SOIL YEASTS, MYCORRHIZAL FUNGI AND BIOCHAR: THEIR INTERACTIONS AND EFFECT ON WHEAT (*TRITICUM AESTIVUM* L.) GROWTH AND NUTRITION

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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SUMMARY

In order to test the effect of different plant growth-promoting strategies on *Triticum aestivum* L. (wheat), we investigated the ability of biochar and a grain-associated soil yeast, to improve the growth of this crop. Our first goal was to study the effect of biochar amendments to sandy soil on the growth and nutrition of wheat in the presence of mycorrhizal fungi. This was accomplished by amending soil with 0%, 1%, 2.5%, 5% and 10% (w/w) biochar and cultivating wheat plants in these soil-biochar mixtures. After harvesting, plant growth and mycorrhizal colonization of roots were measured. In addition, we studied the nutritional physiology of these plants with regards to nitrogen (N), phosphorous (P) and potassium (K) concentrations, as well as the growth efficiencies and uptake rates of these nutrients. We found that wheat growth was improved by biochar amendments to soil, probably as a result of elevated K levels in the plant tissues supplied by the biochar amendments.

The second goal of this study was to obtain a soil yeast from the rhizosphere of another monocot in the family Poaceae, i.e. T*hemeda triandra* Forssk. (red grass), and then evaluate this isolate for its ability to improve wheat performance. Three different *Cryptococcus* species were isolated from the rhizosphere of wild grass, i.e. *Cryptococcus zeae*, *Cryptococcus luteolus* and *Cryptococcus rajasthanensis*. Since *C. zeae* was previously isolated from maize, an isolate representing this species was selected to be used in further experimentation. With the ultimate goal of testing the ability of this yeast to improve wheat growth, its effect on wheat germination was investigated and compared to that of two other soil yeasts, i.e. *Cryptococcus podzolicus* CAB 978 and *Rhodotorula mucilaginosa* CAB 826. These three yeasts were subsequently tested for their ability to improve wheat growth in pot cultures in a greenhouse. After one and two months of growth, the culturable yeasts present in the rhizosphere and bulk soil were enumerated. The effects of these yeasts were elucidated by measuring wheat growth in terms of dry weight, as well as root and shoot relative growth rates (RGR). Changes in wheat nutrition were evaluated by determining the concentrations, growth efficiencies and uptake rates for P, K, zinc (Zn) and iron (Fe). During this study, it was found that only *C. zeae* CAB 1119 and *C. podzolicus* CAB 978 were able to enhance seed germination. Similarly, it was shown that *C. zeae* CAB 1119 was able to improve wheat growth during the first and second month of cultivation, whereas *C. podzolicus* CAB 978 only improved growth during the first month, and *R. mucilaginosa* CAB 826 had no effect on growth. This improved

growth could be attributed to *C. zeae* CAB 1119 improving the P, K, Zn and Fe growth efficiency of wheat, which positively influenced the root and shoot RGR, and subsequently wheat growth.

Our final goal was to test whether *C. zeae* CAB 1119 could affect wheat growth and nutrition when cultivated in sandy soil, which contained natural microbial consortia and 10% (w/w) biochar. Plants treated with viable or autoclaved cells of *C. zeae* CAB 1119, were subsequently cultivated in soil only or soil amended with biochar. After one month, plants were harvested and growth was measured with regards to dry weight, root RGR and shoot RGR. In addition, the concentrations of P, K, Zn and Fe were analyzed for these plants, where after the growth efficiencies and uptake rates were calculated for these four nutrients. Results indicated that plants growing in soil amended with biochar, and treated with viable *C. zeae* CAB 1119, showed the best growth. The increased root and shoot RGR witnessed in these plants was probably due to increased concentrations of P and K in the plants. This study opens new avenues of research with regards to the bio-fertilizers of wheat.

OPSOMMING

Die uiteindelike doel van die studie was om die effek van verskillende plantgroei bevorderende metodes op die groei van *Triticum aestivum* L. (koring) te ondersoek. Dus het ons die vermoë van houtskool en ‘n graan-geassosieerde grondgis getoets om die groei van dié plant te bevorder. Die eerste doel van die studie was om die effek van houtskool toedienings tot sanderige grond te evalueer. Dit is bewerkstellig deur 0%, 1%, 2.5%, 5% en 10% (w/w) van die houtskool by die sand toe te voeg en koring in die houtskool-sand mengsels te kweek. Na die verlangde groei tydperk is die koring geoes en die mikorrizale kolonisasie op en in die koring wortels bepaal. Gedurende hierdie studie is die effek van bogenoemde toedienings op die fisiologie van die plante ondersoek deur die konsentrasies, opname tempo’s, en groei ekonomie van die plante vir stikstof (N), fosfaat (P) en kalium (K) te bepaal. Ons het gevind dat die groei van koring deur die toediening van houtskool bevorder is en dit blyk dat dié effek weens die teenwoordigheid van hoë K vlakke in die plantweefsel is.

Die tweede doel van ons studie was om ‘n gis vanuit die risosfeer van ‘n monokotiel wat aan die familie Poacea behoort, naamlik *Themeda triandra* Forssk. (rooigras) te isoleer. Die vermoë van die isolaat om die groei van koring te bevorder was daarna getoets. Drie verskillende *Cryptococcus* spesies was vanuit die risosfeer van rooigras geïsoleer, nl. *Cryptococcus zeae, Cryptococcus luteolus* en *Cryptococcus rajasthanensis.* Omdat *C. zeae* in ‘n vorige studie vanaf mielies geisoleer was, is ‘n isolaat van hierdie spesie gebruik in verdere eksperimente. Met die doel om te bepaal of dié gisspesie koringgroei kan bevorder, was die effek van *C. zeae* op die ontkieming van koring bestudeer en vergelyk met dié van twee ander grond giste, nl. *Cryptococcus podzolicus* CAB 978 en *Rhodotorula mucilaginosa* CAB 826. Hierdie drie giste is ook ondersoek om die groei van koring in ‘n glashuis te bevorder. Na een en twee maande se groei was die getalle van giste teenwoordig in die risosfeer en grond verder weg van die wortels bepaal. Die effek van dié giste op die groei van koring is bepaal in terme van droë gewig asook die relatiewe wortel en halm groei tempos. Veranderinge in die nutrient status van koring is ondersoek deur die konsentrasies, groei-ekonomie en tempo van opname vir P, K, sink (Zn) en yster (Fe) te bepaal. Ons het gedurende dié studie gevind dat *C. zeae* CAB 1119 en *C. podzolicus* CAB 978 die ontkieming van koring kon verbeter. Ons het ook gevind dat *C. zeae* CAB 1119 die groei van koring gedurende die eerste en tweede maand van groei kon bevorder, terwyl *C. podzolicus* CAB 978 dit net gedurende die eerste maand kon vermag en *R. mucilaginosa*

CAB 826 geen effek gehad het nie. Die verbeterde groei kon aan *C. zeae* CAB 1119, wat die P, K, Zn en Fe groei effektiwiteit van die plante verbeter het, toegeskryf word. Die verbetering van groei effektiwiteit het ‘n positiewe invloed op die relatiewe groeisnelheid van die wortels en halms gehad, en dus op koringgroei.

Die laaste doel van die studie was om te bepaal of *C. zeae* CAB 1119 die groei van koring kon bevorder wanneer die koring in sand wat natuurlike mikrobiese populasies bevat en met houtskool aangevul is, gekweek word. Plante is met lewensvatbare of nie-lewensvatbare selle van *C. zeae* CAB 1119 behandel en gekweek in sanderige grond, en/of grond waarby 10% (w/w) houtskool toegevoeg is. Die plante is na een maand geoes en die groei bepaal in terme van droë massa en die relatiewe wortel en halm groei tempos. Die konsentrasies van P, K, Zn en Fe in die plante, asook die fisiologie van die plante, nl. groei ekonomie en tempo van opname, met betrekking tot P, K, Zn en Fe is bepaal, Ons het gevind dat plante wat in die houtskool-grond mengsel gekweek is en met lewensvatbare selle van *C. zeae* CAB 1119 behandel is die beste groei getoon het. Die verbeterde relatiewe groei tempos van die wortels en halms was mees waarskynlik die gevolg van verhoogde P en K konsentrasies in die plante. Hierdie studie toon nuwe resultate in verband met die gebruik van biologiese alternatiewes tot kunsmis.

MOTIVATION

It has been known for some time now that plants benefit from associations with microorganisms found in the rhizosphere. These microorganisms include plant growth promoting rhizobacteria, mycorrhizal fungi and soils yeasts. Interestingly, the plant growth promoting abilities of rhizobacteria (El-Tarabily and Sivasithamparam 2006) and mycorrhizal fungi have been studied more comprehensively than that of soil yeasts. Some studies have demonstrated that soil yeasts can promote plant growth. For example, a *Rhodotorula sp.* was shown to improve *Solanum lycopersicum* L. (tomato) growth and yield (Abd El-Hafez and Shetata 2001). It was also demonstrated that a maize root endophyte, *Williopsis saturnus*, could improve growth of *Zea mays* L. (maize) (Nassar et al. 2005).

Recently it was shown that the common soil yeast *Cryptococcus laurentii* increases root growth of a relatively minor crop of the Western Cape, i.e. *Agathosma betulina* (Berg.) Pillans, also known as buchu (Cloete et al. 2009). It was also demonstrated that this yeast could alter the nutritional physiology of this sclerophyllous shrub (Cloete et al. 2010). Unlike buchu, *Tritcum aestivum* L. (wheat) is the second most produced crop in the world and an important food source (UN, 2010). In order to supply enough food to an evergrowing population crop yield must be increased. This can be accomplished by expanding agricultural land and increasing intensive farming. One of the strategies employed in intensive farming is the addition of inorganic fertilizers to soil (Foley et al. 2011). However, this is a costly and sometimes ineffective practice (FAO, 2005). Therefore, any improvement of wheat growth by soil yeasts during the minimal application of fertilizer would be beneficial, not only to farmers, but also to the economy.

Microorganisms are not the only strategy that can be incorporated to improve wheat growth under low nutrient conditions. It was demonstrated that biochar, the carbon (C) rich material produced through pyrolysis (Nguyen et al. 2008), can improve growth of plants (Chan et al. 2007), including wheat (Blackwell et al. 2007; Solaiman et al. 2010).

With the above as background, the first objective of this study was to determine the effect of biochar amendments to soil on the growth and nutritional physiology of wheat in the presence of mycorrhizal fungi. The second objective was to isolate a potential plant growth promoting yeast from the rhizosphere of another monocot that belongs to the family Poaceae and to establish its effect on wheat germination, growth and nutrition. The final

objective was to evaluate the effect of this yeast isolate on wheat growth and nutrition when cultivated in soil that contained natural microbial consortia and was amended with biochar.

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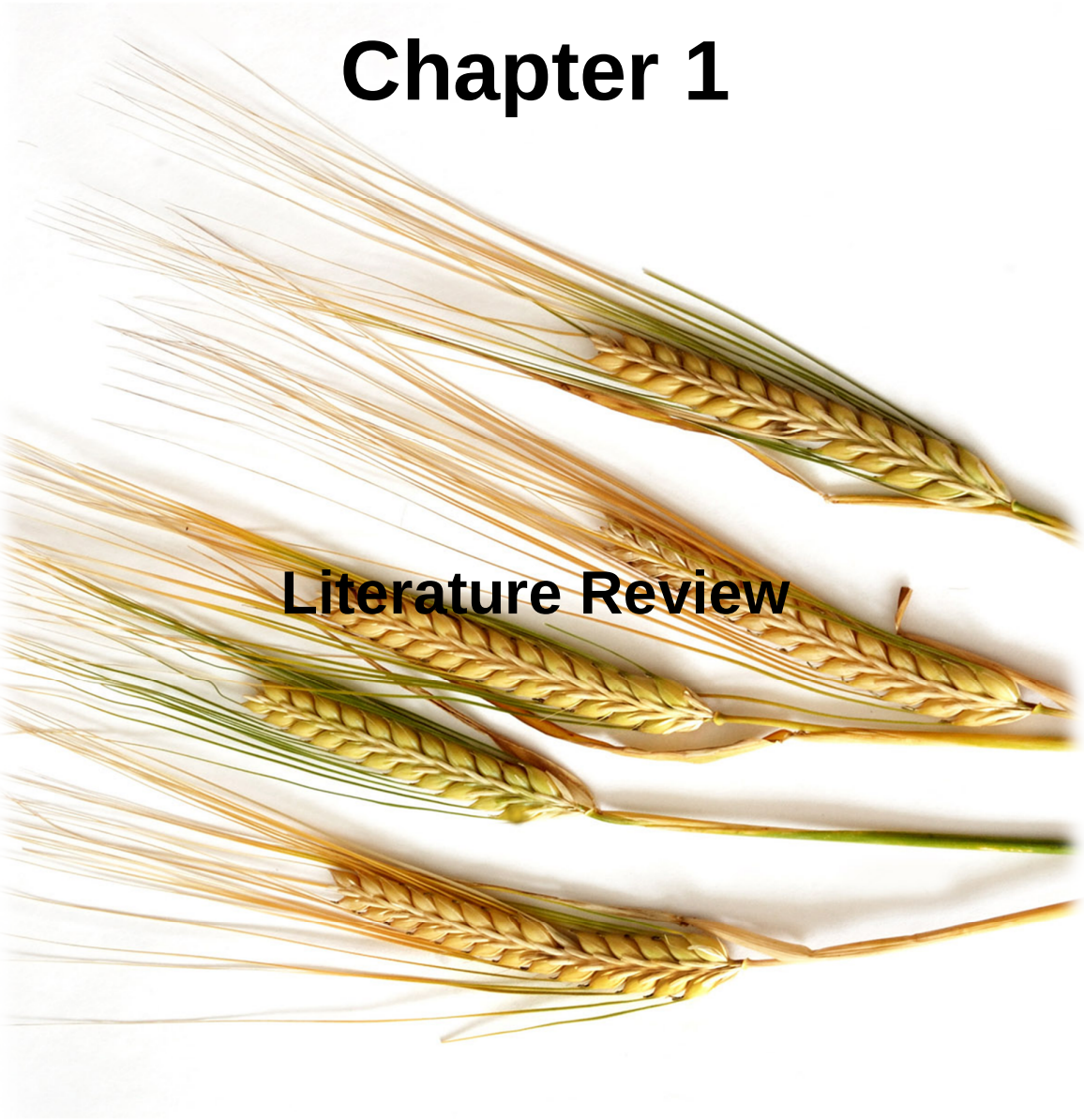
Chapter 1



Literature Review

1. *Triticum aestivum* L. (Bread wheat)

Triticum aestivum L., also known as bread wheat (winter or spring), is the most produced grain of all the wheat species and was first described by Carl Linnaeus in 1753 (Belay 2006; Linnaeus 1753; Percival 1921). In 1895, the genus *Triticum* was placed in the family Poaceae (grass) and after several taxonomic reclassifications, *Triticum aestivum* L. was accepted as the proper name for bread wheat (Belay 2006). The general morphological characteristics (Figure 1) of *T. aestivum* are erect hollow culms (Belay 2006; Simpson 2010; Van Delden et al. 2010) with five to six internodes (Tripathi et al. 2003). The roots may be divided into two groups, where roots that originated from the embryo are known as the seminal roots (Nakamoto and Oyanagi 1996), while the adventitious roots are those that originate from the stem base after germination (Liu et al. 2009). Leaves have an open basal sheath and are linear, distichous and ligulate (Simpson 2010; Van Wyk and Van Oudsthoorn 2012). The blades are bifacial, glaucous and have parallel veins (Bennet et al. 2012; Simpson 2010). Inflorescences of wheat are spikelets aggregated in spikes and bristle-like awns can be found on the apex of the glumes and lemmas (Simpson 2010; Van Wyk and Van Oudtshoorn 2012). Seeds are located in the spikelets and are ellipsoidal, broader at one end and have a central groove on one side (Belay 2006). Bread wheat is also classified commercially, by placing the varieties into distinct categories, based on for example, hardness of the grain, grain colour and whether it is cultivated during the spring or winter seasons (Belay 2006).

Similar to all plants, *T. aestivum* has specific physical and chemical requirements in order to grow optimally. The optimum growth temperature of this crop is between 10 and 24 °C, while at least 200 mm of water is required for good development (Belay 2006). Soil must be deep, properly aerated and drained, have a pH between 6.1 and 6.5, and must contain more than 0.5% organic matter to be suitable for the production of wheat.

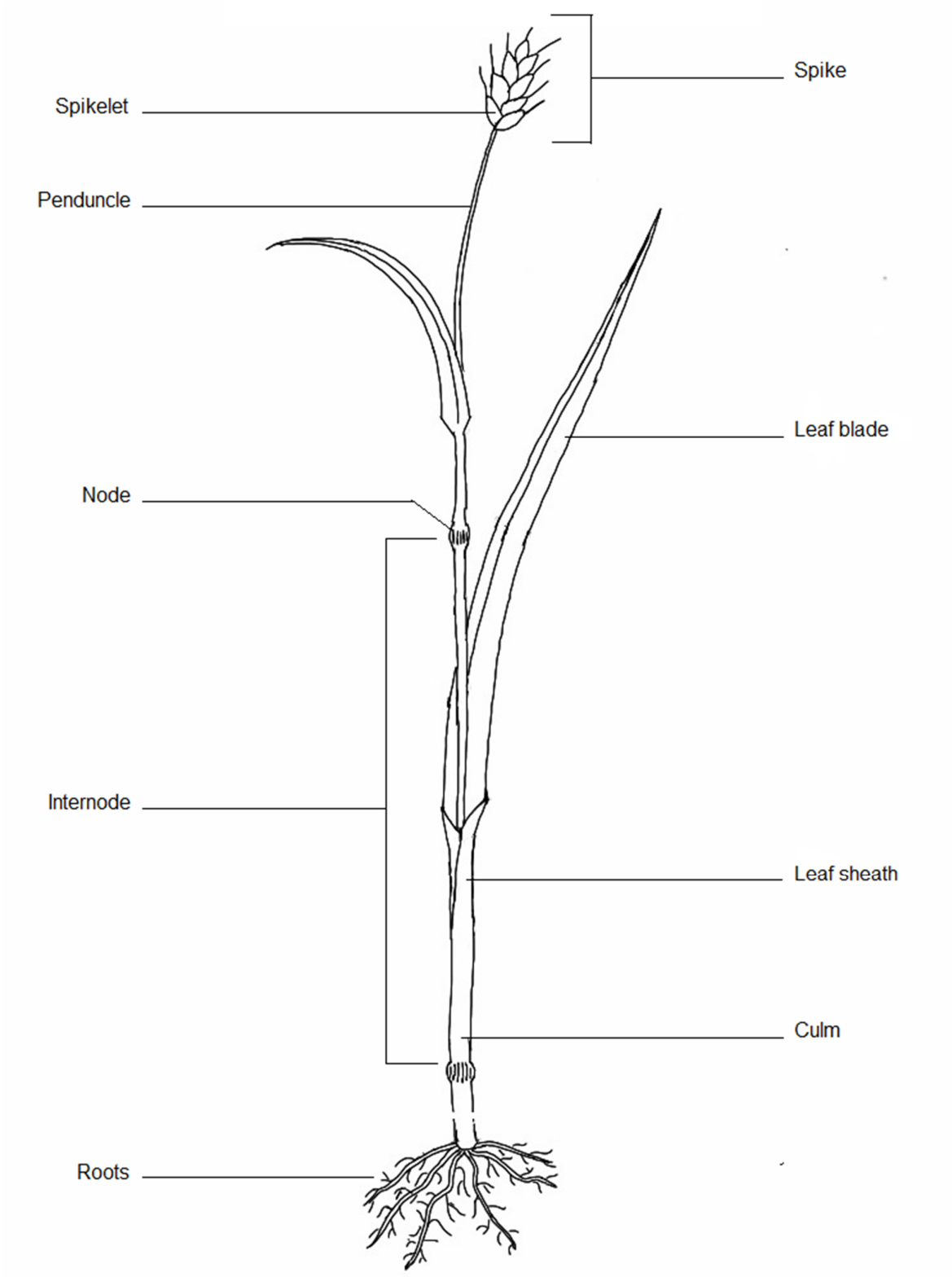


Fig. 1. Line drawing of *Triticum aestivum* L. (wheat), showing the most important morphological characteristics of the plant (Adapted from Glimn-Lacy & Kaufman 2006; Van Wyk and Van Oudtshoorn 2012).

Bread wheat is one of the oldest and the second most produced cereal crop in the world (FAO 2010). It can adapt to grow under a wide range of climatic conditions, as well as in various soil types. It is thus not surprising that it is cultivated in almost every country in the world with the major producers of wheat being Argentina, Australia, Canada, China, the European Union countries and the United States of America (Belay 2006). In the 2009/2010 season approximately 678 million tons of wheat grain was harvested worldwide (IGC 2011).

Wheat is normally grown for its grain, which is ground and used as flour in the baking industry (Belay 2006). However, the grain may be cooked and consumed as an alternative to other grain foods, such as pearl barley, whilst the straw and bran are used as feed for livestock, poultry and prawns. Wheat can also be used for the production of glues, alcohol, as well as gluten (Belay 2006).

2. Food production

It has been estimated that the world population total will reach 9 billion people by 2050 (UN 2010). In order to supply enough food to this population, food production must increase by 70%. This amounts to an increase in cereal production from 2.1 billion to 3 billion tons (UN 2010)..To increase crop production agricultural land must expand or cropping intensity must increase (Foley et al. 2011). Indications exist that expanding agricultural land will not result in a much greater crop production, since most soils that are best suited for agriculture is already used for this purpose (Ramankutty et al. 2002). The remainder of soils are covered by deserts, mountains, ecological reserves, cities and lands that are unsuitable for agriculture (Ellis et al. 2010; Foley et al. 2011). Therefore, recent expansions in agricultural land entailed the deforestation of mostly tropical areas (Foley et al. 2011; Gibbs et al. 2010). Deforestation, however, results in increased greenhouse gasses (Friedlingstein et al. 2010) and the loss of important ecological processes (Foley et al. 2007).

Cropping intensity involves the utilization of irrigation, fertilizers and biocides to increase crop yields (Foley et al. 2011), all of which have a negative impact on the environment. For instance, irrigation depletes water resources (Gleick et al. 2009; Postel et al. 1996), while the excessive use of fertilizers result in the disruption of global nitrogen (N) and phosphorous (P) cycles (Smil 2000; Vitousek et al. 1997) and the eutrophication of water systems. In addition fertilizers are expensive and sometimes inefficient (FAO, 2005). It is

thus not surprising that there has been an increasing interest in alternative methods, e.g. biochar additions and plant growth promoting microorganisms, for increasing crop yield (Adesemoye et al. 2009).

3. Biochar

A promising low-cost alternative to fertilizers is the application of biochar to soil, which can improve soil quality and plant growth. Biochar, or Black Charcoal, is a carbon (C) rich material produced through the incomplete combustion of plant biomass (Nguyen et al. 2008). There has been much interest in biochar since the discovery of *Terra Preta de índio* soils in the Brazilian Amazon. It is thought that the high fertility of these soils are due to high organic C present in the form of char, created through the 'slash and burn' practices of pre-Columbian indigenous people (Chan et al. 2007; Warnock et al. 2007). Biochar has also been discovered in coniferous forest soils and in prairie soils (Spokas et al. 2009), and has since been produced artificially through a process called pyrolysis.

3.1. Biochar Production

Pyrolysis is a thermo-chemical process in which plant material is heated in the absence of oxygen in order to yield solid (biochar), liquid (bio-oil) and/or gaseous (syngas) products (Gaunt and Lehmann 2008; Özcimen and Karaosmanoğlu 2004; Spokas et al. 2009). This yield differs with respect to the type of process, namely fast or slow pyrolysis (Mathews 2008; Spokas et al. 2009). In fast pyrolysis, biomass is exposed to more than 500°C for a few seconds, resulting in the production of more bio-oil and syngas than biochar. Alternatively, the heat applied during slow pyrolysis volatizes mostly hydrogen (H), oxygen (O) and C present within the plant material, resulting in biochar that contains poly-aromatic hydrocarbons and functional groups (Mathews 2008; Warnock et al. 2007).

It has been demonstrated that biochar composition is also influenced by the feedstock type (Chan et al. 2007; Spokas et al. 2009). Research has shown that coniferous biochars generated at temperatures lower that 350°C can contain a larger amount of available nutrients and have a smaller adsorptive capacity for cations (Warnock et al. 2007). In contrast, adsorptive capacity can be increased by using plant species with larger diameter cells in their stem tissues as feedstock. It is thus imperative that the correct pyrolysis conditions, as well as feedstock, are chosen for the production of biochar in order to best suit the intended application.

3.2. Applications and benefits of biochar

Biochar has many proposed applications and benefits. One of these is its ability to reduce greenhouse gasses, especially nitrous oxide (N_2O) and methane (CH_4), when added to soil (Gaunt and Lehmann 2008). Rondon et al. (2005) demonstrated that for soybean plots the production of N_2O was reduced and CH_4 production repressed when 20 mg/ha biochar was added to acidic soils in the Eastern Columbian Plains. Similarly, a 10% (w/w) biochar amendment to Typic Hapludand soils, collected from a grassland in Japan, resulted in 85% reduction in N_2O production (Yanai et al. 2007). The mechanism behind this reduction in greenhouse gasses is still relatively unknown (Spokas et al. 2009). Biochar can also be used to adsorb herbicides as well as chemicals from soil. For example, Yang and Sheng (2003) reported that biochar from burnt wheat and rice residues were 2500 times more effective in absorbing diuron herbicide than other soil organic carbons. Yet, perhaps the most researched benefit of biochar amendments to soil focuses on its ability to improve soil vitality, fertility and overall plant growth.

3.3. Soil amendment with biochar

It is well-known that biochar amendments to soil not only enhances the fertility and vitality of soil, but can also permanently sequester C (Chan et al. 2007; Mathews 2008) in a highly stable (recalcitrant) form due to the aromatic hydrocarbons in its structure (Mathews 2008; Warnock et al. 2007). It thus has the potential to exist in soil for thousands of years (Spokas et al. 2009), with approximately 5000 years as a common estimate (Warnock et al. 2007). Biochar has an impact on greenhouse gasses by removing atmospheric C. For example, plants fix atmospheric CO_2 through photosynthesis and when plants are combusted, as result of forest fires, the remaining char ends up in the soil thus effectively removing CO_2 from the atmosphere.

As mentioned previously, C sequestration is not the only reason for amending soil with biochar. Several studies have shown that the addition of biochar to soil also improves soil fertility and thus plant growth (Kimetu et al. 2008; Spokas et al. 2009). It is important to note that biochar does not improve plant growth when used as a sole fertilizer. This was demonstrated by Chan et al. (2007) who showed that biochar additions to Alfisol did not increase radish yield. However, when the authors added N fertilizers and biochar, radish diameter increased by 266% for 100 t/ ha biochar additions (Chan et al. 2007).

The precise mechanism whereby biochar improves soil fertility and plant growth is still relatively unclear (Chan et al. 2007), but some studies have demonstrated that biochar can alter soil nutrient availability, by influencing the soil physico-chemical properties (DeLuca et al. 2006; Gundale and DeLuca 2006; Matsubara et al. 2002; Tryon 1948). These properties include reduction in tensile strength, increase in field capacity, increase in pH and exchangeable cations (Chan et al. 2007; Major et al. 2005), which may result in enhanced plant performance and elevated tissue nutrient concentrations (Warnock et al. 2007). However, it is known that biochars produced from different feedstocks can have different, and sometimes detrimental, impacts on plant growth (Warnock et al. 2007). For instance, if the pH of the soil is neutral and that of the biochar amendment basic, the pH of the resulting soil may also be too high and the level of bio-available nutrients will be reduced (Havlin et al. 2005). Biochar can be successfully used in low input, as well as high input agriculture, but it must be used in conjunction with fertilizers. It is also important that its chemical composition, as well as impact on soil microorganisms, is tested before adding it to soil. Interestingly, biochar is not the only promising alternative to fertilizers. Recent studies have focused on the enhancement of plant growth by symbiotic soil microorganisms, particularly rhizobacteria, arbuscular mycorrhizal fungi and soil yeasts (El-Tarabily and Sivasithamparam 2006; Gollner et al. 2005; Cloete et al. 2009; Cloete et al. 2010).

4. Soil microorganisms as plant growth promoters

The effects of rhizobacteria on plant growth are well-known and have been extensively studied. They can either affect plant growth directly, through N fixation and production of plant growth regulators, or indirectly by producing metabolites such as siderophores and antibiotics that may inhibit the activity of plant pathogens (El-Tarabily and Sivasithamparam 2006). Similarly, much information is available on the effects of mycorrhizal fungi on plant growth (Boby et al. 2008; Gollner et al., 2005; Mohammad et al. 2004), yet little is known about the effect of biochar on the interactions between these fungi and their host plants.

4.1. Arbuscular mycorrhizal (AM) fungi

Arbuscular mycorrhizal (AM) fungi are a small group of common soil-borne zygomycetic fungi (Schalamuk et al. 2006; Vierheilig et al. 1998) that form symbiotic associations with the roots of approximately 80 to 85% of all terrestrial plant species (Abdel-Fattah 2001;

Schalamuk et al. 2006). During these symbioses, the growth of the host plant is enhanced through increased uptake of mineral nutrients e.g. P and N (George et al. 1995; Gollner et al. 2005; Vierheilig et al. 1998), and in return, the mycobiont receives photosynthetic C from the plant (Cavagnaro et al. 2003; Gollner et al. 2005; Vierheilig et al. 1998). There are two main morphological types of AM fungi, namely the *Arum*- and *Paris*-type, which are distinguished by the presence or absence of intercellular hyphae in colonized roots (Smith and Smith 1997).

It is well-known that mycorrhizal plants grow better in infertile soils compared to non-mycorrhizal plants. This is ascribed to the ability of the extra-radical hyphae of AM fungi to acquire nutrients, especially P, from beyond the root depletion zone (Boby et al. 2007; Boby et al. 2008). It is important to note that the amount of P transported to the plant varies between AM fungi species and thus result in different effects on plant growth (Ravnskov and Jakobsen 1995; Vierheilig and Ocampo 1991b). Nutrient acquisition is not the only benefit of mycorrhizal associations. It has been demonstrated that AM fungi may improve the resistance of the host plant to pathogens, as well as its tolerance to drought, salinity and transplant shock (Boby et al. 2008). The level of plant response to AM fungi colonization depends on the AM species and the degree of infection.

The main factors that influence the degree of root colonization by AM fungi include plant age, climate, P level and finally infectivity of mycorrhizal propagules (spores, colonised root fragments and hyphae). Plant age is an important factor as root colonization is dependent on root growth rates, as well as the initial infection rate and growth rate of the mycobiont in the roots. These three factors are also influenced by the climate (Hetrick and Bloom 1983). This was demonstrated by Daniels-Hetrick et al. (1984), who showed that winter wheat was less colonized by mycorrhizal fungi when plants were cultivated at 10°C in comparison to those that were grown at warmer temperatures. Similarly, Mohammad et al. (1998) showed that an increase in soil temperature resulted in increased root colonization of winter wheat by AM fungi. It is well-known that the extent to which plants are colonized is immensely affected by the concentration of soluble P in the soil. Numerous studies have demonstrated that when the soluble P level increases, the mycorrhizal colonization declines remarkably and *vice versa* (Baon et al. 1992; Boby et al. 2008; Kahiluoto et al. 2000; Khan 1975; Mohammad et al. 2004; Thomson et al. 1991). Finally, colonization by AM fungi is affected by the concentration of the propagules in the soil. The failure of AM fungi to form mycorrhizal associations with wheat plants was shown

to result from low inoculum concentrations (Daniels-Hetrick et al. 1984; Vierheilig and Ocampo 1991a). Furthermore, Schalamuk et al. (2006) showed that tilling decreased AM spore density in agricultural soil, resulting in reduced crop colonization.

Recently, it has been shown that the addition of biochar to soil can affect the symbiosis between mycorrhizal fungi and plants. In most cases biochar additions resulted in an increased colonization, but in a few studies biochar was found to have a negative impact on mycorrhizal colonization (Warnock et al. 2007). This negative effect seems to be largely due to changes in nutrient levels. Gaur and Adholeya (2002) demonstrated that biochar limited the amount of P uptake by plants, while Wallstedt et al. (2002) established that both N and organic C decreased after biochar was added to soil. These negative effects can be prominent if the C/N ratio is high and a portion of biochar is decomposable. This will lead to N-immobilization that will ultimately have a negative impact on plant growth (Warnock et al. 2007). The mechanism whereby biochar influences mycorrhizal fungi, however, is still unclear, but Warnock et al. (2007) proposed four possible mechanisms.

4.1.1. Possible mechanisms whereby biochar may affect mycorrhizal fungi

The first and according to Warnock et al. (2007) the most likely mechanism of influence, is that biochar may alter nutrient availability and/or the soil physico-chemical parameters, ultimately impacting mycorrhizal colonization. As mentioned previously, biochar modifies important physico-chemical properties of soil that might result in a nutrient shift. This could affect mycorrhizal fungi either positively or negatively, as a change in the nutrient balance can alter the activity of mycorrhizal fungi (Miller et al. 2002).

Secondly, biochar additions may result in changes that might be beneficial or detrimental to mycorrhization helper bacteria (MHB) and phosphate solubilising bacteria (PSB). Mycorrhization helper bacteria produce extracellular flavonoids and furans that facilitate growth of mycorrhizal fungi, while PSB, such as *Pseudomonas aeruginosa,* can solubilise plant nutrients, especially phosphate. Biochar itself, or the nutrient that it has absorbed, might serve as a nutrient source for these bacteria. This nutrient source can result in increased proliferation of these bacteria, which would increase the growth and P uptake of mycorrhizal fungi (Warnock et al. 2007).

The third mechanism proposed by Warnock et al. (2007) is that biochar may change the signalling between the host plant and mycorrhizal fungi that may lead to altered root

colonization. Angelini et al. (2003) demonstrated that pH influences certain groups of microorganisms as either a stimulant or inhibitor. However, biochar amendment to soil can alter the pH, which may stimulate or inhibit microorganisms. If biochar with superior absorptive capacity is used, it may absorb the signalling compounds and cause a signalling interference. These absorbed signalling molecules might not have an immediate impact, but may be desorbed later and will be able to stimulate mycorrhizal colonization even further. This can negatively affect colonization, for if the signalling molecules are absorbed it will result in decreased signalling molecules reaching the mycorrhizal fungi (Warnock et al. 2007).

Finally, biochar might serve as a refuge for MHB and mycorrhizal fungi from predators. If hyphae and bacteria colonize biochar, they may be protected from soil predators, especially larger organisms such as mites and protists. For example, if a biochar with a small pore size (> 16 µm) is used and average bacteria (1 – 4 µm) and fungi (2 – 64 µm) colonize these particles, protists (8 – 100 µm) and micro-arthropods (100 µm – 2 mm) may not fit into some of the pores, thus protecting the bacteria and mycorrhizal fungi from predation (Warnock et al. 2007).

4.1.2. AM fungi and wheat

Interestingly, despite wheat being one of the most important crops in the world, relatively few studies have focused on the effect of AM root colonization on wheat performance. This is mainly due to the view that AM fungi are incapable of extensively colonizing this plant's finely branched root system and dense root hairs. The majority of researchers have found that only 10 to 30% of the total root length is colonized by AM fungi (Daniels-Hetrick et al. 1984; Mader et al. 2000; Trent et al. 1989; Vierheilig and Ocampo 1991a). In contrast, a few others have showed that up to 80% of the total root length of wheat can be colonized when plants are grown under controlled conditions or in field trials (Dekkers and Van der Werff 2001; Khan 1975). Recently, it was demonstrated that this mycorrhizal colonization and growth of wheat can be enhanced by biochar amendments to soil during pot trials (Solaiman et al. 2010b) and field trials (Blackwell et al. 2007; Solaiman et al. 2010a). It must be noted however, that wheat can form symbioses with many species of AM fungi, but often no improvement is seen in its growth (Jensen and Jakobsen 1980; Ryan et al. 2002; Vierheilig and Ocampo 1991b). It has been suggested that plant growth might be reduced by these mycobionts due to C drain, especially during the early development of wheat (Graham and Abbott 2000). Even though no positive, and sometimes a negative,

growth response was observed for wheat, the level of P acquisition was still increased by fungal colonization (Graham and Abbott 2000; Zhu et al. 2001). These findings were supported by numerous studies that revealed that mycorrhizal wheat plants absorbed more ^{32}P than non-mycorrhizal plants even though no positive growth response could be observed (Hetrick et al. 1996; Ravnskov and Jakobsen 1995; Schweiger and Jakobsen 1999). However, wheat has shown positive responses when it was cultivated in soil containing a moderate P level (Al-Karaki and Al-Omoush 2002; Thompson 1987; Yao et al. 2001).

Although the positive impact on wheat performance could not always be demonstrated, it is evident that AM fungi play an important role in the ecosystem. Nevertheless, they are not the only fungi known to form associations with plants. Recently, studies have demonstrated the ability of soil yeasts such as *Candida, Rhodotorula, Sporobolomyces, Trichosporon, Williopsis* and *Yarrowia* to also promote plant growth (El-Tarabily and Sivasithamparan 2006; Medina et al. 2004; Nassar et al. 2005).

4.2. Soil yeasts

Soil yeasts are unicellular fungi that not only occur in many different soil types (Botha 2006; El-Tarabily and Sivasithamparam 2006), but are also present in the rhizosphere of plants. It is well-known that plants exude a variety of compounds from their roots. Carbohydrates within these exudates serve as the main C source in the rhizosphere. This can sustain growth of diverse microbial populations, including yeasts belonging to the genera *Cryptococcus*, *Debaryomyces, Lipomyces* and *Schizoblastosporion* (Botha 2006; Cloete et al. 2009; El-Tarabily and Sivasithamparam 2006). As the distance from the root surface increases, the abundance and diversity of yeasts decreases. This is ascribed to the slow diffusion rate of root exudates and the rapid decomposition of nutrients by the soil microbial community (Sauer et al. 2006).

It has been demonstrated that some soil yeast species can promote plant growth, but the mechanisms whereby these yeast genera promote growth differ. *Yarrowia lipolytica,* for example, increases the bio-available P by solubilising rock phosphate, while *Williopsis saturnus* produces indole-3-acetic acid (IAA) and indole-3-pyruvic acid (IPYA) that serve as precursors during auxin production (El-Tarabily and Sivasithamparam 2006). Other genera produce plant growth regulators such as IAA, gibberellins and polyamines, whilst others can alter the nutritional physiology of plants. Recently, it was demonstrated that a

polyamine producing strain of *Cryptococcus laurentii* was able to promote the growth of the sclerophyllous shrub *Agathosma betulina* (Berg.) Pillans (buchu) (Cloete et al. 2009). It was also demonstrated that this yeast altered the nutritional physiology of buchu thereby increasing the P and iron (Fe) content in the plants (Cloete et al. 2010). It is important to integrate the growth and nutritional physiology in plants when investigating the effect of yeasts on plant growth, since growth and more importantly yield are directly linked to nutrient content, uptake and use efficiency. Yet, very little is known about the impact of soil yeasts on plant nutrition.

Despite the importance of wheat, up to date only one study demonstrated the ability of a soil yeast, i.e. *Sporobolomyces roseus,* to improve the growth of this plant (Perondi et al. 1996). It is therefore imperative to investigate the effect of other soil yeasts on wheat growth and nutritional physiology in order to try to increase productivity without the use of fertilizers.

5. Conclusion

Fertilizers have played an important role in the cultivation of wheat. However, the increasing cost and ineffectiveness of fertilizers have resulted in the evaluation of alternative methods for improving crop yield. These alternatives include addition of biochar to soil and exploring the potential of symbiotic relationships between plants and microorganisms. A few studies have demonstrated the ability of biochar to improve wheat growth, but little is known about the effect of emending soil with biochar on wheat nutrition in the presence of mycorrhizal fungi.

Like AM fungi, soil yeasts have been shown to improve plant growth. Yet, very little is known about the way in which these microorganisms affect the growth and nutritional physiology of wheat. It is therefore important that these interactions are studied before biochar and/or soil yeasts can be used to reduce the quantities of inorganic fertilizers used.

6. Research Objectives

With the previous literature in mind, this first aim of the study was to establish the effect of different biochar concentration amendments to sandy soil on mycorrhizal colonization and growth of wheat, with regards to plant biomass and nutrient levels. The second aim was to determine whether a yeast species isolated from the rhizosphere of another

monocot belonging to Poaceae, could affect the germination, growth and nutritional physiology of wheat. Lastly, we sought to evaluate whether this yeast could improve wheat growth and nutrition in sandy soil, containing natural microbial consortia, which was amended with biochar.

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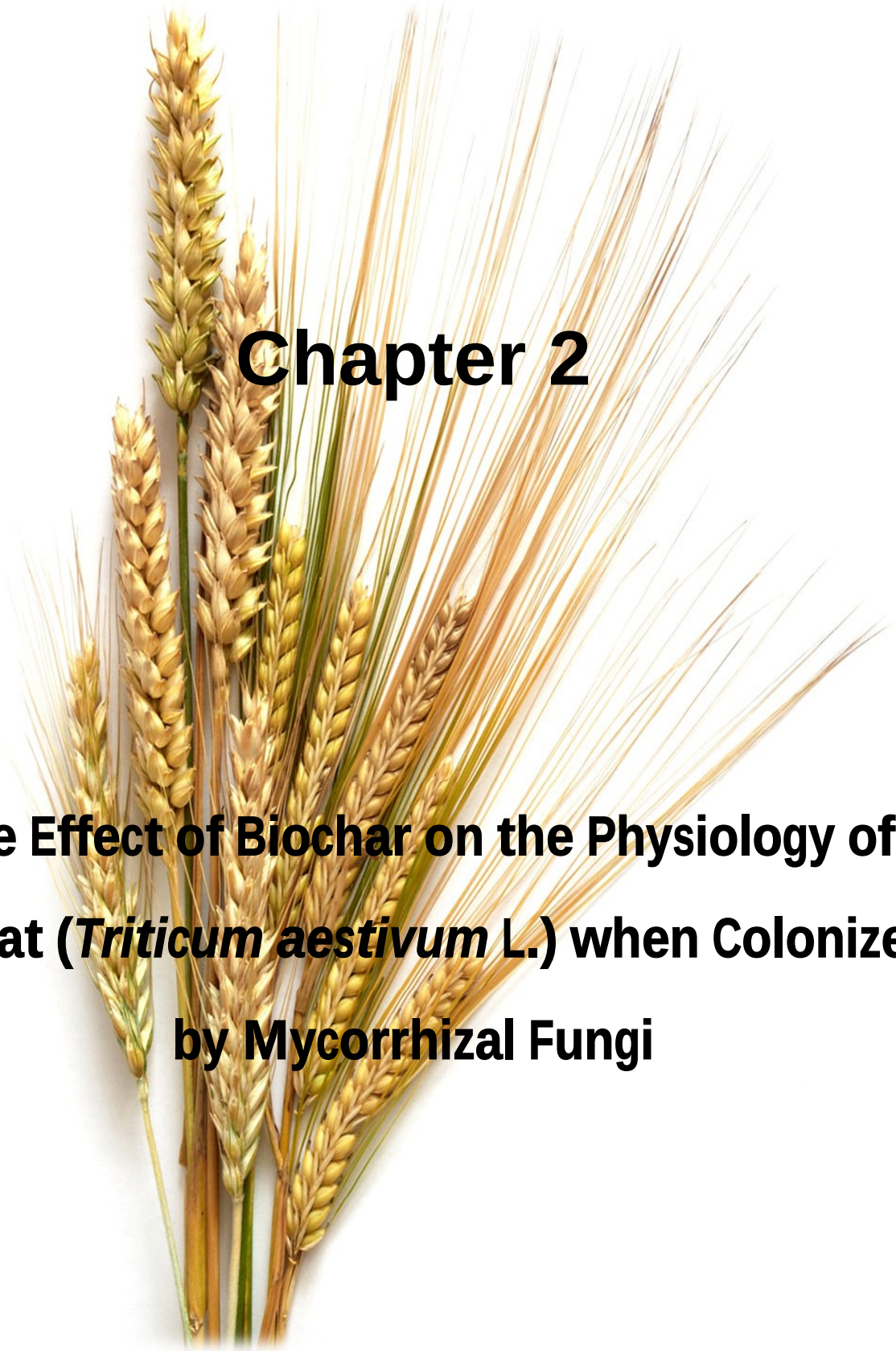

Chapter 2

The Effect of Biochar on the Physiology of Wheat (*Triticum aestivum* L.) when Colonized by Mycorrhizal Fungi

1. Introduction

Triticum aestivum L. (wheat) is one of the oldest and the second most produced cereal in the world (FAO 2010) and can adapt to grow in a wide range of climatic conditions, as well as various soil types. It is thus not surprising that it is cultivated in almost every country, including South Africa (Belay 2006). However, it is known that present production of this cereal is too low to meet the needs of the world's population in the near future (FAO 2010, UN 2010). Therefore, to supply enough food to 9 billion people by 2050, food production must increase by 70% (UN 2010). This amounts to an increase in cereal production from 2.1 billion to 3 billion tons. Expansion of agricultural land, however, will contribute little to increasing crop production, since all over the globe soils best suited for agriculture are already in use. It thus seems likely that most of the increase in food production will be due to increased cropping intensity. One strategy employed in increasing yield is the application of fertilizers to soils, especially underperforming soils (Foley et al. 2011). Fertilizers, however, are expensive, sometimes inefficient and have a negative impact on the environment, thus necessitating the need for alternative methods to improve crop growth and yield (Adesemoye et al. 2009).

One such an alternative is the emendation of soil with biochar (Chan et al 2007). Biochar is a carbon rich material that is produced through slow pyrolysis of plant biomass (Lehmann et al. 2003; Chan et al. 2007; Chan et al. 2008; Glaser 2007; Nguyen et al. 2008; Mathews 2008). Due to its highly porous nature, biochar may enhance nutrient and water retention, as well as serve as a microbial habitat (van Zwieten et al. 2010a; Joseph et al. 2010; Kookana et al. 2011), impacting on microbial N2-fixation (Mathews 2008) and mycorrhizal root colonization (Spokas et al. 2009).

Arbuscular mycorrhizal fungi (AM fungi) is a small group of soil-borne zygomycetes (Vierheilig et al. 1998; Schalamuk et al. 2006) that form symbiotic associations with the roots of up to 85% of all terrestrial plant species (Abdel-Fattah 2001; Schalamuk et al. 2006). It is well-known that during these symbioses the fungus may increase uptake of nutrients such as phosphorus (P), nitrogen (N), and potassium (K) by its host plant. In turn, the plant provides the fungus with photosynthetic carbon (Jakobsen 1995; Gollner et al. 2005). Interestingly, it was demonstrated that addition of biochar to soil can affect these symbioses.

A few studies have demonstrated that application of biochar to soil had a positive effect on mycorrhizal colonization of wheat in both pot trials (Solaiman et al. 2010b), as well as field trials (Blackwell et al. 2007; Blackwell et al. 2010; Solaiman et al. 2010a). In most of these cases however, it was found that although biochar increased the mycorrhizal colonization, no positive effect was seen on wheat growth (Blackwell et al. 2007; Solaiman et al. 2010b). Yet, data on the physiology of wheat in the presence of biochar is inconclusive, since in most of these studies only one or two factors were tested, e.g. N uptake (Van Zwieten et al. 2010a; Van Zwieten et al. 2010b), N uptake and N content (Prendergast-Miller et al. 2011), or P and N uptake (Blackwell et al. 2010). However, to fully understand the effect of biochar on wheat growth it is important to assess the nutritional physiology of all three major macro-elements in plants, i.e. N, P and K. These nutrients are pivotal elements in plant nutrition and any deficiencies thereof may have detrimental effects on growth, as well as yield (Bolland 2001; Edwards 2001; Mason 2001).

Since biochar is known for its nutrient retention (Mathews 2008; Warnock et al. 2007), and known to affect on mycorrhizal colonization under different nutrient conditions (Warnock et al. 2007), it is hypothesized that increased biochar amendments to low nutrient soil may lead to an increased effect on mycorrhizal colonization and the nutritional physiology of N, P and K in wheat. With the above as background we aimed to study the effect of different concentrations of biochar in sandy low nutrient soil on the growth, mycorrhizal colonization and nutritional physiology of N, P and K in *T. aestivum*.

2. Materials and methods

2.1. Biochar and soil analyses

The biochar used in this study was produced from pinewood sawmill waste using slow pyrolysis at approximately 450°C (Allbrick, Thembalethu, South Africa). Before chemical analyses, the biochar was crushed and milled to an average particle size of 2 mm. Excess moisture was removed by drying in an oven at 40°C for 12 h and the biochar was then ball-milled to less than 1 mm diameter particle size. Carbon and N present in the biochar was determined by using the dry combustion method (EuroVector CNH Analyzer). The pH of the biochar was measured in a 1:20 biochar: water ratio, as well as in 1 M KCl as described by White (1997). The cation exchange capacity (CEC) and the plant-available nutrients of the

biochar were determined according to the methods described by Rhoades (1982), and Soltanpour and Workman (1979), respectively. These methods were selected due to the alkaline nature of the biochar. Proximate analysis of the biochar to determine the ash, fixed C and volatile content was performed using thermogravimetric analysis (Perkin Elmer Pyris TGA 7). The sample was heated from 25°C to 600°C at 10°C/min and then from 600°C to 900°C at 20°C/min under nitrogen gas (N_2). After 7 minutes, oxygen at a 15 ml/min flow rate was introduced for the combustion stage. All of these analyses were performed in duplicate. The sandy soil used in this study was collected from an unused field (33° 53' 43.08'' S, 18° 43' 24.24'' E) near Brackenfell in Cape Town (Western Cape, South Africa). This soil was classified (World Reference Base) as a Haplic Stagnosol (Albic), and only the thick E horizon was sampled at a depth of 10 – 100 cm. The sampled soil was texturally classified as pure sand (98% sand) with a medium grade. The C and N content, pH, as well as CEC, of the soil were analyzed according to the methods described by Vreulink et al. (2007). In addition, the plant-available nutrients in the soil were determined by the AB-DPTA method of Soltanpour and Workman (1979).

2.2. Pot preparation and wheat cultivation

To establish which biochar application rate had the best effect on both mycorrhizal colonization and plant growth, the soil and biochar were mixed to create a series of soil mixtures containing 0% (control), 1%, 2.5%, 5% and 10% (w/w) biochar. The pH of these mixtures was measured in a 1:20 biochar: water, as well as 1M KCl ratio (White, 1997). Of these mixtures 800 g was added to pre-cleaned 13 cm diameter plastic pots (12 pots per concentration) and each pot was subsequently saturated to field capacity with modified Long Ashton nutrient solution (Cloete et al. 2009). Wheat seeds (cultivar SST 047) (n = 30) were surface sterilized by submerging them in 70% ethanol for one minute, followed by 40 seconds in 1% (v/v) sodium hypochlorite solution. The seeds were then rinsed in sterile distilled water, planted on quarter strength MS agar (Slater et al. 2008) and allowed to germinate for two days at room temperature, in the dark. After germination, seedlings were planted in the pots and arranged in a well ventilated greenhouse with a 12 h photoperiod of 1000-1100 µmol m^2/s photosynthetic photon flux density. The average day/night temperatures and relative humidity were 23/15°C and 50/80%, respectively. To simulate low nutrient conditions, plants received only sterile distilled water during the two month growth period.

2.3. Harvesting, colonization by mycorrhizal fungi and nutrient analyses

To determine the colonization of wheat roots by mycorrhizal fungi, the wheat plants were harvested and the roots were washed in sterile physiological saline solution (PSS). A sub-sample of the harvested roots was taken from each plant and the wet weight of these sub-samples was determined. Mycorrhizal fungi present on and in the roots were visualized by staining the sub-samples with chlorozol black E (CBE) according to the method of Brundrett (1994), immediately after harvesting. The roots were then cut into 1 cm pieces and arranged on a microscope slide in a such a manner that the mycorrhizal colonization could be determined using the grid-line intersect method (Brundrett 1994). The fresh weight of the remainder of the plants was recorded and the plant material was then dried in an oven for one week at 80°C.

The effect of biochar and mycorrhizal fungi on the growth and physiology of wheat were determined by recording the dry weight of the wheat plants. The dried material was subsequently analyzed for its N, P and K content, according to methods described by Vreulink et al. (2007). The values obtained, together with the dry weights, were used to calculate the nutrient uptake rate and growth efficiency according to the formulae proposed by Mortimer et al. (2005).

2.4. Statistical analyses

Significant differences were analysed by using ANOVA and differences between treatment means were separated using a *post hoc* Fishers least significant difference (LSD) test, using the program Statistica version 10 (Statsoft, Tulsa, OK, USA). In addition, correlation matrixes were created using the same program.

3. Results

3.1. Biochar and soil analyses

The chemical properties of both the biochar and the sandy nature reserve soil were determined before it was used in the experimentation. The most important results are provided in Table 1. The sandy soil was acidic (pH 5.14), deficient in K (critical value is 180 mg kg-1) and P (critical value is 15 mg kg-1), exceptionally low in C and N, and had a low

CEC (Table 1). The biochar was alkaline (pH 9.36) and possessed a relatively high CEC. Proximate analysis of the biochar revealed that it contained 2.7% ash, 78.8 % fixed C and 18.5% volatile matter. When the pH of the different biochar mixtures were measured in water it was found that the pH increased from 5.45 (0% biochar amendment) to 7.58 (10% biochar amendment) and in 1M KCl it increased from 4.46 (0% biochar amendment) to 6.62 (10% biochar amendment).

Table 1. Chemical properties of pinewood sawmill waste biochar and the soil collected from a nature reserve in the Cape Flats region. All analyses were preformed according to Vreulink et al., (2007).

Analysis type	Value for biochar	Value for sand
pH (H_2O)	9.36	5.14
pH (1M KCl)	8.63	4.30
Plant available P (mg/kg)	45.94	3.93
Plant available K (mg/kg)	878.30	10.88
Cation exchange capacity ($cmol_c$ /kg)	118.30	1.96
Total C (%)	76.99	0.16
Total N (%)	0.50	0.03
C:N ratio	154:1	5:1

3.2. Colonization by mycorrhizal fungi and wheat growth

Mycorrhizal colonization of *T. aestivum* roots was determined by dividing the mycorrhizal counts by the total intersections studied and the obtained value was expressed as the percentage colonization (Fig. 1). It was found that wheat roots were more colonized by mycorrhizal fungi when the plants were cultivated in soil amended with biochar compared to those cultivated in soil without biochar ($p < 0.05$).

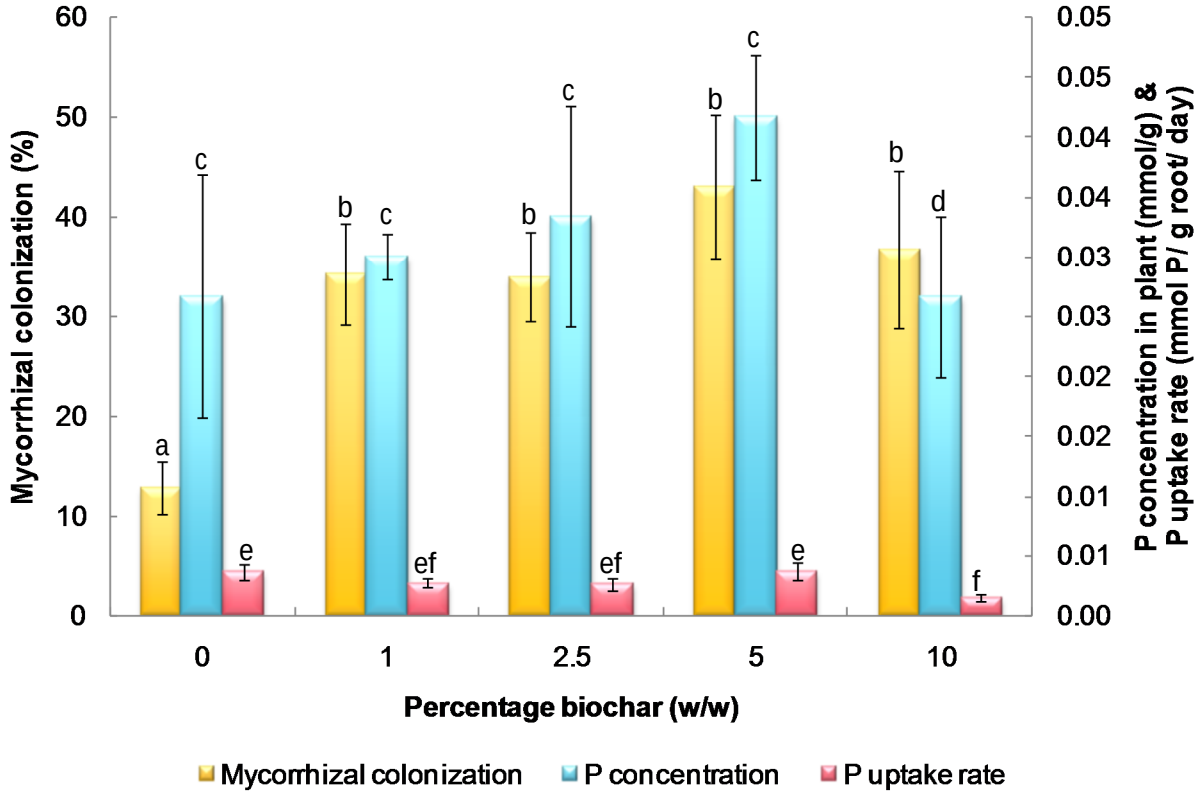

Fig. 1 Comparison of mycorrhizal colonization (%) of wheat roots, P concentration in plants (mmol/g) and P uptake rate (mmol P/g root/day) by wheat when grown in soil amended with different biochar concentrations [0%, 1%, 2.5%, 5% and 10% (w/w) biochar] under greenhouse conditions. Bars represent the mean obtained for six replicates, while the standard error values are displayed on top of each bar. Different letters indicate significant differences among biochar treatments, separated by a Fishers LSD test ($p < 0.05$) (Letters a & b indicate differences in mycorrhizal colonization; c & d indicate differences in P concentration; e & f indicate differences in P uptake rate).

Wheat growth was determined by measuring total plant dry weight and it was found that a 10% (w/w) biochar amendment resulted in an increased dry weight ($p = 0.000$; Fig. 2).

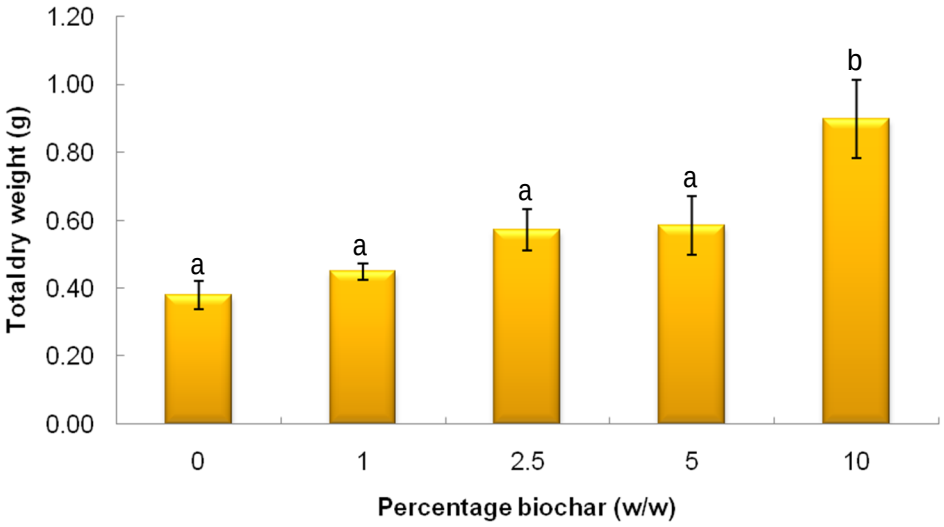

Fig. 2. Total dry weight of wheat plants grown in soil amended with different biochar concentrations (w/w) under greenhouse conditions. Each bar represents the mean obtained for six replicates and the standard error values are displayed on top of each bar. Different letters indicate significant differences among biochar treatments, separated by a Fishers LSD test ($p < 0.05$).

3.3. Nutrient effects

When P concentration and uptake rate in wheat were compared to the mycorrhizal colonization (Fig. 1), it was found that both P concentration and uptake rate increased as the colonization increased, but no significant difference was found. In addition, the P concentration and uptake rate of P was lower for plants cultivated in soil amended with 10% (w/w) biochar compared to those cultivated in soil amended with 5% (w/w) biochar ($p = 0.000$). Comparison of N uptake rate and N growth efficiency revealed that N uptake was lower in the presence of biochar amendments ($p = 0.000$), while N was utilized more efficiently during growth (Fig. 3).

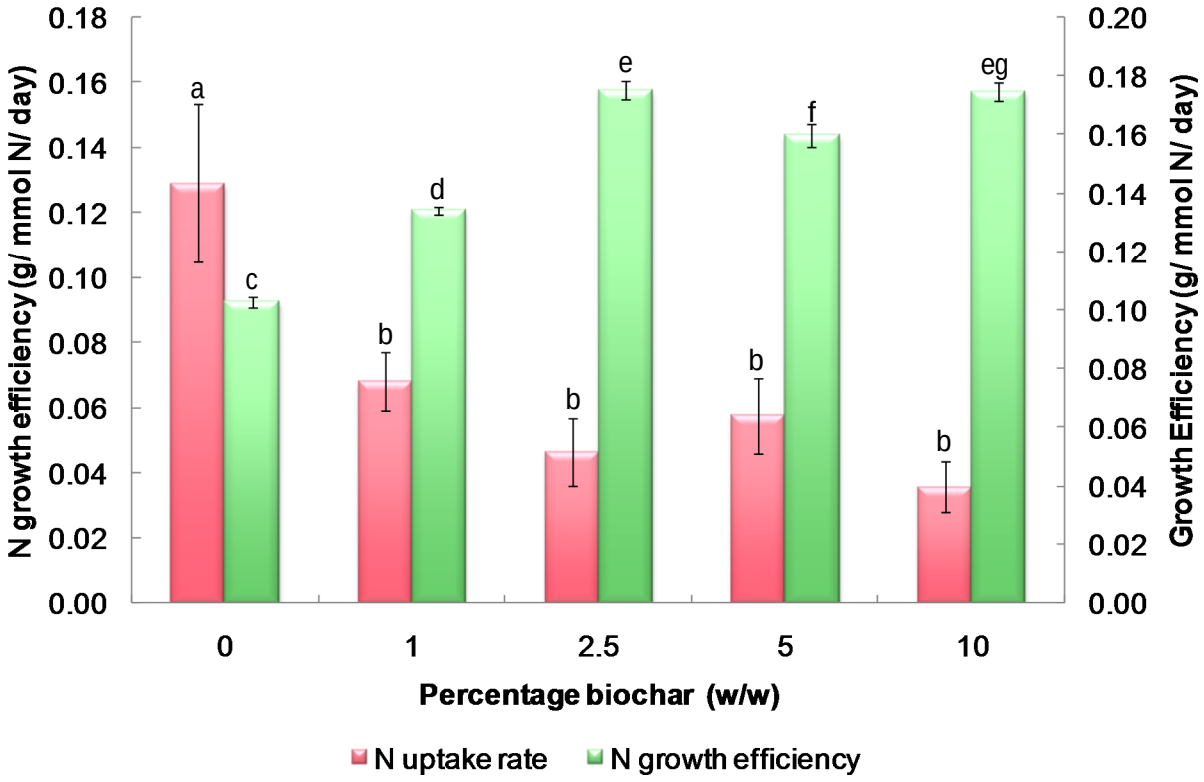

Fig. 3. Comparison of N uptake rate (mmol N/g root/day) and N growth efficiency (g/mmol N/day) for wheat plants cultivated in soil amended with different biochar concentrations (w/w) under greenhouse conditions. Bars represent the mean obtained for six replicates and the standard error values are displayed on top of each bar. Different letters indicate significant differences among biochar treatments, separated by a Fishers LSD test ($p < 0.05$) (Letters a & b indicate differences in N uptake rate; c, d, e, f & g indicate differences in N growth efficiency).

In contrast to data obtained for N, there was no difference in the uptake rate of K for the different treatments. Yet, this uptake rate seemed to be negatively correlated to the use efficiency of K during growth (Fig. 4; $r = - 0.731$, $p < 0.050$). The concentrations of K in wheat corresponded to the high uptake rate of K, while K was utilized less efficiently during growth.

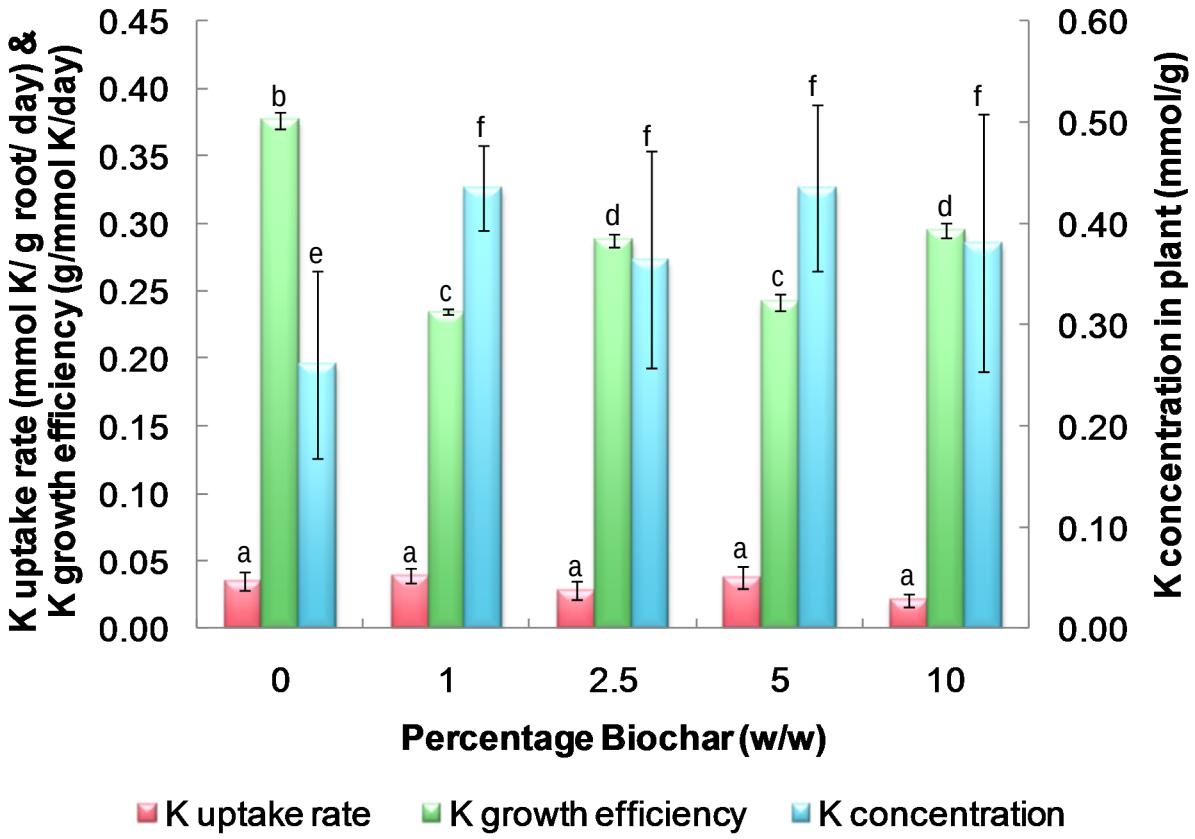

Fig. 4 Comparison of K uptake rate (mmol K/g root/day), K growth efficiency (g/mmol K/day) and K concentration in the total plant (mmol/g) for wheat plants cultivated in soil amended with different biochar concentrations (w/w) under greenhouse conditions. Each bar represents the mean obtained for six replicates, while the standard error values are displayed on top of each bar. Different letters indicate significant differences among biochar treatments, separated by a Fishers LSD test ($p < 0.05$) (Letters a indicates differences in K uptake rate; b, c & d indicate differences in K growth efficiency; e & f indicate differences in K concentration).

4. Discussion

The integration of growth and nutritional physiology in plants are important, since growth and yield are directly linked to nutrient content, nutrient uptake and nutrient use efficiency. Yet, knowledge on the effect of biochar on these processes in wheat, especially in the presence of mycorrhizal fungi, is limited. We therefore sought to evaluate the effect of different biochar amendment concentrations on wheat growth in the presence of mycorrhizal fungi, with regards to N, P and K.

4.1. Colonization by AM fungi, wheat growth and nutrient effects

After wheat plants were cultivated in soil amended with different biochar concentrations, it was found that these additions had a positive effect on mycorrhizal colonization (Fig. 1). This agreed with results of some previous studies that demonstrated that biochar application positively affected mycorrhizal colonization (Blackwell et al. 2007; Blackwell et al. 2010; Solaiman et al. 2010a). Although the mechanism by which biochar improves colonization is still unclear, it is generally accepted that biochar can alter the soil physico-chemical parameters and/or nutrient availability (Warnock et al. 2007). In this regard, it has been suggested that biochar may decrease bio-available nutrients in soil (Gaur and Adholeya 2000), which may force plants to rely more on nutrient acquisition by mycorrhizal fungi, and thereby resulting in greater root colonization.

It was found that the total dry weight of wheat was increased at a 10% (w/w) biochar amendment (Fig. 2). This indicates that biochar might have had a positive effect on wheat growth, possibly by altering the physiology of the plants. Addition of the alkaline biochar to the acidic soil resulted in an increased pH from 5.45 to 7.58 in water. The increased pH most likely resulted in more bio-available P (Bolland 2001). It is apparent, however, that at 10% (w/w) biochar amendment, there was a decrease in the uptake rate of P and mycorrhizal colonization. This can be ascribed to the pH rising above 7 in the 10% biochar amendment (pH 7.58), since P is less available in basic soils (Havlin et al. 2005).

When N uptake rate and use efficiency were compared for the biochar concentrations it was found that at higher amendment concentrations the N uptake rate decreased (Fig. 3). This decrease may be ascribed to immobilization of N by the biochar used in our study (Table 1), since such reactions are typical for high C: N ratio biochars (Warnock et al. 2007). As plant available N increases, the N use efficiency decreases and N uptake rate increases (IFA 2007). In addition, we found that there was an inverse relationship between the N uptake rate and N use efficiency (Fig. 3). This ability to utilize N more efficiently is desirable; since it means that less fertilizer would be needed, thus reducing farming cost.

It was found that there was no difference in the uptake rate of K when wheat was cultivated in soil or in soil amended with biochar (Fig. 4). In addition, plants cultivated in soil amended with biochar utilized K less efficiently during growth. The high potassium concentration in

plants cultivated in soil amended with biochar, was most likely due to the high concentration of plant available K present in the biochar (Table 1). As mentioned previously, K plays various roles in plants, one of which is the maintenance of turgor (Mengel and Arneke 1982). Some studies have demonstrated that K facilitates leaf expansion, and higher levels of K in plants resulted in increased photosynthesis (Pervez et al. 2006). This increased leaf expansion was most likely the main driver for increased growth.

5. Conclusion

In this study we found that the biochar amendments seemed to improve wheat growth and increase mycorrhizal colonization of roots. However, it seems that the improved growth was not solely due to increased mycorrhizal colonization and uptake of P, but rather due to elevated concentrations of K in the plants. It is tempting to speculate that K was obtained from the biochar and that high levels of this nutrient in the plants may have facilitated shoot growth. It thus seems feasible to incorporate this biochar into soil management practices in order to improve wheat growth and potentially yield when cultivated in low nutrient soils. It is likely that the effect of biochar may differ for different wheat cultivars and therefore future studies must elucidate the effect of biochar on different wheat cultivars. In addition, future studies must focus on the amount of fertilizer and biochar needed in order to produce the maximum wheat yield, without negatively affecting the plant's physiology.

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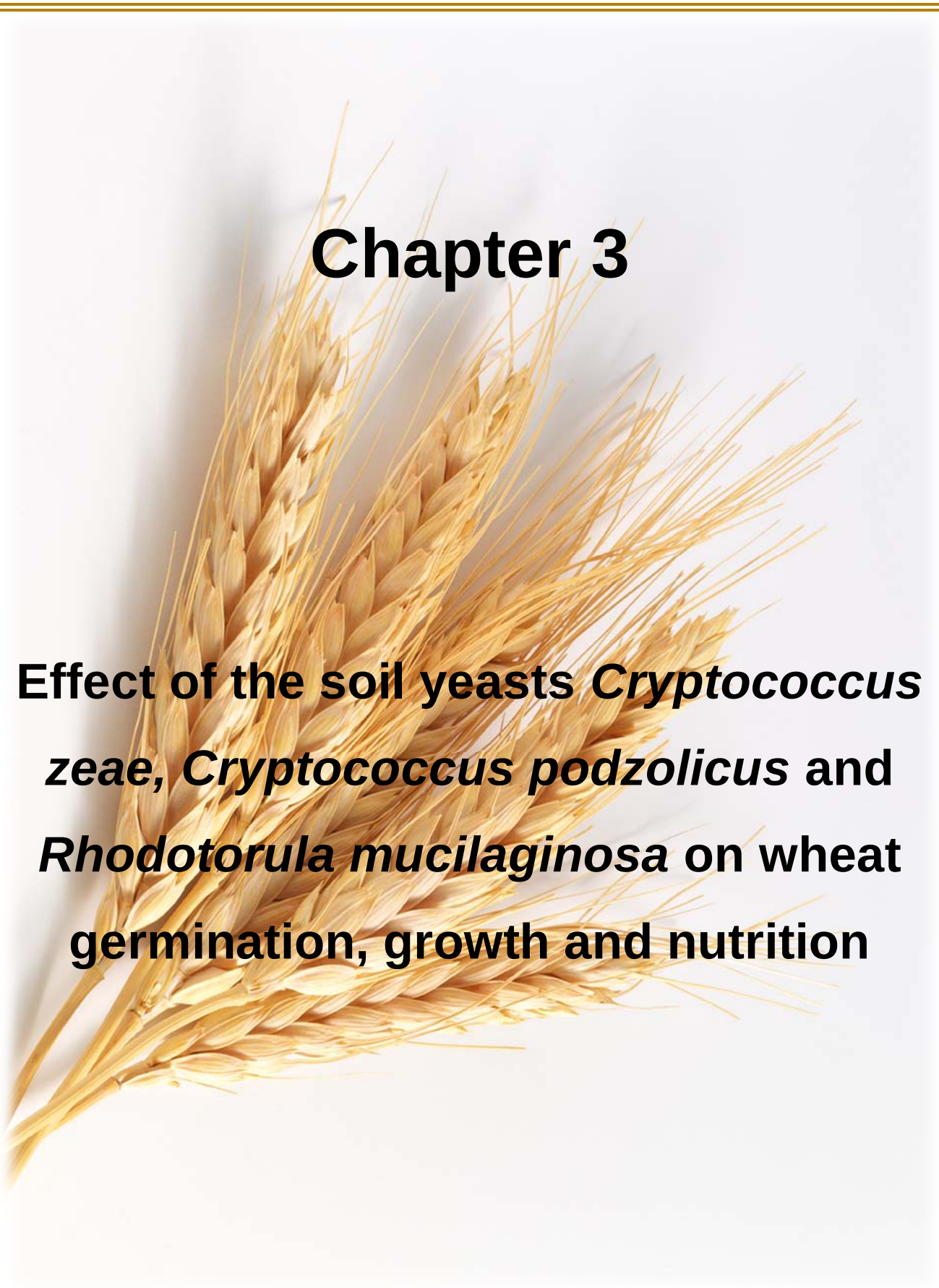

Chapter 3

Effect of the soil yeasts *Cryptococcus zeae*, *Cryptococcus podzolicus* and *Rhodotorula mucilaginosa* on wheat germination, growth and nutrition

1. Introduction

Triticum aestivum L. (wheat) is one of the oldest and the second most produced cereal crop in the world (FAO 2010). At present, wheat production is too low to meet food demands of the world's population in the future (FAO 2010; UN 2010). To supply enough food to 9 billion people by 2050, food production must increase by 70% (UN, 2010). With current agricultural practices, however, this increase cannot be met. Therefore, agricultural land must expand or crop intensity must be increased. Indications exist that expansion of agricultural land will have little contribution to yield increases (Foley et al. 2011) and thus cropping intensity, such as fertilizer application, must increase. Yet, fertilizers have a negative impact on the environment, such as the eutrophication of water bodies and over-all pollution (Matson et al. 1997; Vorosmarty et al. 2000). These factors together with the increasing cost of fertilizers resulted in the evaluation of alternative methods to improve crop yield (Adesemoye et al. 2009). These methods include biochar amendments to soil (Warnock et al. 2007) and the use of plant growth promoting microorganisms, e.g. mycorrhizal fungi (Gollner et al. 2005) and soil yeasts. In Chapter 2, it was demonstrated that biochar application to sandy soil positively affected wheat growth and mycorrhizal colonization.

Mycorrhizal fungi are not the only soil microorganisms known to form associations with plants and promote plant growth. Numerous studies have demonstrated the ability of various soil yeast species to promote growth of crop plants (Chapter 2). In one of these studies, it was demonstrated that the germination of cabbage seeds were stimulated by *Torulopsis,* now known as *Candida* (Bab'eva and Belyanin 1966). Another study found that a species of *Rhodotorula* improved tomato (*Solanum lycopersicum* L.) growth and yield (Abd El-Hafez and Shehata 2001). In addition, two studies conducted on maize (*Zea mays* L.) demonstrated that soil yeasts, i.e. *Candida glabrata, Candida maltosa, Candida slooffii, Rhodotorula rubra and Trichosporon cutaneum*, (El-Mehalawy et al. 2004), as well as the maize root endophyte *Williopsis saturnus* (Nassar et al. 2005), improved growth of this crop. Despite the importance of wheat, only one study has been conducted to investigate the ability of yeasts to improve the growth of this crop (Perondi et al. 1996). It was found that *Sporobolomyces roseus* improved wheat growth by 16-30%.

All of the above-mentioned studies lack experimentation to determine the effect of yeasts on the nutritional physiology of plants. As mentioned in Chapter 2, it is important to incorporate nutritional physiology in plant growth promoting studies. The importance of nutritional physiology was demonstrated in a study by Cloete et al. (2010). They showed that an isolate of the soil yeast *Cryptococcus laurentii*, originating from the rhizosphere of a wild stand of *Agathosma betulina* (Berg.) Pillans (buchu) in pristine Fynbos, was not only able to improve growth of this plant, but it also altered its nutritional physiology.

With the above as background, the first aim of this study was to isolate a potential plant growth promoting yeast from the rhizosphere of another monocot belonging to the same family as wheat, growing in a pristine grassland. Secondly, the ability of one of the isolated yeasts, i.e. a strain of *Cryptococcus zeae,* to improve wheat germination was tested and compared to germination results of two other soil yeasts, *Cryptococcus podzolicus* and *Rhodotorula mucilaginosa*. Lastly, the effect of these three yeasts on the growth and nutritional physiology of wheat was determined and compared.

2. Materials and Methods

2.1. Isolation of yeasts

Four grass plants belonging to the family Poaceae, i.e. *Themeda triandra* Forssk. (red grass) were collected from a pristine grassland next to the N4 highway near Malelane, Mpumulanga, South Africa, by uprooting each plant and transporting it to the laboratory, while keeping it at *ca*. 15°C. The roots were subsequently placed in tubes containing sterile physiological saline solution (PSS) and vortexed for 10 min to dislodge any yeast cells. The resulting suspensions were used to create dilution plates using thymine-mineral-vitamin (TMV) agar (Cornelissen et al. 2003). After five days of incubation at 26°C, 31 yeast colonies were randomly selected from the TMV plates using a modification of the Harrison's disc method (Harrigan and McCance 1967). The yeast isolates were purified and tested for the inability to ferment glucose, the ability to produce starch and assimilate inositol, according to the methods described by Kurtzman & Fell (2000). Isolates that showed these typical cryptococcal characteristics were then identified using molecular methods. This was accomplished by extracting the genomic DNA and amplifying the D1/D2 region of the ribosomal RNA (rRNA) gene, according to the methods described by Vreulink et al. (2010).

Sequences were obtained using an ABI Prism (model 3100) genetic sequencer (Applied Biosystems, Johannesburg, South Africa). The sequences were compared to known sequences on GenBank using BLAST (http://www.ncbi.nlm.nih.gov/blast).

2.2. Effect on germination

Of the eight isolates that were obtained from red grass, the majority was found to be *C. zeae*. Therefore, isolate *C. zeae* CAB 1119 together with *C. podzolicus* CAB 978, isolated from Fynbos soil, and *R. mucilaginosa* CAB 826, isolated from pristine Fynbos soil at Tygerberg Nature Reserve, Cape Town, South Africa (Vreulink et al., 2010), were tested for their ability to improve *T. aestivum* L. (wheat) germination. This was accomplished by cultivating each of the three yeasts in 100 ml conical flasks containing 25 ml yeast malt extract (YM) broth on a rotary shaker (100 rpm) at 26°C for two days. The cells were harvested by centrifugation (38000 *xg*; 5 min) and washed twice with sterile PSS. A haemacytometer (Superior, Germany) was then used to determine the concentration of cells in the final suspensions, of which the volume was adjusted with sterile PSS to give a final concentration of log 9 yeast cells/ml.

A dilution series was subsequently prepared from the three yeast suspensions, resulting in concentrations ranging from log 6 to log 9 yeast cells/ml. Four controls were included in this experiment, namely seeds coated with autoclaved suspensions of the three yeasts and seeds that received no yeast inoculum. Wheat (cultivar SST 047) seeds were surface sterilized by submerging them in 70% ethanol for 1 min, followed by 40 s in 1% (v/v) sodium hypochlorite solution, and then rinsed in sterile distilled water. Yeast coated seeds were prepared by dipping surface sterilized wheat seeds (n = 175) into the seven (four control and three yeast inoculums) different inoculums (25 seeds per inoculum, representing five repetitions of five seeds each). The seeds were then removed from the inoculums and allowed to dry in sterile Petri-dishes for 20 min at 22°C. After drying, the seeds were planted in Petri-dishes (five seeds per Petri-dish) containing quarter-strength Murashige and Skoog (MS) agar (Slater et al. 2008) and allowed to germinate at room temperature in the dark for two days. Each day, the seedlings were inspected for contamination by filamentous fungi and since no statistical difference could be obtained when the percentage germination was analyzed, the number of roots produced was used instead.

2.3. Pot preparation and wheat growth

Wheat growth in sand was studied under hygienic[1] conditions using pot cultures arranged in a well-ventilated greenhouse with a 12 h photoperiod of 1000–1100 µmol m^2/s photosynthetic photon flux density. The average day/night temperatures and relative humidity were 23/15 °C and 50/80%, respectively. Silica sand (grain diameter ranging from 250 to 355 µm) was obtained from Consol Glass, Western Cape, South Africa. The sand, as well as plastic pots (13 cm in diameter), tubes, rubber stoppers and drainage chips, were acid washed with 0.1 M HCl, rinsed three times with distilled water and dried. The bottom of the pots (n = 144) were then covered with drainage chips and they were filled with 800 g of the silica sand. Pots were individually sealed in plastic bags and gamma radiated [minimum absorbed dose, 25 kGy (1kGy = 0.1Mrad) per kilogram].

From the germination data (Figs. 1 and 2) it was determined that the best inoculation concentration for *C. zeae* CAB 1119 and *C. podzolicus* CAB 978 were log 9 cells/ml and log 8 cells/ml for *R. mucilaginosa* CAB 826. To obtain these desired concentrations, the three yeasts were cultivated, harvested and the volume of the suspensions was adjusted as described in section 2.2. The controls that were included in this experiment consisted of autoclaved cell suspensions of the three yeasts. Wheat (cultivar SST 047) seeds (n = 1080, 180 seeds per suspension) were surface sterilized as described in section 2.2, and 180 of these seeds was dipped in either autoclaved or viable suspensions of *C. zeae, C. podzolicus* and *R. mucilaginosa*. The seeds were allowed to dry in sterile Petri dishes for 20 min at 22°C where after they were planted on quarter-strength MS agar plates and allowed to germinate for two days at room temperature in the dark.

Two-day-old wheat seedlings of similar size were planted in the gamma-radiated pots (24 pots per treatment, where those intended for 1 month or 2 months of cultivation contained four seedlings or two seedlings per pot, respectively. Plants were watered up to field capacity with sterile quarter-strength Long Ashton nutrient solution (Cloete et al. 2009).

To prevent the contamination of pots by airborne microorganisms, hygienic[1] conditions were created. Plants was then cultivated for one and two months, whilst receiving nutrients and sterile distilled water weekly.

2.4. Harvesting and nutrient analyses

2.4.1. Harvesting

A half of the plants (n = 72) were harvested after one month of growth and the other half after two months of growth. The plastic beads were removed from the top of the pots using a sterile spatula. Plants were then gently uprooted from the pots and their roots were washed in test tubes containing sterile PSS. A sub-sample of the bulk soil was placed in a sterile Petri-dish and stored at 15°C, for three hours for later analysis of the yeast numbers present in bulk soil. Plants were subsequently dried at 80°C for a week and the dry weights were recorded.

2.4.2. Yeast enumeration and identification

In order to enumerate the yeasts present in the rhizosphere, a dilution series using TMV plates were prepared of the PSS root washings. For the bulk soil, 1 g of the soil sub-sample was added to PSS test tubes and a dilution series using TMV plates were prepared. After one week of incubation at 26°C, the colonies on the plates were counted and the number of yeasts present in the rhizosphere and bulk soil was determined. Since it was apparent that more than one colony type was present on the plates, yeast-like colonies were randomly selected from plates, prepared from both the one month and two-month-old plants, by using the modified Harrison's disc method (Harrigan and McCance 1967).

After the colonies were purified, they were grouped into two groups, namely red-pigmented colonies and non-pigmented colonies, whilst ensuring that those arising from the one month and two-month-old plants were kept separately. For each of the four groups 30 colonies were randomly picked as representatives and these 120 isolates were classified by subjecting them to restriction fragment length polymorphism (RFLP) analysis. This was accomplished

[1] With hygienic conditions it is implied the conditions created to minimize the exposure of pots to airborne microorganisms, by covering sterilized pots with sterile plastic beads and using a plastic tube covered with a rubber stopper to supply the plants with sterile nutrients and distilled water.

by amplifying the internal transcribed spacer (ITS) region of the ribosomal gene cluster, using colony PCR and the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). The cultures were cultivated overnight in YM broth on a tissue culture roll drum (10 rpm) at 26°C. The 50 µl PCR mixture contained 25 µl of master mix (2x) (Fermentas International Inc., Burlington, Ontario, Canada), 2 µl of each primer (10 µmol/L) (Inqaba biotech Industries, Pretoria, South Africa) and 2 µl of the culture. Amplification was performed in a Perkin-Elmer 2400 thermal cycler at an initial denaturation of 95°C for 3 min, 36 cycles consisting of denaturation at 95°C for 45 s, annealing at 52°C for 45 s and extension at 72°C for 1 min, as well as a final extension at 72°C for 7 min.

To obtain RFLP profiles of the representing isolates, the amplified ITS region was digested with the restriction endonucleases *Hin*61, *Hin*f1 and *Mbo*11 according to the manufacturer's specifications (Fermentas). The resulting fragments were separated on a 2% (w/v) agarose gel containing ethidium bromide and photographed (Gene Flash, Syngene Bio Imaging, Cambridge, UK). Banding patterns, as well as sizes of the fragments were compared to a 100-bp DNA Ladder (GeneRuler, Fermentas).

Yeast isolates, representative of each RFLP profile, were identified by analysing the D1/ D2 region of the rRNA gene. The isolates were cultivated overnight in YM broth as described earlier and the D1/D2 region was amplified using colony PCR and the forward primer F63 (5'-GCATATACAATAAGCGGAGGAAAAG-3'), and the reverse primer LR3 (5'-GGTCCGTGTTTCAAGACGG-3') (Fell et al. 2000). The 50 µl PCR reaction was set up as described above and amplification was performed in a Perkin-Elmer 2400 thermal cycler. The amplification parameters were an initial denaturation at 95°C for 3 min, 35 cycles consisting of denaturation at 95°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 1 min. A final extension at 95°C for 4 min was also included. The nucleotide sequences for the D1/D2 region were obtained using an ABI Prism (model 3100) genetic sequencer (Applied Biosystems). The sequences were aligned with those of *C. zeae* CAB 1119, *C. podzolicus* CAB978 and *R. mucilaginosa* CAB 826 with DNAMAN for Windows, version 4.13 (Lynnon Corp., Quebec, Canada). To identify the unknown yeast isolates, sequences were compared to known sequences on GenBank using the program BLAST (http://www.ncbi.nlm.nih.gov/blast). The results were used to determine the relative quantities

of yeast species among the non-pigmented and red-pigmented yeast for both the one-month and two-month-old plants.

2.4.3. Nutrient analyses

After the dry weight of the wheat plants were recorded, the phosphorous (P), potassium (K), iron (Fe) and zinc (Zn) content of the plants were measured according to the methods described by Vreulink et al. (2007). The root relative growth rate (RGR), shoot RGR, uptake rates of P, K, Zn and Fe, as well as nutrient use efficiency of P, K, Zn and Fe, were calculated for three growth periods. These three periods were the first 30 days of growth, the second 30 days of growth and the total growth period (60 days). All of the above were calculated according to the formulae proposed by Mortimer et al. (2005).

2.5. Statistical analyses

Significant differences in dry weights, relative growth rates, nutrient concentrations, uptake rates and growth efficiency were analysed by using ANOVA and differences between treatment means were separated using a *post hoc* Fishers least significant difference (LSD) test, using the program Statistica version 10 (Statsoft, Tulsa, OK, USA). Additionally, correlation matrixes comparing the nutrient concentrations, uptake rates and growth efficiency for P, K, Zn and Fe were constructed.

3. Results

3.1. Isolation of yeasts from wild grass

The fermentation, starch production and inositol assimilation test indicated that eight of the 31 isolates obtained from the dilution series prepared from the grass samples belonged to the genus *Cryptococcus*. The blast search of the obtained sequences revealed that four of these isolates were *Cryptococcus zeae* (isolates 6-9D), three were *Cryptococcus. cf. luteolus* (isolates 2-4D) and one was *Cryptococcus rajasthanensis* (isolate 5D). It was decided to use *C. zeae* CAB 1119 (isolate 6D) in further experimentation.

3.2. Effect on germination

The three yeasts *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826 were tested for their ability to inhibit growth of filamentous fungi during seed germination. It was found that concentrations of log 9 cells/ml for *C. zeae* and log 7-9 cells/ml for both *C. podzolicus* and *R. mucilaginosa,* fully inhibited the growth of contaminating filamentous fungi (Fig. 1).

The average number of roots per seedling, on MS agar after two days at 22°C, was measured as an indication of germination (Fig. 2). It is apparent that a concentration of log 9 cells/ml for *C. zeae* and *C. podzolicus* had a superior effect on the germination of the seedlings. *Rhodotorula mucilaginosa,* however, had no effect on the number of roots produced per seedling. Seedlings had more roots when they were coated with autoclaved yeast suspensions compared to the control containing no yeast (Fig. 2).

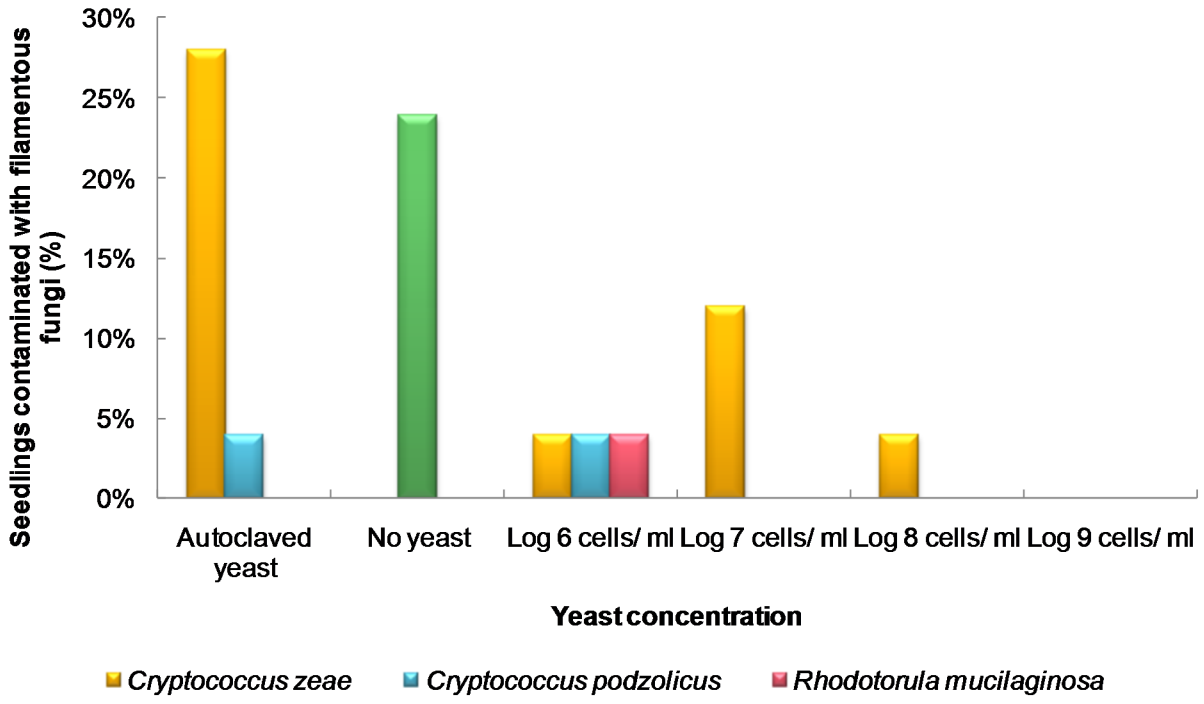

Fig. 1. A comparison of the percentage contamination of seedlings with filamentous fungi. Seeds were coated either with no yeast, autoclaved (control) yeast suspensions or viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826 at concentrations ranging from log 6 cells/ml to log 9 cells/ml. Bars represent the mean values obtained for five replicates.

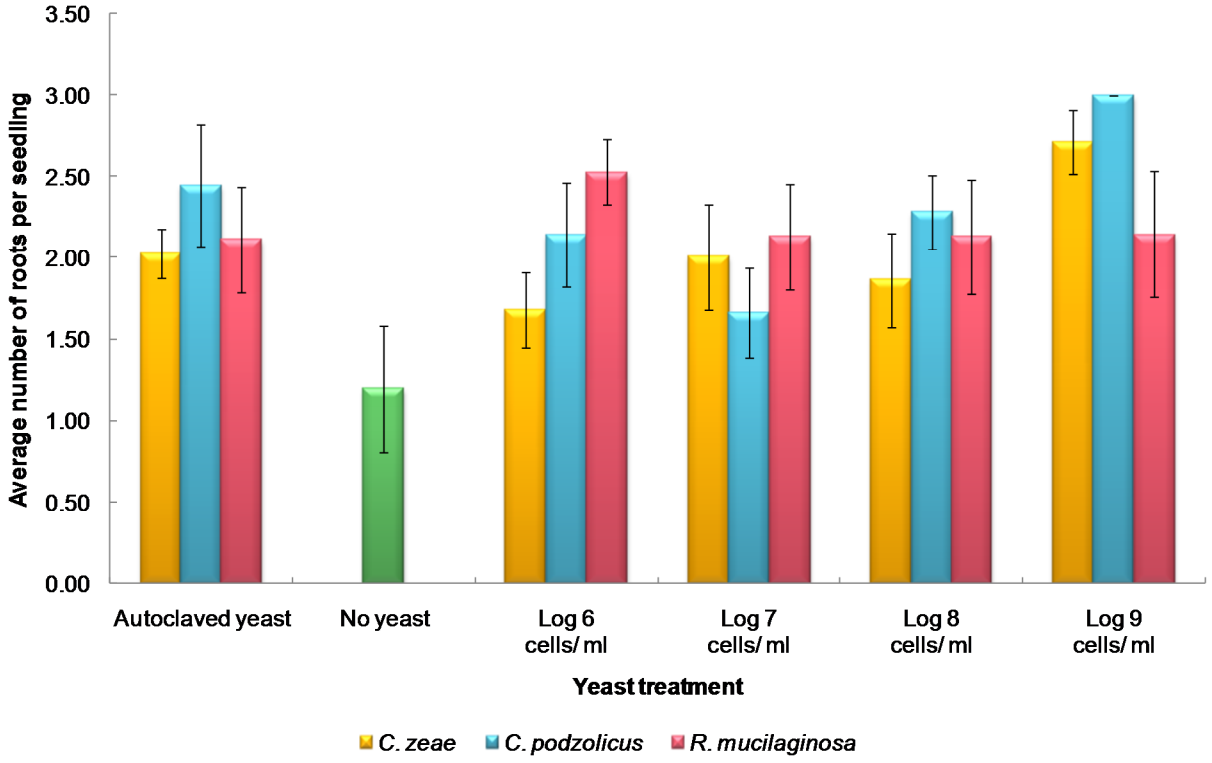

Fig. 2. Comparison of the average number of roots produced per seedling during germination in the presence of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826. Four controls were included, i.e. autoclaved cells of the three yeasts and one treatment containing no yeast. Bars represent the mean values obtained for five replicates and standard error values are displayed on top of the bars.

3.3. Wheat growth

Analyses of the dry weight of wheat plants showed that one and two-month-old plants that were coated with viable cells of *C. zeae* displayed superior growth compared to those coated with autoclaved cells of *C. zeae* (Fig. 3). Plants coated with viable *C. podzolicus* cells showed greater growth only during the first month, compared to plants coated with autoclaved cells of this yeast. Growth was not increased by coating seeds with viable cells of *R. mucilaginosa* (Fig. 3).

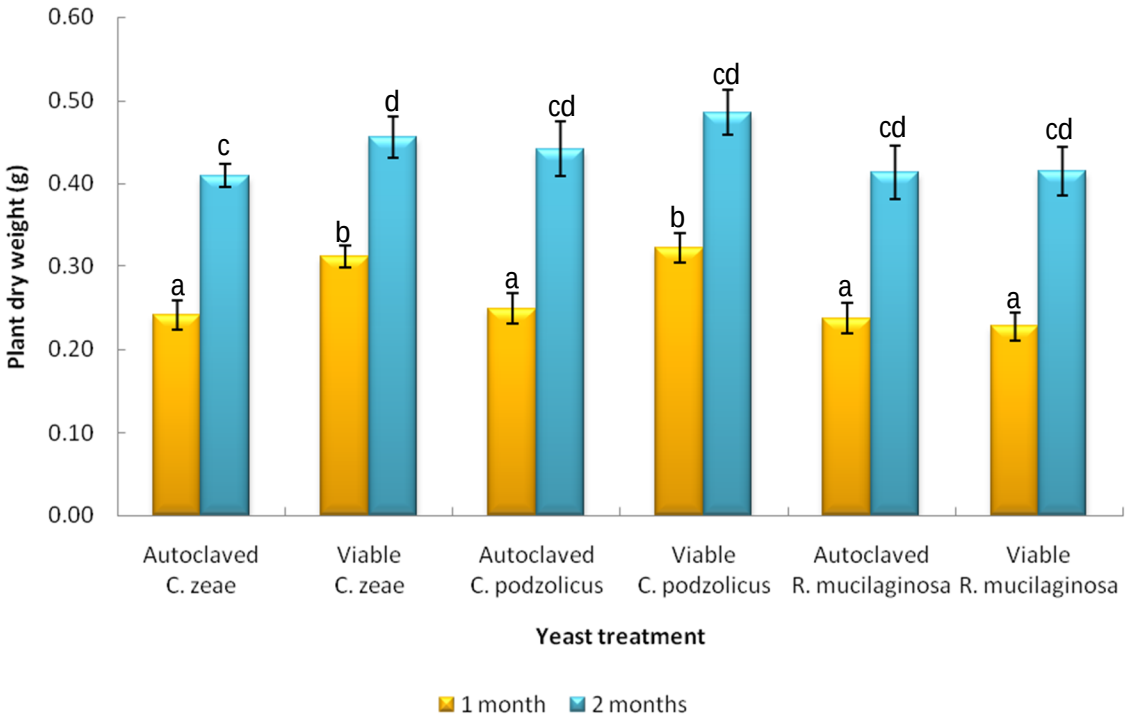

Fig. 3. Total dry weight (g) of wheat plants coated with autoclaved or viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826. Plants were cultivated for one or two months under hygienic conditions in a greenhouse. Each bar represents the mean obtained for 12 replicates, while the standard error values are displayed on top of each bar. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$) (Letters a & b indicate differences in plant dry weight for the first month of growth; c & d indicate differences in plant dry weight for the second month of growth).

When the relative growth rates (RGR) of the roots were calculated, it was found that the root RGR decreased during the second month of growth (Fig. 4B). Yet, during the total growth period, the root RGR increased (Fig. 4C). It is evident that plants coated with viable *C. zeae* cells had a greater root RGR during both the first month and the total growth period (Fig. 4A & C).

Similar to the root RGR results, plants coated with viable *C. zeae* cells had a greater shoot RGR than other plants during the initial 30 days and the total growth period (Fig. 5A & C). During the second month (Fig. 5B), the shoot RGRs decreased, while it increased during the total growth period.

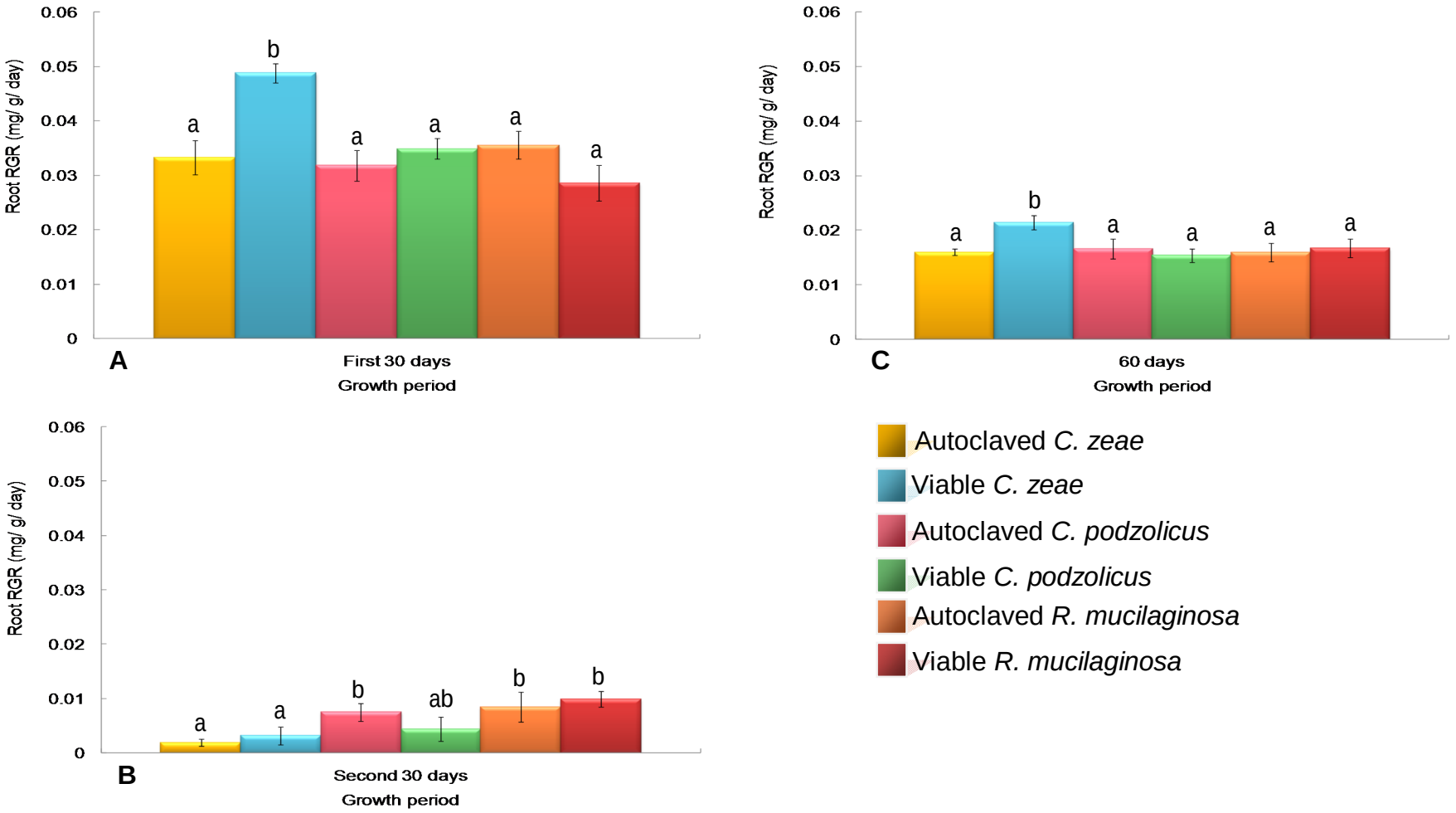

Fig. 4. Relative growth rate (RGR) of roots for the three different growth periods i.e. the first 30 days (A), the second month (second 30 days, B) and the total growth period (60 days, C). Seeds were coated with autoclaved or viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826. Plants were cultivated under hygienic conditions in a greenhouse. Each bar represents the mean obtained for 12 replicates and the standard error values are depicted on top of the bars. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$).

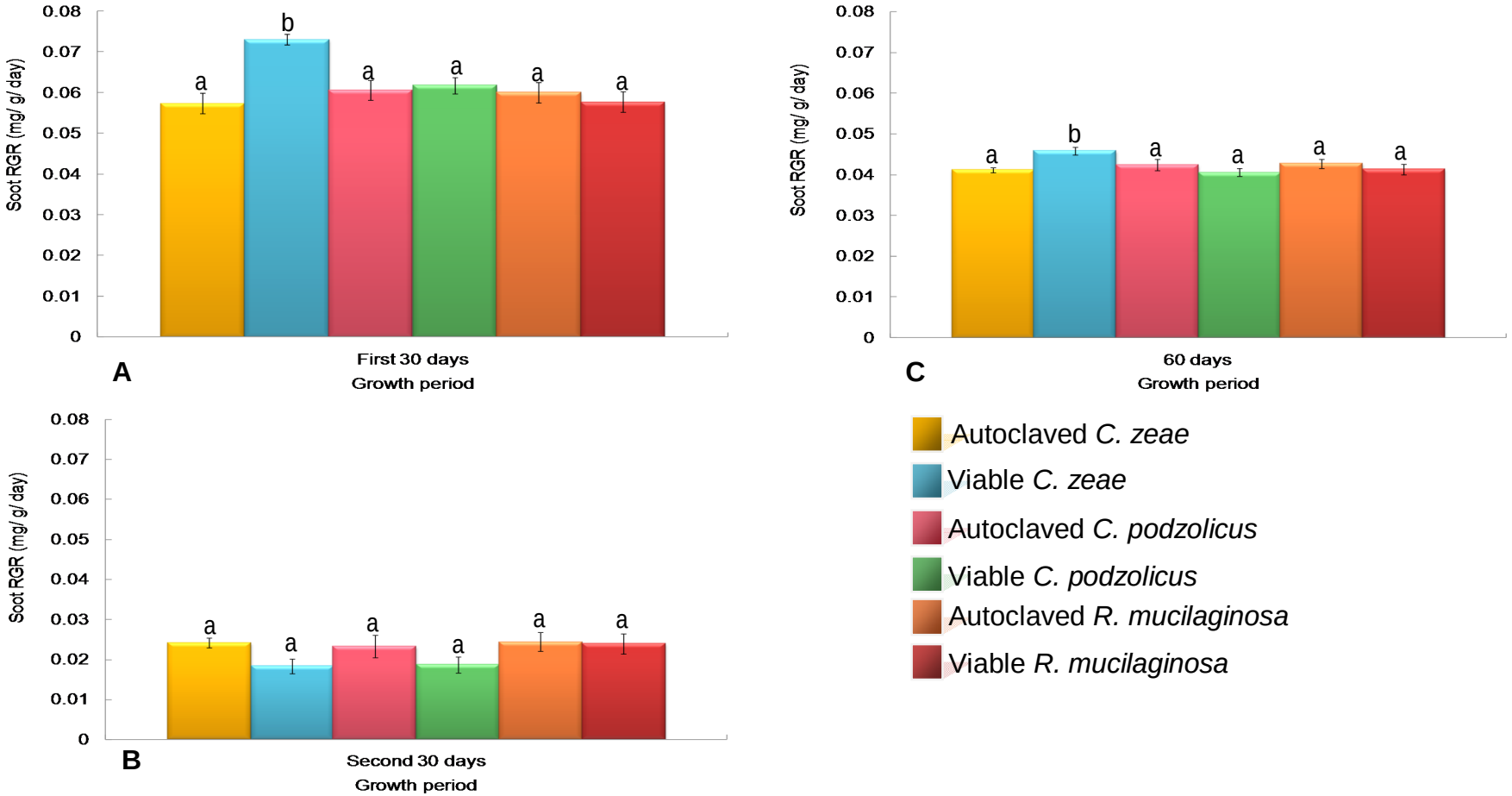

Fig. 5. Shoot relative growth rate (RGR) of wheat plants for the three different growth periods i.e. the first 30 days (A), the second 30 days (B) and the total growth period (60 days, C). Plants were cultivated under hygienic conditions in a greenhouse. Seeds were coated with autoclaved or viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826. Each bar indicates the mean obtained for 12 replicates and the standard error values are displayed on top of the bars. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$).

3.4. Yeast enumeration and identification

When culturable microorganisms, forming yeast-like colonies, in the rhizosphere were enumerated, it was found that plants of which the seeds were coated with autoclaved and viable cells of *C. zeae* and viable cells of *C. podzolicus* had more non-pigmented microorganisms in their rhizosphere than red-pigmented microorganisms (Fig 6). In contrast, plants of which the seeds were coated with viable cells of *R. mucilaginosa* had more red-pigmented microorganisms in their rhizosphere than non-pigmented microorganisms (Fig. 6). There were more non-pigmented microorganisms present in the rhizosphere of two-month-old plants compared to one-month-old plants, coated with autoclaved cells of *C. podzolicus*. Red-pigmented microorganisms present in the rhizosphere of all plants decreased from one month to two months.

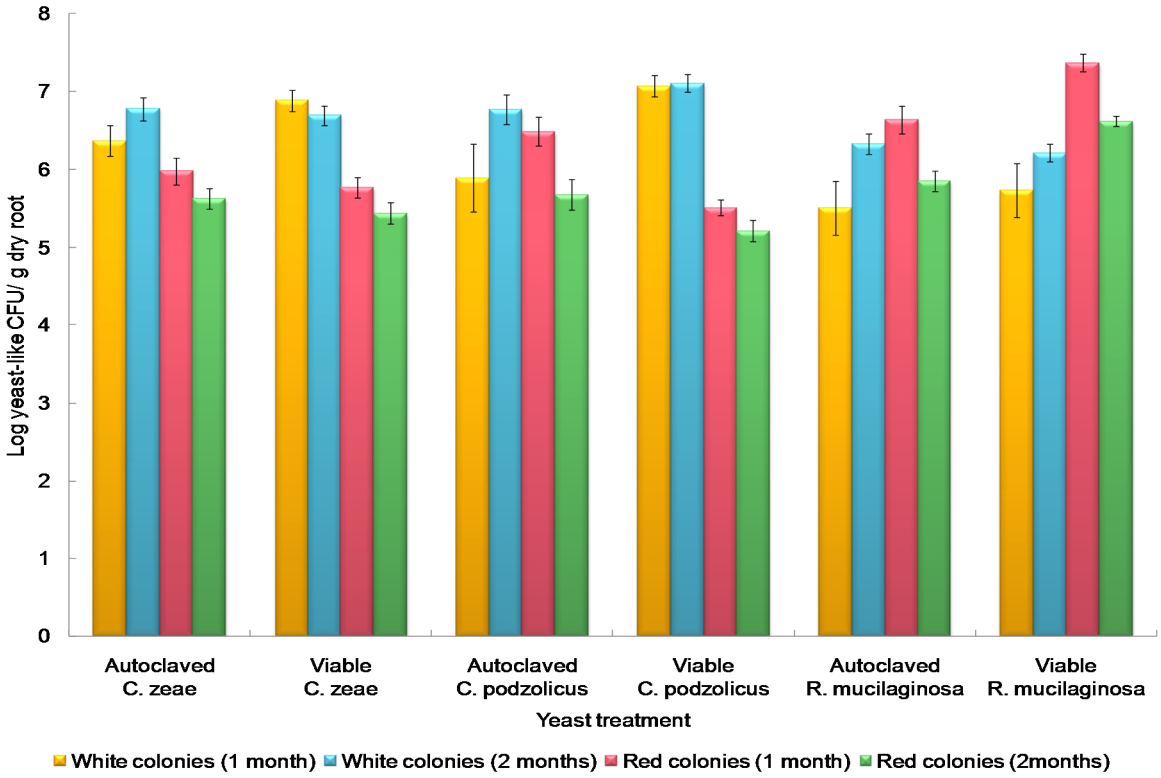

Fig. 6. Microorganisms forming non-pigmented and red-pigmented yeast-like colonies [log colony forming units (CFU)/ g root] present in the rhizosphere of one month and two-month-old wheat plants coated with autoclaved or viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826. Plants were cultivated under hygienic conditions in a greenhouse. Each bar represents the mean obtained for 12 replicates, while the standard error values are displayed on top of the bars.

The number of microorganisms, forming yeast-like colonies was lower in the bulk soil (Fig. 7) than in the rhizosphere (Fig. 6). In addition, the dominance of non-pigmented microorganisms in the rhizosphere of plants inoculated with viable *C. zeae and C. podzolicus,* and the dominance of red-pigmented microorganisms in the rhizosphere of plants inoculated with *R. mucilaginosa,* was not as obvious in the bulk soil (Fig. 7). The number of culturable non-pigmented microorganisms was only dominant for one-month-old plants inoculated with viable *C. zeae* cells and two-month-old plants that were treated with autoclaved and viable cells of *R. mucilaginosa* (Fig. 7). Pigmented microorganisms were only dominant in the bulk soil of one-month-old plants treated with autoclaved cells of *C. zeae* (Fig. 7).The numbers of non-pigmented microorganisms in the bulk soil of plants inoculated with viable cells of *C. zeae* and *C. podzolicus* decreased from one month to two months (Fig. 7). In addition, non-pigmented microbial numbers increased in the bulk soil of plants coated with autoclaved *R. mucilaginosa* cells.

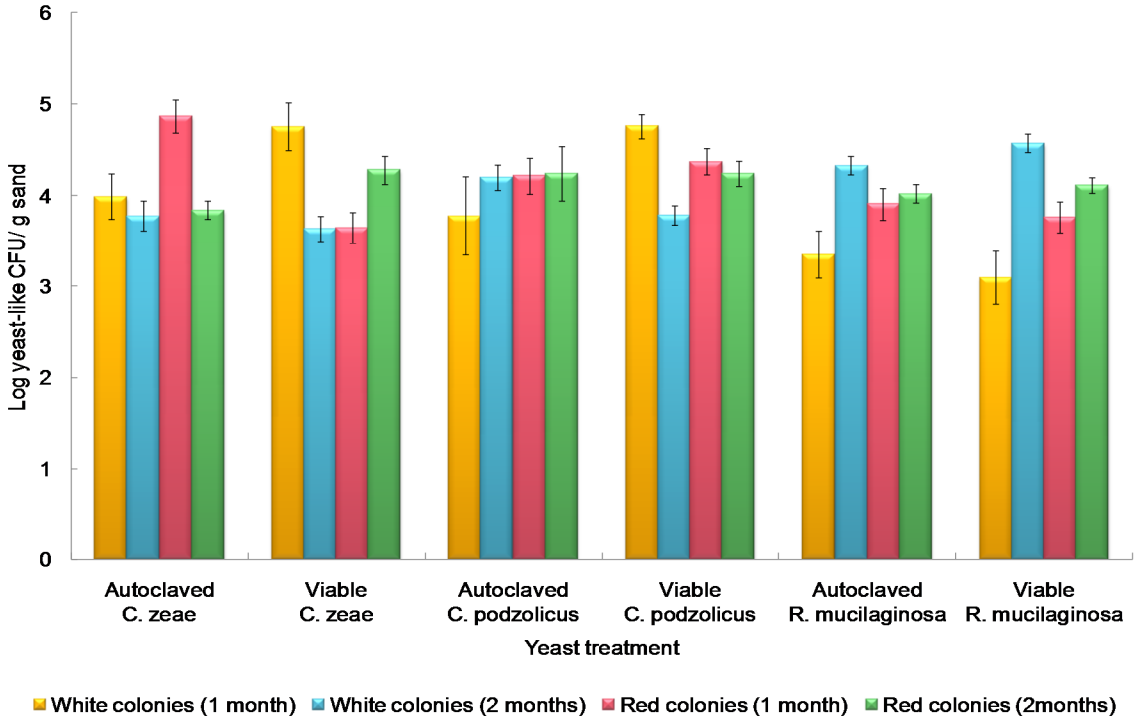

Fig. 7. Microorganisms forming non-pigmented and red-pigmented yeast-like colonies in the bulk soil [log colony forming units (CFU)/g sand] of wheat pot cultures cultivated for one and two months under hygienic conditions, in a greenhouse. Seeds were coated with autoclaved or viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826. Each bar represents the mean obtained from 12 replicates and the standard error values are depicted on top of the bars.

It seems that other microorganisms, forming yeast-like colonies, might have been present in addition to the yeast inoculums in the pot cultures, seeing that hygienic conditions were provided during cultivation. RFLP analyses and molecular identification were therefore used to obtain an indication of the yeast diversity in the rhizosphere (Table 1) and bulk soil (data not shown). We found that the percentage of *C. zeae* cells decreased from one month to two months for all treatments, except for plants inoculated with viable *R. mucilaginosa* cells (Table 1). The abundance of *R. mucilaginosa* also decreased from one month to two months for all yeast treatments, except for plants inoculated with viable *C. podzolicus* cells. In the rhizosphere of plants coated with viable yeasts it was found that even though there were a number of different yeast species present, the dominant yeast species was that used to coat seeds before germination (Table 1). During the second month of cultivation an increase was noticed in the numbers of non-pigmented bacteria present in the rhizosphere, forming colonies similar to that of cryptococci.

Table 1. Percentage of different yeasts relative to the total number of yeast-like colonies randomly selected from the enumeration plates used to calculate yeast numbers in the rhizosphere of wheat. Plants were cultivated for one and two months under hygienic conditions in a greenhouse. Seeds were coated with autoclaved or viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826.

Yeast treatment	**1 Month**						**2 Months***				
	○CZ	○CF	○CP	○PG	○CA	●RM	○CZ	○CF	○CP	○PG	●RM
Autoclaved *C. zeae*	48%	8%	NP	NP	16%	28%	NP	NP	NP	NP	7%
Viable *C. zeae*	84%	NP	NP	NP	NP	16%	58%	NP	NP	NP	4%
Autoclaved *C. podzolicus*	16%	NP	NP	5%	NP	79%	10%	10%	NP	22%	37%
Viable *C. podzolicus*	22%	NP	65%	11%	NP	2%	NP	NP	71%	7%	8%
Autoclaved *R. mucilaginosa*	18%	NP	NP	18%	NP	64%	26%	NP	NP	NP	30%
Viable *R. mucilaginosa*	3%	NP	NP	NP	NP	97%	16%	NP	NP	NP	76%

*Some non-pigmented colonies were identified as bacteria (93% for Autoclaved *C. zeae*; 38% for Viable *C. zeae*, 21% for Autoclaved *C. podzolicus*, 14% for Viable *C. podzolicus*, 44% for Autoclaved *R. mucilaginosa* and 8% for Viable *R. mucilaginosa*).

○ Non-pigmented yeast, ● Red pigmented yeast

CZ – *C. zeae*, CF – *C. flavescens*, CP – *C. podzolicus*, PG – *P. guillermondii*, CA – *C. albidus*, RM – *R. mucilaginosa* and NP – Not present.

3.5. Nutrient analyses

When one-month-old wheat plants were subjected to three different yeast treatments, the P concentrations in the plants did not differ, except for plants treated with viable *C. podzolicus* cells, which had a lower P concentration than the rest (Fig. 8). It was found that two-month-old wheat plants had lower concentrations of P in their tissues than one-month-old plants.

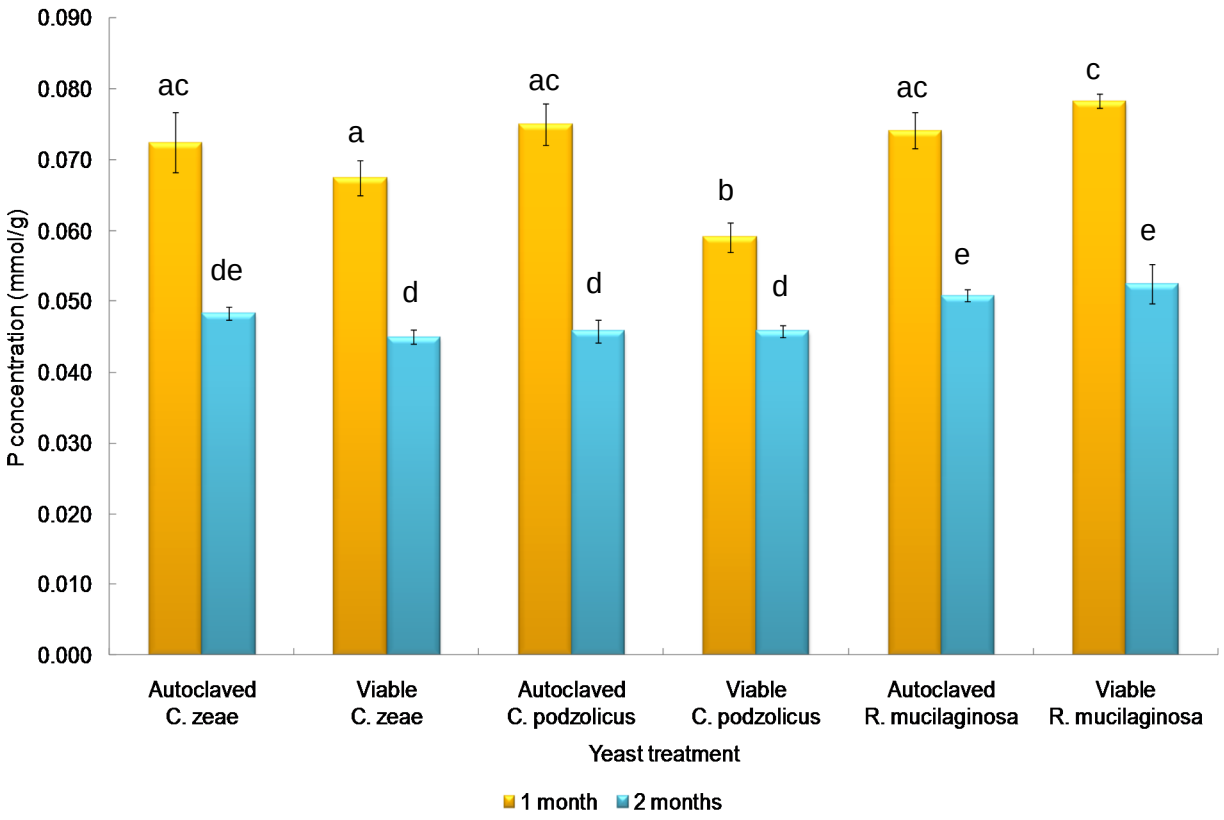

Fig. 8. A comparison of the P concentration in one and two-month-old wheat plants. Seeds of the plants were coated with autoclaved and viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826 before cultivation in a greenhouse. Bars represent the means obtained for 12 replicates, while the standard error values are shown on top of the bars. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$) (Letters a, b & c indicate differences in P concentration in plants for the first month of growth; d & e indicate differences in P concentration in plants for the second month of growth).

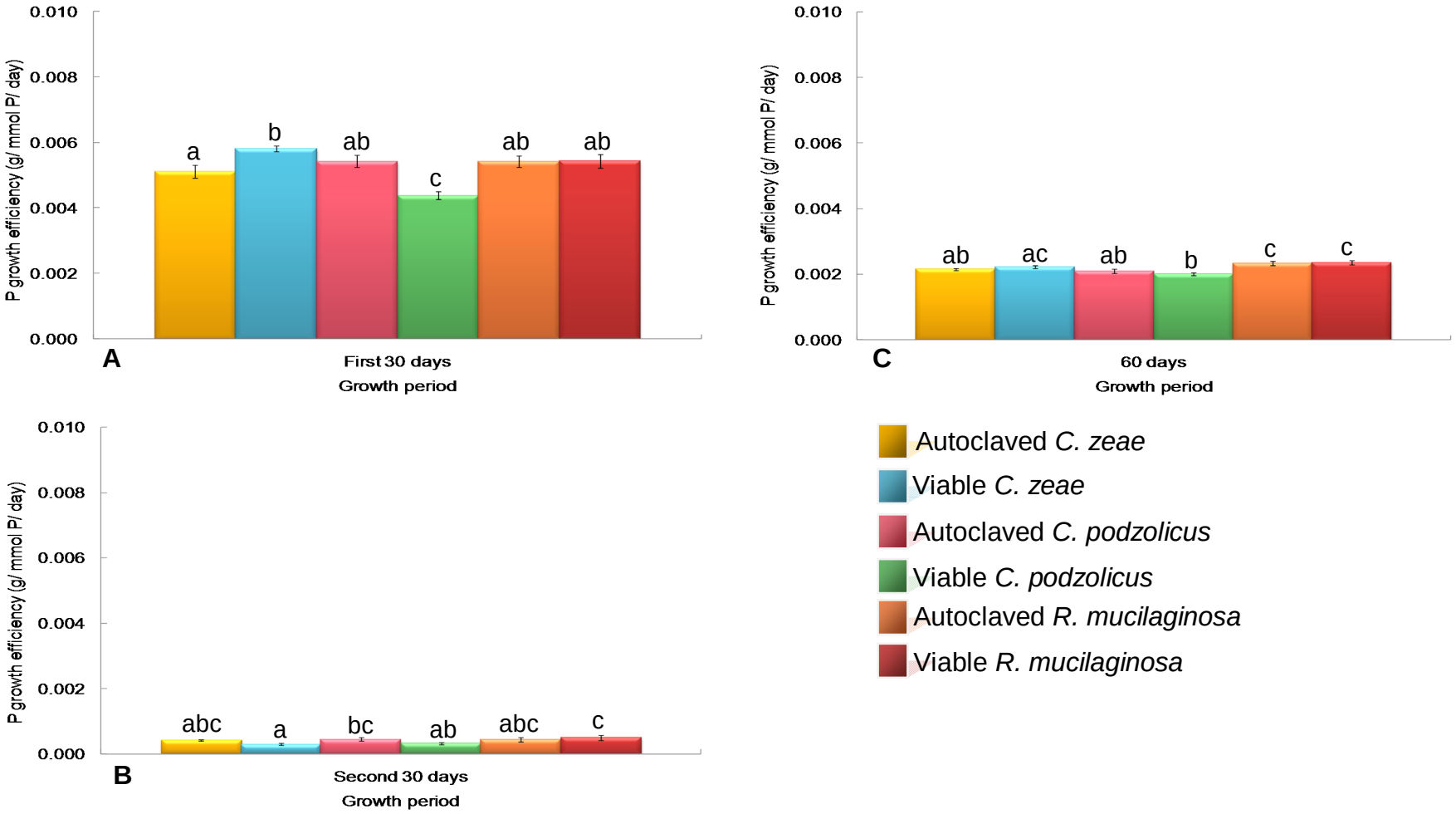

Fig. 9. Comparison of the P growth efficiency of wheat plants for the three different growth periods i.e. the first 30 days (A), the second 30 days (B) and the total growth period (60 days, C). Seeds were coated with autoclaved and viable cells of *C. zeae* CAB 1119*, C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826*.* Seedlings were cultivated under hygienic conditions in a greenhouse. Each bar represents the mean obtained for 12 replicates, whilst standard error values are shown on top of the bars. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$).

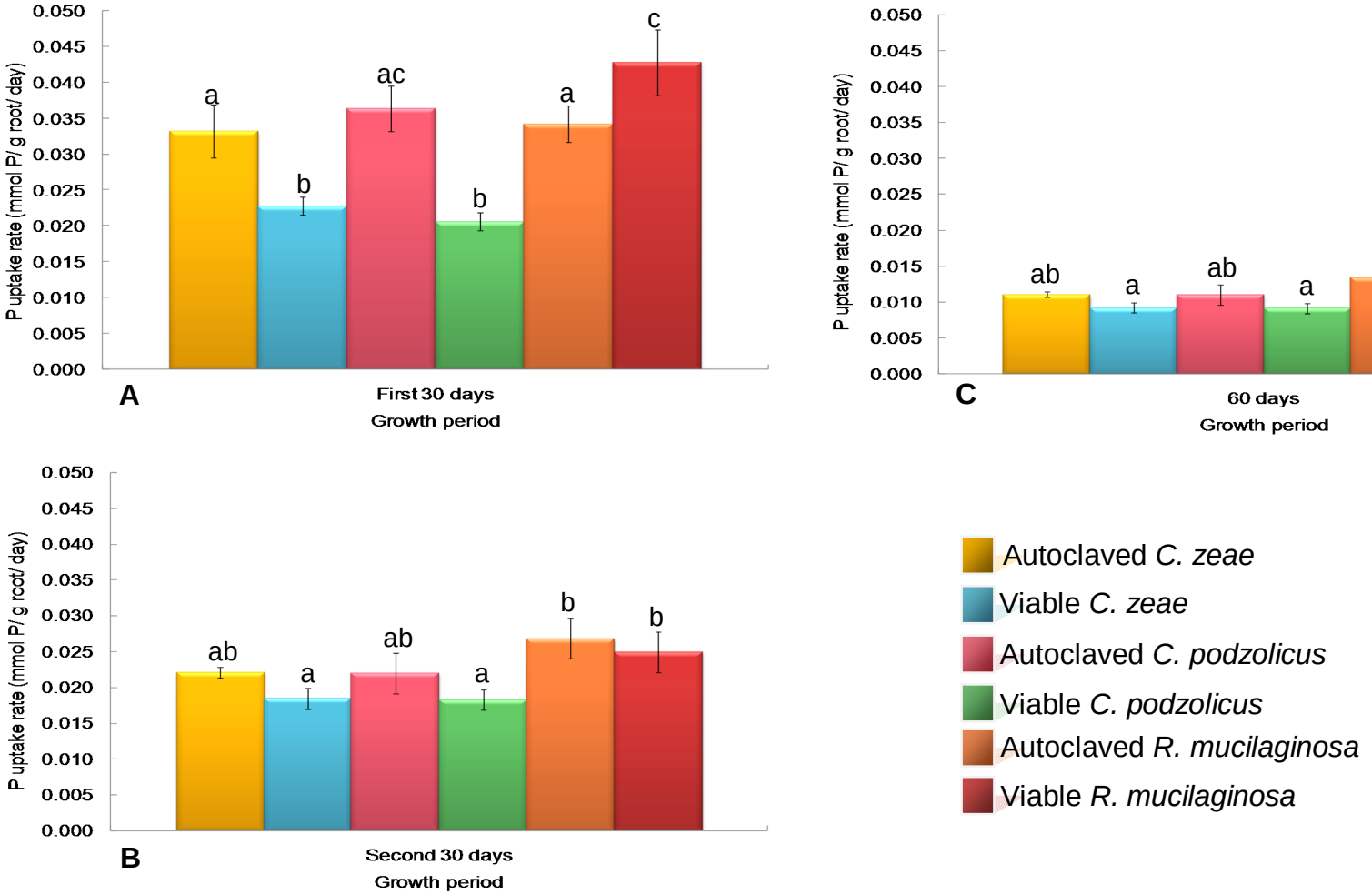

Fig. 10. A comparison of the P uptake rate of wheat plants for the three different growth periods i.e. the first 30 days (A), the second 30 days (B) and the total growth period (60 days, C). Seeds were coated with autoclaved and viable cells of *C. zeae* CAB 1119*, C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826*.* Seedlings were cultivated under hygienic conditions in a greenhouse. Bars represent the mean obtained for 12 replicates, whilst standard error values are displayed on top of the bars. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$).

It was also found that plants inoculated with viable *C. zeae* cells utilized P most efficiently during the first 30 days of growth, whilst those treated with *C. podzolicus* cells utilised P least efficient (Fig. 9A). All plants utilized P less efficiently during the second month of growth compared to the first 30 days of growth (Fig. 9A & B). During the total growth period, plants treated with viable yeast cells did not utilize P more efficiently than those treated with autoclaved cells (Fig. 9C).

In contrast to P growth efficiency, the P uptake rate for plants inoculated with viable *C. zeae* cells was lower ($p = 0.018$) than that for plants treated with autoclaved *C. zeae* cells during the first 30 days of growth (Fig. 10A). During all three growth periods (first 30 days, second 30 days, and the whole growth period) plants that were treated with viable *R. mucilaginosa* cells displayed the greatest uptake rate compared to those treated with viable cells of *C. zeae* ($p = 0.000$, $p = 0.040$, $p = 0.040$, respectively) and *C. podzolicus* ($p = 0.000$, $p = 0.035$, $p = 0.035$, respectively). The uptake rate was lower for plants cultivated during the second month and even lower during the total growth period (Fig. 10B & C). Correlation matrices revealed that there was a negative correlation between the P uptake rate and growth efficiency for all three growth periods, i.e. first 30 days, second 30 days, and the whole growth period ($r = -0.334$, $r = -0.508$, $r = -0.483$ respectively; $p < 0.050$). In addition, for all three growth periods (first 30 days, second 30 days, and the whole growth period), P concentration was positively correlated to P uptake rate ($r = 0.691$, $r = 0.374$, $r = 0.374$ respectively; $p < 0.050$) and P growth efficiency ($r = 0.272$, $r = 0.323$, $r = 0.320$ respectively; $p < 0.050$). It was also found that that the root and shoot RGR correlated positively with P growth efficiency ($r = 0.757$, $r = 0.770$ respectively; $p < 0.050$).

It can be seen in Fig. 11 that inoculation with viable cells of *C. zeae, C. podzolicus* and *R. mucilaginosa* resulted in increased K concentration in one-month-old plants, compared to plants treated with autoclaved cells ($p = 0.013$, $p = 0.022$, $p = 0.010$, respectively). The K concentration in two-month-old plants inoculated with viable *R. mucilaginosa* was greater than that of plants treated with autoclaved cells of *R. mucilaginosa* ($p = 0.003$). Similar to P concentration, the K concentration of two-month-old plants inoculated with viable *C. zeae* and *C. podzolicus* cells were lower than that of one-month-old plants (Fig. 11).

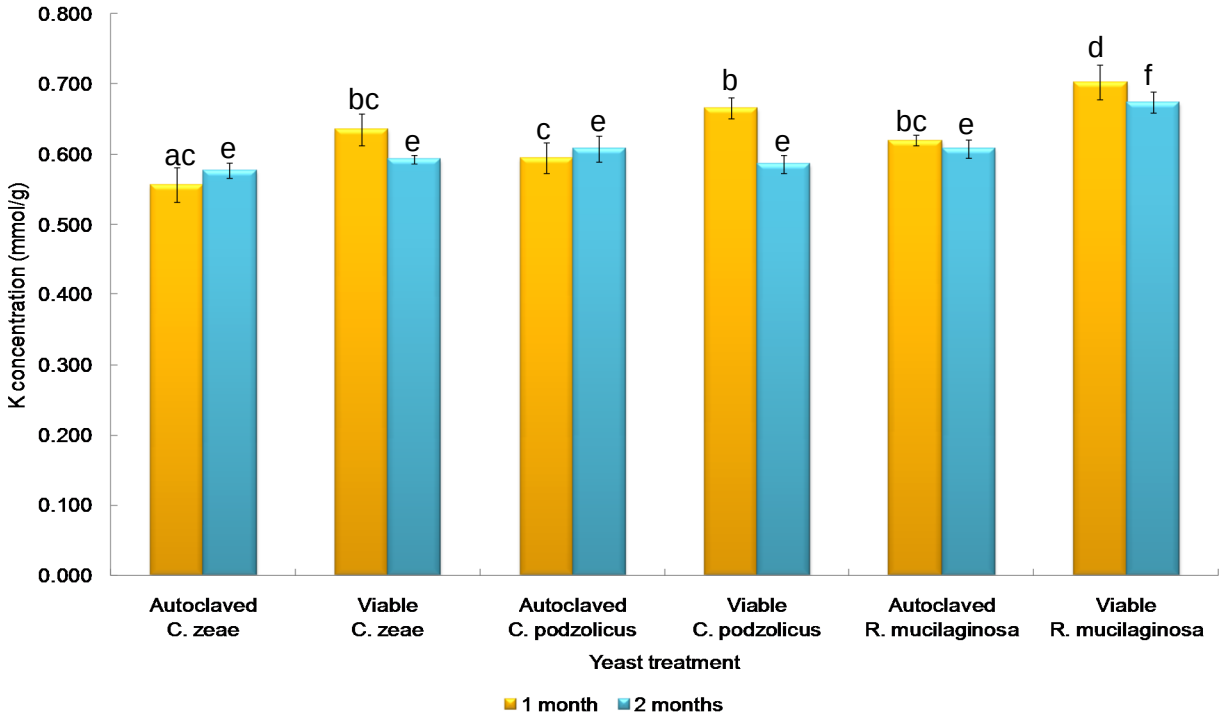

Fig. 11. Comparison of the K concentration in wheat plants, which were coated with autoclaved and viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826. Seedlings were cultivated under hygienic conditions for one and/or two months in a greenhouse. Each bar represents the mean obtained for 12 replicates and the standard error values are depicted on top of each bar. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$) (Letters a, b, c & d indicate differences in K concentration in plants for the first month of growth; e & f indicate differences in K concentration in plants for the second month of growth).

From Fig. 12A it is apparent that wheat plants inoculated with viable *C. zeae* and *C. podzolicus* cells utilized K more efficiently compared to those treated with autoclaved *C. zeae* ($p = 0.000$) and *C. podzolicus* ($p = 0.003$) cells, respectively during the first 30 days of growth. All plants utilized K less efficiently during the second month of growth, whilst during the total growth period, those inoculated with viable *C. zeae* and *R. mucilaginosa* cells utilized K most efficiently compared to those treated with viable *C. podzolicus* cells ($p = 0.000$, $p = 0.020$ respectively, Fig. 12B & C). A positive correlation was observed between shoot RGR and K growth efficiency for plants inoculated with viable cells of *C. zeae* and *C. podzolicus* during the first month of growth, ($r = 0.878$; $p < 0.050$). Similarly, a correlation was observed between shoot RGR and K growth efficiency, calculated over the total growing period, for plants inoculated with viable *C. zeae* cells ($r = 0.875$; $p < 0.050$).

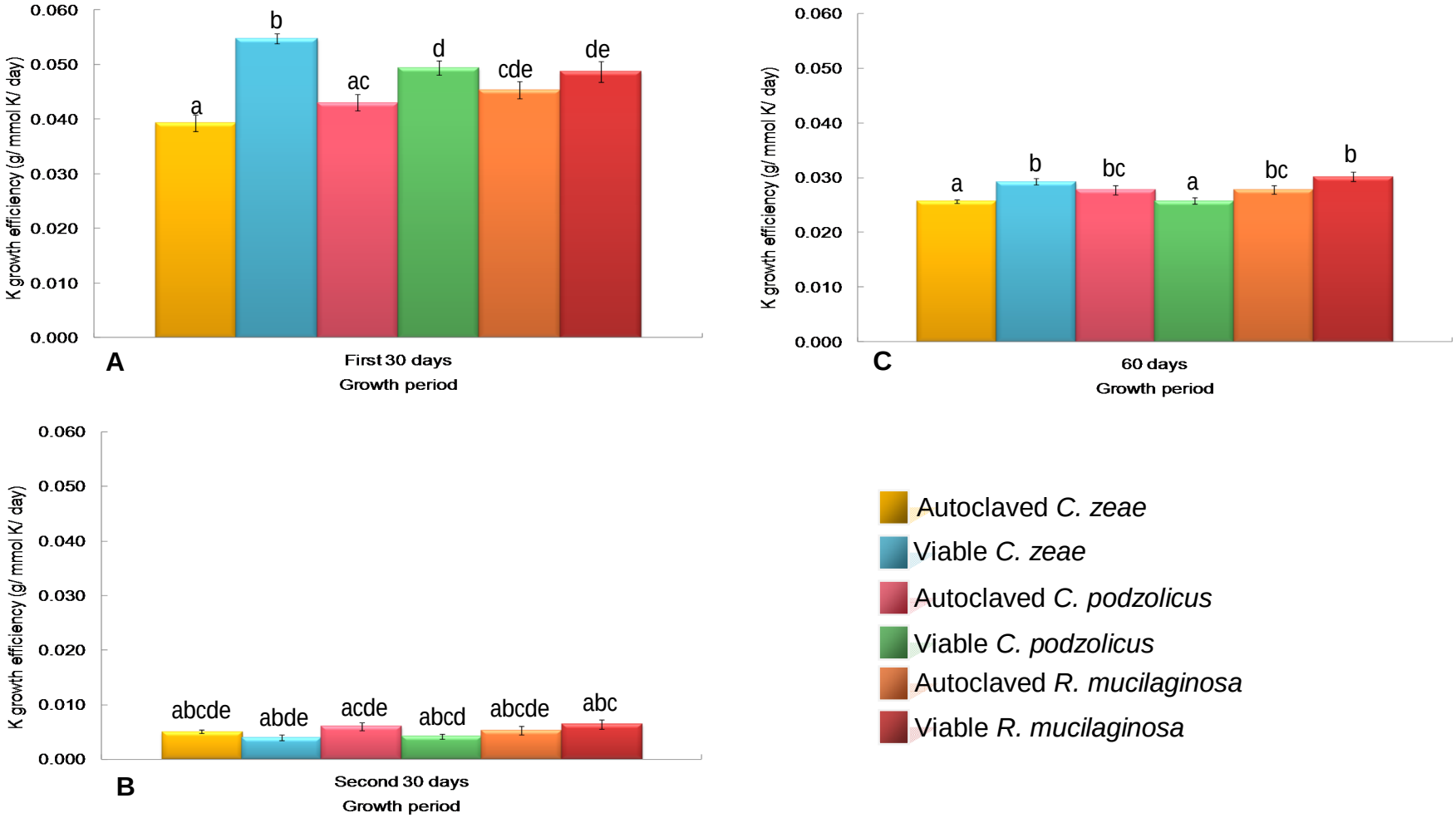

Fig. 12. A presentation of K growth efficiency of wheat plants for the three different growth periods i.e. the first 30 days (A), the second month (B) and the total growth period (60 days, C). Autoclaved and viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826 were used to coat seeds. Seedlings were cultivated in a greenhouse under hygienic conditions. Each bar represents the mean obtained for 12 replicates, whilst standard error values are depicted on top of the bars. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$).

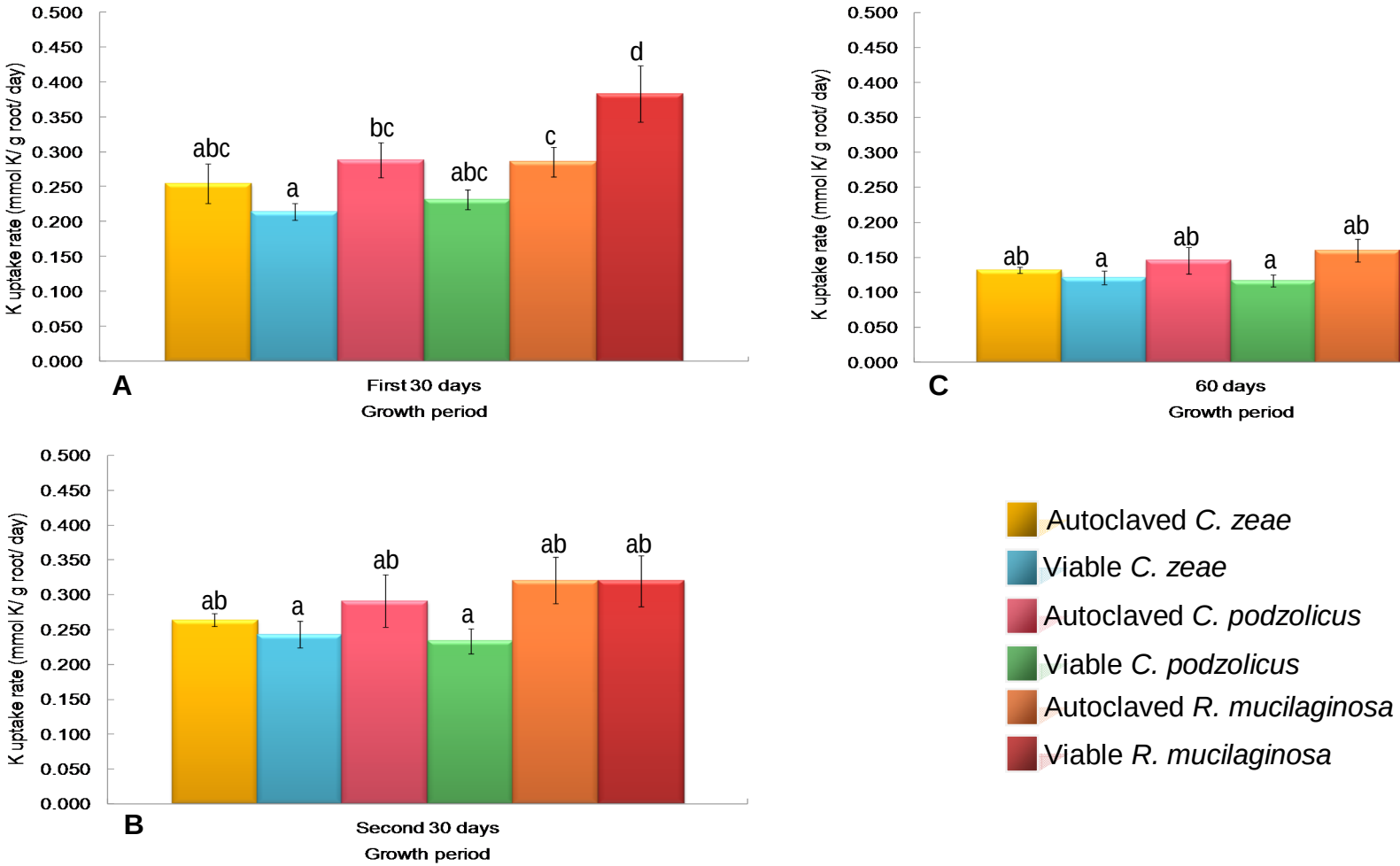

Fig. 13. Comparison of the K uptake rate of wheat plants for the three different growth periods i.e. the first 30 days (A), the second 30 days (B) and the total growth period (60 days, C). Autoclaved and viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826 were used to coat seeds. Seedlings were cultivated under hygienic conditions in a greenhouse. Bars represent the mean obtained for 12 replicates and standard error values are depicted on top of the bars. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$).

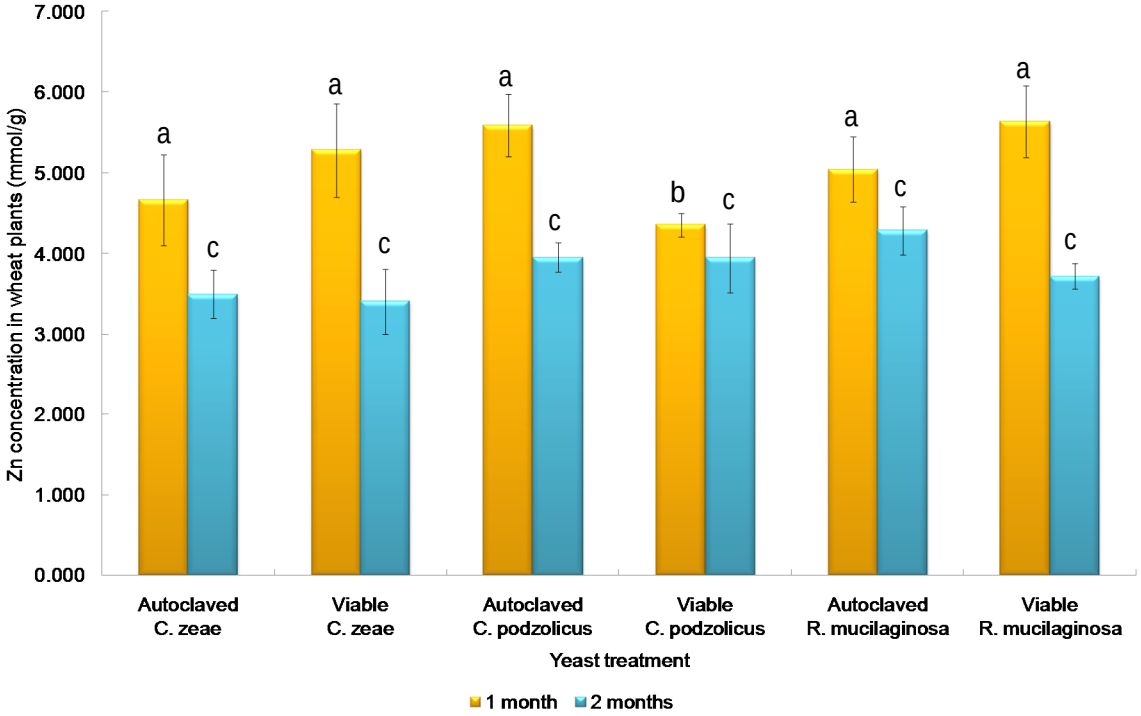

Fig. 14. Comparison of the Zn concentration in one and two-month-old wheat plants. Seeds of the plants were coated with autoclaved and viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826 before cultivation in a greenhouse. Bars represent the mean obtained for 12 replicates, while the standard error values are shown on top of the bars. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$) (Letters a & b indicate differences in Zn concentration in plants for the first month of growth; c indicates differences in Zn concentration in plants for the second month of growth).

During the first 30 days, plants inoculated with viable *R. mucilaginosa* cells had the highest K uptake rate compared to other plants treated with autoclaved or viable yeast cells ($p = 0.010$, Fig. 13A). The K uptake rate of plants inoculated with viable *C. zeae* and *C. podzolicus* cells remained similar to that of plants treated with autoclaved cells of these two yeasts. All plants had a lower K uptake rate during the second month of growth (Fig. 13B). Over the total growth period, the uptake rate of K was higher for plants treated with viable *R. mucilaginosa* cells compared to that of plants inoculated with viable *C. zeae* ($p = 0.032$) and *C. podzolicus* ($p = 0.031$) cells (Fig. 13C). During all three growth periods (first 30 days, second 30 days, and the whole growth period) a negative correlation was detected between K uptake rate and growth efficiency ($r = -0.635$; $r = -0.603$; $r = -0.598$ respectively, $p < 0.050$). Similar to P, a positive correlation occurred between K concentration and K growth efficiency for all three

growth periods, i.e. first 30 days, second 30 days, and the whole growth period ($r = 0.505$, $r = 0.295$, $r = 0.0486$ respectively; $p < 0.050$).

Similar to P concentration, the Zn concentration did not differ between one-month-old plants, except for those treated with viable *C. podzolicus* cells, which had lower Zn levels in their tissues compared to those treated with autoclaved *C. podzolicus* cells ($p = 0.000$; Fig. 14). The Zn concentration of two-month-old plants was lower than that of one-month-old plants, except for those inoculated with viable *C. podzolicus* cells and those treated with autoclaved *R. mucilaginosa* cells (Fig. 14).

Wheat plants that were inoculated with viable *C. zeae* cells utilized Zn more efficiently during the first month of growth, compared to plants treated with autoclaved or viable yeast cells ($p = 0.000$, Fig. 15A). Plants inoculated with viable *C. podzolicus* cells utilized Zn less efficiently than those treated with autoclaved *C. podzolicus* cells during the same growth period ($p = 0.000$). Similar to data for P and K, plants utilized Zn less efficiently during the second month of growth (Fig. 15B). The treatment that resulted in the most efficient utilization of Zn over the total growth period was coating the seeds with autoclaved cells of *R. mucilaginosa* ($p = 0.014$, Fig. 15C). The root and shoot RGR was found to positively correlate to the Zn growth efficiency ($r = 0.741$, $r = 0.810$ respectively; $p < 0.050$) during the first 30 days of growth and the total growth period.

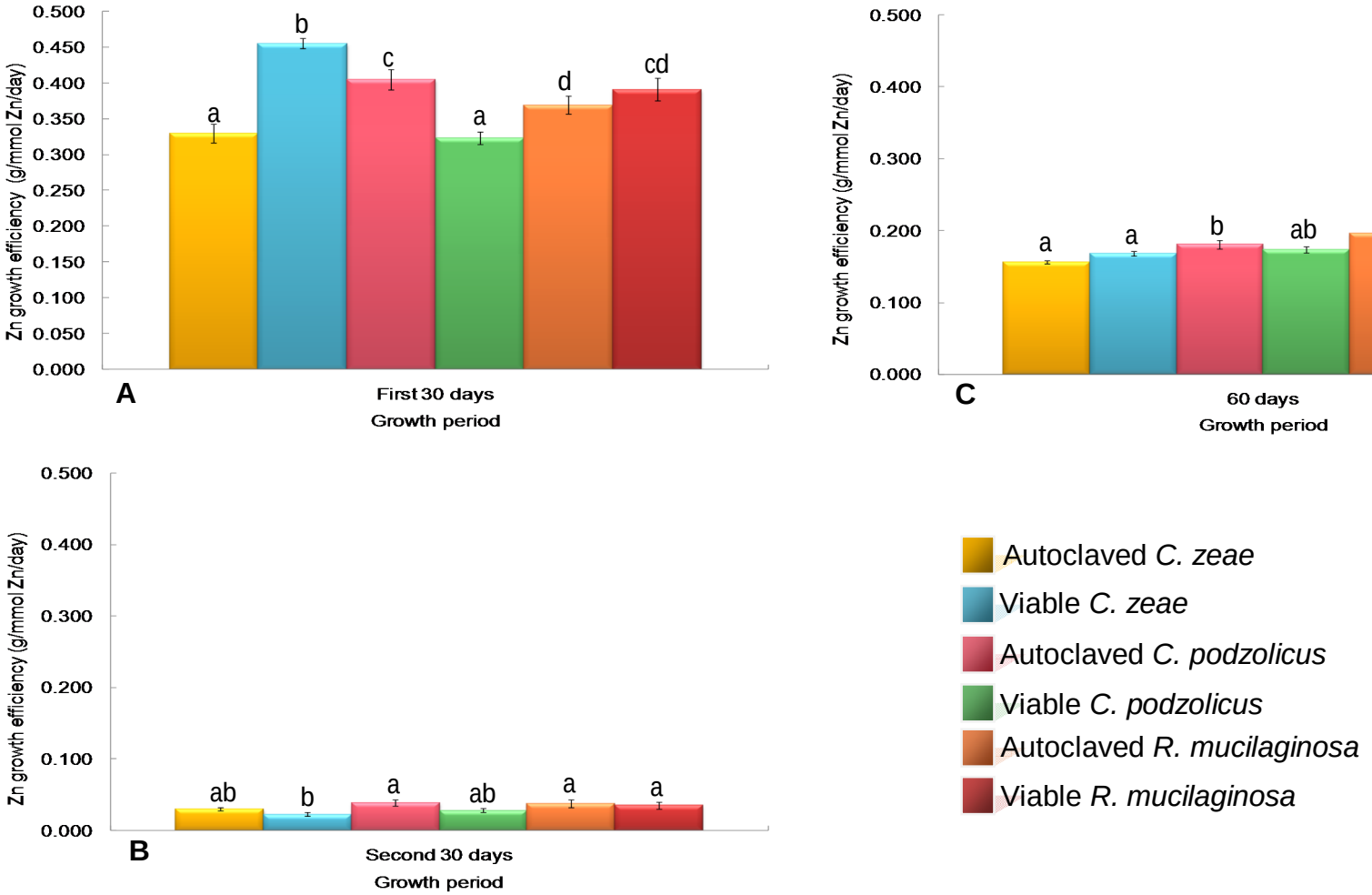

Fig. 15. A comparison of the Zn growth efficiency of wheat plants for the three different growth periods i.e. the first 30 days (A), the second 30 days (B) and the total growth period (60 days, C). Seeds were coated with autoclaved and viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826. Seedlings were cultivated under hygienic conditions in a greenhouse. Each bar represents the mean obtained for 12 replicates and standard error values are shown on top of the bars. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$).

From Fig. 16A it is evident that, during the first 30 days, plants inoculated with viable *R. mucilaginosa* cells had a greater uptake rate of Zn compared to those treated with viable *C. zeae* and *C. podzolicus* cells ($p = 0.000$, $p = 0.014$, respectively). During this same period plants inoculated with viable *C. podzolicus* demonstrated the lowest uptake rate of Zn (Fig. 16A). Similar to the uptake rates of P and K, the uptake rate of Zn calculated over the total growth period was notably lower than the uptake rate for this nutrient calculated over the shorter growth periods. Not surprisingly, there was a negative correlation between the uptake rate and growth efficiency of Zn for all three growth periods, i.e. first 30 days, second 30 days, and whole growth period ($r = -0.266$; $r = -0.525$; $r = -0.402$ respectively, $P < 0.005$) for all three growth periods. Similar to P, for all the growth periods (first 30 days, second 30 days, and whole growth period) the Zn content was found to be positively correlated to the Zn growth efficiency ($r = 0.528$, $r = 0.317$, $r = 0.872$ respectively; $p < 0.050$) and Zn uptake rate ($r = 0.556$, $r = 0.348$, $r = 0.348$ respectively; $p < 0.050$).

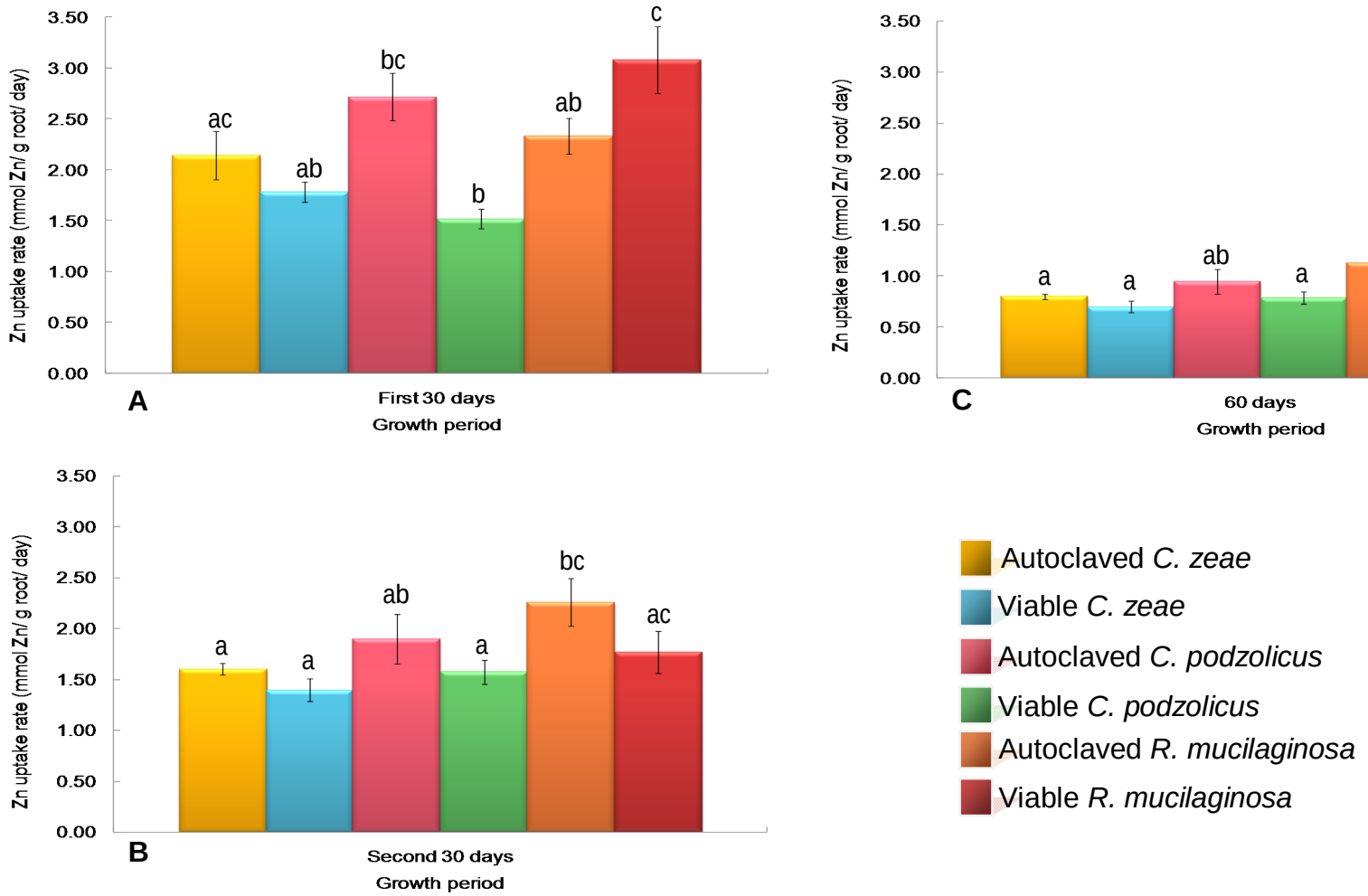

Fig. 16. Comparison of the Zn uptake rate of wheat plants for the three different growth periods i.e. the first 30 days (A), the second 30 days (B) and the total growth period (60 days, C). Seeds were coated with autoclaved and viable cells of *C. zeae* CAB 1119*, C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826*.* Seedlings were cultivated in a greenhouse under hygienic conditions. Each bar represents the mean obtained for 12 replicates, whilst standard error values are depicted on top of the bars. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$).

Analysis of Fe levels within the wheat plants revealed that all of the plants had similar concentrations of Fe in their tissue, and these concentrations decreased from one month to two months (Fig. 17).

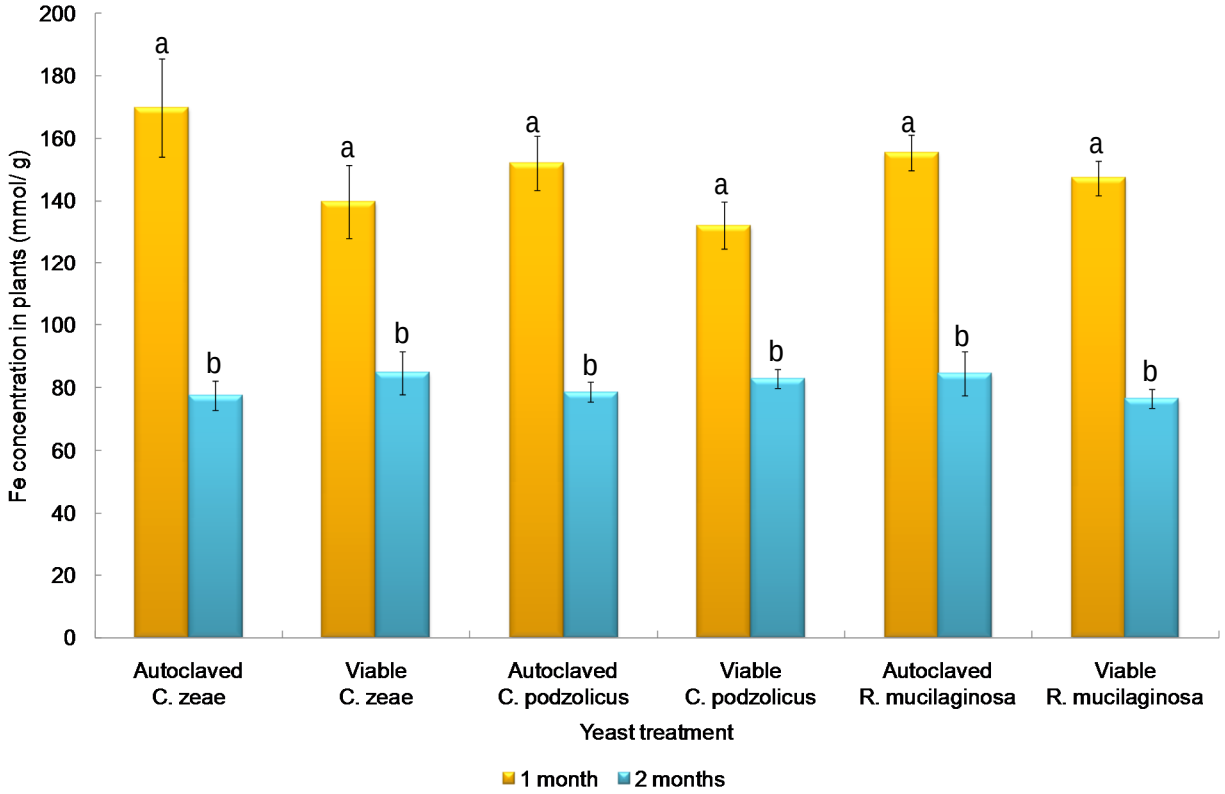

Fig. 17. Comparison of the Fe concentration in wheat plants, which were treated with autoclaved and viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826. Seedlings were cultivated under hygienic conditions for one and two months in a greenhouse. Values represent the means of 12 replicates and the standard error values are depicted on top of each bar. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$) (The letters a indicates differences in Fe concentration in plants for the first month of growth; b indicates differences in Fe concentration in plants for the second month of growth).

During the first 30 days of growth, plants inoculated with viable *C. podzolicus* and *R. mucilaginosa* cells utilized Fe less efficiently than those treated with autoclaved cells of *C. podzolicus* ($p = 0.018$) and *R. mucilaginosa* ($p = 0.025$), respectively (Fig. 18A). Similar to data for the other three nutrients, Fe was used less efficiently during the second month of growth (Fig. 18B). Over the total growth period, plants inoculated with viable *C. zeae* cells utilized Fe more efficiently than those treated with viable cells of *C. podzolicus* ($p = 0.000$) and *R. mucilaginosa* ($p = 0.000$, Fig. 18C). For all treatments the shoot and root RGR

positively correlated with the Fe growth efficiency ($r = 0.878$, $r = 0.794$ respectively; $p < 0.050$).

Plants inoculated with viable *C. zeae* and *C. podzolicus* cells demonstrated a lower Fe uptake rate than plants subjected to the other treatments during the first 30 days of growth (Fig. 19A). However, the Fe uptake rate calculated over the second month of growth, as well as over the whole of the growth period, revealed no differences between the plants that received the different treatments (Fig. 19B & C). Similar to the data obtained for P, K and Zn, a negative correlation was observed between Fe growth efficiency and Fe uptake rate for the first and second month of growth, as well over the total growth period ($r = -0.449$; $r = -0.781$; $r = -0.591$ respectively, $p < 0.050$). Unlike the other nutrients, the Fe concentration only correlated with Fe uptake rate ($r = 0.571$; $p < 0.050$) and Fe growth efficiency ($r = 0.347$; $p < 0.050$) for the first 30 days of growth.

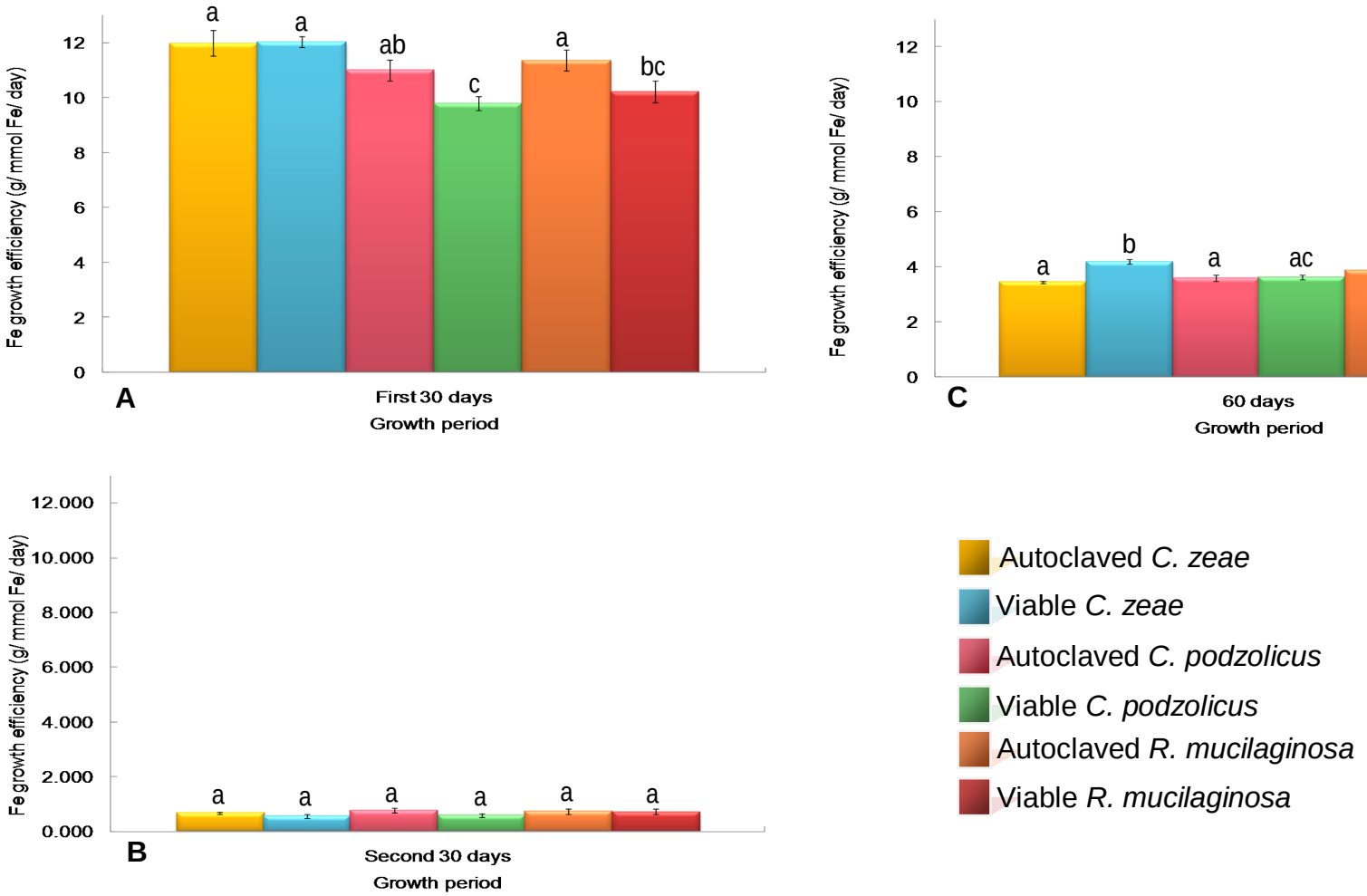

Fig. 18. A presentation of Fe growth efficiency of wheat plants for the three different growth periods i.e. the first 30 days (A), the second month (B) and the total growth period (60 days, C). Seeds were coated with autoclaved and viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826. Seedlings were cultivated in a greenhouse under hygienic conditions. Bars represent the mean obtained for 12 replicates, whilst standard error values are shown on top of the bars. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$).

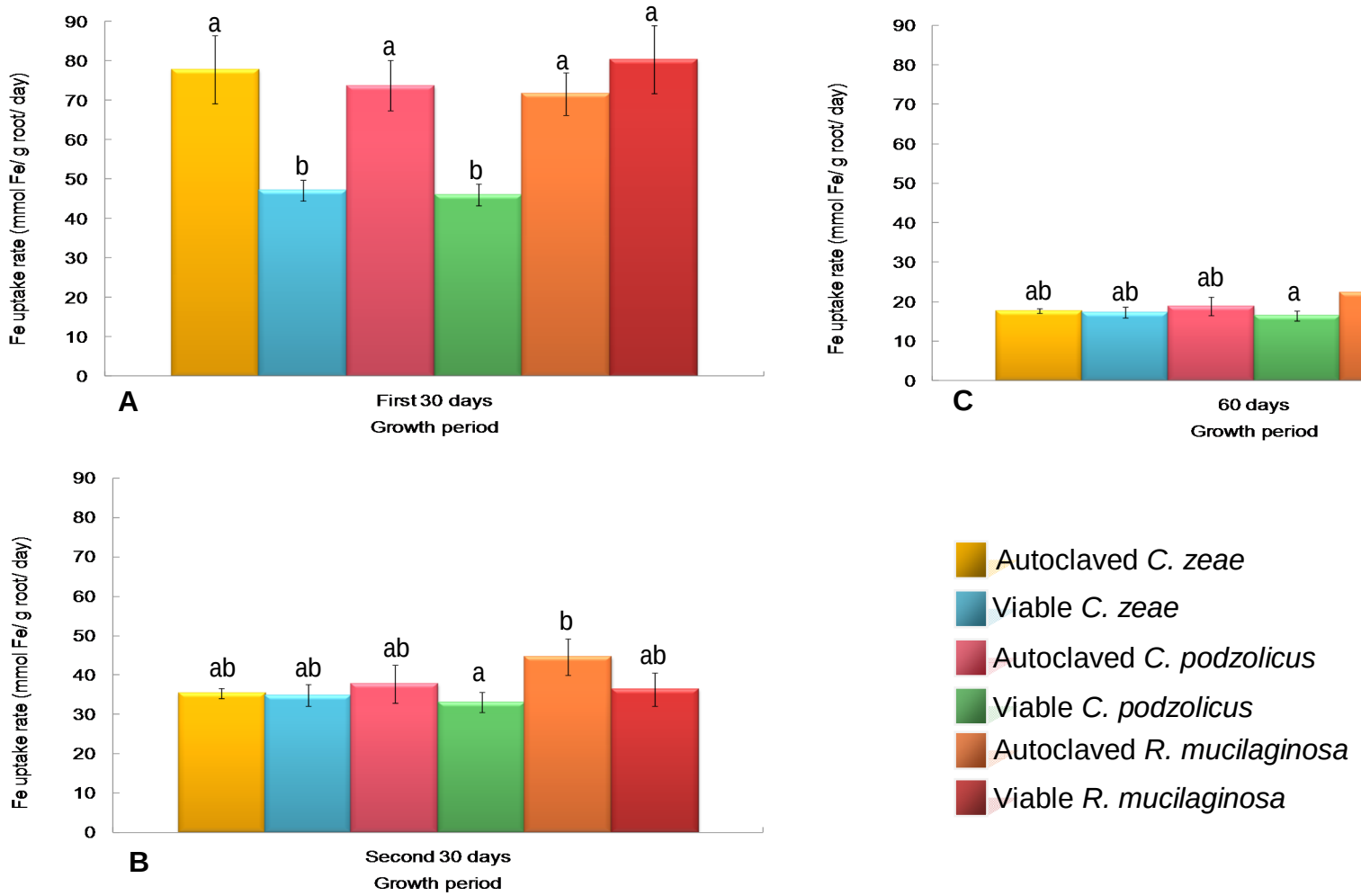

Fig. 19. Comparison of the Fe uptake rate of wheat plants for the three different growth periods i.e. the first 30 days (A), the second 30 days (B) and the total growth period (60 days, C). Autoclaved and viable cells of *C. zeae* CAB 1119, *C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826 were used to coat seeds. Seedlings were cultivated under hygienic conditions in a greenhouse. Bars represent the means obtained for 12 replicates and standard error values are depicted on top of the bars. Different letters indicate significant differences among yeast treatments, separated by a Fishers LSD test ($p < 0.05$).

4. Discussion

Several studies have evaluated soil microorganisms as plant growth promoters. However, very little is known about the ability of soil yeasts to promote wheat growth and to affect wheat nutrition. Since red grass and wheat are both monocots that belong to the same family (Poaceae) (Hoisington et al. 1999) and a plant growth promoting strain of *C. laurentii* was isolated from the rhizosphere of buchu growing in the wild (Cloete et al. 2009), we aimed to isolate potential plant growth promoting cryptococci from the rhizosphere of red grass growing in a pristine area. Furthermore, we sought to evaluate a representative *Cryptococcus* isolate for its ability to improve wheat germination by comparing its performance to that of two other soil yeasts. In addition, we studied the effect of the three soil yeasts on growth and physiological nutrition of wheat during the first two months of growth in a greenhouse.

4.1. Isolation of yeasts from wild grass

Cryptococci have previously been isolated from many different grasses (Botha, 2006; Fonseca & Inácio, 2006; Marquez et al., 2007). We were able to isolate three different *Cryptococcus* species from the rhizosphere of red grass, namely *C. zeae, C. luteolus* and *C. rajasthanensis.* Up to date *C. zeae* has not been extensively isolated from the environment. The first authors to report of this yeast were Molnár and Prillinger in 2006. They isolated *C. zeae* from the gut of corn pests, healthy corn stems and leaves. It was found that the closest relative of this yeast is *Cryptococcus luteolus,* a yeast that has been isolated from cacti (Rosa et al. 1995) and the phylloplane of Hawaiian plants (Marchant & Towers 1987). The second isolation of *C. zeae* was conducted by Čadež et al (2010). Only one strain of *C. zeae* was isolated, namely ZIM 607 from grape berries. The taxonomic determinative D1/D2 region of the rRNA gene of the four *C. zeae* isolates that were obtained from the rhizosphere of wild grass during our study shared a 99% homology with *C. zeae* ZIM 607. Due to the association of *C. zeae* with crop plants such as maize, we decided to use one of our isolates representing this species, i.e. *C. zeae* CAB 1119, in further experimentation.

4.2. Effect on germination

Seeds were coated with different concentrations of *C. zeae* CAB 1119*, C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826 and allowed to germinate. Concentrations of log 9 cells/ml for *C. zeae* and log 7-9 cells/ml for *C. podzolicus* and *R. mucilaginosa* effectively inhibited the growth of filamentous fungi (Fig. 1). It is well-known that soil yeasts can inhibit the growth of filamentous fungi and yeast species belonging to genera such as *Saccharomyces, Sporobolomyces, Rhodotorula* and *Cryptococcus* have been employed in the bio-control of plant pathogenic fungi (El-Terabily 2004).

In our study, we found that the number of roots per seedling was increased by coating the seeds with viable suspensions of *C. zeae* and *C. podzolicus* (Fig. 2). This is not surprising, since several studies have demonstrated that yeasts capable of inhibiting fungal pathogens also affect the germination of seedlings. For example, El-Mehalawy (2004) demonstrated that in the presence of the fungal pathogen, *Fusarium oxysporum,* the yeasts *Saccharomyces unispora* and *Candida steatolytica* were able to increase the germination of kidney bean. Similarly, it was found that *S. cerevisiae,* used as a bio-control agent against *Fusarium,* was able to increase the germination of sugar beet seeds (Shalaby and El-Nady, 2008).

Autoclaved suspensions of all three yeasts evaluated in our study, i.e. *C. zeae* CAB 1119*, C. podzolicus* CAB 978 and *R. mucilaginosa* CAB 826, also increased the number of roots per seedling (Fig. 2). Thus, it is likely that the yeasts might have produced a metabolic factor that influenced germination of the wheat seeds. This factor might be related to two diterpenoids, i.e. fusicoccin and cotylenin A, respectively produced by the fungi belonging to the genera *Fusicoccum* and *Cladosporium*. Cotylenin A has been implicated in the improvement of seed germination (Yamamoto-Yamaguchi et al. 2001) and its structure resembles that of fusicoccin. It has been shown that fusicoccin is a plant growth regulator and an important property of this metabolite is its ability to induce and speed up seed germination (Muromtsev et al. 1989; Muromtsev et al. 1994). The structure of fusicoccin resembles that of the well-known phytohormone gibberellin (Muromtsev et al. 1994). It has been demonstrated that the soil yeasts *Candida valida, Rhodotorula glutinis* and *Trichosporon asahii* were able to produce gibberellic acid, a simple gibberellin (El-Tarabily 2004). Since it was demonstrated that these

phylogenetically diverse soil yeasts might be able to produce gibberellic acid, it seems likely that the three yeasts used in our study may also be able to produce similar compounds, influencing seed germination in the process.

4.3. Wheat growth, yeast numbers and nutrient effects

When the dry weight of wheat plants was analyzed, we found that when seeds were coated with viable *C. zeae* cells, plants demonstrated superior growth during the first and second month of cultivation (Fig. 3). Plants coated with viable *C. podzolicus* cells only demonstrated increased growth during the first month of cultivation, whilst *R. mucilaginosa* had no effect on wheat growth (Fig. 3). The increased growth witnessed for *C. zeae* inoculated plants can be explained by the superior root and shoot RGR of these plants, when calculated over the entire growth period, but especially during the first 30 days of growth (Fig. 4A & C and Fig. 5A & C).

The increased plant growth observed during this study agrees with what has been found in other studies, demonstrating the ability of different yeast species to promote the growth of various plants (Amprayn et al. 2011; Medina et al. 2004; Mucciarelli et al. 2003; Nassar et al. 2005). To the best of our knowledge, there are only two previous studies on members of the genus *Cryptococcus* and their ability to promote plant growth. They demonstrated that *C. laurentii* CAB578 could improve the growth of buchu and colonize its rhizosphere (Cloete et al. 2009; Cloete et al. 2010).

When pigmented and non-pigmented yeasts present in the rhizosphere of wheat plants were enumerated, non-pigmented yeasts were found to be predominant on the roots of plants treated with autoclaved and viable cells of *C. zeae* and *C. podzolicus.* Red-pigmented yeasts tended to be more dominant in the rhizosphere of plants treated with autoclaved and viable *R. mucilaginosa* cells (Table 1, Fig. 6). In contrast to the dominance in the rhizosphere of yeasts with the same pigmentation than those used to treat the plants, the numbers of pigmented and non-pigmented yeasts in the bulk soil were mostly similar. The only exceptions were the dominance of non-pigmented yeasts in the bulk soil of one-month-old plants treated with viable *C. zeae* cells, and two-month-old plants treated with autoclaved and viable cells of *R. mucilaginosa*. Red-pigmented yeasts in the bulk soil were only dominant in one-month-old plants treated with autoclaved cells of *C. zeae* (Fig. 7). In general, yeast numbers were higher

(Fig. 6 and Fig. 7) in the rhizosphere than in the bulk soil. This is not surprising, since it is well-known that yeast abundance decreases as the distance from the roots increases (Botha 2006; Cloete et al. 2009). Yeast diversity in the bulk soil may also differ to that found in the rhizosphere, since it has been shown that the rhizosphere can select for specific microbial populations (Botha 2006). Therefore, it was decided to focus on the effect of rhizosphere yeasts on wheat growth.

As mentioned previously, there was more than one yeast morphotype in the rhizosphere. The RFLP profiles showed that the dominant yeast isolated from one month and two-month-old plants treated with viable *C. zeae* cells was indeed *C. zeae* (Table 1). For one month and two-month-old plants coated with viable cells of *C. podzolicus,* the dominant yeast was *C. podzolicus.* Similarly, *R. mucilaginosa* was dominant for one month and two-month-old plants coated with viable cells of *R. mucilaginosa* (Table. 1). It is therefore possible that the differences seen in plant dry weight, RGRs and plant nutrition was mostly due to the effect of the yeasts used to treat the seeds before germination. It is tempting to speculate that the decreased abundance of *C. zeae* and *R. mucilaginosa* in the rhizosphere of two-month-old plants, compared to one-month-old plants, may be explained by decreased metabolic activity that culminated in decreased root RGR (Fig. 4).

The impact of the yeasts, used in the inoculums, on wheat metabolism was studied by testing the response of plant nutritional physiology to coating the seeds with viable cells of *C. zeae, C. podzolicus* or *R. mucilaginosa.* The P concentration in wheat plants were not affected by coating the seeds with viable *C. zeae* and *R. mucilaginosa* cells. Plants that were inoculated with viable *C. podzolicus* cells however, had a lowered P concentration during the first month of growth (Fig. 8). Since there was a positive correlation between the P concentration and the P growth efficiency, it seems that the lowered P concentration can be ascribed to the lowered P growth efficiency (Fig. 9A) of plants inoculated with viable cells of *C. podzolicus*. During the same growth period, plants inoculated with viable *C. zeae* cells utilized P more efficiently than plants subjected to the other treatments (Fig. 11A). This efficient use of P by these plants most likely contributed to the increased wheat growth, since it was found that there was a positive correlation between the root and shoot RGR and P growth efficiency. This phenomenon is known to occur in plants colonized by mycorrhizal fungi. For example, Monzón and Azcón (2001) demonstrated that the P use efficiency and

growth of alder trees belonging to the species *Alnus cordata, Alnus incana* and *A. glutinosa* were improved by the mycorrhizal fungi *Glomus mosseae* and *Glomus intraradices*. It was shown that P use efficiency could be improved by these mycobionts via the solubilisation of soil P or by increasing the sorption area of the roots (Shenoy and Kalagudi 2005). It is well-known that many microorganisms can solubilise P by either producing organic acids (Pradhan and Sukla 2005) or phosphatases. It was demonstrated that cryptococci can produce these extracellular enzymes (Greenwood and Lewis 1976; Garcìa-Martos et al. 2001), which can catalyse the hydrolysis of organic phosphates to inorganic phosphates (Mubyana et al. 2002), rendering them available to plants (Miyasaka and Habte 2001). A recent study conducted by Cloete et al. (2010) demonstrated that the soil yeast *C. laurentii* CAB 578 increased P concentration in buchu roots. It is therefore likely that *C. zeae* could produce either organic acids or phosphatases thereby increasing the P uptake and P concentration of wheat.

When comparing plants treated with autoclaved cells of the three yeasts to those inoculated with viable cells, it was found that viable *C. zeae* and *C. podzolicus* increased the K concentration in plants only during the first month of growth, while *R. mucilaginosa* increased the K concentration in plants during both months of growth (Fig. 11). It seems likely that the increased K concentration in plants treated with viable *C. zeae* and *C. podzolicus* cells was due to the efficient use of K during the first 30 days of growth (Fig. 12A).The elevated K concentration in the one and two-month-old plants inoculated with viable cells of *R. mucilaginosa* can be ascribed to the higher K uptake rate of these plants during the first 30 days and over the total growth period (Fig. 13A & C). It seems that under the experimental conditions of this study the increased K concentration had no effect on wheat growth, since the growth of plants treated with viable *R. mucilaginosa* cells did not increase (Fig. 3). In addition, the K concentration of two-month-old plants inoculated with viable *C. zeae* cells did not increase, but growth of both one and two months old plants, inoculated with this yeast, did increase. This increased growth may be attributed to the plants utilizing K more efficiently during growth, due to the shoot RGR and the K growth efficiency correlating positively for plants treated with *C. zeae* and *C. podzolicus* during the first month of growth and plants treated with *C. zeae* during the total growth period. Unlike P and N, K does not form part of structural components, but is rather shunted through the plant to maintain important processes such as photosynthesis, transportation of sugars and maintenance of turgor (Edwards, 2001). Therefore, an efficient use of K by plants will be beneficial.

When the data for Zn nutrition was analyzed, it was found that during the first 30 days of growth, plants inoculated with viable cells of *C. zeae* utilized Zn more efficiently (Fig. 15A) and had a lower Zn uptake rate in comparison to those treated with autoclaved *C. zeae* cells (Fig. 16A). In contrast, plants inoculated with viable *R. mucilaginosa* cells had an increased Zn uptake rate, but the Zn growth efficiency remained similar to that of plants treated with autoclaved cells of *R. mucilaginosa* (Fig. 15A). Similar results were obtained when Zn growth efficiency, were calculated for *C. zeae* treated plants over the total growth period. However, plants treated with viable cells of *R. mucilaginosa* utilized Zn less efficiently in comparison to those coated with autoclaved cells of this yeast (Fig. 15C). Yet, there was a positive correlation between the root and shoot RGR and the Zn growth efficiency during the first 30 days of growth and the total growth period. Therefore, the efficient use of Zn by plants inoculated with viable *C. zeae* cells during the first 30 days of growth, as well as during the total growth period, positively affected the root and shoot RGR and thus wheat growth. Plants inoculated with viable *R. mucilaginosa* cells however, used Zn less efficiently during the total growth period, which may have negatively affected the root and shoot RGR and wheat growth. This is in contrast to results obtained by Baon (1996), who demonstrated that mycorrhizal fungi decreased the Zn use efficiency in *Coffea arabica* L. (coffee). It has been shown, however, that mycorrhizal fungi can increase the Zn uptake rate of plants, by increasing the absorptive surfaces of the roots (Manjunath and Habte 1988).

During the first 30 days of growth, plants that were inoculated with viable cells of *C. zeae* and *C. podzolicus* had a lower Fe uptake rate compared to plants treated with autoclaved yeast cells (Fig. 19A), even though the Fe concentration was similar for all plants (Fig. 17). Similar to data obtained for P and K, the Fe concentration in the tissues of two-month-old plants was lower than that of one-month-old plants (Fig. 17). This lower concentration of Fe is likely due to a lower Fe uptake rate calculated for the total growth period (Fig. 19C). The most efficient utilization of Fe during this period occurred in plants inoculated with viable *C. zeae* cells; while plants coated with viable *R. mucilaginosa* cells utilized Fe less efficiently in comparison to those treated with autoclaved *R. mucilaginosa* cells (Fig. 18C). Since there was a positive correlation between the Fe growth efficiency and the root and shoot RGR, it seems that wheat growth was positively affected by *C. zeae,* probably as a result of its ability to induce efficient Fe utilization, resulting in improved plant growth (Fig. 3). In contrast, *R. mucilaginosa* could have had a negative effect on Fe utilization and hence shoot and root

RGR, which may explain why this yeast did not improve wheat growth. Little is known about the mechanisms involved in the improvement of Fe utilization by yeasts. Mycorrhizal fungi can solubilise insoluble Fe by the production of siderophores (Haselwandter, 1995). However, there are indications that cryptococci may not able produce siderophores, such as in the case of *C. neoformans* (Howard, 1999). Yet, it has been demonstrated that the soil yeast *C. laurentii* was able to increase the Fe concentration in *A. betulina* plants, when colonizing the rhizosphere of this sclerophyllous shrub (Cloete et al. 2010). These authors speculated that this unicellular fungus might have increased the Fe concentration in these plants by polyphosphates binding to the Fe cation, which is then transported to the host, or by the bioaccumulation of this cation by this yeast.

From the above it seems likely that the increased wheat growth observed in our study was due to *C. zeae* CAB 1119, improving the efficiency of the plant to utilize P, K, Zn and Fe. This finding may contribute to meeting the global demand for wheat in the near future, since it has been envisaged that improvement of nutrient use efficiency and other soil management practices will increase crop yield drastically across the globe (Foley et al. 2011).

5. Conclusion

We have demonstrated that soil yeasts isolated from the rhizosphere of wild grasses can be used to inoculate wheat, influence nutrition and improve plant growth. We have also found that there are differences between soil yeasts regarding their ability to influence the germination and growth of wheat seedlings. These differences between soil yeasts to influence wheat growth most likely stem from different physiological responses in wheat induced by different yeast species.

The isolate of *C. zeae*, isolated from red grass, seemed superior in its ability to improve wheat performance compared to the strains representing other soil yeasts, i.e. *C. podzolicus* and *R. mucilaginosa*. It seemed that *C. zeae* could improve wheat growth by affecting the growth efficiency and subsequently the relative growth rates of the plants More isolates representing this yeast should therefore be screened in future for their ability to enhance wheat performance. Future studies should also include the testing of carbon (C) and nitrogen (N) concentrations in plants cultivated in the presence of these yeasts, since the data

obtained can be used to determine the carbon cost of the yeasts. In addition, the effect of *C. zeae* on grain yield should be investigated. However, it is important to first evaluate the growth promoting qualities of *C. zeae* in the presence of natural soil microbial consortia. Thus, the ability of *C. zeae* to improve wheat growth should be conducted with wheat planted in unsterilized soil. The ability of *C. zeae* to enhance wheat growth in the presence of natural soil microbial consortia will be reported on in the next chapter.

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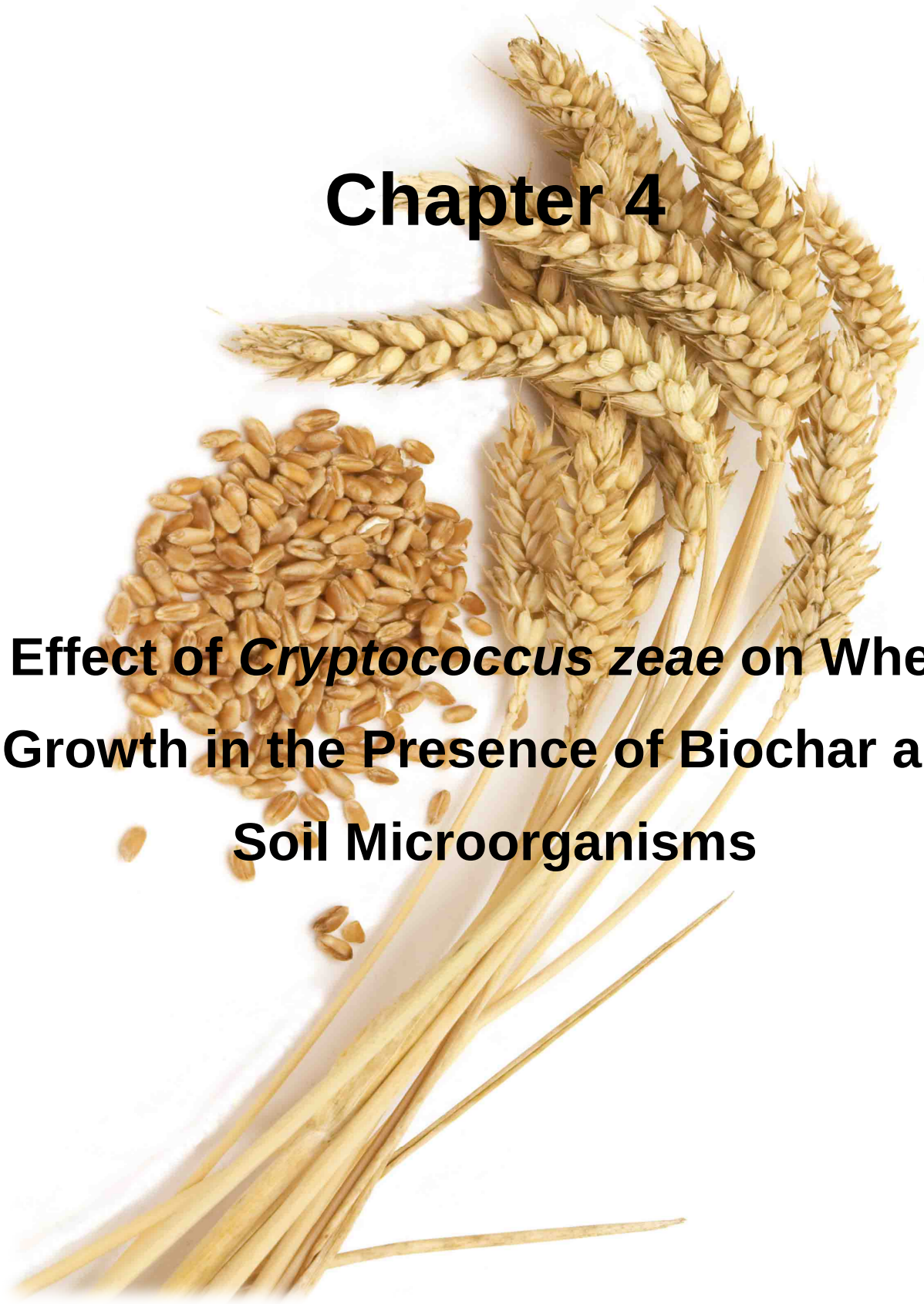

Chapter 4

Effect of *Cryptococcus zeae* on Wheat Growth in the Presence of Biochar and Soil Microorganisms

1. Introduction

As mentioned in previous chapters, *Triticum aestivum* L. (wheat) is an important food source in the world (FAO 2010). However, the production of this crop needs to be increased to eleviate the global food shortages invisaged in the near future (UN 2010). A manner in which this could be accomplished is to increase cropping intensity by using inorganic fertilizers (Foley et al. 2011). Unfortunately, fertilizers are costly and have a negative impact on the environment. It is therefore not surprising that there has been increasing interest in alternative plant growth promoters, such as biochar and soil microorganisms (Adesemoye et al. 2009).

We found that amendments of biochar to sandy soil improved mycorrhizal colonization, as well as wheat growth (Chapter 2). These findings supported the results of others who also reported the ability of biochar to improve wheat growth and affect mycorrhizal colonization (Blackwell et al. 2010; Solaiman et al. 2010). However, mycorrhizae are not the only fungi known to have a positive effect on plant growth. It was demonstrated that soil yeasts, such as those belonging to the genera *Cryptococcus, Yarrowia* and *Williopsis*, are able to improve plant growth (Cloete et al. 2009; Nassar et al. 2005; Medina et al. 2004). Similarly, we showed that the soil yeasts *Cryptococcus podzolicus* and *Cryptococcus zeae* could improve wheat growth under hygienic conditions in acid washed sand (Chapter 3). Yet, the impact of biochar on the ability of soil yeasts to improve wheat growth in natural soil is still unknown.

Therefore, the first aim of this study was to test the hypothesis that *C. zeae* could improve wheat growth and nutrition in a sandy low nutrient soil containing natural microbial consortia. The second aim was to study the effect of a biochar amendment on the ability of *C. zeae* to improve wheat growth, as well as nutrition, in this sandy soil.

2. Materials and methods

2.1. Yeast inoculum and seed preparation

Cryptococcus zeae CAB 1119 was used in this experiment which aimed to ascertain the effect of *C. zeae* on wheat growth in the presence of other soil microorganisms and biochar. The yeast inoculum was prepared by cultivating the yeast in yeast-malt (YM) broth on a rotary shaker (100 revolutions per minute) for 48 hours at 26°C. The cells were harvested by centrifugation (38 000 *xg*; 5 min) and washed twice with sterile physiological saline solution

(PSS). A haemacytometer (Superior, Germany) was then used to determine the concentration of cells in the final suspension. Since it was demonstrated that wheat germination was superior when treated with viable *C. zeae* cells at a concentration of log 9 yeast cells/ml (Chapter 3), the volume was adjusted with sterile PSS to obtain this concentration of cells. Wheat (cultivar SST 047) seeds were surface sterilized in 70% ethanol for 1 min, followed by submersion in 1% (v/v) sodium hypochlorite solution for 40 seconds. The seeds were then rinsed in sterile distilled water and coated with the yeast. This was accomplished by submerging 240 seeds in 5 ml of the yeast suspension. The seed coating of the control plants was prepared by submerging 240 seeds in a 5 ml suspension of autoclaved cells. Seeds were removed from the suspensions and allowed to dry at 22°C for 15 min. After drying, the seeds were planted on sterile Murashige and Skoog (MS) agar plates (Slater et al. 2008) and allowed to germinate for two days at 22°C in the dark.

2.2. Pot preparation and wheat growth

The sandy low nutrient soil was collected from an unused field near Brackenfell in Cape Town, South Africa (Chapter 2). Pre-cleaned 13 cm diameter plastic pots (n = 24) were filled with 800 g of this soil. Since results in Chapter 2 indicated that wheat growth was superior at 10% (w/w) biochar amendment, 800 g of a mixed substrate consisting of the soil and 10% (w/w) biochar (Allbrick, Thembalethu; Chapter 2) was transferred to each of a series of 24 plastic pots. All pots were watered up to field capacity with quarter strength Long Ashton nutrient solution (Cloete et al. 2009).

Two day old seedlings of similar size were planted in the pots (four seedlings per pot), resulting in 24 pots (12 containing soil only, 12 containing soil with 10% biochar added) planted with seedlings inoculated with viable yeast cells, and 24 pots (12 containing soil only, 12 containing soil with 10% biochar added) planted with seedlings treated with autoclaved yeast cells. The plants were allowed to grow for one month in a well-ventilated greenhouse with a 12 h photoperiod of 1000–1100 µmol m^2/s photosynthetic photon flux density. The average day/night temperatures and relative humidity were 23/15 °C and 50/80%, respectively. Low nutrient conditions were simulated, by supplying plants only with sterile distilled water twice weekly.

2.3. Harvesting and nutrient analyses

After one month, the wheat plants were harvested and their roots washed in sterile PSS. These plants were then dried in an oven at 80°C for one week, where-after the dry weight of the roots and shoots were recorded. Dry mass of the plants were analysed for phosphorous (P), potassium (K), zinc (Zn) and iron (Fe) concentration, according to the methods described by Vreulink et al. (2007). The obtained data was used to calculate different physiological parameters, i.e. root relative growth rate, shoot relative growth rate, nutrient use efficiency and nutrient uptake rate according to the formulae proposed by Mortimer et al. (2005).

2.4. Statistical analyses

Significant differences between treatment means were analyzed using ANOVA and separated using a *post hoc* Fishers least significant difference (LSD) test, using the program Statistica version 10 (Statsoft, Tulsa, OK, USA). In addition, correlation matrixes comparing the nutrient concentrations, uptake rates and use efficiency for P, K, Zn and Fe were constructed using the same software program.

3. Results

3.1. Wheat growth

Wheat growth was increased by both the biochar amendment and the treatment with viable yeast cells (Fig.1). Compared to the other treatments, plants inoculated with viable *C. zeae* cells and cultivated in 10% (w/w) biochar amended soil, showed the best growth.

When cultivated in soil alone, root relative growth rate (RGR) was higher for plants inoculated with viable yeast cells compared to plants treated with non-viable cells ($p = 0.000$; Fig.1). The root RGR of plants cultivated in soil amended with 10% (w/w) biochar was higher than the root RGR of plants cultivated in soil alone and treated with viable yeast ($p = 0.006$). Similar to the results obtained for plant growth, plants that were treated with viable *C. zeae* cells and cultivated in 10% (w/w) biochar amended soil, showed the highest root RGR. In addition, these plants also demonstrated a higher shoot RGR, whilst the shoot RGR of the other treatments remained similar ($p > 0.050$; Fig. 1).

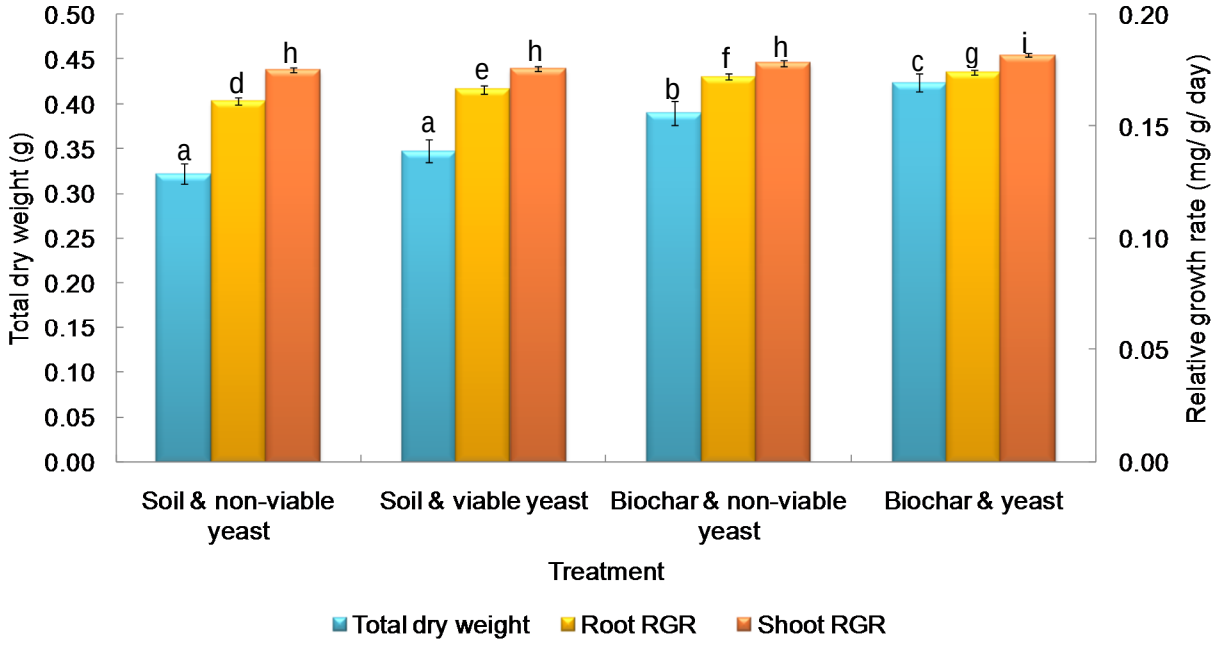

Fig. 1. Comparison between the the total dry weight, root relative growth rate (RGR) and shoot RGR of wheat plants cultivated in soil and soil amended with 10% (w/w) biochar. Seeds were treated with autoclaved cells (non-viable yeast) or viable cells (log 9 cells/ml) of *Cryptococcus zeae* CAB 1119 before germination, and cultivated under greenhouse conditions for 1 month. Each bar represents the mean obtained for 12 replicates, while the standard error values are indicated on the top of the bar. Different letters indicate significant differences among yeast and biochar treatments, separated by a Fishers LSD test ($p < 0.05$) (Letters a, b & c indicate differences in total dry weight; d, e, f & g indicate differences in root RGR; h & i indicate differences in shoot RGR).

3.2. Nutrient analyses

Analyses of P concentration in the wheat plants showed that plants cultivated in 10% (w/w) biochar-amended soil had higher concentrations of P in their tissue than plants cultivated in soil alone (Fig. 2). Plants utilized P more efficiently during growth when the seeds were treated with viable yeasts and cultivated in both in soil alone ($p = 0.000$) and in the biochar soil mixture ($p = 0.000$). In addition, plants treated with viable yeast and cultivated in the biochar soil mixture had a greater P growth efficiency than those cultivated in soil alone ($p = 0.000$). The P uptake rate was lower in plants cultivated in the biochar-amended soil than in plants growing in soil alone (Fig. 2).

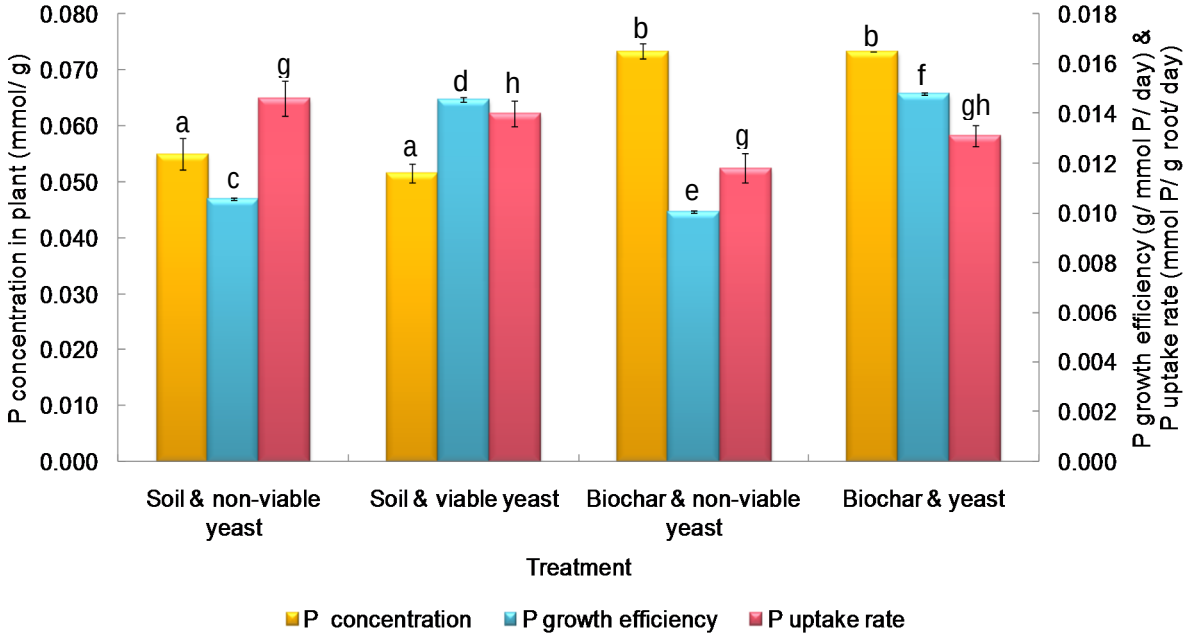

Fig. 2. Comparison of P concentration, P growth efficiency and P uptake rate of wheat plants, treated with autoclaved (non-viable yeast) or viable yeast (log 9 cells/ml), after cultivation in either soil or soil amended with 10% (w/w) biochar, under greenhouse conditions for 1 month. Each bar represents the mean obtained for 12 replicates, while the standard error values are indicated on the top of the bar. Different letters indicate significant differences among yeast and biochar treatments, separated by a Fishers LSD test ($p < 0.05$) (Letters a & b indicate differences in P concentration; c, d, e & f indicate differences in P growth efficiency; g & h indicate differences in P uptake rate).

Similar to the results obtained for P concentration of the wheat plants, the K concentration was found to be higher in plants cultivated in the biochar-amended soil than in plants cultivated in soil alone (Fig. 3). In the latter case, the viable yeast treatment had no effect on the K concentration in the plants compared to plants treated with non-viable yeast cells ($p > 0.050$). In addition, these plants treated with viable yeast and growing in soil alone, utilised K less efficiently ($p = 0.001$) and had a lower uptake rate ($p = 0.021$) than plants treated with non-viable yeast cells and growing in the soil alone. In the presence of biochar plants treated with viable yeast showed a higher K concentration than plants treated with non-viable yeast cells ($p = 0.000$). Similarly, these plants growing in the presence of biochar and treated with viable yeast utilized K more efficiently than those growing in the presence of biochar and treated with non-viable cells ($p = 0.000$), whereas the K uptake rate remained similar ($p > 0.050$).

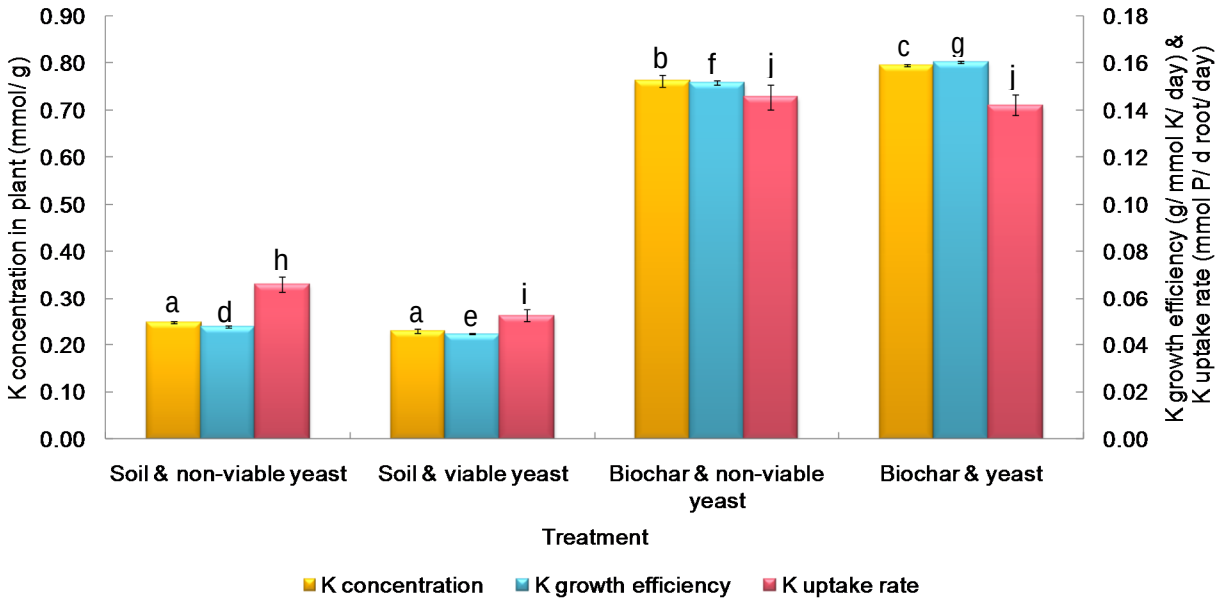

Fig. 3. Comparison between the K concentration, K growth efficiency and K uptake rate of wheat plants cultivated in soil and soil amended with 10% (w/w) biochar. Wheat seeds were treated with autoclaved (non-viable yeast) or viable *C. zeae* cells (log 9 cells/ml) before planting. Each bar represents the mean obtained for 12 replicates, while the standard error values are indicated on the top of the bar. Different letters indicate significant differences among yeast and biochar treatments, separated by a Fishers LSD test ($p < 0.05$) (Letters a, b & c indicate differences in K concentration; d, e, f & g indicate differences in K growth efficiency; h, i & j indicate differences in K uptake rate).

In contrast to the results obtained for the P and K concentration of the wheat plants, the Zn concentration was less in plants cultivated in the biochar-amended soil than in plants cultivated in soil alone (Fig. 4). In the latter case, Zn concentration, Zn use efficiency and Zn uptake rate was higher for plants treated with non-viable yeast than those treated with viable yeast ($p = 0.000$, $p = 0.000$, $p = 0.000$ respectively). However, there was no difference in Zn concentration, growth efficiency and uptake rate of plants growing in biochar-amended soil concentration and treated with viable yeast and plants cultivated in the same soil and treated with non-viable yeast cells ($p > 0.050$).

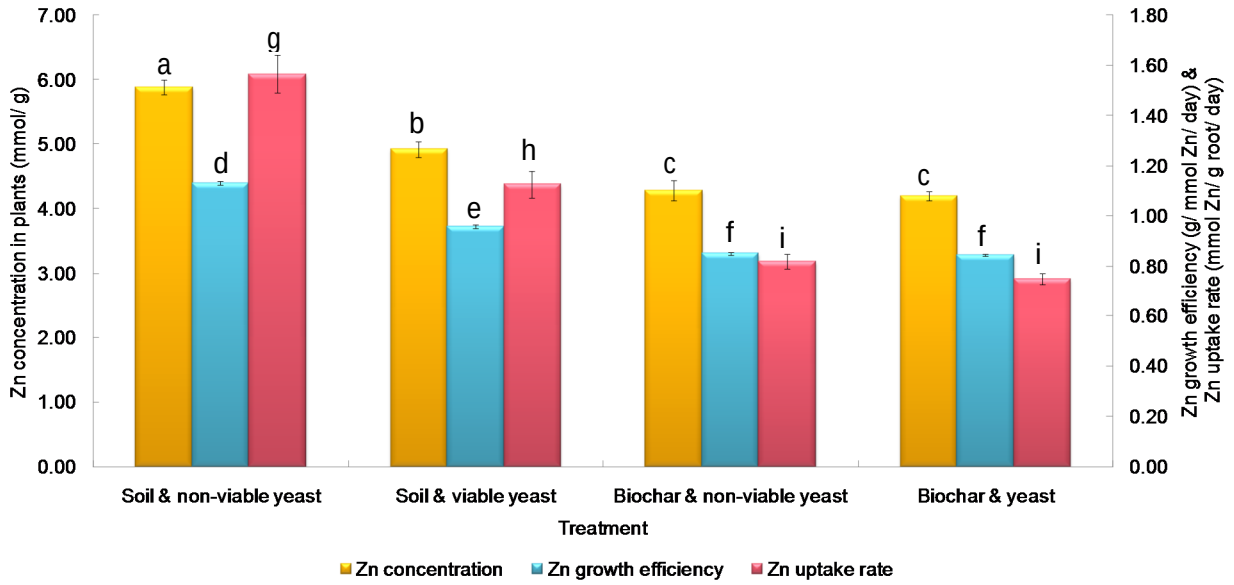

Fig. 4. Comparison between the Zn concentration, Zn growth efficiency and Zn uptake rate of wheat plants, treated before germination with autoclaved (non-viable yeast) or viable *C. zeae* cells (log 9 cells/ml). Seedlings were cultivated in soil either with or without 10% (w/w) biochar amendments for 1 month under greenhouse conditions. Each bar represents the mean obtained for 12 replicates, while the standard error values are indicated on the top of the bar. Different letters indicate significant differences among yeast and biochar treatments, separated by a Fishers LSD test ($p < 0.05$) (Letters a, b & c indicate differences in Zn concentration; d, e & f indicate differences in Zn growth efficiency; g, h & i indicate differences in Zn uptake rate).

Plants cultivated in biochar-amended soil had a lower Fe concentration compared to those cultivated in soil alone (Fig. 5). Plants growing in soil alone and treated with viable yeast cells showed a higher Fe concentration ($p = 0.000$), Fe growth efficiency ($p = 0.000$) and Fe uptake rate ($p = 0.000$) than plants growing in soil and treated with non-viable cells. Plants cultivated in biochar-amended soil and treated with viable yeast showed a lower Fe concentration ($p = 0.042$) and growth efficiency ($p = 0.000$), whilst the Fe uptake rate remained similar ($p > 0.050$) to that of plants treated with non-viable yeast and growing in the presence of biochar.

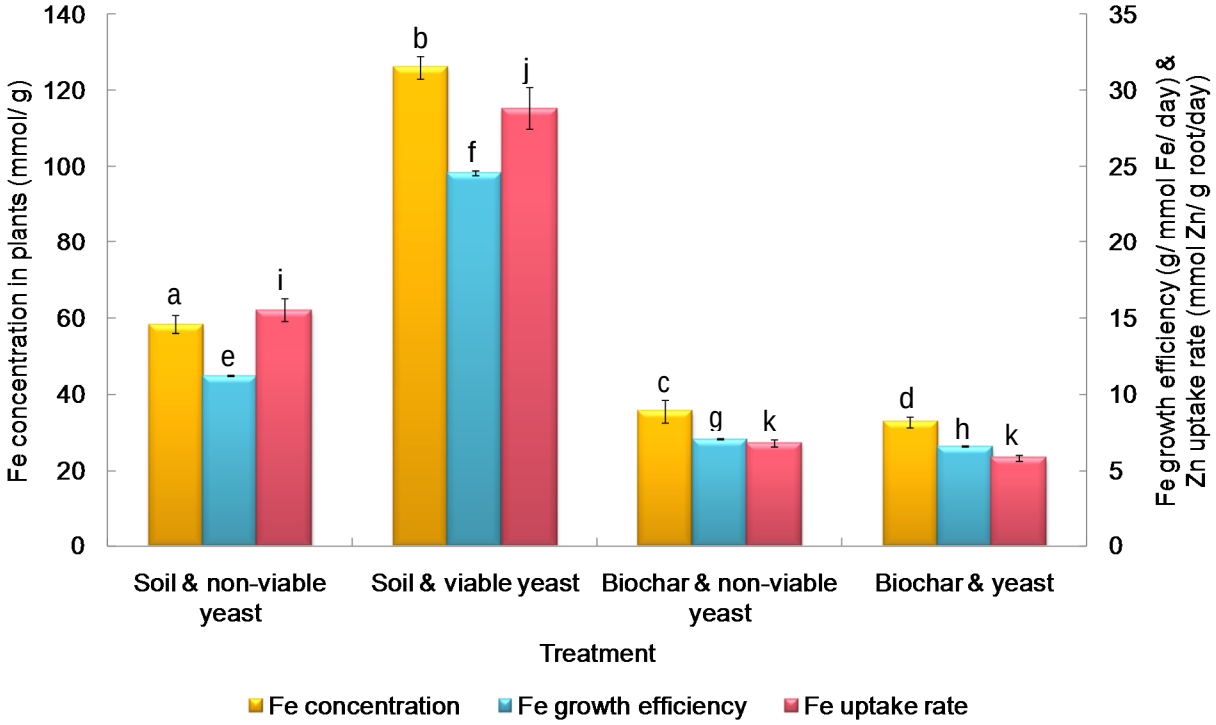

Fig. 5. Comparison between the Fe concentration, Fe growth efficiency, Fe uptake rate of wheat plants. Seeds were treated with autoclaved (non-viable) or viable *C. zeae* cells (log 9 cells/ml) and seedlings were cultivated in soil and 10% (w/w) biochar amended soil for 1 month under greenhouse conditions. Each bar represents the mean obtained for 12 replicates, while the standard error values are indicated on the top of the bar. Different letters indicate significant differences among yeast and biochar treatments, separated by a Fishers LSD test ($p < 0.05$) (Letters a, b, c & d indicate differences in Fe concentration; e, f, g & h indicate differences in Fe growth efficiency; i, j & k indicate differences in Fe uptake rate).

4. Discussion

Since knowledge on the effect of biochar and soil yeasts on wheat growth and nutritional physiology is limited, it is important to evaluate these before incorporation in soil management practices. Therefore, we firstly sought to test whether *C. zeae* can improve wheat growth and nutrition in sandy soil containing natural microbial consortia. Secondly, we wanted to study the effect of a biochar amendment on the ability of *C. zeae* to improve wheat growth and nutrition in this sandy soil.

4.1. Wheat growth and nutrient effects

Similar to the results presented in Chapters 2 and 3, we found that both seed treatment with viable *C. zeae* and biochar emendation of the sandy soil, positively affected wheat growth (Fig. 1). Interestingly, the best growth was observed in plants of which the seeds were treated with the viable yeast and that were growing in biochar-amended soil. Thus, the yeast was able to stimulate plant growth beyond the enhancing effect of the biochar amendment in the presence of natural soil microbial consortia. Root RGR was enhanced by treating plants with viable cells when cultivated in soil alone. Amending the soil with biochar also increased root RGR, whilst treating plants with viable cells and cultivated in biochar had the greatest root RGR. Thus, it seems that both yeast treatment and biochar resulted in plants investing more in root growth, than plants growing in soil alone. Plants treated with *C. zeae* while growing in biochar-amended soil also had a greater shoot RGR, thus investing more in shoot growth than other plants.

Nutrient concentration analyses revealed that the P concentration of wheat plants cultivated in 10% (w/w) biochar was more than the P concentration of wheat plants growing in soil alone (Fig. 2). The biochar, containing *ca*. 46 mg/kg bio-available P (Chapter 2; Table 1), could have acted as a P fertilizer increasing the P concentration of the plants. Yet, it was demonstrated in Chapter 2 that 10% (w/w) biochar increased the soil pH to 7.58. At this soil pH, P becomes less available in soil (Havlin et al. 2005). This should result in a decreased P uptake rate, which was witnessed in this study for plants cultivated in soil amended with 10% (w/w) biochar when compared to those cultivated in soil alone (Fig. 2). This decrease as a result of biochar emendation was not as pronounced for plants treated with viable *C. zeae* cells compared to the plants treated with non-viable yeast cells. Even though the P uptake rate of plants growing in biochar-amended soil was lower in comparison to that of plants cultivated in soil alone, it seems that the yeast enhanced the bio-availability of P despite the increased soil pH. It is well-known that many microorganisms can solubilise P by either producing organic acids (Pradhan and Sukla 2005) or phosphatases. It has been demonstrated that cryptococci can produce these extracellular enzymes (Greenwood and Lewis 1976; Garcìa-Martos et al. 2001), which can catalyse the hydrolysis of organic phosphates to inorganic phosphates (Mubyana et al. 2002), rendering them plant available (Miyasaka and Habte 2001). A recent study conducted by Cloete et al. (2010) demonstrated

that the soil yeast *C. laurentii* CAB 578 increased P concentration in the roots of the sclerophyllous shrub *Agathosma betulina* (Berg.) Pillans (buchu). It is therefore likely that *C. zeae* could produce either organic acids or phosphatases thereby increasing the P uptake and P concentration of wheat, in the presence of biochar. Plants that were treated with viable *C. zeae* cells utilized P more efficiently during growth compared to plants treated with non-viable yeast cells, regardless of whether they were planted in soil alone or biochar-amended soil (Fig. 2). This is in agreement with the results obtained in Chapter 3, where it was found that wheat plants treated with viable *C. zeae* cells utilized P more efficiently than those treated with autoclaved cells. This phenomenon is similar to that reported for mycorrhizal fungi, which was found to improve plant P use efficiency by solubilising soil P or by increasing the sorption area of the roots (Shenoy and Kalagudi 2005).

We previously reported that the biochar used in this study contains high levels of plant available K (*ca.* 878mg/kg K; Chapter 2, Table 1), which may increase the K levels in wheat plants growing in soil amended with this biochar. These results were confirmed in the present study when we found that wheat plants cultivated in 10% (w/w) biochar amended soil had a higher K concentration than wheat plants cultivated in soil alone (Fig. 3). When cultivated in soil amended with 10% (w/w) biochar, the K concentration and the K growth efficiency of plants treated with viable yeast cells were higher, compared to plants treated with non-viable yeast. The presence of viable *C. zeae* therefore seemed to render K more available to the plant. The mechanism how this enhanced bio-availability as a result of the yeast occurs is still unknown, however, it has been demonstrated that some bacteria are able to solubilise K by the production of organic acids (Friedrich et al. 1991). It was demonstrated that another member of the genus *Cryptococcus,* i.e. *C. laurentii,* is able to produce organic acids such as citric acid, lactic acid succinic acid, formic acid and acetic acid (Freitas et al. 1999). It thus seems possible that *C. zeae* could solubilise K via acidification of its microenvironment, but this must still be confirmed in future research.

In contrast to plants growing in the presence of biochar, plants cultivated in soil alone and treated with viable yeast had a lower K concentration than plants treated with non-viable yeast and growing in soil alone (Fig. 3). This is also in contrast to results obtained in Chapter 3, since it was found that K concentration was higher in plants treated with viable *C. zeae* cells compared to those treated with autoclaved *C. zeae* cells. Since the soil is deficient in K

(*ca.* 11 mg/kg K, Chapter 2, Table 1) it is likely that the yeasts and the plants competed for K. This probably resulted in K being utilized less efficiently with a lower K uptake rate for plants growing in soil alone and treated with viable yeasts, than for plants growing in soil alone and treated with non-viable yeast.

It seems likely that the emendation of soil with biochar rendered soil Zn less available to the wheat plants (Fig. 4). It is likely that Zn was less available in soil amended with 10% (w/w) biochar, since we found that biochar emendation of this soil increases soil pH (Chapter 2), which may result in decreased bio-availability of soil Zn (Clark and Zeto 1996). Wheat plants treated with non-viable yeast cells and cultivated in soil alone, showed the greatest Zn concentration, use efficiency and uptake rate, while plants cultivated in soil amended with 10% (w/w) biochar and treated with viable yeast cells had the lowest uptake rate. Yet, the Zn concentration and use efficiency of these plants did not differ from plants growing in biochar emended soil that was treated with non-viable yeast (Fig. 4). In contrast, plants cultivated under hygienic conditions that were treated with viable *C. zeae* cells utilized Zn more efficiently during growth compared to those treated with autoclaved *C. zeae* cells. Therefore, it seems possible that the yeasts and other soil microbiota competed with the wheat plants for Zn, since Zn is an important micronutrient for yeast (Stehlik-Tomas et al. 2004).

Similar to the results obtained with Zn levels, it seemed that biochar also rendered soil Fe levels less bio-available (Fig.5). Since it is known that Fe is less bio-available in alkaline and sandy soils (Fageria 2001), the low Fe concentration of plants growing in 10% (w/w) biochar may be due to increased soil pH (Chapter 2). There are indications that under the low Fe conditions induced by the biochar the yeast competed with the plants for this nutrient resulting in low Fe concentrations in the plant (Fig. 5). However, when the plants were growing in soil alone the presence of viable *C. zeae* resulted in increased Fe concentration, growth efficiency and uptake rate compared to plants treated with non-viable yeasts. Similarly, it was found that plants treated with viable *C. zeae* cells utilized Fe more efficiently than those treated with autoclaved *C. zeae* cells (Chapter 3). Similar results were obtained by Cloete et al. (2010), who found that root colonization by the soil yeast, *C. laurentii,* increased the Fe concentration of buchu. The authors stated that this might be accomplished by Fe binding to polyphosphates, which may be accumulated by the yeast or by the bioaccumulation of Fe by the unicellular fungus.

Since this study was conducted with soil containing natural soil microbiota, it is not surprising that differences in the nutritional physiology was observed between plants treated with viable *C. zeae* whilst cultivated under hygienic conditions (Chapter 3), and those treated with viable *C. zeae* cells whilst cultivated in soil (this study). It is known that microorganisms compete with each other, as well as with plants, for nutrients in the rhizosphere (Botha, 2006), thus altering the nutrition of the plants.

5. Conclusion

In this study, we found that biochar amendments to sandy soil, together with plants treated with *C. zeae* resulted in increased wheat growth. This increased growth may be attributed to the increased root and shoot RGR witnessed for these plants. Elevated K levels in the plant tissue, most likely originating from the biochar, seemed to be the main driver for the increase in shoot RGR, which is supported by the results obtained in Chapter 2. In addition, the efficient utilization of P during growth by plants treated with viable *C. zeae* cells could possibly have influenced wheat growth in a similar manner as mycorrhizal fungi. This improved growth efficiency is desirable, since plant growth and potentially yield can be increased, while the impact of agriculture on the environment is decreased.

Future research should focus on also including carbon, magnesium, manganese and boron in plant analyses in order to determine the effect of biochar and *C. zeae* on carbon cost and also the nutrition of wheat with regards to micronutrients. Furthermore, the impact of biochar on the growth and ability of *C. zeae* to colonize the rhizosphere should be investigated in pot and field trials.

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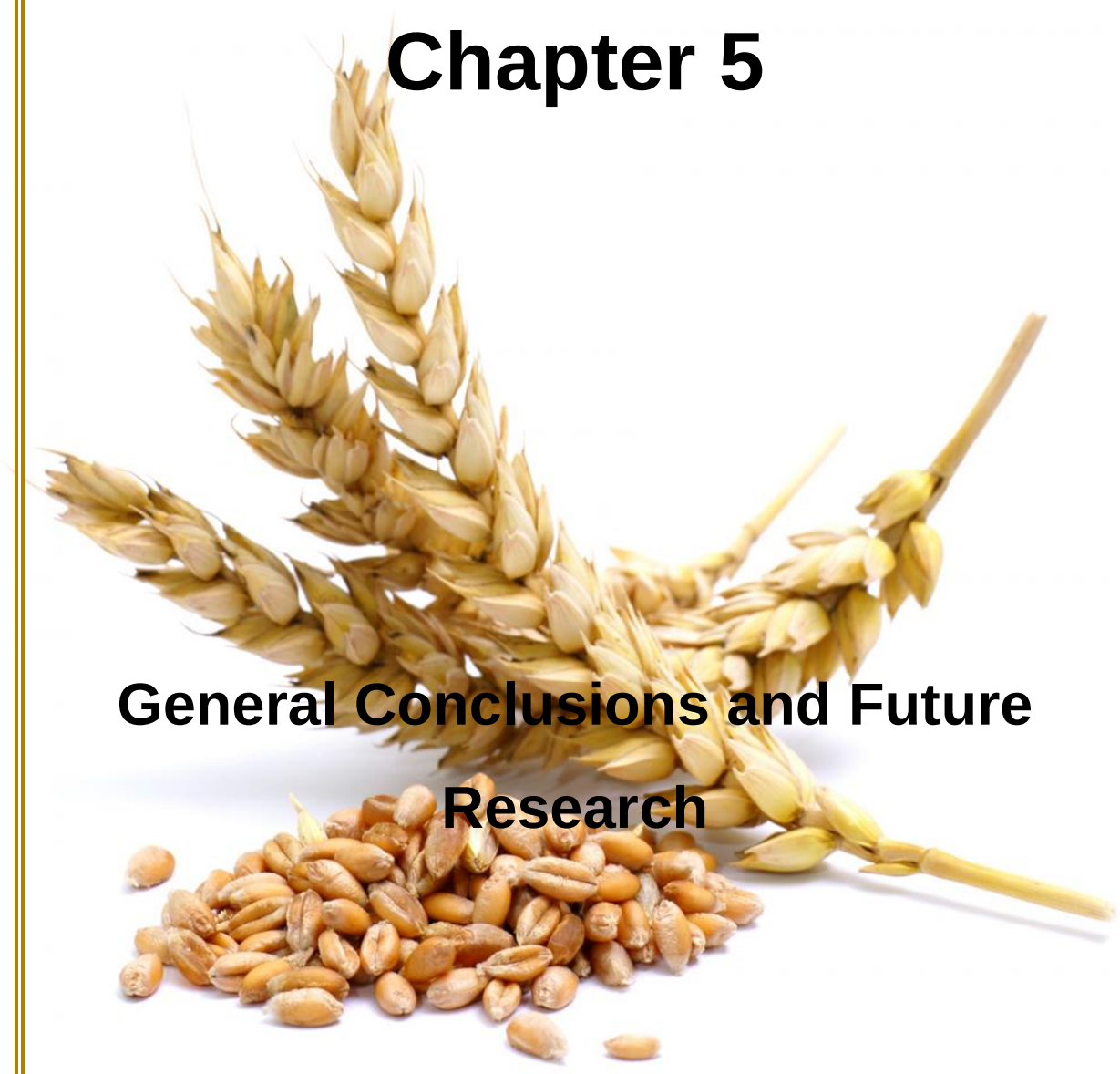
Chapter 5



General Conclusions and Future Research

1. General conclusions and future research

It is well-known that the world-population is increasing, with a projected population total reaching 9 billion people by 2050 (UN, 2010). To supply enough food to the population, food production must increase. Therefore, agricultural land must expand or cropping intensity increased (Foley et al. 2011). One strategy employed in increasing cropping intensity is the application of fertilizers, especially to underperforming soils. However, this is a costly and sometimes inefficient practice (Adesemoye et al. 2009).

There has thus been increasing interest in alternative methods to improve crop growth. These methods include the emendation of soil with biochar and utilizing soil microorganisms, such as mycorrhizal fungi and yeasts, to improve plant growth. Even though several studies could improve crop growth by using biochar applications (Kimetu et al. 2008; Mathews 2008; Spokas et al. 2009) or inoculating plants with soil yeasts (Abd El-Hafez and Shehata 2001; El-Mehalawy et al. 2004; Nassar et al. 2005), few studies have focused on the promotion of wheat growth, despite its importance as a food source.

Results obtained in the present study demonstrated that biochar amendments to sandy soil had a positive effect on wheat growth under low nutrient conditions. This concurred with other studies conducted with wheat and biochar (Solaiman et al. 2010). We found that the improved wheat growth was most likely due to the increased potassium (K) concentrations in the tissue of the plants. The increased concentrations might have facilitated shoot growth, since K has been implicated in the expansion of leaf area (Pervez et al. 2006).

During this study, we found that soil yeasts differed in their ability to improve wheat germination and growth. The isolate of *Cryptococcus zeae*, obtained from the rhizosphere of another monocot belonging to the same family, seemed superior with regards to improving wheat germination and growth, when compared to other soils yeasts, i.e. *Cryptococcus podzolicus* and *Rhodotorula mucilaginosa.* It seems likely that *C. zeae* improved wheat growth by altering the nutritional physiology of the plants, since it was found that plants treated with this yeast utilized P, K, Zn and Fe more efficiently during growth. This is supported by the findings of similar studies, where it was demonstrated that the soil yeast *Cryptococcus laurentii* could not only improve the growth, but also alter the nutritional physiology of *Agathosma betulina* (Berg.) Pillans (Cloete et al. 2009; Cloete et al. 2010).

We also found that *C. zeae* could improve wheat growth when cultivated in sandy soil, containing natural occurring microbiota, which was amended with biochar. It seems that this yeast and the biochar amendment had a synergistic effect on the growth of wheat. This increased wheat growth might be attributed to the increased root and shoot RGR witnessed for these plants. It seemed that the elevated K levels within the plant tissues, most likely originated from the biochar, and the efficient utilization of P during growth was possibly the main factors influencing the root and shoot RGR. This is in accordance with the previous results, where it was found that biochar influenced the K concentrations, whilst *C. zeae* increased the P growth efficiency. The ability of *C. zeae* to improve wheat growth by improving plant growth efficiency of plants does have potential to improve crop yield, while reducing the environmental impact of agriculture.

Future research should include field studies employing both biochar and *C. zeae* to improve wheat growth. In addition, the impact of biochar on the growth and ability of *C. zeae* to colonize the rhizosphere should be investigated. During these field studies yield, as well as grain quality, should also be monitored. In addition, the effect of *C. zeae* and biochar on carbon cost should be investigated. Furthermore, it should be determined which fertilizer concentration and biochar amendment concentration would result in the best wheat growth in the presence of *C. zeae*, without negatively affecting plant nutrition.

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