Nutrient seed treatments to improve abiotic stress tolerance in ***Brassica napus*** **L.**

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Summary

Poor germination and limitations during early plant growth are widespread constraints for oilseed rape (OSR; *Brassica napus* L.) with increasing importance due to a rising frequency of weather extremes related with global climate change. In this study, efforts have been made to improve health and stress resistance of OSR by exploring perspectives of cost-effective application techniques for micronutrients with stress-protective functions to cover increased demands of these nutrients under stress conditions. After preliminary screening experiments, special emphasis was placed on zinc (Zn) seed treatments including seed priming (SP) and seed dressing (SD). Effects on seedling performance during early growth were recorded at optimal conditions for plant growth in terms of temperature, nutrient and water supply and also under drought stress for winter OSR and under low root zone temperature (RZT) stress in spring OSR.

Higher seedling biomass and healthy seedling development highlighted the most promising seed treatment potential for $ZnSO_4$ among zinc (Zn), manganese (Mn), iron (Fe) and boron (B) priming treatments in a pre-selection experiment conducted as petri-dish germination test at 8°C, applied as a stress factor. Accordingly, $ZnSO_4$ was used in two model experiments with ZnSP and ZnSD treatments under soil conditions. The presence of more than the double amount of the inherited Zn seed reserves in the shoot tissue of five-day old seedlings indicated a massive demand for Zn during early seedling development to be mainly supplied via root uptake. A positive effect on plant growth, and particularly on root development, regardless of the application method (SP or SD) during the first 10 days of seedling growth suggested suboptimal Zn supply via root uptake at this developmental stage even under optimum growing conditions with respect to temperature (23°C), nutrient availability and water supply. Seed priming treatments (Zn 50 µM) and a seed dressing treatment (Zn 50 µM at 1.2 ml kg^{-1} seeds) achieved the highest growth responses without posing any adverse effect on germination. A similar functional equivalence of SP and SD treatments was confirmed under field conditions but the best performance was recorded with Zn 25 µM treatments in this case. The magnitude of improvement was even higher at low root zone temperatures (12°C) tested in spring OSR. Increased contents particularly of sparingly soluble nutrients (such as Mn and P) in the shoot can be regarded as secondary effect, resulting from better root growth in response to the Zn seed treatments. A decline in fine root development (30%) during winter under field conditions, which was further reduced to 70% in presence of temporal waterlogging as additional stress factor, indicated the need for a well-established root system also in winter OSR (Chapter 2).

The present study also suggests that the combination of Zn seed priming with the placement of ammonium-phosphate starter fertilizers can support the expression of plant traits beneficial for field establishment of winter rape and spring rape as well. In a rhizobox experiment on a soil with moderate P availability, it was demonstrated that even in absence of environmental stress factors, such as temperature extremes or drought stress, Zn seed priming could improve early plant development in terms of root elongation and stimulation of seedling growth in winter rape. This improved the exploitation of an underfoot ammonium-phosphate fertilizer depot with root attracting properties, placed at a soil depth of 8 cm. No negative side effects of depot fertilization

on simultaneous rooting of deeper soil layers, important for winter hardness, were observed. However, at 5 weeks after sowing, the P status of the plants with homogenous NPK supply was critical, while optimum P shoot concentrations were recorded in the depot fertilizer variants. However, the largely improved P status was not associated with excessive shoot growth, known to be detrimental for winter survival. Also under field conditions, 11.6 % higher yields were recorded in a combination of ZnSD (25 µM) with striptill sowing and placement of di-ammonium phosphate fertilization in comparison with standard sowing without fertilizer placement.

In spring rape exposed to reduced root zone temperatures (12°C) to simulate low temperature stress in spring, the combination of Zn seed priming with ammonium-phosphate depot fertilization stimulated shoot biomass production by up to 30%, Plants with homogenous nutrient supply were severely P deficient, while the P status was optimal in the depot variants. Since no root growth effects were detectable in this case, it is concluded that Zn priming promoted root activity and root-induced P acquisition via the well-documented ammonium-induced rhizosphere acidification. The improved nutrient status obviously supported seedling establishment of spring OSR exposed to low root zone temperatures as frequently recorded in spring (Chapter 3).

Crop production is also increasingly affected by water limitation even in temperate climates due to a rising frequency of drought periods, related with global change. Application of stress-protective nutrients belongs to the measures discussed as mitigation strategies. Therefore, this study investigated potential drought-protective effects of nutrient seed treatments (NST), applied as seed dressings based on calcium (Ca), potassium (K), Fe, Zn and Mn on early growth of OSR. Responses were investigated in five winter-OSR hybrids under greenhouse conditions on two soils with contrasting properties (sandy-loam pH 5.6 vs. silty-loam pH 6.9). A 7-days drought period with reduced soil moisture level (40% soil water-holding capacity WHC) significantly reduced shoot and root growth and caused irreversible wilting and leaf necrosis (27-46%), depending on the investigated genotype and was particularly expressed on the sandy-loam with lower WHC compared to the silty loam soil. The seed treatments increased shoot-, and particularly root growth and shoot nutrient accumulation but reduced the proportion of irreversibly damaged leaves to 17-21%, with the largest expression in the strongly drought-affected genotypes under the more challenging conditions on the sandy-loam soil. Analysis of physiological stress indicators revealed increased accumulation of phenolics, total antioxidants and increased activities of ascorbate peroxidase (APX) in the leaf tissue, counteracting drought-induced oxidative stress. Moreover, APX activity was positively correlated with root length development and indole acetic acid (IAA) accumulation, suggesting a protective effect on drought-induced oxidative IAA degradation with inhibitory effects on root growth. Increased levels of abscisic-, jasmonic-, and salicylic-acids during drought-stress recovery point to stress priming effects, strengthening the natural adaptive responses to water limitation (Chapter 4).

Accordingly, both, ZnSP and ZnSD may offer practical, economically low-cost application methods to improve early seedling establishment particularly under challenging environmental conditions, to improve the perspectives for conversion into higher economic yields and could be equally attractive for small-scale on-farm use and rape seed industry.

Zusammenfassung

Reduzierte Keimung und Einschränkungen in der Jugendentwicklung sind weitverbreitete Probleme im Rapsanbau, die aufgrund einer durch den Klimawandel bedingten Erhöhung der Häufigkeit von Witterungsextremen zunehmend an Bedeutung gewinnen. In der vorliegenden Arbeit wurden daher Perspektiven für die kostengünstige Anwendung von Mikronährstoffen mit Schutzwirkungen gegen Stressfaktoren untersucht, um einen erhöhten Bedarf dieser Nährstoffe unter Stressbedingungen nach Möglichkeit auszugleichen. Basierend auf den Ergebnissen einführender Screeningversuche. wurden bevorzugt Ansätze zur Saatgutbeizung und zum Saatgutpriming, sowohl unter optimalen Anzuchtbedingungen im Hinblick auf Temperatur, Wasser und Nährstoffangebot sowie Trockenstressbehandlungen bei Winterraps untersucht, während der Einfluss niedriger Wurzelraumtemperaturen bei Sommerraps betrachtet wurde.

In Petrischalenkeimtests mit Zink (Zn), Mangan (Mn), Eisen (Fe), und Bor (B)-Priming ergaben sich erhöhte Biomasseentwicklung und verbesserte Keimlingsgesundheit bei Saatgutbehandlungen mit $ZnSO_4$ bei einer Keimtemperatur von 8°C als Stressfaktor. Daher wurden zunächst ZnSO-Saatgutbehandlungen in Form von Saatgutpriming und Saatgutbeizung in weiterführenden Modellexperimenten in Bodenkultur untersucht. Mehr als doppelt so hohe Zn-Sprossgehalte im Vergleich zu den Samenreserven bereits bei fünf Tage alten Keimlingen weisen auf einen hohen Zinkbedarf hin, der schon in den frühen Phasen der Keimlingsentwicklung durch Wurzelaufnahme gedeckt werden muß. Gleichermaßen positive Effekte von Saatgutbeizung und Saatgutpriming-Behandlungen auf Sprosswachstum und besonders auf die Bewurzelung während der ersten 10 Tage der Keimlingsentwicklung, belegen eine suboptimale Zn-Versorgung durch die Wurzelaufnahme in dieser Entwicklungsphase, die selbst unter optimalen Wachstumsbedingungen im Hinblick auf Temperatur, Wasser-, und Nährstoffangebot auftritt. Zinkkonzentrationen von 50 µM in den Lösungen für Priming-, und Beizapplikationen erzielten die höchsten Biomasseeffekte ohne Beeinträchtigung der Keimraten. Eine ähnliche Äquivalenz von Saatgutpriming-, und Beizbehandlungen wurde auch in begleitenden Feldversuchen beobachtet, wobei hier die besten Effekte mit Zinkkonzentrationen von 25 µM erreicht wurden. Besonders ausgeprägte Wachstumsstimulierungen wurden bei niedrigen Wurzelraum-temperaturen (12°C) bei Sommerrapst erreicht. Ein verbesserter Ernährungsstatus auch im Hinblick auf andere Nährstoffe wie Phosphat (P) und Mangan kann als Sekundäreffekt bedingt durch das verbesserte Wurzelwachstum durch Zn Saatgutbehandlungen betrachtet werden. Eine Abnahme der Feinwurzellänge um 30% während der Auswinterung unter Feldbedingungen, die sich bei zusätzlichen Stressfaktoren wie Staunässe auf 70% erhöhte unterstreicht die Wichtigkeit einer optimalen Wurzelentwicklung z.B. bei Winterraps (Kapitel 2).

Die vorliegende Arbeit lieferte auch Hinweise darauf, dass durch die Kombination von Zn-Saatgutbehandlungen mit platzierter Ammoniumphosphat-Starterdüngung die Bestandsetablierung sowohl von Winterraps als auch von Sommerraps unterstützt werden kann. Dabei wurde in Rhizoboxversuchen gezeigt, dass selbst in Abwesenheit von Stressfaktoren wie Temperaturextreme oder Trockenheit, durch Zn-Saatgutpriming die Wurzelentwicklung und dadurch die Erschließung von Ammoniumphosphatdepots mit Wurzel-Lockwirkung in 8 cm Bodentiefe beschleunigt

und die frühe Pflanzenentwicklung bei Winterraps gefördert wurde. Dabei wurde die für die Ausprägung der Winterhärte wichtige Durchwurzelung tieferer Bodenschichten nicht inhibiert. Nach einer Wachstumsperiode von fünf Wochen bei homogener Düngerausbringung war P-Mangel bei den Versuchspflanzen nachweisbar während die Depotdüngungsvarianten eine optimale P Versorgung aufwiesen. Der deutlich verbesserte P Status führte jedoch nicht zu überschüssigem Sprosswachstum was sich negativ auf die Winterhärte auswirken würde. Auch unter Feldbedingungen wurde in einer Kombination aus Zink-Saatgutbeizung (25 µM) mit Striptill-Aussaattechnik und Diammoniumphosphat-Platzierung eine Ertragssteigerung von 11.6 % im Vergleich zu herkömmlicher Saattechnik ohne Depotdüngung beobachtet.

Bei Sommerrapsanzucht mit niedriger Wurzelraumtemperatur (12°C) zur Simulierung niedriger Bodentemperaturen im Frühjahr, förderte die Kombination von Zn-Saatgutpriming mit Ammoniumphosphat-Depotdüngung das Sprosswachstum um bis zu 30%. Pflanzen mit homogenem Düngerangebot zeigten starken P-Mangel während der P Status in den Depotdüngungsvarianten im Optimalbereich lag. Da in diesem Fall keine Wurzelwachstumsstimulierung nachweisbar war, wird angenommen, dass Zn-Priming die Wurzelaktivität und damit die Ammonium-induzierte Protonenabgabe zur P-Mobilisierung förderte. Der verbesserte P-Ernährungsstatus unterstützte so die Keimlingsentwicklung unter dem Einfluss niedriger Bodentemperaturen, wie sie häufig im Frühjahr auftreten (Kapitel 3).

Wassermangel ist selbst in gemäßigten Breiten ein Stressfaktor mit zunehmender Bedeutung, wegen des gehäuften Auftretens von Trockenperioden unter dem Einfluss des Klimawandels. Daher untersuchte die vorliegende Arbeit auch Perspektiven für Saatgutbehandlungen durch Beizung mit Nährstoffen wie Calcium (Ca); Kalium (K), Fe, Zn und Mn mit Funktionen bei der Anpassung an Trockenstress im Jugendwachstum von Winterraps. Die Versuche wurden mit fünf Winterrapshybriden unter Gewächshausbedingungen auf zwei Böden mit unterschiedlichem Eigenschaften (einem sandigen Lehm, pH 5,6 und einem schluffigen Lehm pH 6.9), durchgeführt. Eine 7-tägige Trockenstressbehandlung mit reduzierter Bodenfeuchte (40% der Bodenwasserhaltekapazität, WHC) führte zu einer signifikanten Verminderung der Sprossbiomasse, des Wurzelwachstums und einer irreversiblen Blattschädigungsrate von 27- 46% je nach untersuchtem Genotyp, die besonders auf dem sandigen Lehm mit einer verminderten Wasserhaltekapazität gegen über dem schluffigen Lehmboden ausgeprägt war. Die Saatgutbehandlungen förderten das Spross-und besonders das Wurzelwachstum, erhöhten die Spross-Nährstoffgehalte und verminderten die irreversiblen Blattschäden auf 17-21%. Diese Effekte waren am stärksten bei den gegenüber Trockenstress empfindlichsten Genotypen auf dem sandigen Lehmboden ausgeprägt. Die Analyse physiologischer Stressindikatoren ergab erhöhte Akkumulation von Phenolen und Antioxidanzien sowie erhöhte Aktivitäten von Ascorbatperoxidase (APX) mit Funktionen bei der Entgiftung freier Radikale im Blattgewebe. Die APX-Aktivität war darüber hinaus positiv mit der Wurzellängenentwicklung und der Akkumulation von Indolessigsäure (IAA) korreliert, was auf einen Trockenstress-bedingten oxidativen Abbau von IAA mit Hemmwirkung auf die Wurzelentwicklung hinweist, dem durch Antioxidanzien und erhöhte APX Aktivitäten entgegengewirkt wurde. Erhöhte Level von Abscisin-, Jasmon-, und Salicylsäure, die auch nach Erholung von der Trockenstressbehandlung nachweisbar

waren, weisen auf Stresspriming-Effekte zur Stärkung der Pflanzeneigenen Abwehr gegenüber Trockenstress hin (Kapitel 4).

Sowohl Zn-Saatgutpriming als auch Zn-Saatgutbeizung könnten also praktische und ökonomisch günstige Ansätze bieten, um die Keimlingsentwicklung besonders unter abiotischen Stressbedingungen zu fördern, als Grundlage für eine optimierte Bestandsentwicklung und verbesserte Ertragsbildung. Die Methoden bieten Perspektiven sowohl für direkte On-Farm Anwendungen als auch für großtechnische Ansätze in der Saatgutindustrie.

Chapter 1

1. General Introduction

1.1. Oilseed rape

Oilseed rape (*Brassica napus L*.) has internationally grown importance over time, especially in Europe (Abbadi and Leckband, 2011), China and Canada. It is the world-leading oilseed crop after soybean regarding production. In the Genus *Brassica*, it is the most widely used species. Oilseed rape is rated as a highly economical crop due to its extensive use for food, feed and fuel (Melut et al., 2012, van Duren et al., 2015). The last three decades showed an enormous hike that resulted in the doubling of its production worldwide. The total area under oilseed rape cultivation, in Germany, doubled between 1991-2006 (Christen and Friedt, 2007). Although the total cultivated area is reduced for other oil crops (e.g. Soybean and Sunflower) in the world, oilseed rape cultivated area was increasing, particularly in the European Union (Christen and Friedt 2007). A graphical illustration of oilseed crops (oilseed rape, sunflower, soybean) production shows an increase in the production of oilseed form 2008-2017 (Fig 1.1). Total production of 3132 thousand tons and a production area of 870 thousand hectares in Germany was forecasted for the year 2019 (Coceral, 2019).

Previously, oilseed rape was considered unsuitable for human consumption due to the accumulation of high amounts of glucosinolates, having bitter and antinutritive properties, and Erucic acid. High erucic acid rapeseed cultivars contain 45-60%, while moderate cultivars contain 35-45% (MvVetty and Scarth, 2002). Erucic acid intake causes coronary disorders in humans (Knutsen et a., 2016) and potentially changes the liver and kidney weight in animals. Therefore, rapeseed oil, could only be used for industrial purposes and house lamps. However, scientists succeeded in reducing these two toxicants and produced high-quality cultivars in the early 1970s through a global integrated breeding program. The invention of double low or double zero “00” cultivars made it suitable for human consumption because of low in Erucic acid and glucosinolate contents. These double low cultivars have been named Canola (Canadian Oilseed Low Acid). It contributes of approx. 40-45% oil, 3.5% fat and 47% protein (Asghari et al., 2011). The invention of double zero “00” cultivars contained

very low levels of erucic acid and glucosinolates, making them suitable for human consumption. EU sets a threshold of 2% erucic acid contents in oil for human consumption (Brooks, 2003). Double 00 cultivars contributed enormously to the rapeseed dominance over other oilseeds (Zanetti et al. 2009). Almost completely shifting to double low cultivars since 1991 documents the success of this breeding approach. In Central Europe, winter oilseed rape is more widely distributed (Lääniste et al. 2007) as compared to Spring rape. It gives higher yields because of its more extended growing period (Olesen et al., 2011).

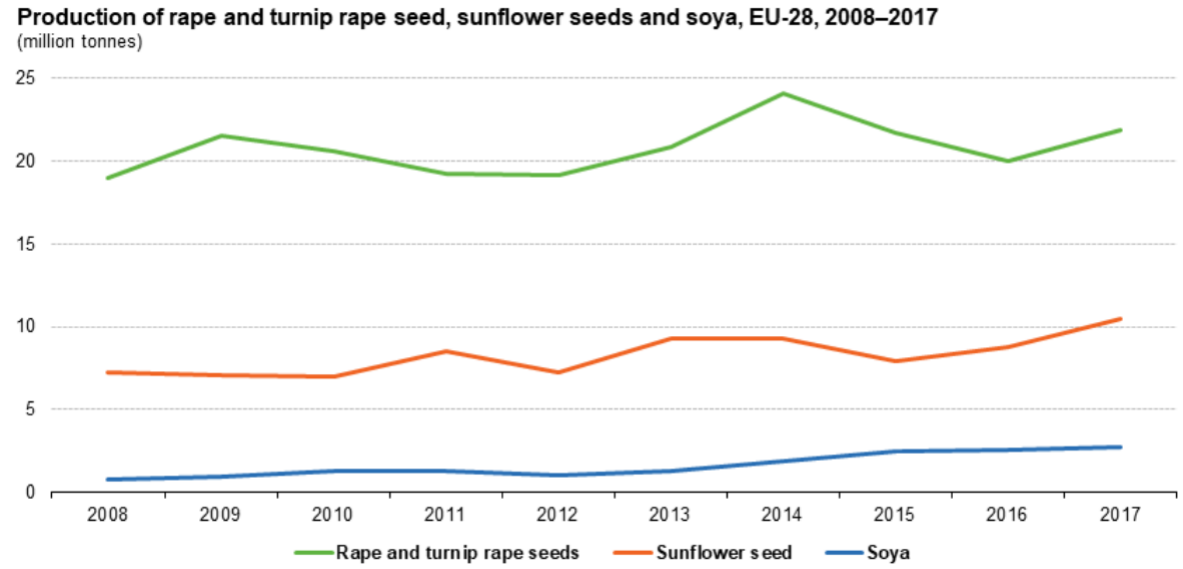

Fig. 1.1: Oilseed crops production in EU-28, 2008-21017 (Eurostat, 2017).

1.1.1. Root growth traits in oilseed rape

Oilseed rape root can extend from 1.5 to 2.4 meters in the soil (Peltonen et al., 2011), a well-developed taproot serves to store nutrients particularly crucial for winter rape. Since Brassica napus L. is a non-mycorrhiza plant species, the dependency on chemical strategies to mobilize nutrients, more significant fine root formation and longer root hair, is higher (Föhse et al., 1991; Hoffland et al.,1992). Under optimal conditions, the root biomass of oilseed rape increases quickly right from seeding till the early flowering stage and reaches the maximum at late flowering and then until maturity. Most of the oilseed rape root biomass (approx. 70%) achieved during 5-6 weeks after sowing/drilling and early root growth development is now considered one of the yield determent variables. Even at early growth stages,1-2 leaf and 2-4 leaf, root

length was found to be positively correlated with grain yield (Kosenley et al., 2012). Additionally, it was observed that the cultivars having greater root growth before winter have the better ability to regenerate in summer, improves Leaf area index (LAI) and potentially the yields.

Different oilseed rape cultivars are different in their response to water and nutrients search and are highly versatile in root vertical and horizontal distribution (Peltonen-Sainio et al. 2009a; Peltonen-Sainio et al. 2011; Wang et al. 2009a; Wang et al. 2009b). Root plasticity could increase water nutrient availability. Shallow roots preferentially contribute to the acquisition of nutrients more abundant in the topsoil layer but are more sensitive to water shortage by drying of the topsoil, while the taproot can reach deeper soil layers with particular importance for water uptake (Duncan and Carrow 1997; Gahoonia and Nielsen 2004b). Therefore, an improved root development is a desirable trait, particularly essential under stressful plant growth conditions, to supply water and nutrients appropriately. However, oilseed rape root growth and development are encountered by numerous challenges in the field which are depicted in Fig 1.2, 1.3, 1.4 & 1.5.

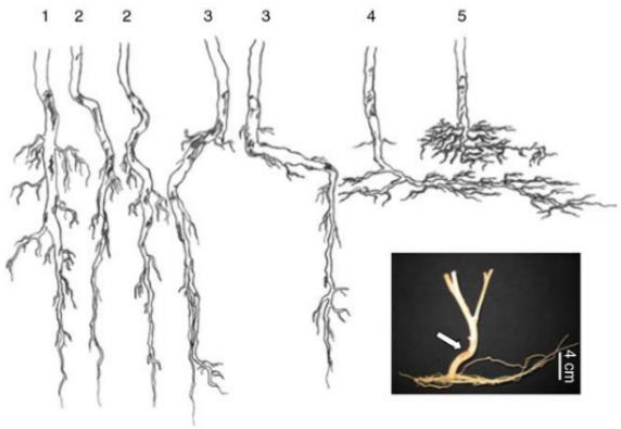

Fig. 1.2: (Left): Soil compaction affected oilseed rape root growth. Optimally grown root (1), slight root growth restriction(2), hard soil layers caused horizontal root growth folloed by the vertical grwth after finiding the space to penetrate(3), root encountered a very hard barrier which completely restrict the root vertical growth (4), roots grown in an impermiable soil layer resulting in blokage of vertical root growth. All the root growth occures in upper 2-4cm soil (5) (Peltonen-Sainio et al. 2011).

Fig. 1.3: (Right): Stunted root growth when encountered with highly acidic soil layer (both left)

(DAFWA, 2013)

Fig. 1.4: (Left): Club root affected oilseed rape roots (right) and without disease (left) (FarmingUK, 2011).

Fig. 1.5: (Right): OSR root system affected by soil moisture and sowing date. Well-developed roots under optimal soil condition and sown at optimal dates (middle), roots grown in excessive soil moisture (left), and after sowing too late (right) (Rapool Wurzelfiebel, 2012).

1.1.2. Low temperature in oilseed rape

Winter oilseed rape is sown from 1st August to the 30th of September (Lääniste et al. 2007). The sowing date and early seedling growth and development is crucial for winter survival. A plant with 6-8 leaves in the form of a deep-lying rosette has a better chance to cope with frost damages since the rosette gives a protective cover to the growing tissue. A taproot diameter up to 1 cm, before the plant would face an adverse winter temperature, ensures sufficient resources for plant growth and development (Christen and Friedt 2007). A well-developed tap root acts as a storage organ for the accumulation of carbohydrates or sugar required to tolerate the winter stress. Sugars are involved in plants cold tolerance as it plays a vital role in lipid membrane stabilization and potentially regulating cold-induced gene expressions. For winter survival, a plant must have to attain a minimum dry weight of 1-2 g before the winter commence (Mendham and Scott 1975). Supra-optimal nitrogen applications in autumn speeds up stem elongation and leads to an increased vulnerability to frost damage. Therefore, Excessive shoot growth before winter is not desirable as it makes the plant more vulnerable to frost damages and decreases the winter tolerance as well as limit regeneration capacity of the plant. However, it can be managed by avoiding excess nitrogen (N) applications. Oilseed rape plants with smaller shoots (Rathke et al., 2006) and intense root development exhibit highest winter survival rate. In addition to the

Fig. 1.6: Winter oilseed rape freeze injury a) close view b) field view (She et al., 2015)

winter rape, spring rape could also be subjected to sub-optimal temperature may be due to the early sowing or extended winter. Moreover, sub-optimal soil temperature at the time of germination, in other parts of the world such as Canada, is also a limiting

factor for normal germination and early seedling growth and development. Oilseed rape exposed to the very low temperature experienced reduction in photosynthetic efficiency and membrane damages (Megha et al., 2018a).

1.1.3. Drought in oilseed rape

Drought is one of the main abiotic stresses for crop plants affecting plant morphological, physiological, and reproductive growth process and eventually reducing final yields. According to an estimation, 40-60% of world crop yield is affected due to drought (Shao et al., 2008). In a rapidly changing climate, the topic of drought is getting highlighted around the world as low precipitation is already a pending problem and has been predicted to further increase in the future (Bates et al., 2008 & Li Y et al., 2009). According to Collins et al., 2009, the occurrence of drought and water shortage conditions is getting more and more relevant even in temperate climates. A severe wave of drought in Central and Northern Europe in spring and summer 2018 was a recent example and remained an issue during the last two years. Furthermore, a further increase in the frequency of drought stress events similar to the year 2018 has been predicted (EU Science Hub, 2019). The need for innovative strategies in agriculture to mitigate drought stress is, therefore, urgently needed and is inevitable.

In case of oilseed rape, detrimental drought effects on seed germination, shoot, root and leaf growth leading to poor seed yields have been previously documented (Yang et al., 2007, Qaderi et al., 2006, Mehanna et al., 2013, Hadi et al., 2014 & Raza et al., 2015a). A decreased germination percentage and germination rate is inevitable in drought conditions (Shahverdikandi et a., 2011), which has been recently recorded as an increasing problem due to late summer or autumn drought in Central and Eastern Europe. Cell/stem elongation is hindered and results in restricted shoot growth and length (Gul Ahmed, 2004). Along with the shoot growth, root growth is also severely affected but at mild drought, root growth parameters (root length, root fresh and dry weight) are improved. Ashraf et al., 2013 found an enhanced root length, root fresh and dry weight when grown in mild drought conditions similarly reported by Mehanna et al., 2013. Although a reduction in the plant root growth, when grown in the severe water limiting conditions, is well understood (Hadi et al., 2014).

In addition to morphological consequences of drought, physiologically, drought can limit the production of chlorophyll, manipulate ABA production affecting stomatal conductance. Increase in ABA production leading to stomatal closure and inhibition of shoot growth is part of the drought stress adaptation to reduce transpiration. It is the central regulator for the upregulation of oxidative stress defence and osmotic adjustment. Addionally, less production of chlorophyll leads to lower chlorophyll contents (Sharma et al., 1993; Zhang et al., 2014). Both forms of chlorophyll, “a” and “b”, are affected and decreased 38% chlorophyll contents (Sharma et al., 1993). Habibi, 2015 reported a higher decrease in chlorophyll “a” (57%) compared to the chlorophyll “b” (31%) under water deficit conditions. Chlorophyll has a clear linkage to the photosynthesis; a reduction in chlorophyll contents reduces the photosynthetic activity, vital for plant growth. Reactive oxygen species (ROS) like superoxide radical (O^-) hydrogen peroxide (H_2O_2), singlet oxygen (O^{-1}) and hydroxyl radical (OH^-) are harmful for the plants. High concentration of ROS directly damages cell organelles like mitochondria and chloroplast, causing lipid peroxidation of membranes, chloroses, necroses and finally cell death (Ebrahimian & Bybordi, 2014). However, there are several other factors hindering photosynthesis under water limiting situation, i.e., decrease in CO_2 availability and stomatal conductance, leaf relative water content and increase in ABA (Pasban Eslam et al., 2000, Emam and Niknejad, 2004, Ashraf and Harris & 2013, Nasab et al., 2014; Achard et al., 2006).

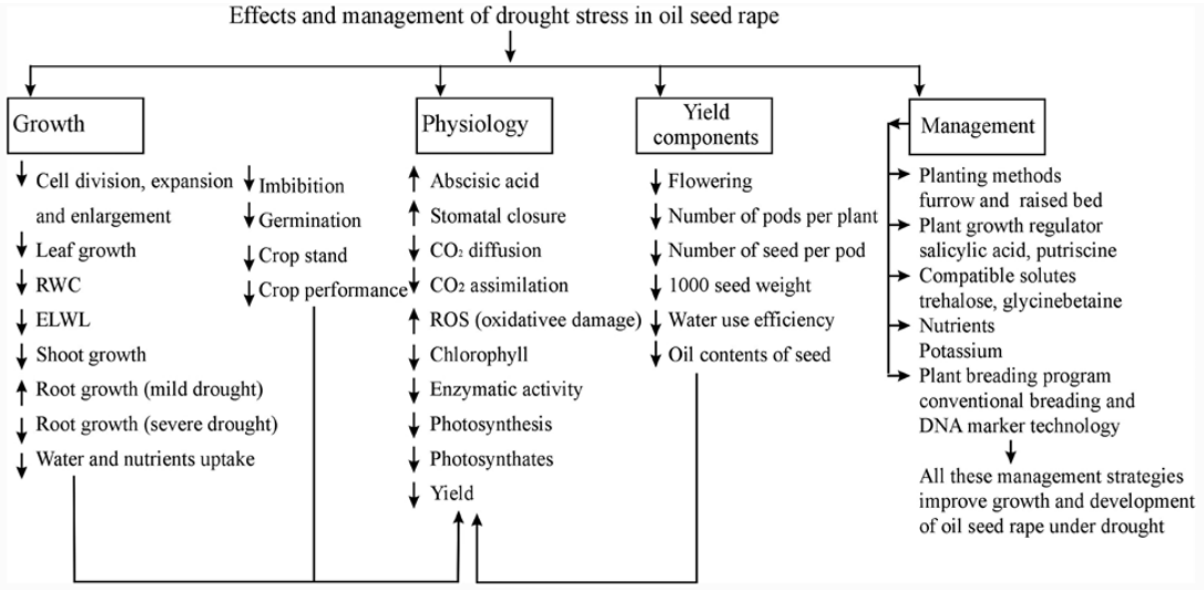

Fig. 1.7: Effect of drought on growth, physiology, and yield components of oilseed rape (*Brassica napus L*). (adapted from Raza et al., 2017).

All drought-induced morphological and physiological dysfunctionalities described above, eventually lead to the reduction in ultimate seed yields. As a result of drought, a decrease in the number of pods per plant, flowers, seed size and oil contents were recorded (Malcolm and Doug, 2002; Rahnema & Bakhshandeh, 2006). A drought-induced reduction in biological and seed yield by 17.9-32.1% and 18.5-38.7% has been reported, by Gunaskara et al., (2006). Although both vegetative and reproductive plant growth stages of oilseed rape are affected by water limitation, drought prevalence at the reproductive growth stage has higher detrimental effects on yield formation (Ghobadi et al., 2006). An overview illustration of growth, physiology and yield components affected by drought stress is shown in Fig 1.7.

1.2. Stress-protective micronutrients

The role of essential macro and micro-nutrients in plant growth and development is well known. Although there are some plant essential nutrients which play a vital role in plant protective functions under stressful plant growth conditions. Some of these stress-protective micronutrients are Zinc (Zn), Manganese (Mn), Iron (Fe), Copper (Cu) and Boron (B).

Zinc is an essential nutrient for plants as well as humans. It is an integral part of various plant enzymatic reactions, including enzymes involved in protein synthesis and many other plant physiological functions. Zinc is taken up by the plants as divalent Zn^{2+} cation in normal growth conditions. During zinc transport in xylem, it is either transported as a free divalent form or bound to organic acids (Marschner, 2012). A high concentration of zinc in phloem sap occurred probably due to its binding with low weight organic solutes (Kochian, 1991).

Zinc is a critical metal as part of enzymes of all six enzyme classes in a biological system. These enzyme classes include oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (Sousa et al., 2009). Three are four different roles that are associated with zinc; (i) catalytic, (ii) structural, (iii) co-catalytic, and (iv) protein interface. Alcohol dehydrogenase, carbonic anhydrase and superoxide dismutase are the well-known enzymes having the structural and functional role of zinc. The deficiency of zinc could negatively affect root functions due to the reduced functioning of alcohol dehydrogenase (Moore und Patrick, 1988). Carbonic anhydrase is present

in chloroplast and cytoplasm and is required for the conversion of CO_2 to HCO_3^-. Carbonic anhydrase functions differently in C3 and C4 plants and has a prominent functional role in C4 plants rather than C3 plants. Therefore, zinc-deficient conditions may affect the photosynthesis rate more in C4 plants than C3 plants (Burnell et al., 1990).

The third and most popular enzyme with higher relevancy to abiotic stress resistance in plants is superoxide dismutase (SOD). In-plant cells, the CuZn dismutase (CuZn SOD) is the most frequently present SOD. Copper (Cu) acts as catalytic. Zinc associated with two histidines and one aspartate provides structural stability (Abreu and Cabelli, 2010). There are three different identified isoforms of CuZn SOD (Yruela, 2009), localized in the cytosol, chloroplast and peroxisomes. There is a direct correlation between plant zinc status and CuZn SOD activity. Zinc deficiency reduces enzyme activity which could be immediately reverted by zinc supply, explaining the essential structural role of zinc for normal CuZn SOD functioning (Cakmak & Marchner, 1988c). Due to the strong relationship of CuZn SOD activity with zinc supply, the enzyme activity is therefore presumed a better indicator of zinc deficiency tolerance than total leaf zinc concentration (Cakmak et al., 1997b; Yu et al., 1999b; Hacisalihoglu et al., 2003). A decrease in CuZn SOD activity leads to an increase in the rate of free oxygen radicals (O^-) at the same time, suggesting a critical role of CuZn SOD. An increase in the rate of toxic free O^- radicals increases membrane permeability and cause membrane lipid peroxidation (Cakmak and Marschner, 1988c). However, overexpression of CuZn SOD in transgenic plants could be a valuable tool to enhance abiotic stress tolerance (Cakmak, 2000; Kim et al., 2010). There are many other enzymes in which zinc serves as a metal component (Coleman, 1992, 1998). Some of them are; alkaline phosphatase, phospholipase, carboxypeptidase, and RNA polymerase.

Manganese, an essential plant micronutrient, plays an essential role in enzyme structure/activation, photosynthesis and cell division/elongation. It activates about 35 different enzymes involved in plant metabolism (Brunell, 1988). Hydrolytic reactions, Oxidation-reduction and carboxylation processes are catalyzed by most of these enzymes. Mn-containing enzymes like Mn protein in PS 11, oxalate oxide and Mn superoxide dismutase (MnSOD) are not present in greater numbers (Marchener 2012). Superoxide dismutase (SOD) are important to scavenge harmful oxygen

species, produced in aerobic organisms (Fridovich, 1983; Elster, 1982), in abiotic stress conditions. There are different members of SODs, FeSOD, MnSOD, CuZnSOD (Sadam and Böger 1983) which also contributes towards plant protection. In Mn-containing enzymes, MnSOD is one of the critical enzymes present in with relevancy to abiotic stress. Increased plant tolerance against drought, salinity and Mn deficiency was observed with an enhanced MnSOD production (Tanakka, 1999; Yu et al., 1999b; Wang et a., 2005a).

Generally, the most prominent role of Fe is in chloroplast development and photosynthesis, and as a constituent of redox systems. Presence of FeSOD in the chloroplast (Kwaitowsky et al., 1985) and may be some other parts of the cell like mitochondria or peroxisomes helps the plant to detoxify stress-induced free oxygen radicals (Droillard and Paulin, 1990). Reduced activity of FeSOD can be observed in the plants with low Fe levels (Iturbe-Ormaetxe, 1995). Additionally, catalase and peroxidase are also Fe-containing heme enzymes. Catalase takes part in photorespiration, glycolate pathway and superoxide dismutase processes. Peroxidase is involved in cell functions in different ways one of them is as ascorbate peroxidase (APX) to catalyse the process of detoxification in the chloroplast.

Copper (Cu) is involved structurally and functionally in plant cell. It plays the role in biosynthesis of proteins and chlorophyll and used in mitochondria and chloroplast to catalyse the redox reaction (Lombardi & Sibastiani, 2005). As a redox catalyst, Cu plays its role in more than 30 enzymes in different metabolic pathways (Harrison et al., 1999). Along with the above-mentioned function, as CuZn SOD, Cu performs a vital role in respiration and C and N metabolism. It has been noted that the stress tolerant genotypes have the higher activity of CuZn SOD under abiotic conditions similarly observed in Staria Italica under salinity stress, is one of the examples (Sreenivasulu et al., 2000). A lower activity of CuZn SOD in a stress sensitive genotypes (Hernandez et al., 1995) was also observed.

Boron (B) performs critical functions in the plant cell wall structure, membrane integrity and functioning, shoot growth and root elongation. Boron is the integral constituent of the cell wall structure but its involvement in cell wall synthesis is still in question. Plant membranes are the one oxidatively damaged when abiotic stress occurs. However, membrane functions caused by the B deficiency can be restored in few minutes after B application (Goldbach et al., 1990; Barr et al., 1993). The restored functions may

include membrane permeability and oxidative activity (Cakmak and Römheld, 1997; Blevins and Lukaszwiski, 1998). Root elongation function of B may also alter plant capacity to deal with stressful plant growing conditions.

1.3. Nutrient seed treatment

1.3.1. Benefit and limitations

Seed treatments were used to protect the seeds from diseases and pests by treating with fungicides, and insecticides, primitively. Seed treatments offered great results with such a small quantity. With the advancement in seed treatment technology, seeds are now being treated with beneficial microbial inoculants and plant growth regulators due to their plant growth-related benefits. Treating the seeds with nutrients is another form of seed treatment technology where seeds are treated with essential plant macro and preferably, micronutrients. Nutrient seed treatments (NST) offer a great variety of benefits. Both nutrient seed priming (NSP) and nutrient seed dressing (NSD) improves the seed nutritional status and give the plant the desired starter nutrition for a good crop stand establishment. Healthy crop seedlings are less susceptible to diseases and pathogens. Mineral nutrients are needed in minute amounts and therefore is a cost-effective technique. Furthermore, treating the seeds with the nutrients (K, Ca, Zn, Fe Mn, B, Cu) involved in the stress tolerance mechanism not only serves as an essential nutrient but also protect the plants from plant growth limiting conditions, i.e., sub-optimal temperature, drought & salinity.

Despite the benefits, there are several limitations of treating the seeds with the essential plant nutrients. High quantities of treating nutrients could harm the seeds (Fraooq et al., 2012; Miraj et al., 2013); therefore, the treatment dose needs to be optimized before wide applications. Phytotoxic situation for the growing seedling may arise when applying with high dose rates. Treating with NSP requires additional evaluation of priming time duration to hydrate the seed and activate germination related metabolic activities. NSP also requires an extensive re-drying after the application which needs extra labor and mechanization.

1.3.2. Water priming

In seed priming, seeds are soaked in water for particular time duration and then dried back to initial moisture contents. Water-soaked seeds trigger specific processes like; biochemical activity, breaking dormancy and enzyme activation, crucial for seed germination. Seeds priming allows the seeds to complete its pre-germination metabolic activities (Tizazu et al., 2019) and therefore enhance seedling germination and germination speed upon exposure to the favourable germinating environment. Additionally, primed seeds reduce seedling heterogeneity and help in good crop stand establishment (Rows, 1995). However, the whole germination process is divided into three stages; a- imbibition, b- metabolic processes activation, c- radical emergence and growth. As a result of priming, the first two stages of germination are completed. Seeds are taken out of the water before the radical emergence. The soaking time duration should be optimized for each crop species. If not optimized, radical emergence before drying can damage seed germination and viability.

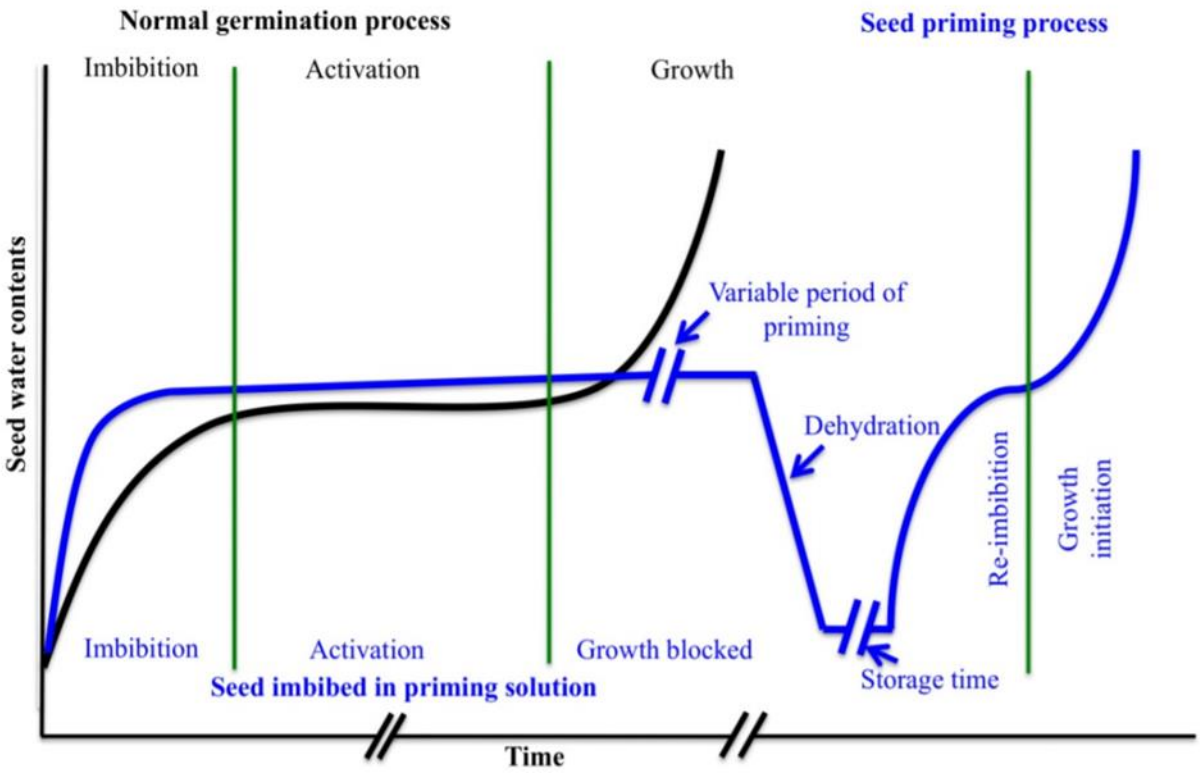

Fig. 1.8: Schematic diagram of normal germination process and germination process after seed priming (Imran, 2013)

Seed priming of various species showed positive germination, growth-promoting and yield-enhancing effects. Better crop establishment and improved germination in rice,

maize, sorghum and chickpea were noted (Harris, 1996; Harris et al., 2000; Harris et al., 1999). In addition to the germination, Harris et al., 1999 reported a higher number of flowers and higher grain yields in maize. Furthermore, primed Barley seeds have shown improved seedling growth over unprimed seed (Ajouri, 2004).

1.3.3. Nutrient seed priming

Nutrient Seed-priming is a cost-effective method of seed treatment. It is soaking of seed in a nutrient solution for an identified period and afterward the seeds are re-dried and stored for further use. Although, seeds could be treated with both macro and micro-nutrients but treating with micro-nutrients allows using a little amount of nutrients for more exceptional outcomes. Zinc, Manganese, Boron, Copper and molybdenum are potentially viable and have been used in seed priming (Masuthi et al., 2009**,** Jhonson et al., 2005, Johansen et al., 2006a, Foti et al., 2008). Although, Zinc sulfate ($ZnSO_4.7H_2O$) has a vast reported potential, as a primed media, in several crop species like rice, wheat, barley, maize, lentil and chickpea (Salton et al., 2001; Harris et al., 2008; Ajouri et al., 2004; Harris et al., 2007; Johnson et al., 2005). Seed-priming with Zn-solution improved grain yield of rice, wheat, barley chickpea and lentils (Harris et al., 2008; Johnson et al., 2005; Ajouri et al., 2004). In a trial, it was investigated that the mean grain-yield of chickpea increased by 19% and wheat yield by 14 % by priming with zinc (Harris et al., 2008). Zn seed-priming prominently improved grain-zinc concentration by 29% in chickpea and 12% in wheat as compared to unprimed (Harris et al., 2008).

Instead of optimal plant growth conditions, micro-nutrient seed priming and particularly zinc seed priming under sub-optimal growth conditions showed more significant stress alleviatory effects on the plant (Imran et al., 2015; Imran et al., 2018). Salt-stress, drought and low temperature are some of the typical yield-limiting abiotic stresses for crops, decreasing plant growth and nutrient uptake. Under salt-stress circumstances, the production of maize plants biomass from Zn priming was 25% greater as compared to water-priming treatment (Imran et al., 2018). In another experiment, it was shown that Zn seed-priming along with Manganese (Mn) improved maize grain-yield by 15%, signifying the potential for long-term effects of nutrient seed-priming (Imran et al., 2015).

Although there is very little literature available on oilseed rape priming but it was found that oilseed rape (Brassica napus L.) was also positively responsive to seed priming, particularly at sub-optimal plant growth conditions. Water priming of oilseed rape (canola) improved 39% of germination and reduced time to 50% emergence when compared with non-primed control (Zheng et al., 1994). Potassium nitrate (KNO_3) priming improved seedling performance and induced pronounced positive effect on canola plant at the highest drought levels (Mohammadi & Amiri, 2010). The potential of zinc seed priming in oilseed rape has not been fully explored yet which is the principal goal of the current study.

1.3.4. Seed dressing/coating

Seed dressing is another commercially viable and acceptable method to treat the seeds. The seed industry very quickly adopted innovative seed dressing/ coating methods in the recent past and it is a rapidly growing market. According to an estimation, the treated seed market value of 6.1 USD in 2016 will be of approx. double of its size (11.3 USD) by the end of 2022 (Marketandmarket, 2020). Although the seed priming is also a competitive approach, but the seed industry inclination towards seed dressing is for several advantages that it delivers. For example, Seed dressing does not require additional labor and mechanization for re-drying, offers a great variety of flexibility for the seed treatments to be combined with seed protective agents like., fungicides and insecticides, gives an opportunity to combine chemical-biological seed treatments innovatively and lastly can be easily stored.

Seed treatment with plant protective agents is getting rapid popularity. In addition to the fungicide and insecticides, the efforts are being made to explore new plant protective agents that can help the plants against biotic and abiotic stresses. Nutrient based formulations are also now available in the market for seed dressing purposes. Seed dressing, coating, or pelleting are being used interchangeably. In terms of micronutrient seed coating, the effectiveness mainly depends on the selected micronutrient, soil type and nutrient to seed ratio (Halmer, 2008). Rice seeds coated with zinc sulfate and zinc oxide improved rice yields by 27.59% and 29.62%, respectively, when treated with 2% w/w each (Shivay et al., 2008). Adhikari (2016) reported a positive seedling growth effect on maize and soybean when coated with

zinc oxide. Inclusion of micronutrients (Cu, Mn & Zn) in wheat seed coating enhanced dry matter and grain yields by 13.4-20.7% and 436-706kg hec^{-1}, respectively (Pawel Wiatrak, 2013). Seed coating with Borax improved 37% grain yields of cowpea (Masuthi et al., 2009). Furthermore, reports are also available on molybdenum seed coating exhibiting positive plant-growth-promoting and yields enhancing effects in common beans and soybean (Biscaro et al., 2009 & Remesh and Thitumurugang, 2001).

1.4. Study question and Hypothesis

The current study was conducted to evaluate nutrients seed treatment role to alleviate the abiotic stresses (the sub-optimal temperature drought) in oilseed rape. The potential of nutrient seed treatment has been documented in many other crops but not fully explored in oilseed rape. Therefore, the role of nutrient seed treatment in germination, early seedling development, plant nutrient status under low RZT and drought conditions was investigated.

Hypothesis 1

Micronutrient seed treatments have beneficial effects on germination and early seedling growth of oilseed rape.

Hypothesis 2

Micronutrient seed treatments have beneficial effects on early root development resulting in improved nutrient acquisition.

Hypothesis 3

Micronutrient seed treatments show protective effects under abiotic stress conditions.

Hypothesis 4

Responses to nutrient seed dressing treatment show genotypic variations.

Hypothesis 5

Seed priming and seed dressing treatments are equivalent in terms of efficiency.

Chapter 2

Micronutrient seed treatments to improve seedling establishment in Oilseed Rape (Brassica napus. L)

2.1. Abstract

Poor germination and limitations during early plant growth are widespread constraints for oilseed rape (Brassica napus. L) grown around the world with increasing importance due to increased frequency weather extremes related to global climate change. In the current study, efforts have been made to enhance health and stress resistance by exploring perspectives of cost-effective application techniques for micronutrients with stress-protective functions to cover potentially increased demands under stress conditions. After preliminary screening experiments, special emphasis was placed particularly on zinc (Zn) seed treatments including seed priming (SP), and seed dressing (SD). Effects on seedling performance during early growth were recorded at optimal conditions for plant growth in terms of temperature, nutrient and water supply for winter OSR and under low root zone temperature (RZT) stress conditions in spring OSR.

Higher seedling biomass and healthy seedling development highlighted the most promising seed treatment potential for $ZnSO_4$ among Zn, Mn, Fe and B priming treatments in a pre-selection experiment conducted as petri-dish germination tests at 8°C, applied as a stress factor. Accordingly, $ZnSO_4$ was used in two model experiments with ZnSP and ZnSD treatments under soil conditions. The presence of more than the double amount of the inherited Zn seed reserves in the shoot tissue of five-day-old seedlings indicated a massive demand of Zn during early seedling development to be mainly supplied via root uptake. A positive effect on plant growth, particularly on root growth regardless of the application method (SP or SD) during the first 10 days of seedling growth suggested suboptimal Zn supply via root uptake at this developmental stage even under optimum growth conditions with respect to temperature (23°C) nutrient availability and water supply. Seed priming treatments Zn 50 µM and seed dressing treatment Zn 50 µM (1.2 ml kg^{-1} seeds) achieved the highest possible growth responses without posing any adverse effect on germination. A similar functional equivalence of SP and SD treatments was

confirmed under field conditions, but the best performance was recorded with Zn 25µM treatments in this case. The magnitude of improvement was even higher at low root zone temperatures (12°C) tested in spring OSR. A decline in fine root development (30%) during winter under field conditions, which was further decreased to 70% in the presence of temporal waterlogging as an additional stress factor, indicated the need for a well-established root system in winter OSR. Improved contents, particularly of other sparingly soluble nutrients (such as Mn, and P) in the shoot can be regarded as secondary effect resulting from better root growth in response to the seed treatments.

Accordingly, both, ZnSP and ZnSD may offer practical, economically low-cost application methods to improve early seedling establishment particularly under challenging environmental conditions, to improve the perspectives of conversion into higher economic yields and could be equally attractive for small-scale on-farm use and rape seed industry.

2.2. Introduction

Oilseed rape (OSR) is one of the most widely grown oil crops, especially in temperate climates (Bauer, 2011). In Europe, it accounted for 77% of all the oilseed production in 2010/2011 (Milke, 2011). Winter OSR is the predominant form of OSR in Central Europe (Rapacz et al., 2000) and Germany as well. Germination and early seedling establishment are prominent features with economic impact in crop species (Rajjou et al., 2012). A rapid and proper seedling establishment can contribute to a better crop stand and economic yields (Ellis, 1992). In case of winter OSR, the seedlings must attain 1-2 g of dry weight during autumn (Mendham and Scott 1975) and a 6-8 leaves stage in the form of a deep-lying rosette, which gives a protective cover to the growing tissue for better winter survival. As a non- mycorrhizal plant species, OSR is highly dependent on a well-established root system for nutrient acquisition as well as for the expression of any strategy regarding nutrient mobilization and even nutrient storage in taproots (Föhse et al.,1991, Hoffland et al., 1989, 1992). Therefore, a proper establishment of the root system before winter is highly desirable for survival and re-growth in spring. Furthermore, Koscielny & Gulden, 2012 reported a correlation of early root length development at the 3-4 leaf stage with final seed yields.

Meanwhile, various seed enhancement techniques with expected positive effects on germination, seedling health, stress resistance & plant establishment attract increasing popularity in the face of climate-change-related weather extremes and a declining availability of chemical plant protection agents. Soaking of seed in water for a specified period to initiate the seed metabolic activities before germination and then drying back to initial moisture is termed as seed priming (Bradford, 1986). Seed priming (SP) is known to enhance germination and seedling growth in various field crops, and in varying plant growth conditions, i.e., limited moisture conditions and P and Zn deficiency (Ajouri et al., 2004; Mena et al., 2013). Hydro-, nutrient-, chemical- and bio-priming are the standard forms of SP. Seed dressing (SD) is another seed enhancement approach in which fungicides, insecticides, or any growth-promoting chemical or biological agents adhere to the seed coat (Ellis, 2004, Stendhal,2005) aiming to enhance seed performance.

Nutrient seed priming (NSP) is a seed treatment technique in which seeds are soaked in a specific nutrient solution for a particular time duration. Seeds can be primed either with macro or micronutrients. However, since micronutrients are needed in smaller quantities, priming with micronutrients usually has longer-lasting effects. Nutrient seed priming with micronutrients improved early seedling nutritional status in maize (Imran et al., 2013; 2015) providing growth-promoting and stress-protective effects. Plants are continuously exposed to environmental stress challenges in variable intensities. Although micronutrients, such as Zinc (Zn), Manganese (Mn), Iron (Fe), Copper (Cu) and Boron (B) are required in small amounts, vital roles in protection against different stresses are documented (Fig.2.1).

The availability of these micronutrients in the soil and the plant is important under stress conditions to perform stress-protective functions in membrane integrity, signalling, synthesis of protective secondary stress metabolites, growth hormones, and many more. Micronutrients like Zn, Mn, Cu and Fe are well known to be co-factors of various enzymes (superoxide dismutases, peroxidases, catalses) involved in detoxification of reactive oxygen species produced in excess amounts in response to various environmental stress factors (Jaleel et al, 2009).

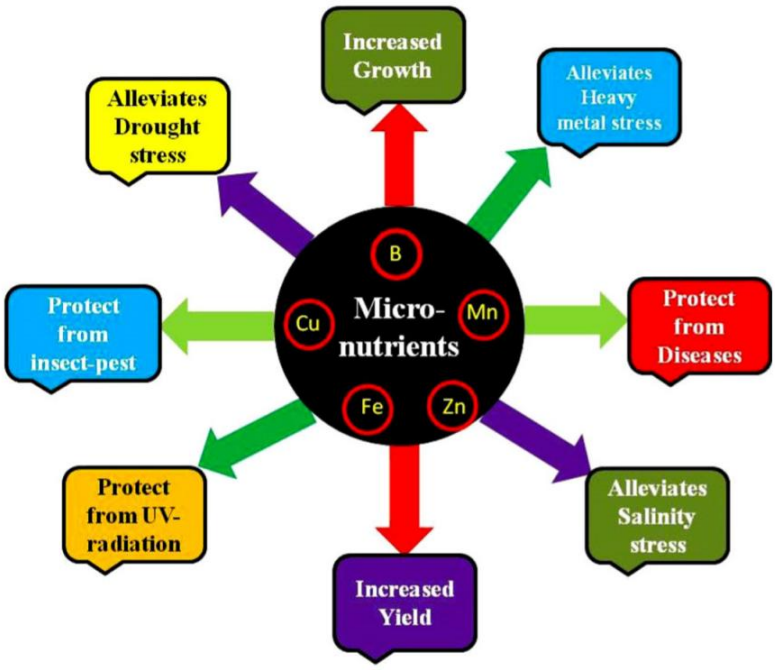

Fig. 2.1: Protective role of micronutrients (Zn, Mn, Fe, Cu and B) in different biotic and abiotic stresses (Tripathi et al., 2015)

Manganese (Mn), Cu and B are also important cofactors for many enzymes and metabolic pathways involved in the biosynthesis of phenolics with widespread functions in stress adaptations, such as lignification, antibiotic activity, signals for beneficial micro-organisms, UV light protection and functions as antioxidants. Copper takes part in the regulation of various plant physiological and metabolic processes**.** Enzymes, in which copper act as a cofactor, play pivotal roles in respiration, cell wall lignification and phenolic compounds metabolism. Manganese (Mn) is discussed as a micronutrient with central relevance for the antibiotic activity in terms of disease resistance in plants (Huber and Graham, 1999; Heckman et al., 2003). Lignin and subrin biosynthesis is important for the formation of mechanical barriers to resist pathogen invasion, i.e., fungal attack. Manganese activates various enzymes in the shikimic acid and phenylpropanoid pathway (Marchner, 1995) regulating the biosynthesis of lignin and phenolic compounds in general (Romheld and Marchner 1991; Vidhyasekaran, 1997).

The involvement of Boron in lignin, polyphenol and flavonoid synthesis highlights it as another important stress-protective micronutrient (Dixit et al., 2002). Additionally, the role of B in plant-microbe interactions to establish a symbiotic relationship (Reguera et al., 2010) and cell wall construction (0`Neill et al., 2004) further highlights its

essential role for plant development. The role of micronutrients in different metabolic pathways is illustrated in Fig.2.2.

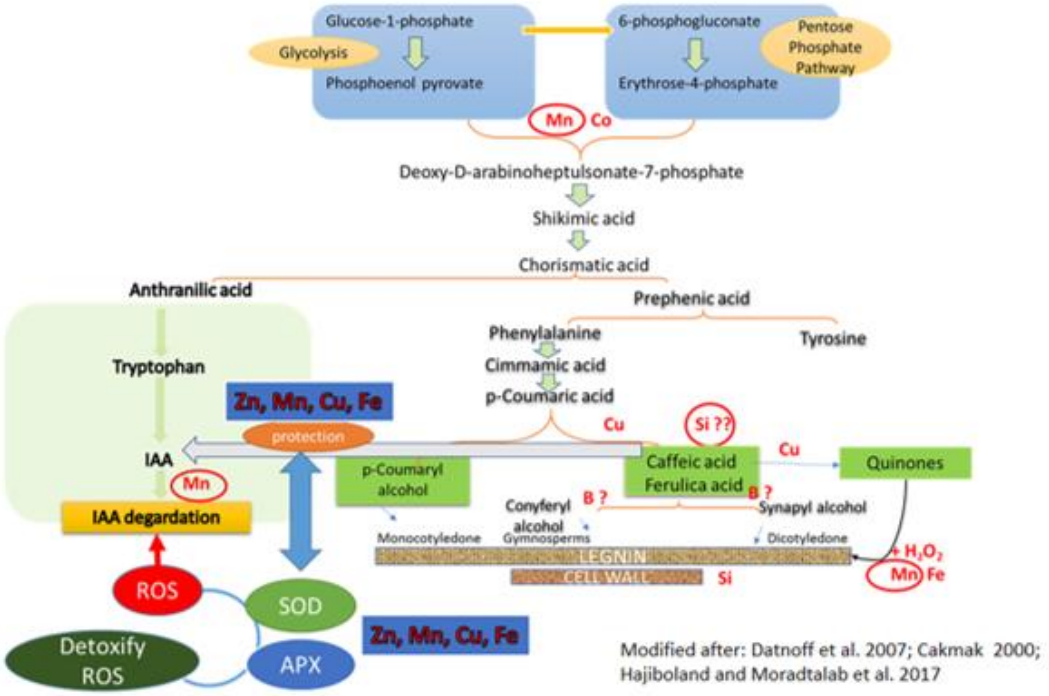

Fig. 2.2: Role of micronutrients in physiological and biochemical processes.

In the present study, micronutrient seed priming and seed dressing approaches were compared for seed nutrient enrichment. The perspective of micronutrients to be used as primed and dressed nutrients was determined. Benefits of SP and SD could only be achieved after attaining an appropriate dosage of the primed and coated media. Therefore, dosage optimization concerning early plant development (root and shoot growth) and its alleviatory stress effects under low-temperature, exemplarily selected as stress factor was investigated in OSR. It was hypothesized that;

a) Micronutrient seed treatments increase seed nutrient reserves.
b) Micronutrient seed treatments exhibit germination improvements.
c) A seedling from micronutrient treated seeds exhibits better plant growth under optimal and low RZT conditions.
d) Plants produced from micronutrient treated seeds exhibit an improved plant-nutritional status (Zn, Mn, Fe, P).
e) Seed priming and seed dressing effects are comparable.

2.3. Materials & Methods

2.3.1 Seed treatments

Nutrient seed priming is a technique in which the seeds are soaked in a specific nutrient solution for a particular time duration, dried back, and stored for further use. Initially, seeds were primed with various micronutrients (Zinc, Manganese, Iron, Copper & Boron) to select the most effective micronutrient for further experiments. $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, Fe-EDTA, $CuSO_4.5H_2O$ and H_3BO_3 solutions of different concentrations were used to prime the seeds with Zn, Mn, Fe, Cu, and B, respectively (Table 1). Seeds were provided by Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (NPZ, Holtsee, Germany). For priming, five grams of OSR seeds were soaked in 50 ml of the respective solution of each concentration for a predetermined time duration of 24 hours. Seeds were rinsed carefully to remove any adhering material on the seed surface. Afterwards, the seeds were dried at room temperature and stored at 2^0C in a cooling room.

Table 2.1: Seed priming solution concentrations.

Micronutrients	Concentration (mM)
$ZnSO_4.7H_2O$	0.01, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, 20
$MnSO_4.H_2O$	0.01, 0.1, 0.2,5, 0.5, 0.75, 1, 2, 3, 4, 15
Fe-EDTA	0.5, 2, 4, 6, 8, 20
$CuSO_4.5H_2O$	0.1, 0.2, 0.3, 0.4, 1.5
H_3BO_3	0.1, 1

Primed seeds were grown on a Whatman No 1 moist filter paper, in the 9 cm round Petri dishes. Ten seeds were grown in each Petri dish in four replicates at low-temperature conditions (8^0C ± 1). Seedling biomass and germination were recorded after 10 days.

For the model experiments, various concentrations of Zinc sulfate ($ZnSO_4.7H_2O$) solution was used (Table 2.2). For seed dressing treatment, the seed of OSR were treated with Zinc sulfate $ZnSO_4.7H_2O$ concentration of 25 and 50 µM, at various doses, by NPZ (Holtsee, Germany). A solution of 25 µM $ZnSO_4$ was supplied with the help of adhesive material on the seed surface at three dosage rates of 1.2, 4 and 8 ml kg^{-1} seeds, while 50 µM $ZnSO_4$ was applied with 1.2 and 4 ml kg^{-1} of seed. Untreated seeds were used as a control in all the experiments. Seeds were stored at 2^0C before use.

Table 2.2: Zinc seed priming (ZnSP) and Zinc seed dressing (ZnSD) treatments in all three experiments. Columns “a & b” represent two independent experiments under controlled growth chamber conditions while column “c” represents the treatments used in greenhouse culture with low root zone temperature (RZT). UT = unprimed control; WP = water-primed control.

	a	**b**	**c**
	Exp-1	**Exp-2**	**Exp-3**
	Priming Treatments	**Dressing Treatments**	**Priming Treatments**
1	UT	UT	UT
2	WP	Zn 25 µM (1.2 ml kg^{-1} seeds)	WP
3	-	Zn 25 µM (4 ml kg^{-1} seeds)	Zn 10 µM
4	Zn 25 µM	Zn 5 µM (8 ml kg^{-1} seeds)	Zn 25 µM
5	Zn 50 µM	Zn 50 µM (1.2 ml kg^{-1}seeds)	Zn 50 µM
6	Zn 75 µM	Zn 50 µM (4 ml kg^{-1}seed)	Zn 75 µM

2.3.2 Experiment 1 & 2

Two experiments were conducted in the growth chamber under controlled environmental conditions. For both experiments, environmental condition comprised average air temperatures of 23 + 2 °C, 15/9 h light/dark periods with a light intensity of 400 mol m^{-2} s^{-1} and relative air humidity of 60-80%. Five hundred grams of a sandy loam soil pH 5.6 was fertilized 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 was filled in each pot covered with thin layer of quartz sand (100 g) to reduce surface evaporation. Ten seeds of the respective treatments were directly sown at 2 cm soil depth. Untreated (UT) and water primed (WP) seeds were taken as a control in the experiment “1” and for experiment “2” only an untreated (UT) control was included (Table 2.2). Soil moisture contents were maintained gravimetrically at 70% substrate water-holding capacity (WHC) throughout the experiment. The plants were sequentially harvested at 5, 10 &15 days after sowing (DAS) for experiment “1” and 10 DAS for experiment “2”.

2.3.3. Experiment 3

2.3.3.1. Low root zone temperature (RZT) treatment

A rectangular wooden box with styrofoam isolation, filled with moist peat was used to construct a controlled root zone temperature system. Cooling pipes connected to the cooling thermostat (Thermomix 1480/Frigomix 1497, Braun, Melsungen, Germany), were installed inside the peat layer vertically and horizontally to provide a uniform temperature distribution and to avoid any temperature gradient (Bradacova et al., 2016). Sealed pots were then inserted into the thermostated peat layer. Soil temperature was maintained at 12°C $\pm$ 2 throughout the experiment.

2.3.3.2 Plant culture

The experiment was conducted in a greenhouse at average air temperature 14.0^0 C. Average humidity ranged between 60-80% and the light intensity from 250 – 400 µmol m^{-2} s^{-1} PAR. A field soil already fertilized for Winter OSR cultivation was taken from University of Hohenheim research station “Heidfeldhof”. The soil had the following properties; clay loam soil with pH 6.6, $N_{min\ CFA}$ 27.4 mg kg^{-1}, P_{CAL} 170 mg kg^{-1}, K_{CAL} 200 mg kg^{-1}, Mg $_{CaCl2}$ 150 mg kg^{-1}, Fe_{CAT} 165 mg kg^{-1}, Mn_{CAT} 231 mg kg^{-1}, $B_{hot\ water}$ 0.312 mg kg^{-1}, Zn_{CAT} 2.89 mg kg^{-1}, Cu_{CAT} 3.51 mg kg^{-1}.No mineral fertilizers were additionally applied. The soil was sieved through 2 mm mesh size and thereafter, pots were filled with 1.5 kg of soil each. Ten primed seeds were sown at 2 cm soil depth in each pot. Pots were then placed in the cooling system for germination. Moisture content was adjusted to 18% by W/W basis and kept constant throughout the experiment by daily gravimetric control. Plants were harvested five weeks after sowing (WAS).

2.3.4. Field Experiments

One field experiment was conducted at university Hohenheim research station “Heid-feldhof” to observe the root establishment under field conditions. A non-destructive method for monitoring root growth through root observation windows (Neumann et al., 2009; 2014) was employed. The experiment was conducted in a pre-fertilized oilseed rape stand with soil properties as specified in experiment 3. Twelve root observation

windows were installed according to the method described by Neumann et al. (2009) and monthly recordings of the root development along the observation windows were performed from October to March.

A comparative study on Zn seed priming and seed dressing approaches under field conditions was conducted by the industrial partner NPZ Innovation GmBH in Hohenlieth. The research station Hohenlieth is located in the Nort-Western part of Schleswig Holstein in Northern Germany with an average annual precipitation level of 815 mm and an average annual temperature of 8.9°C. The soil type is characterized as sandy-loam, pH 6.5. Ultra-late sowing was conducted by the end of September 2014, comparing standard sowing (NSTP1) and striptill sowing, including a starter fertilization with diammoniumphosphate applied as fertilizer placement at a depth of 20 cm with 100 kg ha^{-1} (NSTP2). Seed treatments (SP and SD) were conducted with two concentrations of $ZnSO_4$ (25 and 50 µM) with two OSR hybrids (WR1 and WR2) in four replicates per treatment. WR1 was also compared for ZnSD including a fungicide treatment Thiram-TMTD + Dimethomorph-DMM (meanwhile banned) with ZnSP. Recordings of seedling emergence and scoring of plant performance were conducted twice before winter and final yield determination in 2015. Sampling times are summarized in Table 2.3.

Table 2.3: Samplings for comparative field testing of ZnSP and ZnSD treatments.

Series	Sowing System	Sowing	Emergence 1	Emergence 2	Autumn development
NSTP1	Standard	24.9.	01.10	21.1	21.1
NSTP2	Striptill	24.9.	01.10	21.1	21.1

2.3.5. Plant Analysis

Shoot and root dry biomass was determined gravimetrically after oven-drying at 60°C at final harvest. Total root length was determined for all experiments. Washed root samples were preserved in 30% (v/v) ethanol solution. After separating root samples in a water film on transparent perspex trays, scanning of the root systems was performed with a flatbed scanner (Epson Expression 1000XL, Epson, Tokyo, Japan) at a resolution of 400 dpi. Scanned images were analyzed, and total root length was

measured using a root analysis software WinRhizo (WinRHIZO, Pro V. 2009, Regent Instruments, Quebec, Canada). Root development along the root observation windows was recorded by digital photographs with subsequent analysis using the WinRhizo System (Neumann et al. 2014).

Dried shoot material (500 mg) was ashed in a muffle furnace at 500 °C for 5 hrs. Samples were then cooled and extracted twice with 2.5 mL of 3.4 M HNO_3. To precipitate SiO_2 samples are evaporated until drying. After dissolving ash in 2.5 mL of 4 M HCl, the solution was diluted ten times with deionized water and boiled for two minutes. Fe, Mn and zinc were measured using atomic absorption spectrometry (UNICAM 939, Offenbach/Main Germany) after adding 0.1 mL Cs/La buffer to 4.9 mL ash solution while phosphorous was measured spectrophotometrically according to the method of Gericke and Kurmis (1952).

2.3.6. Statistical analysis

Experiments were conducted using a completely randomized design. Data are presented as means and standard errors (SE). Significant differences were evaluated by one-way analysis of variance ($p < 0.05$) using Sigma Plot Version 11.0. Software (SYSSTAT Software Inc., Erkrath, Germany).

2.4. Results

2.4.1 Selection of the most efficient micronutrients for OSR seed treatments

For the selection of efficient micronutrients for OSR seed priming, seeds were germinated in petri dishes at low temperature (8^0C) selected as a stress factor to promote the expression of protective micronutrient effects. Zinc (Zn), manganese (Mn), iron (Fe), copper (Cu) and boron (B) were used as primed nutrients to select the most efficient micronutrient for OSR. A first priming experiment was conducted with

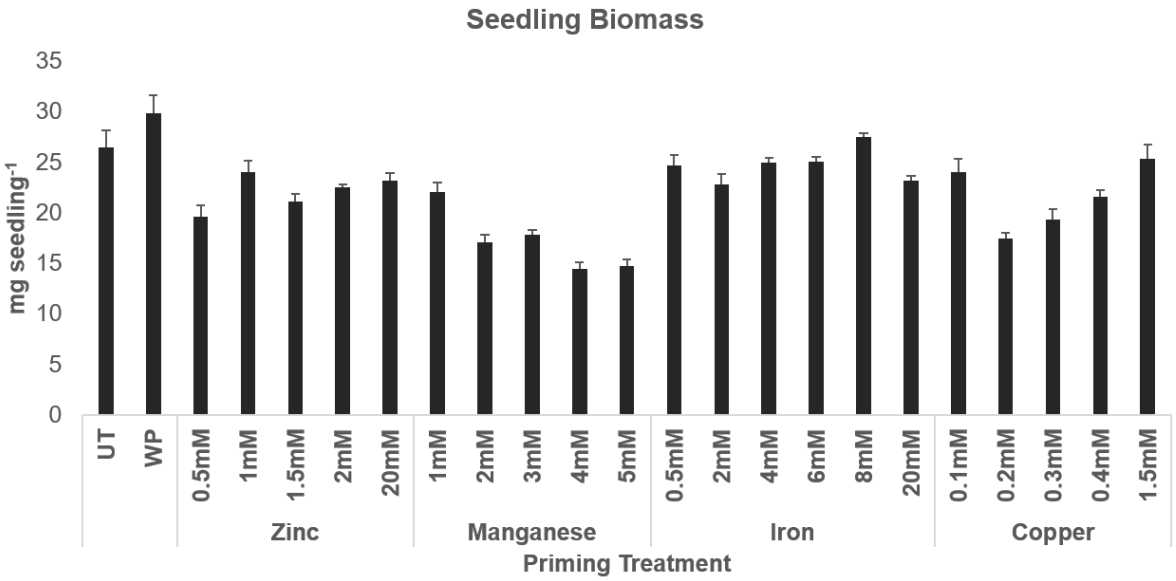

Fig. 2.3: Seedling biomass at 8^0C with micronutrient Zn. Mn. Fe and Cu seed priming. High concentration treatments. Data represent the mean and SE of 4 replicates.

concentrations of Zn, Mn and Fe reported to be effective in maize (Imran et al., 2013). However, compared with the untreated control (UT) no or even negative effect of the applied nutrients on seedling biomass were recorded (Fig. 2.3), suggesting unsuitable priming concentrations.

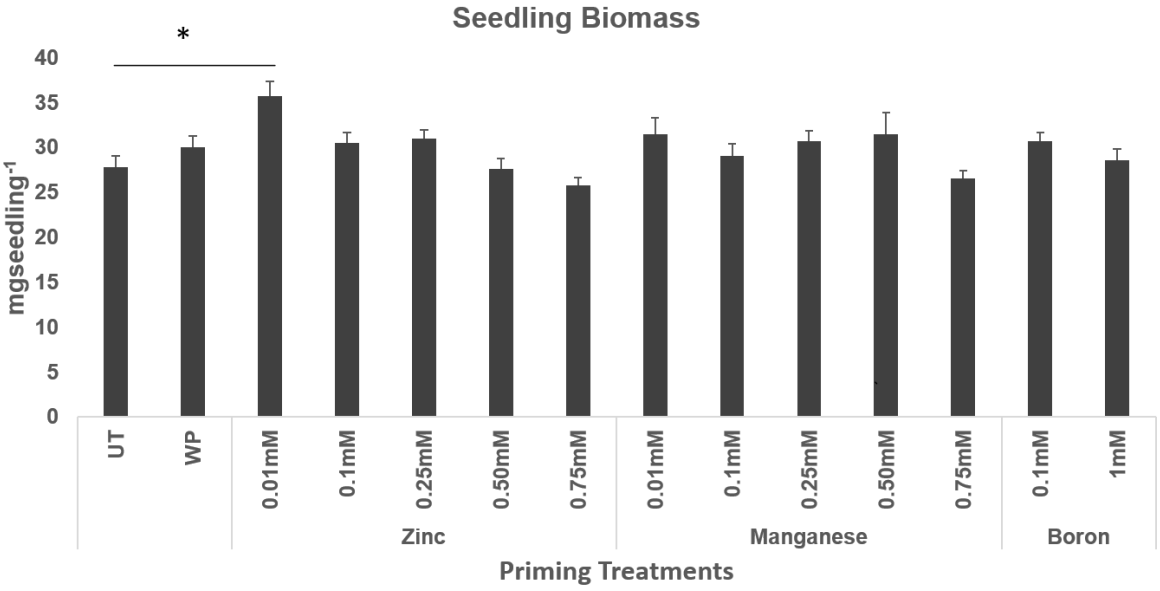

Fig. 2.4: Seedling biomass at 8°C with micronutrient Zn. Mn. Fe and Cu seed priming. Low concentration treatments. Sign (*) indicates a statistical difference at $P<0.05$.

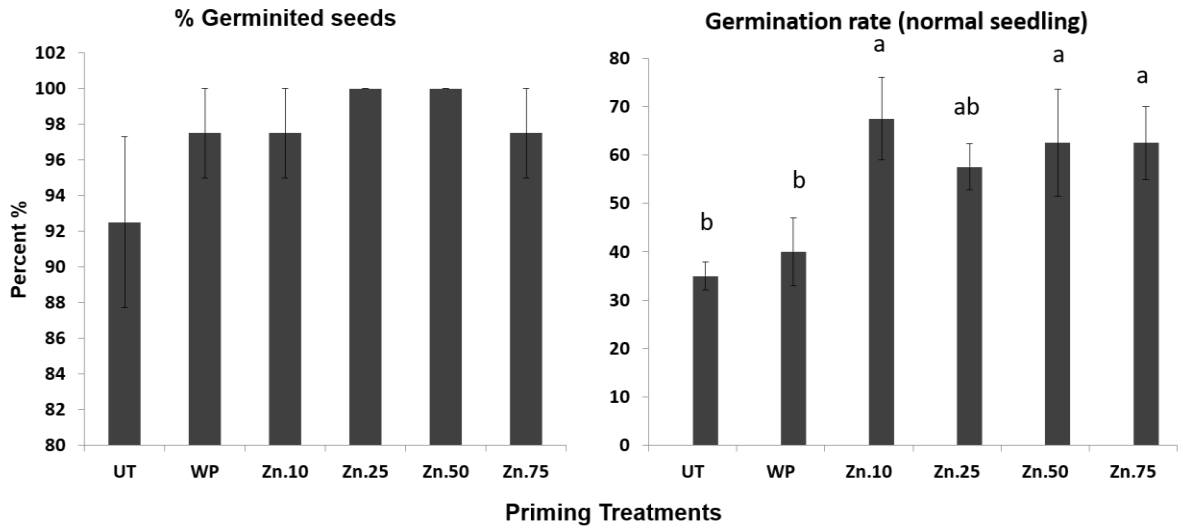

Fig. 2.5: Germination percentage (left) and germination rate (normal seedling development) at 8^0C. Data represent means and SE of 4 replicates. Different characters indicate significant differences between the treatments.

Therefore, further optimization with lower nutrient concentrations was performed (Fig. 2.4). At lower concentrations, zinc appeared to be the most impactful nutrient at the concentration 0.01mM (10µM) with respect to biomass production (Fig. 2.4).

Normal seedling development under low-temperature stress was also improved already at 0.01mM or (10µM) Zn concentration (Fig. 2.5). The aforementioned results suggest zinc as the most effective micronutrient for OSR under the given conditions, which was later tested in further experiments.

2.4.2 Comparison of Zn seed priming and Zn seed dressing effects on seedling development of OSR

Nutrients may be supplemented to the seed either through seed priming or seed dressing, which is technically more feasible and simpler to perform. Therefore, a set of model experiments was conducted in co-operation with NPZ (Holtsee, Germany) to compare the effects of Zn seed priming (ZnSP) with Zn seed dressing (ZnSD) on early seedling growth in OSR under optimal growth conditions.

Zinc reserves appeared to be highly variable in different OSR hybrids (Table 2.3) due to the genotypic variability or to seed lot effects. Seeds with lower zinc reserves may be supplemented through ZnSP or ZnSD.

Table 2.3: Zinc seed reserves of different spring rape hybrids. Data represent the mean and SE of 4 replicates. Different characters indicate significant differences.

	SR1	SR2	SR3	SR4	SR5	SR6
Zinc seed reserves (µg seed^{-1})	50.5+1.0 a	39.1+1.0 b	26.2+0.4 d	51.8+2.0 a	37.9+1.7 b	33.5+1.1 c

2.4.2.1 ZnSP effect on OSR seedling development

In a selected OSR hybrid with low Zn seed reserves (0.19 µg seed^{-1}) seed Zn contents and concentrations were significantly increased by Zn priming treatments. Zn.25, Zn.50, and Zn.75 enhanced the seed zinc concentrations by 57, 221 and 277%, respectively as compared with an unprimed control (UT) (Table 2.4).

Zn priming significantly accelerated and increased particularly root biomass and root length development even under optimal temperature conditions (23°C) by up to 30% compared with the untreated control at Zn concentrations of 75 µM (Table 2.5, Fig. 2.6) particularly during the first 10 d of seedling development.

Table 2.4: Seed zinc concentrations and contents of winter oilseed rape (WR1) after zinc seed priming. Data represent means and SE of 3 replicates. Different characters indicate significant differences between treatments.

Treatments	Concentration [µg g^{-1}]	Contents [µg seed^{-1}]
UT	35.26±0.6 d	0.19±0.00 d
WP	38.14±0.4 d	0.21±0.01 d
Zn.25	55.26±0.5 c	0.30±0.01 c
Zn.50	113.17±0.5 b	0.62±0.01 b
Zn.75	133.11±2.7 a	0.73±0.01 a

Shoot nutrient analysis revealed a particularly high micronutrient (Zn/Mn) demand particularly in the seedling stage with seed reserves lasting for less than 5 DAS until additional root-mediated Zn acquisition was detectable by analysis of Zn and Mn shoot contents, which exceeded the seed reserves already at 5 DAS. By contrast the reserves of P as macronutrient lasted longer, and a significant contribution of root uptake was detectable at 10 DAS. Better root growth induced by Zn priming obviously translated into improved P acquisition detectable at 15 DAS (Table 2.6).

Table 2.5: Shoot and root biomass production of oilseed rape plants grown at 23 ± 2^{0}C for 5, 10 & 15 days after sowing (DAS). Data represent means and SE of six independent replicates of each treatment. Different characters indicate significant differences between treatments.

	Shoot dry biomass [mg plant^{-1}]			Root dry biomass [mg plant^{-1}]		
Treatments	**5 DAS**	**10 DAS**	**15 DAS**	**5 DAS**	**10 DAS**	**15 DAS**
UT	5.14±0.32 a	14.35±0.70 a	41.94±1.68 a	1.66±0.05 a	4.81±0.13 bc	13.22±0.57 a
WP	5.20±0.15 a	14.39±0.49 a	41.81±1.85 a	1.72±0.04 a	5.42±0.16 b	13.83±1.16 a
Zn.25	5.26±0.15 a	14.88±0.30 a	43.67±1.16 a	1.75±0.02 a	5.52±0.16 ab	15.26±1.13 a
Zn.50	5.31±0.18 a	15.23±0.65 a	45.10±1.54 a	1.78±0.02 a	5.78±0.21 ab	16.06±0.84 a
Zn.75	5.35±0.13 a	15.61±0.53 a	47.62±2.23 a	1.82±0.03 a	6.30±0.30 a	16.80±0.88 a

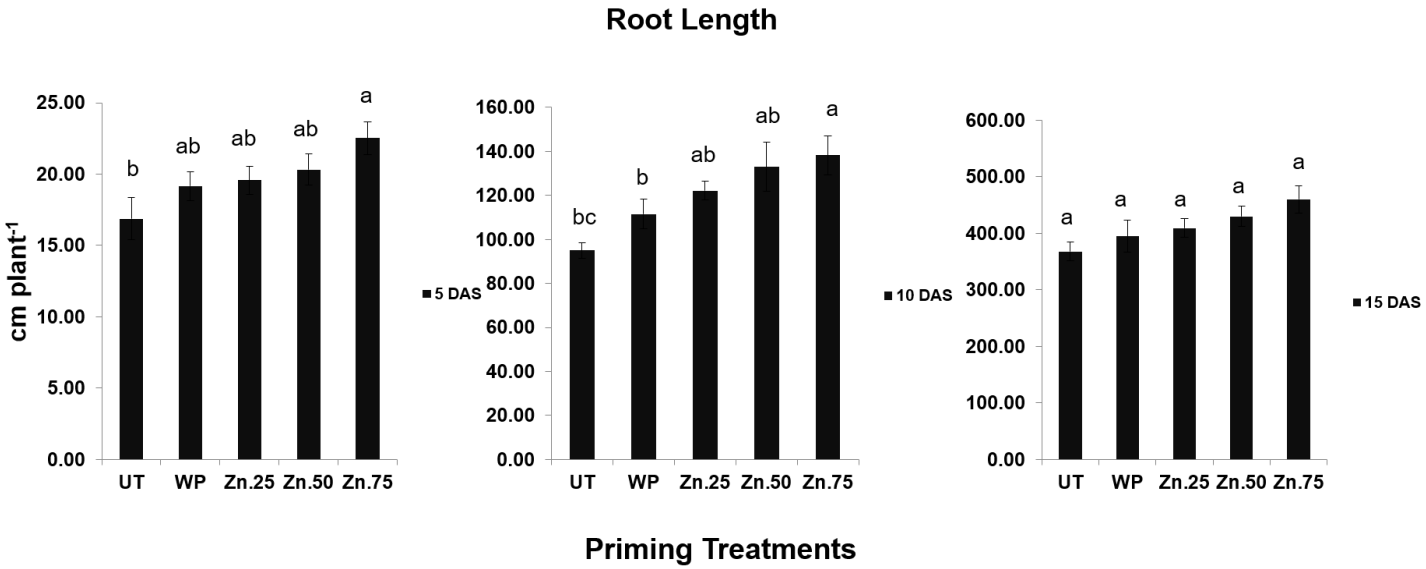

Fig. 2.6: Root length development of oilseed rape plants grown at 23 ± 2^{0}C for 5, 10 and 15 days after sowing (DAS) on a sandy loam pH 5.6. Data represent means and SE of five independent replicates of each treatment. Different characters indicate significant differences between treatments.

Table 2.6: Shoot mineral concentrations and contents of oilseed rape seedlings grown in a sandy loam soil (pH 5.6) at 23 ± 2°C for 5, 10 and 15 days after sowing (DAS). Data represent means and SE of 3 replicates of each treatment. Different characters indicate significant differences between treatments.

	Concentrations				**Contents**			
	Zn [µgg^{-1}]				**Zn [µgplant^{-1}]**			
	0 Day (Seed)	**5 DAS**	**10 DAS**	**15 DAS**	**0 Day (Seed)**	**5 DAS**	**10 DAS**	**15 DAS**
UT	35.26±0.61 e	67.64±0.54 d	62.34±1.11 a	49.62±1.20 a	0.19±0.00 e	0.35±0.00 d	0.90±0.02 b	2.06±0.05 a
WP	38.13±0.39 d	61.23±0.52 e	57.36±0.69 b	46.53±0.24 a	0.21±0.00 d	0.32±0.00 e	0.83±0.01 c	1.97±0.01 b
Zn.25	55.26±0.49 c	71.09±1.11 c	60.62±0.88ab	41.10±0.23 b	0.30±0.00 c	0.37±0.01 c	0.90±0.01 b	1.80±0.01 c
Zn.50	113.17± 0.45 b	76.17±0.56 b	61.29±0.58 ab	39.29±1.16 b	0.62±0.00 b	0.40±0.00 b	0.93±0.01 b	1.77±0.05 c
Zn.75	133.11±2.70 a	82.65±0.67 a	63.99±0.89 a	40.10±0.63 b	0.73±0.01 a	0.44±0.00 a	1.00±0.01 a	1.91±0.03 b
	Mn [µgg^{-1}]				**Mn [µgplant^{-1}]**			
	0 Day (Seed)	**5 DAS**	**10 DAS**	**15 DAS**	**0 Day (Seed)**	**5 DAS**	**10 DAS**	**15 DAS**
UT	22.96±0.26 a	52.16±0.59 a	42.22±0.33 a	36.21±0.21 a	0.13±0.00 a	0.27±0.00 a	0.61±0.00 bc	1.50±0.01 bc
WP	23.77±0.26 a	52.04±0.47 a	42.53±0.15 a	35.00±0.27 a	0.13±0.00 a	0.27±0.00 a	0.61±0.00 bc	1.48±0.01 c
Zn.25	23.79±0.32 a	52.20±0.41 a	43.82±0.58 a	35.84±0.54 a	0.13±0.00 a	0.28±0.00 a	0.65±0.01 ab	1.56±0.02 b
Zn.50	24.30±0.04 a	51.18±1.10 a	42.50±0.29 a	34.73±0.05 a	0.13±0.00 a	0.27±0.01 a	0.65±0.00 ab	1.57±0.00 b
Zn.75	23.54±0.05 a	53.06±0.99 a	42.74±0.13 a	35.62±0.51 a	0.13±0.00 a	0.28±0.01 a	0.67±0.00 a	1.70±0.02 a
	P [mgg^{-1}]				**P [mgplant^{-1}]**			
	0 Day (Seed)	**5 DAS**	**10 DAS**	**15 DAS**	**0 Day (Seed)**	**5 DAS**	**10 DAS**	**15 DAS**
UT	7.5±0.02 a	8.64±0.12 a	8.17±0.04 a	6.68±0.02 b	41.21±0.10 a	44.39±0.63 a	117.19±0.19 a	277.49±0.54 e
WP	7.56±0.10 a	8.71±0.20 a	8.01±0.03 a	6.76±0.03 ab	41.58±0.55 a	45.27±1.06 a	115.22±0.07 a	285.82±0.38 d
Zn.25	7.23±0.09 a	8.39±0.21 a	8.10±0.03 a	6.69±0.02 b	39.77±0.48 a	44.12±1.08 a	120.48±0.09 a	292.30±0.41 c
Zn.50	7.46±0.03 a	8.38±0.13 a	7.95±0.023 a	6.66±0.03 b	41.03±0.18 a	44.48±0.71 a	121.08±0.06 a	300.22±0.35 b
Zn.75	7.47±0.02 a	8.68±0.09 a	8.08±0.017 a	6.82±0.02 a	41.10±0.13 a	46.45±0.45 a	126.26±0.05 a	324.77±0.28 a

2.4.2.1.2. ZnSD effect on OSR seedling development

Due to the limitations of seed priming treatments for practical applications (e.g. technical requirements for re-drying, difficult to combine with standard seed dressing treatments) also perspectives for Zn seed dressing were investigated.

Similar to seed priming, also seed dressing significantly increased particularly root development at 23°C by 47% (biomass) and 20% (length) with the most effective treatment of 1.2 ml of 50 µM $ZnSO_4$ Kg^{-1} of seeds (Table 2.7).

Zn seed dressing not only increased Zn shoot contents during early seedling growth but also Mn and P accumulation, while Fe, which was present in high concentrations close to the toxicity threshold (approx. 500 µg g^{-1} shoot DM) was decreased by the Zn dressing treatments (Table 2.8).

Table 2.7: Germination, shoot and root biomass production, and root length of OSR plant grown at 23 ± 2^0C for 10 DAS after zinc seed dressing on a sandy loam soil pH 5.6. Data represent means and SE of 10 replicates. Different characters indicate significant differences between treatments.

Zn dressing Treatments	Germination [%]	Shoot dry biomass [mg plant^1]	Root dry biomass [mg plant^1]	Root Length [cm plant^1]
UT	81 a	21.01±0.61 a	1.03±0.09 b	111.17±5.29 b
25µM.(1.2ml)	88 a	22.73±0.61 a	1.30±0.09 ab	125.79±3.03 ab
25µM.(4ml)	86 a	22.02±0.80 a	1.23±0.10 ab	123.44±3.04 ab
25µM.(8ml)	82 a	22.85±0.56 a	1.08±0.04 b	123.22±3.58 ab
50µM.(1.2ml)	85 a	24.22±0.91 a	1.52±0.09 a	133.90±4.66 a
50µM.(4ml)	80 a	23.56±0.71 a	1.32±0.06 ab	119.12±4.08 ab

Table 2.8: Shoot mineral concentrations and contents of oilseed rape plant grown in a sandy loam soil (pH 5.6) at 23 ± 2^0C for 10 days after sowing (DAS). Data represent means and SE of 3 replicates of each treatment. Different characters indicate significant differences between treatments.

	Concentration [µg g^{-1}]				Contents [µg $plant^{-1}$]			
Zn dressing Treatments	**Zn**	**Fe**	**Mn**	**P [mg g^{-1}]**	**Zn**	**Fe**	**Mn**	**P [mg $plant^{-1}$]**
UT	44.56±0.54 a	437.11±5.42 a	96.53±1.05 b	8.03±0.08 cd	0.94±0.01 b	9.18±0.11 b	2.02±0.02 bc	0.17± 0.0 c
25µM.(1.2ml)	47.09±1.03 a	433.66±1.55 ab	97.79±0.71 b	8.56±0.13 bc	1.07±0.02 ab	9.85±0.04 a	2.22±0.02 b	0.20± 0.0 b
25µM.(4ml)	48.12±1.43 a	418.44±3.79 b	104.17±0.60 a	8.87±0.05 b	1.06±0.03 ab	9.21±0.08 b	2.29±0.01 a	0.20±0.0 b
25µM.(8ml)	46.57±0.88 a	367.70±5.77 c	103.29±0.79 a	9.41±0.03 a	1.06±0.02 ab	8.40±0.13 c	2.36±0.02 a	0.22±0.0 a
50µM.(1.2ml)	46.66±1.37 a	344.77±5.24 d	97.81±1.20 b	8.66±0.02 bc	1.13±0.03 a	8.35±0.13 c	2.37±0.03 ab	0.21±0.0 a
50µM.(4ml)	48.01±4.44 a	309.60±4.44 e	96.73±0.89 b	9.15±0.05 ab	1.13±1.61 a	7.30±0.11 d	2.28±0.02 ab	0.22±0.0 a

2.4.2.2. Comparison of ZnSP and ZnSD under field conditions

Since benefits of the nutrient treatments on seedling establishment of winter OSR were also observed under field conditions (Fig. 2.7). A comparative study on Zn seed priming and seed dressing approaches under field conditions was conducted by the industrial partner NPZ Innovation GmBH in Hohenlieth.

Fig. 2.7: Seedling establishment of the winter OSR hybrid Atora at 53 DAS under field conditions with (right) and without (left) nutrient seed treatment, (source: Rapool DE/CZ/HU).

The experiments were based on the outcome of the model experiments in greenhouse culture and included two sowing techniques (standard vs Striptill with the placement of DAP starter fertilizer) and two OSR hybrids (WR1 and WR2).

Fig. 2.8: Seedling emergence in standard and striptill sowing at the experimental station Hohenlieth.

The comparison between ZnSP with ZnSD in WR2 revealed comparable emergence scorings before winter but in both sowing systems higher relative yields with a $ZnSO_4$ priming dosage of 25 µM compared with 50 µM with a trend for better performance of ZnSD (Table 2.9).

Standard Sowing						
Cultivar	**Treatments**		**Yields**	**Emergence 1**	**Emergence 2**	**Autumn development**
	ZnSP	**ZnSD**	**Rel.**	**Number of plants in 2m²**	**Number of plants in 2m²**	**Notation 1-9**
WR1	25µM		91	25	23	6.5
WR1		50µM. (2ml)	94	24	22	6.6
WR2	25µM		101	26	22	7.3
WR2		25µM. (4ml)	104	29	26	7.8
WR2		50µM. (4ml)	99	25	23	6.8
		Grand Mean	49.1	25.7	23	7.1
		CV	5.4	18.5	16.6	9
		LSD	3.9	7	5.6	0.9
		Total Reps	4	4	4	4

Striptill Sowing						
Cultivar	**Treatments**		**Yields**	**Emergence 1**	**Emergence 2**	**Autumn development**
	ZnSP (uM)	**ZnSD (uM)**	**Rel.**	**Number of plants in 2m²**	**Number of plants in 2m²**	**Notation 1-9**
WR1	25µM		94	23	19	6.5
WR1		50µM. (2ml)	96	24	20	6.6
WR2	25µM		102	24	20	7.4
WR2		25µM. (4ml)	105	26	20	7.3
WR2		50µM. (4ml)	97	28	22	7.6
		Grand Mean	54.8	24.7	20.3	7.2
		CV	3.3	16.8	14.8	7.5
		LSD	2.7	6.1	4.4	0.8
		Total Reps	4	4	4	4

Table 2.9: Effects of $ZnSO_4$ seed priming and seed dressing treatments on field establishment and yield of winter OSR hybrids (WR2, WR1) with striptill versus conventional sowing (Source: S. Goertz, NPZ Innovation GmbH, Hohenlieth, Germany).

For WR1 no differences between ZnSD and ZnSP treatments were recorded with a generally lower relative yield as compared with WR2. In both sowing systems the yield

level ranged above the average of 3.3 t ha^{-1} recorded in Germany between 1995-2019 (Ahrens, 2020) with 11.6% higher yields in the striptill system as compared with conventional sowing (Table 2.9). This aspect is discussed more in detail in Chapter 3, addressing options for Zn seed treatments in fertilizer placement strategies.

2.4.3. ZnSP effects under stress conditions – Example cold stress

2.4.3.1 Importance of cold-stress protection in OSR

Although winter OSR in Central Europe is usually sown already in late summer by mid of September, appropriate root establishment during autumn is essential for winter survival. This aspect was investigated in a field experiment using the installation of root windows (Neumann et al. 2009) for non-destructive monitoring of root growth under field conditions (Fig 2.9A).

Even on a clay loam soil, winter OSR exhibited intense fine root development along the root observation window in early autumn was detectable at 7 weeks after sowing in a soil depth > 50 cm (Fig. 2.9B).

Fine root length declined by 30%, without additional stress factors during winter until February but was rapidly regenerated until March (Table 2.10). However additional stress factors such as waterlogging after heavy rainfall events or shoot feeding damage by snail attack reduced the fine root development by 70% and more already before winter (Fig. 2.9) and no further evaluation of plant growth was possible in this case, highlighting the importance of a well-developed root system for winter hardness

Table 2.10: Time course of root length development along with root observation windows between October and March. Data represent Means and SD of 3 replicates.

Month	**Root length along root window (cm)**
October	166 ± 31
November	243 ± 48
December	228 ± 39
January	202 ± 40
February	171 ± 42
Marz	302 ± 47

Fig. 2.9: A field view of installed root windows (A) Fine root development along with root observation windows of field-grown Winter OSR 7 weeks after sowing (Both); Lower row: root development along with observation windows with (right) and without (left) exposure to waterlogging (Experimental Station Heidfeldhof – Uni Hohenheim).

2.4.3.2 ZnSP effect on seed zinc status, germination & seedling growth at low RZT

Apart from winter hardening of Winter OSR, cold stress resistance may also play a role in spring OSR, although germination at soil temperatures as low as 5°C is possible (Luo et al., 2018). However, the adaptation to low temperatures does not exclude additional beneficial effects of Zn seed priming under cold stress conditions. Accordingly, the response of the spring OSR hybrid “SR1” was investigated under conditions of moderate cold stress with a controlled root zone temperature of 12°C.

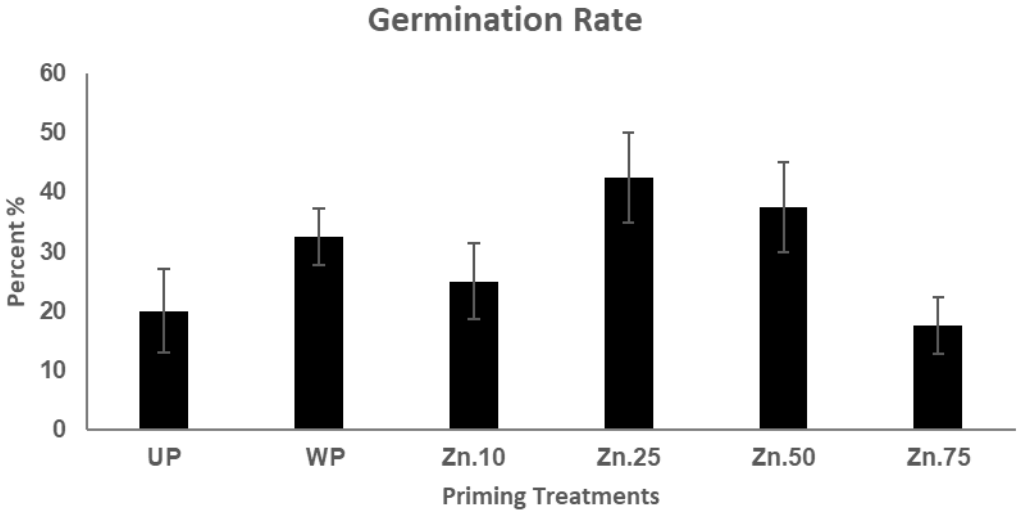

Fig 2.10: Germination rate (normal seedlings) of Spring OSR grown at 12 + 2 0C root zone temperature on a clay loam soil pH 6.6. Data represent means and SE of 4 independent replicates of each treatment.

Zn priming at concentrations between 25 and 50 mM tended to increase the germination rate (normal developed seedlings) from 20 to 40% in Spring OSR exposed to reduced soil temperature (12°C) (Fig 2.10). This was associated with a more homogenous seedling development at 5 weeks after sowing (Fig. 2.11)

The improved seedling development after Zn seed priming was reflected in significant increases in shoot biomass (up to 91%), root biomass (up to 125%) and root length (up to 106 %) (Table 2.11). A closer look on root morphology revealed beneficial effects of Zn seed priming particularly on fine root development (Fig. 2.11)

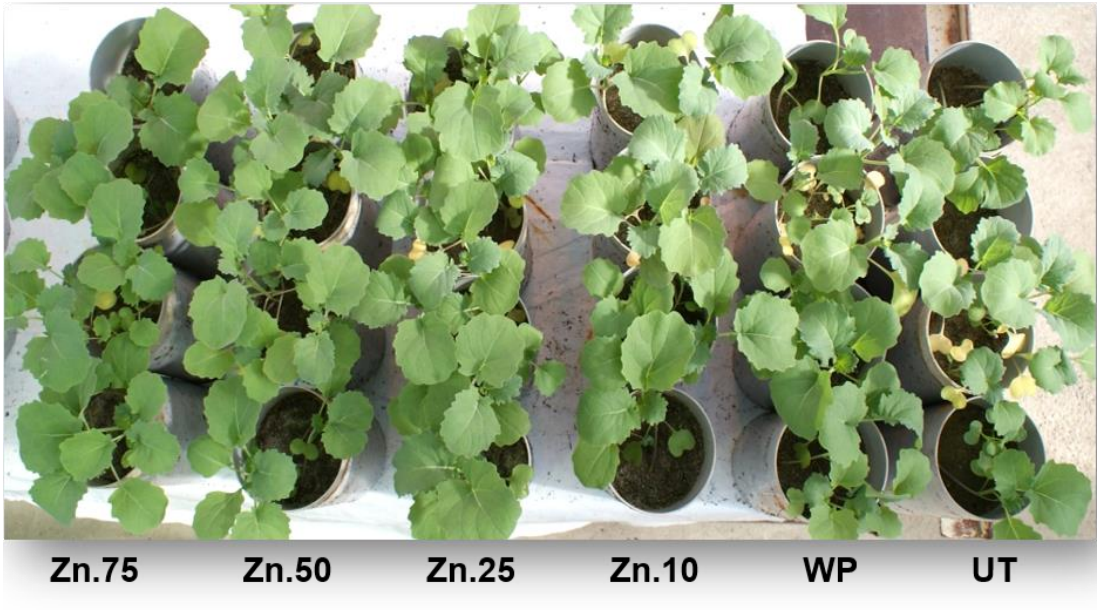

Fig 2.11: Plant development of Spring OSR grown at low RZT (12 ± 2^0C) for 5 WAS on a clay loam soil pH 6.6. UT (Untreated), WP (Water primed), Zn.10, Zn.25, Zn.50, Zn.75µM (Zn primed)

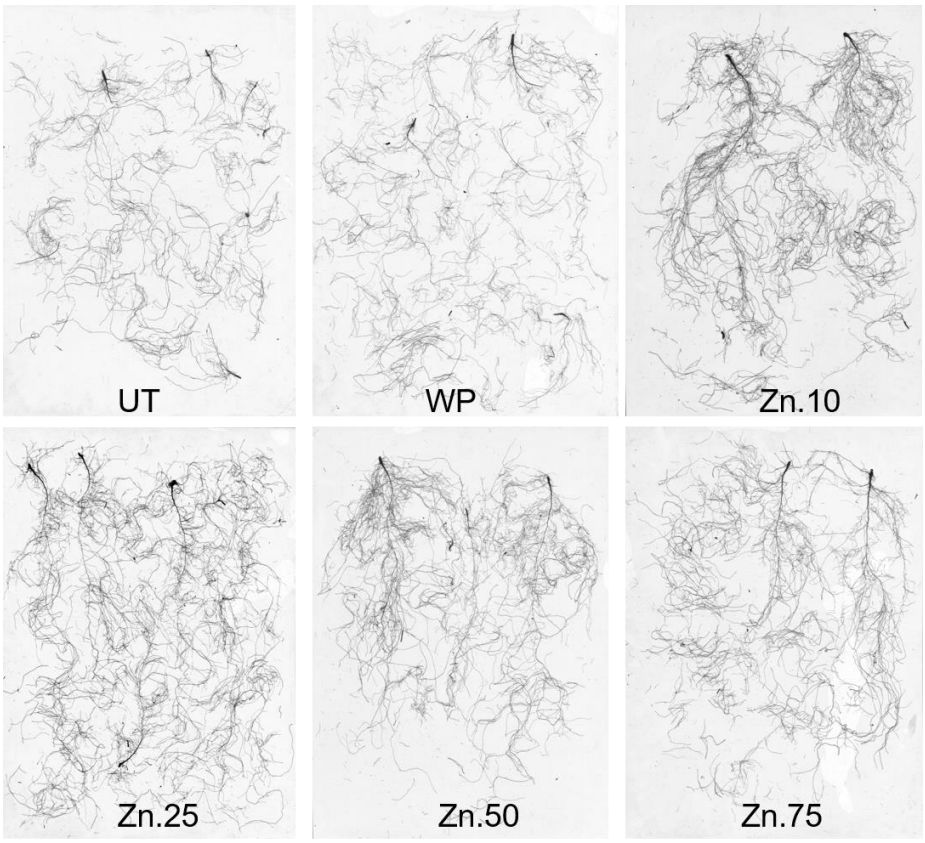

Fig 2.12: Fine root development in Spring OSR grown at low RZT (12 ± 2^0C) for 5 WAS on a clay loam soil pH 6.6. UT (Untreated), WP (Water primed), Zn.10, Zn.25, Zn.50, Zn.75µM (Zn primed).

Table 2.11: Shoot and root biomass production, and root length of Spring OSR grown under low RZT (12 + 2^0C) for 5 Weeks after nutrient seed priming on a clay loam soil pH 6.6. Data represent means and SE of 4 independent replicates of each treatment. Different characters indicate significant differences between the treatments.

	Shoot dry biomass [g plant^{1}]	**Root dry biomass [g plant^{1}]**	**Root length [cm plant^{1}]**
UT	0.12±0.02 c	0.04±0.1 cd	430.42±19.70 cd
WP	0.17±0.01b	0.06±0.00 c	634.67±32.45 bc
Zn.10	0.18±0.00 b	0.06±0.00 bc	679.44±17.00 b
Zn.25	0.20±0.01 ab	0.07±0.00 b	779.57±46.33 ab
Zn.50	0.23±0.01a	0.07±0.01 b	790.09±21.59 ab
Zn.75	0.22±0.01 a	0.09±0.00 a	890.80±34.88 a

Higher root length and fine root production induced by Zn priming translated into a generally higher shoot accumulation of sparingly soluble nutrients such as P, Fe, Zn and Mn (Table 2.12).

Table 2.12: Shoot mineral concentrations and contents of oilseed rape plant grown in a silty loam soil (pH 6.9) at the low RZT (12 ± 2^{0}C) for 5 Weeks. Data represent means and SE of 4 independent replicates of each treatment. Different characters indicate significant differences between the treatments.

	Concentrations [µgg^{-1}]				Contents [µgplant^{-1}]			
Priming Treatments	**Zn**	**Fe**	**Mn**	**P [mgg^{-1}]**	**Zn**	**Fe**	**Mn**	**P [mg plant^{-1}]**
UT	22.64±1.08 a	85.9±7.78 a	29.2±0.77 a	3.86±0.29 a	2.62±0.28 bc	9.92±1.31 b	3.41±0.41 bc	0.44±0.03 c
WP	17.09±0.74 bc	79.8±5.95 a	25.4±0.28 b	3.28±0.09 a	2.79±0.20 bc	13.07±1.41 ab	4.14±0.17 bc	0.53±0.01 c
Zn.10	16.47±0.43 bc	79.2±3.55 a	24.9±0.40 b	3.26±0.14 a	2.93±0.04 bc	14.16±0.91 ab	4.45±0.12 b	0.58±0.01 bc
Zn.25	17.16±0.38 bc	77.9±3.86 a	24.5±0.23 b	3.25±0.04 a	3.36±0.13 b	15.40±1.46 ab	4.82±0.29 ab	0.63±0.04 b
Zn.50	17.72±0.35 b	79.9±3.61 a	25.2±0.38 b	3.37±0.03 a	4.13±0.22 ab	18.8±1.87 a	5.89±0.38 a	0.78±0.04 ab
Zn.75	20.49±0.61 ab	71.5±1.49 a	24.9±0.37 b	3.77±0.17 a	4.51±0.19 a	15.82±1.04 ab	5.50±0.26 ab	0.83±0.06 a

2.5. Discussion

2.5.1. Selection of most effective micronutrients for seed priming

Oilseed rape was treated with five stress-protective micronutrients including zinc, manganese, iron, copper and boron at various concentrations (Table 2.1). The dosage range of micronutrients, which was effective in maize (Imran et al., 2013) limited OSR seedling growth and germination (Fig. 2.3), indicating the applied dose not only ineffective but even toxic resulting in negative effects on seedling growth. Excessive salts concentration in the primed media could negatively affect the seed by creating a toxicity situation for the respective micronutrient. However, in face of the applied salt concentrations in the lower mM range, direct salinity stress effects by excess osmotic potentials, water limitation etc., are unlikely in this case. However competitive effects between cationic micronutrients induced by the priming treatments as similarly reported by Imran (2015) cannot be excluded. Overproduction of ROS and lipid peroxidation are the indicators of cellular damage (Lanza Castelli et al., 2010) and may be one of the reasons which lead to poor germination and limited seedling growth. Finally, at lower application levels, out of all tested priming treatments, zinc was the only micronutrient with potential to improve seedling biomass and germination rate at 8^0C already at the lowest dose rate of 0.01 mM (Fig. 2.4, 2.5) underlining its protective functions under abiotic stress conditions (Hassan et al., 2020; Ahmed et al., 2017; Cakmak, 2000). Zinc was therefore, selected for the further model experiments in this study.

2.5.2. Utilization and nutrient demand for zinc during early plant growth

Zinc (Zn) is a vital mineral nutrient required to regulate the normal functioning of the plant physiological processes because of its role in enzymatic activity. The current study revealed a high dependency on soil zinc supply just after the seed germination. Increasing zinc contents in above-ground parts of OSR seedlings, exceeding the Zn seed reserves by 84% just 5 DAS and by approx. 375 % at 10 DAS demonstrated a high zinc demand largely dependent on root uptake already during the early stages of seedling development (Table 2.6). The effect is even larger since zinc determination in below-ground plant parts was not included in this study. During further plant development, the Zn demand of OSR seedlings is efficiently covered by root uptake

as indicated by Zn shoot concentrations in the sufficiency range (Campbell et al., 2013) also in unprimed controls (Table 2.6). However, any stress factor limiting root growth and root activity during this phase, such as temperature extremes, drought, soil compaction, waterlogging can severely affect seedling establishment with mitigation potential by Zn seed treatments (Figs.2.9; 2.11 Table 2.1).

Therefore, seed zinc enrichment through seed priming could give the plant a starter push and thus can contribute to early seedling health and a better plant stand establishment as similarly reported in many other crops, i.e., wheat, barley, chickpea (Rehman et al., 2015; Arif et al., 2007; Ajouri et al., 2004), although OSR is considered as a comparatively Zn-efficient crop. Also for Mn, which has not been introduced in this experiment, the mineral nutrient analysis similarly revealed its higher demand exceeding the seed reserves already during the first 5 DAS (Table 2.6), suggesting potentially an even stronger growth effect to the seedling by combined Zn and Mn application.

2.5.3. Seed nutrients

Different OSR hybrids showed a high variation in seed Zn reserves (Table 2.3). The varying Zn seed reserves may be the result of genetic variability of different OSR hybrids or a consequence of seed lot effects resulting from zinc-deficient growth conditions, i.e. Zn deficient soil or interrupted Zn supply due to environmental stress factors (Cakmak, 2010). In this context, Zn seed treatments may offer a perspective for supplementation of seed lots with low Zn seed reserves (Table 2.3).

Priming doses Zn.25, Zn.50, & Zn.75µM, improved the seed Zn reserves up to +17.9, +37.2 and +60.3 µg, respectively (Table 2.4). Although no reports on OSR zinc seed priming are currently available in the literature, similar improvements in zinc seed reserves in barley (Ajouri et al., 2004) and in maize (Imran et al., 2013 & 2015) have been previously discussed. The tissue-specific and intracellular localization of the primed zinc within OSR seeds is still an open question. In maize (monocots) it is now evident through DTZ (1,5-diphenylthiocarbazone) staining method that primed Zn but also natural Zn seed reserves seed are localized mainly in the aleurone layer and partially in the embryo (Imran et al., 2018) and may, therefore, available to the plants right from the beginning of germination. In cereals, the P storage metabolite“phytate”

is mainly localized in the aleurone layer and the embryo (Lin, Ockenden, & Lott, 2005; Raboy, 2000) and is also acting as an effective chelator for metal cations, such as Zn^{2+} due to six phosphate residues bound in the molecule (Raboy, 2000). This may explain also the compartmentation of primed zinc in the aleurone and embryo in cereals. Although the primed Zn distribution in OSR seeds is still unknown, the presence of phytate inside the globoids in the protein bodies of the radicle and cotyledons (Yiu et al. 1983) may indicate Zn^{2+} compartmentation. However, the majority of Zn (63%) in primed maize seeds remained on the seed coat and the plant availability of this Zn fraction during further seedling development under soil conditions still remains an open question. In contrast to seed priming (SP), in seed dressing (SD), the treated nutrients are exclusively supplied to the seed coat and are expected to become available as soon the seed starts absorbing water from the soil for germination. In this context, water diffusion kinetics could be very crucial, since the speed of water movement from the soil into the seed may also affect the amount of applied nutrients entering the seed. However, similar effects on root growth promotion (Tables 2.5; 2.7) and Zn shoot contents (Tables 2.6; 2.8) in seeds treated with SP and SD, respectively, suggest that both application strategies are largely equivalent. This has been similarly demonstrated under field conditions (Table 2.9) and also in earlier studies with maize exposed to low root zone temperatures (Imran et al., 2013; Bradacova, 2015; Bradacova et al. 2016), provided that Zn uptake into the plant or seeds can take place before the onset of the stress period. By contrast, even continuous supply of all essential nutrients, freely available in unlimited amounts in a nutrient solution experiment during the cold-stress period directly to the roots of the maize seedlings, had no protective effect, demonstrating the limitations in root uptake of protective nutrients during the stress period (Bradacova et al., 2016).

2.5.4. Germination rate

Zinc seed priming (ZnSP) improved germination rate in petri dish germination tests at low temperature (8^0C), indicating a protective effect of primed Zn (Fig 2.5). Additionally, in soil culture, the same concentrations tended to improve germination rate (normal seedling development) by 20-40% in spring OSR at low RZT (12^0 C), associated with a more homogenous seedling development as compared with untreated controls (Fig 2.10). Furthermore, seed priming (SP) has been reported to

speed up germination time up to 50% in barley (Ajouri et al., 2004). Germination improvements associated with micronutrient seed priming have been documented in several crops under growth-limiting conditions, i.e., salinity stress in mustard (Begum et al., 2014), late sowing of maize (Mahboob et al., 2015) and Stevia under drought conditions (Gorzi et al., 2018). However, appropriate nutrient composition and concentration of priming media, as well as a suitable timing of priming treatments needs to be evaluated individually under controlled conditions to attain positive seed germination and plant establishment effects prior to the field application.

2.5.5. Effect of seed zinc application on seedling growth

A continuous decline in the winter OSR root length during winter months indicated a massive die-back particularly of fine roots, further accelerated by additional stress factors, as recorded in the root window experiment under field conditions (Table 2.10) This underlines the significance of proper root establishment in winter OSR before winter with particular importance in late sowing (Table 2.9) . Therefore, ZnSP and SD treatments improving root growth in all model experiments conducted in this study, suggest a promising perspective for winter OSR in this context. At optimal plant growth temperature (23^0C) both ZnSP and ZnSD improved seedling growth particularly root growth; biomass & length during seedling establishment (Table 2.5, 2.7). Since Zn is required for the biosynthesis of tryptophan, a precursor of Indole acetic acid (IAA) as a major hormonal regulator for lateral root development, an active role of Zn in the production of IAA may be assumed (Alloway, 2004 & Brennan, 2005). Due to the very limited Zn seed reserves in OSR (Table 2.8) seed zinc supplementation via SP or SD may therefore have enhance the auxin levels during OSR seedling establishment, particularly under conditions when root-mediated Zn acquisition is not fully active during early stages of root development or under the influence of stress factors counteracting root growth and activity.

As an example, for the latter scenario, plants subjected to low RZT are affected by limited root and shoot growth caused by sub-optimal plant growth conditions. However, the detrimental low root zone temperature (RZT) effect was mitigated with ZnSP treatment and increased shoot and root biomass and root length up to 91, 125 and 106 %, respectively (Table 2.10). One of the first response to almost all plant

stresses including low temperature is the oxidative stress (Blokhina et al., 2003) which is attributed to the overproduction of reactive oxygen species (ROS), i.e., superoxide, hydrogen peroxide and the hydroxyl radical, causing membrane damage, enzyme inactivation, DNA damage and also oxidative degradation of IAA (Cakmak 2000; Allen and Ort, 2001). Zinc, being the co-factor of various enzymes involved in ROS detoxification, such as superoxide dismutases (SOD) is therefore essential for enzymatic ROS detoxification, and particularly impaired in Zn deficient plants (Cakmak & Marschner 1988; Cakmak, 2000). External Zn application through SP or SD may play a crucial role in this context by mediating an improved enzymatic ROS detoxification under abiotic stress conditions. The protective effect on oxidative auxin degradation can maintain a higher auxin level improving shoot and root elongation at low RZT (Table 2.11. Fig. 2.12) as similarly described for ZnSD treatments in maize exposed to low RZT (Bradacova et al., 2016; Moradtalab et al., 2018) and also in drought stress experiments reported in chapter 4 of this thesis.

In response to stimulated root production induced by the SP/SD treatments, the plant could improve spatial nutrient acquisition, resulting in a better shoot nutrient status, particularly for sparingly soluble available nutrients like Zn, Mn, Fe and P (Table 2.12), as similarly reported by Imran et al. (2013) and Bradacova et al. (2016) in maize. An improved nutritional status with stress-protective micronutrients may increase plant resistance to better cope with stress, as a secondary effect of Zn seed treatment. Improvement of shoot P status suggests an improved spatial acquisition of available P in the rhizosphere through enhanced root length and fine root production (Table 2.12). Since Mn is the co-factor of various enzymes involved in the biosynthesis of phenolic compounds with anti-oxidative properties, an increased shoot Mn status could additionally contribute to ROS protection under low RZT and other stress factors requiring ROS detoxification. Furthermore, Koscienly & Gulden, 2012 have correlated higher root length at 3-4 leaf stage of OSR seedling with higher economic yields.

Generally, Zn seed treatments obviously can promote seedling health and performance in different ways. Beneficial effect of micronutrient, particularly, zinc seed priming (ZnSP) is already well documented in several field crops i.e., enhanced root dry weight in rice (Prom-u-thai et al., 2012) and improved maize root and shoot growth at suboptimal root zone temperature 12 ^{0}C (Imran et al., 2013). On the other hand, Zinc seed coating improved yields in chickpea and had growth-promoting and yield

improving effects on maize, wheat and sunflower (Masuthi et al., 2009; Singh, 2007; Bradacova et al., 2016; Moradtalab et al., 2018).

In the current study, zinc application has positively contributed to the plant growth, particularly root growth, in all three experiments, regardless of the method of application and varying plant growing conditions. Furthermore, the results suggest that a very pronounced effect of zinc application can be observed under sub-optimal plant growth conditions especially, at low RZT. Out of the tested dose rates for ZnSP and ZnSD, 50 µM $ZnSO_4$ treatment concentrations appeared to be highly responsive under the given conditions. The suggested dose rates should be further tested in various plant growing conditions to evaluate their spectrum of applicability. The prospect of Mn SP or SD in combination with Zn could be tested and may further improve the beneficial effects as reported by Imran et al. (2014) and Bradacova et al. (2016) in maize.

2.5.6. Conclusion

This is the first report highlighting the high zinc demand of OSR at the initial phase of seedling development and optimizing its application dose to explore the highest possible outcome in two different plant growth scenarios for oilseed rape. Priming and dressing techniques for seed zinc enrichment offer a great diversity of application fields from on-farm use to the commercial seed industry. The physiological background of the effects at a molecular level still needs to be further elucidated. The inexpensiveness of the offered approaches suggests the possibility for the development of economically practical and viable application methods without much increasing the input costs.

Chapter 3

Zinc seed priming promotes fertilizer depot exploitation and nutrient acquisition in oilseed rape

3.1. Abstract

The present study suggests that the combination of Zn seed priming with the placement of ammonium-phosphate starter fertilizer can support the expression of plant traits beneficial for field establishment of winter rape and spring rape as well.

In a rhizobox experiment on a soil with moderate P availability, it was demonstrated that even in absence of environmental stress factors, such as temperature extremes or drought stress, Zn seed priming could improve early plant development in terms of root elongation and stimulation of seedling growth in winter rape. This improved the exploitation of an underfoot ammonium-phosphate fertilizer depot placed at a depth of 8 cm with root attracting properties. No negative side effects of depot fertilization on simultaneous rooting of deeper soil layers, important for winter hardness in comparison with homogenous application of NPK fertilizers were observed. However, at 5 weeks after sowing, the P status of the plants with homogenous NPK supply was critical, while optimum P shoot concentrations were recorded in the depot fertilizer variants. However, the largely improved P status was not associated with excessive shoot growth, known to be detrimental for winter survival.

In spring rape exposed to reduced root zone temperatures (12°C) to simulate low temperature stress in spring, the combination of Zn seed priming with ammonium-phosphate depot fertilization stimulated shoot biomass production by up to 30%. Plants with homogenous nutrient supply were severely P deficient, while the P status was optimal in the depot variants. Since no root growth effects were detectable in this case, it is concluded that Zn priming promoted root activity and root-induced P acquisition via the well-documented ammonium-induced rhizosphere acidification. The improved nutrient status supported seedling establishment of spring wheat exposed to low root zone temperatures as frequently recorded in spring.

3.2. Introduction

Brassica napus L., commonly known as Rape seed is widely cultivated for its oil importance (Melut et al., 2012) and for human as well as animal consumption (Downey et al., 1974). Rape seed is produced in approx. 62.4 million tons (Mt) around the globe, while the total production area is reported to be 33.6 million hectares (FAOSTAT, 2011). Winter rape is a major cultivar in cold areas like central Europe (Lääniste et al. 2007), having long vegetation periods and comparatively gives double yield than spring rape (Butruille et al., 1999). Being a non-mycorrhizal fungal association, the plant is highly dependent on a well-developed fine roots network and root hairs along with the nutrient mobilizing chemical strategies which fulfils the requirement for adequate nutrient supply (Föhse et al., 1991, Hoffland et al., 1989; 1992). The root system of rape seed reaches 1.5- 2.4m down the soil layers and take up water and nutrients (Peltonen et al., 2011).

3.2.1. Fertilization Strategies

Nitrogen (N) and phosphate (P) fertilizers are among the most critical inputs in crop production systems, with the potential to increase agricultural productivity by up to twenty folds (Brennan et al., 2014). Adapted nitrogen fertilization represents an important component to control field establishment of rape seed and plays a significant role in determining plant size, shoot branching, inflorescence formation, fruit and seed setting and protein contents (Grant et al., 2011). Insufficient N and P supply during vegetative growth in autumn or spring could severely limit the establishment of rapeseed (Sieling and Kage, 2010; Bauer 2011). Shoot growth regulation is also crucial for winter tolerance and regeneration capacity of rapeseed after winter. In this context, certain fungicides, especially the ones used against Phoma and Sclerotinia disease, inhibit shoot growth by interacting with gibberellic acid (GA) metabolism, are used for the regulation of shoot growth (Graf and Krueger, 2009).

Nitrogen and P fertilization strategies are not only determining shoot development but can be also employed to modulate development and morphology of the root system. The most suitable mineral nutrients to be adopted in this context are ammonium and phosphate, having root attracting properties. Low mobility of these nutrients in the soil allows control of root growth and development by fertilizer placement strategies.

(Sommer et al., 1993; Bauer, 2011; Bischoff, 2012). Among the fertilizer placement approaches, underfoot placement (4-6cm deep) of diammonium-phosphate improves rooting and efficient nutrient uptake at the topsoil layer during early plant growth while deep fertilizer placement (14-17cm deep) encourages deep rooting promoting winter hardness and drought resistance (Fig.3.1, Bauer, 2011). Accordingly, proper root development during seedling establishment is predictive for greater seed yields in OSR (Koscielny & Gulden, 2012).

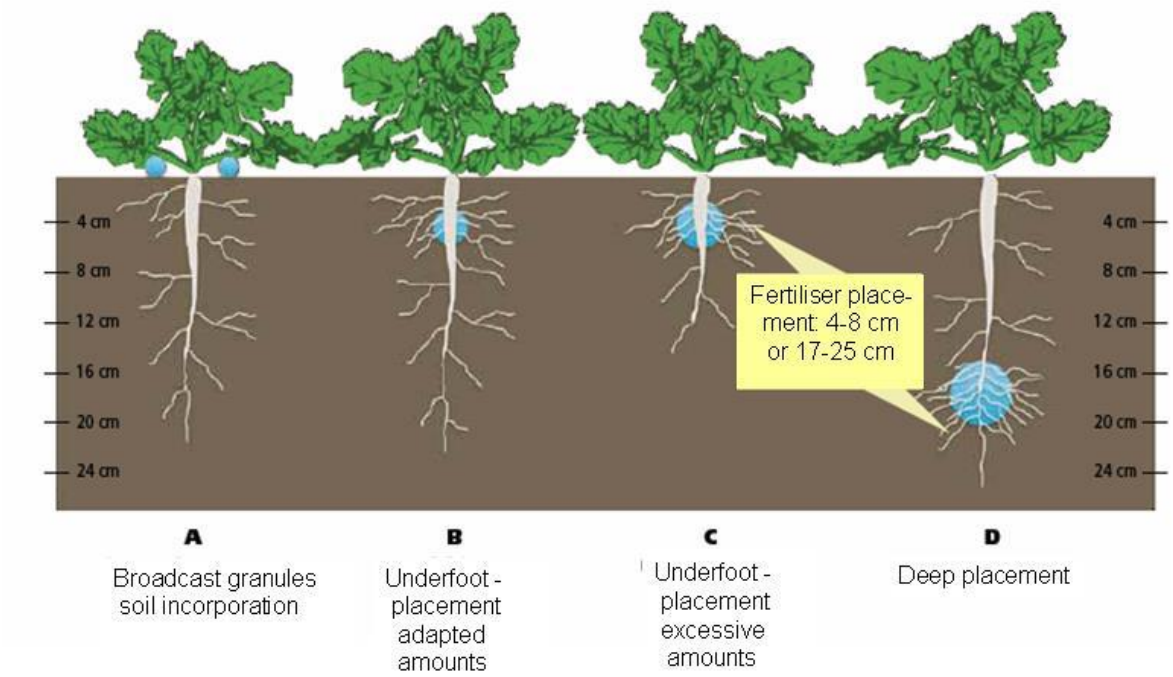

Fig. 3.1: Oilseed rape response to N/P fertilizer depot (adapted from Bauer, 2011)

However, apart from the importance for the acquisition of water and nutrients, root systems also have important functions in the regulation of shoot development. In addition to GA, hormonal control of shoot growth is mediated by cytokinins (CKs), a group of phytohormones preferentially produced in the roots and transported to the shoot, where they stimulate shoot branching, cell division and expansion. In this context, nitrogen (N) is an important factor in regulating the biosynthesis and transport of CKs from the roots to shoot and therefore shoot growth as well (Walch-Liu et al. 2000; Rahaju et al. 2005). Nitrate (NO_3^-) is the most important N-form for cytokinin mediated growth and development of shoots. In contrast to ammonium-N, the presence of nitrate in the growth medium induces the expression of IPT genes in the root tissue encoding isopentenyl transferases as key enzymes for the biosynthesis of

cytokinins. (Miyawaki et al., 2004), Similar shoot growth-promoting effects associated with increased shoot cytokinin supply have been related with phosphate nutrition, and P deficiency (similar to N deficiency) is usually associated with inhibition of shoot growth and a declining cytokinin status of the shoot tissue (Martin et al., 2000). Lateral shoot outgrowth in tomato and tillering in barley was enhanced with an increasing nitrate to ammonium/urea ratio in the growing media (Bauer, 2004; Römheld et al., 2008). In tomato culture, when nitrate was replaced by ammonium, the cytokinin xylem transport to the shoots substantially declined already 2h after ammonium application and reduced the leaf expansion rate to 50% after 6h (Rahayu et al., 2005).

This raises the question whether Ammonium and phosphate applied as a stabilized depot fertilization strategy could be employed to regulate both, shoot growth and root development in a way to optimize field establishment of rapeseed depending on the culture system (e.g. winter rape vs spring rape culture).

3.2.2. Nutrient Seed Priming

Even after advancement in fertilization strategies soil and climate conditions, e.g., temperature extremes and drought stress, are widespread stress factors for early growth and germination of plants like winter and spring rape despite having adaptations for temperate climates (Kremer, 2011; Bollermann, 2011). Many recent studies have reported the potential benefits of Zn availability supplied at the seedling stage through nutrient seed priming and fertigation for alleviating problems like cold stress during early growth and germination of maize (Asim, 2012; Imran et al., 2013; Bradacova et al, 2016; Moradtalab et al., 2018).

In this context, nutrient seed priming is a seed enhancement technique which potentially improves seed nutrient contents. In nutrient seed priming seeds after being soaked in nutrient solutions for a particular time are dried back to their original moisture content. It enhances the speed and uniformity of seed germination as well as seedling growth (Copeland and McDonald, 2002; Butler et al., 2009). Improved seed performance and early seedling growth have been reported in wheat, rice and maize (Tabassum et al., 2017; Hussain et al., 2017; Imran et al., 2013). Basra et al., 2003 and Abdolahi et al., 2012 also reported beneficial effects of different seed priming techniques on oilseed rape. Furthermore, root stimulating properties of Zn priming

reported for different plant species, including rape seed (Neumann et al. 2014; Bradacova et al., 2016; Moradtalab et al. 2018) make it a suitable tool also for the current study. This study investigated the perspectives of adapting seed enhancement and fertilization strategies, i.e. nutrient seed priming as zinc priming combined with the localized application of stabilized ammonium as N form close to the seeds, to stimulate early root development, to exploit the fertilizer depot, and regulate shoot growth in spring and winter rape plants. For winter rape, improved nutrient acquisition (particularly N and P) by stimulation of root development was expected, also contributing to winter hardness and at the same time avoiding excessive shoot growth induced by ammonium dominance of the N nutrition. Particularly improved P supply as a critical nutrient under low root zone temperatures and stimulatory effects on root growth were expected to stimulate seedling establishment in spring rape, exposed to suboptimal soil temperatures.

3.3 Materials and Methods

3.3.1. Test plants

Two-hybrid lines of oilseed rape (Brassica napus, L), i.e., winter OSR (cv WR1) and spring OSR (cv SR1) provided by Nord Deutsche Pflanzenzucht Hans-Georg Lembke KG, Holtsee, Germany. Seeds were stored at 2^0 C and sorted for homogeneous seed size before use.

3.3.2. Seed Priming

Nutrient seed priming is a technique in which seeds are soaked in a specific nutrient solution for a specific time and dried back to the initial condition. For optimization of zinc priming nutrient concentration, a range of Zinc sulfate ($ZnSO_4.7H_2O$) dose rates 25µM, 50µM and 75µM were primed for a time duration of 24 hours (predetermined in pilot experiments). Forty (40) ml of respective $ZnSO_4.7H_2O$ solutions were used to soak one gram of seeds at room temperature for each treatment. Thereafter, seeds were rinsed in distilled water for one minute to clean the seed surface from any adhered priming solution. After air drying, the seeds were stored at 2^oC for further use.

3.3.3. Plant Culture

Rhizoboxes with a size of (35 cm×10 cm×2 cm) containing 500 g soil were used for the observation of root growth and development. Rhizoboxes are specialized rectangular or square-shaped PVC boxes equipped with transparent root observation windows, which are customized to measure root development and rhizosphere chemistry non-destructively in soil-grown plants (Neuman *et al*., 2009). The experiments were conducted under greenhouse conditions at two different temperature regimes. The average green-house temperature was 18^{o}C during the experiments (Fig. 3.2). Additionally, illumination was applied with a light intensity of 250 and 400 mol m^{-2} s^{-1} PAR for 16/8 hours day/night and relative humidity ranged between 60-80%. Winter and spring OSR were cultivated for 5 and 7 weeks, respectively. A controlled cooling system (Frigmix 1497, Thermomix 1480) was connected with the water bath to keep the temperature constant throughout the growth period (Fig. 3.3).

3.3.4. Soil Preparation and Fertilizer Placement

A sandy loam soil (pH 6.0), with Potassium (K), Magnesium (Mg) and Phosphorus (P) at concentrations of 27, 17 and 2 mg Kg^{-1} (CAL extractable fraction), was used for the experiments. Nitrogen was added as Ca $(NO_3)_2$ for homogeneous application (BC) and $(NH_4)_3PO_4$ in fertilizer depot treatments both at the rate of 100 mg N kg^{-1} soil. Additionally, fertilizers with the following concentrations were added to the soil; 50 mg P kg^{-1} soil as $Ca(H_2PO_4)_2$ (in the nitrate variant only) and 80 mg K kg^{-1} soil as KCl. Initially, five seeds were sown at a depth of 2 cm and later thinned to two plants for further cultivation. In order to prepare a fertilizer depot, 235 mg of $(NH_4)_3PO_4$ was mixed with 20 g of soil from a depth, 8cm below the seed. 50 µL of 10 times diluted nitrification inhibitor (3,4 dimethylpyrazole phosphate (DMPP, provided by BASF, Ludwigshafen, Germany)) was added to the depot soil mixture at a rate of 10 µL 100 mg^{-1} of Nitrogen, according to the recommendations of the manufacturer. The observation window was covered with black plastic foil in order to prevent light exposure. The rhizoboxes were placed in a wooden stand at an angle of 45°, with the observation window facing downwards to promote root growth along the observation

plane. A soil moisture content of 18% was adjusted gravimetrically, by watering the plants through back slits of rhizoboxes daily.

Table 3.1: Various priming treatments, fertilization and growing temperatures used for the experiments for both OSR hybrids.

	Treatments	Nitrogen Source	Root Zone Temperature
SR1	Homogeneous N/P (Unprimed)	Ca $(No_3)_2$	12 ± 2^0C
Localized N/P	Control (Unprimed) (UP)	$(NH4)_3PO_4$+ DMPP(NI)	12 ± 2^0C
	Water primed (WP)	$(NH4)_3PO_4$+ DMPP(NI)	12 ± 2^0C
	Zn 25 µM primed (25µM)	$(NH4)_3PO_4$+ DMPP(NI)	12 ± 2^0C
	Zn 50 µM primed (50µM)	$(NH4)_3PO_4$+ DMPP(NI)	12 ± 2^0C
	Zn 75 µM primed (75µM)	$(NH4)_3PO_4$+ DMPP(NI)	12 ± 2^0C
WR1	Homogeneous N/P (Unprimed)	Ca $(No_3)_2$	Ambient
Localized N/P	Control (Unprimed) (UP)	$(NH4)_3PO_4$+ DMPP(NI)	Ambient
	Water primed (WP)	$(NH4)_3PO_4$+ DMPP(NI)	Ambient
	Zn 25 µM primed (25µM)	$(NH4)_3PO_4$+ DMPP(NI)	Ambient
	Zn 50 µM primed (50µM)	$(NH4)_3PO_4$+ DMPP(NI)	Ambient
	Zn 75 µM primed (75µM)	$(NH4)_3PO_4$+ DMPP(NI)	Ambient

Initially, five seeds were sown at a depth of 2 cm and later thinned to two plants for further cultivation. The observation window was covered with black plastic foil in order to prevent light exposure. The rhizoboxes were placed in a wooden stand at an angle of 45°, with the observation window facing downwards to promote root growth along the observation plane. A soil moisture content of 18% was adjusted gravimetrically, by watering the plants through back slits of rhizoboxes daily.

3.3.5. Temperature Regimes

Winter OSR (cv WR1) was grown at an average ambient green-house day temperature of 18^{o}C. A maximum temperature of 25^{o}C at day-time and a minimum of 9^{o}C at night was recorded during the experiments (Fig. 3.2) comparable with temperature fluctuations during sowing time of winter rape under field conditions in temperate climates. Spring OSR (cv SR1) was grown in greenhouse culture with reduced root zone temperature (12°C RZT) after placing the rhizoboxes sealed in plastic bags into a temperature-adjusted water bath (Fig. 3.3) to simulate low soil temperatures during spring.

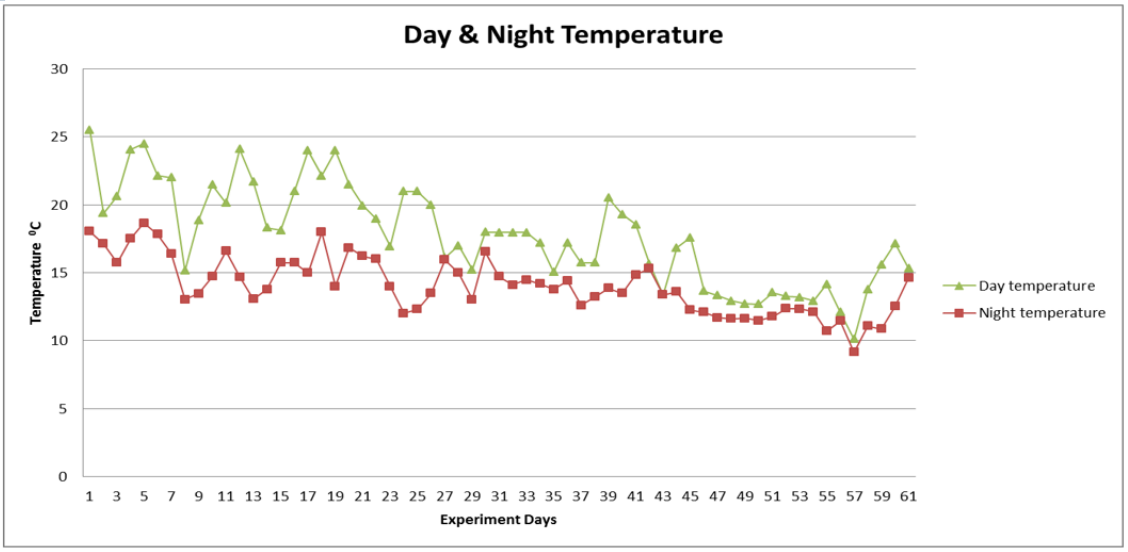

Fig. 3.2: Temperature regime during greenhouse culture.

3.3.6. Plant Analysis

3.3.6.1. Plant Biomass

At the final harvest, root and shoot fresh biomass was noted. Roots were preserved in 30% ethanol for further root length evaluation. Depot root biomass was taken from 8-14 cm from the soil surface for both hybrids. After drying at 65°C root and shoot dry biomass was recorded.

3.3.6.2. Root Analysis

A video microscope (Stemi 200-C, Zeiss. Oberkochen Germany) and the Zeiss Axiovision 3.4.1 software (Zeiss, Oberkochen, Germany) were used to digitize and analyze the root hair development. For root analysis, rhizoboxes were sectioned into three compartments; from surface of the soil to 8cm, from 8 to 14cm and from 14cm to the bottom of the rhizobox. Nine photos were taken from each rhizobox weekly,

Fig. 3.3: Thermostated water bath incubator for rhizobox culture.

three from each divided compartment, average root hair length and density of analysed photos was presented. Roots were sketched on a transparent foil weekly for non-destructive monitoring of root length development along with the root observation windows. Root sketches and previously stored roots were scanned with an EPSON Perfection V700 PHOTO dual-lens scanner (Epson, Nagano-ken, Japan) and then evaluated with WinRhizo software V22009c (Regent Instruments, Quebec, Canada).

3.3.6.3. SPAD Measurement and Plant Height

SPAD-Meter 502 Plus (Konica Minolta, Osaka, Japan) was used to determine the leaf chlorophyll content by measuring green values (SPAD) of cv WR1 and cv SR1 at 5 and 7 WAS, respectively. At 1, 2, 3 and 4 weeks after sowing, plant height was also recorded.

3.3.6.4. Mineral Nutrient Analysis

After drying, the shoot material was ground to a fine powder (Labor Scheibenschwingmühle TS-100A, Sieb Technik GmbH, Mühlheim-Ruhr, Germany). Dry plant material (250 mg) was ashed at 500°C for 5 hours using a muffle furnace. After cooling, 1 ml of 3.4 M HNO_3 was used to extract the samples twice and

evaporated completely to precipitate silicates (SiO_2). 1 ml of 4 M HCL was used to dissolve the ash, which was later diluted with ten times hot deionized water followed by two minutes of boiling converting meta- and pyrophosphates to orthophosphates. The final volume was brought to 25ml. 0.1mL of Cs/La buffer was added to each 4.9mL of ash solution and Zn, Fe & Mn concentrations were calculated using atomic absorption spectrometry (UNICAM 939, Offenbach/Main, Germany). Molybdate vanadate colour reagent was added to the solution and orthophosphate was determined by spectrophotometry as described by Gericke & Kurmis (1952).

3.3.6.5. Statistical Evaluation

This experiment was carried out with a completely randomized design with four replications. One-way analysis of variance ($p < 0.050$) was carried out to determine significant differences using the Sigma Plot Stat version 3.1 software (SYSTAT Software Inc., Erkrath, Germany).

3.4. Results

3.4.1. Seed Zn status

Zinc seed priming increased Zn seed contents and concentrations significantly at all applied concentration levels in both genotypes. However, the effects were most expressed in winter oilseed rape (cv WR1) with a 3-4-fold increase, while in spring oilseed rape (cv SR1) Zn levels increased up to 2-fold (Table 3.2).

Table. 3.2: Seed zinc concentrations and contents after nutrient seed priming. Data represent Means and SE of 4 replicates. Different characters indicate significant differences between treatments. Cont (unprimed control), WP (Water primed), 25, 50, 75µM ($ZnSO_4$ primed).

	WR1		SR1	
Treatments	**Concentration [µg g^{-1}]**	**Contents [µg $seed^{-1}$]**	**Concentration [µg g^{-1}]**	**Contents [µg $seed^{-1}$]**
UT	35.26 ± 0.61d	0.19 ± 0.00d	50.40 ± 1.09d	0.24 ± 0.01d
WP	38.14 ± 0.39d	0.21 ± 0.01d	52.83 ± 2.67d	0.25 ± 0.01d
25 µM	55.26 ± 0.49c	0.30 ± 0.01c	68.34 ± 0.52c	0.32 ± 0.00c
50 µM	113.17 ± 0.45b	0.62 ± 0.01b	98.19 ± 2.09b	0.46 ± 0.01b
75 µM	133.11 ± 2.70a	0.73 ± 0.01a	122.69 ± 0.76a	0.58 ± 0.00a

3.4.2. Winter rape – ambient temperature

3.4.2.1. Plant habitus and shoot growth

At five weeks after sowing, no visible differences in aboveground plant development were detectable. In all treatments rooting occurred over the full length of the rhizoboxes with clear root attracting effects in the depot zones (Fig.3.4).

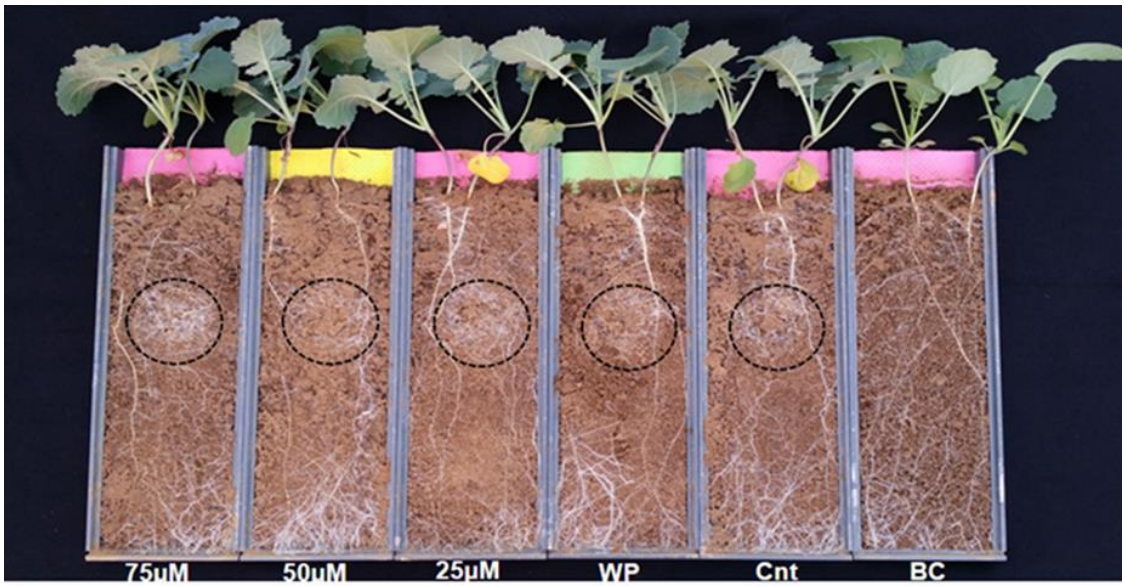

Fig. 3.4: Shoot and root development of winter oilseed rape (cv. WR1) at 5 weeks after sowing (WAS) grown in rhizoboxes at greenhouse temperature. BC: (NO_3 homogeneous), Cnt (unprimed control, NH_4 depot), Seed priming variants (NH_4 depot): WP (Water primed), 25, 50, 75µM (Zn primed).

No significant treatment effects on shoot dry matter were recorded at five weeks after sowing, although depot fertilization showed a trend for higher values than homogeneous fertilizer supply (Fig. 3.5).

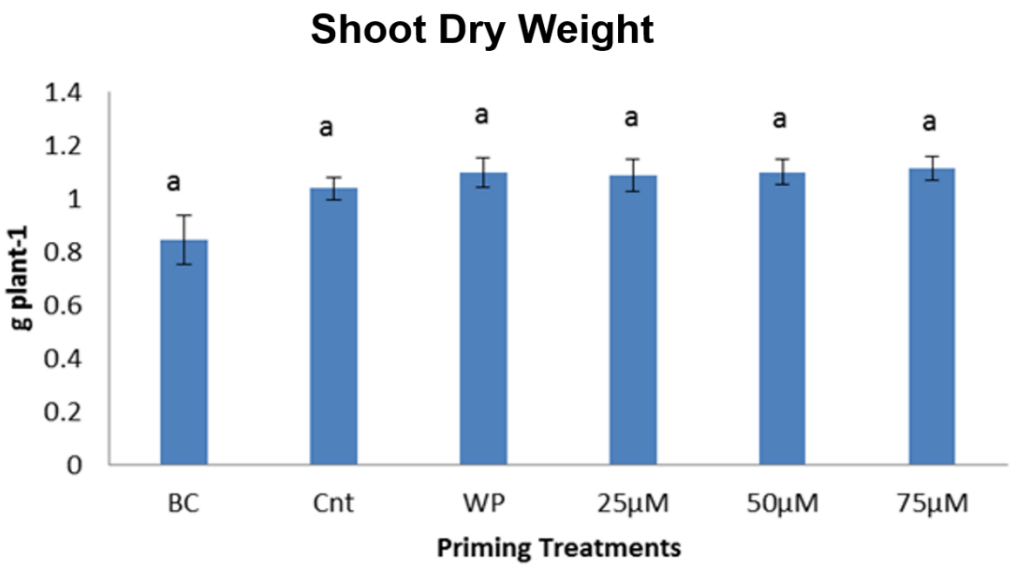

Fig. 3.5: Shoot dry weight of 5 weeks old winter oilseed rape (cv. WR1) grown in rhizoboxes at ambient greenhouse temperature. Data represent means and SE of 4 replicates. BC (NO3 homogeneous), BC: (NO_3 homogeneous; Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP (Water primed), 25, 50, 75µM (Zn primed).

However, depot fertilization and particularly seed priming increased plant height during two weeks after sowing with declining effects in later stages of plant development. No significant treatment differences were recorded at the end of the five weeks culture period (Fig. 3.6).

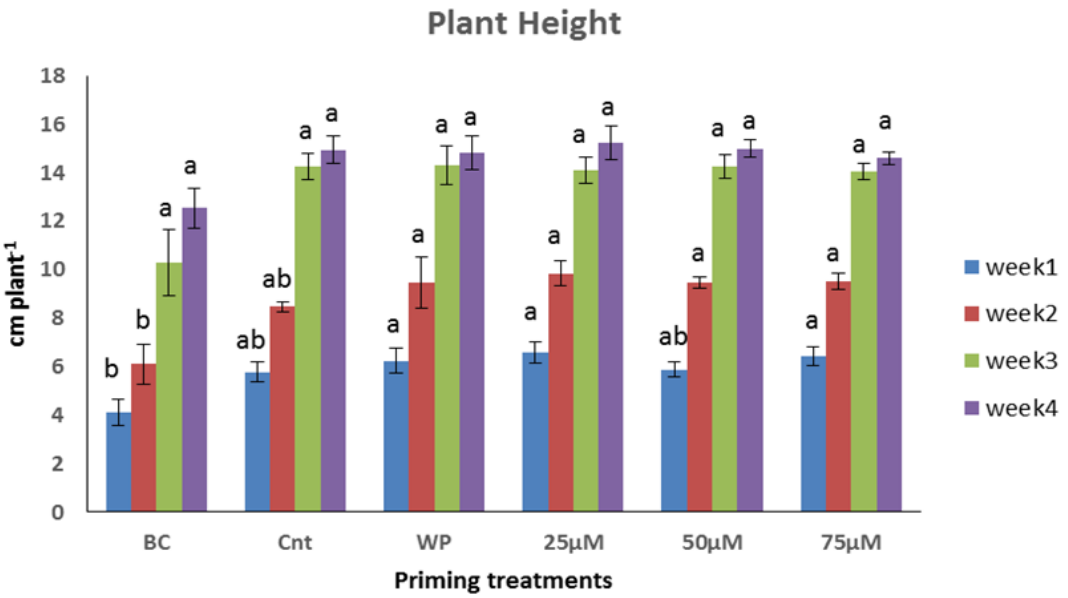

Fig. 3.6: Plant height of winter oilseed rape (cv. WR1) during 1 – 4 weeks after sowing grown in rhizoboxes. Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments. BC: (NO_3 homogeneous, Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

4.4.2.2. Root development

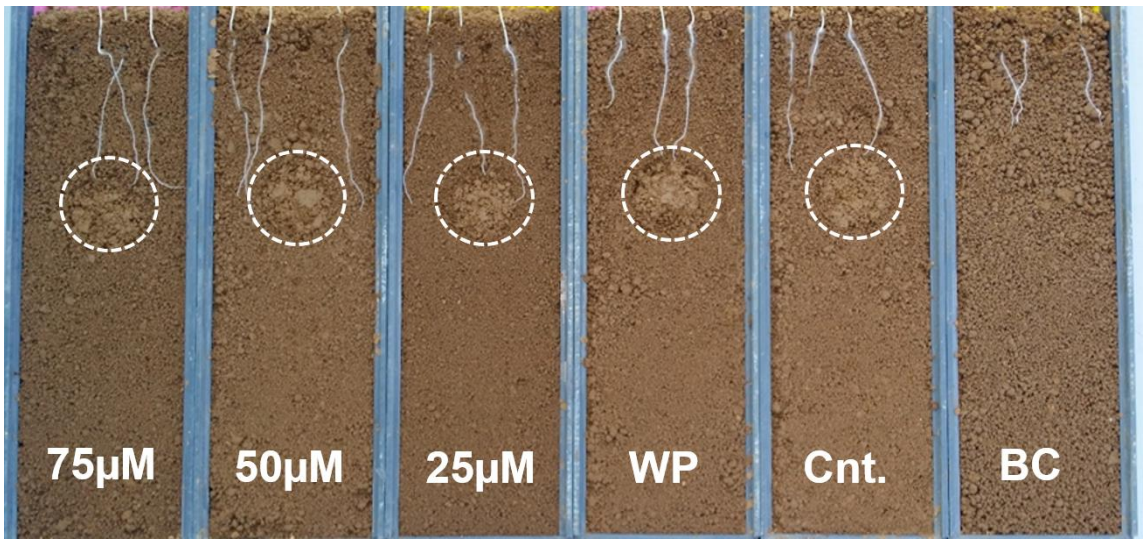

Fig. 3.7: Root development of winter oil seed rape (cv. WR1) along the root observation window of rhizoboxes at 5 days after sowing. BC: (NO_3 homogeneous), Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

Depot fertilization and particularly Zn priming stimulated root elongation during five days after sowing (Fig. 3.7). Accordingly, Zn-primed plants reached the fertilizer depot zone earlier than the unprimed plants

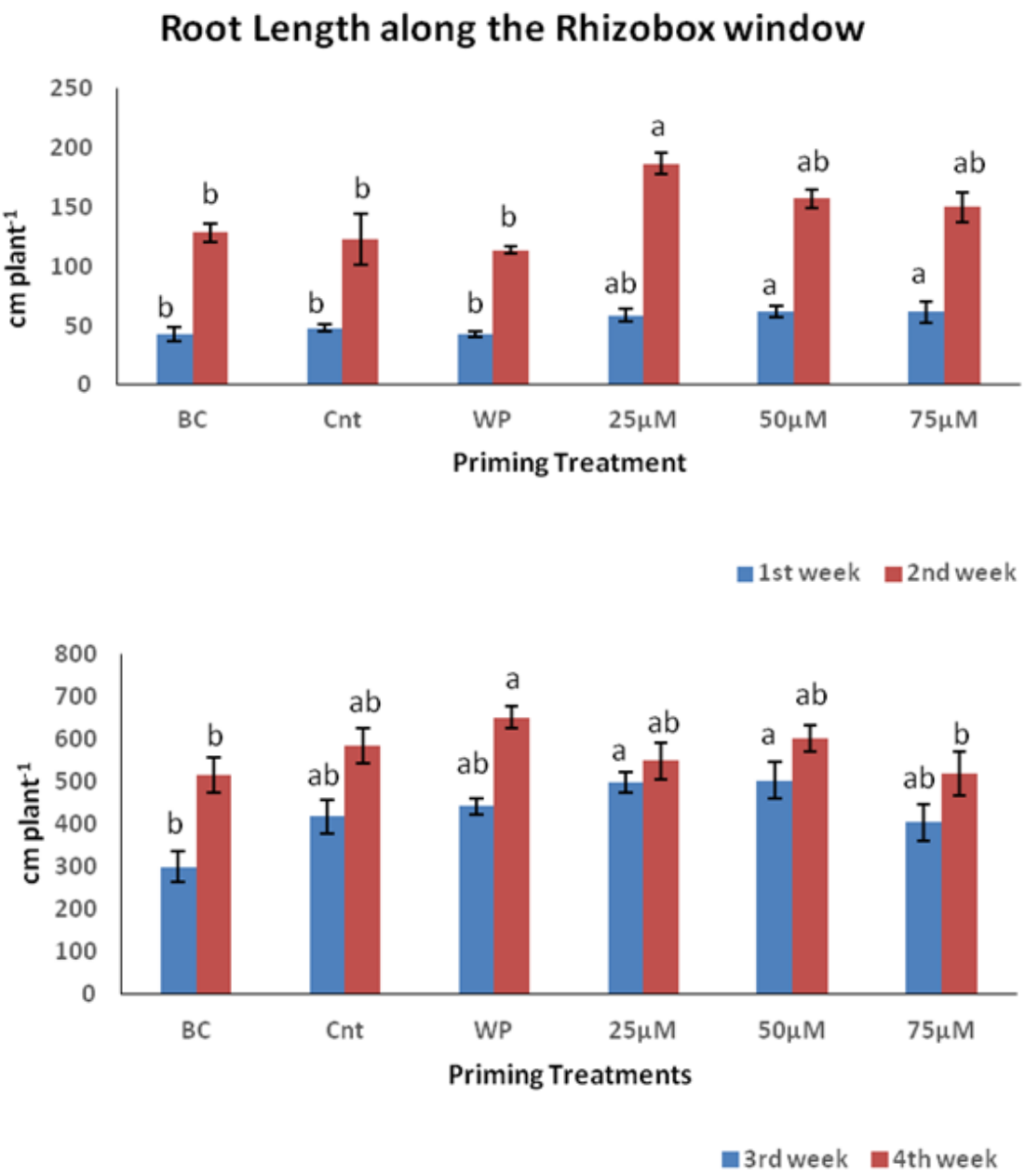

Fig. 3.8: Root length development of winter oilseed rape (cv. WR1) along the root observation window of rhizoboxes during 1 - 4 weeks after sowing (WAS) Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments. BC: (NO_3 homogeneous, Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

Root growth stimulation along the root observation window induced by fertilizer placement and Zn seed priming proceeded during three weeks of seedling development but the treatment differences disappeared until 4 weeks after sowing (Fig. 3.8).

Accordingly, also total root length and total root biomass, recorded after the excavation of the whole root systems at final harvest at five weeks after sowing, did not show significant treatment differences.

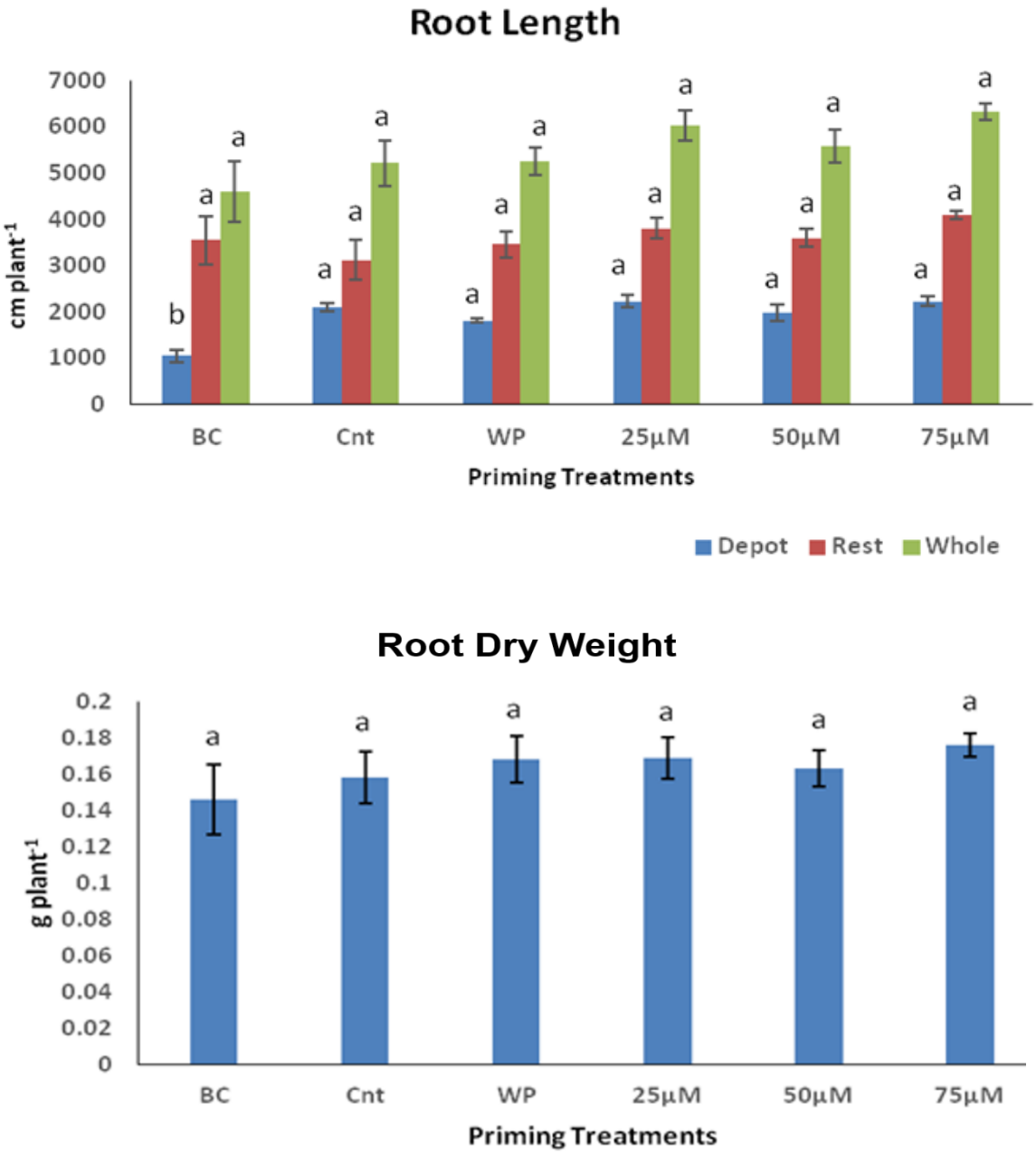

Fig. 3.9: Root length development inside and outside of the depot zone, and total root biomass of winter oilseed rape (cv. WR1) at five weeks after sowing. Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments. BC: (NO_3 homogeneous), Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

However, fertilizer placement promoted root length development in the depot zone, without additional effects by Zn seed priming (Fig. 3.9). The stimulatory effect of

fertilizer placement on root growth in the depot zone did not affect root development in the remaining parts of the rhizobox and also deep rooting in the lower parts of the culture vessels was not influenced (Fig. 3.9).

At the end of the five weeks culture period a certain stimulatory effect (approx. 25%) of the Zn seed priming treatments was recorded for root hair density (Fig. 3.10), while no significant effects on average root hair length ranging between 1.8 and 2.2 mm where recorded (data not shown).

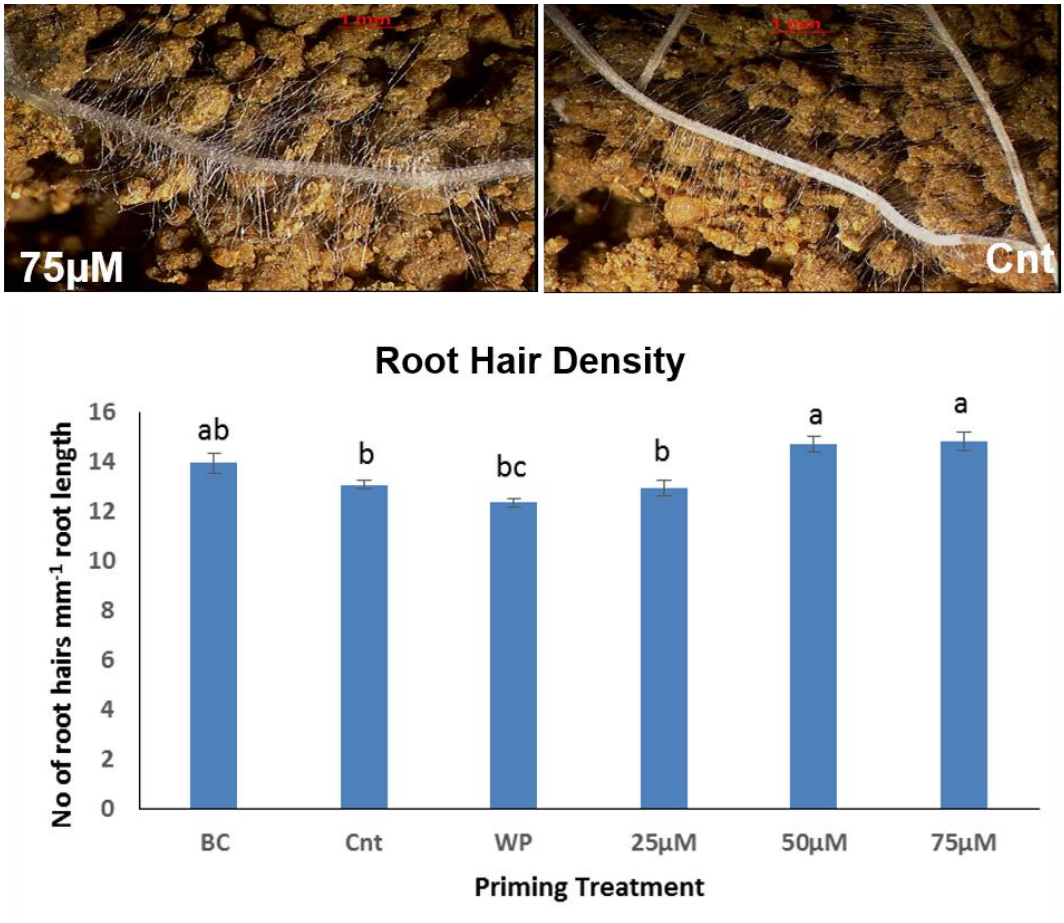

Fig. 3.10: Root hair density winter oilseed rape (cv WR1) at five weeks after sowing. Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments. BC: (NO_3 homogeneous, Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

3.4.2.3. Plant nutritional status

Chlorophyll content as indicator for the N and micronutrient status, measured by SPAD recordings in young and fully developed leaves at final harvest at five weeks after sowing revealed no significant treatment differences (Fig.3.11) and no chlorosis or deficiency symptoms were detectable.

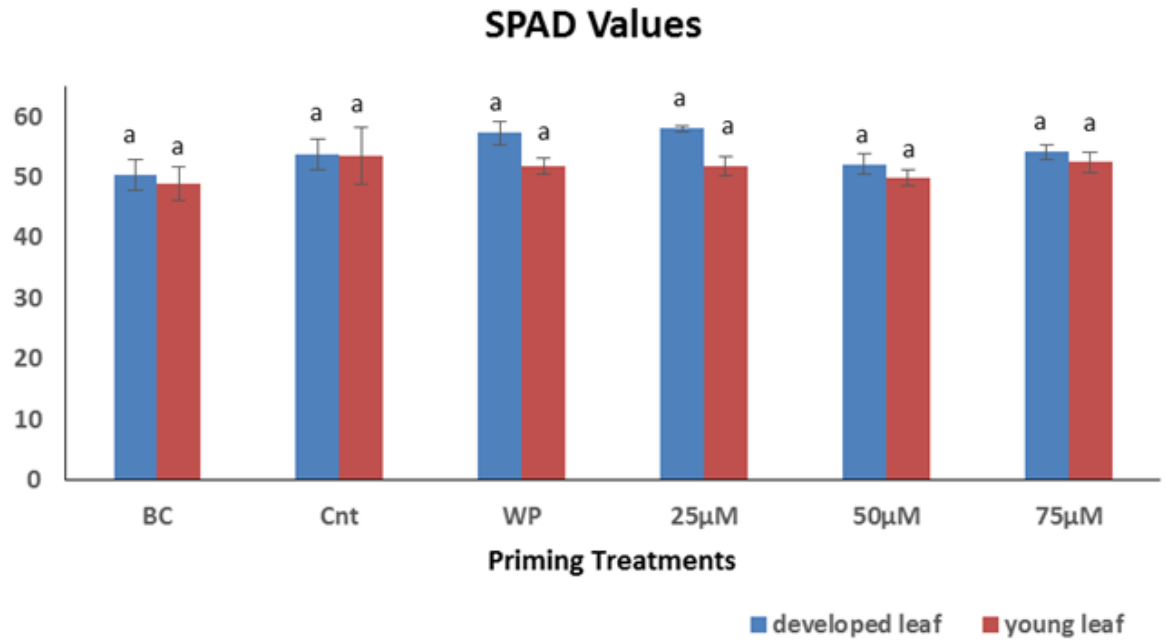

Fig. 3.11: SPAD values of fully developed and young leaves in winter oilseed rape (cv. WR1) at 5 weeks after sowing. Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments. BC: (NO_3 homogeneous, Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

This was confirmed also by mineral nutrient analysis of the shoot tissue with micronutrient (Zn, Mn, Fe) concentrations above the critical deficiency thresholds (Campbell 2013) and no differences between the fertilizer treatments, However, the P nutritional status in the treatment with homogeneous nitrate fertilization (BC) with 3.2 mg P g shoot DM^{-1} was critical but optimum levels were recorded in the treatments with depot fertilization without additional effects by Zn seed priming (Table 3.3).

Table 3.3: Shoot mineral nutrient concentrations and contents after 5 weeks old winter oilseed rape (cv. WR1) grown in rhizoboxes at greenhouse temperature. Data represent Means and SE of 4 replicates. Different characters indicate significant differences between treatments. BC: (NO_3 homogeneous, Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

	Concentration [µg g^{-1}]				**Contents [µg $plant^{-1}$]**			
Treatments	**Zn**	**Mn**	**Fe**	**P [mg g^{-1}]**	**Zn**	**Mn**	**Fe**	**P [mg $plant^{-1}$]**
BC	47±3.0 a	132±5 a	93±9 a	3.2±0.1 b	21±2.3 a	55±6.1 a	38±3.1 a	1.37±0.11 b
Cnt	39±0.6 a	105±4 b	88±7a	8.9±0.3 a	22±1.0 a	54±1.2 a	45±2.0 a	4.60±0.01 a
WP	38±1.9 a	116±7ab	91±9ab	7.6±0.5 a	21±1.5 a	63±5.6 a	49±2.7 a	4.10±0.07 a
25 µM	39±2.5 a	107±1 b	88±8 a	8.0±0.4 a	21±2.0 a	58±3.6 a	48±6.3 a	4.30±0.24 a
50 µM	40±4.4 a	112±2ab	85±6 a	8.1±0.2 a	20±3.8 a	61±3.3 a	46±2.1 a	4.47±0.22 a
75 µM	44±3.9 a	109±4 b	81±3 a	8.5±0.2 a	20±3.2 a	60±2.6 a	45±1.3 a	4.74±0.09 a

3.4.3. Spring rape (cv SR1) – reduced root-zone temperature (12°C)

3.4.3.1. Plant habitus and shoot growth

At seven-weeks after sowing, a stimulation of shoot growth induced by depot fertilization and particularly Zn seed priming was recorded in spring rape (Fig. 3.12 and 3.13).

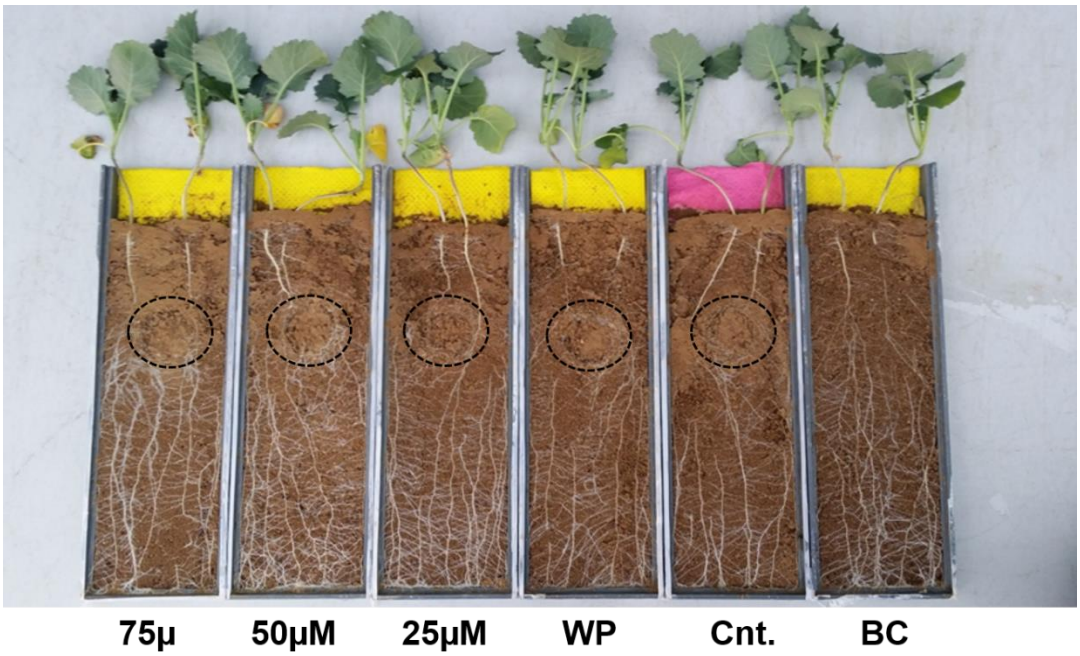

Fig. 3.12: Shoot and root development of winter oilseed rape (cv. SR1) at 7 weeks after sowing (WAS) grown in rhizoboxes at reduced root zone temperature 12^0C. BC: (NO_3 homogeneous), Cnt (unprimed control, NH_4 depot), Seed priming variants (NH_4 depot): WP (Water primed), 25, 50, 75µM (Zn primed).

Shoot dry weight (SDW) was increased by 24 and 34%, respectively in the zinc priming treatment compared to water priming in depot fertilization and the unprimed variant with homogeneous (BC) fertilization (Fig 3.13). Accordingly, plant height in depot fertilization and zinc seed priming treatments (25, 50, 75µM) was increased during 2^{nd} 3^{rd} and 4^{th} WAS, (Fig 3.14). However, no further improvements at the later plant growth stage till 7^{th} week was found (data not shown).

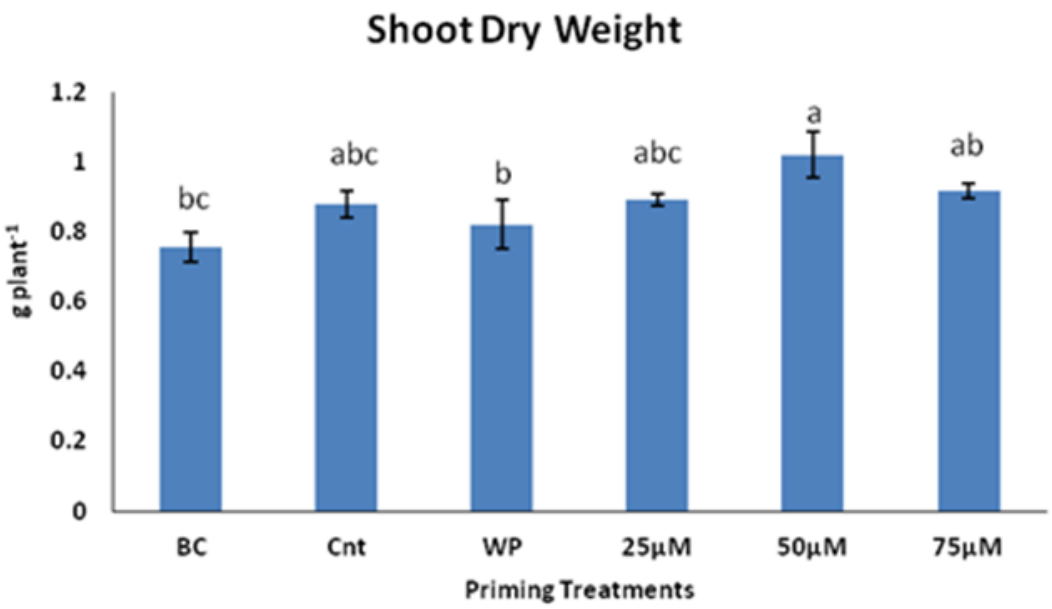

Fig. 3.13: Shoot dry weight of Spring oil seed rape (cv. SR1) at 7 weeks after sowing (WAS) grown in rhizoboxes at reduced root zone temperature 12^{0}C. BC: (NO_3 homogeneous), Cnt (unprimed control, NH_4 depot), Seed priming variants (NH_4 depot): WP (Water primed), 25, 50, 75µM (Zn primed).

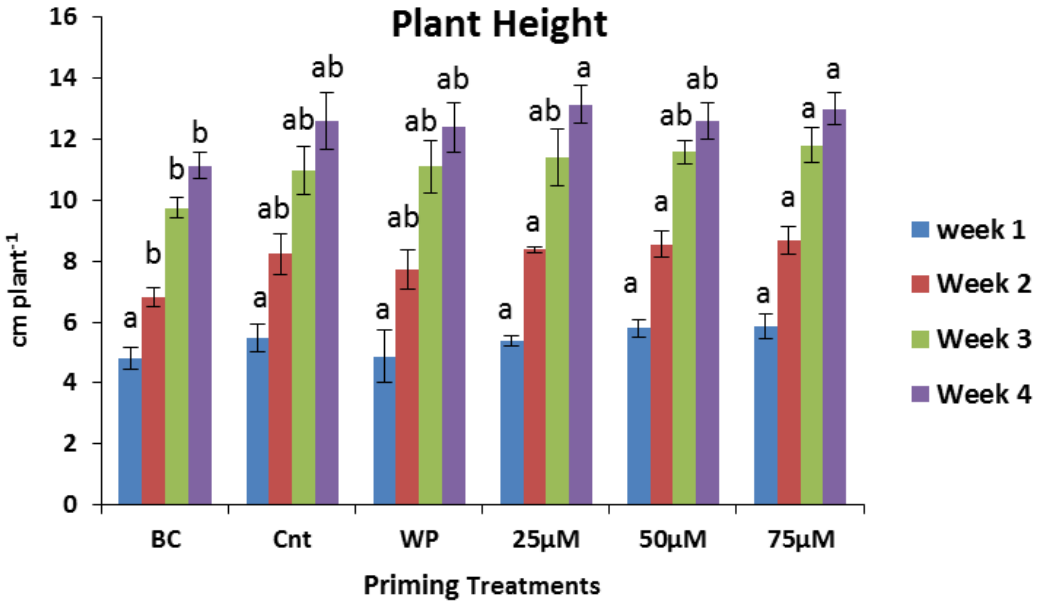

Fig. 3.14: Plant height of Spring oilseed rape (cv. SR1) during 1 – 4 weeks after sowing grown in rhizoboxes reduced root zone temperature 12^{0}C. Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments. BC: (NO_3 homogeneous, Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

3.4.3.2. Root Development

The root length evaluation along the rhizobox observation window at 1st, 2nd 3rd and 4th WAS, only showed positive trend for Zn priming treatment at 1st and 2nd WAS stage which was not significant and disappeared completely at later stages of plant growth (Figs 3.15 and 3.16).

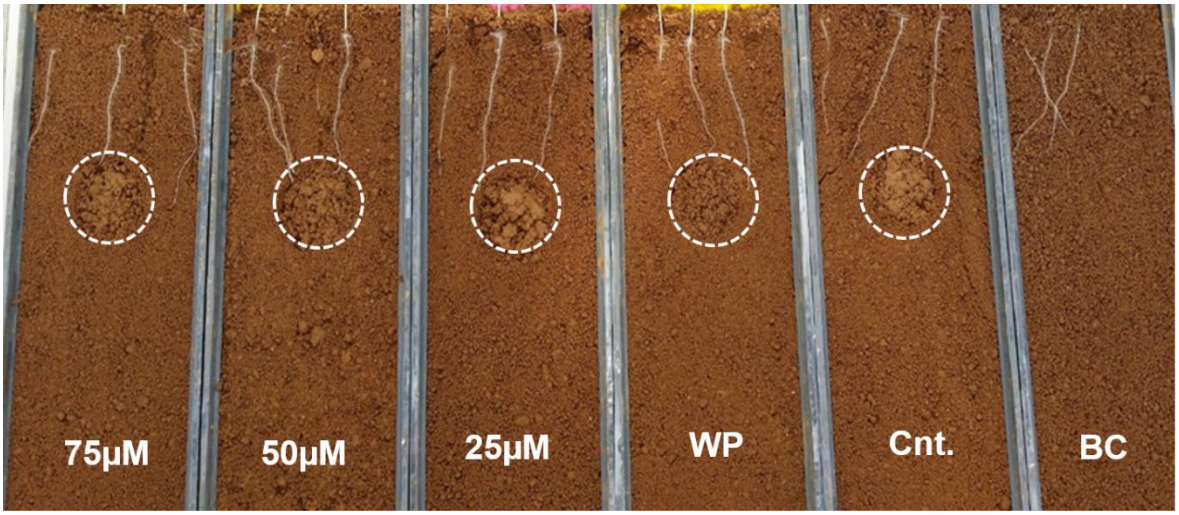

Fig. 3.15: Root development of Spring oilseed rape (cv. SR1) along with the root observation window of rhizoboxes at 7 days after sowing. BC: (NO_3 homogeneous), Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

Similarly, total root length evaluation after harvest (7 WAS) confirmed no improvement in the root length either inside or outside the depot (Fig 3.17). Furthermore, no improvement in root dry weight (RDW) even when evaluated separately for depot zone only.

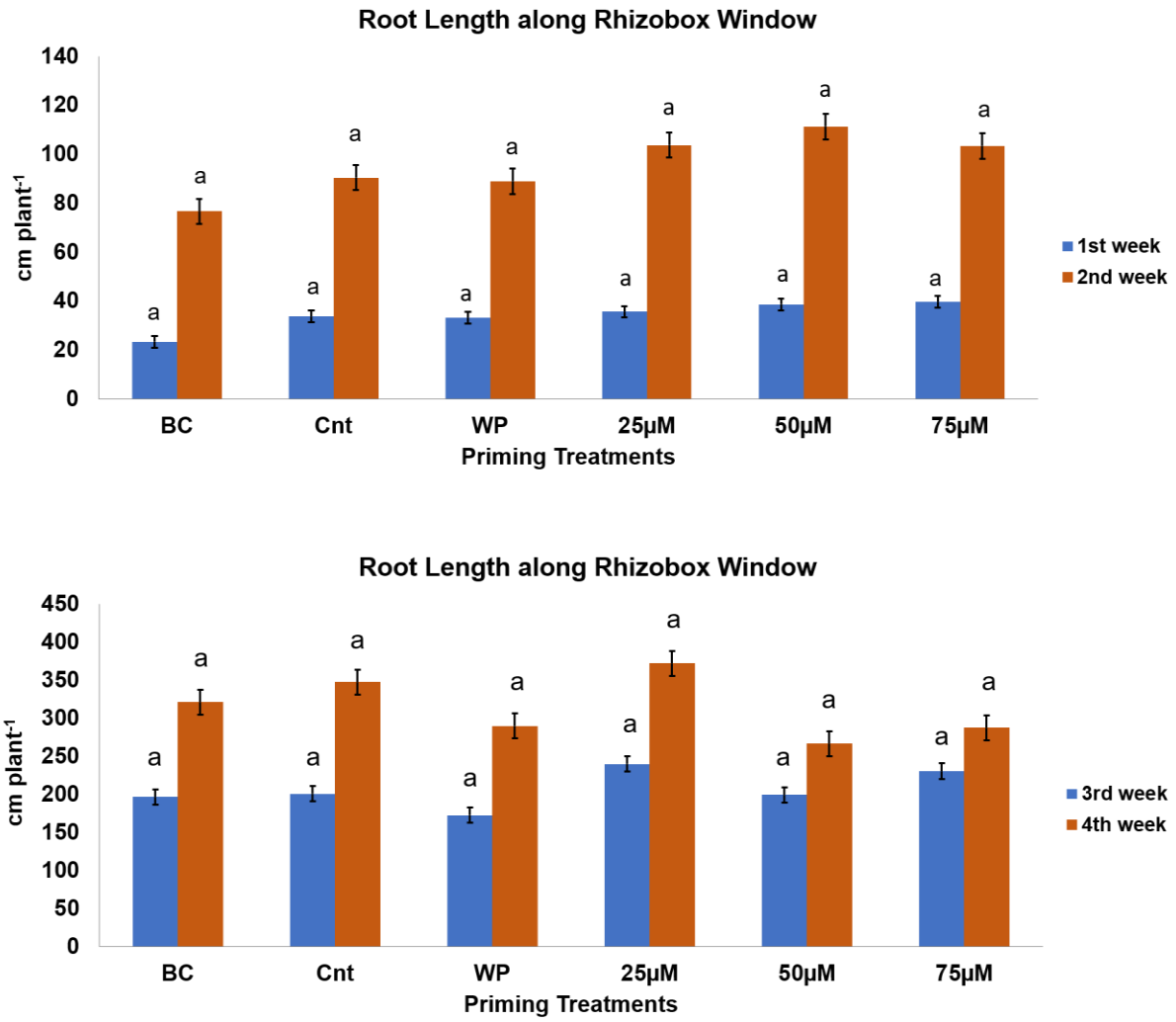

Fig. 3.16: Root length development of Spring oilseed rape (cv. SR1) along with the root observation window of rhizoboxes during 1 - 4 weeks after sowing (WAS) Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments. BC: (NO_3 homogeneous), Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

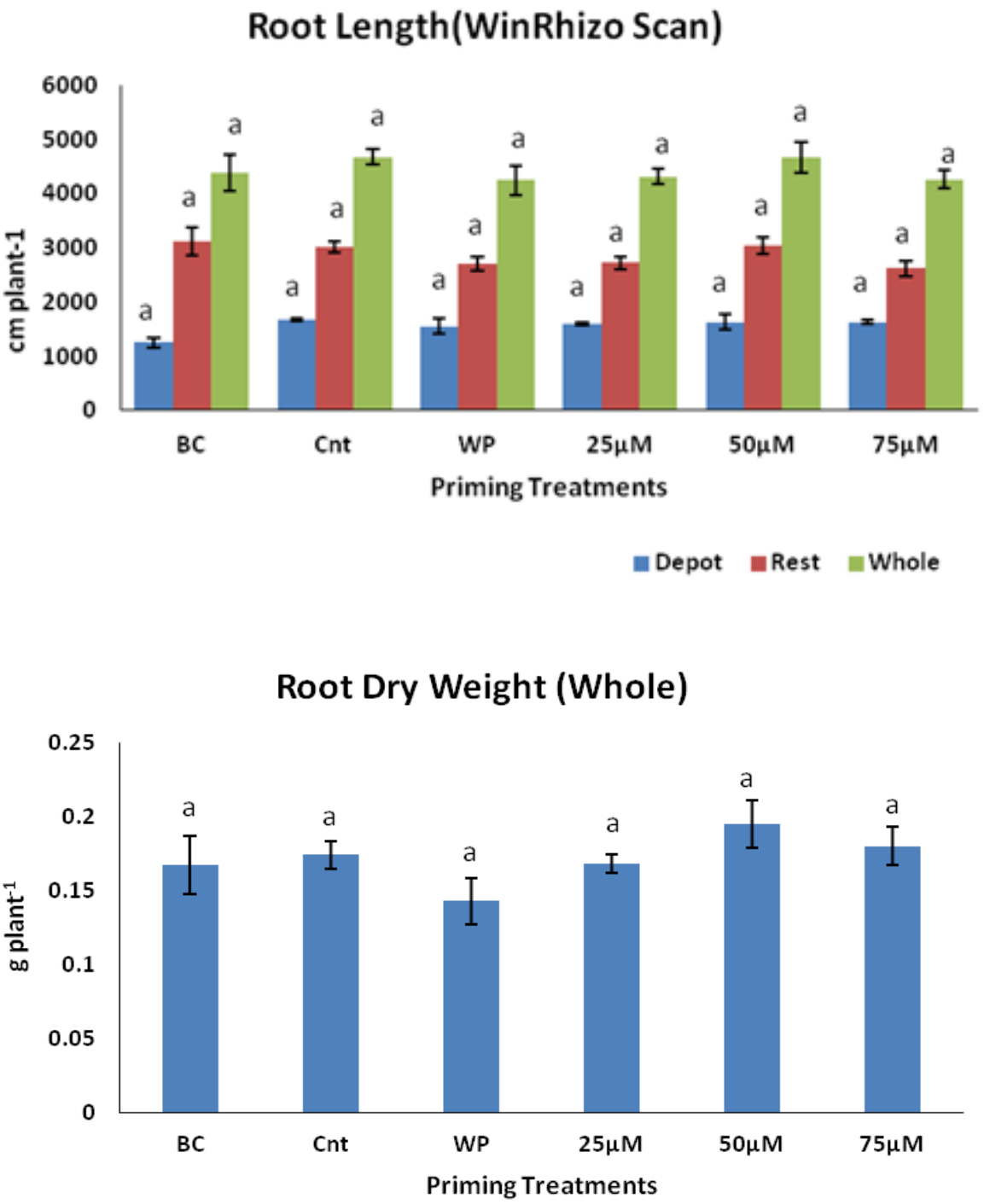

Fig. 3.17: Root length development inside and outside of the depot zone, and total root biomass of Spring oilseed rape (cv. SR1) at 7 weeks after sowing. Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments. BC: (NO_3 homogeneous), Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

Similar to the other root growth indicators, depot fertilization and zinc seed priming had no significant effects on root hair density or root hair length (Fig, 3.18).

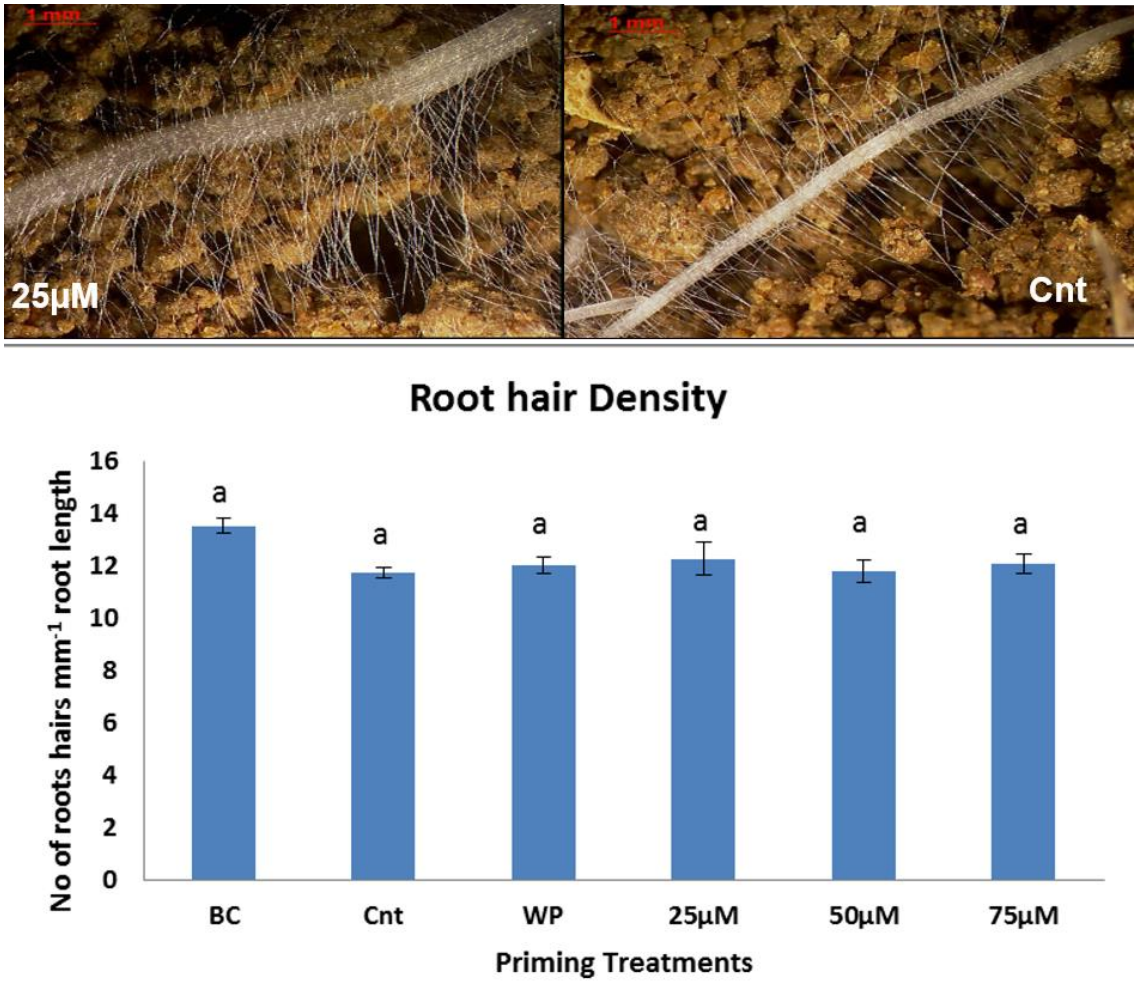

Fig. 3.18: Root hair density winter oilseed rape (cv SR1) at 7 weeks after sowing. Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments. BC: (NO_3 homogeneous), Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

3.4.3.3. Plant Nutritional status

After the 7-weeks period at reduced RZT at 12°C. the winter rape plants were clearly P deficient (Campbell, 2013). Depot fertilization improved the P status to optimum concentrations (Table 3.4). In all treatments, the micronutrient status was sufficient and SPAD values also indicate no treatment differences in the N status (Fig. 3.19). Zinc seed priming had no effects on the plant nutritional status at final harvest

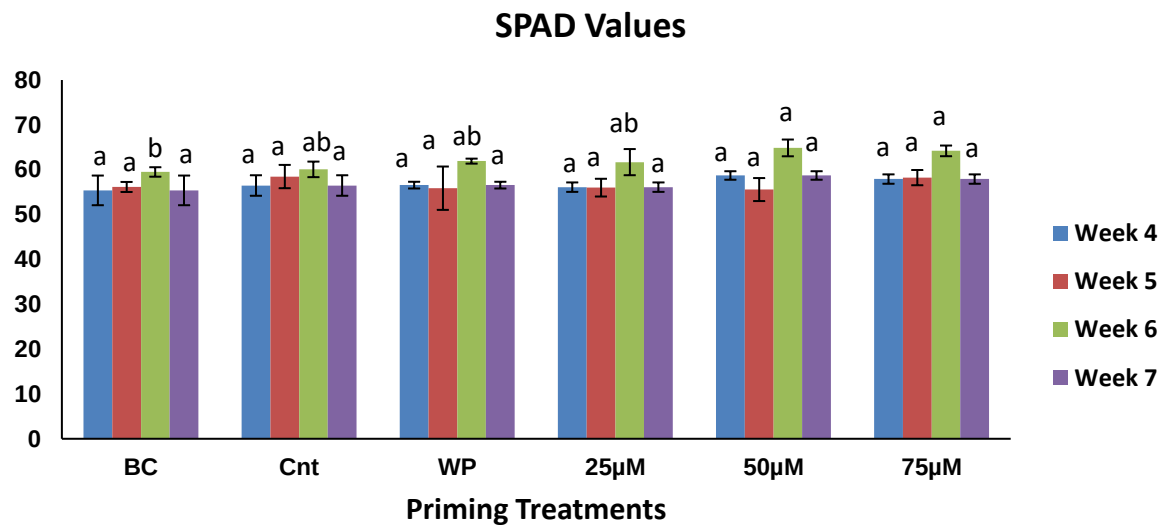

Fig. 3.19: SPAD values of fully developed leaves in Spring oilseed rape (cv. SR1) at 7 weeks after sowing. Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments. BC: (NO_3 homogeneous, Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed)

Table 3.4: Shoot mineral nutrient concentrations and contents after 7 weeks old winter oilseed rape (cv. SR1) grown in rhizoboxes at reduced root zone temperature 12^0C. Data represent Means and SE of 4 replicates. Different characters indicate significant differences between treatments. BC: (NO_3 homogeneous, Cnt: (unprimed control, NH4 depot), Seed priming variants (NH4 depot): WP: (Water primed), 25, 50, 75µM (Zn primed).

	Concentration [µg g^{-1}]				**Contents [µg $plant^{-1}$]**			
Treatments	**Zn**	**Mn**	**Fe**	**P [mg g^{-1}]**	**Zn**	**Mn**	**Fe**	**P [mg $plant^{-1}$]**
BC	61±3.8 a	211±5.5 a	90±4.9 a	2.7±0.05 b	27±0.9 a	94±4 a	40±2 a	1.25±0.08b
Cnt	52±3.2 a	168±10 a	84±1.2 a	7.2±0.2 a	23±0.3 a	76±2 b	38±1 a	3.3±0.1 a
WP	56±4.3 a	166±21 a	85±5.6 a	7.9±0.5 a	23±1.7 a	69±9 b	35±1 a	3.3±0.2 a
25 µM	53±1.7 a	163±16 a	80±4.8 a	7.7±0.6 a	22±1.0 a	68±5 b	33±1 a	3.3±0.3 a
50 µM	54±3.5 a	193±10 a	116±30 a	6.9±0.3 a	20±1.8 a	74±5 b	44±11a	2.6±0.1 a
75 µM	54±2.3 a	167±4.1 a	69±3.8 a	7.0±0.3 a	23±2.1 a	71±3 b	29±1 a	2.9±0.09a

3.5. Discussion

3.5.1. Winter rape

Winter rape culture at ambient greenhouse temperatures, comparable to the open field temperature regimes in temperate climates during sowing time in September (Fig. 3.2), was associated with root growth promotion, particularly in the zinc (Zn) priming treatments compared to the unprimed control in the depot variants. The stimulatory effect was apparent during early seedling establishment, enabled faster depot exploitation already during the first week after sowing and persisted until 3 weeks after sowing (Fig 3.7 & 3.8). This finding confirms the limited Zn seed reserves of oilseed rape lasting for less than 5 DAS until additional root-mediated Zn acquisition is required (Asim 2017), which was obviously suboptimal, even under the selected growing conditions avoiding environmental stress factors with detrimental effects on root growth. The well-documented root attracting effect of the ammonium phosphate depot, enabling efficient nutrient exploitation (Ying et al. 2010; Nkebiwe et al. 2016), was detectable until the end of the culture period. At the same time, deep rooting was not negatively affected by depot fertilization in upper soil layers. Therefore, in comparison with homogeneous fertilization, the selected fertilization strategy based on ammonium phosphate depot fertilization combined with Zn seed priming obviously supported root traits required for the successful establishment of winter rape in autumn, combining the efficient acquisition of starter fertilizers and deep rooting to improve winter hardness (Bauer, 2011).

Limitations of root growth are particularly critical for nutrients with limited mobility in soils, such as micronutrients (Fe, Zn, Mn) at soil pH levels > 5.0 and phosphate (Neumann and Römheld, 2002). Macronutrients (N, K, Mg) were supplied in sufficient amounts during starter fertilization, while the P fertilization level of 50-70 mg P kg^{-1} soil resulted in a moderate P supply on the selected soil with low P availability. Analysis of the plant nutritional status at the end of the culture period revealed shoot micronutrient concentrations in the sufficiency range for all treatments, and the lowest levels for Zn among the investigated micronutrients, reaching shoot concentrations between 38 – 47 µg g^{-1} DM, closest to the deficiency threshold of approx. 30 µg g^{-1} DM (Campbell, 2013). However, this does not exclude Zn limitation during early seedling growth when the root system is not yet completely developed, which was obviously supplemented

by Zn seed priming resulting in improved root growth. Beneficial effects of micronutrient Zn/Mn) seed priming on root growth have been similarly reported also in maize (Bradacova et al., 2016) associated with increased auxin production as a major regulator of lateral root development (Moradtalb et al., 2020). However, the most striking effect on the plant-nutritional status was a critically low P level (3.2 mg g^{-1} DM) of the plants supplied with homogeneous NPK fertilization with nitrate as N form (Campbell, 2013). This implicates that apart from Zn limitation also P limitation can be expected during early seedling growth, particularly on soils with a moderate P status. By contrast, the P status was luxury (7.6 - 8.9 mg g^{-1} DM) in all ammonium-phosphate depot fertilization variants. Rhizosphere acidification induced by the roots in an ammonium-dominated N nutrition can mediate solubilisation of Ca-P and counteract P fixation (Neumann and Römheld, 2002) and this effect can be further promoted by fertilizer placement due to localized root proliferation (Jing et al., 2010; Bischoff, 2012). Root growth stimulation supported more rapid depot exploitation and consequently improved P acquisition in the Zn-primed variants, which also explains the shoot growth promotion observed during the first three weeks of seedling establishment since P limitation inhibits shoot growth (Martin et al., 2000). However, despite a largely improved P nutritional status, excessive aboveground plant development with negative side effects on winter hardness was not observed in later stages of seedling growth. This may be attributed to reduced cytokinin production reported under ammonium-dominated fertilization in various plant species (Walch-Liu et al., 2000; Rahayu et al., 2005; Römheld et al., 2008), which counteracted shoot growth promotion.

Interestingly, in a similar experimental setup with ZnSD under field conditions an 11.6 % yield increase at a surprisingly high yield level of 5.5 t ha^{-1} in a strip till system with ammonium phosphate placement at a soil depth of 20 cm in comparison with standard sowing without fertilizer placement, was noted (Table 2.9, Chapter 2). Therefore, the benefits of Zn seed treatments in combination with fertilizer placement recorded in the model experiments described above may be at least partially responsible for the observed yield benefits under field conditions. Taken together the results support the hypothesis that ammonium phosphate depots combined with micronutrient seed priming could provide a promising starter fertilization strategy for winter rape to

promote early root establishment and nutrient acquisition worthwhile for further investigations under real field conditions.

3.5.2. Spring Rape

The situation in spring rape exposed to low root zone temperatures (RZT) of 12°C during the culture period was different. In contrast to winter rape grown at ambient greenhouse temperature, shoot growth of spring rape under cold stress was clearly promoted by approximately 30% even at the end of the 7-weeks culture period, with significant effects detectable only in the Zn-primed ammonium-phosphate depot variants compared with homogeneous NPK-nitrate fertilization (Fig. 3.13), At the same time, no significant effects on root growth or depot exploitation by local root proliferation were detectable. This is contradictory to the results of an earlier study conducted with the same cultivar (SR1) grown at 12°C RZT supplied with homogeneous nitrate based NPK fertilization. Here a pronounced positive Zn priming effect on root length was noted (Neumann et al., 2014) as similarly reported also for maize exposed to low RZT, both with nitrate and ammonium dominated fertilization (Bradacova et al., 2016; Ahmed, 2017; Moradtalab et al., 2018). A major difference of the present study was the limited P availability, as indicated by shoot P concentrations in the deficiency range (2.7 mg g^{-1} DM; Campbell, 21013) in the variants with homogeneous NPK fertilization (Table 3.4). Particularly at low soil temperatures, impaired P acquisition represents a major problem due to limited root growth and activity (Bradacova et al., 2016), and placement of P fertilizers close to the roots is a widespread measure to counteract this problem (Nkebiwe et al., 2016). Interestingly, also Gomez-Munoz et al. (2018) demonstrated that cold-protective effects of Zn supply in maize were not detectable under P-limited conditions. However, in our study, the plant P status in the ammonium-phosphate depot variants finally reached optimum levels between 6.9 –7.9 mg g^{-1} DM) even without significant root growth effects. This finding suggests that the improved P acquisition was rather related to root-induced rhizosphere acidification triggered by predominant ammonium nutrition and root growth stimulation was less important in this context. Apart from beneficial effects on auxin-mediated root growth promotion, beneficial effects of Zn seed priming have been also reported on detoxification of free radicals and the production of cold-stress

protectants (Moradtalab et al. 2018). This may promote the metabolic activity of the roots and root activity during early seedling development under cold stress conditions, finally leading to efficient root-induced P mobilization to overcome P-limitation, reflected in improved shoot growth. This means that Zn seed priming combined with ammonium-phosphate depot fertilization could overcome cold-stress induced limitations of critical nutrients, thereby promoting seedling establishment.

3.5.3. Concluding remarks

The present study suggests that the combination of Zn seed priming with the placement of ammonium-phosphate starter fertilizer can support the expression of plant traits beneficial for field establishment of winter rape and spring rape as well. This includes promotion of root development for efficient exploitation of the fertilizer depot and improvement of the plant-nutritional status without excessive stimulation of shoot growth in winter rape before winter and improved acquisition of critical nutrients (e.g. P) at low soil temperatures in spring rape. For further evaluation, replacement of seed priming by technically more feasible seed dressing treatments with field testing under different soil conditions would be required. This also includes more detailed characterization of related rhizosphere processes, hormonal profiling and expression of physiological stress indicators for a better understanding of the underlying mechanisms.

Chapter 4

Drought-protective effects of nutrient seed treatments during early growth of oilseed rape

4.1. Abstract

Crop production is increasingly affected by water limitation even in temperate climates due to a rising frequency of drought periods, related with global change. The stress-protective nutrients, discussed as a mitigation strategy, were investigated for their potential drought-protective effects of nutrient seed treatments, based on Ca, K, Fe, Zn, Mn on early growth of oilseed rape (OSR).

Responses were observed in five OSR hybrids under greenhouse conditions on two soils with contrasting properties (sandy-loam pH 5.6 vs. silty-loam pH 6.9) in two independent pot experiments. A 7-days drought period with reduced soil moisture level (40% soil water-holding capacity. WHC) inhibited shoot and root growth and caused irreversible wilting and leaf necrosis (27-46% of total leaf area) particularly on the sandy-loam with lower WHC, depending on the investigated genotype. Nutrient seed treatment increased shoot (10-15%) and particularly root growth (14-23%) as well as nutrient accumulation, but also reduced the proportion of irreversibly damaged leaves to 17-21%, with the largest effect in strongly drought-affected genotypes under the challenging conditions on the sandy-loam soil. Analysis of physiological stress indicators revealed increased accumulation of phenolics (23-28%), antioxidants (14-47%) and higher activities of ascorbate peroxidase (APX) (23-87%) in the leaf tissue, counteracting drought-induced oxidative stress. Moreover, APX activity was positively related with root length ($R^2$0.8953), suggesting a protective effect on drought-induced oxidative IAA degradation with inhibitory effects on root growth. Increased levels of absisic, jasmonic and salicylic acids during drought stress recovery point to stress priming effects, strengthening the natural adaptive responses to water limitation.

4.2. Introduction

Among the plant-based oil and protein sources for food and feed use, oilseed rape (OSR) is one of the most important crops worldwide (FAO STAT, 2017) but is being increasingly affected by water deficit or drought conditions around the globe. Related with climate change, the problem of water shortage increases not only in semi-arid and arid parts of the world but is also getting more and more relevant even in temperate climates (Collins et al., 2009). Moreover, other stress factors directly or indirectly associated with drought conditions, particularly heat and light, soil compaction or salinity stress lead to further aggravation of the drought stress syndrome (Whitemore & Whalley, 2009; Dreesen et al., 2012).

In OSR, mild to severe drought conditions can limit germination and seedling establishment (Yang et al., 2007) and caused a decrease in above-ground dry matter biomass by 18-32%, while seed yields were reduced by 19-39% associated with significant economic losses (Gunaskara et al., 2006). Impaired photosynthesis via reduced stomatal aperture to minimize transpiratory water losses, limited production of chlorophyll (Alam et al., 2014) and excessive formation of reactive oxygen species (ROS) leading to lipid peroxidation and membrane damage (Mirzae et al., 2013), which further impairs photosynthetic efficiency, associated with nutrient limitations due to root growth inhibition (White et al., 2015) are the main physiological drought responses mediating the substantial decline in yield formation.

In OSR the degree of detrimental drought effects leading to final yield losses is very much related to the sensitivity of the plant developmental stage. Oilseed rape is most sensitive to early summer drought at anthesis or at the stem elongation phase, while some genotypes are most sensitive at the pod filling stage (Richard & Thurling 1978a). However, a drought period in spring can severely affect the recovery phase of Winter OSR or seedling establishment of Spring OSR, which can be similarly affected by late summer drought in Winter OSR. Under these conditions, optimal root growth is of great importance, particularly for non-mycorrhizal plants such as OSR exclusively dependent on their root system to support the plant for water and nutrients. This applies not only for the drought period itself. A well-established root system in Winter OSR is imminent for winter hardiness, tap-root nutrient storage, plant survival in winter and regrowth and

nutrient acquisition after winter (Föhse et al.,1991, Hoffland et al., 1989, 1992**).** However, root development of both tap and lateral roots is impaired by water deficit conditions (Richard and Thurling,1978b; Hadi et al., 2014).

Apart from plant breeding, various agronomic approaches, such as planting methods suitable for water conservation i.e. drill sowing, raised bed planting and furrow planting (Zhang et al., 2007; Kukal et al., 2010; Aiken et al., 2015), application of nutrients (Fanaei et al., 2009) and growth regulators with protective functions (Ullah et al.,2012, Ahmadi et al., 2015) including foliar application of salicylic acid (SA) and abscisic acid (ABA) have been proposed to ameliorate detrimental effects of water limitation on plant performance. Among the strategies based on application of stress-protective nutrients, nutrient seed treatment, seed priming, and seed dressing have beendescribed as approaches providing beneficial growth-promoting effects under water deficit conditions, with a clear preference of seed dressings for industrial applications. In seed dressing, seeds are treated with insecticides, fungicides or growth-promoting biological agents (Ellis, 2004; Stendhal, 2005) and combined applications are possible. Seed priming (soaking + redrying) with potassium (K) and Zinc (Zn) improved drought tolerance, germination and early seedling growth in wheat and faba bean, respectively (Farooq e al., 2013; Farooq e al., 2021). Seed priming with $ZnSO_4$ and osmopriming with $CaCl_2$ helped to mitigate the drought stress by enhancing antioxidants in *Nigella sativa* and lentils, respectively (Sina et al., 2018; Farooq et al., 2020). Imran et al. (2018) explored positive $ZnSO_4$ priming effects under salt stress conditions in maize with beneficial effects also on field establishment and yield formation in response to cold stress during spring (Imran et al., 2013). Neumann et al. (2014) reported improved seedling establishment and root development after $ZnSO_4$ seed priming in Spring OSR exposed to low root zone temperatures.

In face of the numerous reports on stress protection induced by nutrient seed treatments, this study was initiated to investigate potential drought protective effects on early growth also in OSR and to elucidate related modes of action under controlled conditions. A meanwhile commercialized seed dressing formulation (Wurzel-Plus Beizung, Rapool Ring GmbH; Isernhagen Germany) based on a combination of stress-protective nutrients including Ca, K, Zn, Mn, and Fe, was selected for nutrient seed treatment (NST). We hypothesized that a combined application of different drought

protective nutrients could provide complementary or even synergistic benefits as drought protectants. To consider also potential genotypic differences in the responsiveness to drought and NST applications, five different Winter OSR hybrids were investigated in two experiments conducted under controlled greenhouse conditions on two soils with contrasting properties (sandy loam pH 5.6 vs. silty loam pH 6.9).

4.3. Materials & Methods

4.3.1 Seed Material

Treated winter oilseed rape (*Brassica napus*.L) seeds of five hybrids (WR2, WR3, WR4, WR5, WR6) were provided by NPZ Innovation GmbH, Holtsee, Germany. All seeds were treated with a fungicide combination of Thiram (TMTD) against damping off (*Rhizoctonia solani*) and Dimethomorph (DMM) against downy mildew (*Hyaloperonospora parasitica*) including the seeds used as a control treatment. Seed nutrient status was enhanced by nutrient seed treatment (NST) as seed dressing with a pool of selected micronutrients (chelated Zn, Mn, Fe) and macro-nutrients (Ca, K) applied in a commercial formulation (Wurzel-Plus Beizung, RAPOOL-RING GmbH, Isernhagen, Germany). Seeds were homogenized by passing through a seed grading sieve and finally a seed size of 2 mm was selected for the experiments.

4.3.2 Plant Culture:

Experiment 1:

A silty loam soil pH 6.9 was fertilized 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 and sieved through 2 mm mesh size. Plastic pots were filled with 2 kg of fertilized soil substrate. Ten seeds each of three different hybrids of winter OSR (WR2, WR3, WR4) were sown directly at 2 cm depth, the pots were transferred to a growth chamber and cultivated with a 15 h light period (200 µmol m^{-2} s−1) , 60-80% rel. humidity and a 23 ±2 °C temperature regime.

Soil moisture was maintained at 70% Water Holding Capacity (WHC) for 3 weeks, followed by 1 week of drought stress (40% WHC). Thereafter, plants were subjected

to 70% WHC moisture conditions again for a one-week recovery period before harvesting, at 35 DAS. For each treatment, additional 5 replicates were grown as a positive control at 70%WHC during the whole duration of the experiment.

Soil water holding capacity (WHC) was determined according to the method described by Öhlinger *et al.* (1996). A plastic cylinder of 15cm height and 4 cm inner diameter, with a bottom fitted 0.5 mm mesh, was used. Three cylinders were filled with the substrate and dipped in water, upto the level of the soil inside the cylinder, for 24 hours. In order to drain all the gravitational water, the cylinders were kept standing on a moist sand bath for 48 hrs. After weighing, the soil was dried at 105 °C until a constant weight was achieved. The WHC was calculated using the following formula and was maintained on weight basis (W/W) throught the experiments.

$$\text{WHC\%} = \frac{\text{Weight of the water held against gravity in saturated soil}}{\text{Weight of the dried soil}} \times 100$$

Experiment 2:
A similar setup was used to explore the NST responses of OSR on two different soils; a sandy loam pH 5.6 and a silty loam pH 6.9. Ten seeds each of three different hybrids (WR2, WR5, & WR6) were sown and thinning to 5 plants pot^{-1} was performed at 10 DAS after carefully selecting homogenous seedlings. All the other plant culture details remained the same as in experiment 1.

4.3.3 Plant Analysis:
After three days recovery from drought stress, the youngest fully developed leaves from each replicate was harvested and stored at -80°C to be further analyzed for physiological stress markers. After the one-week recovery phase at 35 DAS, any necrotic, chlorotic, or stunted leaf was determined and classsified as “irreversible damaged.” and the percentage damaged leaves relative to the number of total leaves was determined. Shoot and root dry matter was recorded after oven drying at 60°C. Root length was determined after digitalization using the WinRHIZO root analysis software (Regent Instruments Inc., Quebec, Canada) of root samples previously washed out from the soil and preserved in 30% (v/v) ethanol solution.

4.3.4 Plant mineral analysis:

Aliquots of 250 mg dried shoot material were transferred to crucibles and subsequently ashed for 5 hours in a muffle furnace at 500°C. The samples were cooled and extracted with 2.5ml of 3.4 M HNO_3 two times. For precipitating SiO_2, samples were evaporated till dryness and subsequently dissolved in 2.5 mL of 4 M HCl. The volume was increased ten times with hot deionized water and subsequently boiled for at least 2 min to convert meta- and pyrophosphates to orthophosphate. Ash solution was determined for Mg, Zn, Mn, Fe with atomic absorption spectrometry (ATI Unicam Solaar 939, Thermo Electron, Waltham, USA) after adding 0.1 mL Cs/La buffer to 4.9 mL ash solution while phosphate was measured spectro-photometrically (Hitachi U-3300 spectrophotometer, Hitachi Ltd. Corporation, Tokyo, Japan) after adding molybdate-vanadate color reagent according to Gericke and Kurmis (1952. Flame photometry (ELEX 6361, Eppendorf, Hamburg, Germany) was used to determine Ca and K in the ash solution.

4.3.5 Determination of stress metabolites

4.3.5.1 Determination of total phenolics

Concentration of total phenolics was measured by Folin-Ciocalteau reagent, using gallic acid as standard, based on the spectrophotometric method as described by Panico et al. (2009). The extraction buffer was a methanol-HCl solution at a ratio of 98:2. One hundred mg of the leaf samples were homogenized in 1 ml of extraction buffer using mortar and pestle and centrifuged at 3,000 g for 20 min. For the reaction buffer, Folin-Ciocalteau reagent was diluted 1:10 (v/v), and sodium carbonate (Na_2CO_3) 7.5 % (w/w) was boiled in 100 ml of ultrapure H_2O for 5 min. Finally, 1 ml of ultrapure H_2O, 500 µl of diluted Folin-Ciocalteau reagent, 1 ml of 7.5 % Na_2CO_3, and 100 µl of supernatant of the leaf were pipetted into the reaction cells. Absorbance was measured at 765 nm after 10 min of incubation. Total phenolic concentration was expressed as gallic acid equivalents in mg g^{-1} fresh weight. The concentration of gallic acid was ascertained from a calibration curve using gallic acid dissolved in the methanol-HCl solution (98:2).

4.3.5.2 Determination of total antioxidant capacity by DPPH assay

For the spectrophotometric determination of total antioxidants after Panico et al. (2009) the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was used to evaluate the free radical scavenging activity of antioxidants. One hundred mg of the leaf samples were homogenized in 1 ml of extraction buffer (1:1 ethanol: H_2O) using mortar and pestle and centrifuged at 3,000 g for 20 min. For the reaction buffer, a 3 mM DPPH-solution was prepared and stored in a dark flask covered with aluminum foil at 4°C, in between the single runs of the measurements. Finally, the reaction cells contained 28 µl of DPPH-solution, 944 µl of ethanol, and 28 µl of leaf extract supernatant. After 10 min incubation in the dark at room temperature, the reaction was assayed by reading the absorbance at 515 nm, whereas the base was represented by the absorbance value of cuvettes containing the same mixture of reagents, but containing an equivalent amount of ethanol instead of supernatant. The percentage decrease in absorbance was recorded for each sample. The percentage quenching of DPPH-radical was calculated based on the observed decrease in absorbance according to the following formula:

$$\% \text{ Inhibition} = [(A0 – A1) / A0] \times 100$$

where A0 was the absorbance value of the base, and A1 is the absorbance value of the sample solution.

4.3.5.3 Determination of proline

The extraction buffer used for the spectrophotometric determination of proline (following Bates et al. (1973) was 3 % aqueous sulfosalicylic acid. Leaf samples (100 mg) were homogenized with extraction 1 ml buffer using mortar and pestle, followed by centrifugation at 3,000 g for 20 min. For the reaction buffer, proline reagent was prepared by dissolving 1.25 g ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid. Two millilitres of proline reagent, 2 ml of glacial acetic acid and 1 ml of supernatant were pipetted into glass reaction vials and incubated at 100 °C for 1 h, using an electro-thermal heater. After cooling back to 25 °C, 4 ml of toluene were added and mixed for 30 s until two phases had formed. From the upper (pink) phase, containing the chromophores, 1.5 ml were transferred to cuvettes, and the absorbance was measured at 500 nm every 30 s for 5 min against a blank cell filled with 1.5 ml toluene. The proline concentration was determined via external standardization with a standard curve and calculated on a fresh weight basis (Bates et al. 1973).

4.3.6 Determination of ascorbate peroxidase activity

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to the spectrophotometric method described by Boominathan and Doran (2002). One hundred mg of leaf material was homogenized using mortar and pestle in 1 mL 100 mM phosphate buffer (pH 7.5) containing 0.2 mM EDTA at 4°C, followed by centrifugation at 12,000 g for 15 min.

Reaction cells were filled with 325 µl of 50 mM potassium phosphate buffer, 200 µl of 0.5 mM ascorbic acid, 200 µl of 1.25M bovine serum albumin (BSA) and 200 µl of supernatant. To start the reaction, 50 µl of 0.5 M H_2O_2 were added and oxidation of ascorbic acid was monitored recording the decrease in absorbance at 290 nm during 1 min after starting the reaction, whereas the blank was represented by the absorbance value of cuvettes containing the same mixture of reagents but containing an equivalent amount of buffer instead of supernatant. Ascorbate peroxidase activity was calculated according to lambert-Beer´s law, using an absorbance coefficient for ascorbic acid of 2.6 mM^{-1} cm^{-1}. One unit of APX is defined as the amount of enzyme oxidizing ascorbic acid at a rate of 1 µmol min^{-1} at 25 °C.

4.3.7 HPLC-MS analysis of phytohormones:

One gram of frozen shoot samples was homogenized twice with 2.5 ml of 80% methanol in falcon tubes after grinding them to a fine powder using liquid nitrogen. Further homogenization was performed by ultrasonication (Micra D-9 homogenizer, Art, Müllheim Germany) at 10,000 rpm for 1 min and 15 s. Methanol extracts were added to the microtubes, and the samples were centrifuged for 5 min at 5,645 × g. Ultra-pure water was added to the supernatant and centrifuged again for 5 min at 5,645 × g. Membrane filtration (Chromafil R O20/15 MS) cleaned the supernatant and shifted to HPLC vials. Velos LTQ System (Thermo Fisher Scientific, Waltham, Massachusetts, USA), was used to perform UHPLC-MS analysis, which was fitted with Synergi Polar column, 4µ, 150 ∗ 3.0 mm, (Phenomenex, Torrance, California, USA). The flow rate was set to 0.5 ml min^{-1} with mobile phase (a): water and 5% acetonitrile and mobile phase (b) acetonitrile and a gradient profile, for gradient elution. The gradient profile was 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 including 3-indoleacetic-acid, (+/–)-abscisic acid,

gibberellic acid, trans-zeatin, salicylic acid, (+/–)-jasmonic acid, were purchased from Sigma Aldrich, (Sigma Aldrich, St. Louis, Missouri, USA).

4.3.8 Statistical analysis:

The experiments were arranged in a completely randomized design and with five and four replicates for each treatment in experiment 1 and 2, respectively. Data represent Mean + and SE represents. One-way analysis of variance $p < 0.05$) was performed with the Sigma Plot version 11.0. Software (SYSSTAT Software Inc., Erkrath, Germany) to evaluate the statistical significance of differences between the means of treatment groups.

4.4. Results

4.4.1 Experiment 1

The potential benefits of nutrient seed treatments (NST) on early plant growth under drought conditions was investigated with three Winter-OSR hybrids (WR2, WR3, WR4) grown on a silty loam soil pH 6.9. Germination ranged between 74% (WR4) and 90% (WR2) without significant differences between NST and untreated controls. After a drought stress period of 7 days at a reduced soil moisture level of 40% WHC, detrimental drought effects on oilseed rape plants were detectable in all three genotypes. The habitus of drought-affected plants was characterized by reduced leaf area (Fig. 4.1 and 4.2), wilting, rolling, and necrosis of leaves.

Fig. 4.1 Habitus of well-watered (70% WHC) and one-week drought-affected OSR plants (40% WHC), genotypes WR2-WR4, 28 DAS with and without nutrient seed treatment (NST).

During the drought stress period, all genotypes expressed development of a blue-grey coloured cuticular layer at the upper leaf side, which was particularly intense in the NST variants associated with a general trend for enlarged leaf area (Fig 4.2)

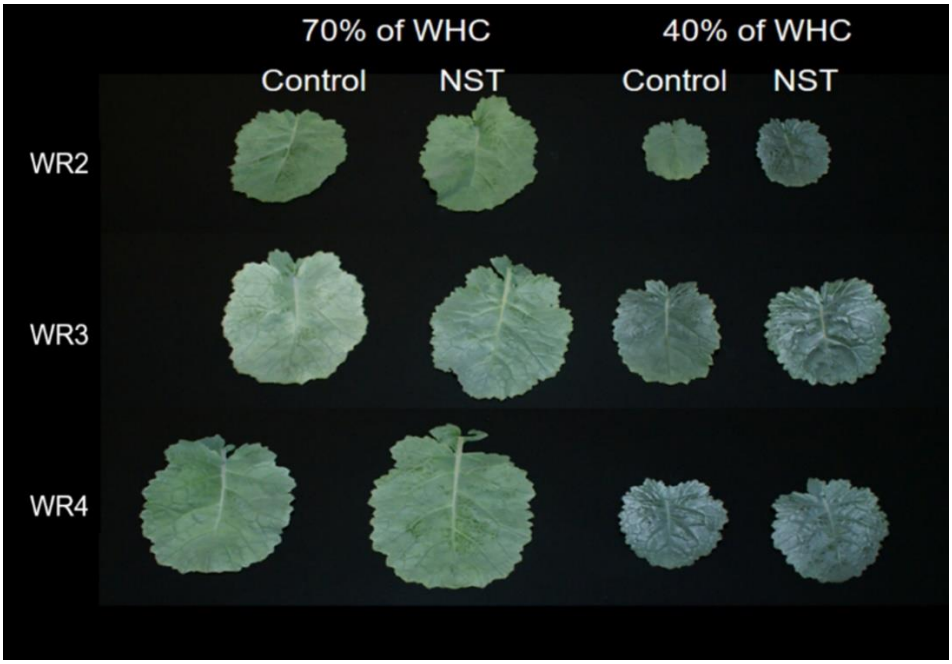

Fig. 4.2 Leaf habitus and leaf colouration of well-watered (70% WHC) and one-week drought-affected OSR plants (40% WHC); genotypes: WR2-WR4, 28 days after sowing (DAS) with and without NST.

Microscopic examination revealed a thicker cuticula layer of the drought-stressed plants, which was more homogenous without cracks in the NST-treated plants (Fig. 4. 3).

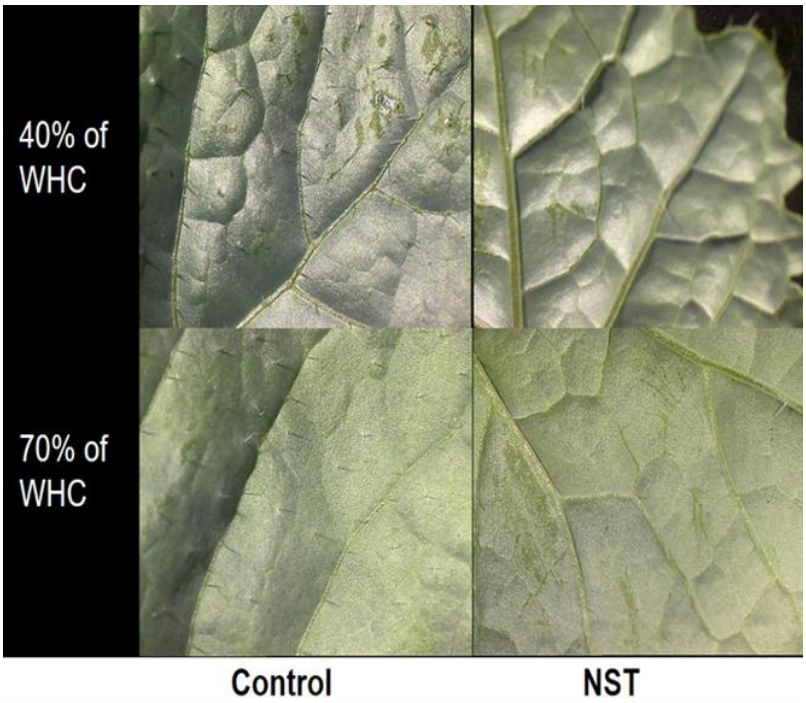

Fig. 4.3 Cuticular development of well-watered (70% WHC) and one-week drought-affected OSR plants (40% WHC), genotype WR4, 28 DAS with and without NST.

Drought stress-induced irreversible leaf damage (chlorosis, necrosis, wilting) decreased in the order WR3 (43% affected leaves) > WR2 (38%) > WR4 (27%). Application of NST reduced the leaf damage in WR3 by 24% followed by WR2 (21%) and then WR4 (5% n.s) (Table 4.1). Under drought stress, shoot and root biomass, as well as root length, significantly declined in WR2 and WR4 with a similar trend in WR3. Nutrient seed treatment fully compensated these reductions in shoot and root growth in WR3 and WR4 but not entirely in WR2. Nutrient seed treatment also stimulated root and shoot growth already in unstressed plants with significant effects in WR3 (8-17%) and WR2 (5-15%) (Table 4.1).

In all treatments, the nutritional status of WR2 and WR3 was critical for Ca (< 20 mg g^{-1} DM), K (< 35 mg g^{-1} DM), Zn (< 33 µg g^{-1} DM) and Cu (< 5 µg g^{-1} DM) at 35 DAS, and the Mn-status declined below the deficiency threshold (< 30 µg g^{-1} DM) under drought stress (Campbell, 2013), while Mg and P were in the sufficiency range. There

was a general trend for increased shoot accumulation of nutrients induced by the NST treatments, although the effects were not significant in all cases (Table 4.2).

Table 4.1: Shoot, root dry biomass production, and root length of oilseed rape (genotype WR2, WR3, WR4), grown at 23 ± 2^{0}C for 5 Weeks with and without nutrient seed treatment (NST). Data represent means and SE of 5 replicates. Different characters indicate significant differences between treatments.

			Germination [%]	Damaged leaves [%]	SDW [g plant^{-1}]	RDW [mg plant^{-1}]	RL [cm plant^{-1}]
70 % WHC	**WR2**	**Cont.**	90±2 a		0.47±0.03 b	54.98±0.43 b	1640.76± 2.90 b
		NST	95±3 a		0.54±0.01 a	59.12±0.73 a	1722.75±19.80 a
	WR3	**Cont.**	84±3 a		0.23±0.01 b	62.99±1.36 b	1865.3± 11.57 b
		NST	91±2 a		0.27±0.00 a	70.79±0.95 a	2020.72±37.86 a
	WR4	**Cont.**	74±5a		0.54±0.02 a	37.04±1.0 a	1632.84±59.58 a
		NST	78±5 a		0.6±0.02 a	40.01±0.92 a	1725.87±50.42 a
40 % WHC	**WR2**	**Cont.**		38.1 a	0.32±0.01 a	44.7±1.52 a	1311.28±15.48 b
		NST		17.1 b	0.36±0.01 a	49.38±1.69 a	1399.73±21.97 a
	WR3	**Cont.**		43.1 a	0.22±0.01 b	55.66±1.15 b	1679.61± 50.74 b
		NST		19.3 b	0.3±0.02 a	61.45±0.73 a	1810.89±15.76 a
	WR4	**Cont.**		26.9 a	0.42±0.02 b	33.36±1.18 b	1446.74± 79.40 a
		NST		21.4 a	0.6±0.04 a	40.14±1.74 a	1732.67±47.18 b

Table 4.2: Shoot mineral concentrations and contents of oilseed rape (genotype WR2, WR3, WR4), grown on a silty loam soil (pH 6.9) at 23 ± 2^{0}C for 5 Weeks with and without NST. Data represent means and SE of 5 replicates. Different characters indicate significant differences between treatments.

			Zn		Mn		Cu	
			[µg g^{-1}]	[µg $plant^{-1}$]	[µg g^{-1}]	[µg $plant^{-1}$]	[µg g^{-1}]	[µg $plant^{-1}$]
WR2	70 % WHC	Cont.	30.01±0.62 a	14.17±0.78 a	42.87± 0.76 a	18.31±0.34 a	4.93±0.09 a	2.32±0.11 a
		NST	28.82±0.33 a	15.71±0.22 a	37.35±0.59 b	20.36±0.47 a	4.78±0.15 a	2.61±0.09 a
	40 % WHC	Cont.	29.94±0.41 a	8.31±0.33 b	20.18±1.01 a	12.75±0.59 b	5.34±0.22 a	1.49±0.12 a
		NST	31.74±0.67 a	10.07±0.26 a	20.36±0.35 a	15.30±0.68 a	5.52±0.28 a	1.76±0.12 a
WR3	70 % WHC	Cont.	29.32±0.97 a	7.00±0.34 a	45.74±0.34 a	9.58±0.43 a	4.27±0.25 a	1.07±0.05 b
		NST	28.62±0.67 a	7.83±0.20 a	48.07±0.47 a	11.24±0.24 a	4.15±0.19 a	1.39±0.07 a
	40 % WHC	Cont.	28.32±0.45 b	6.27±0.28 b	12.71±0.59 a	9.24±0.44 b	4.27±0.21 a	0.940±0.02 b
		NST	32.78±0.73 a	9.79±.52 a	15.30±0.68 a	12.77±0.66 a	4.15±0.14 a	1.24±0.08 a
WR4	70 % WHC	Cont.	43.36±0.75 a	23.52±0.63 b	50.72±0.72 a	27.56±1.07 a	7.82±0.31 a	4.23±0.08 a
		NST	45.50±0.69 a	27.32±0.67 a	49.91±0.61 a	30.04±1.24 a	7.94±0.29 a	4.78±0.26 a
	40 % WHC	Cont.	35.50±0.73 a	15.10±0.86 b	51.51±0.63 a	21.94±1.33 a	7.35±0.30 a	3.12±0.18 a
		NST	33.43±0.55 b	20.29±1.76 a	42.52±0.67 b	25.74±1.96 a	7.01±0.19 a	4.24±0.31 a

			Ca		K		Mg		P	
			[mg g^{-1}]	[mg $plant^{-1}$]	[mg g^{-1}]	[mg $plant^{-1}$]	[mg g^{-1}]	[mg $plant^{-1}$]	[mg g^{-1}]	[mg $plant^{-1}$]
WR2	70 % WHC	Cont.	13.47±0.47 b	6.37±0.46 b	17.36±0.45 b	8.16±0.34 b	3.05±0.07 a	1.43±0.06 b	5.50±0.12 a	2.59±0.10 a
		NST	17.11±0.46 a	9.33±0.28 a	19.32±0.30 a	10.53±0.15 a	3.03±0.03 a	1.70±0.05 a	5.32±0.10 a	2.89±0.06 a
	40 % WHC	Cont.	16.83±0.21 a	4.67±0.20 a	25.70±1.09 a	7.14±0.45 a	4.01±0.10 a	1.11±0.03 a	7.22±0.32 a	2.01±0.15 a
		NST	15.81±0.33 a	5.04±0.26 a	26.61±0.99 a	8.46±0.44 a	3.85±0.06 a	1.22±0.05 a	7.08±0.09 a	2.25±0.07 a
WR3	70 % WHC	Cont.	21.22±1.07 a	5.07±0.30 a	20.93±1.19 a	4.97±0.21 a	3.60±0.07 a	0.85±0.03 a	5.63±0.27 a	1.35±0.08 a
		NST	21.62±1.41 a	5.91±0.38 a	18.69±1.04 a	5.10±0.17 a	3.49±0.04 a	0.95±0.02 a	5.68±015 a	1.55±0.06
	40 % WHC	Cont.	16.45±0.53 a	3.64±0.15 a	18.88±1.42 a	4.16±0.36 a	3.53±0.07 a	0.78±0.03 b	5.91±0.29 a	1.31±0.07 a
		NST	15.22±0.50 a	4.53±0.19 a	15.68±0.94 a	4.66±0.33 a	3.43±0.09 a	1.02±0.05 a	5.49±0.25 a	1.64±0.10 a
WR4	70 % WHC	Cont..	27.44±0.73 a	14.90±0.64 a	37.03±2.31 a	20.24±1.81 a	5.19±0.12 a	2.81±0.12 a	9.00±0.48 a	4.88±0.22 a
		NST	26.59±0.47 a	15.97±0.49 a	37.63±1.47 a	22.71±1.50 a	4.92±0.06 a	2.97±0.13 a	9.45±0.37 a	5.71±0.42 a
	40 % WHC	Cont.	23.16±0.48 a	9.85±0.54 b	32.29±1.37 a	13.65±0.53 a	4.69±0.06 a	1.99±0.13 a	9.20±0.22 a	3.92±0.28 a
		NST	21.46±0.33 a	12.97±0.94 a	26.78±0.70 a	16.30±1.59 a	4.29±0.09 a	2.61±0.23 a	8.34±0.23 a	5.03±0.35 a

4.4.2. Experiment 2

An additional experiment was conducted to test the drought protective NST effects, evaluated in the first experiment, on two different soil types; a silty loam pH 6.9 and a sandy loam pH 5.6. Apart from the highly NST-responsive genotype WR2 identified in experiment 1, two additional genotypes WR5 and WR6 were included in the setup of experiment 2.

Table 4.3: Seed mineral nutrient contents of the tested OSR genotypes, WR2, WR5 & WR6

OSR genotypes	Ca	P	K	Mg	Fe	Mn	Cu	Zn
	[mg g^{-1}]	[mg g^{-1}]	[mg g^{-1}]	[mg g^{-1}]	[µg g-^{1}]	[µg g^{-1}]	[µg g^{-1}]	[µg g^{-1}]
WR2	4.15	5.28	7.06	2.48	62.90	28.90	3.60	36.16
WR5	4.03	8.24	7.56	3.09	75.00	39.10	3.62	49.52
WR6	4.95	7.40	7.65	2.68	49.70	18.60	3.95	30.82

A comparative analysis of the native seed mineral nutrient concentrations revealed the lowest levels of Fe, Zn, and Mn in WR6 (Table. 4.3) as micronutrients supplemented in the NST variants.

4.4.2.1. Plant growth and nutritional status

Generally, reduced plant growth (Fig. 4.4) and lower shoot biomass production on pH 5.6 soil as compared to pH 6.9 was detectable for all genotypes even before onset of the drought stress treatment but also after drought recovery (Table 4.4), The highest shoot and root biomass production was recorded for WR6, which was lowest in WR2 (Table 4.4). Also stress symptoms during the drought period (wilting, leaf rolling) were more intensively expressed on the sandy loam pH 5.6 (Fig. 4.4). At the end of the 7d recovery period on both soils, irreversible leaf damage (permanent wilting, chlorosis, necrosis) was most strongly expressed in WR2 (45-46%) followed by WR5 (34-39%) and finally WR6 (22-31%). Significant NST-mediated reductions in drought-induced leaf damage were recorded for WR5 on the pH 5.6 soil and WR2 on the pH 6.9 soil with a similar trend for the soil with pH 5.6. Nutrient seed treatment significantly

increased shoot and root dry matter production in all three genotypes on the pH 5.6 soil. This was associated with a higher root length development, which was detectable in WR2 also on the pH 6.9 soil (Table 4.4). A significant increase in shoot biomass production on the pH 6.9 soil was detectable only in WR6. Generally, NST-induced growth responses were most strongly expressed in WR2, and the lowest responsiveness was recorded in WR6.

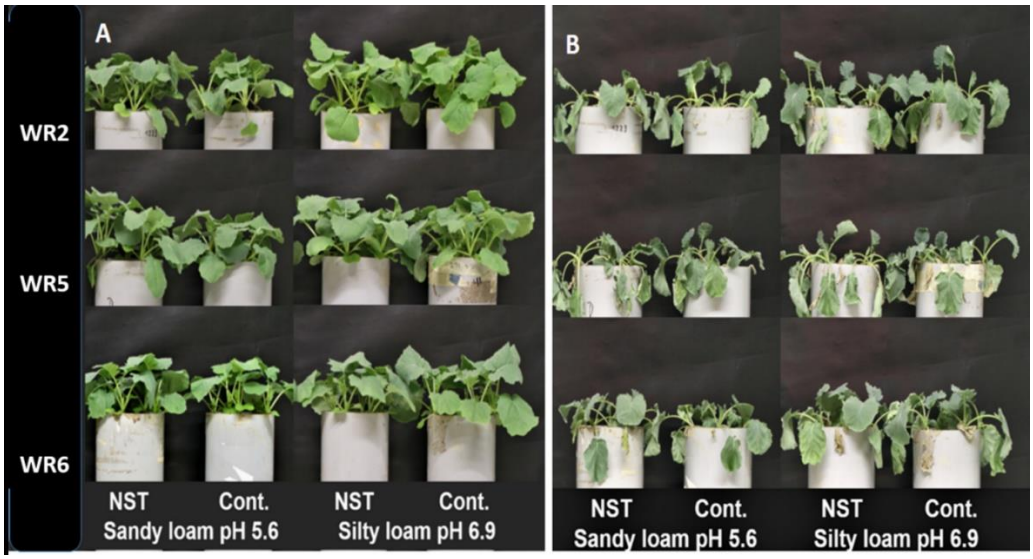

Fig. 4.4: Shoot growth and expression of drought stress symptoms of three OSR genotypes (WR2, WR5, WR6) before (A) and after (B) 7d of growth at a soil moisture level of 40% WHC.

After drought recovery, the nutritional status for all investigated nutrients (P, K, Ca, Mg, Zn, Mn, Fe & Cu) reached the sufficiency range in all treatments and all genotypes (Campbell, 2013). High Mn concentrations were recorded on the acidic pH 5.6 soil (Table 4.5).

Table 4.4: Shoot, root dry biomass production, root length an activity of ascorbate peroxidase in leaves of oilseed rape hybrids (WR2, WR5, WR6) grown on a sandy loam soil pH (5.6) & a silty loam soil (pH 6.9) at 23 ± 2°C for 5 weeks with and without NST. Data represent means and SE of 5 replicates. Different characters indicate significant differences between treatments.

			Leaf number plant^{-1}	Damaged leaves [%]	SDW [mg plant^{-1}]	RDW [mg plant^{-1}]	RL [cm plant^{-1}]	Leaf APX activity [Units g^{-1} FW]
WR2	**Sandy loam pH 5.6**	**Cont.**	4.0±0.14 a	45.0 a	240.41±3.64 b	34.42±1.01 b	1395.41±27.74 b	149.8±8.05 b
		NST	4.2±0.10 a	36.1 a	275.35±6.93 a	38.80±0.99 a	1606.02±35.47 a	238.15±11.10 a
	Silty loam pH 6.9	**Cont.**	4.7±0.13 a	46.2 a	350.76±6.69 a	41.62±1.49 a	1723.04±38.91 b	281.46±17.07 b
		NST	4.5±0.05 a	34.8 b	344.42±11.3 a	45.94±1.45 a	1884.7±40.92 a	345.91±13.78 a
WR5	**Sandy loam pH 5.6**	**Cont.**	4.4±0.14 a	38.6 a	253.34±5.62 b	34.91±0.83 b	1376.92±26.95 b	158.9±9.66 b
		NST	4.6±0.17a	26.4 b	287.91±1.68 a	43.24±0.99 a	1538.91±22.97 a	258.38±4.63 a
	Silty loam pH 6.9	**Cont.**	4.7±0.13 a	34.0 a	353.23±2.84 b	46.23±1.48 a	1678.66±51.63 a	311.64±6.56 b
		NST	4.6±0.13 a	34.1 a	388.54±8.76 a	51.15±1.05 a	1738.88±27.52 a	349.94±11.43 a
WR6	**Sandy loam pH 5.6**	**Cont.**	4.0±0.05 a	21.5 a	264.87±3.90 b	28.11±0.58 b	1349.5±32.37 b	116.37±2.76 b
		NST	3.8±0.08 a	22.4 a	275.48±6.81 a	32.40±0.65 a	1495.75±45.55 a	217.21±4.56 a
	Silty loam pH 6.9	**Cont.**	4.2±0.05 a	31.3 a	393.59±10.10 a	35.23±1.10 a	1836.71±35.28 a	257.03±6.90 b
		NST	4.2±0.17 a	31.3 a	395.46±15.90 a	36.44±0.39 a	1862.81±41.76 a	326.96±8.25 a

Table 4.5: Shoot mineral concentrations and contents of oilseed rape hybrids (WR2, WR5 & WR6) grown on a sandy loam soil pH (5.6) & a silty loam soil (pH 6.9) at 23 ± 2^{0}C for 5 Weeks with and without NST. Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments.

		Zn				**Mn**			
		Sandy loam pH 5.6		**Silty loam pH 6.9**		**Sandy loam pH 5.6**		**Silty loam pH 6.9**	
		[µgg^{-1}]	**[µgplant^{-1}]**	**[µgg^{-1}]**	**[µgplant^{-1}]**	**[µgg^{-1}]**	**[µgplant^{-1}]**	**[µgg^{-1}]**	**[µgplant^{-1}]**
WR2	**Cont**	58.95± 1.42 a	14.12± 0.55 a	31.26± 2.04 a	10.93± 0.67 a	289.76± 14.10 a	69.41± 4.23 b	78.80± 2.15 a	27.60± 1.54 a
	NST	56.55± 2.06 a	15.53+ 0.93 a	32.26± 1.19 a	11.10± 0.74 a	294.04± 3.68 a	80.77± 3.42 a	77.15± 2.18 a	26.58± 2.06 a
WR5	**Cont**	62.90± 3.19 a	15.88± 0.97 b	32.72± 1.32 a	11.56± 0.47 a	316.17± 22.99 b	79.87± 6.99 b	97.24± 0.29 a	34.34± 0.61 a
	NST	61.30± 1.26 a	17.59± 0.53 a	32.88± 2.40 a	12.76± 1.05 a	341.13± 10.92 a	97.83± 2.66 a	101.96± 6.0 a	39.61± 3.59 a
WR6	**Cont**	63.62± 3.68 a	16.53± 0.76 a	34.20± 1.76 a	13.46± 1.07 a	306.81± 7.80 a	79.77± 1.11 a	88.52± 3.74 a	34.79± 1.40 a
	NST	59.18± 2.17 a	16.29± 1.07 a	32.43± 1.07 a	12.82± 1.30 a	304.18± 8.21 a	83.60± 2.33 a	83.13± 4.97 a	32.91± 4.08 a
		Fe				**Cu**			
		Sandy loam pH 5.6		**Silty loam pH 6.9**		**Sandy loam pH 5.6**		**Silty loam pH 6.9**	
		[µgg^{-1}]	**[µgplant^{-1}]**	**[µgg^{-1}]**	**[µgplant^{-1}]**	**[µgg^{-1}]**	**[µgplant^{-1}]**	**[µgg^{-1}]**	**[µgplant^{-1}]**
WR2	**Cont**	159.36± 7.46 b	38.18± 2.48 b	136.85± 13.88 a	47.87± 4.65 a	12.35± 0.42 a	2.96± 0.12 b	9.51± 0.49 a	3.33+ 0.18 a
	NST	173.83± 2.75 a	47.77± 2.56 a	148.46± 11.97 a	51.09± 4.59 a	12.53± 0.29 a	3.45± 0.24 a	7.84± 0.02 b	2.70± 0.18 b
WR5	**Cont**	304.05± 49.46 a	76.63± 11.51 a	356.16± 8.15 a	125.83± 4.81 a	12.84± 1.41 a	3.23± 0.29 b	10.37± 0.62 a	3.67± 0.25 a
	NST	329.27± 36.38 a	94.44± 10.33 a	268.53± 27.31 b	104.06± 9.25 a	14.19± 0.46 a	4.07± 0.10 a	10.49± 0.47 a	4.08± 0.33 a
WR6	**Cont**	490.2± 10.48 a	127.47± 10.33 a	222.46± 3.56 b	87.55± 5.56 b	14.25± 0.30 a	3.70± 0.11 a	9.84± 0.02 a	3.87± 0.20 a
	NST	350.29± 35.57 b	96.14± 2.52 b	304.80± 7.38 a	120.58± 12.52 a	13.50± 0.98 a	3.71± 0.30 a	9.50± 0.44 a	3.76± 0.44 a

		Ca				K			
		Sandy loam pH 5.6		Silty loam pH 6.9		Sandy loam pH 5.6		Silty loam pH 6.9	
		[mgg^{-1}]	[mgplant^{-1}]	[mgg^{-1}]	[mgplant^{-1}]	[mgg^{-1}]	[mgplant^{-1}]	[mgg^{-1}]	[mgplant^{-1}]
WR2	**Cont**	23.10± 0.49 a	5.54± 0.25 b	33.27± 0.99 a	11.64± 0.43 a	45.68± 1.52 a	10.95± 0.59 a	49.31± 2.68 a	17.25± 0.89 a
	NST	24.38± 0.93 a	6.70± 0.29 a	32.34± 0.80 a	11.14± 0.76 a	45.10± 1.45 a	12.40± 0.10 a	53.63± 3.27 a	18.48± 1.82 a
WR5	**Cont**	24.52± 0.72 a	6.19± 0.31 a	30.05± 2.23 a	10.61± 0.77 a	40.56± 3.10 a	10.24± 0.95 b	43.84± 2.58 a	15.62+ 0.45 a
	NST	23.53± 0.16 a	6.75± 0.11 a	31.19± 3.02 a	12.08± 0.99 a	40.16± 0.47 a	11.52± 0.25 a	43.17± 0.43 a	16.76± 0.88 a
WR6	**Cont**	22.93± 0.77 a	5.97± 0.28 b	28.69± 1.36 a	11.28± 0.66 a	51.82± 0.76 a	13.48± 0.37 a	47.42± 2.07 a	18.67± 0.25 a
	NST	23.8± 0.43 a	6.54± 0.33 a	30.63± 1.68 a	12.07± 0.55 a	47.43± 0.32 a	13.05± 0.66 a	50.01± 1.73 a	19.72± 0.21 a
		Mg				**P**			
		Sandy loam pH 5.6		Silty loam pH 6.9		Sandy loam pH 5.6		Silty loam pH 6.9	
		[mgg^{-1}]	[mgplant^{-1}]	[mgg^{-1}]	[mgplant^{-1}]	[mgg^{-1}]	[mgplant^{-1}]	[mgg^{-1}]	[mgplant^{-1}]
WR2	**Cont**	5.91± 0.11 a	1.42± 0.04 a	6.28± 0.49 a	2.19± 0.18 a	5.34± 0.08 a	1.28± 0.05 a	6.85±0.10 a	2.4± 0.11 a
	NST	6.09± 0.24 a	1.67± 0.05 a	6.47± 0.25 a	2.23± 0.19 a	5.62± 0.57 a	1.54± 0.19 a	7.42± 0.25 a	2.55± 0.13 a
WR5	**Cont**	6.65± 0.15 a	1.68± 0.07 b	7.14± 0.08 b	2.52± 0.06 b	5.37± 0.29 a	1.36± 0.08 b	6.0± 0.18 b	2.14± 0.05 b
	NST	6.72± 0.17 a	1.93± 0.04 a	7.56± 0.18 a	2.93± 0.11 a	5.36± 0.05 a	1.54± 0.02 a	6.59± 0.20 a	2.56± 0.17 a
WR6	**Cont**	6.38± 0.18 a	1.66± 0.07 a	6.94± 0.25 a	2.73± 0.15 a	5.15± 0.28 a	1.34± 0.06 a	6.20± 0.36 a	2.43± 0.12 a
	NST	5.91± 0.11 a	1.61± 0.08 a	6.71+ 0.21 a	2.65± 0.26 a	5.03± 0.31 a	1.38± 0.04 a	6.12± 0.16 a	2.41± 0.17 a

4.4.2.2. Physiological stress indicators

The analysis of physiological drought stress indicators revealed a significantly increased shoot activity of ascorbate peroxidase (APX) induced by NST on both soils and all genotypes. The activity was generally lower on the pH 5.6 soil as compared with pH 6.9 (Table 4.4). However, the relative NST-induced increase in APX activity was higher on the sandy-loam pH 5.6 (59-87%) than on the clay-loam pH 6.9 (12-27%).

Total phenolics, total antioxidants, proline, and the phytohormonal status were investigated in the two most contrasting genotypes with respect to the NST response, WR2, and WR6. There was a general trend for increased concentrations of phenolics and total antioxidants but a reduced accumulation of proline in the youngest-fully developed leaves, although the differences were not significant in all cases. The most drought-sensitive and NST-responsive genotype WR2 shows the highest NST-induced increase in the concentration of total phenolics and antioxidants under the most challenging growth conditions on the pH 5.6 soil but the lowest accumulation of the drought stress metabolite proline (Table 4.6).

Concerning the hormonal status, the IAA concentration in the youngest fully developed leaves (source tissue) was significantly increased by NST in all tested genotypes. Interestingly, in the less drought-sensitive genotype WR6, also the concentrations of GA and the stress hormones ABA, Salicylic acid (SA) and Jasmonic acid (JA) were substantially increased by NST in both soils (Table 4.7).

Table 4.6: Total phenolics (gallic acid equivalents), total antioxidants and proline status of the youngest fully developed leaf of the oilseed rape (WR2 & WR6) three days after recovery from the drought phase. Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments.

			Total phenolics [mg g^{-1}FW]	Total Antioxidants [%] Inhibition	Proline [mg g^{-1}FW]
WR2	**Sandy loam pH 5.6**	**Cont.**	1.18±0.03 b	38.14±1.47 b	2.13±0.06 a
		NST	1.51±0.03 a	56.33±1.86 a	1.38±0.08 b
WR6	**Sandy loam pH 5.6**	**Cont.**	1.44±0.03 b	52.24±1.71 a	1.55±0.07 a
		NST	1.77±0.08 a	57.16±0.80 a	1.35±0.03 a
	Silty loam pH 6.9	**Cont.**	2.06±0.07 a	63.30±0.94 b	1.10±0.04 a
		NST	2.28±0.05 a	72.38±1.32 a	0.73±0.05 b

Table 4.7: Hormone-status of the youngest fully developed leaf of oilseed rape (WR2 & WR6) three days after the recovery from the drought phase grown at 23 ± 2^0C. Data represent means and SE of 4 replicates. Different characters indicate significant differences between treatments.

			IAA	Zeatin	GA	ABA	JA	SA
			[ng g^{-1}]	[pg g^{-1}]	[ng g^{-1}]	[ng g^{-1}]	[pg g^{-1}]	[ng g^{-1}]
WR2	**Sandy loam pH 5.6**	**Cont.**	1.13+ 0.04 b	23.74±1.15 a	1.09+0.02 a	0.68+0.01 a	6.45±0.11 a	1.12+ 0.07 a
		NST	1.41+ 0.09 a	25.89±0.29 a	0.97+ 0.06 a	0.64+0.01 a	6.17±0.23 a	1.21+ 0.08 a
WR6	**Sandy loam pH 5.6**	**Cont.**	1.58+ 0.04 b	25.41±0.77b	0.98+ 0.04 b	0.62+0.02 b	5.84±0.04 b	1.31+0.02 b
		NST	2.95+ 0.15 a	49.86±1.22 a	1.85+0.09 a	1.38+0.03 a	13.10±0.83 a	2.50+ 0.06 a
	Silty loam pH 6.9	**Cont.**	0.69+ 0.03 b	11.63±0.45 b	0.38+ 0.01 b	0.28+0.01 b	8.53±0.25 b	0.11+ 0.02 b
		NST	2.76+ 0.03 a	52.52±1.72 a	1.69+ 0.02 a	1.32+0.02 a	11.8±0.39 a	2.30+ 0.08 a

4.5. Discussion

This study was conducted to analyze the potential role of nutrient seed treatment (NST) and its implications on drought stress responses during early growth in different OSR genotypes, on two different soils (sandy loam pH 5.6 vs silty loam pH 6.9).

4.5.1 Drought responses

In accordance with previous studies (Ashraf et al., 2013), the investigated OSR genotypes exposed to the drought conditions reduced the root and shoot biomass (5-32%) and leaf expansion (Table 4.1 and 4; Figs. 4.2 and 4.3). Reduction in shoot growth is a general response of drought or water deficit conditions due to the higher production of the stress hormone ABA (Achard et al., 2006, Qadri et al., 2006), causing inhibitory growth effects via declining levels of the growth hormone gibberellic acid, leading to increased accumulation of DELLA proteins as transcription factors triggering shoot growth inhibition (Liu and Hou, 2018; Wu et al., 2020). During the early stages of water limitation, this is an active adaptive response to reduce the surface area involved in transpiratory water losses (Daszkowska-Golec, 2016). In this context, ABA also triggers closure of stomata (Achard et al., 2006) and the deposition of leaf cuticular waxes (Kosma et al., 2009), as indicated also in this study (Fig. 4.2). Additionally, ABA is involved in stimulation of root growth to improve nutrient and water acquisition under moderate drought stress (Yuan et al., 2020). Moreover, ABA acts in a cross talk with jasmonic acid (JA) and salicylic acid (SA) as a central regulator of defense reactions against drought-induced over-production of reactive oxygen species (ROS), which leads to oxidative damage of membranes, photosynthesis pigments and other cell structures. (Kharadmand et al., 2014, Mondal & Khujuria 2000), observed as irreversible leaf damage (chlorosis, necrosis, wilting) also in this study (Table 4.1 and 4.4). However, under long-term water deficit, ABA-induced stomatal closure affects photosynthesis (Sharma et al., 1993) and finally carbon supply to the root system, leading to inhibitory effects on root growth.

The finding that in this study, both, shoot and root growth (Table 4.2 and 4.4) were affected by the one-week drought stress treatments, associated with irreversible oxidative leaf damage even after one-week recovery under well-watered conditions (Table 4.1 and 4.4), demonstrates that the intensity of drought stress already exceeded

the adaptive potential of the investigated OSR genotypes in terms of ROS detoxification. However, the genotype-dependent expression in severity of stress symptoms points to genotype or seed lot effects, with impact on the efficiency of the adaptive tolerance mechanisms. With respect to the intensity of stress symptoms, WR2 and WR3 were characterized as particularly sensitive, while WR4 and WR6 as more tolerant. To answer the question whether these observations are reflecting seed lot effects or true genotypic differences, additional tests with different seed lots of the same genotype would be required.

Additionally, the expression of drought stress symptoms was also affected by the soil type. Reduced plant growth even prior to drought stress and later a stronger expression of drought stress symptoms was noted in a sandy loam soil pH 5.6 as compared with a silty loam pH 6.9 (Fig. 4.4, Table 4.4). This may be explained by the higher sand content of the sandy loam, which limits the ability to retain water, resulting in a lower WHC as compared with the clay loam soil (sandy loam 21 %, silty loam 25 %) and more rapid and intense expression of water limitation, Nutrient limitation on the investigated pH 5.6 soil seems to be unlikely in face of a full N-P-K-Mg fertilization and a sufficient micro-and macronutrient status (Campbell, 2013), recorded even after recovery from the drought stress treatment (Table 4.5). However, high Mn shoot concentrations (> 300 µg g^{-1} shoot DM) due to high Mn solubility on the low-pH soil (Table 4.5) may be related with moderate toxicity effects, limiting plant growth particularly under well-watered conditions, since high soil moisture levels further promote Mn solubility (Marschner. 1995).

4.5.2 Effects of NST on growth responses and nutritional status

In both experiments, NST showed protective effects against drought stress in terms of reduced oxidative leaf damage, root growth promotion and shoot biomass production which were influenced by the genotype and the soil type. Generally, the expression of drought-protective NST effects was most apparent in genotypes with high drought sensitivity (WR2 and WR3) and under soil conditions promoting the expression of drought stress (sandy loam soil pH 5.6).

In both experiments, NST consistently increased root length development in all genotypes and to some extent even under well-watered conditions (Tables 4.2 and

4.4). Similar results were found also in the experiments conducted with Zn priming and Zn seed dressing winter rape and spring rape, exposed to optimal (23°C) and sub-optimal (12°C) root zone temperatures (Neumann et al., 2014), suggesting that the Zn component of the NST formulation provided an essential contribution to the protective effects. Particularly under water deficit conditions, an extended root system is crucial for water (Zhang et al., 2014; White et al., 2015) and nutrient uptake. The beneficial effect of Ca and Zn seed priming was also reported under water deficit conditions in lentils and faba beans, respectively (Farooq e al., 2020; Farooq e al., 2021). Mackay and Barber (1985a) reported that especially the acquisition of sparingly soluble nutrients under severe drought stress is much stronger limited by root growth inhibition (50-70%) than by the drought-induced limitation in the solubility of the respective nutrients (10-30%). Accordingly, beneficial effects of NST on root growth, recorded for all tested genotypes in experiment 1 (Table 4.2) translated into a general trend for improved shoot accumulation of all investigated nutrients, although not significant in all cases (Table 4.2). Significant effects were mainly found in the most drought affected genotypes. Also, the improved NST-induced plant performance on the sandy loam soil pH 5.6 in experiment 2 could be associated with increased root length development observed across the genotypes (WR2 29%, WR5 22%, WR6 20%) (Table 4.3), enabling a more efficient exploration of a larger soil volume, for the acquisition of water and essential sparingly soluble nutrients. This aspect is particularly important for non-mycorrhizal plant species, such as OSR, completely dependent on root-mediated nutrient acquisition and consequently lacking also drought-protective physiological effects of arbuscular-mycorrhizal associations (Begum et al., 2019).

4.5.3 Interactions of NST with physiological stress indicators

The increased root length formation induced by NST was associated with increased leaf ascorbate peroxidase (APX) activity, particularly expressed on the most drought-affected sandy loam soil, which acts as a critical enzyme for drought stress-induced ROS detoxification (Caverzan et al.,2012). The protective enzymatic system for ROS detoxification (superoxide dismutase, catalase, ascorbate peroxidase etc.) depends on various sparingly soluble micronutrients (Zn, Mn, Cu, Fe) acting as co-factors (Cakmak 2000; Caverzan et al.,2012), which were at least partially provided by the NST formulation. The same holds true also for non-enzymatic ROS detoxification via

compounds with antioxidative properties (e.g. ascorbic acid and phenolics), requiring Mn and Cu as co-factors for their biosynthesis (Datnoff et al., 2007). Accordingly, also the shoot accumulation of phenolics and antioxidants was increased by NST (Table 4.6) particularly under the most severe drought stress conditions (WR2 on pH 5.6 soil). Due to low solubility, micronutrient acquisition is particularly affected in dry soils, even when the supply is sufficient under well-watered conditions as indicated in Table 4.5. Excessive stress-induced ROS formation leads to oxidative cell damage, indicated e.g. by irreversible chlorosis and necrosis of leaves, which was obviously mitigated by NST-mediated micronutrient supply (Table 4.4). However, also Ca seed treatments as another component of the NST formulation can contribute to membrane protection and oxidative stress alleviation (Maathuis et al., 2009; Hussain et al., 2016).

Moreover, excessive ROS production can induce also oxidative degradation of indole acetic acid (IAA) as a major shoot-borne regulator of root growth (Cakmak et al. 1989). Accordingly in our study, a positive correlation (R^2=0.8953) between root length and APX activity may suggest APX-mediated ROS detoxification induced by NST (Fig. 4.5), thereby reducing oxidative auxin degradation, and finally resulting in elevated auxin concentrations (Table 4.7) triggering the observed stimulation of root growth (Table 4.4).

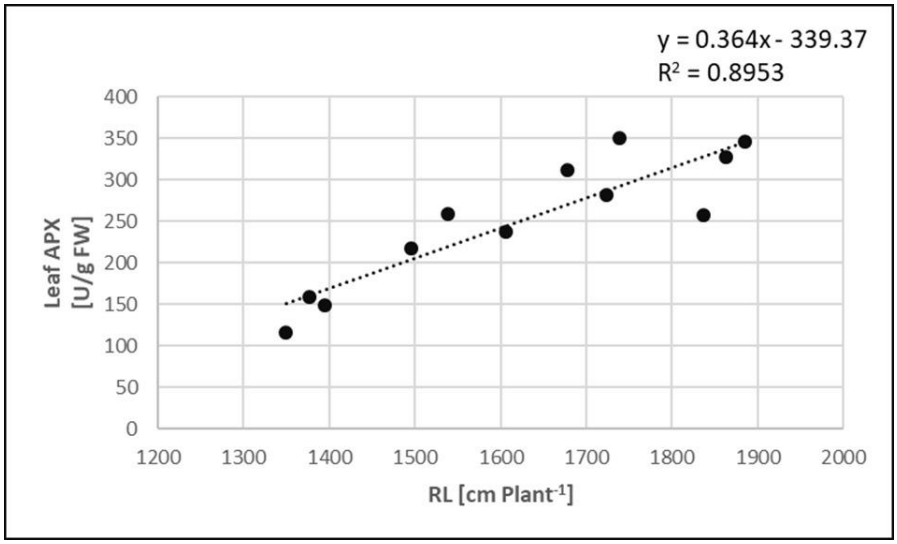

Fig. 4.5: Relationship between leaf ascorbate peroxidase (APX) activity and root length (RL) in the investigated OSR plants after 7d recovey from a 7 d-drought stress period at 40% soil water-holding capacity.

Therefore, micronutrient supplementation by nutrient seed treatments may support ROS detoxification, providing sufficient auxin supply for the stimulation of shoot and

root growth, also documented in the literature (Cakmak et al., 1989, Moradtalab et al., 2018). Since APX as many other peroxidases is an iron-protein (Caverzan et al.,2012), the Fe component of the NST formulation may be of particular importance in this context, although at the end of the recovery period, the Fe status was sufficient in all cases. This underlines the importance to perform intermediate nutrient analyses also during the drought period for future studies.

Also the stress hormone ABA, which acts as a central regulator of adaptive drought stress responses in a cross-talk with jasmonic acid and salicylic acid (Riemann et al. 2015) was upregulated together with JA and SA by NST at least in the less drought-sensitive genotype WR6 (Table 4.7). Apart from the down-regulating of stomatal aperture and shoot growth, ABA is also involved in drought-adaptive root growth stimulation (Yuan et al., 2020), induction of ROS defense responses (Mittler & Blumwald, 2015), and increased formation of cuticular waxes to reduce transpiration (Martin et al., 2017). Higher NST-induced levels of ABA, JA and SA may therefore indicate involvement in the induction of these adaptive responses and also explain the more intense and homogeneous cuticula formation observed in the drought-affected OSR plants (Figs. 4.1 and 4.2). The same situation (upregulation) also applied for the growth hormones zeatin and gibberellic acid (Table 4.7) known to stimulate shoot growth, which may indicate a better drought stress recovery also reflected by lower accumulation of the stress metabolite proline (Kaur & Asthir, 2015) in the NST variants (Table 4.6).

4.5.4 Stress priming effects

The finding that increased levels of the stress hormones ABA, JA and SA were detectable after recovery from the drought stress treatment may also point to a longer-lasting stress priming effect of NST, expressed at least in the less stress-affected WR6 genotype, which may improve the adaptive responsiveness also to potentially up-coming stress events. Overlapping signaling events of drought stress adaptations also with other stress factors such as salinity, heat or cold stress (Verma et al. 2016) may therefore suggest a broad stress-protective spectrum of NST-based applications as indicated also by literature reports (Mondal and Bose, 2019; Ullah et al. 2019). The longevity of the observed priming effects requires further investigation. However, recent reports on the effects of similar multi-nutrient seed treatments used in this study

(Wallenhammar & Stoltz, 2019), revealed shoot growth-promoting effects in field trials with spring rape detectable until early flowering (BBCH 60). The finding that the nutrient status in general and not only the treated nutrients were elevated (Table 4.5) points to a more indirect mode of action via beneficial NST effects on root system establishment during early growth, finally contributing to improved nutrient acquisition.

Nevertheless, the beneficial NST effects on early plant establishment do not always result in final yield improvements (Moradtalab et al., 2018; Wallenhammar and Stoltz, 2019) and the translation into yield benefits may depend also on the expression of the compensatory potential of a given genotype to recover after a stress event. However, due to the extremely low amounts of nutrients and active ingredients required, NST-based starter fertilization strategies can be regarded as cost-effective and resource-efficient approaches with prophylactic stress-protective potential for the early growth of OSR.

Chapter 5

5. General Discussion

Seed treatment technologies are a rapidly growing market with a current market share of USD 6.4 billion in 2020 and is expected to reach USD 11.3 billion by 2025, with and annual growth rate of 12.1% (Markets & markets, 2020).

Seed treatments involve the application chemical, physical and biological agents applied prior to sowing to improve the health of crops. It can help to control seed and soil-borne diseases and pests, reduce germination time, promote seedling establishment and resistance to environmental stress factors finally contributing to improved overall productivity. On the basis of function, seed treatments can be classified into seed protection agents including fungicides and particularly insecticides with the currently highest market share of 60%. Targeting the the diseases and pests with lower dosages of active ingredients per hectare make it a sustainable agricultural practice, using these products and processes (Allied Market Research, 2020). However, according to recent developments, the highest growth rate forecasts are made for the second group of agents termed as seed enhancers. These products comprise biologicals including beneficial microorganisms with pathogen-suppressive and biofertilizer properties, micronutrients, plant growth regulators, seed priming and seed disinfection technologies. The increasing interest is related with rising awareness of environmental problems related to regular use of chemical plant protection agents and resistance problems resulting in more stringent governmental regulations. Moreover, an increasing impact of environmental stress factors such as drought, temperature extremes or heavy rainfall events related with climate change are interesting application fields with prospects for seed enhancers. Seed treatments are mostly used in high productivity cereals and grain crops, such as wheat and maize or oilseed crops like soybean or oilseed rape (OSR). In OSR chemical seed protection was the dominant application for seed treatments but in the recent past also seed enhancement is attracting increasing interest for the reasons mentioned above. Growth limiting environmental factors like temperature extremes, drought and occasional waterlogging demand a continuous effort to be made in the direction of reduction and alleviation of detrimental environmental effects. In the current study, the

emerging nutrient seed priming (particularly zinc), and nutrient seed dressing techniques were characterized and adapted for optimal dosage for oilseed rape (Brassica napus L.) evaluated in terms of the potential for alleviation of abiotic stress factors like low temperature and drought, as a contribution to product development, meanwhile used as a standard treatment for OSR hybrid seeds in practical applications.

5.1 Seed priming and seed dressing

Among the various seed treatment technologies including seed priming, seed coating, seed dressing, and seed pelleting, seed priming (SP) and seed dressing (SD) were addressed in the present study. Seed priming is a technique in which seeds are hydrated by soaking up to the point that is just before the radical protrusion (Chen et al., 2011).

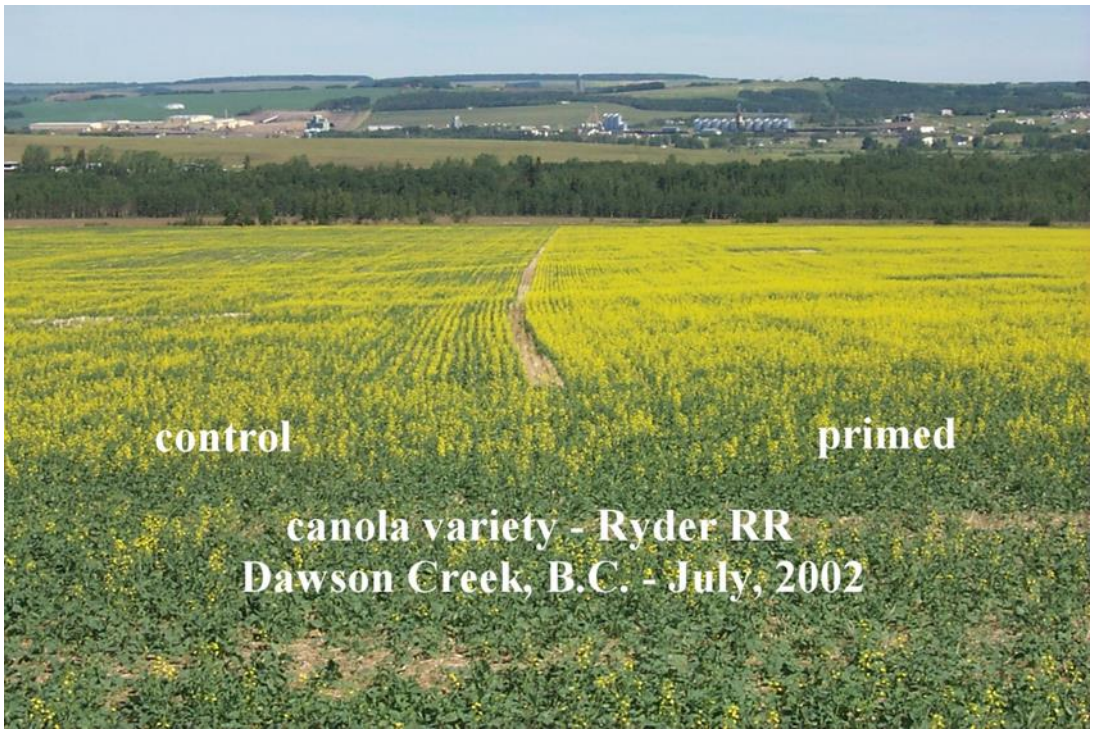

Fig. 5.1 Impact of water priming on the acceleration of OSR plant development under field conditions in Canada (photo by courtesy of Volker Römheld)

The seeds are then dried-back and used for sowing. Extended hydration of seed may harm the germinating seedling while drying (Ajouri et al., 2004). Therefore, the duration of priming should be carefully accessed. Water seed priming helps to break

seed dormancy, initiates the physiological process and mobilizes carbohydrates (Bawely and Black, 1982;). It is also known for reducing germination time, improving germination rate and establishing a uniform crop stand in various field crops (Harris, 1996; Harris and Johnes, 1997; Ajouri et al., 2004). Positive effects on early seedling growth under suitable and growth-limiting conditions have also been observed in maize, sunflower, rice and durum wheat (Rehman et al., 2011; Kaya et al., 2006; Goswami et al., 2013; Fercha et al., 2013).

Nutrient seed priming is the soaking of seeds with essential or protective mineral nutrients, combined with back-drying before sowing. The applied nutrient concentrations but also the duration of the priming period and even the speed of seed imbibition are critical factors determining the magnitude of an outcome (Imran et al., 2016) and therefore, need to be optimized for each crop for attaining highest possible positive plant growth effects. Excess concentrations of the primed nutrients can induce toxicity effects and potentially damage seed and inhibit seed germination (Roberts 1948). Particularly in large-seeded leguminous crops, such as soybean, rapid water uptake during the pre-soaking period can cause imbibition damage (Imran et al., 2016) known as a stress factor also during germination under excess soil moisture levels (Powell and Mathews, 1979), which can impair seed vigor and seedling development. Nutrient seed treatments (NST) have attracted increased interest in recent years. The nutrient seed priming potential, using essential micro and macronutrients, has been tested in various crops, both, under optimal and sub-optimal plant growth conditions. In this context, for seed priming with micronutrients such as zinc but also manganese and iron, also applied in combinations promising results have been reported in recent past. Zinc in the form of $ZnSO_4$ is a frequently used mineral nutrient for seed priming (Begum et al., 2014; Imran et al., 2015, 2018; Rehman et al., 2015). It is an essential plant micronutrient involved in various plant physiological processes with stress-protective properties. Therefore, based on the results of initial testing of different micronutrients including Zn, Mn, Fe Cu and also B, so far considered as one of the most important micronutrients in OSR culture, in the current study, a primary focus was placed on zinc seed priming and its responses to different application doses for oilseed rape (Brassica napus L.) (chapter 1). This strategy was further extended by a comparative approach also considering seed dressing and multiple nutrient seed treatments.

In the present study, an optimum pre-germination soaking time duration of 24 hours was determined for the priming treatments by soaking seeds into the water for 8, 16, 24, and 32 hours (data not shown). Munz et al. (2017) demonstrated the water entrance pathway into the seed between 0 and 24 h, using magnetic resonance imaging (MRI) technique, suggesting 24 hours of soaking time appropriate for seed hydration. However, seed water uptake mainly depends on the seed architecture and seed coat properties, which may differ even in different seed lots. Accordingly, Imran et al. (2016) reported much shorter optimum priming periods of only 12 h in soybean. For determination of the optimal treatment dosage, seeds were tested over a wide range of zinc concentration (10 µM – 100 mM) in the priming media. Out of the tested zinc dose ranges, a potentially viable dose range (10-75 µM) was selected for further optimization and investigating the response under various plant growth conditions, mainly in model experiments and also under field conditions.

5.2 Seed treatment effects on the seed nutrient status

Seed nutrient reserves are crucial for early seedling growth and development but can affect also later stages of plant development either indirectly by promoting proper seedling establishment, resulting in improved health, resilience to environmental stress factors and nutrient acquisition efficiency, which can translate into improved general performance and yield. Due to the low absolute demand, direct longer-lasting effects are possible in case of micronutrients. Accordingly, wheat seeds with low Mn contents produced lower grain yields (Marcar and Graham 1986; Sing and Bharti 1985). Barley seeds with high zinc contents showed better vegetative growth compared to the seeds with low zinc contents (Genc et al., 2000). Since seed zinc reserves have a proven early seedling vigor and growth improvement effects (Grewal & Robin, 1997), nutrient seed priming is a way to improve the seed nutrient reserves. Seeds enriched with zinc through seed priming enhanced the seed nutrient status and final yield in barley and maize (Ajouri et al., 2004; Harris et al., 2007; Imran et al., 2013, 2015).

Similarly, in the present study, the seed zinc status has also been improved through zinc seed priming by up to 141% in high seed zinc inherited hybrid (SR1) and even by 284% in a hybrid (WR1) with low inherited seed zinc (chapter 2). The different uptake

response may be related to genotypic differences of the investigated OSR hybrids. However, even seed lots coming from different fields may express site-specific differences in seed zinc status resulting from different soil types, soil pH, environmental stress factors (chilling, heat or drought) or fertilization strategies. (chapter 3). Also, the localization of the primed zinc in oilseed rape is still unknown. Imran et al.,(2018) recently investigated the primed zinc deposition sites in maize seeds, which were found in the aleurone layer and partly in the seed embryo but a major fraction >60% sticking to the seed coat, representing the zinc deposition is cereals. In non-cereal/ dicots like oilseed rape, the deposition sites may differ. Also, the fate and potential plant availability of primed nutrients on the seed coat requires further investigation.

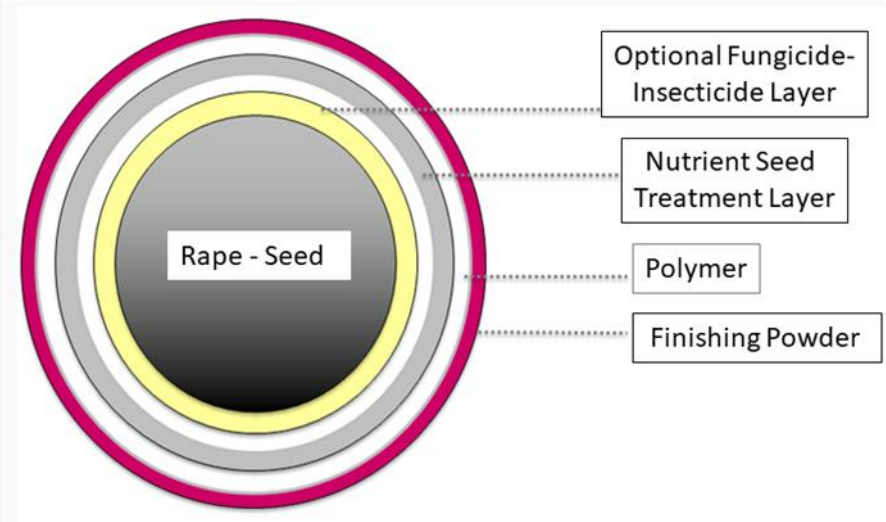

Figure 5.2: Optimized seed dressing formulation used in the experiments (S. Goertz, Rapool STWG)

Along with seed priming, seed dressing/coating is another seed enrichment technique that has been widely adopted particularly for commercial applications since no mechanization and energy requirements are necessary for re-drying, combination with seed protection agents (insecticides, fungicides and generally easier handling is possible. Unlike seed priming, in seed dressing, the seed enriching media are sticking to the outer seed surface without any alteration in the seed physiological status. Various chemical formulations are commercially available for seed treatment containing essential plant nutrients as a dressing/coating media. The beneficial effects of seed dressing have already been documented in sunflower, maize, wheat, cowpea, common beans and soybean (Ramesh and Thirumurugan 2001; Sing, 2007; Biscaro

et al., 2009; Masuthi et al., 2009, Bradacova et al. 2016). Since no data was found on the oilseed rape seed dressing, it was worth testing and reporting the valuable results. In the recent study, nutrient seed dressing was included to evaluate the potential. Seed dressing was performed with zinc as $ZnSO_4$ alone (chapter 2) and in a combination of selected essential plant nutrients (chelated Zn, Mn, Fe) and macro-nutrients (Ca, K) (chapter 4).

5.3 Seed treatment effects on germination

The seed germination process starts with water imbibition, associated with metabolic activation and ends with radicle emergence (Bewley 1997). Seed priming allows the seed to complete the initial and intermediate phases of germination by activating seed metabolism. Subsequently, this process is interrupted by re-drying. The process of priming finishes as soon the seed metabolism is fully activated and ready to protrude the radicle The metabolically pre-activated seeds have obvious advantages over non-primed seeds, reported to speed up the germination process, improve germination rate, early seedling growth and vigor in many crop species (Harris et al., 1996; Harris & Jones, 1997; Ajouri et al., 2004). In oilseed rape, water seed priming increased germination by 33% and reduced germination time by 50% under low-temperature stress (Zheng et al., 1994).

Despite the fact, that for seed priming improved germination has been frequently reported, the current study recorded no clear germination improvements, neither for seed priming nor for seed dressing treatments conducted under optimum temperatures for plant growth and germination (23°C). This may be explained by comparatively high germination rates ranging between 80-88%, where further optimization is difficult to demonstrate However, strong positive germination trends were noted under low root zone temperature (12°C), leading to more homogenous seedling development of spring OSR with Zn seed priming (Chapter 2). Inhomogeneous germination at low soil temperature has been already reported by Li et al., 2013 and Hussain et al., 2016 in wheat and rice, respectively. Causes of a germination heterogeneity at low temperatures comprise membrane injury, overproduction of reactive oxygen species and the reduction of water uptake (Xing & Rajashekar., 2001; Nayyar et al., 2005; Li et al., 2013). Accordingly, at 12°C soil

temperature, untreated seeds reached only germination rates of 20%. Increased germination rates (+112.5%) and (+87.5%) corresponding to the treatments Zn.25 and Zn.50, respectively, indicated the stress-alleviatory s properties of water and zinc priming acting together at low soil temperature (Chapter 2). However, higher priming doses with Zn.75 exhibited a decreasing trend in germination rate, pointing to a potential Zn toxicity effect, which underlines the importance for optimization of the application doses.

5.3. Seed treatments effects on plant growth

a) Shoot and Root development

Both seed priming and seed dressing are known to support plant growth and development, particularly at the initial stages of seedling development A better early seedling growth as a result of ZnSP has been reported in wheat, chickpea rice, maize, and soybean (Abdul Rehman et al., 2015, Aman Ullah et al. 2019, Prom-u-thai et al., 2012; Imran et al. 2013). The magnitude of responses may vary with changing seed treatment techniques and plant growing conditions, seed quality, and target plants.

In the present study, a smaller growth-related response was recorded, when zinc primed. Zinc dressed seeds were grown in optimal plant growth conditions (23°C, full NPK supply and optimum soil moisture) as compared with the presence of stress factors, such as low root zone temperature (12°C) (Chapter 2). However, regardless of the method of treatment, there were no substantial differences in shoot growth in terms of dry shoot weight (SDW) in the experiments conducted under optimal growth conditions (Table 2.2). By contrast, root growth was significantly improved by 31% and 49% corresponding to the best responsive treatments, each in SP and SD, at 10 and 14 days after sowing, respectively (Table 2.5, 2.7). This demonstrates the functional equivalence of both seed treatment techniques, as previously shown also or maize (Imran et al. 2013; Bradacova et al. 2016).

Similarly, also winter OSR plants grown with ammonium-phosphate depot fertilization (Fig. 5.3) preferentially showed improved root growth responses leading to faster exploitation of the fertilizer depot, without simultaneous stimulation of shoot growth in response to ZnSP treatments. Also, root development outside the depot zone and

particularly in deeper soil layers remained unaffected. This pattern of shoot and root development is in accordance with the requirements for the establishment of winter hardness in winter OSR with intense and healthy root development for improved winter survival and regeneration in spring and improved nutrient acquisition but at the same time avoiding excessive shoot growth before winter (Bauer, 2011). In this context, the stabilized ammonium depot as preferential nitrogen source may reduce root-induced cytokinin production known to stimulate preferential shoot growth responses (Walch-Liu et al., 2000; Rahaju et al., 2005; Römheld et al., 2008). At the same time, local ammonium-induced rhizosphere acidification counteracts P fixation and improves P availability (Table 3.3) particularly on neutral – alkaline soils (Jing et al., 2010; Bischoff, 2012).

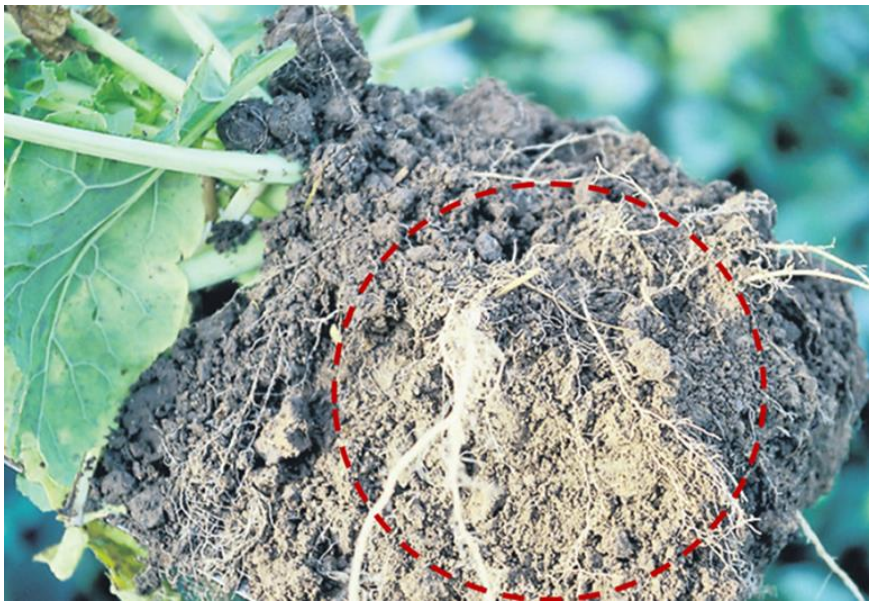

Figure 5.3 Fine root proliferation in the ammonium depot zone under field conditions (Bischoff, 2012).

Taken together, the results suggest that Zn seed treatments may offer an option to further optimize ammonium-phosphate placement strategies in winter OSR to adapt N fertilization in autumn, limit N losses during the winter period, improve the plant P acquisition (Bischoff, 2012; Bibinger, 2018) particularly and speed up seedling development with particular relevance for late sowing approaches.

b) Impact of stress conditions

In contrast to optimal growth temperatures, the plant response to zinc seed priming was much more intensively expressed at low RZT (12^0C), as a common stress factor, e.g. during early seedling development in spring OSR or ultra late sowing approaches in winter OSR. At five weeks after sowing, both, shoot and root growth were highly responsive to the primed zinc treatments suggesting a clear starter push. The highest response was noted in the Zn.75 treatment, SDW (+83%) RDW (+125%) with a doubling of root length and increased fine root production, compared to the untreated seedlings (Table 2.11). The seed priming potential can be explored to its highest in stressful plant growth conditions and it performs better under limiting plant growth conditions (low/high temperature, drought, salinity) compared to the optimal plant growth conditions. ZnSP proved as abiotic stress alleviatory strategy in barley and maize (Ajouri et al., 2004; Imran et al., 2013; Imran et al., 2018). A better seedling growth at low RZT could be a combination of a starter push by the priming and protective zinc effect on plant growth-related hormones, i.e., auxin, resulting in maintaining a suitable level for plant growth. It is now well known that Oxidative damage is expected, as a response of most of the plant stress, including low-temperature stress, by producing harmful reactive oxygen species (ROS) (superoxide radical (O^-) hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). These ROS damages the cell membranes, interrupt enzyme activation and degrade proteins (Allen and Ort, 2001). Since zinc is a co-factor of superoxide dismutase SOD, an enzyme involved in ROS detoxification (Cakmak, 2000), the externally applied zinc may have enhanced SOD activity and minimized the damage caused by the free oxygen radicals under low RZT conditions. This scenario has been recently confirmed for Zn/Mn seed treatments in maize exposed to similar low RZT (12°C) treatments (Bradacova et al., 2016; Moradtalab et al. 2018; 2020). In this case, it was demonstrated that even with high available Zn and Mn supplied in nutrient solutions at 12°C RZT, root uptake of the micronutrients was strongly limited and protective effects were only detectable when the nutrients were supplied in advance by seed treatments. In soil-grown plants, this resulted in higher activities of SOD and increased accumulation phenolics, total antioxidants and auxins, leading to less oxidative leaf damage and improved root and shoot growth.

In a similar scenario, multi-nutrient seed dressing of OSR performed in the present study, including also Zn, Mn and Fe as micronutrients, resulted in improved root development and, stimulation of cuticula formation and reduced irreversible oxidative leaf damage after recovery from a one-week drought stress period at 40 % soil water-holding capacity. Similar to cold stress, uptake of sparingly soluble mineral nutrients (including micronutrients) is also impaired under drought stress conditions by limitations of nutrient diffusion and impairment of root growth and activity (Neumann and Römheld, 2002). The resulting nutrient limitations may be at least partially supplemented by prophylactic nutrient seed treatments as earlier reported for cold stress (Bradacova et al., 2016; Moradtalab et al., 2018). Similarly, in the drought stress scenario, this was associated with increased accumulation of antioxidants and phenolics in the shoot tissue, increased expression of enzymatic ROS defense, indicated by increased activities of the Fe-dependent ascorbate peroxidase as a key enzyme for drought stress-induced ROS defence (Caverzan et al.,2012), which correlated with increased auxin levels as a major regulator of lateral root development. Micronutrients, such as Zn, Mn, Fe and Cu are involved in the biosynthesis of various enzymes involved abiotic stress alleviation. Pants need to regulate the level of reactive oxygen species (Apel and Hirt, 2004), which is generally enhanced under various abiotic and biotic stress conditions Apart from causing oxidative membrane damage, the increase of ROS in the plant tissues can quickly induce oxidative auxin degradation as demonstrated for Zn-deficient plants by Chakmak et al. (1989). These stress-induced escalations of ROS are scavenged by the micro-nutrient-dependent enzymes directly involved in ROS detoxification or in the production of phenolic antioxidants (Datnoff et al. 2007).

Based on the comparison of different OSR hybrids, genotype, or seed-lot-dependent differences in the expression of stress symptoms were observed in this study (Table 4.1). The protective effects of the nutrient seed treatment described above were most distinctly expressed in the more drought-sensitive hybrids and also affected by soil properties with stronger effects on a light sandy loam with a lower water holding capacity as compared with a silty loam (Table 4.4).

Taken together, the results suggest that micronutrient seed treatments can act as a promising approach to counteract the impact of various stress factors during germination and seedling establishment, primarily related with the functions of

micronutrients in ROS detoxification as a common constraint generally related with the stress exposure of plants. In this context, micronutrients internalized prior to the onset of stress exposure can at least supplement limitations of root growth and activity during the stress period.

5.4 Seed treatment effects on the plant nutritional status

Many studies indicate high seed nutrient contents as an assurance to promote early seedling growth and vigor. Genc et al. (2000) and Marcar and Graham 1986 highlighted the importance of seed Zn and Mn reserves for the seedling establishment of barley (*Hordeum vulgare L.)* and Wheat (*Triticum aestivum*) The dependency is greatest until a functional root system contributes to the uptake of nutrients for the growing seedling.

Imran et al., 2015 reported the sufficiency of primed Zn and Mn seed reserves in maize for at least three weeks during a culture period in a Zn/Mn deficient nutrient solution. The present study with unprimed OSR demonstrated a time period of fewer than five days in soil culture before the developing seedling was dependent on root uptake of Zn and Mn, as indicated by the comparison of the seed reserves with the seedling contents (Table 2.6). Even under optimum soil temperatures, water and nutrient availability, the related micronutrient supply was obviously suboptimal, since root growth was further stimulated by Zn seed treatments (Fig. 2.6). Seed priming effects on Zn contents lasted for about 10 days. Thereafter nutrient uptake, in general, was improved, related with the promotion of root growth, which was induced by the priming treatment, finally leading to higher levels also of the other nutrients such as P and Mn. Similar results were observed also for the seed priming treatments in OSR seedlings exposed to low root zone temperature or after Zn seed dressing treatments described in chapter 2 and also for Zn/Mn seed treatments in maize (Bradacova et al., 2016; Moradtalab et al., 2018). These findings suggest that although the time window for direct effects of micronutrient seed treatments on the nutritional status of seedlings is quite limited restricted to a maximum time period of 2-3 weeks, longer-lasting and even broader effects on nutrient acquisition in general can be expected due to root growth-promoting potential of micronutrient seed treatments. This seems to be of particular importance e.g. for small-seeded plant species with limited seed reserves including OSR, depending on root-mediated nutrient acquisition already during very early stages

of seedling development and under conditions when root development and the nutrient uptake capacity is limited by external stress factors. Since plant nutrient acquisition is not only directly determined by root growth and activity but also by plant-microbial interactions in the rhizosphere, the question arises to which extent these interactions are also influenced by nutrient seed treatments. While numerous reports exist on interactions of pesticide seed treatments with the rhizosphere microflora, the aspect of nutrient seed treatments in this context has not been addressed in the literature so far.

5.5 Seed dressing effect on phytohormonal balances

Negative effects of metabolic Zn limitations on the auxin status of higher plants, either by limited Zn availability of the growth substrates, induced Zn limitations due to impaired root activity or particularly high demands under stress conditions has been frequently reported in the literature (Cakmak et al., 1989; Bradacova et al., 2016; Moradtalab et al., 2018; 2020). This has been related with increased oxidative auxin (IAA) degradation as a consequence of impaired enzymatic ROS defence depending on sufficient supply of Zn acting as enzymatic co-factor finally leading to depressions of shoot and root growth due to IAA limitation (Cakmak et al., 1989), which can be mitigated by external micronutrient supplementation via seed treatments (Moradtalab et al., 2018; 2020). The present study demonstrated that this may hold true also for other micronutrients, such as Fe, determining the function of ascorbate peroxidase (Chapter 4) as a key enzyme for ROS detoxification under drought stress (Table 4.4). While protective effects of nutrient seed treatments against drought stress were associated with an improved auxin status of all investigated OSR hybrids (Table 4.7) additionally the levels of stress-related hormones, such as abscisic acid (ABA), jasmonic acid (JA) and salicylic acid SA) were elevated exclusively in the hybrid with the lowest sensitivity to the drought stress treatment. (Table 4.7). This effect was detectable even after recovery from the stress period. Since ABA is regarded as a major regulator of various abiotic stress adaptations in higher plants including stress factors such as drought, salinity, cold stress, metal toxicities etc. (Vishwakarma et al. 2017) involving cross talks also with JA, SA and ethylene (Figure 5.4). This may indicate the induction of a stress priming effect by the nutrient seed treatment acting

after recovery from the stress period and facilitating the expression of adaptive responses to various environmental stress factors. Accordingly, Cakmak et al. (1989)

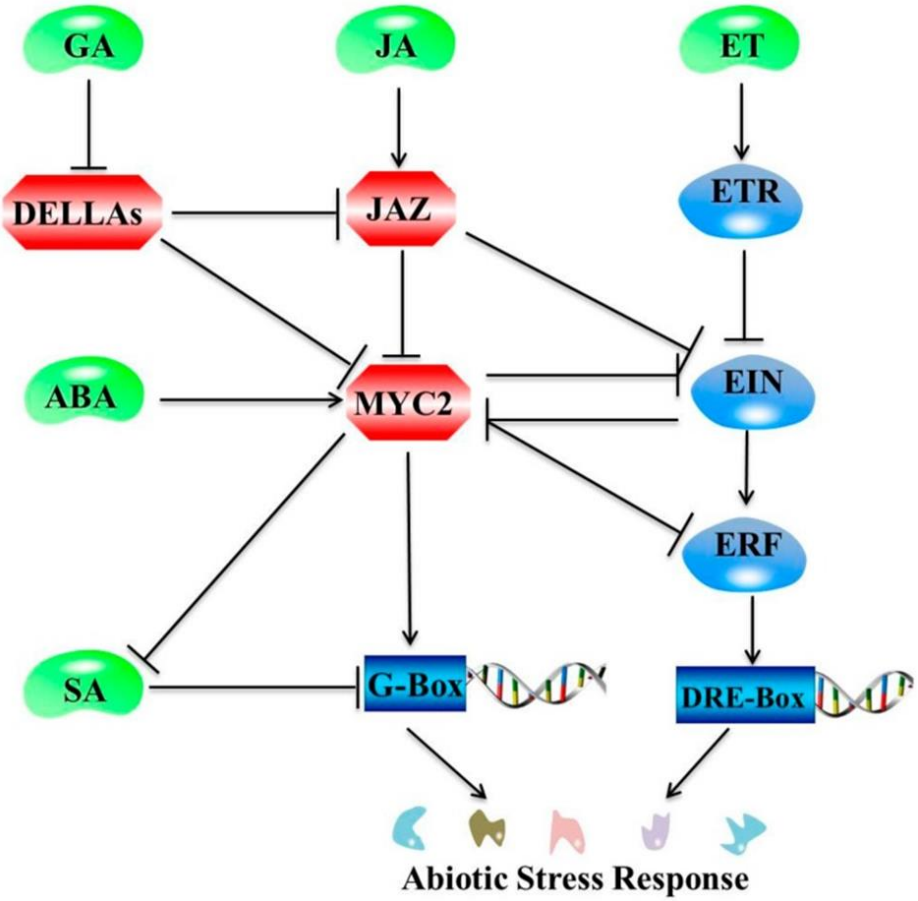

Figure 5.4: Schematic diagram representing the crosstalk of JA with other plant hormone signalling pathways. Note: Positive and negative regulatory actions are indicated by arrows and lines with bars, respectively. MYC2 is the major component involved in interactions between JA and gibberellin (GA). DELLAs interact with JAZ repressors, relieving MYC2 from JAZ repression, and facilitate JA-mediated defense responses by the activation of MYC2. MYC2 is also positively regulated by ABA. Conversely, MYC2 inhibits salicylic acid (SA) regulation of abiotic stress response genes. The JAZ inhibition of EIN mediates JA and ET signalling synergy in plant resistance, whereas the reciprocal counteraction between MYC2 and EIN mediates JA and ethylene (ET) signalling antagonism (Wang et al., 2020)

and more recently, Wang et al. (2012) reported that ABA levels declined in Zn-deficient plants. However, the longevity of the postulated stress priming effects remains to be established. In accordance with a protective effect of the postulated hormonal stress adaptations also the levels of important growth regulators, such as gibberellic acid and cytokinins (zeatin) increased in the respective plants (Table 4.7).

5.6 Potential grain yields

The ultimate goal to improve crop plant health, growth and development is to enhance the final economic yields., So far, several studies reported an increase in seed yields after Zn seed treatment in different crops, such as rice (27.6 - 28.3%) (Slaton et al., 2001; Shivay et al., 2008), wheat (34.4%) (Arif et al. 2007) chickpea (36.0%) (Arif et al., 2007) and maize (27.1%) (Harris et al., 2007). The increase in seed yields could be a result of enhanced germination, better early seedling establishment and stress protection of plants. In the present study, root length was the most prominent trait positively increased in response to most of the tested seed treatments. Accordingly, Kosenley et al., (2012) reported that root length improvement during early seedling growth in oilseed rape coincided with greater grain yields. However, the present study also demonstrated that no direct long-lasting nutritional benefits can be expected from nutrient seed treatments (NST) and the observed growth responses in later stages of plant development must be regarded as indirect consequences of initial direct nutrient effects. Whether these benefits finally translate into yield improvements as a consequence of stress-protective properties of nutrient seed treatments strongly depends on various other factors, such as the compensatory potential of the respective plants, which can also overwrite initial beneficial effects of a nutrient seed treatment (Neumann et al., 2018; Wallenhammar and Stoltz, 2019). Similarly, in the absence of additional environmental stress factors, the limited expression of NST benefits does not translate into yield improvements. However, due to the extremely low amounts of nutrients and active ingredients required (Bradacova et al., 2016), NST-based starter fertilization strategies may provide cost-effective and resource-efficient approaches with prophylactic stress-protective potential for the early growth of OSR. Nevertheless, at the moment NST-based approaches must be regarded rather as complementary stress-protective strategies with plant-strengthening potential and can by far not replace additional measures to cope with the increasing impact of environmental stress factors, particularly during later stages of plant development.

5.7 Future Outlook

Seed treatments are an emerging technology. However, the application potential and viability is by far not completely understood for many crop species. In the current study, the potential of seed priming and seed dressing approaches has been explored in oilseed rape (Brassica napus L.). The results highlighted a distinct response of oilseed rape to the tested seed treatments, particularly for micronutrients, such as Zn. Mn and Fe suggested an improved viability particularly under environmental stress conditions. While the available results point to a central role of the investigated NST strategies in the improvement of root development, ROS detoxification and interactions with hormonal balances, the molecular and physiological basis of the protective effects is far from being completely understood. This applies for related changes of gene expression as well as consequences for the metabolome but also for related processes in the rhizosphere and plant-microbial interactions.

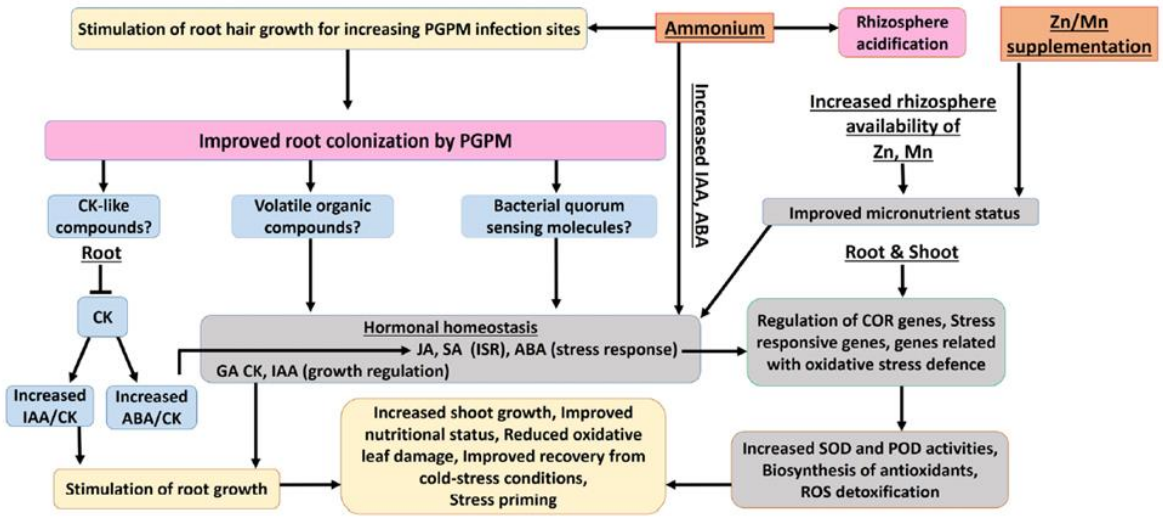

Figure 5.5: Proposed interactions of stabilized ammonium fertilization, PGPM inoculation and Zn/Mn supplementation contributing to increased cold tolerance during the early growth of maize (Moradtalab et al., 2020).

Another promising approach is the exploitation of additive or even synergistic effects of different agents used for NST, including also fertilization strategies. An impressive example was recently published by Moradtalab et al. (2020), dissecting synergistic interactions of micronutrient seed treatments with beneficial microbial consortia used

as inoculants and stabilized ammonium starter fertilization in the alleviation of cold stress effects in maize at the molecular and physiological level (Figure 5.5).

Particularly high OSR yields in combined applications of ZnSD with localized ammonium phosphate starter fertilization in a striptill system may point into a similar direction. Also, further developments of the commercial OSR seed dressing formulation investigated in the present study, by including beneficial microorganisms (Wurzel-Plus Beize natur, Rapool Ring GmbH) are a step forward into this direction.

Seed priming just before sowing may provide an easy and affordable on-farm strategy for seed enhancements, available also for resource-poor farmers, having small landholding (with low soil fertility) in developing countries. However, combination with commercial seed dressings, e.g. with plant protection agents is difficult, and back-drying of seeds after priming treatments is a challenging and cost-ineffective technical process for industrial applications, where seed dressing treatments represent a more suitable approach. The functional equivalence of both seed enhancement techniques demonstrated in this study underlines the flexibility of the proposed strategy.

Chapter 6

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