Rhizosphere engineering

Improving plant tolerance to drought by modifying the physical and biological properties of the rhizosphere

Dissertation

to obtain the Ph.D. degree

In the International Ph. D. Program for Agricultural Sciences in Goettingen (IPAG)

at the Faculty of Agricultural Sciences,

Georg-August-University of Göttingen, Germany

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Göttingen, March 2018

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Date of dissertation: 17 March 2018

“To those

who are ready facing challenges bravery

to follow their dreams”

Summary

Among various biotic and abiotic stresses, drought is one of the most limiting factors compromising plant growth and crop productivity. Mobility and availability of nutrients are controlling factors for plant growth and development and they are strongly limited by soil drying. An additional factor that is negatively influenced by low soil water contents is microbial activity, which is one of the main drivers of nutrient availability to the plants. To cope with soil drying and its adverse direct and indirect effects on water and nutrient availability, it has been proposed that plant roots modify their environment in which they grow, the so-called rhizosphere, to improve the mobility and the availability of nutrients. These modifications of physical and biogeochemical properties of the rhizosphere have been attributed to root exudation and the secretion of mucilage. Mucilage is a bio-polymeric and gel-like substance released by the roots. Mucilage is capable of adsorbing a large volume of water, thereby increasing the water retention of the rhizosphere. It also maintains the contact between the root surface and the soil matrix by enhancing soil aggregation around the roots, as manifested by the formation of the so-called rhizosheath, a cohesive layer of soil particles adhering to the root surface. Rhizosheath has been proposed to play a crucial role in increasing plant tolerance to water deficit by maintaining the contact between roots and soil. A moist rhizosphere and optimal contact between root and soil could facilitate transport of resources to the roots as soil dries. However, recent studies have shown that mucilage makes the rhizosphere of some plant species water repellent, which might limit the fluxes of water across the root-soil interface during repeated drying-wetting. In summary, it is still unclear to what extent mucilage properties and its water repellency influences water dynamics, nutrient uptake and microbial and enzyme activity in the rhizosphere.

The objective of this thesis was to explore strategies for improving plant drought tolerance by modifying the biophysical properties of the rhizosphere. To this end, I focused on potential ways to alter mucilage properties and their impact on rhizosphere physical and biogeochemical processes. The first objective of this thesis was to ascertain if plant tolerance to soil drying could be improved by increasing the mechanical stability of the rhizosheath and if this could be achieved by affecting mucilage swelling and its viscosity. In the second and third sections of this study, I investigated the effects of reduced rhizosphere hydrophobicity on i) microbial and enzyme activities in the rhizosphere and on ii) mobility and availability of nutrients and consequently plant performance under dry condition.

Initially, the concept of rhizoligand, a new way to engineer rhizospheric properties, was developed. A rhizoligand was defined as an additive that: i) interacts with the mucilage network in the rhizosphere increasing its viscosity and thus increasing rhizosheath mass and the contact between roots and soil; and that ii) decreases the water repellency of rhizosphere. A commercial surfactant was tested and selected as prototype of rhizoligands for the experiments carried out in this thesis.

The rhizoligand concept was tested through a series of experiments. Firstly, the capability of the tested surfactant to induce new cross linkage in the network of mucilage was tested using an analogue of root mucilage, mucilage from chia seeds. The surfactant significantly reduced the final swelling of mucilage. Secondly, the ability of the surfactant to enhance the rewetting rate of rhizosphere was tested with lupines growing in sandy soils. To this end, the neutron radiography was used to *in situ* monitor rhizosphere soil water dynamics. The results showed that under dry condition, the rhizosphere became hydrophobic, while the application of the used surfactant reduced its hydrophobicity and homogeneously rewetted the rhizosphere. These two preliminary tests proved that the selected surfactant behaved as rhizoligand.

Then, in the first test with the rhizoligand we addressed the question whether rhizoligand enhances rhizosheath formation and the carbon content in the rhizosheath. White lupins were grown in sand and were exposed to six drying-rewetting cycles. Half of the plants were irrigated with water and the other half with the rhizoligand. The radius of rhizosheath was quantified by scanning the roots and analyzing the rhizosheath using the software WinRhizo. Rhizoligand application increased rhizosheath formation by 1.64 times. Additionally, the total carbon contained in the rhizosheath of plants irrigated with rhizoligand was significantly greater than in the rhizosheath of plants that were not treated with the rhizoligand.

The second part of this thesis addressed the effect of rhizoligand on microbial and enzyme activity in the rhizosphere. It was hypothesized that the reduced hydrophobicity of the rhizosphere and the enhanced formation of rhizosheath created a favorable environment around the roots, with greater moisture and greater amount of carbon and mucilage in the rhizoligand-amended soil, and therefore stimulated microbial and enzyme activities in the rhizosphere. In agreement with this hypothesis, activities of the chitinase, sulfatase, and β-glucosidase were 4, 7.9, and 1.5 times greater in the rhizosphere of plants irrigated with rhizoligand than water. Similarly, microbial biomass C and microbial biomass N increased by 1.57 and 3 times in the rhizosphere of plants under rhizoligand application in comparison to

the rhizosphere of reference plants, respectively. The effects of rhizoligand on the distribution of enzyme activities was also visualized using zymography. Application of rhizoligand i) increased the β-glucosidase and phosphatase activities by 5.3 and 2.9 times in the regions close to the roots (0-0.5 mm distance from the root surface), and ii) enlarged the area with high enzyme activity 1.46-fold for β-glucosidase and 1.2-fold for phosphatase. The enlarged area with high enzyme activity around the roots in amended-rhizoligand plants in comparison to reference plants was attributed to greater rhizosheath thickness of plants irrigated with rhizoligand.

The third section of this thesis addressed the impact of rhizoligand on nutrient uptake. Plants amended with rhizoligand had higher nutrient content on a plant biomass basis (g plant^{-1}) in comparison to control plants (plants not amended with rhizoligand). Fe content increased by 51% and Mn content increased by 46%. Additionally, root biomass was greater in the rhizoligand amended plants relative to control plants. Greater plant nutrient acquisition was explained as a result of multiple factors: i) higher biological activity (as shown in the section above) which lead to increase nutrient availability; ii) greater soil water content in the rhizosphere and consequently greater nutrient mobility; and iii) greater rhizosheath thickness which maintained the roots in contact with the soil and reduced root mortality during severe drying cycles (in fact, rhizosheath acts as a cylindrical protective layer covering the root surface and maintaining roots hydrated).

In conclusion, application of rhizoligand improves plant performance by: i) reducing rhizosphere water repellency, ii) increasing the mechanical stability of the rhizosheath, iii) increasing the microbial and enzyme activities in the rhizosphere, and iv) improving plant nutrient acquisition. Such improvements are triggered by the interaction between mucilage and the applied rhizoligand, which binds the mucilage network and increases its viscosity, creating a new matrix at the root-soil interface. We propose the rhizoligand concept as an effective approach to engineer the rhizosphere properties and to improve plant tolerance to water shortage.

Zusammenfassung

Neben einer Vielzahl von biotischen und abiotischen Stressfaktoren ist Trockenheit einer der wichtigsten Faktoren, der limitierend auf das Pflanzenwachstum und die Produktivität wirkt. Die Nährstoffverfügbarkeit ist ein weiterer Faktor, der das Pflanzenwachstum und deren vollständige Entwicklung kontrolliert und vor allem bei sinkenden Wassergehalten des Bodens stark limitierend wirkt. Des Weiteren wird die mikrobielle Aktivität durch einen sinkenden Bodenwassergehalt stark negativ beeinflusst, welches wiederum Auswirkungen auf die Nährstoffverfügbarkeit hat. Um diese direkten und indirekten negativen Effekte der Wasser- und Nährstofflimitierung zu überwinden, verändern Pflanzenwurzeln ihre Umgebung, die sogenannte Rhizosphäre, in der sie wachsen. Die physikalischen und biochemischen Eigenschaften der Rhizosphäre werden durch Wurzelexsudate (*Mucilage*) modifiziert. Wurzelexsudate sind gelartige Biopolymere, die von den Pflanzenwurzeln abgesondert werden und welche die Eigenschaft haben große Mengen an Wasser zu absorbieren. Diese Eigenschaft ermöglicht es den Wasserspeicher in der Rhizosphäre zu erhöhen, sowie die Kontaktfläche zwischen der Wurzeloberfläche mit der Bodenmatrix durch eine Erhöhung der Bodenaggregate um die Wurzeln herum beizubehalten. Es bildet sich eine kohäsive Schicht aus Bodenpartikeln, die an der Wurzeloberfläche anhaftet, die sogenannte *Rhizosheath.* Es wird vermutet, dass Pflanzen durch den vermehrten Wurzel-Bodenkontakt in der *Rhizosheat* toleranter auf Wassermangel reagieren. Eine feuchte Rhizosphäre und ein optimaler Kontakt zwischen Wurzeln und Boden könnten den Transport von Ressourcen zu den Wurzeln erleichtern, während andere Bodenbereiche trocknen. Aktuelle Studien haben jedoch gezeigt, dass Wurzelexsudate bestimmter Pflanzen die Eigenschaft haben die Rhizosphäre hydrophob werden zu lassen, was den Wasserfluss an der Wurzel-Bodengrenzfläche, während wiederholten Trocken-Feuchten Zyklen des Bodens limitieren könnte. Zusammenfassend ist es jedoch unklar zu welchen Anteilen die Eigenschaften von Wurzelexsudaten die Wasserdynamik, mikrobielle Aktivität und Enzymaktivität in der Rhizosphäre beeinflussen.

Das Ziel der Arbeit war es Strategien zu untersuchen, welche eine erhöhte Trockentoleranz von Pflanzen durch die Modifikation der biophysikalischen Eigenschaften der Rhizosphäre ermöglichten. Zu diesem Zweck habe ich meinen Fokus auf die potentiellen Möglichkeiten einer Änderung der Eigenschaften der Wurzelexsudate und dessen Einfluss auf die physikalischen und biogeochemischen Prozesse in der Rhizosphäre gelegt. Als Erstes sollte

festgestellt werden, ob die Trockentoleranz von Pflanzen erhöht werden könnte durch eine gesteigerte mechanische Stabilität der Rhizosheath, welche durch veränderte Schwelleigenschaften und Viskosität der Wurzelexsudate erzielt wurde. Im zweiten und dritten Abschnitt der Studie habe ich untersucht welchen Einfluss die hydrophoben Eigenschaften der Wurzelexsudate in der Rhizosphäre auf i) die mikrobielle Aktivität und die Enzymaktivität und ii) die Nährstoffmobilität und –verfügbarkeit und somit die Leistung der Pflanzen unter Trockenbedingungen ausüben.

Zunächst wurde das Konzept des *Rhizoligand* entwickelt, welches eine neue Möglichkeit ist Rhizosphäreneigenschaften anzupassen. Ein *Rhizoligand* wurde definiert als sein Additiv welches i) mit den Wurzelexsudaten interagiert, die Viskosität und Masse des *Rhizosheath* und somit auch den Kontakt zwischen Wurzeln und Boden erhöht ii) das Wasserabweisungsvermögen der Rhizosphäre verringert. Für diese Versuche wurde ein kommerzieller oberflächenaktiver Stoff getestet und als Prototyp eines *Rhizoligands* ausgesucht.

Das *Rhizoligand* Konzept wurde durch eine Reihe von Experimenten getestet. Zunächst wurde getestet inwieweit dieser Stoff neue Bindungen mit den Wurzelexsudaten eingehen konnte. Dazu wurden die Exsudate von Chia-Samen getestet, da diese ähnliche Eigenschaften aufweisen wie Wurzelexsudate. Das *Rhizoligand* reduzierte signifikant die letzte Schwellung, bzw. Wasseraufnahme der Exsudate. Des Weiteren wurde der Einfluss des *Rhizoligand* auf die Wiederbenetzungsrate der Rhizosphäre getestet, indem Lupine in einem Sandboden herangezogen wurden. Dazu wurde die Neutron-Radiografie verwendet, um die Bodenwasserdynamik in der Rhizosphäre in-situ zu verfolgen. Die Ergebnisse zeigten, dass unter trockenen Bedingungen die Rhizosphäre hydrophob wurde und ein Zusatz des *Rhizoligand* dies verringern konnte und gleichzeitig ein gleichmäßiges Rückbefeuchten der Rhizosphäre ermöglichte. Durch diese zwei Vor-Tests konnte nachgewiesen werden, dass das verwendete Additiv als *Rhizoligand* verwendet werden konnte.

Im ersten Teil der Arbeit wurde der Frage nachgegangen, ob das *Rhizoligand* die Bildung von *Rhizosheath* und dessen Kohlenstoffgehalt erhöht. Dazu wurden weiße Lupine im Sand angebaut, wobei sechs Trocken- und Feuchtzyklen abwechselnd durchgeführt wurden. Die Hälfte der Pflanzen wurde mit Wasser und die andere Hälfte mit dem Zusatz des *Rhizoligand* bewässert. Der Radius des *Rhizosheath* wurde durch Wurzelscans und die Analyse durch die Software *WinRhizo* bestimmt. Das Applizieren des *Rhizoligand* erhöhte die Bildung von

Rhizosheath um das 1.64 fache und der Kohlenstoffgehalt war bei den Pflanzen signifikant erhöht, die damit bewässert wurden im Vergleich zur Kontrolle

Im zweiten Teil der Arbeit wurde der Frage nachgegangen, welchen Effekt *Rhizoligands* auf mikrobielle Aktivität und Enzymaktivität in der Rhizosphäre haben. Die Hypothese war, dass durch die reduzierte Hydrophobizität der Rhizosphäre und die erhöhte Bildung von *Rhizosheath* verbesserte Bedingungen um die Wurzeln herrschen. Diese gehen mit mehr Feuchtigkeit und höheren Kohlenstoffmengen einher, die wiederum die mikrobielle Aktivität und die Enzymaktivität in der Rhizosphäre stimulieren. Die Hypothese wurde durch folgende Ergebnisse bestärkt, und zwar waren die Aktivitäten in der Rhizosphäre von Chitinase, Sulfatase und β-Glucosidase um das 4, 7.9 und 1.5 fache in der Variante *Rhizoligand* erhöht als mit der Kontrollvariante Wasser. Ähnlich verhielt es sich mit dem Kohlenstoff- und Stickstoffgehalt der mikrobiellen Biomasse unter der Zugabe des *Rhizoligands*, welche jeweils 1.57 - und 3 -fach höhere Werte hatten als bei der Variante Wasser. Die Auswirkung des *Rhizoligands* auf die Verteilung der Enzymaktivität wurde durch Zymografie visualisiert. Die Applizierung des *Rhizoligand* i) erhöhte die β-Glucosidase- und die Phosphataseaktivität um 5.3 und 2.9 in der Umgebung nahe der Wurzel (0-0.5 mm von der Wurzeloberfläche) und ii) erweiterte die Fläche mit hoher Enzymaktivität 1.46 –fach für β-Glucosidase und 1.2 –fach für Phosphatase. Die größere Fläche um die Wurzeln mit erhöhter Enzymaktivität in der *Rhizoligand* Variante wurde durch die höhere *Rhizosheath* Dicke erklärt.

Im dritten Teil der Arbeit wurde der Frage nachgegangen inwieweit die Zugabe des *Rhizoligand* einen Einfluss auf die Nährstoffaufnahme der Pflanze hat. Die Pflanzen in der Variante mit dem *Rhizoligand* hatten erhöhte Nährstoffkonzentrationen auf Biomassebasis (g Pflanze^{-1}) im Gegensatz zu den Kontrollpflanzen. Der Fe-Gehalt war um 51 % und der Mn-Gehalt war um 45.7 % erhöht. Zusätzlich war die Wurzelbiomasse in der *Rhizoligand* Variante erhöht im Vergleich zur Kontrolle. Die erhöhte Nährstoffaufnahme der Pflanzen wurde durch mehrere Faktoren erklärt: i) eine höhere biologische Aktivität, welche zu einer erhöhten Nährstoffverfügbarkeit führt, ii) einen erhöhten Wassergehalt in der Rhizosphäre und daher erhöhte Nährstoffmobilität und iii) eine erhöhte *Rhizosheath* Dicke, welche dazu führt, dass die Wurzeln im Kontakt mit dem Boden bleiben und somit Wurzelsterben während schweren Trockenphasen verhindert (tatsächlich dient die *Rhizosheath* der Wurzel als eine Schutzschicht, indem sie die Wurzeln umgibt und somit die Wurzeln vor dem Austrocknen schütz).

Zusammengefasst verbessert die Anwendung von *Rhizoligands* die Leistung von Pflanzen durch i) die Verringerung des Wasserabweisungsvermögens der Rhizosphäre, ii) die Erhöhung der mechanischen Stabilität der Rhizosphäre, iii) die Erhöhung der mikrobiellen Aktivität, sowie der Enzymaktivität der Rhizosphäre und iv) der Verbesserung der Nährstoffaufnahme. Solche Verbesserungen werden durch die Interaktion, vor allem durch die entstandenen Bindungen zwischen dem *Rhizoligand* und den Wurzelexsudaten hervorgerufen, welche eine erhöhte Viskosität und eine neue Matrix an der Wurzel-Bodenkontaktfläche zur Folge haben. Wir sehen das *Rhizoligand* Konzept als einen effektiven Ansatz, um Rhizosphäreneigenschaften zu verändern und somit Pflanzen eine optimale Umgebung, während einer Trockenphase mit Wassermangel zu schaffen.

Acknowledgements

First of all, I praise God almighty for providing me this opportunity to make one of my old dreams come true and giving me the capability to complete this research successfully. I would like to express my deepest thanks to all people who supported and helped me during this exciting journey toward achieving the PhD degree. My sincere gratitude to Prof. Dr. Andrea Carminati, my main supervisor, for giving me this opportunity and pursuing my research interests freely as well as his continuous support and guidance. I owe my gratitude to Dr Mohsen Zarebanadkouki for his guidance, patience, and his valuable help. I would also like to extend my appreciation to Prof. Dr. Yakov Kuzyakov for his support, guidance, and his professional comments. My sincere thanks also go to Prof. Dr. Michaela Dippold, Dr Bahar Razavi, PD. Dr Evgenia Blagodatskaya, Dr. Stanley J. Kostka and Dr. Kazem Zamanian for their guidance and their insightful comments.

Special thanks to, Mrs Ingrid Osmer and all other technicians and colleagues in the soil science department at University of Götingen for their assistance in laboratory experiments. My warmest thanks also go to my Iranian and international friends for sharing their experience and knowledge and for the wonderful time that we spent together during this period in Germany.

Finally, I would like to express my gratitude and appreciation to my beloved family members as follows: My great husband, Valiollah, for all his supports in different aspects. He respected my decision and stayed behind me and tolerates all loneliness during this time. My mother who always kept the hope in my heart alive and her praying always brought me clarity and peace into my soul. My sweetheart-children, Sara and Mehrdad, whom I experienced true love and happiness with them, those who were the most important source of my motivation, strength and power in my life. My sister, Maryam, and my brothers and most especially my younger wise brother, Ali, who always was ready to help and support me by his valuable guidance. My family has always been full of unconditional love and encouragement and they are the most precious God's gift.

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Chapter One

Extended Summary

1 Introduction

Water scarcity is a major cause of crop yield reduction, poverty and food unsecurity (Godfray et al., 2010; Sposito, 2013). It is forecasted that frequency and severity of drought periods will increase and three billion people will face water shortage by 2050 (Misra, 2014). Productivity of main agricultural crops, such as maize, will be reduced by more than 10-50%, causing increased hunger for 130 million people in sub-Saharan Africa and Latin America (Matthias Ruth, 1998; Misra, 2014). Plant growth and development has long been known to be reduced with reduction of water transport in plant and soil as soil drying progresses. Plants transport approximately 200-1000 times of their dry biomass in the root–stem–leaf pathway to assimilate carbon and energy over their lifetime (Hsiao and Xu, 2000). Numerous plant processes are linked to water transport, including photosynthesis, respiration and nutrient acquisition, which are reduced as a consequence of limited water transport in dry soil. Furthermore, many physico-chemical and biological processes in the soil are adversely influenced by water stress (Chaitanya et al., 2003).

Understanding adverse effects of drought on bio-physico-chemical processes in the soil as well as plant adaptation strategies are essential to sustain crop production under water scarcity. In the following sections, I briefly describe how mobility and availability of water and nutrient in the soil and plant are negatively influenced by soil dryness.

1.1 Root water uptake in dry soil

The ability of plants to capture water depends on root traits (including root architecture) and water availability in the soil surrounding the roots. Water availability to plant roots is strongly linked to the soil hydraulic properties. Water evaporation from leaf stomata generates suction on the water flowing into the leaves and a gradient in water potential that drives an inflow of water from the soil, through the roots and the stem, to the leaves. In wet soils, the soil has a low and almost negligible hydraulic resistance, and root hydraulic conductivity determines water transport. As soils progressively dry, the soil hydraulic conductivity decreases, until it eventually becomes the limiting factor for water transport. Additionally, as severe drying is approached, roots dehydrate and shrink possibly losing contact to the soil. This leads to an even lower soil hydraulic conductivity and a decline in water availability to plants. Consequently water and nutrient uptake are reduced, and so is plant yield (Carminati and Vetterlein, 2013; North and Nobel, 1997).

1.2 Root nutrient acquisition in dry soil

Plant nutrient uptake, a major factor for plant growth and development, is restricted in dry soils because of the reduction of mobility and availability of nutrient, even in enriched-nutrient soil (Lobet et al., 2014; Silva et al., 2011). The mobility of nutrient is associated with availability and transport of water in the soil. Nutrients are transported to the plant roots by mass flow and diffusion. As plants transpire, nutrients in the soil solution are passively transported towards the root and translocated in the plant xylems by mass flow (Cramer et al., 2008; Silva et al., 2011). Gradients in nutrient concentration across the rhizosphere drive the movement of nutrients by diffusion, with coefficients of diffusion decreasing with lower water content. Mass flow mainly governs the transport of some elements such as calcium (Ca), whereas uptake of some elements e.g. phosphorus (P), mainly occurs by diffusion. Transport of nutrient by both mass flow and diffusion are limited during drying events (Hu et al., 2007; Kozlowski, 1972; Silva et al., 2011).

1.3 Biological activities in dry soil

Soil microorganisms contributed to many vital functions for plant growth and productivity, including: i) nitrogen fixation, ii) aggregate stability and, iii) nutrient accessibility to the plants. Soil microorganisms by releasing enzymes solubilize nutrients during decomposition and mineralization. They turn immobile nutrients stored in organic matter into forms that are available for themselves and plants (Singh et al., 2011). As a concequence, soil microorganisms can enhance nutrient availability and therefore increase plant nutrient uptake (Richardson et al., 2011). Following this concenpt, incubating plant roots with beneficial microorganisms has attracted considerable interest in terms of agricultural sustainability and reducing synthetic fertilizer consumption. However, this approach cannot do much against soil drying, as low soil water content suppresses microbial biomass and their associated enzyme activities (Sanaullah et al., 2011a; Sardans and Peñuelas, 2005; Stark and Firestone, 1995). As water stress progresses, the largest pores are drained, the water connected regions become smaller. Hence, the water pathways get tortuous and the films of water around soil particles become thinner . All this reduces the diffusion rate of substrates to microorganism. (Chowdhury, 2011; Ilstedt et al., 2000; Stark and Firestone, 1995). In this case, soil micoorganisms suffer from lack of resources as well as ion toxicity in their surrounding environment (Stark and Firestone, 1995). Enzyme activity is an indicator reflecting the biological properties of soil and microbial activity. A literature review showed a reduce β-glucosidase activity by 10–80% as a result of a 10% reduction of soil water content (Sardans and Peñuelas, 2005).

1.4 Proposed strategies to improve plant tolerance in dry soil

Different strategies have been proposed to improve an efficient consumption of the available water and nutrient resources in drought-prone regions. These strategies aim to increase water and fertilizer use efficiency. Relevant strategies include: i) applying super-absorbing materials to enhance water retention of the soil (Hüttermann et al., 1999), ii) applying new irrigation management techniques, e.g. drip irrigation (Payero et al., 2008), and iii) breeding new varieties with optimal root and desired traits. Optimal root traits include: a) large and deep rooted system contributing to deep-water extraction despite low water availability (Comas et al., 2013; Shao et al., 2008), b) high transpiration efficiency (more carbon fixed per water used) as a water-saving strategy, c) ability to interact with mycorrhiza to increase soil fertility and nutrient availability (Ortíz-Castro et al., 2009; Rengel and Marschner, 2005; Zahran, 1999), and d) a higher C allocation belowground to modify the edaphic properties the soil surrounding the roots, the so called rhizosphere (Huang and Gao, 2000).

1.5 Rhizosphere

The rhizosphere is the region around the roots which is dynamically modified by roots and the associated microorganisms (Carminati et al., 2010; Hinsinger et al., 2009; Spohn and Kuzyakov, 2013; York et al., 2016). Roots exude a large quantity of their photosynthetic compounds into the soil modifying physical, chemical and biological properties of the rhizosphere. Some of these modifications impact water and nutrient uptake. Rhizodeposits are composed of low molecular weight compounds such as amino acids, carbohydrates and carboxylic acids (Farrar et al., 2003; Fischer et al., 2007) and high molecular weight compounds such as mucilage (Jones et al., 2009). Mucilage plays a key role in shaping the physical and hydraulic properties. Understanding the mucilage functions and its behavior in response to water stress can potentially help to identify new avenues to improve rhizosphere properties.

1.6 Mucilage roles and its characteristics

Mucilage is a biopolymer exudated by roots and soil microorganisms. It is mainly composed of polysaccharides, protein and phospholipids (Read et al., 2003). The potential role of mucilage in shaping the rhizosphere and affecting plant growth and productivity is discussed in the following points:

- Mucilage keeps the rhizosphere wetter than the bulk soil during drying.

McCully and Boyer (1997) showed that when mucilage is fully hydrated it can hold an amount of water equal to 1000 times its dry weight. Such a property keeps the rhizosphere wetter than bulk soil and might potentially to help to sustain water and nutrient flow in dry soil (Carminati et al., 2011; Read et al., 2003). Mucilage retains water in the rhizosphere and supplies the plant water requirement when the bulk soil is dry and is not able to compensate sufficient water to plant (Carminati et al., 2011).

- Mucilage increases abundance of soil microorganism in the rhizosphere.

Besides of ability of mucilage to maintain rhizosphere wet, mucilage is mainly composed of long chain polysaccharides and other organic compounds. These organic compounds act as C energy sources for microorganisms feeding in the rhizosphere. Hence, great abundance of soil microorganism in the rhizosphere in comparing to bulk soil attributed to presence of root exudates and in particularly mucilage in the rhizosphere (Brzostek et al., n.d.; Jones et al., 2009)

- Mucilage contributes to formation of rhizosheath.

Mucilage of many plant species, e.g. maize and lupin, shows gelatinous and viscoelastic behavior (Read and Gregory, 1997). This specific physical characteristic of mucilage is due to high level of cohesion within their long polymer network. Mucilage becomes more viscous when it shrinks in response to soil drying (Read and Geogory., 1997). With increasing mucilage viscosity, soil particles will be more strongly bound to the root and will lose their ability to move. Read and Geogory (1997) also indicated that surface tension of mucilage increases with dehydration of mucilage in dry soil. Low surface tension and great viscosity stabilize soil aggregates in surrounding root and develop rhizosheath formation (Czarnes et al., 2000; Read and Gregory, 1997). The rhizosheath is defined as a cohesive soil layer adhering to the root surface and is believed to increase plant resistance to water scarcity (Ahmadi et al., 2017; McCully, 1999; Moreno-Espíndola et al., 2007; Pang et al., 2017; George et al., 2014; Watt et al., 1994, 1993). In dry soil, shrinkage of roots causes the formation of a gap between root and soil (Nobel and Cui 1992; Watt et al. 1993). An air gap between roots and soil results in: i) root dehydration and consequently root mortality and ii) limited water and nutrient transport in the soil (Caryn et al., 1985; Hartnett et al., 2013). It has been proposed that rhizosheath reduces the risk of gap formation and ensures a proper contact between roots and soil, helping to sustain water and nutrient transport in dry soils (Czarnes et al., 2000; North and Nobel, 1997).

- Mucilage induces water repellency in the rhizosphere upon drying.

Although mucilage increases the water retention and maintains the rhizosphere wet during drying, mucilage turns hydrophobic upon drying and it can induce water repellency in the rhizosphere. Recent studies with neutron radiography revealed that the rhizosphere of some plant species (e.g. maize and lupin) temporarily remained dry after rewetting following severe drying. Similarly, greater contact angle of water was observed in the rhizosphere than bulk soil after drying (Moradi et al., 2012). These observations suggested a temporary hydrophobicity of the rhizosphere after drying (Moradi et al., 2012; Spohn et al., 2013). Hydrophobicity of rhizosphere was attributed to mucilage. In turn, mucilage hydrophobicity might be caused by presenting of the lipids (Read et al., 2003).
Hydrophobicity of rhizosphere in response to drought might adversely influence root water uptake. Zarebanadkouki and Carminati 2014 showed that rhizosphere repellency limited root water uptake upon irrigation and this effect persisted for at least a few hours (Zarebanadkouki and Carminati, 2014). However, the effect of rhizosphere water repellency on other aspects of soil-plant interactions, such as nutrient uptake and microbial and enzyme activity, remain research gaps.

1.7 Soil hydrophobicity and surfactant application in dry soils

Soil water repellency has been reported in a wide range of soil types and different climatic conditions worldwide (Debano, 2000; Olorunfemi et al., 2014). Recent reviews have drawn the attention to influence of soil water repellency on decreased water efficiency, increased irrigation requirements and reduced fertilizer performance.

Surfactant application to repellent soil decreases soil hydrophobicity and improves water infiltration. Surfactants are amphiphilic compounds which reduce surface tension of water and the contact angle of water in repellent soils, improving and homogenizing water infiltration in soils. Consequences include improved wettability of soil, irrigation efficiency and uniform water penetration in hydrophobic soils (Debano, 2000; Franklin, 2007; Moore et al., 2010). Application of surfactants is recently increasing in turf and has been tested in a variety of horticultural and agricultural crops to improve water use efficiency. The mechanisms of surfactant interactions with water and nutrient uptake have not been explored, in particular regarding interactions taking place in the rhizosphere.

1.8 Rhizosphere engineering as a pathway to improve plant adaptations in dry soil

Recently considerable attention has been focused on rhizosphere engineering as a strategy to improve plant performance under biotic and abiotic stress. Rhizosphere engineering emphasize on: i) enhancing root growth to increase soil exploration and soil resource extraction; ii) introducing beneficial microorganism to increase nutrient availability; iii) regulating root exudation to improve water and nutrient use efficiency (Shen et al., 2013); and iv) inducing chemical signal of ABA and maximizing water use efficiency by partial root zone drying (PRD) (Dodd, 2009). Rhizosphere engineering also has been proposed in recent years as an approach for shifting from a "high input-high output" perspective toward more optimal use of scarce resources, with a view to advancing sustainability and environmental protection (Shen et al., 2013).

This thesis highlights rhizosphere engineering as a new approach to improve plant adaptation under drought stress. We applied a commercial surfactant to modify bio-physical properties of rhizosphere. As a first step, the capability of the selected surfactant on physical and biological properties of the rhizosphere was tested according to hypotheses described below.

2 Hypotheses and preliminary tests

2.1 Stabilization mucilage in surrounding the root to develop rhizosheath formation

First we tested on potential ways to alter mucilage properties through affecting mucilage swelling and its viscosity. Initially, the concept of rhizoligand, as an additive with the capability to interact with mucilage in the rhizosphere and change its physical structure was developed. Rhizoligand and mucilage both have hydrophilic and hydrophobic functional groups. We assumed that the interactions between functional groups of rhizoligand and mucilage induce additional links in the mucilage polymer network. The new links inhibit swelling of mucilage and increase its viscosity. Higher viscosity results in mucilage accumulation at the root surface, increasing the strength of the bonds between root and soil particles and consequently improves rhizosheath formation. Albalasmeh and Ghezzehei (2014) showed that when the viscosity of polygalacturonic acid (analogue to EPS and similar to mucilage concerning the viscosity) is sufficiently high, it improves stability and rhizosheath formation. As prototype of rhizoligand, a commercial surfactant (ACA1820, Aquatrols Corporation of America, Paulsboro, New Jersey, U.S.A) was tested in the experiments carried out in this thesis. To test our hypothesis, the swelling rate of mucilage extracted from chia seeds in water and water treated with surfactant was compared. Fig. 1 indicates that the maximum swelling of chia mucilage was significantly reduced in the rhizoligand solution.

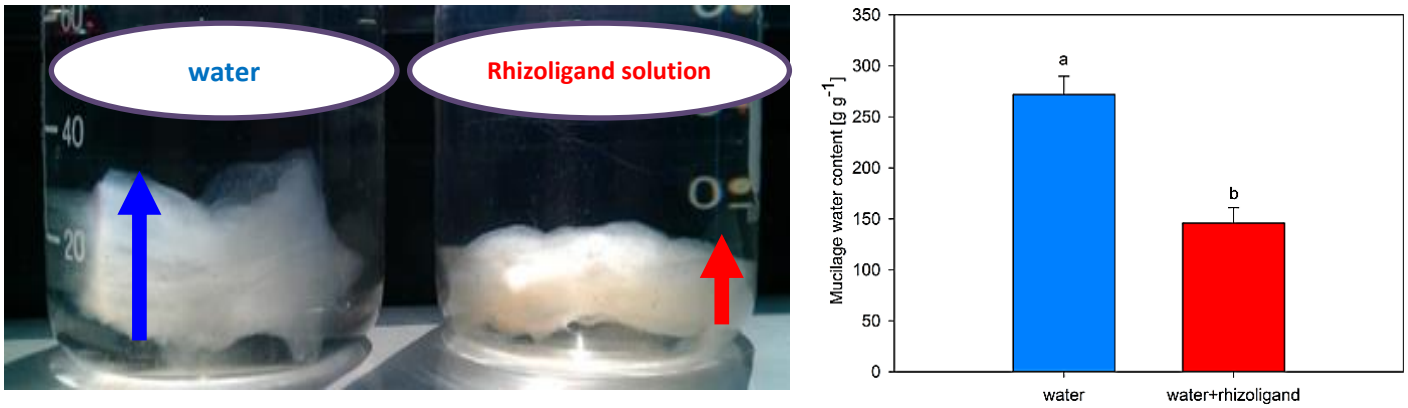

Figure 1. Left) swelling of dried chia mucilage in water and rhizoligand solution. The mucilage was let hydrate for 48 hours. Right) maximum swelling of dried mucilage in water and rhizoligand solution. The results showed that rhizoligands decreased the maximum swelling of mucilage by a factor 1.9. Each value is the average of 5 replications. Different lower case letters indicate a significant difference at $P < 0.05$.

2.2 Increase water retention in the rhizosphere by reducing hydrophobicity of rhizosphere

The underlying hypothesis was that the hydrophobicity of the rhizosphere reduces the water content around the roots during drying and rewetting cycles, causing reduced microbial biomass and nutrient availability in the rhizosphere. We expected that reduced hydrophobicity of the rhizosphere would increase: i) microbial biomass and their associated enzymes and, ii) nutrient transport in dry soil and thus plant nutrient acquisition. Neutron radiography was applied to test the capability of the selected surfactant to rewet the hydrophobic rhizosphere. The rhizosphere of lupin plants irrigated with rhizoligand was homogeneously rewetted after irrigation; whereas, the rhizosphere of reference plants irrigated only by water remained markedly dry (Fig. 2).

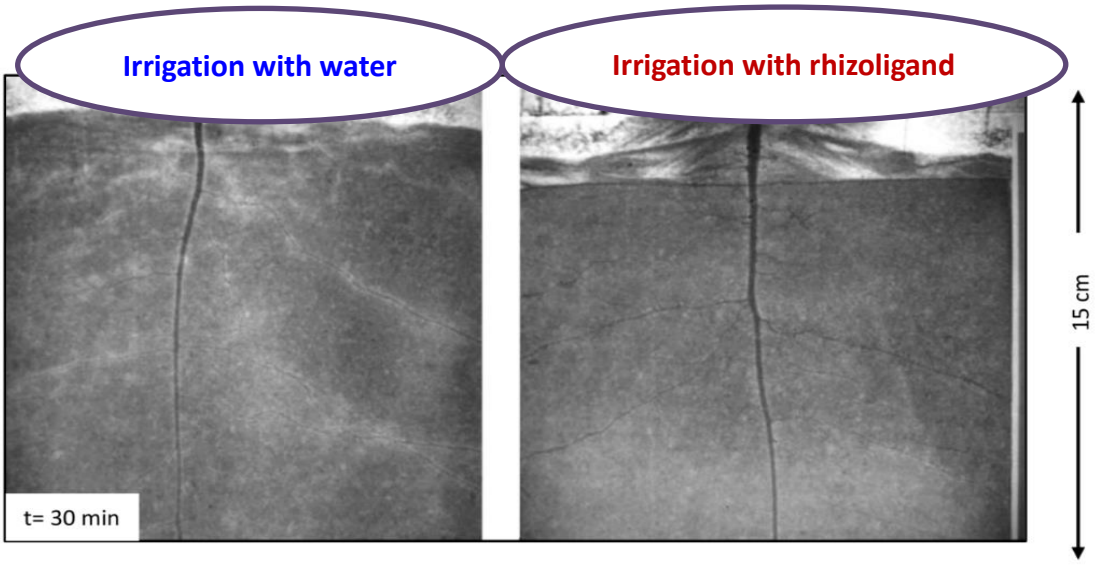

Figure 2. Water distribution in the rhizosphere of 4-weeks old lupin irrigated with water (left side) and irrigated with rhizoligand (right side) 30 min after rewetting. Dark colors show higher water contents, light colors show lower water contents. The rhizosphere of plants irrigated with rhizoligand was homogeneously rewetted after irrigation, whereas, the rhizosphere of plants irrigated with water stayed dry.

3 Objectives

After conducting the preliminary studies, I formulated the following objectives. The main aim of my thesis was to explore strategies for improving plant drought tolerance by modifying the biophysical properties of the rhizosphere.

The specific objectives of this study were:

- To evaluate the effects of rhizoligand addition on rhizosheath formation under dry condition (study 1).
- To estimate the effect of rhizlogand on the carbon content of the rhizosheath (study 1).
- To investigate the effects of reducing rhizosphere hydrophobicity on biological activities in the rhizosphere (study 1, 2).
- To evaluate the consequences of improving rhizosheath formation, rhizosphere wettability and microbial activity on plant nutrient uptake and plant performance under dry condition (study 3).

4 Methods

White lupin and maize were grown in sand and were exposed to six drying-rewetting cycles. Half of the plants were irrigated with water and the other half with the rhizoligand. Thereafter, the following measurements were conducted in the soil of reference plants and rhizoligand-amended plants.

- Analysis of rhizosheath properties

  When lupin plants were 50 days old, the containers were opened, and the roots were gently removed from the container. Then two different methods were employed to quantify the rhizosheath formation including i) The thickness of rhizosheath was estimated by scanning the roots and soil attached to the roots using Winrhizo, ii) the mass of soil attached to the roots was quantified gravimetrically after being removed and dries in the oven.

- Analysis of carbon content in the rhizosheath and bulk soil by VarioMax CNS apparatus according to the Dumas combustion method (study 1).

- Evaluate biological properties of the rhizosphere

  - Assessment activities of four extracellular enzymes consist of β-glucosidase, Chitinase, acid phosphatase and sulfatase in the rhizosphere and bulk soil of lupin using fluorogenically labeled substrates (Razavi et al., 2016), (study 1).

  - Visualization spatial distributions of two enzymes of phosphatase and β-glucosidase around the roots of maize using zymography (study 2).

  - Measurement microbial biomass carbon and microbial biomass nitrogen in the soil of reference and rhizoligand amended maize grown under wetting and drying cycles by the chloroform fumigation-extraction method (Vance et al., 1987), (study 2, 3).

- Evaluate plant nutrient uptake and plant performance

  - The consequences of rhizosphere modifications were further investigated on the nutrient status of plant and soil as well as the plant biomass. Sample preparation

was carried out based on a wet microwave digestion under pressure. Then, the concentration of nutrients in the shoot and roots was determined by ICP-OES (Vista RL, CCD simultaneous ICP-OES, Varian Inc., USA) and atomic absorption spectrometry (220 FS, Varian Inc., USA). The dry weight of roots and shoots were determined gravimetrically after drying in oven for 24 h at 105 °C (study 3).

5 Main results of the thesis

In this thesis, a new concept was introduced and tested to modify bio-physical properties of the rhizospheric soil. It is known that as the soil dries the rhizosphere turns temporarily hydrophobic, which results in a slow and heterogeneously rewetting of the rhizosphere after irrigation. This hydrophobicity is assumed to have an adverse influence on plant nutrient uptake due to: i) reduced transport of water and nutrients in the soil, ii) reduced microbial activity, which results in restricted nutrient availability in drying soil.

Here, we propose to use a commercial surfactant (ACA1820) (referred here as a rhizoligand) to: i) rewet a hydrophobic rhizosphere by reducing the surface tension of water and ii) enhance the rhizosheath formation by cross-linking mucilage polymers and increasing their viscosity.

Monitoring the soil water content around the roots of lupin plants using a neutron radiography technique showed that the tested surfactant could uniformly rewet a hydrophobic rhizosphere upon a drying and subsequent rewetting cycle.

The capability of the selected surfactant to interact with the network of mucilage was tested by comparing the final swelling rate of mucilage extracted from chia seeds in water and rhizoligand solution. The swelling of mucilage was significantly reduced by a factor of 1.9 in comparison to water. It was hypothesized that the rhizoligand thanks to its amphiphilic structure interacted with hydrophobic functional groups of mucilage and induce new cross linking bonds in the network of mucilage and inhibiting swelling.

Thereafter, the effects of rhizoligand in improving plant performance in drying soil were investigated. The main effects are reported below.

5.1 Effect of rhizoligand on mechanical stability of rhizosheath (study 1).

Rhizosheath formation was proposed as a positive trait which helps plants to better tolerate under drought stresses (George et al., 2014a). In drying soil, both roots and soil tend to shrink and therefore an air-filled gap may form at the root- soil interface (Nobel and Cui 1992; Watt et al. 1993).

Rhizosheath could reduce the risk of gap formation and thus could facilitate the transport of water and nutrients by providing a better contact between roots and drying soil (Nobel and Cui, 1992a; Watt et al., 1994). Rhizosheath also protects root against dehydration and mortality in severe drying events (Pang et al., 2017). In this thesis, the effect of rhizoligand on rhizosheath formation was evaluated in lupin plants subjected to six drying and rewetting cycles. The rhizosheath formation was quantified by measuring the weight and the radius of soil attached to the root system of 50 days old lupin plants. Rhizoligand addition increased rhizosheath mass by 1.64 fold as well as rhizosheath radius by 1.55 fold. Such an improvement was attributed to the reduced mucilage swelling in response to rhizoligand addition. A mucilage network with a reduced swelling rate will have a greater viscosity and therefore less prone to move away from the root surface. In this condition, the mucilage network will have stronger strength to bind soil particles to the root surface.

5.2 Effect of rhizoligand on biological properties of the rhizosphere (study 2)

The rhizosphere is known as a hot spot of biological activities with higher abundance of microorganisms and faster processes compared to the adjacent bulk soil (Kuzyakov and Blagodatskaya, 2015). Activities of soil microorganism are restricted in drying soils mainly due to the reduced diffusion rate of resources. Neutron radiography of lupin showed that under dry condition, the rhizosphere of plants irrigated by water was hydrophobic and ununiformly rewet after irrigation. Conversely, a selected surfactant reduced its hydrophobicity and homogeneously rewetted the rhizosphere. We expected that the reduce hydrophobicity of the rhizosphere by rhizoligand application improved biological properties of the rhizosphere. The results of studies 1 and 2 support this hypothesis.

The activities of three enzymes (chitinase, sulfatase and β-glucosidase) were 4, 7.9, and 1.5 folds greater in the rhizosheath of the white lupin under rhizoligand addition in comparing to plants irrigated with water. However, rhizoligand had no effect on phosphatase activity in the rhizosphere and enzyme activities in the bulk soil of lupin plants.

The distribution of enzyme activities was also visualized using zymography. Application of rhizoligand increased the β-glucosidase and phosphatase activities by 5.3 and 2.9 folds, respectively, in the regions close to the Maize roots (0-0.5 mm distance from the root surface). It also enlarged the area with high enzyme activity by 1.46-fold for β-glucosidase and by 1.2-fold for phosphatase. This improvement was attributed to the greater rhizosheath thickness as well as greater moisture of rhizosphere in response to rhizoligand addition.

5.3 Effect of rhizoligand on nutrient uptake (study 3)

A rhizosphere with greater water content thanks to rhizoligand addition was expected to increase the mobility and availability of the nutrients in the soil and in the plants. This hypothesis was tested by measuring the nutrient concentration in plant and rhizospheric soil (sampled as rhizosheath) of plants subjected to several drying and rewetting cycles. The results showed that plants irrigated with rhizoligand had higher nutrient contents (per gram of plant biomass) in comparison to the control plants (plants not irrigated with water), e.g. iron (Fe) increased by 52% in plants under rhizoligand addition. Greater plant nutrient acquisition was explained as a result of several factors: i) higher biological activity which led to increase nutrient availability; ii) greater soil water content in the rhizosphere and consequently greater nutrient mobility; and iii) greater rhizosheath thickness which maintained the roots in contact with the soil.

5.4 Effect of rhizoligand on plant growth (study 1-3)

The consequence of rhizoligand application on plant growth was determined by measuring the root and shoot biomass of plants. The root biomass in both lupin and Maize increased by 28% and 30%, respectively, in the rhizoligand amended plants relative to reference plants. The possible reasons are: i) greater water content in the rhizosphere, ii) greater soil microorganism activity which produces further mucilage, iii) greater rhizosheath radius. All these factors provide a wetter and suitable region in surrounding the root and protect roots from dehydration and mortality during drying cycles.
The conceptual diagram in figure 3 shows the hypotheses and possible mechanisms influencing plant performance in response to rhizoligand application in water stress condition.

6 General conclusion

With this observation we were tempted to conclude that the rhizoligand application impacts on both nutrient and water transport into the roots of plants subjected to several drying and rewetting cycles. Hence, it has potential to improve plant nutrient uptake, particularly of micronutrients and increase plant tolerance in dry soils. The aims of this thesis were inspired from the recent studies showing unexpected water dynamics in the rhizosphere of plants exposed to repeated drying and wetting soils. Here we focused on the capability of the surfactants to rewet water repellent rhizosphere and the effects on microbial activities and nutrient uptake. The experiments were performed under controlled laboratory conditions. To confidently recommend the rhizoligand application further studies in the field are needed. In summary, the main results of my dissertation are depicted in figure 4, which

illustrates the mechanisms of rhizoligand action in improving bio-physical rhizosphere properties and root growth.

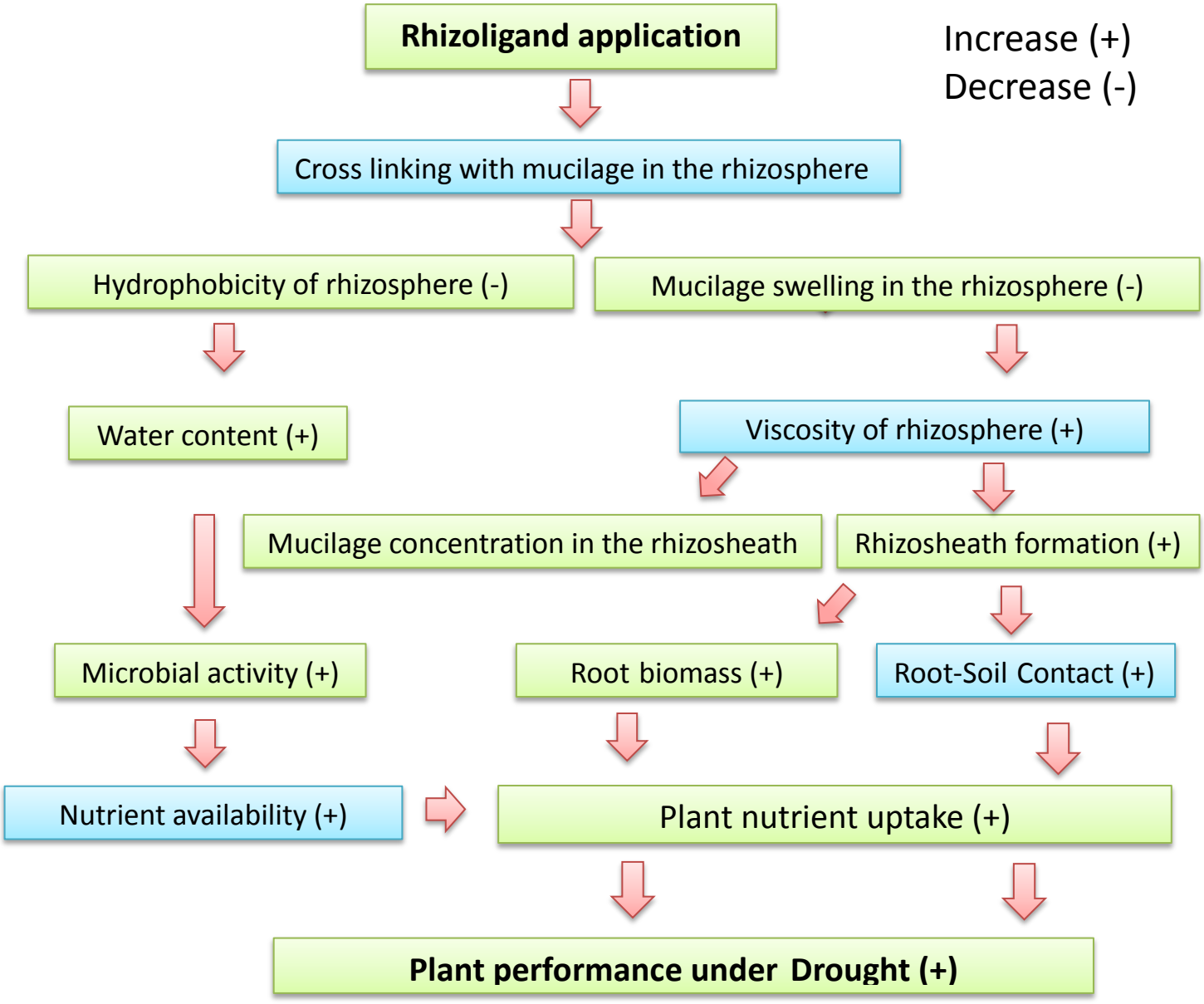

Figure 3. Conceptual diagram showing the mechanism of rhizoligand action which influencing plant nutrient uptake. Altering the bio-physical properties of rhizosphere are expected to improve nutrient acquisition and thus plant performance in water stress condition. The green boxes indicate the factors measured in this thesis, whereas blue color boxes indicate our mechanistic explanations.

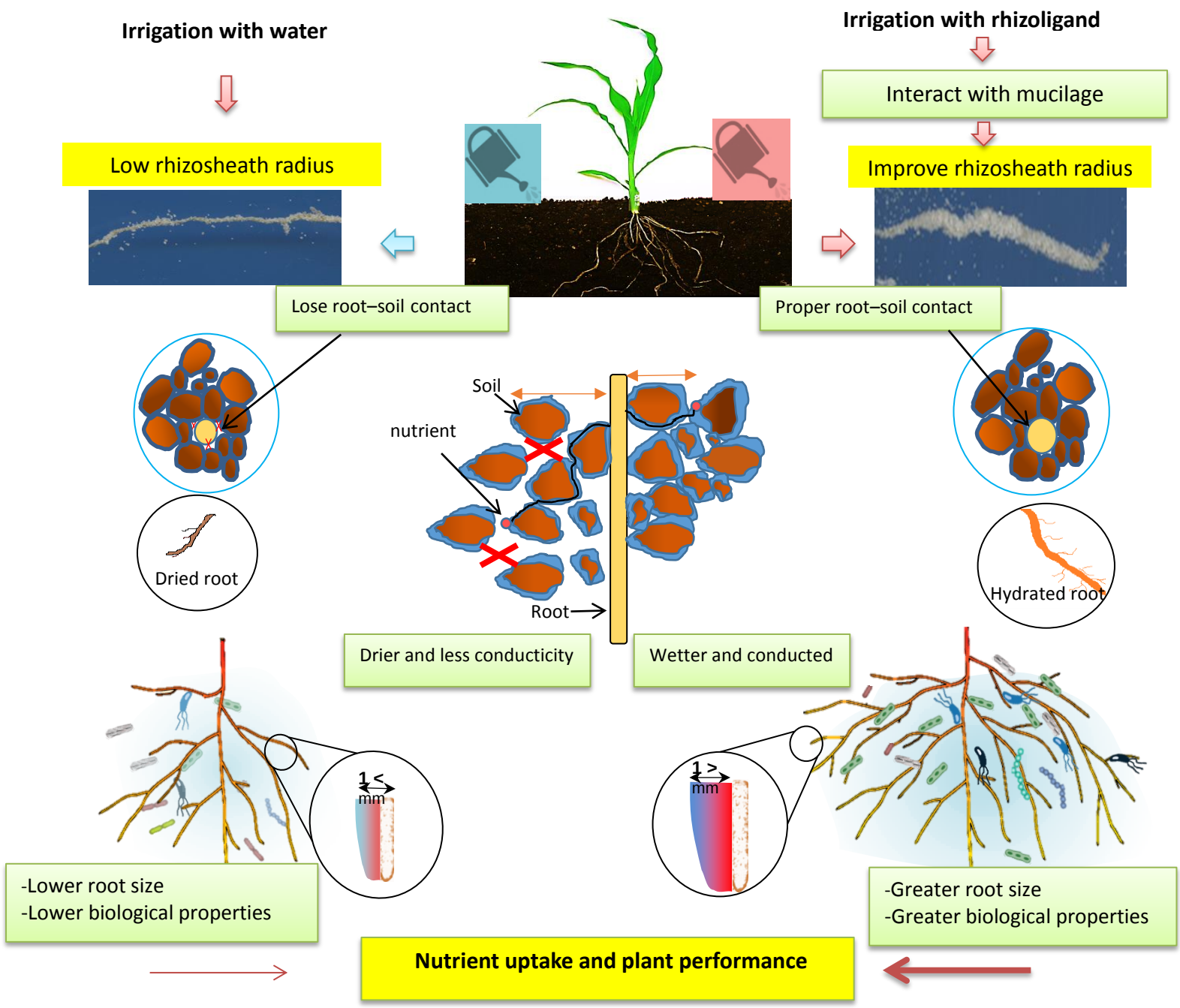

Figure 4. Schematic overview of mechanisms underlying improved bio-physical properties of rhizosphere and increased plant performance after rhizoligand application. The top figures illustrate that soil particles in the rhizosphere are covered with greater mucilaginous compounds in soil treated with rhizoligand (right side). Greater mucilage in surrounding the roots provides a wetter and more conductive environment for root growth as well as sustain nutrient mobility during drying cycles. In contrast the images on the left show an air-filled gap formed at the root-soil interface, where root loses their contact with the soil. As a consequence, root dries during severe drought. Furthermore, due to the loss of hydraulic conductivity of soil particles in vicinity of root, diffusion of nutrient is limited in the rhizosphere.

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Chapter Two

(Study 1)

Rhizosphere engineering: innovative improvement of root environment

As published in Rhizosphere 3 (2017), 176–184

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1 Abstract

The ability of roots to extract water and nutrients from soil depends on the biophysical properties of the rhizosphere, which are strongly influenced by mucilage secretion. The aim of this study was to introduce the concept of rhizoligands to engineer the biophysical properties of the rhizosphere. A rhizoligand is defined as an additive that increases the wettability of the rhizosphere and links the mucilage network to main intimate contact with the root surface. We hypothesize that rhizoligands: i) facilitate the rewetting of the rhizosphere during repeated drying and wetting cycles; ii) enhance rhizosheath formation; iii) increase enzyme activities in the rhizosphere; and iv) increase plant biomass. A commercial surfactant was selected as the prototype rhizoligand to test the effect on the rhizosphere biophysical properties of white lupin grown in quartz sand and subjected to six drying-rewetting cycles. Half of the plants were irrigated with water and the other half with the rhizoligand solution. When plants were 50 days old, we measured: i) soil water content; ii) rhizosheath mass; iii) activity of selected enzymes; iv) carbon content in the rhizosphere; and v) plant biomass. Rhizoligand increased rewetting rate of the rhizosphere after drying and subsequent rewetting, resulting in a greater soil water content. Rhizosheath formation was improved in plants irrigated with rhizoligand and sand particles attached to the roots increased by 1.64 times compared to plants irrigated with water. The activity of the enzymes chitinase, sulfatase, and β-glucosidase were 4, 7.9, and 1.5 times greater in the rhizosphere of plants irrigated with rhizoligand than in the rhizosphere of plants irrigated with water. Plant biomass was 1.2 fold greater in samples irrigated with rhizoligand solution than in samples irrigated with water.

We conclude that application of rhizoligand improves plant performance by influencing the water dynamics in the rhizosphere and the plant, increasing the mechanical stability of the rhizosheaths and increasing the enzyme activities in the rhizosphere. Such effects are probably triggered by the interaction between mucilage and the applied rhizoligand, which reduces mucilage swelling (possibly by cross-linking mucilage polymers) and thus by increasing its viscosity keeps the mucilage close to the root surface. We propose the rhizoligand concept as a strategy to engineer the rhizosphere properties and to improve plant tolerance to water shortage.

Keywords: *Enzyme activity, Mucilage, Rhizosheath, Rhizosphere, Root exudates, Root water uptake, Surfactant.*

2 Introduction

Water shortage has significant direct and indirect adverse effects on plant growth and yield. As the soil dries, the transport of water and nutrients to the roots becomes limited by the low soil hydraulic conductivity. As the soil dries further, roots shrink and air-filled gaps form between soil and roots consequently limiting water and nutrient flow toward the root surface (Carminati et al., 2009; McCully, 1995; Nobel and Cui, 1992b).

During soil drying, also microbial activity decreases (Austin et al., 2004; Sanaullah et al., 2011a). Since plant–microbial interactions play a central role for nutrient availability, soil drying has a further negative impact on the nutrient availability and uptake by plants (Hamilton and Frank, 2001; Hermans et al., 2006; Landi et al., 2006).

Increasing evidences suggest that plants modify their surrounding soil enviroment, the rhizosphere, to better explore water and nutrient resources (Hinsinger et al., 2009; Spohn and Kuzyakov, 2013). Plants release a significant amount of their photosynthetic products into the soil in the process called rhizodeposition (Jones et al., 2009; Pausch et al., 2013). The constituents of these exudates include low-molecular-weight compounds, such as sugars, amino acids, and organic acids (Fischer et al., 2010), and high-molecular-weight compounds, such as mucilage.

Mucilage alters the physical properties of the rhizosphere (Carminati et al., 2010; Moradi et al., 2011; Watt et al., 1994; YOUNG, 1995) and nutrient availability (Belfort et al., 2007; Miransari, 2013; Richardson et al., 2001). Upon secretion from the root tip, mucilage penetrates into the soil pore space and coats the soil particles. As the soil drties, mucilage partly dehydrates, its viscosity increases and it binds the soil particles together and keeps them in contact to the roots (Albalasmeh and Ghezzehei, 2014). The formed layer of soil particles adhering to the roots is commonly called rhizosheath (Watt et al., 1994). Rhizosheath forms at the root surface of many plant species such as cereals, maize, sorghum, cactus, wheat, barly (Delhaize et al., 2012; George et al., 2014b; McCully, 1999). Rhizosheaths formation is strongly correlated to length and density of root hairs (Delhaize et al., 2012). The volume and stability of rhizosheath depend on the plant species, the number of drying and rewetting cycle and sources of secreted mucilage (George et al., 2014b; Nambiar, 1976; Watt et al., 1994, 1993). Rhizosheaths of grasses formed under dry conditions are larger, more coherent, and more strongly bound to the roots than those formed in wet soils (Watt et al., 1994, 1993). The rhizosheath volume of grass was approximately three times bigger in dry compared to wet conditions (Watt et al., 1994, 1993). It was hypothesized that drying and rewetting of soil

stabilize mucilage network close to the root surface and additionally alter the quantity and quality of root and microbial exudates (McCully, 1999; Watt et al., 1994).

Several important functions for water and nutrient uptake have been attributed to the rhizosheath, in particular under water stress condition: i) maintaining the contact between roots and soil during drying (North and Nobel, 1997; Watt et al., 1993; YOUNG, 1995); ii) keeping the soil next to the roots wetter than the bulk soil possibly maintaining the hydraulic connection between the roots and soil (Carminati et al., 2011, 2010; Moradi et al., 2011; Watt et al., 1993; Young, 1995); and iii) providing favorable environment for microbial activity due to larger soil moisture and carbon content around the root (Drenovsky et al., 2004; Kuzyakov and Blagodatskaya, 2015; Pausch and Kuzyakov, 2011). There is a strong correlation between intensity of root exudation, carbon content, soil moisture and enzyme activities in the rhizosphere (Kuzyakov and Blagodatskaya, 2015; Ma et al., 2017a).

Recent experiments with tracing water fluxes by neutron radiography showed that the rhizosphere turns water repellent upon drying (Carminati et al., 2010; Zarebanadkouki et al., 2016a). The main reason of this hydrophobicity is probably the presence of lipids in mucilage (Read et al., 2003). Hydrophobicity of rhizosphere limits the water fluxes across the rhizosphere and consequently reduces root water uptake (Zarebanadkouki and Carminati, 2014).

In the last decade, surfactants have been used to improve irrigation efficiency in hydrophobic soils and to increase water infiltration in the root zone (Chaichi et al., 2015a; Daneshnia et al., 2015; Franklin, 2007; Jafarian et al., 2015a). Density and quality of the turf grass exposed to water stress improved after surfactants were added (Franklin, 2007). Although the application of surfactants to facilitate the rewetting of water repellent soils is a well-established practice, the mechanisms of surfactants' interactions with root exudates and mucilage have not been explored.

The aim of this study was to introduce a concept to engineering the rhizosphere properties to optimize water and nutrient transport as well as biological activity at the root-soil interface. The concept is based on two ideas: i) to stabilize and maintain mucilage and other root exudates in the vicinity of the roots; and ii) to facilitate the rewetting of rhizosphere upon drying cycles. To this end, we tested whether a selected commercial surfactant (ACA1820, Aquatrols Corporation of America, Paulsboro, New Jersey, U.S.A) act as a rhizoligand.

We define rhizoligand a substance that: i) decreases mucilage swelling and ii) facilitates the rewetting of the rhizosphere.

We hypothesize that the application of rhizoligands stabilizes mucilage at the root surface and stimulates rhizosheath formation. The underlying hypothesis is that by decreasing mucilage swelling, rhizoligands maintain mucilage close to the roots increasing the strength of the bonds between the root surface and the soil particles, enhancing rhizosheath fromation. Furthermore, we hypothesize that the higher water content in the rhizosphere of plants treated with rhizoligands enhances microbial activity in the rhizosphere under drought.

Figure 1 shows our conceptual model of the mode of action of rhizoligands. Rhizoligand and mucilage have hydrophilic and hydrophobic functional groups. In an aqueous environment, the hydrophilic heads of rhizoligand link to water molecules, whereas the hydrophobic heads associate with the hydrophobic mucilage groups. Such concept is based on experiments with surfactants and polymeric gels having hydrophobic groups (Goddard, 1994; Hansson and Lindman, 1996). The interactions between hydrophobic and hydrophilic groups of rhizoligands and mucilage form additional bridges between the mucilage polymers limiting mucilage swelling and increasing its stability. After mixing with rhizoligand, mucilage in soil becomes more viscous and remains at greater concentration in the vicinity of the roots. According to Albalasmeh and Ghezzehei (2014) mucilage starts to binding soil particles when its visocisty is sufficiently high. By increasing mucilage viscosity, rhizoligand are expected to improve soil particles binding and therefore rhizosheath formation.

To test our concept, we first investigated the capability of a surfactant to reduce the swelling of mucilage. Then, the effect of rhizoligand on biophysical properties of the rhizosphere of lupins grown in quartz sand was evaluated. The plants were subjected to repeated drying and rewetting cycles and the following parameters were measured: i) the soil water content after rewetting; ii) the rhizosheath formation; iii) the enzyme activities and carbon content in the rhizosphere; and iv) plant dry biomass.

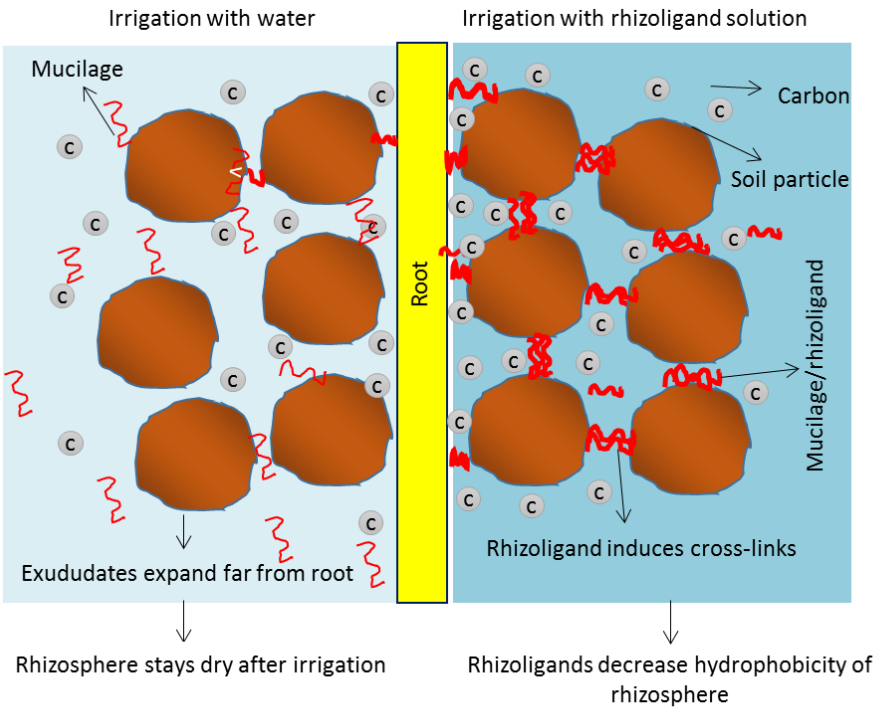

Figure 1. Conceptual model of rhizoligand interactions with mucilaginous compounds secreted by roots in the rhizosphere. The interactions between rhizoligand and hydrophobic mucilage groups reduces mucilage swelling and increases its viscosity. The greater the viscosity of mucilage increases the binding between soil particles and the root surface. The right side of the root in the figure illustrates the effect of rhizoligand on linking mucilage polymers, whereas the left figure indicates the case of a root not treated with rhizoligands.

3 Materials and Methods

3.1 Mucilage swelling

To test our conceptual model, we first measured mucilage swelling. We used mucilage from chia seeds (*Salvia hispanica*), which showed a physical behavior similar to that of mucilage from maize and lupin: it forms a gel upon immersion in water and it turns hydrophobic upon drying (Kroener et al., 2014). However, mucilage from chia seed might differ from root mucilage. The physicochemical properties of root mucilage might depend on many factors, such as age and grwoing conditions, and they are likely to show large variations among plant species (Zickenrott et al., 2016).

Chia seeds were mixed with water at a ratio of 1 to 10 (g seeds/g water) and the mixture was stirred using a magnetic stirrer for 2 hours. The mixture was passed through a series of sieves with size of 0.5 and then 0.2 mm by applying a suction of -800 hPa. Afterwards, 200 g of the extracted wet mucilage were placed in a large petri dish (20 cm in diameter) and they were let dry in a ventilated oven at a temperature of 40 °C. The initial concentration of mucilage was calculated as the dry mass of mucilage (oven dry) divided by the wet mass of mucilage and it was estimated to be 0.6%. The petri dish was covered with a thin layer of paraffin enabling us to easily remove the thin layer of dried mucilage. This procedure resulted in a relatively uniform layer of dried mucilage (with respect to the thickness). A small piece of dried mucilage (1 cm × 2 cm) was weighted and immersed in water (control treatment) and in a selected rhizoligand solution (ACA1820, Aquatrols Corporation of America, Paulsboro, New Jersey, U.S.A) at a concentration of 1 [milliliters of surfactant per liters of water]. This rhizoligand was used in all experiments. Mucilage adsorbed water and swelled until it reached its maximum swelling capacity (which took approximately 2 days). The exceeding water was gently removed, the swollen mucilage was collected and its water content was determined gravimetrically. We have replicated the measurements five times.

3.2 Plant and soil preparation

Seeds of white lupin (*Lupinus albus* L. cv. Feodora) were soaked in 10% H_2O_2 solution for 5 min and then they were thoroughly washed. The seeds were subsequently germinated in the darkness on moist filter papers for 2 days. The seedlings were planted in thin aluminum containers (28 cm width, 30 cm height, and 1 cm thickness) filled with quartz sand (particle size ranging from 50 to 250µm). The quartz sand packing had a bulk density of 1.4 g cm^{-3}. The containers were filled homogeneously, while they were laid horizontally and the sand was passed through a sieve with mesh size of 2 mm in order to reduce soil layering. The germinated seeds were planted at a depth of 1 cm into the containers (one seed per container) and then transferred to a climate chamber under controlled conditions: a daily light cycle of 14 h and 10 h of darkness, a light intensity of 500 µmol m^{-2} s^{-1}, day: night temperature of 24: 19 °C, and relative humidity of 60%.

During the first three days after planting, seedlings were irrigated daily from the top. After the shoots emerged, we covered the soil surface with 1 cm layer of gravel (ca. 2 mm in diameter) to minimize evaporation. The plants were irrigated by capillary rise every fourth day. They were slowly immersed in 15 cm water table for one hour. The containers were then gently

lifted, allowing free drainage of excessive water through the holes at the bottom of the container. This procedure resulted in an average soil water content (volume of water divided by the total soil volume) of 0.25-0.30 cm^3 cm^{-3} after irrigation.

When the plants were two weeks old and the sand had a water content of 0.27 to 0.30 cm^3 cm^{-3}, the first drying cycle was started. The irrigation was stopped and the plants were let dry till a water content of 0.04-0.05 cm^3 cm^{-3}, which was close to the wilting point. Then the plants were divided into two groups: one group was irrigated with water and the second group with water containing rhizoligand (ACA1820) at a concentration of 0.05 g L^{-1}. The drying and rewetting cycles were repeated six times, with the plants being rewetted by capillary rise when they reached a water content of 0.04-0.05 cm^3 cm^{-3}. To determine soil water content during each cycle of drying, the containers were weighted every 12 hours. The two treatments (water versus rhizoligand) were replicated four times.

3.3 Analysis of rhizosheath properties

After the six drying/wetting cycles, when the plants were around 50 days old, we let the plants dry the sand to a water content of 0.04-0.05 cm^3 cm^{-3}. Thereafter, we laid the containers horizontally and opened the detachable plate of each container. The whole root system and the sand attached to the roots were gently removed from the sand and shaken gently to remove the loose sand. The sand adhering to the roots after shaking is referred to as rhizosheath.

We cautiously removed most of the roots from the upper part of the containers and placed them immediately in plastic bags to minimize evaporation and shrinkage of the roots. The whole root segments were spread on an A3 plexiglass tray of the WinRhizo flatbed scanner (Epson STD 4800) equipped with a double light source to avoid root overlapping. The images were acquired using the TWAIN interface at 800 dpi resolution.

Length, radius and volume of roots and rhizosheath were analyzed using the software WinRhizo 2008a image analysis system (Reagent Instruments Inc., Canada). The average radius of the rhizosheath plus root was calculated in treatments with and without rhizoligand. Note that we independently determined the thickness of roots after removing the attached sand in water and water containing rhizoligand samples. For quantification of scanned images by WinRhizo, we first selected the root segments that had no lateral roots and applied a threshold filter to distinguish the root-rhizosheath from their backgrounds. Then, the segmented roots were skeletonized and the length of each root segment was calculated (Zarebanadkouki et al., 2016a). WinRhizo gave the total surface area in each root-rhizosheath segment, A [cm^2].

Assuming that the root-rhizosheath had a cylindrical shape, the average thickness of the root-rhizosheath was calculated as

$$A = 2\pi rL \qquad \text{(Eq.1)}$$

Where L is length of the skeletonized root-rhizosheath segment [mm] and r is the radius of the segmented root-rhizosheath [mm].

In parallel, the weight of roots and rhizosheaths were gravimetrically determined. For removing rhizosheath attached to the root two successive treatments were used: first, the roots were immersed in the distilled water for 24 h and then the sand attached to the root surface was removed through washing and collecting sand. To collect any remaining sand from the root surface, we used a small soft brush. The sand removed from the roots at each step was collected and dried in an oven for 48 h at 104 °C. Then we measured the mass of the sand attached to the roots and normalized it for the root dry mass (Watt et al., 1994).

3.4 Carbon content analysis

The carbon content in one gram of the rhizosheath and bulk soil was analyzed by VarioMax CNS apparatus (VarioMax CNS, Elementar, Germany) according to the Dumas combustion method. The bulk soilwas defined as the sand remaining in the sample containers after removal of the roots – i.e. the not-adhering sand (Chimento et al., 2016).

3.5 Enzyme assays

Extracellular enzyme activities were assayed using fluorogenically labeled substrates (Marx et al., 2005; Razavi et al., 2015). Four enzymatic activities were analyzed: (1) β-glucosidase, which is involved in C-cycle; (2) Chitinase, which is involved in C- and N-cycles; (3) acid phosphatase, which is involved in P-cycle; and (4) sulfatase, which involved in S cycle. In order to assess enzyme activities in rhizosheath and bulk soil, four types of fluorogenic substrates based on 4-methylumbelliferone (MUF) were used (Table 1), (Koch et al., 2007; Stemmer et al., 1998). The MUF-substrates were dissolved in 2-methoxyethanol. Saturation concentrations of fluorogenic substrates were determined in preliminary experiments (Razavi et al., 2015). Pre-dissolved MUF substrates were further diluted with sterile universal buffer [MES ($C_6H_{13}NO_4SNa_{0.5}$)].

One gram of fresh sand was mixed by magnetic stirrer with 50 ml water using low-energy sonication (40 J S^{-1} output energy) for two minutes (Koch et al., 2007; Stemmer et al., 1998). Afterwards, 50 µl of sand suspension was added to 150 µl of each substrate solution (containing either 50 µl universal buffer) in a 96-well microplate (Puregrade, Germany) and incubated for 2 h. Fluorescence was measured in microplates at excitation wavelength of 355 nm, emission wavelength of 460 nm, slit width of 25 nm, with a Victor^{3}1420-050 Multilabel Counter (PerkinElmer, USA).

Each enzyme in three replicates was assayed in each sample (bulk soil and rhizosheath of lupin) at 20 ºC. Calibration curves as well as the controls for the autofluorescence of the substrate were included in every series of enzyme measurements. Enzyme activities were expressed as MUF release in nmol per g dry soil per hour (nmol MUF g^{-1} soil h^{-1}), (Razavi et al., 2015).

Table 1.: Description of the substrates for estimation of enzyme activities in the rhizosheath and bulk soil.

Enzyme	**Substrate**	**Buffer**
C-cycle enzymes		
β-glucosidase	4-methylumbiliferyl-β-D-glucopyranoside	MES*
N-cycle enzymes		
Chitinase	4-methylumbiliferyl-N-acetyl-glucosaminide	MES
P-cycle enzyme		
Acid phosphatase	4-methylumbiliferyl phosphate	MES
S-cycle enzyme		
Sulfatase	4-methylumbiliferyl sulfate potassium salt	Sodium acetate

* MES: ($C_6H_{13}NO_4SNa_{0.5}$)

3.6 Plant biomass measurement

At the end of the drying/wetting experiments, when the plants were 50 days old, we collected roots and shoots. The dry weight of roots and shoots were determined gravimetrically. The roots were separated from the shoots and carefully all the soil particles attached to the roots

were removed prior to weighing. . Both roots and shoots were dried in oven for 24 h at 105 °C and then were weighted individually.

3.7 Statistical analysis

To evaluate statistical differences between two samples, t-test in the software R (version 3.3.2) was applied. The replicates were compared to determine the significant differences between plants irrigated with water and rhizoligand solution. Differences were reported to be significant at an error probability level of $p < 0.05$.

4 Results

4.1 Mucilage swelling

Maximum swelling of chia mucilage significantly decreased with the rhizoligand addition ($P<0.05$) (Fig. 2). Rhizoligand reduced the final swelling of chia mucilage by a factor of 1.89 in comparison to water. One gram of dry mucilage adsorbed 272±18 of water and 144± 14 g of rhizoligand solution.

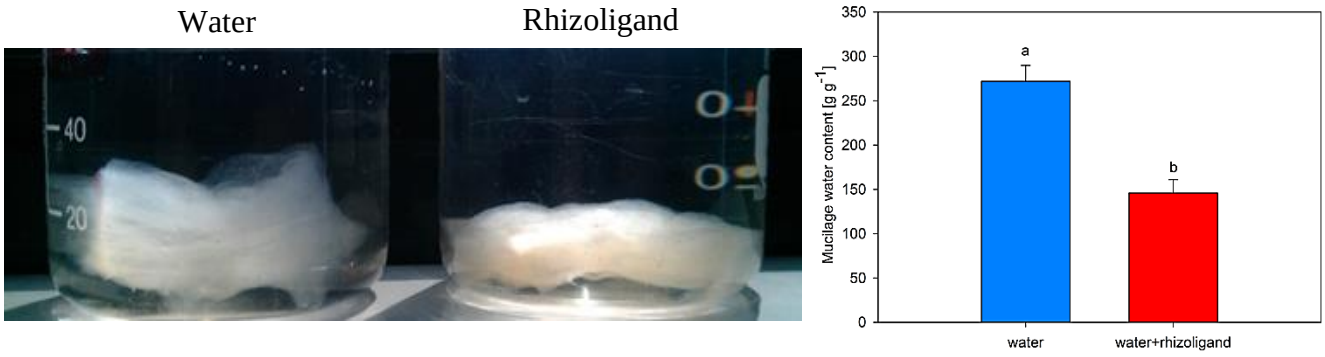

Figure 2. Left) swelling of dried chia mucilage in water and rhizoligand solution. The mucilage was let hydrate for 48 hours. Right) maximum swelling of dried mucilage in water and rhizoligand solution. The results showed that rhizoligands decreased the maximum swelling of mucilage by a factor 1.9. Each value is the average of 5 replications. Different lower case letters indicate a significant difference at $P < 0.05$.

4.2 Wetting and drying cycle

The average soil water content shortly after irrigation was greater in the plants irrigated with the rhizoligand solution compared to water (Fig. 3). Irrigation via capillary rise resulted in an average soil water content of 0.26±0.01 and 0.23±0.01 cm^3 cm^{-3} in plants irrigated with and without rhizoligand, respectively. The differences resulted mainly from the fact that the rhizoligand increased the wettability of the rhizosphere, as shown in Ahmed et al. (2017) using same sand, plant variety and rhizoligand. Note that the drying cycles for plants irrigated with rhizoligand solution were one-two days longer than the plants irrigated with water, because of lower transpiration rates (Ahmed et al. 2017).

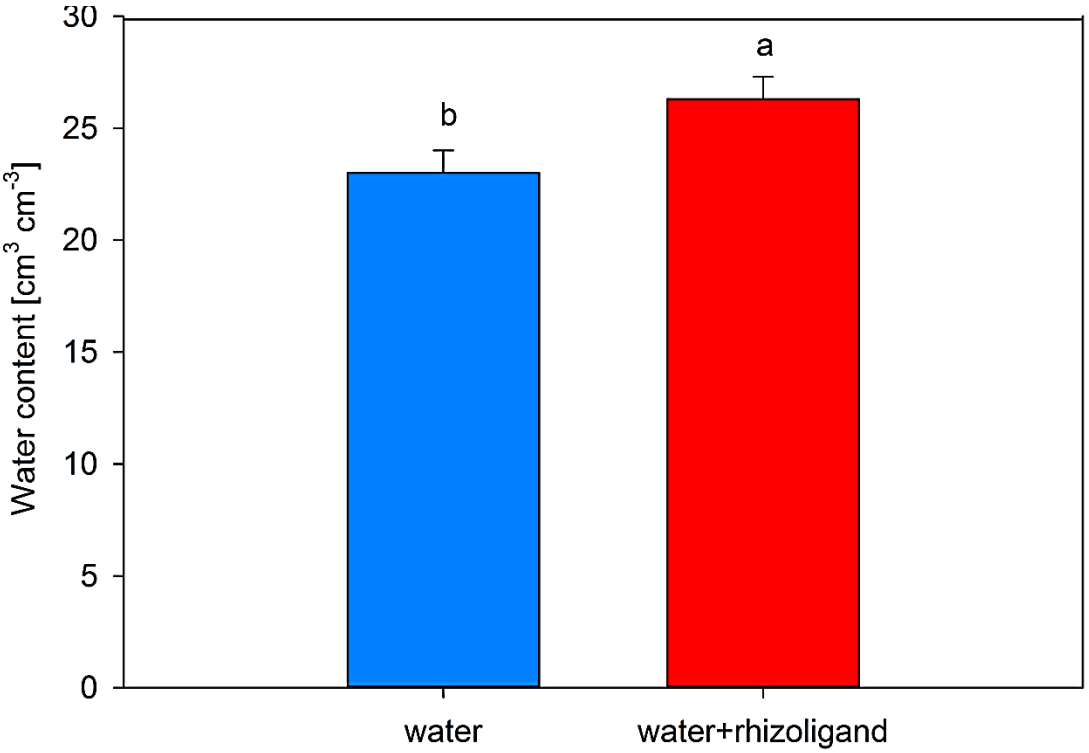

Figure 3. Soil water content shortly after irrigation of a 30-day-old lupin with water and water treated with a rhizoligand. The soil water content was measured gravimetrically by weighing the samples. Each value is the average of four plants. Different lower case letters indicate a significant difference at $P < 0.05$.

4.3 Rhizosheath development

The rhizosheath of plants treated with the rhizoligand were thicker than those of the plants irrigated with water (Fig. 4). For a better illustration of the differences, two roots with and without cluster roots are shown at higher resolution (Fig 5). These figures were obtained from a WinRhizo scanner. The average radius of the root and rhizosheath for the roots without cluster segment was 0.42 ± 0.09 mm in plants irrigated with water and 0.65 ± 0.12 mm in plants treated with the rhizoligand (Fig 6a). The average thickness of roots and their rhizosheaths of clusters root were 0.38 ± 0.08 mm and 0.63 ±0.16 mm, in plants irrigated with water and rhizoligand, respectively (Fig 6b). The root radius did not differ between the treatments. The root radius of the samples irrigated with water was 0.31±0.18 mm and it was 0.31± 0.14 mm in the samples treated with rhizoligand.

In line with these, the mass of the rhizosheath per dry mass of roots was 11 g g^{-1} in the water treatment and 18 g g^{-1} in the rhizoligand treatment (Fig. 6c).

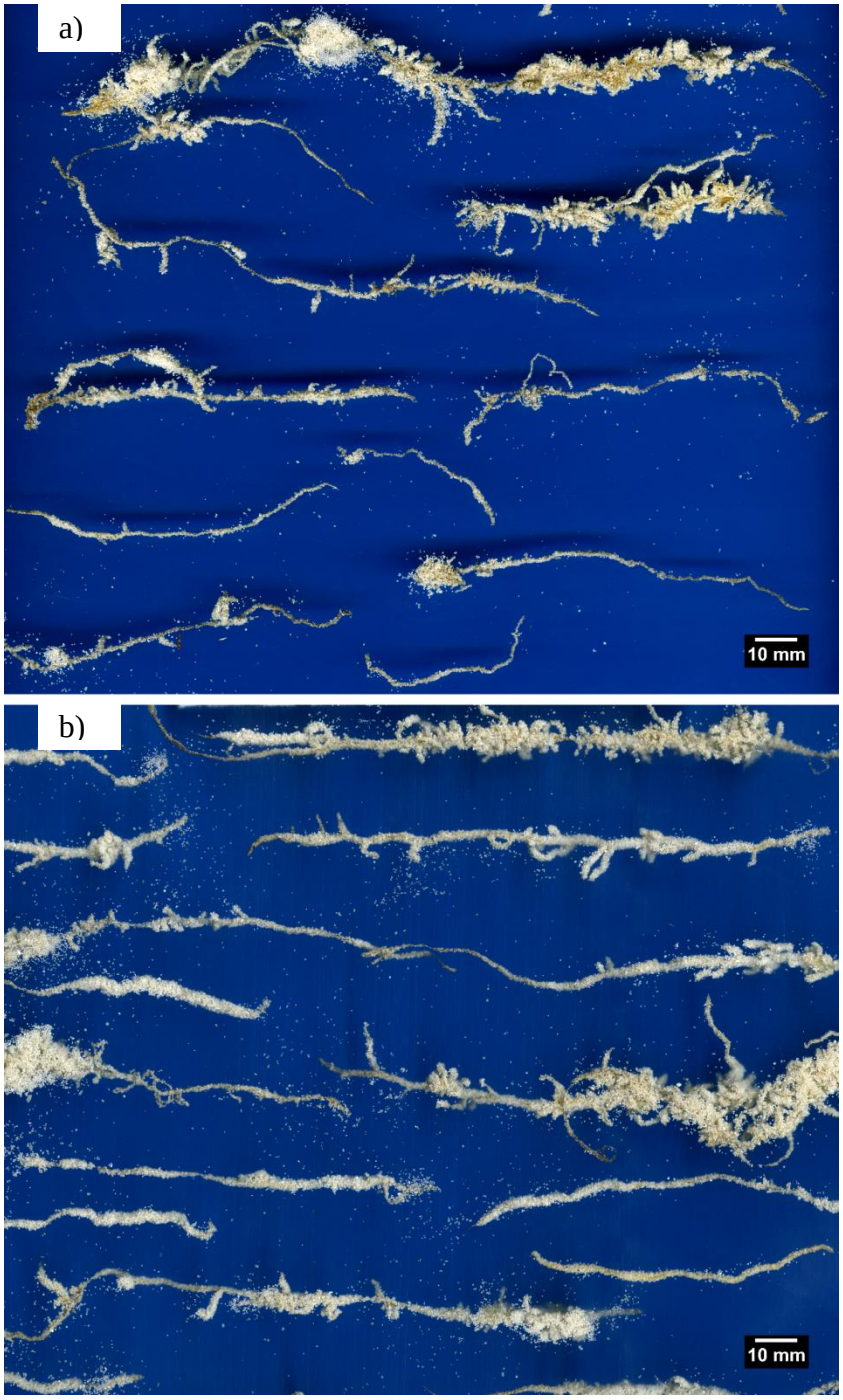

Figure 4. Roots and surrounding rhizosheath scanned with WinRhizo for the plants irrigated with water (a) and water treated with rhizoligand (b). Thickness of rhizosheath in both selected roots with and without cluster roots was greater in plants irrigated with rhizoligand than with water.

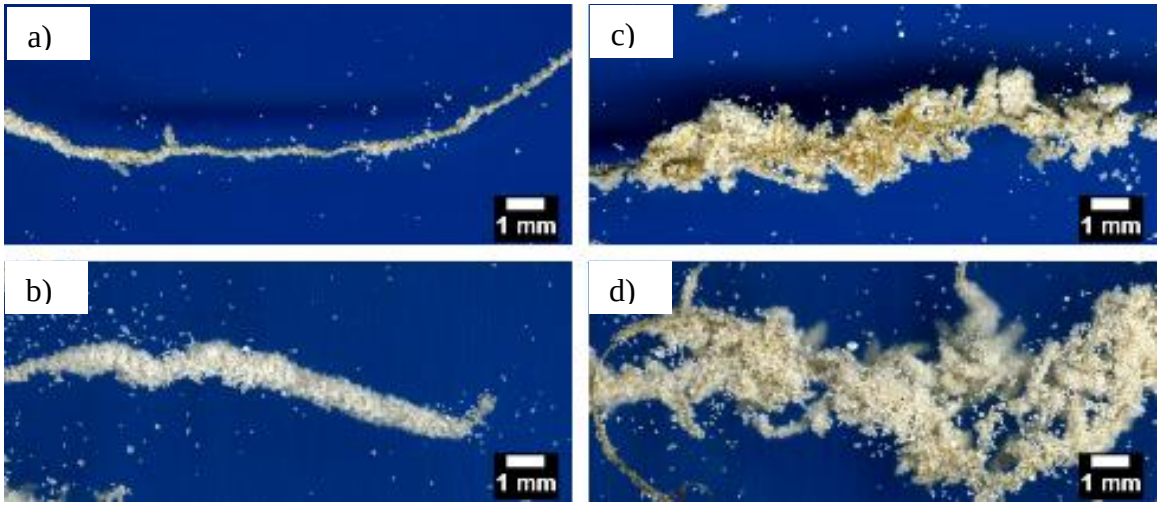

Figure 5. Selected roots and surroundings rhizosheath scanned with WinRhizo for the plants irrigated with water (a, b) and water treated with the rhizoligand solution (c, d). The figures show greater radius of rhizosheath in both bare root (a, c) and root with cluster (b, d).

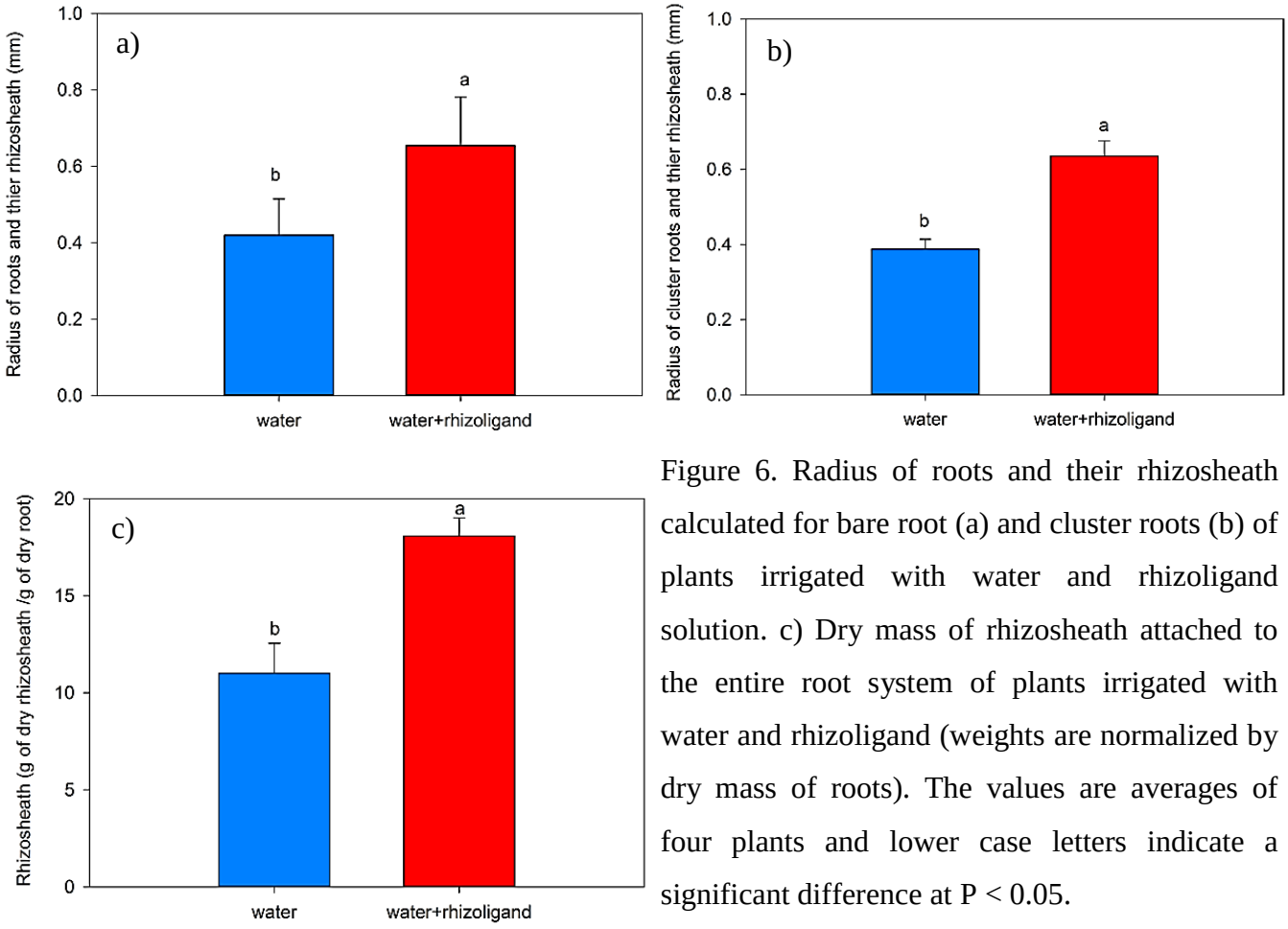

Figure 6. Radius of roots and their rhizosheath calculated for bare root (a) and cluster roots (b) of plants irrigated with water and rhizoligand solution. c) Dry mass of rhizosheath attached to the entire root system of plants irrigated with water and rhizoligand (weights are normalized by dry mass of roots). The values are averages of four plants and lower case letters indicate a significant difference at $P < 0.05$.

4.4 Carbon content and enzyme activities in the rhizosheath

The average carbon content in one gram of rhizosheath was 0.35±0.04 mg g^{-1} in the samples irrigated with water and 0.47±0.16 mg g^{-1} in the samples irrigated with the rhizoligand solution (7a). The difference was not significant. The carbon content in the one gram of bulk soil was significantly lower: it was 0.1±0.01 mg g^{-1} in the bulk soil of samples irrigated with water and 0.13±0.01 mg g^{-1} in the samples irrigated with the rhizoligand solution, respectively (Fig. 7a).

Here, the carbon content was reported as mg of C per g of soil particles attached to the roots. To evaluate the effect of rhizoligand on allocation of carbon in the rhizosphere, it should be taken into account that a greater soil mass was attached to the roots treated with rhizoligand and therefore the carbon content was averaged over a larger volume of soil. The total carbon content in the rhizosheath, calculated by multiplying the carbon content by the dry mass of rhizosheath, was 3.93± 1.04 mg C/ g root in the samples irrigated with water and it was 7.10 ± 0.92 mg C/ g in plants irrigated with rhizoligand. Total carbon content accumulated in the rhizosheath of plants irrigated with the rhizoligand solution was 1.80 times greater than in the rhizosheath of plants irrigated with water and this difference was statistically significant ($P<0.05$) (Fig. 7b).

The activity of four enzymes in the rhizosphere (collected rhizosheath) of plants irrigated with water and rhizoligand was assessed. Activity of three enzymes (chitinase, sulfatase and β-glucosidase) in the rhizosphere of plants irrigated with the rhizoligand solution was significantly greater than in plants irrigated only with water. In contrast, the application of rhizoligand did not affect the activity of phosphatase in the rhizosphere. Enzyme activity in the bulk soil did not change between treatments (Fig. 8).

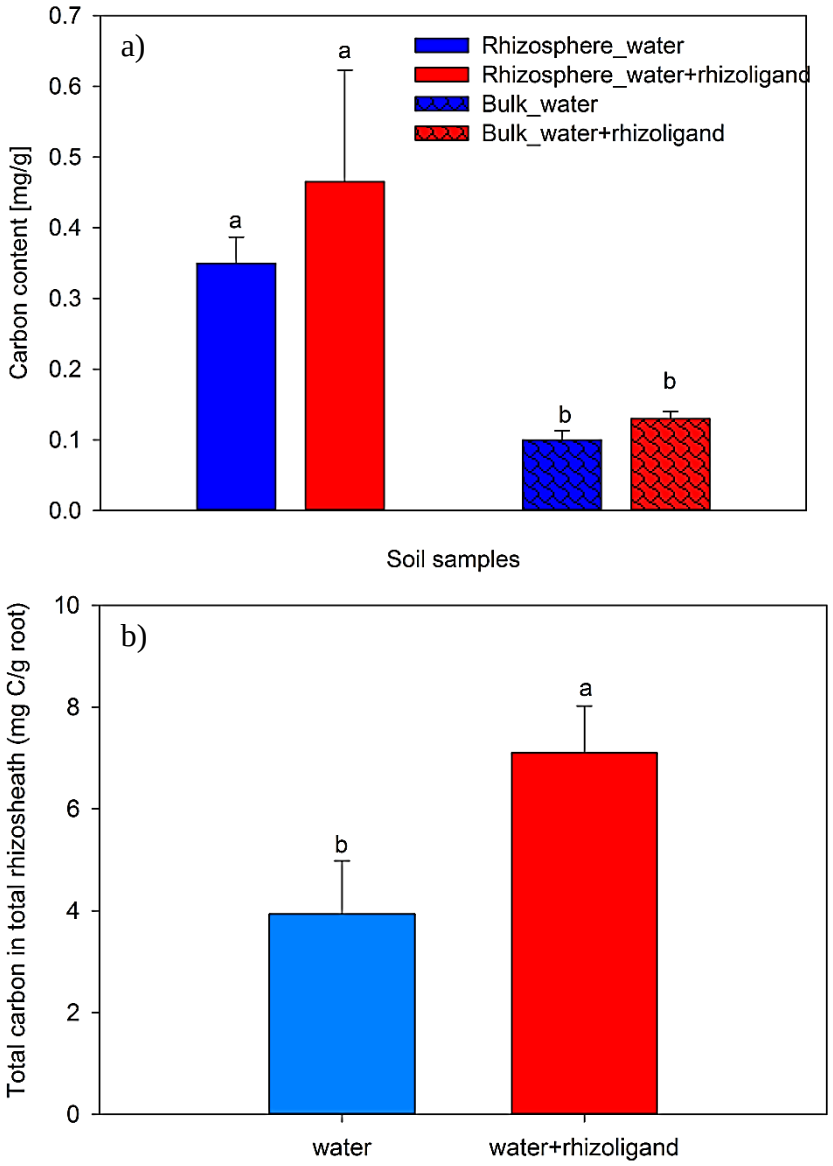

Figure 7. a) Average carbon content (mg) per gram of rhizosheath (two simple patterns in the left-hand side) and bulk soil (two hatch patterns in the right-hand side). b) Total carbon in the rhizosheath of plants irrigated with water and rhizoligand solution. The values are averages of four plants and lowercase letters indicate a significant difference at $P < 0.05$.

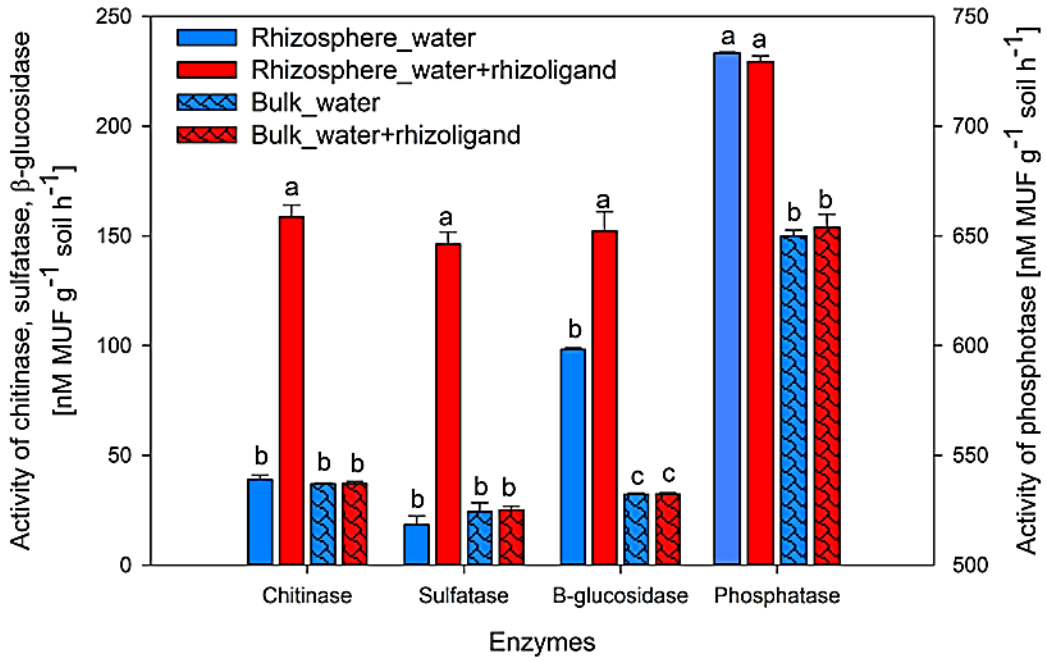

Figure 8. Activity of four enzymes in the rhizosheath and bulk soil of plants irrigated with water and rhizoligand solution. The values are averaged of four samples. Different lower case letters indicate a significant difference at $P < 0.05$.

4.5 Plant biomass

The average dry weight of shoots was 2.33± 0.23 g and 2.43±0.16 g in plants irrigated with water and with rhizoligand solution, respectively (Fig. 9a). The average dry weight of roots was 3.36± 0.64 g and 4.33± 0.22 g in plants irrigated with water and with rhizoligand solution, respectively (Fig. 9a). Dry weight of shoots in plants irrigated with rhizoligand solution was rather similar and did not show a significant difference. In contrast, dry weight of roots in plant irrigation with rhizoligand solution was significantly greater than of the plants irrigated with water. Furthermore, the result showed that the total biomass were 5.70±0.46 g and 6.76±0.23 g in samples irrigated with water and rhizoligand solution, respectively. The weight of total biomass in plants irrigated rhizoligand solution was 1.18 folds greater than samples under water irrigation and the difference was statistically significant ($P<0.05$) (Fig. 9b).

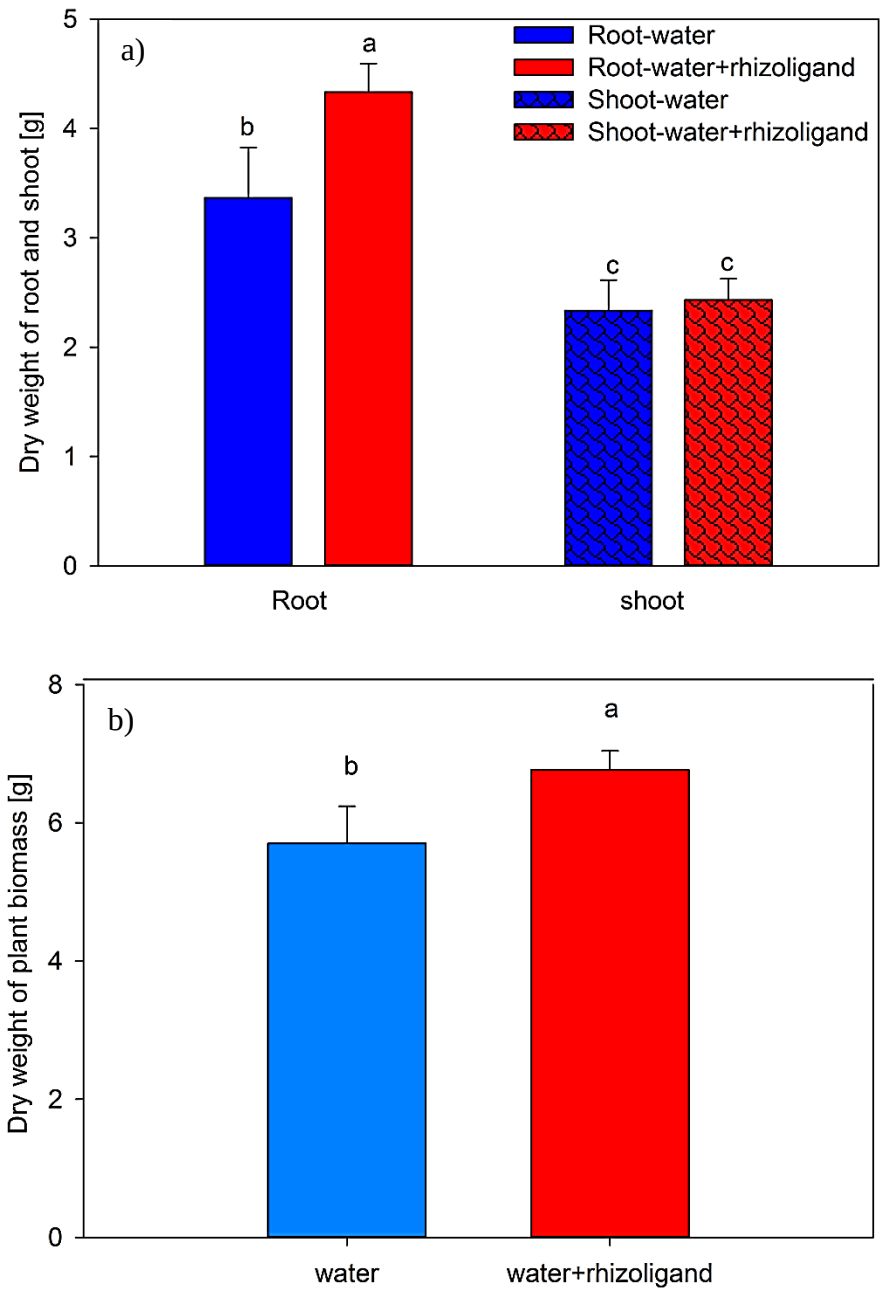

Figure 9. a) Dry weight of root (two simple patterns in the left-hand side) and shoot (two hatch patterns in the right-hand side) of lupins irrigated with water and rhizoligand solution. b) Total biomass of plants irrigated with water (left-hand side) and rhizoligand solution (right-hand side). The data are average of four samples. Different lower case letters indicate a significant difference at $P < 0.05$.

5 Discussion

The selected surfactant (ACA1820) acted as a rhizoligand: it increased the wettability of the rhizosphere and it reduced the final swelling of mucilage (according to the definition of rhizoligand given in the introduction). These effects were concomitant to thicker rhizosheath, higher enzyme activity and larger plant biomass.

The tested hizoligand increased the soil water content in the rhizosphere after irrigation of dry samples (Fig.3). Former studies showed that the rhizosphere of lupins in sandy soils turns hydrophobic and stays temporarily dry after rewetting (Moradi et al., 2012; Zarebanadkouki et al., 2016a). Application of surfactants helps to rewet the water repellent rhizosphere of lupins, as shown in Ahmed et al. (2016). As a consequence, the application of rhizoligand provides greater volume of water available to the plants during repeated drying/wetting cycles. Interestingly, despite of the greater water content of rhizosphere, plants irrigated with rhizoligands transpired less and had a greater water use efficiency (Ahmed et al., 2016). Similar results were by Jafarian et al., (2015) and Chaichi et al., (Chaichi et al., 2015a), who showed that surfactants increase water use efficiency in Alfalfa and corn under water limitation. Further studies are needed to understand why surfactant reduce transpiration.

Rhizoligand significantly increased rhizosheath formation in plants subjected to several drying and rewetting cycles. The rhizosheath thickness was approximately 1.60 times greater in the plants irrigated with rhizoligand compared to the plants irrigated with water (Fig. 6). This finding was attributed to the reduction in the maximum swelling of mucilage after treatment with rhizoligand (Fig. 2). We expected that a lower swelling results in a higher viscosity and therefore in a stronger capacity of mucilage treated with rhizoligand to bind soil particles together, as proposed in the model of Albalasmeh and Ghezzehei (2014). The observation that the tested surfactant reduced mucilage swelling is in line with the observations of Simovic et al., (Simovic et al., 1999), who showed that interaction between non-ionic surfactants and a hydrophobically modified polymer increased stability of this complex. This observation was attributed to the presence of an extra attractive force binding non-ionic surfactants to the hydrophobic groups of the polymer. These interactions increase viscosity of the complex (Simovic et al., 1999). A similar mode of action could have occurred in our samples, but further studies are needed to prove that surfactants cross-link root mucilage.

We expected to observe a greater carbon content in the rhizosheath of plants irrigated with rhizoligand solution. We hypothesized that lower mucilage swelling and its higher viscosity would reduce the diffusion of mucilage and other root exudates far from the roots, result in

greater carbon content in the rhizosheath of the plants treated with rhizoligand. However, the carbon content in one gram of rhizohheath was not different in the two treatments. Anyway, it should be considered that in the rhizoligand treatment, the sampled rhizosheath had a larger volume. The carbon content in the rhizosheath of plants treated with rhizoligand was therefore averaged over a larger distance from the root surface. Since the carbon content typically decreases with increasing distance from the root surface, the fact the in the two treatments the carbon content was similar possibly indicates a higher concentration of carbon content in the rhizoligand treatment. This speculation should be backed up by measurements of carbon content at higher spatial resolution.

Note that the total amount of carbon contained in the rhizoligand structure was much smaller compared to the increase in total carbon content in the rhizosheath of the plants treated with rhizoligand (Fig. 7b). Plants were irrigated six times with the rhizoligand solution at concentration of 0.05 g per liter. Based on the carbon content of the tested surfactant and the total volume of the rhizoligand solution added during irrigation, we estimated that a total of 3.5 mg C was added to the samples. This value is smaller compared to the changes in the total amount of carbon in the rhizosheath of plants irrigated with rhizoligand solution (Fig. 7b).

The activities of three enzymes (chitinase, sulfatase and β-glucosidase) significantly increased in the rhizosheath of the plants treated with rhizoligand (Fig. 8). The greater activity of these three enzymes in the rhizosphere of plants irrigated with rhizoligand solution can be explained by the improved wettability of the rhizosphere and by stimulated microbial activity in the rhizosphere. The greater wettability of the rhizosphere maintains microbial activity during sever drying. Additionally, the rhizoligand might have maintained rhizodeposits close to the root, including mucilage, inorganic and organic substances, and dead root cells, although our data cannot prove it. These components are an energy source for feeding microorganisms an in turn they might increase enzyme activities in the rhizosphere (L. Asmar et al., 1994; Brzostek et al., n.d.; Hinsinger et al., 2009; Jones et al., 2009; Kuzyakov and Domanski, 2000). The effect of rhizoligand on enzyme activity was limited to the rhizospheath and the rhizoligand had no effect on enzyme activity in the bulk soil. Phosphatase activity was not affected by the rhizoligand treatment. Generally, plants and soil microorganism employ many mechanisms to increase availability of phosphorus e.g. releasing extracellular phosphatases. In particular, lupin plants release large quantities of phosphatase as well as carboxylates (mostly citrate and malate) through their cluster roots to increase mobility and availability of phosphorus in soil. Our results showed that activity of phosphatase was greatest among all enzymes. Probably in our experiment, large amounts of phosphatase released by lupin suppressed activity of

microorganism to release phosphatase, as suggested in Landi et al., (2006) and Weisskopf et al. (2006), and this was not affected by the rhizoligand treatment.

The total biomass of plants irrigated with rhizoligand solution was 1.18 folds greater than that of plants irrigated with water (Fig. 9b). The increase in plant biomass shows a positive effect of rhizoligand in plant growth. This effect might arise from multiple processes, such as the greater water content in the rhizosphere, the thicker rhizosheath and the greater enzyme activity in the rhizosphere. All such effects might imply a greater accessibility of water and nutrient resources, particularly when plants experience severe drying as in our experiments. Further studies, with rhizoligands tested with varying plant species and in real soils, are needed to generalize our results and prove that rhizoligands really increase nutrient uptake and plant tolerance to abiotic stresses.

6 Conclusions

We introduced a new concept to engineer the biophysical properties of the rhizosphere. A commercial surfactant was used as a rhizoligand, which was defined as an additive that facilitates the rewetting of the rhizosphere and reduces mucilage swelling. We present experimental evidences that upon addition of rhizoligand the rhizosphere remained wet and mechanically better connected to the root surface (rhizosheath formation) and that enzyme activity in the rhizosphere and plant biomass were greater. We expect that these modifications of the rhizosphere have the potential to improve plant tolerance to abiotic stresses and improve agricultural sustainability in drought-prone areas.

7 Acknowledgements

The authors thank Ingrit Ostermeyer for her technical assistance, Menuka Maharjan for help in the laboratory and Dr. Muhammad Sanaullah and Dr. Bahar Razavi for their helpful comments during the experiment. This work was supported by Aquatrols Corporation of America.

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Chapter Three

(Study 2)

Effects of rhizosphere wettability on microbial and enzyme activities

As submitted for publication to Rhizosphere

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1 Abstract

Drying and wetting cycles of the rhizosphere compared to the bulk soil impact microbial and enzymes activities. For instance, the rhizosphere of some plant species (e.g. *Zea Mays* L. or *Lupinus Albus* L.) becomes water repellent upon drying and this may limit microbial activity during repeated drying and wetting events. The objective of this study was to investigate the effects of rhizosphere water repellency on microbial biomass and enzyme activities. We hypothesized that an increase of rhizosphere wettability by a polymeric surfactant (here referred to as rhizoligand) raises enzyme activities, especially during repeated drying/rewetting cycles.

Maize plants were grown in rhizoboxes and subjected to six drying/rewetting cycles for eight weeks. Half of the plants were irrigated with water and the other half with rhizoligand solution. After six drying/rewetting cycles, we measured: i) enzyme activities and distribution using zymography; ii) microbial biomass carbon; and iii) shoot and root biomass.

Application of a rhizoligand: i) increased the β-glucosidase and phosphatase activities by 5.3 and 2.9 times, respectively, in the regions close to the roots (0-0.5 mm distance from the root surface); ii) enlarged the area with high enzyme activity 1.46-fold for β-glucosidase and 1.2-fold for phosphatase; iii) increased microbial biomass content 1.57-fold; and iv) increased root biomass 1.24-fold.

This general stimulation of microbial activity is connected with the increase in rhizosphere wettability upon rhizoligand application. The higher wettability maintains the stability of microbial habitats and stimulates enzyme activities in the rhizosphere during repeated drying/wetting cycles. We propose that such biophysical rhizosphere interactions could open new avenues to improve plant performance in water deficit condition by rhizoligand application.

Keywords

Microbial activity; Enzyme activity; Soil water repellency; Rhizoligand; Rhizosphere processes; Polymeric surfactant

2 Introduction

Among all biotic and abiotic stresses, drought is one of the most limiting factors compromising plant growth and crop productivity. Many physiochemical and biological soil and plant processes are adversely affected by drought (Chaitanya et al., 2003). It has been proposed that plant roots modify the properties of their surrounding soil, the so-called rhizosphere, in order to better tolerate versus the adverse effects of drought such as reduced nutrients availability (Carminati and Vetterlein, 2013; Walker et al., 2003). Plants release a large proportion of their translocated photosynthetic compounds into the rhizosphere modifying the edaphic properties and facilitating nutrient uptake. Root exudates such as amino acids, carbohydrates, carboxylic acids (Farrar et al., 2003; Fischer et al., 2007) and mucilage (Jones et al., 2009) stimulate microbial activity (Hinsinger et al., 2009; Kuzyakov, 2002) and thus the production of diverse extracellular enzymes (F. Asmar et al., 1994). In fact, The rhizosphere is a hotspot of microbial and enzyme activities in the soil (Drenovsky et al., 2004; Kuzyakov and Blagodatskaya, 2015; Pausch and Kuzyakov, 2011). These organic compounds provide the initial energy sources for microbial activity and trigger the abundance of microorganisms in the rhizosphere. Further, the presence of root exudates in the soil alters the biophysical and biochemical properties of the soil, which in turn favors microbial activity. Root exudates consist of low molecular weight compounds, which are readily degradable carbon sources for microorganisms, and high molecular weight compounds which are more slowly consumed by microorganisms, such as lysates and mucilage (Kuzyakov 2002).

Soil microorganisms are major influencers in the availability and accessibility of nutrients to the plants by producing enzymes that solubilize nutrients during mineralization and decomposition of soil organic matter (SOM). The interactions between roots and associated microorganisms have been suggested to be vital for agricultural sustainability (Ortíz-Castro et al., 2009). Incubation of plant roots with beneficial soil fungi (e.g. *Trichoderma spp*) or beneficial rhizobacteria enhances plant productivity as well as the immune system of plants to increase their resistance to soil-borne and foliar pathogens (Harman et al., 2004; Ortíz-Castro et al., 2009). Thus, the interaction of plant roots with soil microorganisms is a pathway to more optimal use of scare resources, e.g. reduce consumption of synthetic fertilizer and pesticides (Compant et al., 2005; Singh et al., 2011). Extracellular enzyme production represents one of several microbial and plant mining strategies. Soil microbial biomass and enzyme activity are influenced by various abiotic factors such as temperature, pH, organic carbon and soil water content (Nannipieri et al. 2011). Limiting soil water

content is a major factor suppressing microbial biomass and associated enzyme activities (Ilstedt et al., 2000; Stark and Firestone, 1995). For example, a 10% reduction of soil moisture has been shown to reduce β-glucosidase activity by 10–80% (Sardans and Peñuelas, 2005).

Recent studies have shown that during a drying cycle, the rhizosphere remained wetter than its surrounding bulk soil and in contrast, after rewetting, it remained temporarily drier than the bulk soil (Carminati, 2010). The wetter rhizosphere was attributed to the presence of mucilage released by roots and microorganisms. Mucilage, a bio-polymeric gel with a high capacity to absorb large volumes of water, increases water content in the rhizosphere and it might maintain higher root and microorganism activities even when the bulk soil dries. A wetter soil environment in surrounding of the microorganisms could favor the diffusion and increase the transport of nutrients towards the microorganisms as soil dries and the transport properties of the bulk soil drops.

However, as the soil dries, the rhizospheres of lupin and maize were observed to turn water repellent (Ahmed et al., 2015; Benard et al., 2016; Carminati et al., 2010). Moradi et al. (2012) showed greater contact angle of water in the rhizosphere relative to bulk soil after drying. Similarly, temporarily reduction of rewetting rates and thus reduction of root water uptake was observed in the repellant rhizosphere of lupins after irrigation Hydrophobicity of rhizosphere was attributed to the presence of phospholipids substance in the mucilage (Read et al., 2003).

Repellent soils also have been reported in a wide range of soil types and various climatic conditions worldwide. Recent reviews have drawn the attention to the influence of soil water repellency on increase in irrigation requirements and reduced fertilizer performance. Recently, application of surfactant compounds have been suggested to decrease soil hydrophobicity and improve water retention soil (Debano, 2000; Franklin, 2007; Moore et al., 2010).

Our former studies revealed that surfactants reduce hydrophobicity ans thus increases the wettability of the rhizosphere (Ahmadi et al., 2017; Ahmed et al., 2017). Additionally, the selected surfactant was found to decrease mucillage swelling , probably because it corss-linked mucilage polymers. The consequences were that the surfactant increasesd mucilage viscosity and enhanced the formation and size of a stable layer of soil particles adhering to the root surface, the so called rhizosheath (Ahmadi et al., 2017). For this reason we called the selected surfactant rhizoligand. In the presented study, we investigated the effect of

rhizoligand on the microbial properties (i.e. enzyme activities and microbial biomass) of the rhizosphere of plants undergoing repeated drying and wetting cycles.

We hypothesize that rhizosphere hydrophobicity, reducing the water content around the roots, is associated with a decrease in microbial biomass and enzyme activity . Specifically, our working hypotheses are that: i) the increased rhizosphere moisture after treatment with the rhizoligand increases enzyme activity and stimulates soil microbial activity; and ii) rhizoligand will affect spatial distribution of enzyme activity in the rhizosphere. A recently modified imaging technique called direct soil zymography was used as a tool to visualize the activities of enzymes *in situ* in soil. Zymography is a promising technique to visualize and analyze two-dimensional distribution of enzyme activities in the rhizosphere(Ge et al., 2017; Razavi et al., 2017), detritusphere (Liu et al., 2017; Ma et al., 2017b), biopores (Hoang et al., 2016; Razavi et al., 2017)) and other microbial hotspots. We employed this technique to evaluate the spatial distribution of two enzymes, phosphatase and β-glucosidase, which are involved in P and C cycles.

3 Materials and methods

3.1 Soil and plant preparation

Soil was sampled from top 10 cm (Ap horizon) of Reinhausen located 8 kilometers southeast of Goettingen, Germany. The soil consisted of 73% sand, 18% silt, and 9% clay. The total carbon and nitrogen content was 2% and 0.17%; respectively and its pH was 4.9.

The samples were hand-mixed, roots and stones were removed and the remaining soil was collected in tight plastic bags and transported to the laboratory. The soil was dried at a room temperature for 5 to 6 days and it was sieved to a particle size smaller than 2 mm.

Twelve rhizoboxes (inner size of 12.3 × 12.5 × 2.3 cm) were filled horizontally by pouring the soil through a 2-mm sieve to avoid soil layering and achieving a pretty homogeneous packing. The detachable top side was closed, the rhizoboxes were gently turned vertically, and they were slightly shaken to stabilize the soil particle packing.

Maize seeds (*Zea mays* L) were soaked in 10 % H_2O_2 solution for 5 min then washed. Thereafter, the seeds were germinated on filter paper incubated with 10 mL^{-1} $CaSO_4$ solution in the darkness for 72 hours. One seedling was sown at a depth of 1 cm in each rhizobox. The plants were placed in a climate chamber under controlled conditions during the whole growth period with day: night temperature of 24 °C:19 °C, 14 h light cycle and 10 h of dark cycle,

light intensity of 500 µmol m^{-2} s^{-1} at the top of the canopy, and 60% relative humidity. During the entire growth period, the rhizoboxes were at an inclined position (50° angle) to encourage the roots to gravitropically grow close to the lower wall of the rhizobox. Plants were watered daily for the first four days. Subsequently, irrigation was applied every third day until the plants were two weeks old. At this point the drying and rewetting cycles were initiated.

We irrigated the rhizoboxes using capillary rise by immersing the boxes in water (with the water table 5 cm above the bottom of the samples) for half an hour. When plants were two weeks old, the first drying cycle was initiated. The samples were dried (by plant transpiration) to a volumetric soil water content of 0.04-0.06 cm^3 cm^{-3}. The water content was measured gravimetrically by weighing the rhizoboxes and subtracting the dry weight. The first drying cycle took 6-7 days. Plants were then divided into two groups, one group was irrigated with water and another group was irrigated with a selected rhizoligand - an alkyl ether of methyl oxirane-oxirane copolymer with molecular weight of ~ 2500 Da (ACA1820, Aquatrols Corporation of America, Paulsboro, New Jersey, U.S.A) at a concentration of 0.05 g L^{-1} of water. This concentration of the rhizoligand was tested in previous studies (Ahmadi et al., 2017). All samples were irrigated at the same time when the water content in the control samples (average of the samples irrigated with water only) was between 0.04 and 0.06 cm^3 cm^{-3}. Six drying and wetting cycles were applied. Note that the plants were not fertilized during the whole growth period.

3.2 Direct soil zymography

After six drying/wetting cycles, when plants were eight weeks old, we stopped irrigation and allowed the plants to dry once more until the soil water content reached 0.04-0.06 cm^3 cm^{-3}. The rhizoboxes were placed horizontally, opened and the plate of each rhizobox was removed. To assess enzyme activity, we followed the modified protocol described by Razavi et al. (2016). To detect β-glucosidase and phosphatase activity, membranes were saturated with 4-methylumbelliferyl-β-D-glucoside (MUF-G) and 4-methylumbelliferyl-phosphate (MUF-P), respectively. The substrate becomes fluorescent when it is hydrolyzed by enzymes (Dong et al., 2007). Each substrate was separately dissolved to a concentration of 1 mM in buffer [MES ($C_6H_{13}NO_4SNa_{0.5}$); pH: 6.5] (Koch et al., 2007).

For each enzyme and each replicate, a polyamide membrane filter (Tao Yuan, China) was saturated with the respective substrate. Polyamide membrane filters had a diameter of 20 cm

with a pore size of 0.45 μm. The membranes were cut into pieces (12.3 cm × 12.5 cm) to fit within the rhizoboxes. The rhizoboxes were opened from the rooted side and then the saturated membranes were placed directly on the soil surface. After one-hour incubation, the membranes were gently lifted, and any adhering soil was carefully removed using a small, soft brush. Thereafter, the membranes were placed under ultraviolet (UV) illumination with an excitation wavelength of 355 nm and an emission wavelength of 460 nm, in a light-proof room. The samples were fixed at a similar distance from the UV light source during photography using a digital camera (EOS 5D, Canon).

To convert the imaged gray values to enzyme activity, we conducted a calibration test: 4 cm^2 membranes were saturated with the MUF solutions at concentrations of 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM. The volume of MUF absorbed by membrane pieces was calculated according to their size. Thereafter, both membranes and rhizoboxes were imaged and analyzed in the same set up.

3.3 Image analysis and processing

Image processing was carried out using Matlab. Zymograms were transformed into 16-bit images and corrected for light variations and camera noise (Razavi et al., 2016). The gray values of the blank sides of the membrane were used as a reference. After referencing the zymograms, we calculated an average background grayvalue through the zymograms of calibration lines at concentration of zero and subtracted this value from all the zymograms. The contrast between roots and surrounding bulk soil through the zymograms enabled us to easily distinguish roots from the soil. To distinguish the root segments from their backgrounds a threshold filter was applied. Single root segments that had no overlap with other roots and that were entirely visible in the zymograms were selected for further analysis. The segmented roots were first skeletonized and the average gray value was calculated as a function of distance from root surface. Doing so we implicitly assumed a radial symmetry around the roots.

The calibration function was applied to convert the gray values captured on the zymograms into enzyme activity. A linear function was used to relate the gray values of the calibration membranes in a 4 cm^2 area to their substrate (MUF) concentration. To assess the response of plant roots to the treatments in the context of enzyme activities, the total enzyme activities (sum of enzyme activity in each rhizobox) were calculated in each calibrated zymogram individually after subtraction of their background. The extension of the rhizosphere was

calculated as the region with at least 10% higher enzyme activity than the rest of the soil. The extension was calculated as a distance from the root surface. We reported the average activity in the closest 0-0.5 mm to the root surface (the region characterized by the steepest gradients) and at a distance of 2-2.5 mm from the root surface (this is the distance where the profiles are flat, Fig. 3).

3.4 Microbial biomass carbon and plant biomass measurement

Microbial biomass C was measured by chloroform fumigation-extraction method (Vance et al., 1987), and was based on the difference between C extracted from fumigated and nonfumigated soil samples using 0.05 M K_2SO_4. A k_{EC} factor 0.45 was used to convert microbial C flush into microbial biomass C (Joergensen, 1996a).

Dry mass of roots and shoots were gravimetrically determined after applying six drying/wetting cycles. First, the soil attached to the root surface was removed by gently shaking the roots. Thereafter, a small soft brush was used to separate any remaining soil (the rhizosheath) from the root surface. The shoots and roots were dried in oven for 24 h at 105 °C and then weighted.

3.5 Statistics

The normality of the values and homogeneity of variance was tested using Shapiro-Wilk's W test and Levene's test. When data did not meet the normality requirement (e.g. shoot biomass data), the data were transformed by logarithm or square root. The t-test was applied to evaluate statistical differences ($p \leq 0.05$) in enzyme activities, microbial biomass and plant biomass between the maize samples irrigated with water and rhizoligand. All these statistical analyses were performed in R (version 3.3.2).

4 Results

4.1 Soil water content

The soil water content shortly after irrigation until the next irrigation event for one representative drying cycle is shown in figure 1. When plants started to show wilting symptoms, the soil water content in the control treatment (water, in blue) was approximately 0.04 and 0.06 $cm^3\ cm^{-3}$. Soils treated with rhizoligand (red) had greater water content than

soils treated with water during the entire drying period and during all drying cycles. Indeed, the soil of plants irrigated with rhizoligand was initially wetter (after irrigation at time zero in figure 1) than those irrigated with water. This was caused by the rhizoligand capacity to rewet the rhizosphere, which is not quickly rewettable by water alone (Ahmadi et al., 2017; Ahmed et al., 2017; Zarebanadkouki et al., 2016b; Zarebanadkouki and Carminati, 2014). Additionally, the water content in the soil treated with rhizoligand decreased more slowly, indicating lower transpiration rates as previously shown and discussed in Ahmed et al. 2017.

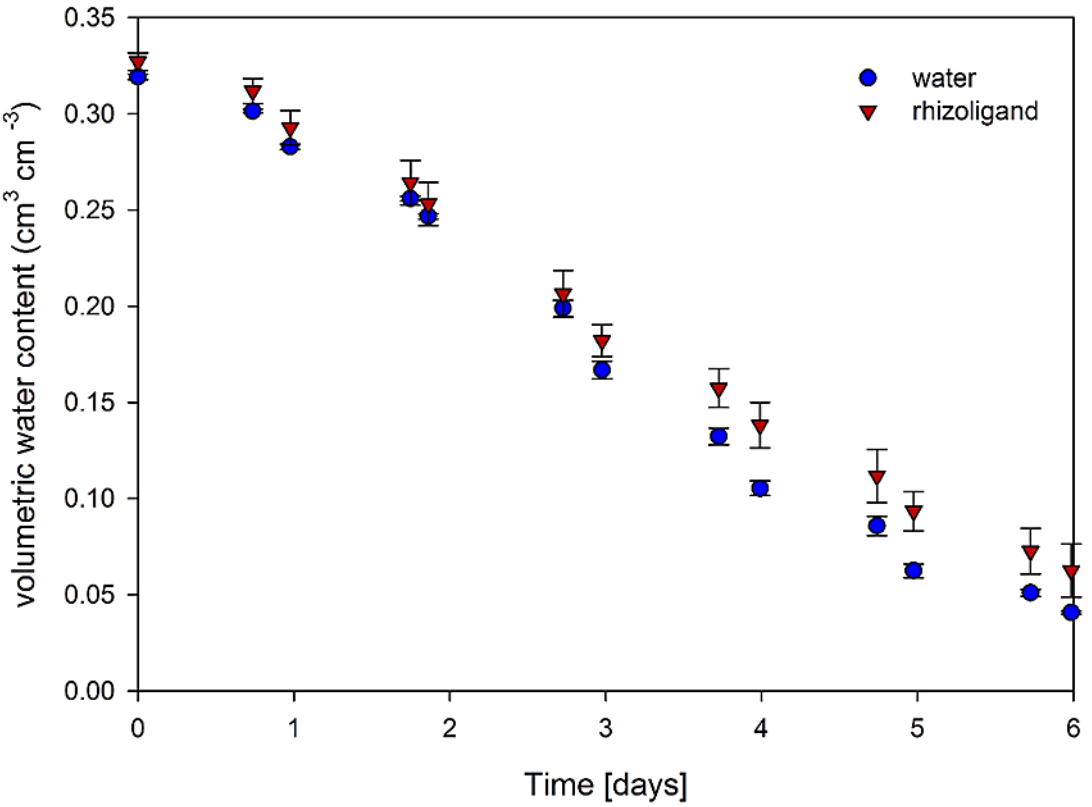

Figure 1. Water content of soils irrigated with water (blue) and with rhizoligand solution (red) during one representative drying cycle after irrigation. The soil water content was measured gravimetrically by weighing the rhizoboxes. The points represent the average of six replicates and error bars indicate.

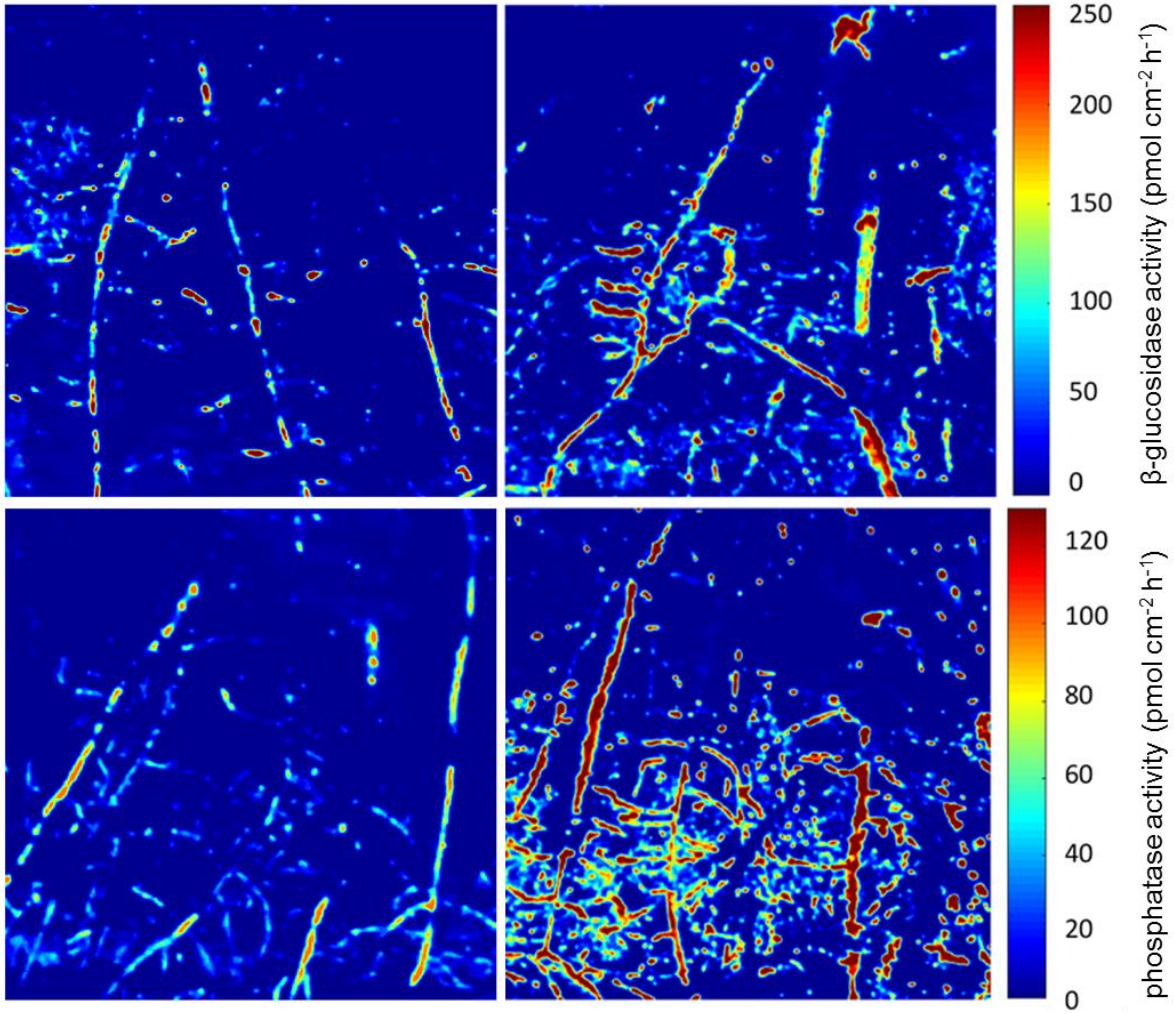

Figure 2. Zymograms of spatial distribution of β-glucosidase (top) and phosphatase (bottom) activities (pmol cm^{-2} h^{-1}) in the soil with maize roots irrigated with water (left) and with rhizoligand solution (right). The color bars at the side indicate the enzyme activities. Plants irrigated with rhizoligand solution showed greater enzyme activity in their rhizosphere.

4.2 Enzyme activities

Enzyme activity increased in the soil of plants treated with rhizoligand compared to the soil of plants irrigated with water alone. A larger extension of the region with high enzyme activity was visible in the soils irrigated with rhizoligand (Figures. 2, 3; Table.1). The extension of the region with high activity was enzyme-specific. The extension of the region with high β-glucosidase activity was 1.46 times greater in the soils irrigated with rhizoligand compared to the soils irrigated with water ($p < 0.06$). In comparison, the extension of the region with high phosphatase activity was only 1.2 fold greater in the soils irrigated with rhizoligand compared to those irrigated with water ($p < 0.005$).

Table 1. Description of the enzyme activity extended from the root surface of plants irrigated with water and rhizoligand. The plants irrigated with rhizoligand solution showed a larger region with higher activity around the roots compare to plants irrigated with water.

Enzyme	Extend of high-activity region of rhizosphere (mm)		Relative increase of high-activity region (mm/mm^{-1})	p value
	Water	Rhizoligand		
β-glucosidase	0.83±0.42	1.22±0.42	1.46±1.01	0.06
Phosphatase	1.05±0.15	1.25±0.17	1.20±0.8	0.005

Average β-glucosidase and phosphatase activities in the region close to the root (0-0.5 mm distance from the root surface) of plants irrigated with rhizoligand increased by 5.3 and 2.9 fold, compared to plants irrigated with water (Fig. 3). This increase was also observed at a distance of 2-2.5 mm. β-glucosidase and phosphatase activities in the bulk soils irrigated with the rhizoligand solution were 11 and 3.5 times greater than in the soils irrigated with water alone. Total β-glucosidase activity in the soils irrigated with rhizoligand was 4.9 fold greater than in soils irrigated with water (averaged throughout the all soil domain) (Fig. 4). A similar trend was found for phosphatase activity: 2.7 fold greater in soils irrigated with rhizoligand compared to soils irrigated with water alone.

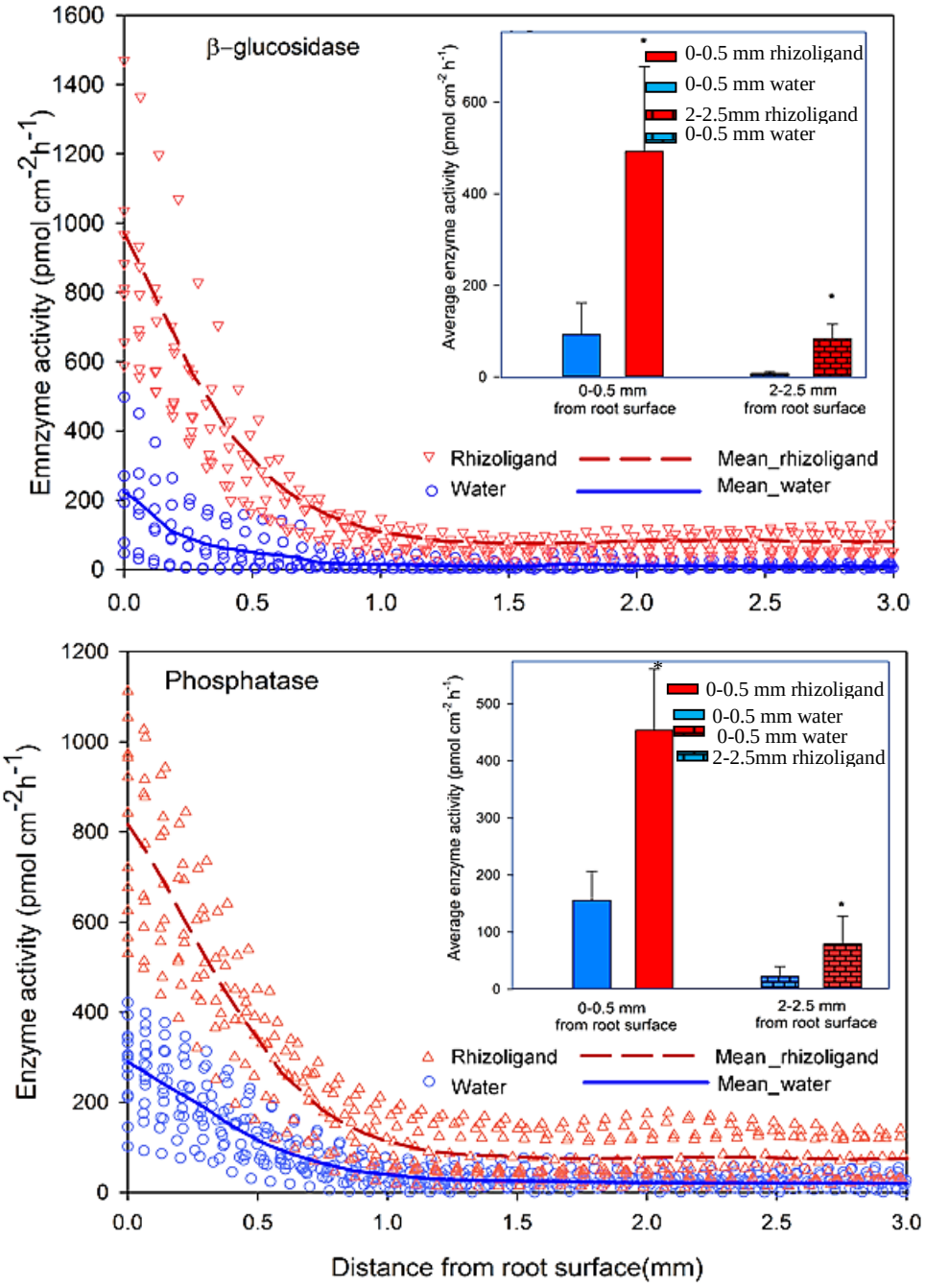

Figure 3. Profiles of β-glucosidase (top) and acid phosphatase (bottom) activities across the rhizosphere of maize roots as a function of distance from root surface. Each circle and triangle curves refer to average enzyme activity across a root segment of ca. 3 cm long. The solid and dash lines show greater enzyme activity as well as greater distribution of enzyme in the rhizosphere of plants irrigated with rhizoligand relative to plants irrigated with water. The bar charts inside of each plot show average β-glucosidase (top) and acid phosphatase (bottom) activities at a distance of 0-0.5 and 2-2.5 mm from the root surface. The data are the average of seven roots selected from three maize plants of eight-weeks-old plants and the error bars

show the standard deviations. The blue color indicates enzyme activity in the soil of the plants irrigated with water, whereas the red color indicates enzyme activity in the soil of the plants irrigated with rhizoligand. Stars indicate differences between the water and rhizoligand addition at $p \leq 0.05$ at a distance of 0-0.5 and 2-2.5 mm from the root surface.

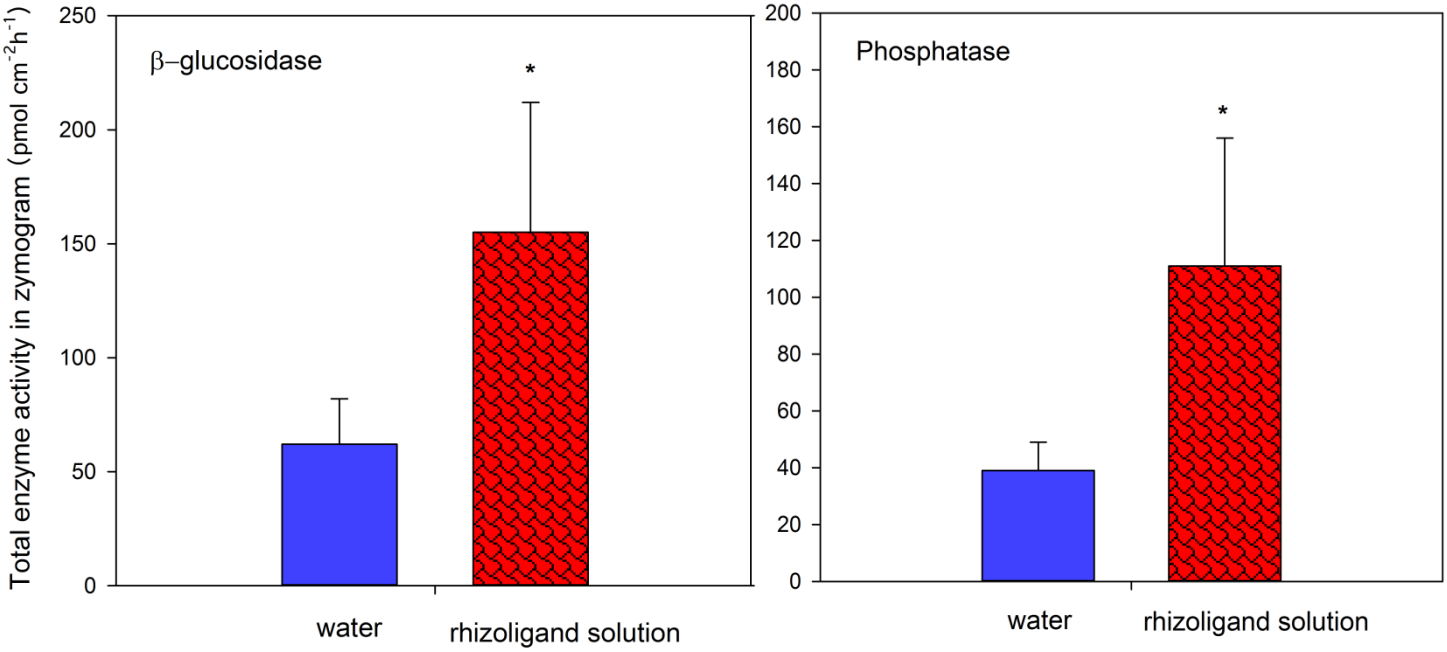

Figure 4. Overall activities of β-glucosidase (left side) and acid phosphatase (right side) obtained from the zymograms of Fig. 2. The blue bar charts indicate β-glucosidase and acid phosphatase activities in the soils irrigated with water, whereas the red color indicates β-glucosidase and acid phosphatase activities in the soils irrigated with rhizoligand. Each column chart is the average from three independent rhizoboxes. Error bars depict standard deviations. Stars indicate differences between the water and rhizoligand addition ($p \leq 0.05$).

4.3 Microbial biomass carbon and plant biomass

Microbial biomass carbon (MBC) was significantly greater ($p \leq 0.05$) in the rhizosphere of plants irrigated with the rhizoligand solution compared to the rhizosphere of plants irrigated with water alone (Fig. 5).

Rhizoligand treatment increased microbial biomass in the rhizosphere by 1.57 fold compared to plants irrigated with water alone. Root dry weight was 0.7 g for plants irrigated with water alone and 0.87 g for plants irrigated with rhizoligand (Fig. 6) ($p \leq 0.05$). The total plant biomass (root plus shoot dry weight) was independent of the irrigation solution (Fig. 6).

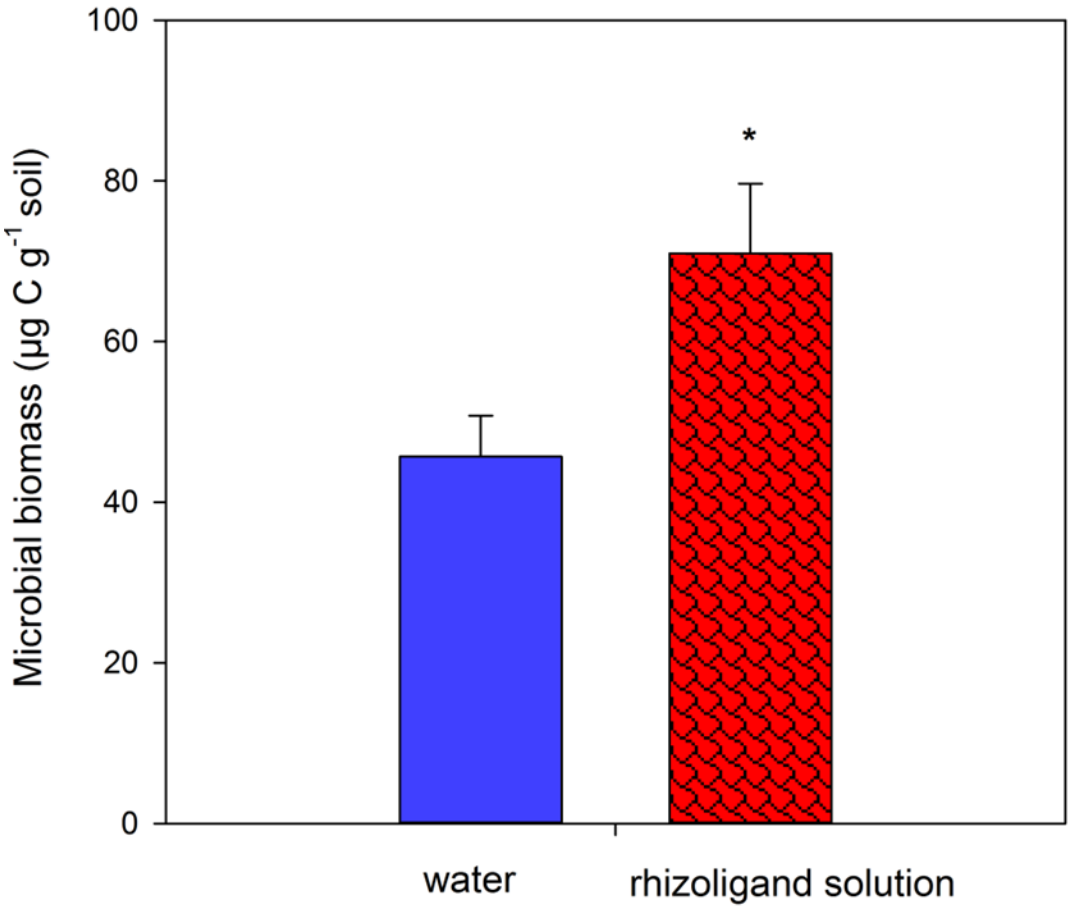

Figure 5. Microbial biomass in the soils irrigated with water (blue left column) and rhizoligand solution (red right column). Each bar chart is the average of six samples. The star indicates a significant difference between water and rhizoligand addition ($p \leq 0.05$).

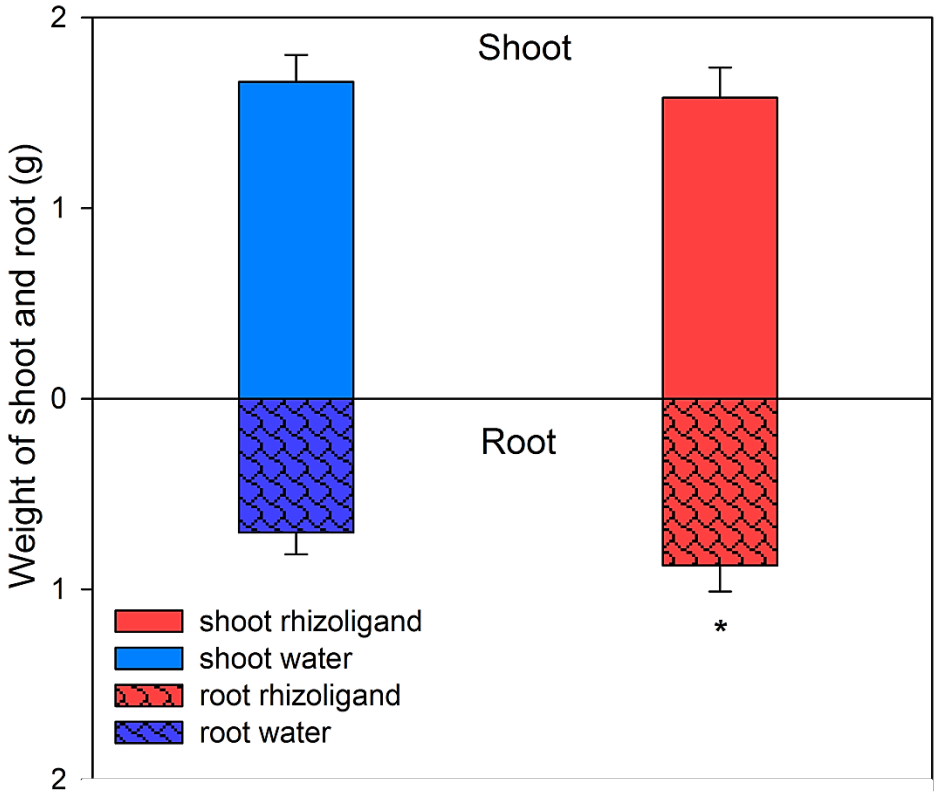

Figure 6. Dry weight of shoots (two simple patterns in the top) and roots (two hatch patterns in the bottom). The blue color indicates the shoot and root weights of plants irrigated with water, whereas the red color indicates the shoot and root weight of plants treated with rhizoligand in eight weeks old maize (vegetative growth stage). Each bar chart presents the average of six samples. A star indicates a significant difference between shoot weight and root weight of control and rhizoligand addition plants ($p \leq 0.05$).

5 Discussion

The findings of this study are conceptualized in figure 7. This figure depicts a conceptual pattern of the root growth, microbial biomass and distribution of enzyme activities in soil surrounding roots maize irrigated with water and rhizoligand solution. These findings support our hypothesis that the reducing hydrophobicity of rhizosphere with rhizoligand addition during irrigation, stimulates microbial properties (microbial biomass and enzyme activity) and root growth in the soil.

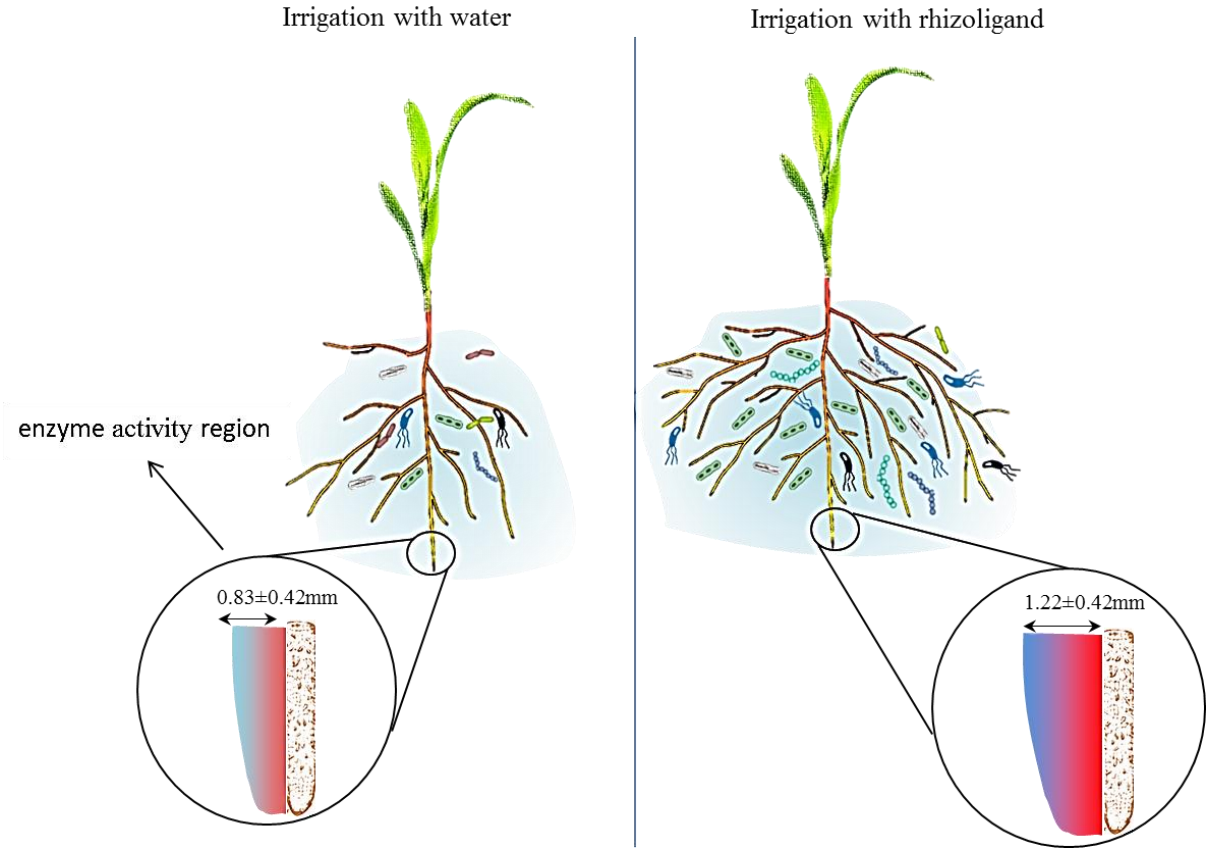

Figure 7. Conceptual patterns of the root growth, microbial biomass and distribution of enzymes activities in soil surrounding maize roots irrigated with water and rhizoligand solution. The left figure shows that plants irrigated with rhizoligand have wetter rhizosphere (darker blue color), higher root and larger microbial biomass. The magnified pictures show distribution of enzyme activities around the root as well as extended of the high-activity region in the rhizosphere under irrigation with rhizoligand (left) and with water alone (right).

We explain the positive responses of enzyme and microbial activity to rhizoligand addition due to the higher wettability of the rhizosphere of the plants treated with rhizoligand. Recent studies showed that as the soil dries, the rhizosphere becomes water repellent and remains temporarily dry upon irrigation (Moradi et al., 2009; Zarebanadkouki et al., 2016b). Application of rhizoligand reduces the contact angle in the rhizosphere and facilitates the rewetting of the rhizosphere (Ahmed et al., 2017). The consequent increase in water content in the rhizosphere is expected to stimulate microbial activity and therefore enzyme activity (Figs. 5 and 4). This speculation is supported by many studies indicating a strong correlation between water content and enzyme activity in the soil (Sanaullah et al., 2011a; Sardans and Peñuelas, 2005; Stark and Firestone, 1995).

An increase in enzyme activity was also observed at 2-2.5 mm distance from the root surface, where no gradients were visible. Thus, our results consistently support the hypothesis that rhizoligand influences the spatial patterns of enzyme activities along the root (Fig. 3). Such an increase is probably the effect of the greater average soil water content in the soils irrigated with the rhizoligand as shown in Fig. 1. Apparently, plants treated with rhizoligand transpired less. This is in line with our former studies showing that the rhizoligand reduced the transpiration rate of lupines undergoing similar drying/wetting cycles (Ahmed et al., 2017). The authors hypothesized that the rhizoligand increased the viscosity of the mucilage mixture reducing the hydraulic conductivity of the rhizosphere. This triggered partial stomatal closure and reduced transpiration. The higher water contents, both close to the root as well as in the adjacent soil, might have therefore caused the greater extension of the region with high enzyme activity as well as the higher enzyme activity further away from the root.

The higher enzyme activity can also be caused by additional interactions of the rhizoligand with the rhizosphere. Ahmadi et al. (2017) hypothesized that rhizoligand promote cross-linkage in the mucilage network, which is likely to increase viscosity of mucilage and which might cause other exudates (i.e. low molecular ones) to remain close to the root, where they are available to soil microorganisms. Additionally, higher mucilage viscosity can affect the microscopic configuration of the liquid phase in the rhizosphere. Recently, Carminati et al. (2017) showed that the combination of high viscosity and low surface tension of mucilage (expressed by the Ohnesorge number) cause the liquid phase to form stable strands that connect particles together maintaining the continuity of the liquid phase across the root-soil interface. Rhizoligand might contribute to the connectivity of the liquid phase across the

rhizosphere by further increasing the viscosity of the mucilage network. This could favor the diffusion through the rhizosphere and increase nutrient uptake and microbial activity.

The rhizoligand solution contains carbon, which is a substrate for microorganisms and so might stimulates enzyme activity in the soil (Hoang et al., 2016). However, the total amount of carbon added to the soil after six irrigations with the rhizoligand solution was around 1.25 mg. This low quantity of carbon could not be the main and sole of increased microbial activity and enzyme activity in the soil irrigated with rhizoligand.

The rhizoligand increased both enzyme activities, but the increase of β-glucosidase activity was greater (factor of 3.14 compared to 1.83). This difference might be linked to the different sources of these two enzymes. Phosphatase is mainly exuded by plants (Grierson and Adams, 2000) rather than by soil microorganisms, while β-glucosidase is exuded by both plants and soil microorganisms (Richardson et al., 2011). This suggests that the elevated enzymatic activity by rhizoligand treatment may be a result of increased microbial biomass. Accordingly, we found that rhizoligand treatment resulted in a positive impact on soil microbial biomass (Fig. 5).

Increased root biomass in response to rhizoligand application could also be caused due to modification of the biophysical properties of the rhizosphere. Increased rhizosphere wettability facilitates water and substrate flow and stimulates microbial growth. It is well documented that microbial activity plays the major role in nutrient availability and improved plant performance (Hermans et al., 2006; Ortíz-Castro et al., 2009). However, shoot biomass did not change in response to rhizoligand solution (Fig. 6). Note that we stopped the experiment when plants were 8 weeks old, while plants were still in the vegetative growth stage. We would expect that by allowing plant grow to the maturity stage, total plant biomass would have increased significantly, as found by Jafarian et al. (2015) in alfalfa, Chaichi et al. (2015b) in maize and Ahmadi et al. (2017) in lupin.

In conclusion, the changes in the rhizosphere wettability upon rhizoligand additions lead to changes in biological activities. The increase of microbial activities sheds new light on the physical and biological interactions in the rhizosphere. Rhizoligand increased the water content and therefore enhanced the diffusion of root exudates in the rhizosphere. So, the rhizoligand maintained high enzyme activity and large microbial biomass. Such effects help plants to better access and mobilize nutrients in drying soils.

6 Acknowledgements

This work was supported by Georg-August University of Goettingen, Germany and Aquatrols Corporation of America, NJ, and USA. The publication was prepared with the support of the "RUDN University program 5-100".

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Chapter Four

(Study 3)

Rhizoligand as a tool to overcome limited nutrient acquisition under drought

In preparation for submission to *Plant and Soil*

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1 Abstract:

Nutrient uptake by roots decreases in dry soils because of the reduced mobility and availability of nutrients. In particular, biophysical processes that alter the water dynamics in the rhizosphere are expected to play a crucial role for controlling nutrient uptake by controlling the microbial activity in proximity of the root. In this study, we tested the hypothesis that the water dynamics close to the root, in particular the water repellency of the rhizosphere, affect plant nutrient uptake under drought. To test this hypothesis we used a surfactant to rewet the otherwise water repellent rhizosphere and we measured its effect on the rhizosphere. The tested surfactant also interacted with root mucilage, stabilizing and increasing the rhizosheath formation – making the tested surfactant a rhizoligand.

Corn plants *(Zea mays L.)* were categorized into two groups, then exposed to six wetting/drying cycles and irrigated with water and rhizoligand solution for 8 weeks. Nutrient content, soil microbial biomass and plant biomass were measured. Corns under rhizoligand amendment exhibited higher nutrient content in total biomass of plants (g $plant^{-1}$) in comparison to water irrigated control plants, e.g. Fe content increased by 51% and Mn content increased by 45.7%. Furthermore, microbial biomass C and, microbial biomass N as well as root biomass increased by a factor of 1.57, 3 and 1.24 times in plants under rhizoligand amendment, respectively.

The observed improvements were attributed to the interactions between the rhizoligand and the mucilage in the rhizosphere and the augmented content of mucilaginous compounds at the root-soil interface. Our interpretation is that the rhizoligand increased the rhizosphere wettability and its water content and enhanced the content of mucilage and other rhizodeposits around the root. This provides a source of C for feeding microorganisms in the rhizosphere and a wet environment for the diffusion of substrate. This study highlights how changes in the biophysical properties of the rhizosphere by rhizoligand amendment affect nutrient acquisition and biological activity in the rhizosphere. It suggests rhizoligands application as a potential approach to improve nutrient use efficiency under drought stress.

Keywords: *Mucilage, plant nutrient uptake, Rhizosphere, Rhizoligand, Microbial biomass, Root-shoot ratio, water stress, nutrient availability*

2 Introduction

Water shortage reduces severely crop productivity which is strongly associated with poverty and food unsecurity (Godfray et al., 2010; Sposito, 2013). Different strategies have been developed to improve plant resistance and sustain plant productivity under water scarcity. Some strategies aim to optimize natural adaptation mechanisms of plants to more efficient consumption of available water and nutrient resources. Plants developed a variety of adaptation mechanisms to enable their survival and growth under water stress (Malinowski and Belesky, 2000), such as reduced transpiration rates, increased root system, increased root to shoot ratio (Comas et al., 2013; Shao et al., 2008), rhizosheath formation (Hartnett et al., 2013), improved soil-microbe interaction (Singh et al., 2011) and higher C allocation belowground (Huang and Gao, 2000). The last adaptation can alter the quantity and quality of root exudates (Henry et al., 2007). These modifications of plants in water scarcity enhance their ability to effectively take up nutrients of low availability. For instance, rhizosheath formation initiates proper contact between root and soil and sustain diffusion rate of nutrients as well as protect roots from severe drought (Caryn et al., 1985), bigger root system with a larger surface area increases nutrient uptake (Comas et al., 2013) and soil-microbe interaction increases nutrient mobilization in soil through mineralization of immobilized nutrients in organic matter into available form (Ortíz-Castro, Hexon Angel Contreras-Cornejo, 2009; Rengel and Marschner, 2005).

Nutrient uptake by plants is strongly limited as a consequence of low water content and nutrient transport in the soil even for the abundant nutrients in the soil (Sanaullah et al., 2011a; Sardans and Peñuelas, 2005). Mass flow and diffusion are the main mechanisms which govern transport of nutrients in the soil towards the plant root. During plant water uptake, ions dissolved in the soil solution and are passively transported towards the root by mass flow. Transpiration is the main driving force of mass flow in the soil but also of translocation of nutrients from the root towards shoot in the plants xylem (Cramer et al., 2008; Hu et al., 2007; Silva et al., 2011). Diffusion occurs when the nutrient concentration at the root-soil interface is reduced and developed a concentration gradient towards the root. Acquisition of some elements occurs by direct connection of the root during growing with the soil colloid contain nutrients, the so-called interception (Waraich et al., 2011). The relative significance of each mechanism of nutrient uptake varies for the individual elements, e. g. 93% of phosphorus and 80% of potassium uptake in maize occur by diffusion, whereas mass flow have the major role for the uptake of elements such as calcium, magnesium and sulfur in

maize, whereas, root interception has little influence to supply of plants nutrient demand; lower that 1% (Cramer et al., 2008).

The root system size and the volume of soil explored by root, which is frequently related to the cumulative length and surface of the root system, is a key factor for plant nutrient acquisition. The importance of root volume is quite noticeable. Plants in the early growth stage (2 to 5 leaf stage) with small root system, despite of low nutrient requirement, exhibit deficiency symptom, but later, when the root system develops, plants are able to fulfill their requirements even if fertilizers are not applied any more. Water availability determines root growth and the length of the root system in the soil. When plenty of water is available in the soil, plants do not need to invest in root growth, because both availability and transport of nutrient are high enough to fulfill their requirement. In contrast, under limited water supply, plants tend to develop their root length to increase nutrient uptake by diffusion and interception (Comas et al., 2013; Cramer et al., 2008).

Soil microbial activity also plays a key role for the mobilization and accessibility of nutrients for plants. Soil microorganism enhance nutrient availability in the soil by a variety of mechanisms including i) release of nutrients during mineralization and decomposition of soil organic matter ii) production of compounds to solubilize nutrient, e.g. enhance iron availability through producing siderophores (Hirsch et al., 2013), or iii) secretion of acids to hydrolyze nutrients from the minerals (Rodríguez and Fraga, 1999).Therefore, plants drive various strategies to stimulate the microbial activity in the soil as well as attract beneficial microbes to interact with their roots such as symbioses with mycorrhiza (Akiyama and Hayashi, 2006; Hassan and Mathesius, 2012). However, survival and activity of soil microorganism is negatively affected by low water contents causing osmotic pressure.

The property of the millimeters of soil surrounding the root, which is called rhizosphere, is altered by root secretions during root growth. Presence of organic compounds in the root exudates makes this tiny area as a hotspot of microbial abundance. Plants and associated microorganisms release a broad range of exudates to modify properties of the rhizosphere and therefore facilitate water and nutrient uptake (Hinsinger et al., 2009; Shen et al., 2013). Mucilage exudates by roots and extracellular polysaccharides of microorganisms have the potential to alter bio-physical and hydraulic properties of the rhizosphere. Mucilage enables to hold large volumes of water and keep the rhizosphere wet and conductive which is a beneficial factor for root growth and microorganism development especially under water scarcity (Carminati et al., 2011; Knee et al., 2001; Read et al., 2003). Furthermore, mucilage has a crucial role to build up rhizosheath, a cohesive soil layer adhering to the root surface

composed of mucilage, root hairs and fungal hyphae (Ahmadi et al., 2017; McCully, 1999; Moreno-Espíndola et al., 2007; Pang et al., 2017 George et al., 2014; Watt et al., 1994, 1993). This layer has the main role to increase plant resistance to abiotic stress in particular water scarcity (George et al., 2014a). In dry soil, soil and root tend to shrink and consequently, roots lose their contact with the soil (Carminati et al., 2010; Nobel and Cui, 1992a; Watt et al., 1993). Such shrinkage leads to i) exposure of roots to dehydration and ii) limited diffusion of nutrients towards the roots. Rhizosheath acts as a cylindrical protector layer which covers the root surface and maintains them wet and therefore alive. In addition the rhizosheath facilitates diffusion of nutrient toward the roots. In fact, rhizosheath by filling the gap between root and soil, provides a continued contact at the root–soil interface and sustains nutrient mobility and ,therefore, nutrient uptake even under drought stress (Czarnes et al., 2000; North and Nobel, 1997).

The objective of this study was to test whether modification of the biophysical properties of the rhizosphere by using a rhizoligand impacts nutrient uptake by roots during repeated drying and wetting cycles. In specific, we tested id altering rhizosphere properties with rhizoligand application, would enhance i) root size system ii) rhizospheric microbial activity and, iii) plant nutrient acquisition. A rhizoligand has been defined in Ahmadi et al (2017) as an additive that increases rhizosphere wettability and it binds mucilage and soil particles enhancing the rhizosheath formation and thereby maintaining the rhizosphere wet and physically connected during repeated severe drying and wetting cycles.

Here, we hypothesize that rhizoligand application: i) reduces root mortality during severe drying cycles, ii) improves microbial activity in the rhizosphere and, iii) reduces the formation of gaps at the root–soil interface. Surfactants act as rhizoligands, and were, for example, recently applied to facilitate water penetration into the soil, when the soil was dry and hydrophobic (Franklin, 2007; Jafarian et al., 2015). Furthermore, rhizoligands enable to interact with hydrophobic functional groups of the mucilage inducing a new corss-link in the network of mucilage in the rhizosphere (Ahmadi et al., 2017; Ahmed et al., 2017). In our former study, we proved that these induced interactions lead to i) reduced mucilage swelling and decrease of its viscosity ii) stabilized mucilage and rhizodeposits close to the root which induces a stronger attachment of the rhizosphere soil to the root (rhizosheath) (Ahmadi et al., 2017; Ahmed et al., 2017). The development of the rhizosheath of lupine by application of rhizoligand supported this conceptual idea (Ahmadi et al., 2017).

Fig. 1 summarizes the conceptual mechanisms underlying improved nutrient uptake of plants in response to rhizoligand application. Firstly, application of rhizoligand keeps the mucilage

closer to the root surface and therefore enhance rhizosheath formation (Ahmadi et al., 2017). Thus, we hypothesize that greater thickness of rhizosheath will maintain the roots wet and protect them against severe drought. Stabilization of mucilaginous components surrounded by easily available C sources leads to our second hypothesis, which is; rhizolihands stimulate microbial activities in the rhizosphere and therefore enhance nutrient availability. Thirdly, the greater the viscosity of mucilage caused by the rhizoligand application enhances the strength and stability of filaments connecting the soil particles and the rhizosphere (as discussed in Carminati et al. 2017), and this is expected to enhance nutrient and substrate mobility during drying cycles.

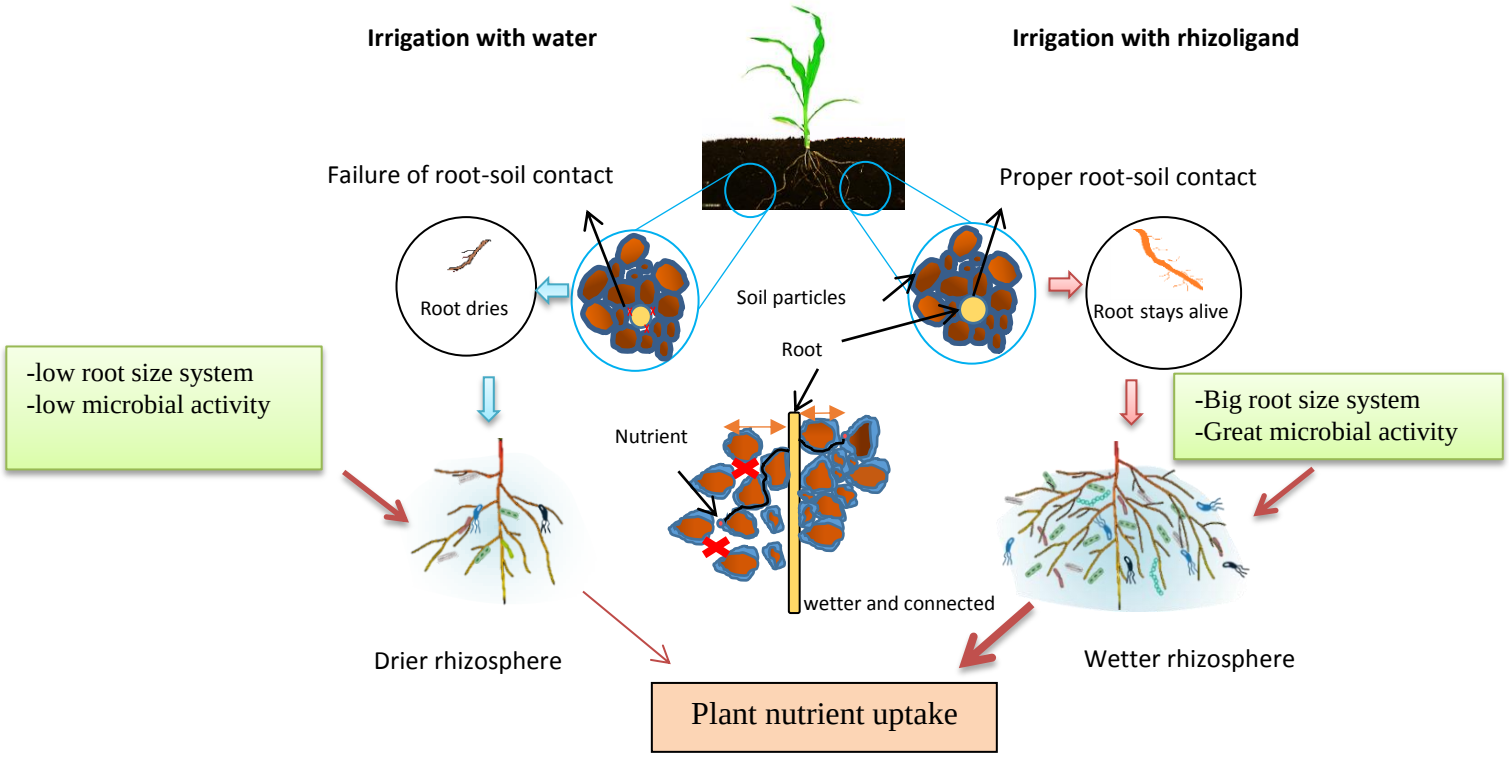

Figure 1. Schematic overview of mechanism underlying improved nutrient uptake of plants in response to rhizoligand application. The top magnitude figures indicate that soil particles in surrounding root cover with greater mucilaginous compounds in soil treated with rhizoligand (right side). Greater mucilage accumulated in surrounding the root provides a wetter and conductive environment which is in favor of root growth and nutrient mobility during drying cycles. In contrast left magnitude figures show an air-filled gap formed at the root-soil interface, where root loses their contact with the soil. As a consequence root dries during severe droughts. Furthermore, due to the loss of soil particle contact in vicinity of root, diffusion of nutrient is limited in the rhizosphere.

3 Materials and methods

3.1 Soil and plant preparation

Soil samples were collected from Reinhausen located 8 km south-east of Goettingen, Germany. The soil contained of 73% sand, 18% silt and 9% clay. The total carbon and nitrogen content was 2%, 0.17%; respectively, and its pH was 4.9.

The soil was dried at a room temperature and thereafter was sieved at a particle size smaller than 2 mm. To avoid soil layering and facilitate homogeneous filling, we filled rhizoboxes horizontally with 2 mm sieved soil. After closing the removable wall, the rhizoboxes were gently turned vertically and were slightly shaken to prepare a stable soil package into the rhizoboxes. Their inner size was 12.3 × 12.5 × 2.3 cm.

Corn seeds (*Zea mays* L) were soaked in 10 % H_2O_2 solution for 5 min and then were washed with water. Thereafter, we put the seeds on the filter paper which were incubated with 10 mL^{-1} $CaSO_4$ solution to germinate in the darkness for 72 h. After germination, one seedling was sown in each rhizobox at a depth of 1 cm. The rhizoboxes were transferred and kept during the whole growth period in a climate chamber under controlled conditions. The following factors were fixed in the climate chamber, including: relative humidity of 60%; day to night temperature of 24: 19 °C; 10/14 hour dark/light cycle, an approximate 500 µmol m^{-2} s^{-1} light intensity at the top of the canopy. In the first four days after plantation, the plants were irrigated every day, then the plants were watered every third day till plants were two weeks old. No fertilizer was applied during the entire growth period. When the plants were two weeks old, we started the first drying and rewetting cycle. The rhizoboxes were irrigated by capillary rise, by being slowly immersed in water (at a depth of 5 cm) for half of an hour till the soil was saturated at the top. Then we started the first drying cycle and the plants were let to dry soil until the soil reached a water content of nearly 5-6%. Afterwards, the samples were splitted into two groups: reference plants were irrigated with water and the rhizoligand-amended plants were irrigated with the rhizoligand from the ACA1820, Aquatrols Corporation of America (Paulsboro, New Jersey, USA) at a concentration of 0.05 g L^{-1} of water. The rhizoboxes were irrigated at a same time, when plants showed welting symptom and soil water content was at the range of 4-6%. Six drying and wetting cycles and two treatments of water and rhizoligand solution were replicated in eight-weeks-old of corn plants. A period within two irrigation events refers as a drying cycle.

3.2 Microbial biomass carbon and nitrogen

The chloroform fumigation-extraction method was used to measure microbial biomass C and N (Vance et al., 1987). The method is based on quantifying the difference between C and N extracted from fumigated and nonfumigated soil samples using 0.05 M K_2SO_4. To correct the none-extractable proportion of the microbial biomass an extraction factor k_{EC} of 0.45 (Joergensen, 1996a) was used for microbial biomass C, whereas a k_{EN} of 0.54 (Joergensen, 1996b) was used for microbial biomass N.

3.3 Element analysis of plant biomass

Element concentrations in plants were determined by ICP-OES (Vista RL, CCD simultaneous ICP-OES, Varian Inc., USA) and atomic absorption spectrometry (220 FS, Varian Inc., USA). Preparation was carried out based on a wet microwave digestion under pressure which was adjusted in the institute of applied plant nutrition in Goettingen University (Tränkner et al., 2016). Firstly, shoots and roots were separately dried in the oven at 105 °C for 24 hours and then the samples were milled. To degrade biological plant tissue, wet digestion method was applied. First, 100 mg of dried plant biomass, 4 ml nitric acid (HNO_3) and 2 ml hydrogen peroxide (30% H_2O_2) were mixed. Then, the mixture was digested in the microwave oven for 75 minutes at a temperature of 200 °C and a pressure of 15 bars. When the vessels were cooled down to room temperature, the digested mixture was diluted to 25 ml by adding water. The samples were analyzed by an inductively coupled plasma-optical emission spectrometer (ICP-OES). The concentrations of Ca, Cu, Fe, K, Mg, P, S were determined in roots and shoots of reference and rhizoligand-amended samples. Then, the concentrations were converted in total nutrient uptake per whole biomass by the total dry weight of the respective plant compartment (shoot and root). Dry weight was received after oven drying for 24 h at 105 °C. Roots were before drying gently shaken and remaining adhering soil was detached by a small soft brush.

3.4 Statistical analysis

The t-test was performed in the software package R (version 3.3.2) to evaluate statistical differences between references and rhizoligand-amended plants. Significant differences were considered at an error probability level of $p \leq 0.05$.

4 Results

4.1 Plant above- and belowground biomass and rhizosphere microbial biomass

The average root biomass of the eight week old corns irrigated with rhizoligand was 1.24 and their root/ shoot ratio was 1.31 fold higher than the control plants irrigated solely with water ($p \leq 0.05$) (Fig. 2). However, such a rhizoligand-induced biomass increase was not reflected in shoot dry biomass and total plant biomass (Fig. 2).

Microbial biomass carbon (MBC) and nitrogen (MBN) were noticeably increased in the rhizosphere of rhizoligand-amended plants relative to the control plants; 1.57 and 3 times higher respectively (Fig. 3). Whereas, the MBC/MBN ratio was greater in the rhizosphere of reference; 11.76±2.82 (µg C / µg N) in comparison to rhizosphere of rhizoligand-amended plant 6.14±1.97 (µg Cg^{-1} soil/ µg Ng^{-1}soil) ($p \leq 0.05$) (Fig. 3).

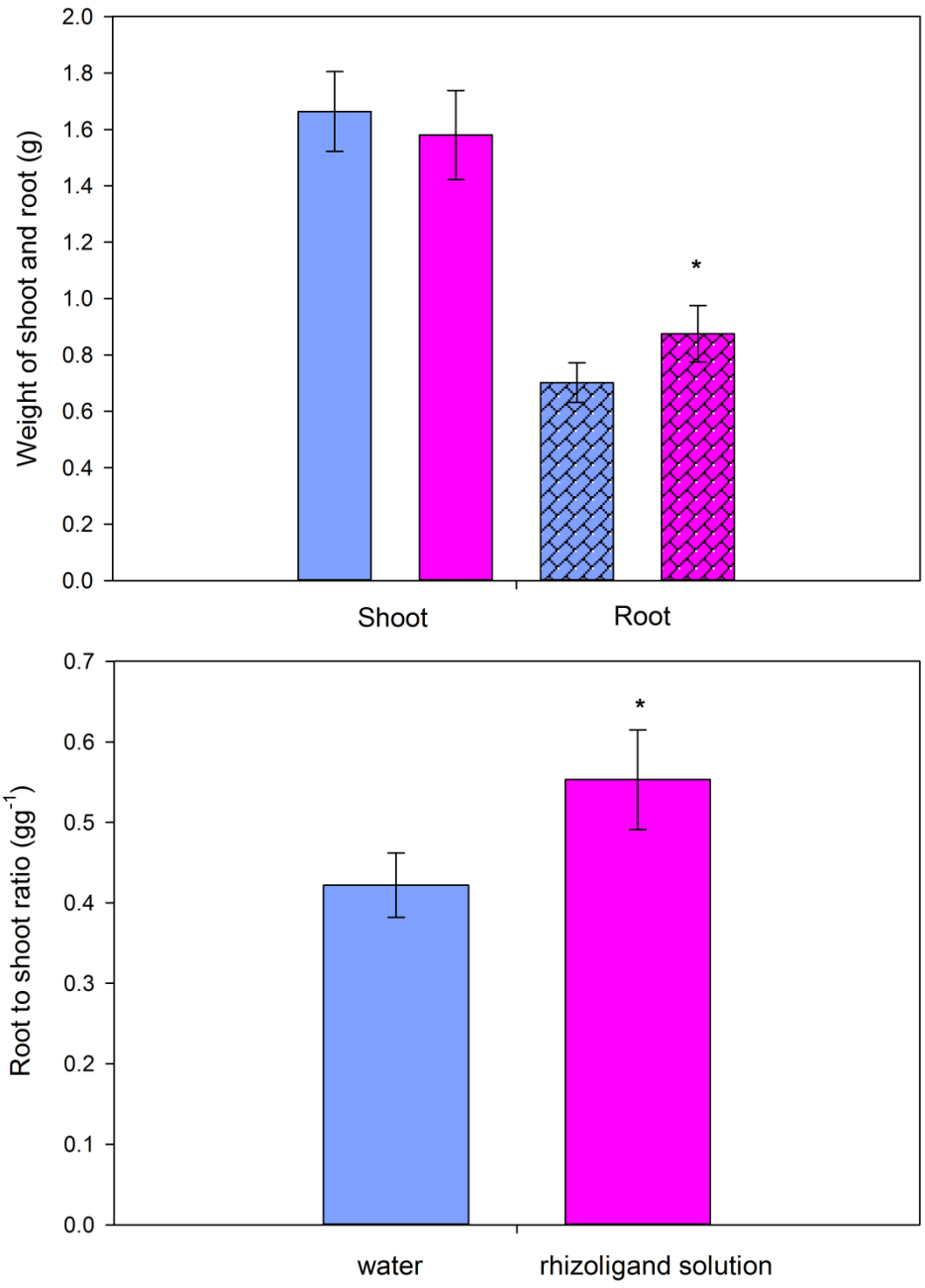

Figure 2. The top figure shows the dry weight of shoots (two simple patterns in the left-hand side) and roots (two hatch patterns in the right-hand sides). The blue color indicates the weight of shoot and root of plants irrigated with water, relative to pink color which indicates the weight of the shoot and root of plants treated with rhizoligand. The bottom figure shows root-shoot ratio (g g^{-1}) in plants irrigated with water (blue) and plants treated with rhizoligand (pink) in eight- weeks-old corn. Each bar chart presents the average of six samples. A star indicates a major difference between the mean of control and mean of rhizoligand addition at $p \leq 0.05$.

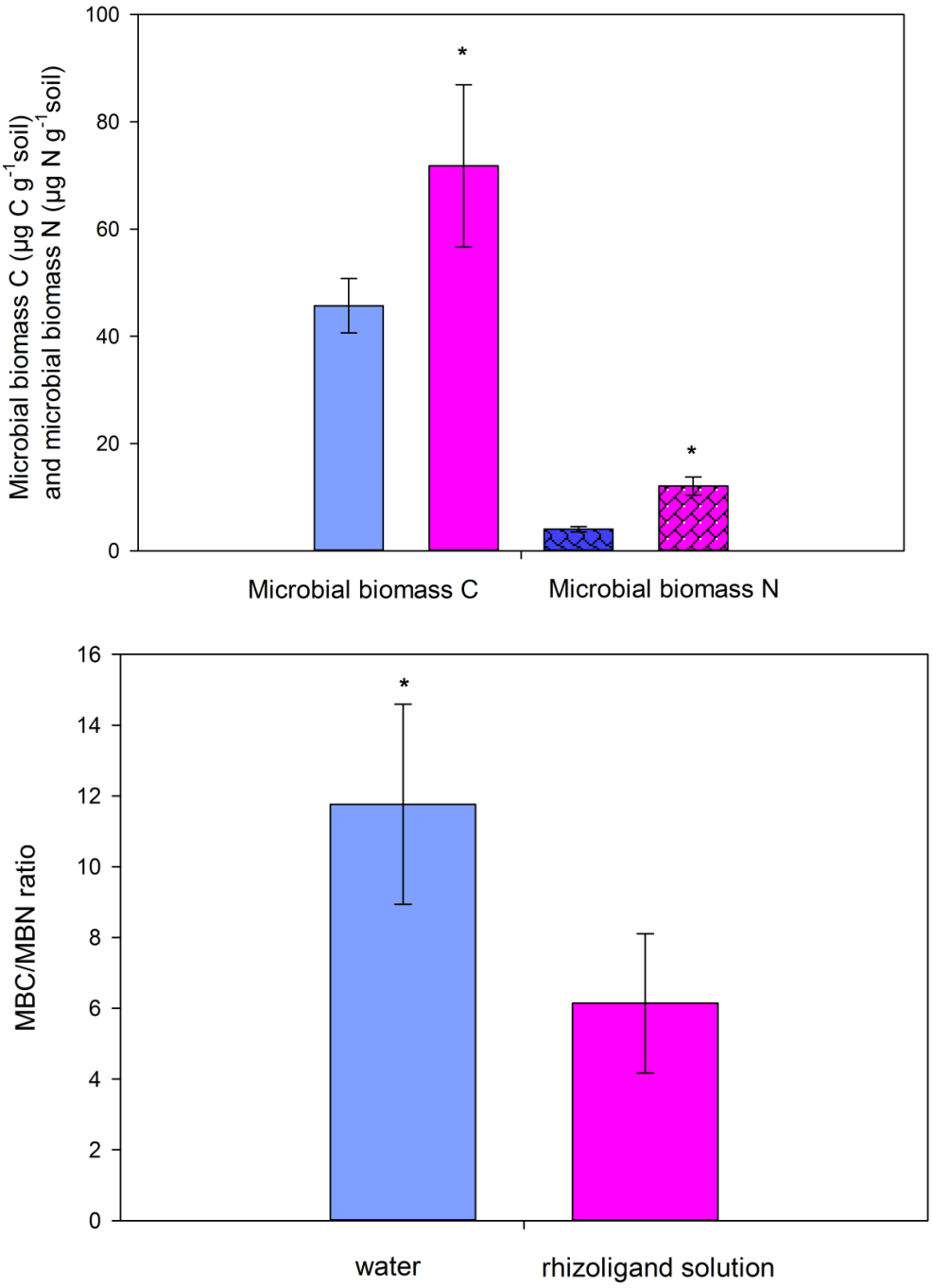

Figure 3. The top figure shows the microbial biomass C (two simple patterns in the left-hand side) and the microbial biomass N (two hatch patterns in the right-hand sides). The bottom figure shows microbial biomass C to N ratio in the rhizosphere of plant irrigated with water (blue) and rhizoligand amended plants (pink) in eight-weeks-old corn. A star indicates a major difference between the mean control rhizosphere and rhizoligand addition rhizosphere at $p \leq 0.05$.

4.2 Element analysis in plant

A clear absolute increase in nutrient content per plant was observed as a consequence of the rhizoligand amendment (Fig. 4), especially the relative increase of some microelements in response to rhizoligand addition was noticeably high, e.g. iron (Fe) increased by 52% ($p < 0.01$) and manganese (Mn) increased by 45.7% ($p < 0.01$), this increase in this two micronutrients also was noticeably greater per gram of plant (Mg g^{-1}) and individually in root and shoot compartments relative to the reference plants. Whereas the relative increase of some macro elements were lower e.g. potassium 6.8% ($p< 0.05$) and phosphorus 9.9% ($p\leq 0.05$), (Fig. 5).

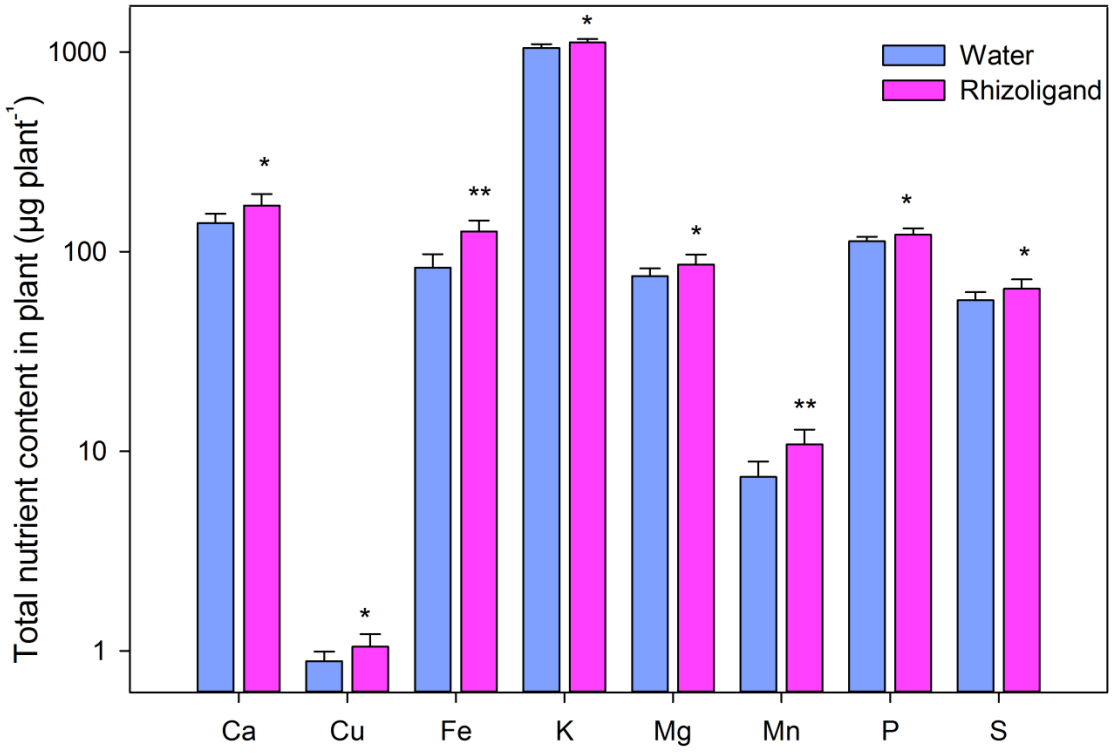

Figure 4. Total nutrient content in entire biomass of of 8-week-old corn irrigated with water (blue color) and rhizoligand solution (pink color). Each column represents the average of the six replicates. Different numbers of stars indicate different p level (**$p\leq 0.01$, ** $p\leq 0.05$) between control and rhizoligand amended samples according to the t-test.

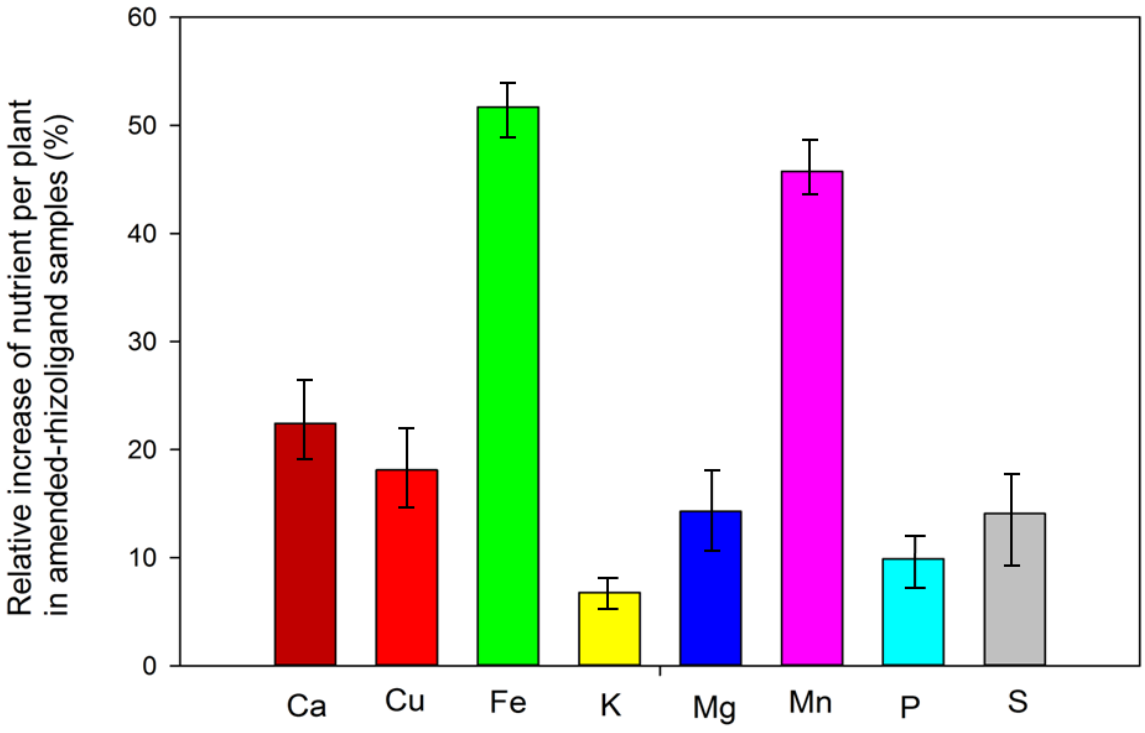

Figure 5. Relative increase of nutrient in total plant biomass (root and shoot) of 8-week-old corn amended with rhizoligand solution in comparison to control plants. The relative increase (%) varied between the elements and was statistically different according to the t-test at the significance level of $p \leq 0.01$ and $p \leq 0.05$. Each column compares the relative increase between six replicates from reference and rhizoligand amended plants.

5 Discussion

In agreement with our hypothesis, altering bio-physical properties of rhizoshere by rhizoligand application improved plant performance through enhancing root biomass and plant nutrient uptake, and stimulate biological activities in the rhizosphere in water scarcity. These improved changes attributed to two main consequences of rhizoligand application; firstly, increase wettability by reducing hyrophobicty of the rhizosphere after drying cycles. Neutron radiography of lupin and maize roots showed after drying cycles, the rhizosphere , where mucilage is present, becomes hydrophobic and water penetration reduces in the soil surrounding roots and therefore, soil stays temporarily dry after irrigation (Carminati et al., 2010; Moradi et al., 2012), whereas the rhizosphere of plants which irrigated with rhizoligand were rewetted more homogeneously and had greater water content in their rhizosphere (Ahmed et al.,

2017). Secondly, rhizoligand application stabilizes mucilage and other rhizodeposition close to the root surface by inducing new interactions in the mucilaginous network in the rhizosphere.These interactions increased the viscosity of mucilage and therefore maintained the root exudates in the vicinity of the root and thus improved rhizosheath formation (Ahmadi et al., 2017; Ahmed et al., 2017).

Here, the results divided into three parts, first discussing the effect on microbial biomass itself, then on the root growth and finally on crop nutrition.

5.1 Effects of rhizoligand amendment on microbial biomass

Enhanced microbial biomass C and microbial biomass N by 1.57 and 3 fold in response to rhizoligand amended (figs. 3) explained by i) greater wettability of rhizosphere providing better habitat properties for microbial growth (Sanaullah et al., 2011b; Stark and Firestone, 1995) and, ii) greater rhizosheath thickness (Ahmadi et al., 2017) containing higher available C sources for microbial activity (Drenovsky et al., 2004; Landi et al., 2006; Pang et al., 2017). These two factors maintain the roots wet and alive against severe drought. Vital plant with greater roots would have higher exudates (Přikryl and Vančura, 1980) and stimulates microbial activities in the rhizosphere (Drenovsky et al., 2004; Landi et al., 2006). Additionally, the result of MBC to MBN ratio at 6.01, close to optimum range for microbial activity, in rhizoligand-amended plants, demonstrated a more favorable environment for soil microbes in rhizoligand amended plants over the control plants (fig. 3).

5.2 Effects of rhizoligand amendment on root growth

The quantity of root biomass and root-to-shoot biomass positively increased by 1.24 and 1.31 fold in the plant under rhizoligand irrigation ($p \leq 0.05$) (Fig. 2). The increase of root biomass (the size of the root system) of the plants treated with rhizoligand attributed to greater rhizosheath development in plants under rhizoligand amended (Ahmadi et al., 2017). Roots with improved rhizosheath maintain a good contact between root and soil and reduce the risk of air-filled gap formation particularly as soil dries and both roots and soil tend to shrink. Since, rhizosheath composed of mucilage and soil organic matter is wetter and protects roots from dehydration and reduces

danger of roots damage during drying cycles and keep the roots alive (Carminati et al., 2010; Nobel and Cui, 1992b; Watt et al., 1994, 1993).

A possible explanation for the greater root-to-shoot biomass in response to rhizoligand amendment could be relevant to reduce the hydraulic conductivity of rhizosphere of rhizoligand-amended plants (Ahmed et al., 2016). The reduction of the hydraulic conductivity might induces a moderate stress and thus activates the natural defense mechanism of plants. Under such conditions, plants increase their root to shoot ratio for a more efficient use of water and nutrients (Cramer et al., 2008; Hu et al., 2011; Silva et al., 2011). Moderate stress is a new technique to induce ABA in the plant and reduce transpiration rate under drought stress. Partial root-zone drying (PRD) is an example to control the transpiration rate and improve the water use efficiency through induction of ABA in the root and its translocation to the leaves consequently decreases stomatal conductance there (Bramley et al., 2009; Hu et al., 2011; Huang and Gao, 2000; Stoll et al., 2000). Reduction of transpiration rate in lupine and maize (Ahmed et al., 2017) and increase ABA concentration in the leaves of the lupine after a tested rhizoligand application, are consistent with this assumption (unpublished observation of Ahmed et al., 2016). The result of an increased root to shoot ratio by a factor of 1.31 supports this assumption ($p \leq 0.05$) (Fig. 2).

However, this tendency was not observed in shoot biomass and total plant biomass between water and rhizoligand irrigated plants (Fig. 2). Concerning that this experiment was stopped, when plants were still in the vegetative stage (8-week old), the stage which plants increase their root biomass to enable increase their productivity in reproductive stage. Therefore, it is logical to note that if the plants were allowed growing to reproductive growth stage, total plant biomass would statistically increased. This speculation was supported with recent studies which showed increase plant yield in rhizoligand-amended plants in compare to control plants in lupin (Ahmadi et al., 2017) and in corn plants (Chaichi et al., 2015b; Mehrvarz et al., 2013).

5.3 Nutrient acquisition

The result clearly demonstrated that the concentration of the macronutrients P, K, Ca, Mg, S and the micronutrients Cu, Fe, Mn, Zn were noticeably increased in the total biomass of 8-week-old corn in response to rhizoligand addition (Fig. 4). The increase in nutrient acquisition in response to rhizoligand application can be the consequence of

three key processes which explained in detail in conceptual diagram in figure 1 and consist of : i) the increased root biomass acquiring nutrients from a higher soil volume ii) the improved diffusion rate of nutrients across the root-soil interface iii) and the enhanced microbial activities mobilizing nutrients. All these factors would especially increase the mobility and availability of nutrient in the rhizosphere under water stress (Fig. 1). Root with a larger surface area would explore larger quantity of soil to take up nutrient and would have greater nutrient uptake by mass flow and diffusion as well. Furthermore, plants with greater root biomass secret more mucilage, organic acids as well as enhance beneficial root-microbe interactions to increase solubility and availability of nutrient (Huang and Gao, 2000).

Root biomass of plants amended with rhizoligands increased by 30% in comparison to control plants (Fig. 2). An increase of root size in plants under rhizoligand addition would expect to increase approximately 15%-20% greater nutrient acquisition, which is the half of the growth period of plant. Since the increase of root system occurs over the entire period of plant growth (Shen et al., 2013). This predicted extend of increased nutrient acquisition was observed for Zn, S, Mg, Cu and Ca. However, increased concentration of P and and K were lower %10 and 7% whereas Fe and Mn were higher 51% and 45.7% of this predicted range; respectively. A unexpectedly high relative increase of some elements such as Fe; 51% and, Mn; 45.7% in comparison to other elements in response to rhizoligand addition is likely to be associated with greater microbial activities which have the teremendes effect to availability of this two elements (Marschener, 1998; Shen et al., 2013)

Furthermore, the higher nutrient accumulation in the plants treated with rhizoligand might be explained by homogenous distribution of water after irrigation with rhizoligand. Such a homogenous rewetting of the rhizosphere with rhizoligand application might maintain the continuity of the water flow at the soil-root interface and improve the diffusion rate of nutrients towards the root.

The higher microbial biomass in the rhizosphere of plants irrigated with rhizoligand also could be another factor of enhancing plant nutrient uptake. Soil microbial activity, as a sink of available nutrient, is an indicator showing the quality and fertility of soil, since they play a crusial role for the availability and accessibility of nutrient by plants. Soil microorganism with a variety of manners enhance nutrient availability in the soil including i) release nutrients during mineralization and decomposition of soil organic matter ii) produce specific chemical compositions to soluble nutrient, e.g. enhance iron

availability through producing siderophores (Carvalhais, 2013; Bar-Ness et al., 1992; Marschener, 1998).

All the possible mechanisms as well as measurments influencing on plant nutrient uptake in response to rhizoligand application are summarized in figure .6.

6 Conclusion

Rhizoligand application, which rewets the rhizosphere, stabilizes mucilage around the root surface and increases the rhizosheath, enables to protect roots from dehydration during drying cycles. Thus, plants with greater root size system have more capability to efficiently take up nutrient in dry soil. Furthermore, greater microbial biomass as a result of rhizoligand amendment helps to maintain nutrient availability in the soil. Such a modification of the rhizosphere could be a new tool to increase nutrient efficiency in drying soils and might provide a new way to reduce the large quantity of synthetic fertilizer application and improve agricultural sustainability.

7 Acknowledgement

This work was supported by Georg-August University of Goettingen, Germany and Aquatrols Corporation of America.

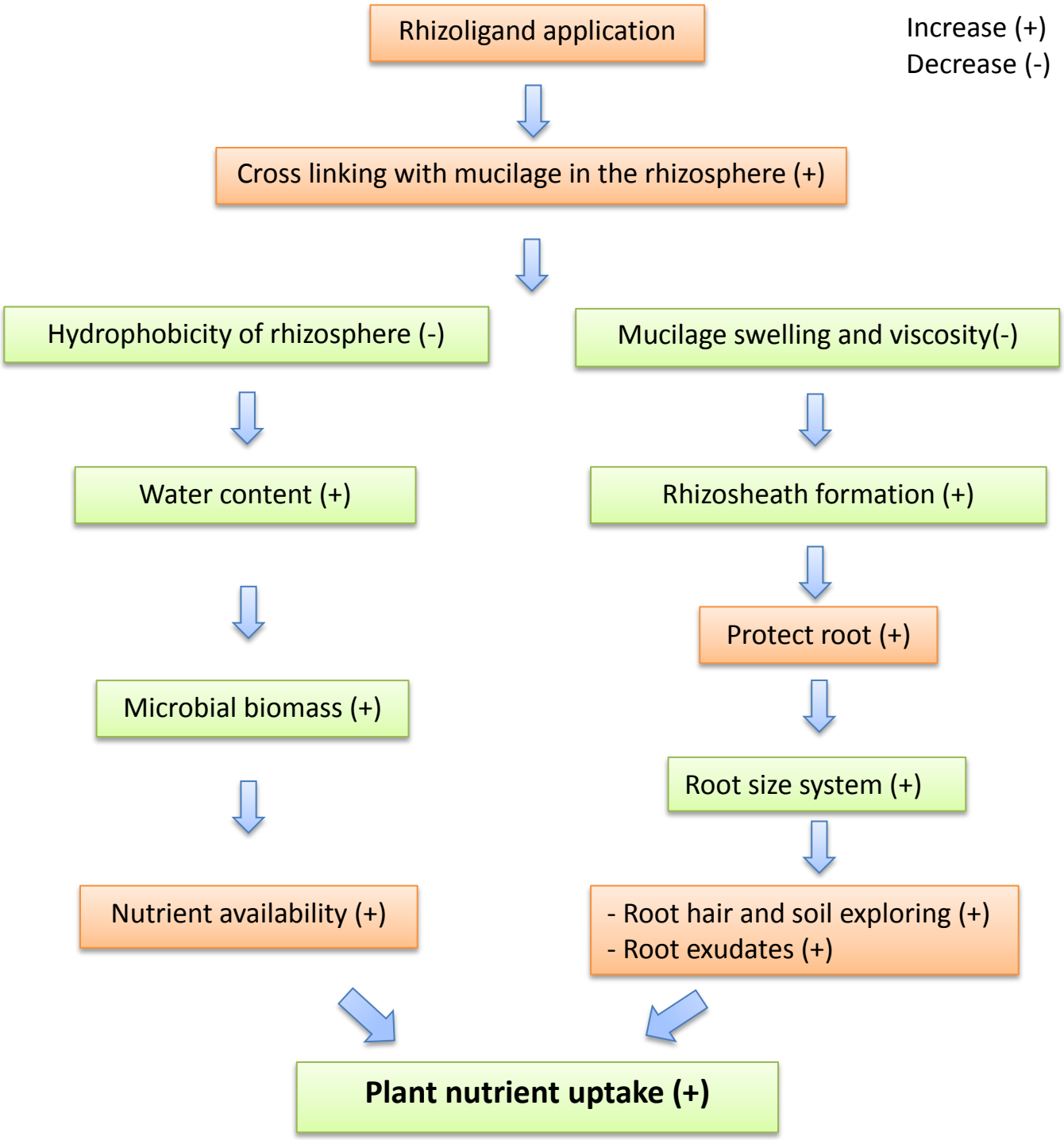

Figure 6. The conceptual diagram showing the possible mechanisms influencing plant nutrient uptake in response to rhizoligand application. It shows the main factors affecting on bio-physical properties of rhizosphere and improve nutrient acquisition in water stress condition. The green boxes indicate respective factors which measured during experiments, whereas, orange color boxes indicates our mechanistic explanations.

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Additional Studies

Engineering rhizosphere hydraulics: pathways to improve plant adaptation to drought

As published in Vadose Zone Journal Vol. 17 No. 1 (2016)

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1 Abstract

Developing new technologies to optimize the use of water in irrigated croplands is of increasing importance. Recent studies have drawn attention to the role of mucilage in shaping rhizosphere hydraulic properties and regulating root water uptake. During drying mucilage keeps the rhizosphere wet and conductive, but upon drying it turns hydrophobic limiting root water uptake. Here we introduced the concept of rhizoligands, defined as additives that 1) rewet the rhizosphere and 2) reduce mucilage swelling hereby reducing the rhizosphere conductivity. We then tested its effect on rhizosphere water dynamics and transpiration.

The following experiments were carried out to test if selected surfactants behave as a rhizoligand. We used neutron radiography to monitor water redistribution in the rhizosphere of lupine and maize irrigated with water and rhizoligand solution. In a parallel experiment, we tested the effect of rhizoligand on the transpiration rate of lupine and maize subjected to repeated drying and wetting cycles. We also measured the effect of rhizoligand on the maximum swelling of mucilage and the saturated hydraulic conductivity of soil mixed with various mucilage concentrations. The results were then simulated using a root water uptake model.

Rhizoligand treatment quickly and uniformly rewetted the rhizosphere of maize and lupine. Interestingly, rhizoligand also reduced transpiration during drying/wetting cycles. Evaporation from the bare soil was of minor importance. Our hypothesis is that the reduction in transpiration was triggered by the interaction between rhizoligand and mucilage exuded by roots. This hypothesis is supported by the fact that rhizoligand reduced the maximum swelling of mucilage, increased its viscosity, and decreased the hydraulic conductivity of soil-mucilage mixtures. The reduced conductivity of the rhizosphere induced a moderate stress to the plants reducing transpiration. Simulation with a reduced hydraulic conductivity of the rhizosphere reproduced well the experimental observations.

Rhizoligands increase the rhizosphere wetting kinetics and decrease the maximum swelling of mucilage. As a consequence, root rehydration upon irrigation is faster, a larger volume of water is available to the plant and this water is used more slowly. This slower water consumption would allow the plant to stay turgid over a prolonged dying period. We propose that by managing the hydraulic properties of the rhizosphere, we can improve plant adaptation to drought.

Keywords: Transpiration, Root water uptake, Mucilage, Soil water repellency, Soil-plant interactions

Declaration

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form.

   Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

   Göttingen, March, 2018

   .....
   (Katayoun Ahmadi)

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorised aid.

   Göttingen, March, 2018

   .....
   (Katayoun Ahmadi)