stress protectants during early growth of maize under field condition. German Plant Nutrition 2016 International Conference. September 2016, Stuttgart-Hohenheim, Germany.
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