THE INFLUENCE OF SORGHUM PHYSIOLOGY ON RHIZOSPHERE INTERACTIONS AND THEIR EFFECT ON ROOT DISEASE

BY

HUNG YU CHUNG

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In the Faculty of Natural and Agricultural Sciences

Department of Plant Sciences (Division of Plant Pathology)

University of the Free State

Bloemfontein, South Africa

Promoters: Prof. W. J. Swart

Prof. N. W. Mclaren

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PREFACE

Grain sorghum (*Sorghum bicolor* L.) is one of the most important cereal crops grown worldwide and also known for its potent allelopathic properties on surrounding plants after a period of continuous monoculture. The present study investigated the allelopathic properties of grain sorghum by using both *in vitro* and *in vivo* methods. This thesis is a compilation of five independent chapters including a literature review and was written in manuscript format.

Chapter one is a literature review covering the importance of plant allelochemicals in cropping systems focusing mainly on grain sorghum. The various types and concentrations of allelochemicals in allelopathic plants, factors affecting allelochemical production and impacts of plant allelochemicals on surrounding plants, microorganisms and macroorganisms are reviewed. The impacts, secondary effects and various application methods of plant allelochemicals in cropping systems are also discussed.

Chapter two described the screening of 22 sorghum genotypes for allelochemicals and investigated the direct effects of these allelochemicals on soil-borne pathogens by using *in vitro* techniques. The phenolic extracts of four sorghum genotypes with varying plant and seed colours in addition to sorgoleone were selected for further experiments. The phenolic extracts and sorgoleone were incorporated in agar and tested against eight soil-borne pathogens isolated from diseased sorghum roots.

Chapter three outlines the direct effect of four phenolic extracts and sorgoleone on soil microbial populations and soil-borne pathogens by using *in vitro* methods. A field trial was also conducted to investigate the inhibitory effect of 22 sorghum genotypes on soil microbial populations. Microbial populations in microcosms were analysed by using FDA and PLFA analysis. The differences in pathogenicity of the eight pathogens were done by vegetative assessment and ergosterol analysis.

Chapter four describes the indirect *in vitro* effect of four phenolic extracts and sorgoleone on soil microbial populations and soil-borne pathogens. A field trial was also conducted to investigate root rot severities of 22 sorghum genotypes. The changes in microbial populations were analysed using PLFA analysis. The differences in pathogenicity of the eight pathogens in the microcosms and the root rot severity in the field trial were analysed by vegetative assessment and ergosterol analysis.

Chapter five describes the effect of crop rotation with two sorghum genotypes on soil microbial populations, sorghum root rot and yield. Differences in microbial populations were analysed by using FDA and PLFA analysis, while root rot severity and yield was analysed using vegetative assessment and ergosterol analysis.

This study will hopefully improve our understanding of sorghum allelochemicals and their effects on soil microbial populations and soil-borne pathogens. Due to the fact that each individual chapter of this thesis is independent, repetition of some results, discussion and references were unavoidable.

CHAPTER 1

THE ROLE OF PLANT ALLELOCHEMICALS IN CROPPING SYSTEMS WITH SPECIFIC REFERENCE TO SORGHUM

1.0 Introduction

Allelochemicals are biological compounds produced by plants during secondary metabolism that influence surrounding plants and other organisms (Peng, Wen and Guo, 2003). Allelopathy affords plants an advantage during competition with other plants and protection against invasion by pests and pathogens (Weir, 2007). The effect of allelochemicals was first documented by Theophrastus (ca. 300 B.C.) who noticed a negative interaction between cabbage and vine plants due to the "odours" produced by the cabbage (Willis, 1985). This effect was later termed allelopathy and defined more comprehensively as "the positive and negative effects of chemical compounds produced mainly from the secondary metabolism of plants, microorganisms, viruses and fungi that have an influence upon the growth and development of agricultural and biological ecosystems" (de Albuquerque *et al.*, 2010).

Weeds are amongst the major causes of reduced yield and productivity and weed control methods have been documented since the beginning of agriculture. These weed control methods have advanced from basic hand removal to modern methods of herbicide application, which are far more effective in limiting weed development and improving crop productivity (Jabran *et al.*, 2015). Modern agricultural weed control methods are however moving away from synthetic herbicides, due to health concerns, and the focus has shifted to searching for natural, environmentally friendly alternatives (Jabran *et al.*, 2015). Manipulating allelopathic interactions between crops and weeds has been identified as a possible alternative for applying herbicides and several allelochemicals have shown potential for being developed into commercial herbicides. Most allelochemicals are partially water-soluble, exhibit bioactivity at low concentrations and have shown profound effects on inhibiting the germination and growth of certain weeds (Vyvyan, 2001; Ilori and Ilori, 2012). Allelochemicals can influence surrounding plants both positively and negatively through

direct or indirect effects (Won *et al.*, 2013). Several studies have documented the effect of allelopathic plants such as rice (*Oryza sativa* L.), sorghum (*Sorghum bicolor* L. Moench), amaranth (*Amaranthus palmeri* L.) and mustard (*Brassica nigra* L.), on growth inhibition of weeds (Cheema and Khaliq, 1999; Turk and Tawaha, 2002; Hejl and Koster, 2004; Kong *et al.*, 2004; Xuan *et al.*, 2004; Won *et al.*, 2013).

Allelochemicals do not only affect weeds, since many other organisms can be negatively or positively influenced (Huang and Chou, 2005; Rattan 2010; Li *et al.*, 2014). Studies have shown that allelochemicals play an important role in influencing soil chemical properties and ecological processes such as decomposition and nitrogen fixation which are facilitated by microorganisms in the rhizosphere (Inderjit and Weiner, 2001; Wang *et al.*, 2011). Soil microorganisms are extremely sensitive to physical and chemical inputs and are regulated by plant species through the rhizosphere effect (Kong *et al.*, 2008). Changes in soil microorganism functional diversity or community profiles may ultimately influence soil chemical and physical properties which in turn will influence subsequent plant populations (Kong *et al.*, 2008).

In this review, the following aspects of allelopathy with special reference to grain sorghum will be discussed: a) allelochemicals produced by plants; b) the agricultural application of allelochemicals; c) secondary effects of applied allelochemicals on macro-/microorganisms; d) the effect of environmental factors on the production and effectiveness of allelochemicals.

2.0 Definition of plant allelochemicals

An early definition of plant allelopathy by Rice (1984) states that it is: “all effects of plants on neighbouring plants through the release of chemical compounds into the environment”. This definition was considered to be too broad and as studies in allelopathy

continued, several other definitions with more specificity appeared (Inderjit and Weiner, 2001). The term allelopathy was later reviewed by the International Allelopathy Society (IAS) and is currently defined as "any process involving secondary metabolites (allelochemicals) produced by plants and microorganisms that influence the growth and development of agricultural and biological systems (excluding animals), including positive and negative effects" (Eljarrat and Barcelo, 2001).

Allelopathy can be divided into a) the direct effect of a plant's allelochemicals inhibiting a range of "target" plants and b), the indirect effect of a plant's allelochemicals affecting soil ecological processes within its immediate vicinity, ultimately affecting surrounding plants as well as succeeding plants (Inderjit and Weiner, 2001; Wang *et al.*, 2011). To inhibit surrounding plants, a variety of allelochemicals are released by the "donor" plant. This could vary between water soluble organic acids, straight-chain alcohols, aldehydes, ketones, simple unsaturated lactone, long-chain fatty acids, flavonoids, tannins, terpenoids, steroids, amino acids, peptides, alkaloids or glucosinolates depending on the "donor" plant (Wang *et al.*, 2011).

2.1 Allelochemicals produced by plants

Grain sorghum is one of the most important cereal crops grown in several parts of the world. Sorghum species such as *Sorghum bicolor* subsp. *bicolor, S. vulgare, S. bicolor* var. *sudanese and S. halpense* are well known for their phytotoxic allelopathic effect on surrounding plants which may persist until the following year, injuring other crops but also suppressing weeds (de Albuquerque *et al.*, 2011). Due to its allelopathy, sorghum is often used in integrated pest management systems as green manure or cover crop which induces herbicidal effects to suppress weeds (Dayan, Howell and Weidenhamer, 2009). Sorghum produces several allelochemicals that are found throughout the entire plant, though its most

studied allelochemical, sorgoleone, is exudated from the roots (de Albuquerque *et al.*, 2010; Luthria and Liu, 2013).

2.1.1 Allelochemicals in the vegetative plant parts

Grain sorghum contains a wide variety of allelochemicals in its vegetative parts depending on the genotype. Studies have shown that a mature sorghum plant may contain more than nine water soluble allelochemicals that have been shown to be phytotoxic towards a wide variety of weeds (Cheema and Khaliq, 1999). The contents of sorghum allelochemicals consist mainly of phenolic acids, simple and complex phenolics (flavanoids and glycosides) and tannins (Dykes, Rooney and Rooney, 2013). Although these phenolic allelochemicals are just as potent as the allelochemical found in root exudates, few studies have been done to investigate their variability and the metabolic pathways involved (Won *et al.*, 2013). The wide variety of sorghum phenolics is sometimes referred to as total phenolics since they differ among genotypes of *S. bicolor* (Sene, Dore and Pellissier, 1999; Chiremba *et al.*, 2011; Won *et al.*, 2013). Within these total phenolics, Sene *et al.* (1999) identified eight phenolic acids (ferulic, *p*-coumaric, *p*-hydroxybenzoilc, vanillic, protocatechuic, syringic, caffeic and gentisic) and their associated aldehydes in soil samples previously planted with *S. bicolor* (Variety $CE_{145\text{-}66}$). Won *et al.* (2013) identified five phenolic compounds from sorghum (Variety SS-450) crude extracts: *p*-hydroxybenzoic acid, *p*-coumaric acid, *trans*-cinnamic acid, ferulic acid and kampferol.

Grain sorghum phenolics are found in significant amounts (1-2 %/dry weight) throughout the major vegetative parts of the sorghum plant, from shoot to root (Weston, Alsaadawi and Baerson, 2013). The amounts of phenolics found in the vegetative parts are also different depending on the growth stage of grain sorghum. A study done by Wong *et al.* (2013) showed significant changes in the amount of phenolic compounds in crude extracts

during different growth stages of *S. bicolor* (Variety SS-450) in 15-day-intervals for a period of 105 days.

2.1.2 Allelochemicals in root exudates

Sorgoleone, found in the root exudate of grain sorghum is one of the most studied plant allelochemicals. Sorgoleone was originally discovered in 1986 while searching for a secondary plant metabolite that triggered the germination of *Striga asiatica* (L.) Kuntze (witchweed) (Netzly and Butler, 1986; Netzly *et al.*, 1988; Dayan *et al.*, 2010). Sorgoleone is an active *p*-benzoquinone and is described as 2-hydroxy-5-methoxy-3-[(8′Z,11′Z)-8′,11′,14′-pentadecatriene]-*p*-benzoquinone [CAS 105018-76-6], it is one of the major components of the hydrophobic oily substance in the root exudates of grain sorghum (Dayan *et al.*, 2010). Sorgoleone has received lots of attention from scientists due to its allelopathic potency at very low concentrations and the fast rate at which it is produced from grain sorghum roots (de Albuquerque *et al.*, 2010).

Sorgoleone and its resorcinol analogue occurs in a 1:1 ratio and can make up 80-95 % of the total component of the entire root exudate produced by sorghum seedlings (de Albuquerque *et al.*, 2010; Weston, Alsaadawi and Baerson, 2013). The amount of sorgoleone produced by *S. bicolor* can differ significantly between genotypes and between different growth stages. In an evaluation study between seven sorghum accessions, Czarnota, Rimando and Weston (2003) showed significant differences in the amount of root exudates ranging from 0.5 mg/g (sorgoleone/root fresh weight) to 14.75 mg/g (sorgoleone/root fresh weight) between several *S. bicolor* genotypes. Another study by Uddin *et al.* (2010), showed significant differences in both sorgoleone concentrations and total root exudate content changes as the sorghum plant (cultivar Chalsusu) ages over a period of 40 days.

2.2 Inhibition by allelochemicals of surrounding plants

The direct inhibitory mechanism of allelopathy is also referred to as direct plant-plant allelopathic interference, where "plant 1" produces an allelochemical compound that influences "plant 2". This is also referred to as plant allelopathy in the narrow sense (Inderjit and Weiner, 2001). This phytotoxic reaction can be caused by two different modes of release of plant allelochemicals, in the case of the grain sorghum the allelochemicals could: firstly, be released in the form of allelopathic root exudates from living plant parts; or secondly, be released from decomposing vegetative material in the form of phenolic compounds (Weston, Alsaadawi and Baerson, 2013). Several interactions are involved in these inhibitory mechanisms and in the following section the mechanisms of the two major types of sorghum allelopathy is discussed.

2.2.1 Phenolic compounds

A wide variety of phenolic allelochemicals are found throughout the vegetative parts of grain sorghum. These allelochemicals are normally released either by decomposing sorghum residues from the previous season or sorghum mulch (Weston, Alsaadawi and Baerson, 2013). These phenolic compounds have an inhibitory effect on seedling germination and plant growth of a wide variety of weeds found in such as *Amaranthus retroflexus* L.*, Chenopodium album* L., *Convolvulus arvensis* L., *Echinochloa crus-galli* L., *Rumex denatatus* L., and *Phalaris minor* Retz. (Cheema and Khaliq, 1999; Won *et al.*, 2013). This inhibition of germination can be associated with direct inhibitory properties of phenolics and tannins such as inhibition of plant root elongation and plant cell division (Ilori and Ilori, 2012; Won *et al.*, 2013). Won *et al.* (2013) demonstrated significant growth inhibitory effects of up to 79.2 % for *A. retroflexus* and 53.6 % for *E. crus-galli* by foliar sprays containing phenolic extracts of sorghum leaves. This inhibition of weeds can also be caused

by inhibition of other plant processes such as photosynthesis, and ion uptake as demonstrated by Yu *et al.* (2002) who used phenolic cucumber (*Cucumis sativus* L.) root extracts containing benzoic and cinnamic acids to inhibit the growth of maize (*Zea mays* L.) and soybean (*Glycine max* L.).

2.2.2 Sorgoleone

Sorgoleone is a major component of root exudates produced by living grain sorghum. It has the ability to suppress a very wide variety of small seeded weeds and is potent even at extremely low concentrations of 10 µM treatments (Einhellig and Souza, 1991). This inhibitory property of sorgoleone is comparable to a pre-plant incorporated herbicide that gives sorghum seedlings an advantage during competition with surrounding plants (Milchunas *et al.*, 2011).

Sorgoleone inhibits plant growth at a molecular level. The allelochemical significantly inhibits the photosynthetic and the mitochondrial electron transport of the target plant (Dayan *et al.*, 2010). This results in the inhibition of photosynthesis in the target plant and the effects are found to be most effective on seedlings 4 days or younger (Dayan *et al.*, 2010). Dayan Howell and Weidenhamer (2009) discovered that compared to young seedlings, older plants do not translocate sorgoleone acropetally. Sorgoleone is also able to inhibit plant growth by inhibiting the H^+-ATPase activity of the target plant which ultimately leads to impaired water uptake by the target plant (Weston, Alsaadawi and Baerson, 2013).

2.3 Effects of plant allelochemicals

Recent studies on plant allelochemicals have focused on utilising plant allelopathy as an alternative pesticide by manipulating their plant–plant, plant–insect and plant–macro-microorganism inhibitory effects. Soil applications of plant allelochemicals in the form of

mulches, crop residue or cover crops significantly affect soil ecology within an agroecosystem (de Albuquerque *et al.*, 2010). Several studies have shown that plant allelopathy significantly impacts soil microorganisms, ultimately influencing decomposition, nutrient cycling, mineral transformation and fertility recycling within soil (Kong *et al.*, 2008; Gimsing *et al.*, 2009; Li *et al.*, 2014). Soil micro- and macroorganisms are mostly responsible for these ecological cycles and have been shown to be extremely sensitive to physical and chemical changes in the soil (Inderjit *et al.*, 2011). Several studies have shown the pesticidal effects of several allelochemicals on soil micro- and macroorganisms (Kong *et al.*, 2004; Zhang *et al.*, 2008; Gong *et al.*, 2013).

2.3.1 Effect of plant allelochemicals on soil organisms

2.3.1.1 Impact on microorganism diversity

Several studies have investigated the "soil sickness" phenomenon caused by allelopathy in fields that have been subjected to monocropping for several successive seasons (Weston, Alsaadawi and Baerson, 2013). Many of these studies related this "sickness" to the build-up of plant allelochemicals that severely affected potential crop yield. Recent studies however, have related the effect to the changes in microorganism facilitated ecological processes (Inderjit and Weiner, 2001; van der Heijden, Bardgett and Straalen, 2008).

Allelochemicals can affect soil microorganisms in two ways, either by inhibiting them or promoting their growth (Kong *et al.*, 2008; Gimsing *et al.*, 2009). Either of these effects ultimately influences the diversity and structure of soil microorganisms. Li *et al.* (2014) investigated the yield loss in replanted ginseng (*Panax ginseng* Meyer) crops due to changes in soil ecological processes. Following metabolic and molecular analysis, a severe decrease in microorganism diversity was shown that significantly changed the intrinsic carbon metabolic functions in the replanted soil compared to control treatments. Kong *et al.* (2008)

investigated allelopathy of rice root exudates and showed that allelochemicals produced by allelopathic rice seedlings are able to reduce the soil microorganism population and influence their community structure in a rice paddy compared to non-allelopathic rice. It was suggested that the allelopathic rice modified the microbial community to its advantage.

2.3.1.2 Impact on soil-borne fungal pathogens

Soil-borne fungal pathogens such as *Fusarium* spp., *Rhizoctonia solani* Kühn and *Pythium* spp. are some of the most resilient plant pathogens with a wide range of potential host plants. They can cause a wide variety of diseases such as root rot, stem rot, damping-off and wilting and can remain dormant within plant residues for several years without the presence of host plants (Huang and Chou, 2005; Wu *et al.*, 2008; Wu *et al.*, 2013). Strategies for controlling these pathogens often involve sterilising soil, applying fungicides and rotation management systems. Recently however, studies on the potential herbicidal effect of plant allelochemicals on weeds have demonstrated the inhibitory effect of allelochemicals on soil-borne fungal pathogens (Xuan *et al.*, 2004; Zhang *et al.*, 2008).

Several studies have shown significant inhibitory effects of plant allelochemicals on the mycelial growth, conidia formation and spore germination in soil-borne fungal pathogens such as *Bipolaris sorokiniana* Sacc.*, Gaeumannomyces graminis* Sacc.*, Pyricularia oryzae* Cavara and *R. solani* (Kong *et al.*, 2004; Qi, Zhen and Li, 2015). Kong *et al.* (2004) demonstrated significant inhibition of the growth and spore germination of two soil-borne pathogens *P. oryzae* and *R. solani* by salicylic acid extracts from rice plants. Salicylic acid successfully inhibits spore germination, hyphal growth and conidia formation of *Fusarium oxysporum* f.sp. *niveum* (Wu *et al.,* 2008). Due to the changing nature of allelochemicals in soil however, most studies have only observed these effects *in vitro* under controlled environments. Other studies have shown successful inhibition of soil-borne fungal pathogens

in the field by using plants extracts and the incorporation of plant residues in soil, in addition to planting cover crops (Zhang *et al.*, 2008; Zhang *et al.*, 2011). Xuan *et al.* (2004) screened the effects of more than 30 species of allelopathic plants against weeds and soil-borne pathogens under field conditions and observed successful inhibition of *Fusarium solani* Sacc., *Pyricularia grisea* Sacc. and *Rhizoctonia stolonifer* Kühn by allelochemical extracts from *Piper methysticum* Forst.

2.3.1.3 Impact on nematodes

Nematodes inhabit all types of soil with sufficient moisture and survive by consuming microorganisms or attacking plants both below- and aboveground (Kleynhans *et al.*, 1996). Several species of nematodes are important soil-borne pathogens on a wide variety of crop plants (Hooks *et al.*, 2010). Control methods usually include the use of chemical nematicides (Akhtar and Malik, 1999) and although applying nematicides is a quick and effective way to control nematodes, they have several hazardous effects on the environment (Akhtar and Malik, 1999).

The use of crop rotation and green manure to control nematodes has been practiced by farmers for centuries (Akhtar and Malik, 1999) oblivious of the fact that allelochemicals were involved. Several crops such as neem (*Azadirachta indica* Juss.), marigolds (*Tagetes* spp.), rapeseed (*Brassica napus* L.), lemon-scented gum (*Eucalyptus citriodora* Hook.), and chinaberry (*Melia azedarach* L.) have suppressive allelopathic effects on nematodes (Akhtar and Malik, 1999; Gong *et al.*, 2013) due to allelochemicals released by decomposing plant residues and plant root exudates (Ruess *et al.*, 1997). Viaene and Abawi (1998) reported suppression of the northern root-knot nematode (*Meloidogyne hapla* Chitwood) on lettuce by using sudangrass (*Sorghum sudanense* L.) as a green manure incorporated into the soil, and rye (*Secale cereale* L.) and oats (*Avena sativa* L.) as alternative crops. Suppression of the

root-knot nematode (*Meloidogyne incognita* Chitwood) on tomato using garlic (*Allium sativum* L.) straw incorporated into soil has also been reported (Gong *et al.,* 2013).

2.3.1.4 Impact on insects

For the past century, the most effective method of insect control has been the use of chemical insecticides (Haouas *et al.*, 2011; Karbache, Mouhouche and Fleurat-Lessard, 2011). The major disadvantages of chemical insecticides are residues that remain on the crop after harvesting (Rattan, 2010). Over the past few decades, countless studies have focused on finding alternative substitutes for chemicals that cause less environmental stress and pose less harm to humans (Haouas *et al.*, 2011; Gahukar, 2012). A large number of plants that produce allelochemicals with potent insecticidal properties have been discovered (Klocke, 1989; Gahukar, 2012). Insecticidal allelochemicals reduce insect pest damage by either having a deterrent effect or a toxic effect that kills herbivorous insects and their larvae when feeding on the plant (Rattan, 2010). Two of the most effective plants with insecticidal properties are Indian neem and chrysanthemums (*Chrysanthemum spp.)* (Gopal *et al.*, 2006; Haouas *et al.*, 2011). Extracts of *Chrysanthemum macrotum* (D.R.) Ball leaves mixed in an artificial diet fed to cotton leafworm (*Spodoptera littoralis* Boisduval) caterpillars showed a significant reduction of up to 5.03 mg/mg/day in the growth rate and up to 100 % mortality rates in the caterpillars (Haouas *et al.,* 2011). Extracts from neem kernels sprayed on rice plants and fed to the nymphs of the brown planthopper (*Nilaparvata lugens* Stål), induced a significant mortality rate of up to 70 % and reduction in weight of up to 22 % in the target nymphs compared to the control (Nathan *et al*., 2006). Bean extracts (*Phaseolus vulgaris* L.) mixed with artificial meal fed to cowpea weevils (*Callosobruchus maculatus* Fabricius) showed inhibitions in fecundity to < 25 % and oviposition rates to 50 % in the target weevils compared to the control (Karbache *et al*., 2011). Plant phenols, flavanoids, and α,β-

unsaturated carbonyl compounds are found to inhibit the detoxification enzyme (glutathione *S*-transferases) in the midgut of fall army worm (*Spodoptera frugiperda* Smith) larvae ultimately killing the larvae (Yu and Abo-Elghar, 2000). Appel and Maines (1994) investigated the *in vivo* effects of plant leaves on gypsy moth (*Lymantria dispar* L.) caterpillars and related decreases found in midgut pH of the caterpillars with tannins present within the leaves, resulting in significantly increased caterpillar mortality (Appel and Maines, 1994).

3.0 Exploitation of allelopathy in agriculture

Allelopathy is often exploited as an alternative weed control method that is inexpensive and environmentally friendly (Milchundas *et al.*, 2011; Mhlanga *et al.*, 2014; Jabran *et al.*, 2015). There are two basic methods of exploiting allelopathy in agroecosystems, firstly, the application of allelopathic plant material or allelochemical extracts to soil and secondly, by means of cover cropping, intercropping or in rotation with other crops.

3.1 Application of allelopathic extracts and plant material

Allelochemicals can be applied directly to soil by means of extracts or the incorporation of green plant material. Several potent allelochemicals can be extracted by simple methods and a distinction is made between water-soluble extracts and hydrophobic extracts (Eljarrat and Barcelo, 2001). Some phenolic compounds found within living and decomposing plant material of allelopathic plants such as *S. bicolor* are water-soluble and can easily be extracted by soaking in water for a period of time (Weston, Alsaadawi and Baerson, 2013). Water extracts of mature sorghum plant material applied to weeds such as *Fumaria indica* Hauskn., *Phalaris minor* Retz., *Rumex denatatus* L. and *Convolvulus arvensis* L. by

means of foliar sprays showed significant mortality rates of up to 40.8 % and reductions of up to 56 % weed dry mass in the treated weeds compared to the control (Cheema and Khaliq 1999). Water extracts of *Cassiope tetragona* D.Don and *Empetrum hermaphroditum* L. leaves applied to soil nematode communities resulted in significantly lower nematode diversity and showed maturity indexes (MI) ranging between 1.48 and 1.76 in extract treated soils compared to 2.02 in the untreated control (Ruess *et al.,* 1997). Water extracts of black mustard *(Brassica nigra* L.) plant parts (leaf, stem, root and flower) applied to wild oats (*Avena fatua* L.) resulted in significant reductions in seed germination and emergence of up to 56.1 % compared to the control (Turk and Tawaha, 2002). Water extracts of roots, stems and leaves of ginger (*Zingiber officinale* Rosc.) applied to soybean and chives showed significant reductions in seed germination and seedling growth of up to 100 % in both crops (Han *et al.*, 2008). Water extracts of ground alfalfa (*Medicago sativa* L.) leaves showed autotoxic effects in inhibitions of > 95 % in root length of both alfalfa and barnyard grass compared to the control (Chon *et al.*, 2002). Water extracts of sugarcane straw (19 to 64 g/L water) applied to arrowleaf sida (*Sida rhombifolia* L.) showed significant inhibitions of up to 56.2 % in the root length of the treated plant compared to the control (Sampietro *et al.,* 2007).

Hydrophobic allelochemicals require the use of methanol or acetone for extraction and the crude extract can then be mixed with a dispersing liquid e.g. water or dried into powder form (Uddin *et al.*, 2010). The inhibitory effects of such crude formulations have been demonstrated extensively in several studies. Sesquiterpernoids extracted from the aerial parts and seeds of the *Maytenus* species and fed to *Drosphila melanogaster* Meigen larvae, showed significant insecticidal activity of mortality rates of up to 100 % during three days of feeding with concentrations ranging between 8.3 to 37.6 µmol/ml artificial diet (yeast, glucose, agar and propionic acid mixed in water) (Alarcon, Lamilla and Cespedes, 2012). Extracts from leaves of *Chrysanthemum macrotum* (D.R.) Ball applied to caterpillars of

Spodoptera littoralis Boisduval displayed significant insecticidal effects of up to 100 % with concentrations ranging between 0.1 to 10 mg/g artificial diet (Haouas *et al.*, 2011). Ethanol extracts from roots of *Solidago canadensis* L. applied to fungal plant pathogens such as *R. solani* and *Pythium ultimum* Trow in petri dish, greenhouse and field experiments showed suppression in the growths of up to 84 % (*R. solani*) and 91 % (*P. ultimum*) at 25 µl/ml agar compared to the control (Zhang *et al.*, 2008). The extracts also showed significantly reduced mortality rates of 43.63 % (*P. ultimum*) and 36.67 % (*R. solani*) and reduced damping-off rates of 81 % (*P. ultimum*) and 50.34 % (*R. solani*) in treated tomato plants compared to the control (Zhang *et al.*, 2008). Root exudate extracts of sorghum ranging between 10 to 100 µM applied to six field weed seedlings (*Abutilon theophrasti* Medik, *Amaranthus retroflexus* L., *Datura stramonium* L., *Digitaria sanguinalis* (L.) Scop, *Echinochloa crusgalli* (L.) Beauv., and *Setaria viridis* (L.) Beauv.) in an aqueous growth system for a duration of 10 days showed a lower plant weight in all weeds of up to 78 % compared to the control (Einhellig and Souza, 1991). Ethanol extracts (between 25 to 200 mg/L algae) of ground tree bark from *Ailanthus altissimoa* (Mill.) Swingle applied to an environmentally hazardous algal species (*Microcystis aeruginosa* Kütz.) showed significant inhibition of up to 91.83 % algal growth compared to the control (Meng *et al.*, 2015). Root exudate extracts of taro (*Colocasia esculenta* Schott) showed significant autotoxic growth inhibition of up to 46 % in plant fresh weight and root length in treated taro seedlings compared to the control (Asao *et al.*, 2002). Methanol extracts of cucumber root exudate showed significant autotoxic inhibition effects of up to 87 % in leaf transpiration and up to 83 % in photosynthesis in treated cucumber seedlings compared to the control (Yu *et al.*, 2002).

Allelochemicals can also be applied in the form of plant material and release the allelochemical through the decomposing plant material or ground plant material that can be consumed by macroorganisms (Gopal *et al.*, 2006; Karcache, Mouhouche and Fleurat-

Lessard, 2011; Qi, Zhen and Li, 2015). In a study done by Gong *et al.* (2012) the allelopathic effect of soil incorporation of garlic straw on root-knot nematodes showed mortality rates of up to 98 % and decreased egg masses of up to 51.9 % in treated soils compared to the control.

3.2 Intercropping, cover crop and crop rotation

Intercropping or multi-cropping implies planting two or more crops in the same planting season and in the same field (de la Fuente *et al.*, 2014). Compared to commercial monocropping, there are several benefits of intercropping that include reduced disease and pest pressure, improved biological diversity and the creation of natural buffers against the spread of pests and pathogens (Gomez-Rodriguez *et al.*, 2003; Qin *et al.*, 2012; Weston, Alsaadawi and Baerson, 2013). Intercropping with allelopathic crop plants can enhance these benefits by also inhibiting weeds (Fenández-Aparico, Sillero and Rubiales, 2006; Jabran *et al.*, 2015).

Intercropping with allelopathic plants will release allelochemicals both during the planting season and from decomposing debris after the primary crop has been harvested in a minimum tillage cropping system (Weston, Alsaadawi and Baerson, 2013). Sunflower intercropped with soybean showed a land equivalent ratio (LER) of 1.27 compared to mono crop sunflower and soybean indicating an overall yield increase without increasing the species richness and abundance of surrounding weeds and insects (de la Fuente *et al.,* 2014). Rice intercropped with water chestnut (*Eleocharis dulcis* (Burm. f.) Trin. ex Hensch.) can suppress both rice sheath blight and rice blast improving their fresh weights to up to 33.9 % and showed a LER of 1.70 compared to monocrop rice (Qin *et al.,* 2012). Maize intercropped with peanuts (*Arachis hypogaea* L.) showed a significant improvement in microbial functional diversity, composition, enzyme activity and soil nutrients compared to monocrop systems (Li *et al.*, 2015). Fenugreek (*Trigonella foenum-graecum* L.) intercropped

with other legumes showed a significant suppressive effect in the infection of an important holoparasitic plant boomrape (*Orobanche* spp.) of up to 57 % under laboratory conditions and up to 40.5 % under field conditions compared to the control (Fernández-Aparicio, Emeran and Rubiales, 2007). Rice intercropped with watermelon (*Citrullus lanatus* Trunb.) suppressed *F. oxysporum* f.sp. *niveum* infection of watermelons to 0 % infection and mortality compared to the 66.7 % infection and 44.4 % mortality of the monocrop watermelon. In addition, the fresh weight of the intercropped watermelon plants were significantly improved to 10.64 g compared to the 3.72 g of the monocrop control (Ren *et al.*, 2007). Several *Desmodium* species intercropped with sorghum showed significant inhibition of up to 75 % weed emergence of the parasitic weed *Striga hermonthica* (Delile) Benth. by the allelopathic root exudate of *Desmodium* spp. under field conditions compared to the monocrop control (Hooper *et al.*, 2015).

Cover cropping implies growing a fast growing crop plant between cropping seasons which is killed off and either worked into the soil or used as a mulch to improve soil nutrients, increase soil organic matter and prevent soil and water erosion (Milchunas *et al.*, 2011; Brust, Claupein and Gerhards, 2014). Planting allelopathic cover crops not only reduces weed infestation but also reduces nematode and fungal pathogen build-up and improves soil microbial properties during crop intervals (Viaene and Abawi, 1998; Hooks *et al.*, 2010). The crops operate by releasing allelochemicals from living plants as well as from decomposing plant material when it is incorporated as green manure or a mulch (Weston, Alsaadawi and Baerson, 2013; Jabran *et al.*, 2015). The incorporation of sudangrass (*S. sudanense*) as green manure on lettuce (*Lactuca sativa* L.) can successfully inhibit nematode egg production to 710 eggs per root compared to 3217 eggs per root of the untreated control (Viaene and Abawi, 1998). The planting and incorporation of oat as a cover crop showed significantly reduced emergence of weeds such as *Lolium* spp., *Papaver rhoeas* L., *Stellaria*

media L., *Fumaria officinalis* L., *Veronica persica* L. and *Galium aparine* L. to 10.2 plants/m² compared to 54.2 plants/m² of the control. In addition, the incorporation of hairy vetch (*Vicia villosa* Roth.) as a cover crop significantly improved yield of tomatoes (*Lycopersicon esculentum* Mill.) of up to 99.7 t/ha compared to 77.7 t/ha of the control (Campiglia *et al.*, 2009). The planting of rye cover crops as well as applying compost inoculated with the soil antagonist *Trichoderma virens* (J.H. Mill., Giddens & A.A. Foster) Arx, reduced both grassy and broadleaf weeds to 17 g dried weed mass/m² compared to 412 g dried weed mass/m² of the compost treated control in pumpkins (Heraux, Hallett and Wellar, 2005). The planting and converting of oats from a cover crop into mulch, significantly reduces the density of weed such as *Sinapis arvensis* L., *P. rhoes*, *S. media*, *V. persica*, *F. officinalis*, *G. aparine*, *Ammi majus* L., and *Lolium* spp. of up to 97 % in pepper (*Capsicum annuum* L.) compared to the control treatment without the oat cover crop (Radicetti, Mancinelli and Campigila, 2012). The incorporation of mustard (*Sinapis alba* subsp. *mairei*) winter cover crop residue in olive groves showed a delayed appearance and significant decrease of up to 60 % in the density of summer weed *Amaranthus blitoides* S.Wats. and *Chenopodium album* L. compared to the untreated control (Alcántara, Pujadas and Saavedra, 2011). The incorporation of mulches as undersown crops of several cover crops (*Pisum sativum* L., *Trifolium repens* L., *Trifolium subterraneum* L., *Trifolium pratense* L., *Festuca rubra* L., *Cannibis sativa* L., *Raphanus sativus* (L.) Domin, and *Avena strigosa* Schreb.) significantly reduced biomass of several weeds such as *Alopecurus myosuroides* Huds., *Abutilon theophrasit* Medik., *G. aparine*, and *Lamium purpureum* L. of up to 91 % in barley (*Hordeum vulgare* L.) and up to 70 % in wheat (*Triticum aestivum* L.) compared to the untreated control (Rueda-Ayala, Jaeck and Gerhards, 2015).

The correct choice of crops for intercropping and cover cropping is therefore of utmost importance and several studies have demonstrated the negative effects of allelopathy

affecting current or succeeding crops (Sene, Dore and Pellissier, 1999; Dayan, Cantrell and Duke, 2009; de Albuquerque *et al.*, 2010). Well known allelopathic crops such as sorghum for example are found to not only suppress weeds but also inhibited the growth and germination of peanuts, alfalfa, wheat and lucerne that are planted in the following season (Sene, Dore and Pelissier, 1999; Weston, Alsaadawi and Baerson, 2013). The incorporation of different concentrations (1, 2 and 4 %) of raw garlic straw mulch water extracts (2:1 w/w) was found to not only have a higher relative control efficacy (RCE) of up to 82 against the root-knot nematode (*Meloidogyne incognita* Chitwood) in tomato crops compared to the control but the higher concentration mulch was also found to inhibit the tomato seedlings of up to 19 cm in plant height and 0.23 cm in stem diameter compared to the untreated control (Gong *et al.*, 2013). Dhima *et al.* (2012) investigated the effects of multiple sunflower (*Helianthus annuus* L.) hybrids with allelopathic properties rotated with lentils (*Lens culinaris* Medik.) and a weed known as ivy-leaved speedwell (*Veronica hederifolia* L.). The authors found significant inhibition of up to 22.6 % in plant number and 28 % in plant fresh weight of the ivy-leaved speedwell and significant reduction of up to 7.3 g in plant dry mass and 13 % seed yield of the lentils by the sunflower residues compared to the control.

4.0 Secondary effects of allelochemicals

Most studies on the application of allelochemicals have focused on the direct inhibitory effects of one plant on a range of "target" plants, insects and soil-borne pathogens. Recent studies have however focused on several indirect interactions involved in allelopathy (Inderjit and Weiner, 2001). Allelochemicals can affect the surrounding agroecosystem via indirect toxicity on soil organisms, thereby affecting their diversity, and also via indirect changes to soil chemical content (Inderjit and Weiner, 2001).

Indirect allelopathic effects can sometimes be neglected and result in "soil sickness" often observed in monocultured crops. Decreases in growth and yield of cereals such as rice and sorghum due to continuous planting have been related to build of pathogens, depletion of nutrients and build up toxic allelochemicals (Nishio and Kusano, 1975; Einhellig and Souza, 1991; Nie *et al.*, 2008; Weston, Alsaadawi and Baerson, 2013). Li *et al.* (2014) demonstrated that soil sickness associated with peanuts is caused by changes in soil microbes due to the plant allelochemicals and not by the direct phytotoxicity of the peanut allelochemical. Extracts from peanut root exudates applied to simulated rhizosphere soil showed disappearances of plant-growth-promoting rhizobacteria such as *Burkholderia soli* and *Mitsuaria chitosanitabida* and the increase in abundance of *Fusarium oxysporum* Schlecht., *Didymella macrostoma* Mont. and *Bionectria ochroleuca* (Schwein.) Schroers & Sameuls with the gradual addition of the root extracts. Similarly, Lorenzo, Pereira and Rodríguez-Echeverría (2012) showed that the functional and genetic diversity of soil bacteria are significantly changed by plant allelochemicals present within the natural canopy leachate of an invasive species of tree legumes (*Acacia dealbata* Link) in pine forests, which ultimately contributes to their process of invasion.

4.1 Edaphic factors affected by allelochemicals

Both the planting of allelopathic plants and the incorporation of allelopathic plant material into soil can significantly change the physical, chemical and nutrient properties of soil (Inderjit and Weiner, 2001). During the growth of a plant, soil moisture, texture and structure can change due to the development of plant roots, cover provided by the plant canopy, the absorption of soil water by the plant and the incorporation of plant residues after harvesting (Mhlanga *et al.*, 2014; Jabran *et al.*, 2015). These changes can either be beneficial or devastating to an agroecosystem.

Plant allelochemicals such as phenolic acids and terpenoids can directly affect the accumulation of N, Fe, Mn and Al in soil, lowering soil pH and affecting the solubility of Mn and Fe in soil (Inderjit and Weiner 2001). A significant decrease of up to 1.91 % in organic matter, up to 0.062 mg/100g (soil) in phosphate, up to 31.2 mg/100g (soil) in Fe^{3+}, up to 2.37 mg/100g (soil) in Mn^{2+} and up to 0.797 mg/100g (soil) in Al^{3+} was observed in soil amended with water extracts of five phenolics compounds (ferulic, p-courmaric, p-hydroxybenzoic, catechol, and protocatechuic acids) compared to the control treatment (Inderjit and Mallik, 1996). The invasive allelopathic plant *Prosopis juliflora* (Sw.) DC. on the other hand showed higher soil organic matter (13.56 %), K (2.85 mEq/L), N (1.9 ppm) and P (0.15 ppm) in soil beneath the *P. juliflora* canopy compared to the adjacent soil (El-Keblawy and Abdelfatah, 2013).

4.2 Effects of allelochemicals on surrounding plants

Indirect allelopathic effects on surrounding plants can either promote or inhibit them. Surrounding plants can be affected by changes in microorganism mediated soil ecological processes (van der Heijden, Bardgett and Straalen, 2008) or by changes in the availability of nutrients in the soil that can influence the presence of soil-borne pathogens and pests. The indirect effect of plant allelochemicals on soil ecosystem processes are often more important for surrounding plant communities than the direct effects (Inderjit and Weiner, 2001; Gomez-Rodriguez *et al.*, 2003; Weir, 2007). For example, reduced weed infestation could be due to a shading effect by adjacent intercrops, changes in microbial communities and changes in root colonization by arbuscular mycorrhizal fungi (Fenandez-Aparicio, Sillero and Rubiales, 2006).

5.0 Factors affecting allelochemical production

Due to the hazardous nature of synthetic pesticides, the need for finding environmentally friendly alternatives have led to numerous studies focused on determining the effectiveness of plant allelochemicals as herbicides (Hooks *et al.*, 2010; Haouas *et al.*, 2011; Karbache, Mouhouche and Fleurat-Lessard, 2011). Although several studies have successfully demonstrate the inhibitory effects of allelochemicals comparable to synthetic pesticides, experiments are often done *in vitro* and inhibition is inconsistent with *in vivo* trials (Ruess *et al.*, 1997; Wu *et al.*, 2013). These inconsistencies can be due to either inefficient application methods, mineralisation of allelochemicals by microorganisms or various biotic and abiotic factors that can influence the effectiveness of the applied allelochemical (Peng, Wena and Guo, 2004; Inderjit *et al.*, 2011). The production and effectiveness of allelochemicals are also dependent on the physiological state of the plants, as determined by their natural biotic and abiotic environment. Changes in temperature, moisture, photoperiod, mineral nutrients, plant pests, and soil properties, can, for example, either increase or decrease the production or allelochemicals and/or effectiveness of allelopathy in general (Rivero *et al.*, 2000; Rivoal *et al.*, 2011).

5.1 Factors influencing allelopathy

The production of allelochemicals and the allelopathic potential of plants are directly related to the growth of a donor plant and is mainly dependent on two factors: the current growth stage or age of the plant and external forces that influence the plant's physiological processes (Peng, Wen and Guo, 2004; Uddin *et al.*, 2010). Allelopathic plants produce the highest concentration of allelochemicals during their seedling stage and the inhibitory effect of allelochemicals can in fact improve survivability of the donor's seedlings (Uddin *et al.*, 2010; Won *et al.*, 2013). The allelochemical concentration in root exudates of sorghum

plants is highest in a 5-day-old seedling and decreases sharply as the plant ages (Uddin *et al.*, 2010). This implies that sorghum seedlings can be grown as a cover crop to take full advantage of its strong allelopathy during its seedling stage.

Changes in photoperiod or changes in ambient temperature and soil properties (nutrient content, pH, structure, moisture content or texture) directly affects the physiological processes of a plant that can either stress it or enhance growth (Karageorgou, Levizou and Manetas, 2002; Lobon *et al.*, 2002; Rivoal *et al.*, 2011). The allelopathic potential of gum rockrose (*Cistus ladanifer)* showed that high temperature and prolonged photoperiod increased the inhibitory effects of allelochemicals (Lobon *et al.*, 2002). It is also well known that allelopathic plants experiencing environmental stress increase the production of allelochemicals that improves the plant's competitiveness and survivability (Blanco, 2007). Studies have shown that seasonal and weather changes significantly influence the allelopathic potential of plants (Peng, Wen and Guo, 2003).

Another important factor that can affect the allelopathic potential of a plant is the availability of nutrients in the soil. A lack of mineral nutrients due to poor agricultural management or competition between plants can increase the production of allelochemicals in allelopathic plants similar to the defense response of allelopathic plants suffering from pathogen and pest attacks (Song *et al.*, 2008). For example, the allelopathic potential of some rice cultivars is enhanced by low N conditions (Song *et al.,* 2008). In contrast, the allelopathic potential of some plants can be enhanced by the addition of nutrients. This phenomenon can be found in plants suffering from other natural stress factors that utilise nutrients to increase the concentration or amount of allelochemicals produced (Karageorgou, Levizou and Manetas, 2002). Karageorgou *et al*. (2002) demonstrated that the allelopathic potential of *Dittrichia viscose* was highest in plants that suffered from water stress and had sufficient nutrients compared to plants suffering from both water and nutrient stress.

5.2 Factors influencing allelochemicals

Natural factors can indirectly affect the effectiveness of applied allelochemicals in several ways. Firstly, by changing the rate of microbial decomposition of the plant material; secondly, by influencing the mineralisation rate of the released allelochemical post decomposition; and thirdly, by influencing the target plant (Peng, Wen and Guo, 2004; Gimsing *et al.*, 2009; Inderjit *et al.*, 2011; Li *et al.*, 2014).

Soil microorganisms are extremely sensitive to fluctuations in their surrounding environment such as changes in temperature, moisture, mineral nutrients, and soil properties. These changes can impact directly on the diversity and biomass of soil biota (Carrera *et al.*, 2007; Chazarenc, Brisson and Merlin, 2010). Both microbial decomposition and mineralisation are dependent on the diversity and biomass of soil microorganisms and can influence the effectiveness of applied allelochemicals (Gimsing *et al.*, 2009; Al Harun *et al.*, 2014). Enhanced mineralisation by microbes reduces the longevity of allelochemicals that will directly influence their effectiveness (Gimsing *et al.*, 2009). Gimsing *et al.* (2009) found soil microorganisms with the ability to utilise allelochemicals as a carbon source that drastically decreased their persistence in soil.

Enhanced microbial decomposition can on the other hand introduce phytotoxic effects of allelopathic plants that can indirectly promote or inhibit surrounding plants and microorganisms. Bonanomi *et al.* (2005) compared the phytotoxic effects of aqueous extracts from decomposing plant materials of 25 Mediterranean plant species belonging to four different functional groups (grass-sedges, N-fixer, woody and forbs) against *Lepidium sativum* L. and found phytotoxic inhibition ranging between 20 % to 80 % on the root growth of *L. sativum* by the extracts of 22 plant species compared to the control. In a study by Bonanomi *et al.* (2011) on the allelopathic effects of aqueous extracts from decomposing alfalfa residues showed both phytotoxicity inhibition of up to 100 % on the root growth of *L.*

sativum compared to the control and inhibition of up to 100 % on the mycelial growth of 17 fungal species that consisted of soil-borne pathogens, airborne pathogens, saprophytes and antagonists. However, natural factors such as temperature and moisture can directly affect microbial decomposition resulting in changes in the allelopathic potential of applied plant materials (Al Harun *et al.*, 2014). Al Harun *et al.* (2014) compared differences between and phytotoxic effects of decomposing boneseed (*Chrysanthemoides monilifera* subsp. *monilifera*) under different temperatures. Their results showed higher concentrations of > 100 mg/L water soluble phenolics in decomposing boneseed exposed to 25 to 35°C compared to decomposing boneseed exposed to 5 to 15°C.

6.0 Conclusions

The main focus of agriculture has always been on enhancing the productivity/quality of crops and reducing the cost of production by improving disease and pest control. Recently however, the focus of modern agricultural management has been more on human health benefits and technologies that are environmentally safe. Insect pests and microbial pathogens as well as weeds are however more difficult to control when synthetic pesticides are rejected.

Allelochemicals are naturally produced by certain plants and have several benefits compared to synthetic pesticides. Studies focusing on the potential of allelochemicals demonstrate that they can effectively control several microbial pathogens, insect pests and weeds compared to synthetic pesticides (Dayan, Cantrell and Duke, 2009; Heleno *et al.*, 2014). Several plant allelochemicals however, have a short half-life and are easily mineralised by microbial decomposers compared to some synthetic pesticides (de Albuquerque *et al.*, 2011). Therefore, the application of allelochemicals in the form of cover crops, inter-crops or green manure can still result in effective exploitation of their inhibitory properties (Jabran *et al.*, 2015).

Recent studies on allelochemicals have mostly focused on their primary inhibitory mechanisms while secondary effects on the surrounding agroecosystem are not well understood. Similar to synthetic pesticides, allelochemicals often affect a wider range of organisms other than the intended target organisms. Thus, a herbicidal allelochemical has the potential of affecting not only weeds but also soil microorganisms. The method of application can also have a secondary effect on surrounding organisms. Current application methods are either by planting allelopathic plants or incorporating soil with allelopathic plant material. These application methods can however, alter several key aspects e.g. soil properties, microclimate and macro/microorganism community structure of the agroecosystem ultimately benefitting/ hindering the health of the crop plant (Inderjit and Weiner, 2001; Gomez-Rodriguez *et al.*, 2003; Li *et al.*, 2014).

Past and current studies in allelopathy reflect many inconsistencies between the results of *in vitro* studies and *in vivo* experiments. The main reasons for these inconsistencies are complex interactions with various biotic and abiotic factors in the agroecosystem that disguise the mechanisms at play. Abiotic factors such as temperature, moisture, photoperiod and soil properties and biotic factors such as plant pathogens, insect pests and soil microorganisms are found to affect both the production and the effectiveness of agriculturally applied plant allelochemicals. Furthermore, current studies on allelopathy and its potential application in agriculture are still very limited in terms of addressing biotic and abiotic interactions. Future studies with a more multifaceted approach on enhancing the agricultural benefits of allelochemicals in agroecosystems should therefore be more carefully planned and performed.

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CHAPTER TWO

EVALUATION OF SORGOLEONE AND TOTAL PHENOLICS CONTENT OF SORGHUM GENOTYPES AND THEIR IN VITRO EFFECT ON SOIL-BORNE FUNGAL PATHOGENS

Abstract

Sorghum phenolics and sorgoleone are known for their phytotoxic properties while their effects on surrounding organisms and plant pathogens are not well understood. The aim of this study was to determine the phenolic and sorgoleone content of 22 sorghum genotypes and the effects of four phenolic and one sorgoleone extracts on eight soil-borne pathogens *in vitro*. Phenolics were extracted from 2-week-old seedlings with an aquaeous acetone on acidified methanol solution and the concentration was determined by using a spectrophotometer at 760 nm. Root exudate containing sorgoleone was extracted from 7-day-old seedlings with methanol (1:20 w/v) and the amount of root exudate was determined by weighing the dried root exudates. Phenolic and sorgoleone extracts were incorporated into potato dextrose agar (PDA) seeded with agar plugs of the pathogens where after colony diameter of each pathogen was recorded after 7 days. Most pathogens were significantly stimulated by PDA containing phenolics. Phenolic extracts of genotype RTx436 had the most stimulating effect on the pathogen growth while sorgoleone displayed the least stimulating effect.

Introduction

Plant allelochemicals are biological compounds produced by plants during secondary metabolism that can influence surrounding plants and other organisms by providing an advantage during competition with other plants and protection against pests and pathogens (Peng, Wen and Guo, 2003; Weir, 2007). Due to environmental concerns and health hazards, modern weed and pest control methods have shifted away from synthetic pesticides and are moving towards finding more natural and environmentally friendly alternatives (Jabran *et al.*, 2015). Plant allelochemicals are often found to be partially water-soluble, exhibit bioactivity at low concentrations and have shown profound effects on inhibiting the germination and growth of weeds (Vyvyan, 2001; Ilori and Ilori, 2012).

Grain sorghum (*Sorghum bicolor* L. Moench) in particular, is known for containing multiple phytotoxic compounds within the vegetative parts. These compounds include many phenolics present in aerial vegetative parts and allelochemicals in root exudates (Won *et al.*, 2013; Dykes, Rooney and Rooney, 2013). Total phenolics in some genotypes can consist of multiple phenolic acids such as, *p*-coumaric acid, *p*-hydroxybenzoilc acid, vanillic acid, protocatechuic acid, syringic acid, caffeic acid, gentisic acid, and ferulic acid or kampferol (Sene, Dore and Pellissier, 1999; Chiremba *et al.*, 2012; Won *et al.*, 2013).

Phenolics are usually released into the sorghum plant's environment either by means of decomposing residues from the previous season or sorghum mulch (Weston, Alsaadawi and Baerson, 2013). These phenolics have an inhibitory effect on both seedling germination and plant growth of a wide variety of weeds (Won *et al.*, 2013). In some cases a chemically induced burning effect may occur on sensitive plant species (Won *et al.*, 2013).

The major component of root exudates produced by sorghum seedlings is an allelochemical referred to as sorgoleone, which can comprise up to 80-95 % of the total root exudate volume (Weston, Alsaadawi and Baerson, 2013). Sorgoleone is an active *p*-

benzoquinone known as 2-hydroxy-5-methoxy-3-[(8′Z,11′Z)-8′,11′,14′-pentadecatriene]-*p*-benzoquinone [CAS 105018-76-6] (Dayan *et al.*, 2010). It is a hydrophobic, oily substance and currently one of the most extensively studied plant allelochemicals due to its potency at very low concentrations and its high concentration within the root exudates of grain sorghum (de Albuquerque *et al.*, 2010).

Sorgoleone has the ability to suppress a wide variety of small seeded weeds and is most affective against younger growth stages (Einhellig and Souza, 1991). Sorgoleone significantly affects the photosynthetic ability and mitochondrial electron transport of the target plant by mimicking their natural electron acceptors plastoquinones and ubiquinone. (Dayan *et al.*, 2010). This inhibitory effect is comparable to a pre-plant chemical herbicide and provides sorghum seedlings with a competitive advantage during emergence (Milchunas *et al.*, 2011).

The concentration of phenolics and sorgoleone produced by sorghum plants is dependent on the specific genotype and growth stage (Won *et al.*, 2013). Czarnota, Rimando and Weston (2003) investigated in the amount of sorgoleone containing root exudates produced by seven sorghum genotypes and found root exudates ranging between 0.5 mg/g (root fresh weight) to 14.75 mg/g (root fresh weight) between the genotypes. Sene, Dore and Pellissier (1999) and Won *et al.* (2013) conducted studies on two sorghum genotypes (Variety $CE_{145\text{-}66}$ and Variety SS-450) and identified different phenolic compound spectra from respective plant extracts. Won *et al.* (2013) also demonstrated significant changes in the concentration of specific phenolic compounds during different growth stages. Similarly, Uddin *et al.* (2010) showed that the amount of sorgoleone containing root exudate produced by sorghum is significantly different between growth stages ranging from 40 µg/mg (root dry weight) in 5-day-old seedlings to 9.2 µg/mg (root dry weight) in 40-day-old seedlings.

The inhibitory effects of sorgoleone are not only limited to surrounding plants but also affect microorganisms within the soil environment. The "soil sickness" phenomenon, caused by allelopathy in fields that have undergone monocropping for several succeeding seasons is related to a build-up of allelochemicals and their effect on soil microorganisms (Inderjit and Weiner, 2001; van der Heijden, Bardgett and Straalen, 2008; Weston, Alsaadawi and Baerson, 2013). Soil microorganisms are either inhibited or stimulated, thereby ultimately changing their community diversity and structure. (Kong *et al.*, 2008; Gimsing *et al.*, 2009). Numerous studies have demonstrated both inhibitory and stimulatory affects of allelochemicals on soil-borne fungal pathogens (Wu *et al.,*2008; Zhang *et al.*, 2008; Zhang *et al.*, 2011; Qi, Zhen and Li, 2015). For example, Kong *et al.* (2004) reported significant inhibition of spore germination of *Pyricularia oryzae* by allelochemical extracts from rice.

The objectives of the present study were firstly, to determine the concentration of phenolics and sorgoleone present within extracts of 22 *S. bicolor* genotypes and secondly, to investigate whether these extracts have promoting or inhibiting effects on the growth of eight soil-borne plant pathogens.

Materials and Methods

Selection of sorghum genotypes

A total of 22 genotypes of *S. bicolor* were selected for allelochemical extraction because they represent a wide range of phenotypic characteristics associated with commercial sorghum, including variation in plant and grain colour (Table 1).

Extraction of total phenolics

Sorghum seeds of each genotype were grown separately in rectangular plastic containers (500 ml) containing vermiculite in an incubator set at ± 28ºC and 12 hr day/night cycles. Seedlings were watered daily to field water capacity. After two weeks, seedlings were removed from the containers, roots were excised and air dried with the remaining vegetative material at room temperature for 7 days before being ground into a fine powder using liquid nitrogen. The powder (0.125 g) was placed in a 50 ml tube containing 6.25 ml of a 75 % aquaeous acetone/acidified methanol (1 % HCl per 100 ml methanol) solution. The mixture was shaken in a Multi Reax shaker (Heidolph, Labotec) for 2 hr at 600 rpm and subsequently centrifuged at 3500 rpm for 6 min. The supernatant (1 ml) was added to a 100 ml volumetric flask that contained 70 ml deionised water and 5 ml of Folin-Ciocalteu's reagent (Sigma-Aldrich, Merck). The flasks were left for 1 min before 15 ml of a sodium carbonate solution (anhydrous sodium carbonate/deionised water, 20 % m/v) was added and then made up to volume (100 ml) with deionised water. After standing for further 2 hr at room temperature the liquid in each flask was added to a cuvette and absorbance was measured with a spectrophotometer (T60 UV VIS spectrophotometer, PG Instruments) at 760 nm. Gallic acid was used to develop a standard curve and served as a standard to estimate the total phenolic concentration within the extracts. Results were expressed as mg (gallic acid equivalent) per g (sample dry mass).

Extraction of sorgoleone

Sorghum seeds from 22 genotypes were germinated in a closed rectangular plastic container (500 ml) between two sheets of sterilised tissue paper. The tissue paper were wetted with sterilized water until holding capacity and sprayed with sterilized water daily during the incubation period. The container was incubated at ± 25 ºC for 7 days in the dark.

Seedlings were removed from the tissue paper and roots were retained for sorgoleone extraction according to the methods of Netzly and Butler (1986) and Uddin *et al*. (2010). Freshly cut seedling roots were weighed and placed in a 50 ml tube and immersed in methanol (1:20 w/v) for 30 sec after which the extract was filtered using filter paper (Selecta faltenfilter nr. 595½, Schleicher & Schüll). The filtered extracts were evaporated under a fume hood at room temperature. The amount of root exudate containing sorgoleone was determined by physically weighing the exudate powder according to a method by Czarnota, Rimando and Weston (2003). The exudate powders were weighed with a scale (RADWAD Wagi Elektroniczne, Lasec) and determined proportionally to the weight of the original roots. The results were expressed as mg (root exudate) per g (sample fresh weight).

Effect of allelochemicals on soil-borne fungal pathogens

The experimental design was a factorial randomised block with five sorghum extracts and eight soil-borne pathogens as factors. Eight soil-borne fungal pathogens *(Sarocladium strictum* Gams*, Alternaria alternata* Keissl.*, Curvularia trifolii* Boedijn*, Didymella macrostoma* Mont.*, Colletotrichum capsici* Butler & Bisby*, Fusarium thapsinum* Klittich and *Fusarium equiseti* Corda*, Fusarium oxysporum* Schlecht.) previously isolated from diseased sorghum roots were used for this study (van Rooyen, 2012). Phenolics were extracted from four sorghum genotypes: RTx 430, RTx 436, Rtam 428, SCAY 21 and sorgoleone was extracted from genotype BTx 3197. Genotypes for phenolic extracts were selected due to differences in plant colour while the genotype used for sorgoleone was selected for producing the most sorgoleone based on the results of the previous experiment.

Phenolic extraction was adapted from the method of Won *et al*. (2013). Dried plant material was finely ground in liquid nitrogen and placed in 50 ml tubes before being immersed in methanol (1:50 w/v) for 24 hr at room temperature. Dried sorgoleone extracts

were prepared as described previously and reconstituted in methanol (1:20 w/v). All extracts were filtered through filter paper (Selecta faltenfilter nr. 595½, Schleicher & Schüll) before being incorporated into an agar medium. Potato dextrose agar (PDA) (Biolab, Merck) was prepared by adding the agar powder into water previously mixed with an antimicrobial agent (CHLORCOL, Adcock Ingram Limited) to prevent bacterial contamination. The agar mixture was autoclaved for 20 min at 120 ºC and cooled to 55 ºC in a water bath before the extracts were added. A concentration of 1 mg phenolic extract per ml agar and 0.1 mg sorgoleone per ml agar was prepared by adding the methanol/extract mixture to the PDA. A control was prepared by adding 1 ml methanol per 100 ml agar to the PDA. The agar mixtures were then poured into 90 mm Petri dishes and allowed to solidify before being stored at 4 ºC. Agar plugs of the eight fungal isolates were transferred to Petri dishes containing extracts. All treatments including the control were replicated three times. Petri dishes were incubated in the dark at 25 ºC for a period of 7 days. The diameter of each fungal colony was measured with a micrometer (Lasec, Laboratory & Scientific Equipment Co. Pty. Ltd.). A GLM-ANOVA was conducted to determine differences in fungal colony diameter between treatments using statistical software NCSS2007 (Hintze, 2007). The means were compared using Fisher's least significant difference (LSD) and significant differences were determined at ($P < 0.05$).

Results

Total phenolic content

Total phenolic content of the different genotypes ranged between concentrations of 0.29 mg/g (gallic acid equivalent) to 4.11 mg/g (gallic acid equivalent) (Table 2). The highest phenolic content within the range was recorded for genotypes BTX ARG-1 (tan plant, red seed), RTam 2566 (purple plant, white seed), RTx 436 (tan plant, red seed), SCAY 14

(tan plant, lemon-yellow seed) and BTx 635 (tan plant, white seed). The lowest phenolic content was recorded for genotypes S5C719-11E (purple plant, red seed) and SCAY 21 (tan plant, yellow seed). A higher average phenolic content occurred in tan coloured genotypes compared to genotypes that had red plant colour. A higher average phenolic content was recorded in genotypes with red coloured seed compared to white or yellow seed.

Sorgoleone production

Root exudates containing sorgoleone produced by the different genotypes ranged between amounts of 6.25 mg/g to 43.77 mg/g (Table 2). The highest amount of sorgoleone within the range was produced by genotype BTx 3197 (purple plant, white seed), while genotype Tx 2911 (red plant, red seed) yielded the lowest amount. Sorghum genotypes with a purple colour produced the highest amount of sorgoleone, while red coloured sorghum genotypes produced the lowest amount of sorgoleone. Genotypes with white seed produced the highest amount of sorgoleone, while genotypes with a red seed produced the lowest amount.

***In vitro* effect of phenolic and sorgoleone extracts on soil-borne fungal pathogens**

Phenolic and sorgoleone extracts significantly ($P < 0.05$) affected the colony diameter of the soil-borne pathogens growing on amended PDA compared to the control treatment. The different pathogen isolates were found to have significantly ($P < 0.05$) different colony diameters. Significant extract x soil-borne pathogen interactions ($P < 0.05$) were recorded.

Colonies were generally larger for most pathogens growing on sorgoleone treated PDA compared to the control treatment. A significantly smaller ($P < 0.05$) average fungal colony was found in *C. capsici* and *F. equiseti* inoculated on PDA and amended with sorgoleone compared to the control (Figure 1, Figure 2). The largest mean colony diameter

for most soil-borne pathogens were observed on PDA treated with phenolic extracts of RTx 430 and RTx 436.

Discussion

In the present study, both total phenolic content and sorgoleone production varied between the 22 sorghum genotypes selected for this study. This is consistent with several other papers on the phenolic and sorgoleone contents of sorghum genotypes (Nimbal *et al*.,1996; Czarnota, Rimando and Weston, 2003; Awika and Rooney, 2004; Chiremba *et al*., 2012; Dykes, Rooney and Rooney, 2013). Czarnota, Rimando and Weston (2003) evaluated sorghum root exudates of seven sorghum genotypes and found variation in the amount of root exudates produced by the sorghum genotypes ranging between 0.5 mg/g (root fresh weight) to 14.75 mg/g (root fresh weight). Chiremba *et al*. (2012) evaluated phenolic extracts of grains in several sorghum cultivars and found significant differences in total phenolic contents ranging between 0.63 g (catechin equivalent)/100 g to 0.96 g (catechin equivalent)/100 g amongst the sorghum genotypes. This difference in phenolic content between genotypes of the same plant species is not only found in sorghum, but also in several other crops such as strawberries (*Fragaria vesca* L.), apples (*Malus pumila* (L.) Mill), bacaba-de-leque (*Oenocarpus distichus* Mart.), barberry (*Berberis* spp.), Chilean guava (*Ugni molinae* Turcz.), soybean (*Glycine max* L.) and spiked ginger lily (*Hedychium spicatum* Sm. in A. Rees) (Scalzo *et al*., 2004; Kumar *et al.*, 2009; Holderbaum, Kon and Guerra, 2014; Carvalho *et al*., 2016; Hassanpour and Alizadeh, 2016; Pena-Cerda *et al*., 2017; Rawat *et al*., 2017). Pena-Cerda *et al.* (2017) found significant differences ranging between 157.8 mg (gallic acid equivalent)/g to 260.6 mg (gallic acid equivalent)/g in the total phenolic content of leaf extracts between 10 Chilean guava genotypes. Similarly, Hassanpour and Alizadeh (2016) found significant differences ranging between 261.68 mg (gallic acid equivalent)/g to

623.07 mg (gallic acid equivalent)/g in the total phenolic content of extracts from 20 different barberry (*Berberis vulgaris* L.) genotypes.

Variation in phenolic content and root exudates between genotypes could be related to physiological differences between the sorghum genotypes. Chiremba *et al*. (2012) investigated the phenolic content between sorghum genotypes varying in grain hardiness and found more phenolics present in sorghum genotypes with hard grains compared to those with soft grains. Dykes, Rooney and Rooney (2013) investigated the phenolic content of black sorghum hybrids and found strong correlations between the 3-deoxyanthocyanidin and flavone contents and the difference in pericarp colour of the genotypes. Similar relationships between plant physiological differences and differences in phenolic contents are also found between genotypes of other crops. Colak *et al.* (2017) investigated the phenolic content of eight bilberry (*Vaccinium myrtillus* L.) genotypes and found that the phenolic acid profiles between these genotypes were closely associated with the difference in berry colour. Chen, McClung and Bergman (2017) investigated the phenolic content of 25 rice (*Oryza sativa* L.) genotypes and found significantly higher average total phenolic content in rice genotypes with red (49.2 mg (gallic acid equivalent)/g) and purple (25.6 mg (gallic acid equivalent)/g) bran colour compared to genotypes with white (2.7 mg (gallic acid equivalent)/g), light brown (3 mg (gallic acid equivalent)/g) and brown (4.7 mg (gallic acid equivalent)/g) bran colours.

The direct effects of five phenolic extracts on eight soil-borne pathogens revealed significantly stimulated fungal growth in treated colonies compared to the control treatment. This stimulation of fungal growth could be caused by phenolics specific to sorghum pathogens since all fungal pathogens were isolated from grain sorghum roots. Wu *et al*. (2013) investigated the effect of phenolic extracts on *F. oxysporum* and observed a significant increase of biomass for *F. oxysporum* f. sp. *niveum* when treated with phenolic extracts from

watermelon. These results showed that extracts from resistant watermelon significantly reduced biomass while extracts from susceptible watermelon increased biomass. Wu *et al.* (2013) indicated that the phenolic acids that stimulated *F. oxysporum* were vanillic and syringic acids. These phenolic acids were found to not only increase the growth of *F. oxysporum* but also their pectinase, protease, cellulase and amylase activities which play vital roles in the breaking down of plant tissue. These findings support the results of the present study, since vanillic and syringic acids are also found in sorghum total phenolics (Sene, Dore and Pellissier, 1999; Chiremba *et al.*, 2012; Won *et al.*, 2013).

In conclusion, the present study demonstrated variation between the phenolic content and the amount of sorgoleone produced between 22 sorghum genotypes with plant and seed colour potentially influencing phenolic concentration. These results may be useful in determining genotypes with higher levels of phenolics or sorgoleone for natural herbicidal uses in crop systems. Further investigation to explain the possible relationship between sorghum genotypes and allelochemical properties is therefore required to improve our understanding of sorghum allelochemicals and their possible uses. The present study also demonstrated significant growth stimulation of certain soil-borne pathogens by phenolic extracts. This observation suggests that sorghum phenolic build-up in soil may contribute to pathogen build-up in monocrop systems. Further investigation into the phenolics responsible for stimulating soil-borne pathogens is necessary to develop rotation systems that could reduce weeds and soil-borne pathogens.

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Table 1. Grain sorghum genotypes used to evaluate sorgoleone and total phenolic content with regards to plant and seed colour.

Line No	Line	Pl. Col.	Pericarp	Mesocarp	Testa	Spread.	Plant	Seed colour
1	BTx378	PPQQ	RRYYII	zz	b1b1B2B2	SS	purple	red, chalky
2	BTx3197	PPQQ	RRyyii	zz	b1b1B2B2	SS	purple	white, chalky
3	RTx430	PPQQ	RRyyll	ZZ	b1b1b2b2	SS	purple	white, pearly, yellow endosperm
4	RTam428	PPqq	rryyll	ZZ	b1b1B2B2	ss	red	white, pearly
5	RTx436	ppQQ	RRyyii	ZZ	B1B1b2b2	--	tan	white
6	BTx635	ppQQ	RRyyii	ZZ	--	--	tan	white pearly food grad sorghum
7	BTX ARG-1	ppQQ	RRyyii	ZZ	--	--	tan	waxy endosperm
8	RTx2917						tan	red
9	SC630-11EII	PPQQ	RRYYII	ZZ	b1b1B2B2	SS	purple	dark red, pearly
10	SC630-11 Eii	PPQQ	RRYYii	ZZ	b1b1B2B2	SS	purple	light red, pearly
11	SC748-5	PPQQ	rrYYIl	ZZ	b1b1B2B2	ss	purple	lemon yellow, pearly
12	SC109-14E	PPQQ	RRyy--	zz	B1B1B2B2	ss	purple	white pearly, purple testa
13	S5C719-11E	PPQQ	RRYYii	zz	B1B1B2B2	ss	purple	red, chalky,
14	SC103-12E	PPQQ	RRYYII	zz	B1B1B2B2	SS	purple	dark brown-red, chalky
15	Dobbs	PPQQ	RRyyii	zz	B1B1B2B2	SS	purple	brown, chalky
16	Hegari	PPQQ	RRyyii	zz	B1B1B2B2	ss	purple	white, chalky
17	RTam2566	PPQQ	RRYYII	ZZ	B1B1B2B2	ss	purple	dark brown-red, pearly
18	Tx2911						red	red, chalky
19	SCAY13						tan	lemon-yellow
20	SCAY16						tan	lemon-yellow
21	SCAY21						tan	red
22	SCAY14						tan	lemon-yellow

Table 2. Sorgoleone and total phenolics content of 22 grain sorghum genotypes.

Line	**Total phenolic content (mg/g gallic acid equivalent)**	**Sorgoleone content (mg/g)**
BTx378	2.85	11.94
BTx3197	1.69	43.77
RTx430	1.32	13.77
SC630-11EII	1.15	12.97
SC630-11 Eii	1.26	19.95
SC748-5	1.08	14.53
SC109-14E	2.81	11.35
S5C719-11E	0.30	11.34
SC103-12E	1.04	18.95
Dobbs	1.26	12.64
Hegari	2.59	13.78
RTam2566	2.97	27.46
RTam428	0.80	10.86
Tx2911	2.58	6.25
RTx436	2.95	13.70
BTx635	2.95	10.75
BTX ARG-1	4.11	9.26
SCAY13	2.34	15.66
SCAY21	0.29	14.81
SCAY14	2.98	18.39
RTx2917	2.08	12.12
SCAY16	1.62	12.27

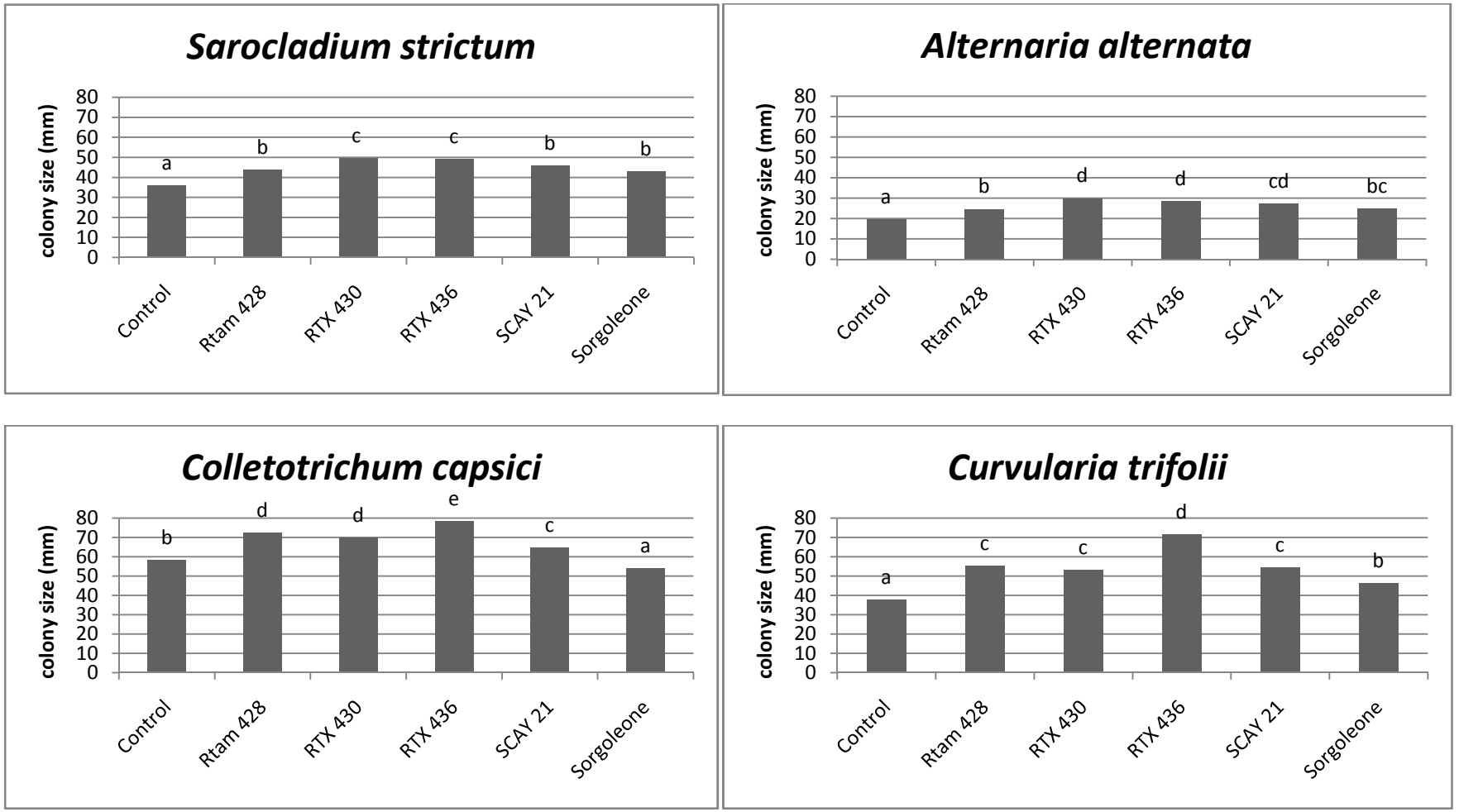

Figure 1. Average fungal colony size of *Sarocladium strictum, Alternaria alternata, Colletotrichum capsici,* and *Curvularia trifolii* affected by phenolic extracts of 4 sorghum genotypes and sorgoleone. Bars with the same lowercase letter/s are not significantly different ($P > 0.05$).

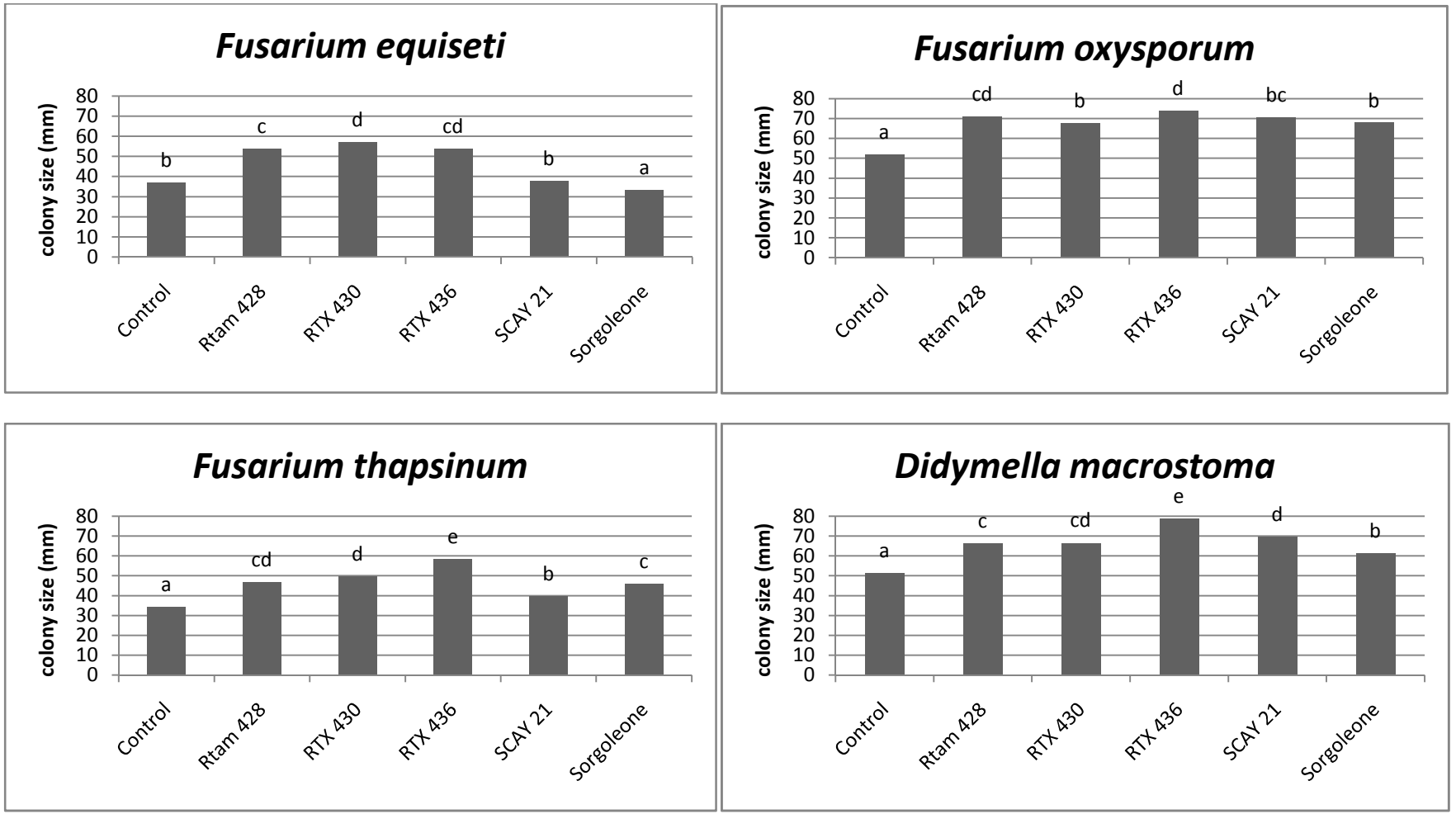

Figure 2. Average fungal colony size of *Fusarium equiseti, Fusarium oxysporum, Fusarium thapsinum* and *Didymella macrostoma* affected by phenolic extracts of 4 sorghum genotypes and sorgoleone. Bars with the same lowercase letter/s are not significantly different ($P > 0.05$).

CHAPTER THREE

DIRECT EFFECTS OF SORGHUM PHENOLIC EXTRACTS AND SORGOLEONE ON SOIL MICROBIAL ORGANISMS AND SOIL-BORNE FUNGAL PATHOGENS

Abstract

Plant phenolics and other plant products with allelopathic properties are known for their direct effects on surrounding organisms and provide plants with an advantage during competition with other plants, as well as pests and pathogens. The present study investigated the direct effects of allelochemicals produced by various grain sorghum genotypes on eight soil-borne plant pathogens. Phenolics obtained from sorghum and the allelochemical sorgoleone were applied to natural, unsterilised and sterilised loam soils. Soils were inoculated in a greenhouse experiment with eight soil-borne pathogens and planted with sorghum seedlings (PAN 8806) to investigate direct effects of the sorghum allelopathic extracts on soil microbial diversity and the pathogens. On average, the glasshouse experiment revealed significantly shorter leaf length (78 %), longer root length (63 %) and reduced root rot (88 %) in sorghum plants inoculated with eight soil-borne pathogens compared to the untreated control. On average, phenolics and sorgoleone extracts were most effective in reducing the root rot caused by *Alternaria alternata* (20 %), *Curvularia trifolii* (16 %) and *Fusarium thapsinum* (16 %) and least effective in reducing the root rot caused by *Sarocladium strictum* (< 5 %) and *Colletotrichum capsici* (< 5 %). Sorghum phenolic SCAY 21 0.5x and sorgoleone 1x extracts were most effective against all of the soil-borne pathogens. A field trial to determine the effect of 22 sorghum genotypes on soil microorganisms revealed significant differences in soil microbial activities in the rhizosphere of each genotype.

Introduction

Continuous cultivation in a cropping system often leads to "soil sickness" that severely affects crop yield. This phenomenon has often been associated with the build-up of allelochemicals from plants but recent studies have associated it with changes in ecological processes in the soil (Inderjit and Weiner, 2001; van der Heijden, Bardgett and Straalen, 2008; Weston, Alsaadawi and Baerson, 2013). Rhizosphere microorganisms are extremely sensitive to their surroundings and their population diversity and community structure is determined by soil type and associated plant species (Kong *et al.*, 2008; Xu *et al.*, 2008). They can be either stimulated or inhibited by allelochemicals that can alter their abundance and diversity structure (Kong *et al.*, 2008; Gimsing *et al.*, 2009). Kong *et al.* (2008) observed significant modifications between the phospholipid fatty acid (PLFA) profiles of soil microbial populations by allelopathic rice varieties compared to non-allelopathic rice varieties. They related this modification to the allelochemicals within the root exudates with lower microbial populations in soils of allelopathic rice compared to soils of non-allelopathic rice. Li *et al.* (2014) discovered significantly lower microbial diversity in soil replanted with ginseng (*Panax ginseng* Meyer). This lower microbial diversity was found to negatively influence the intrinsic carbon metabolic functions of the soil microorganisms in the replanted soil.

Allelochemicals can promote or inhibit soil-borne fungal pathogens (Xuan *et al.*, 2004; Zhang *et al.*, 2008). Several *in vitro* studies have shown direct inhibitory or stimulatory effects of multiple allelochemicals such as benzoic, salicylic, cinnamic, coumaric, caffeic, vannilic, syringic acids as well as flavones and cylohexenones on mycelial growth, spore germination and mycotoxin production in several fungal pathogens (Kong *et al.*, 2004; Wu *et al.*, 2008; Wu *et al.*, 2013; Qi, Zhen and Li,

2015). Kong *et al.* (2004) observed significant inhibition of mycelial growth and spore germination of *Pyricularia oryzae* Cavara and *Rhizoctonia solani* Kühn by rice extracts. Wu *et al.* (2008) found significant inhibition of *Fusarium oxysporum* f.sp. *niveum* Schlecht. mycelial growth, spore germination and conidia formation by salicylic acid. They also discovered an increase in mycotoxin production and related this increase to salicylic acid.

It is sometimes difficult to determine whether allelochemicals are the major factor directly affecting soil-borne fungal pathogens in *in vivo* studies. Several studies have however successfully demonstrated inhibition of pathogens in the field by applying plant extracts, incorporating plant residues in soil or planting cover crops that are allelopathic (Xuan *et al.,* 2004; Zhang *et al.*, 2008; Zhang *et al.*, 2011; Qin *et al.*, 2012). Allelopathic extracts from kava (*Piper methysticum* Forst.) were shown to be inhibitory to *Rhizopus stolonifer* Vuillemin, *Taphrina deformans* (Berk.) Tul., *Pyricularia grisea* Sacc., *Thanatephorus cucumeris* Donk and *Fusarium solani* Sacc. under field conditions with *R. stolonifer* being the most inhibited by the kava extracts (Xuan *et al.,* 2004). Similarly, Gomez-Rodriguez *et al.* (2003) found significant reductions in foliar damage caused by *Alternaria solani* Sorauer. on tomato plants intercropped with allelopathic marigold (*Tagetes erecta* L.) and pigweed (*Amaranthus palmeri* Wats.) compared to the tomato plants in the monoculture control.

The objectives of the present study were to investigate: (i) the direct *in vitro* effect of grain sorghum phenolic and sorgoleone extracts on rhizosphere microorganisms; (ii) the direct *in vitro* effect of sorghum phenolic and sorgoleone extracts on eight soil-borne fungal pathogens; (iii) the direct *in vivo* effect of sorghum genotypes in the field on rhizosphere microorganisms.

Materials and methods

Inoculum preparation

Eight soil-borne fungal pathogens *(Sarocladium strictum* Gams*, Alternaria alternata* Keissl.*, Curvularia trifolii* Boedijn*, Didymella macrostoma* Mont.*, Colletotrichum capsici* Butler & Bisby*, Fusarium thapsinum* Klittich*, Fusarium equiseti* Corda*, Fusarium oxysporum* Schlecht.) previously isolated from diseased sorghum roots were used for this study (van Rooyen, 2012). A medium for growing inoculum was prepared by placing sorghum grain (100 g) in glass beakers and soaking them in deionised water (100 ml) for 24 hr. The excess water was then decanted and the seeds were autoclaved twice for 25 min at 121 ˚C. Agar plugs of each fungal isolate were then placed in the beaker and incubated under UV light at room temperature for 3~4 weeks. The beakers were shaken every two days to promote fungal growth. The inoculum was then removed from the beaker, dried at 25 °C for 72 hr, ground into a fine powder and stored in sterile rectangular plastic containers (500 ml) at room temperature.

Phenolic and sorgoleone extraction

Phenolics were extracted from four sorghum genotypes: RTx 430 (purple plant), RTx 436 (tan plant), Rtam 428 (red plant) and SCAY 21 (tan plant) and sorgoleone was extracted from genotype BTx 3197. Phenolic extraction was adapted from the method of Won *et al*. (2013). Sorghum seeds of each genotype were grown separately in rectangular plastic containers (500 ml) containing vermiculite in an incubator set at ± 28 ºC and 12 hr day/night cycles. Seedlings were watered daily to field water capacity. After two weeks, seedlings were removed from the containers, roots were excised and the remaining vegetative material were air dried at room

temperature for 7 days before being ground into a fine powder using liquid nitrogen. Powder of each genotype was placed in 50 ml tubes and immersed in methanol (1:50 w/v) for 24 hr at room temperature.

Sorghum seeds of BTx 3197 were germinated in a closed rectangular plastic container (500 ml) between two sheets of sterilised tissue paper. The tissue paper were wetted with sterilized water until holding capacity and sprayed with sterilized water daily during the incubation period. The container was incubated at ± 25ºC for 7 days in the dark. Seedlings were removed from the tissue paper and roots were retained for sorgoleone extraction according to the methods of Netzly and Butler (1986) and Uddin *et al*. (2010). Freshly cut seedling roots were weighed and placed in 50 ml (1:20 w/v) methanol for 30 sec after which the extract was filtered using filter paper (Selecta faltenfilter nr. 595½, Schleicher & Schüll). The filtered extracts were evaporated under a fume hood at room temperature. The amount of root exudate containing sorgoleone was determined by physically weighing the exudate powder according to the method of Czarnota, Rimando and Weston (2003). The exudate powder was weighed with a scale (RADWAD Wagi Elektroniczne, Lasec) and determined proportionally to the weight of the original roots.

Effect of phenolic extracts and sorgoleone on soil microorganisms

Two greenhouse experiments were conducted to determine the effects of sorghum extracts on soil microbial diversity and soil-borne pathogens. In both experiments phenolic extracts and sorgoleone were applied at two different concentrations prior to the planting of the sorghum seedlings (Table 2).

The first greenhouse experiment was conducted to determine the effect of sorghum extracts on soil microbial activity and diversity. Microcosms were prepared

using 18 cm plastic pots filled with sieved natural loam soil purchased in Bloemfontein. Each extract was mixed with water (< 3 % methanol extract/ L water) to their respective concentrations and the water mixtures (200 ml) were applied directly onto the soil. A control treatment was prepared without sorghum extracts and each treatment was replicated three times. Sorghum seeds (PAN 8806) were pre-germinated and seedlings were planted in all microcosms to simulate field conditions. Seedlings were cultivated at a mean temperature of 24 ˚C and watered daily. After 6 weeks cultivation, the sorghum plants were removed and two soil samples were collected directly from the rhizosphere of each sorghum plant. The first soil sample was stored at 4 ˚C for fluorescein diacetate analysis (FDA) and the second soil sample was stored at -80 ˚C for phospholipid fatty acid analysis (PLFA). FDA and PLFA were performed to determine microbial activity and diversity.

Effect of phenolic extracts and sorgoleone on soil-borne pathogens

The second greenhouse experiment was conducted to determine the effect of sorghum extracts on the eight aforementioned soil-borne pathogens. Microcosms were designed using 12 cm plastic pots filled with sieved sterilised loam soil. Inoculum powder equal to 0.3 % of the soil mass was mixed into the pots 2 weeks prior to the application of the sorghum extracts and watered weekly. Each extract was mixed with water (< 3 % methanol extract/ L water) to their respective concentrations and the water mixtures (200 ml) were applied directly onto the soil. A control treatment was prepared without sorghum extracts and each treatment was replicated three times. Sorghum seeds (PAN 8806) were pre-germinated and seedlings were planted to simulate field conditions. Seedlings were cultivated at a mean temperature of 24 ˚C and watered daily. After 6 weeks cultivation, the sorghum plants were

carefully removed from the soil, washed and leaf length, root length, plant weight and the severity of root disease were assessed. The roots were then dried at room temperature and ground into powder using liquid nitrogen. A GLM-ANOVA was conducted to determine differences in root mass, root-rot rating and grain mass between samples using statistical software NCSS2007 (Hintze, 2007). The means were compared using Fisher's least significant difference (LSD) and significant differences were determined ($P < 0.05$).

Effect of sorghum genotype on soil microorganisms

A total of 22 genotypes of *Sorghum bicolor,* representing a range of phenotypic characteristics associated with grain sorghum, including variation in plant and grain colours were selected (Table 1).

The study was conducted on a farm situated in the Alma district, in the Limpopo province of South Africa (-24°28'59.99"S, 28°03'60.00"E) during 2014 and 2015. Seed was sown in October 2014 and sorghum plants were harvested in April 2015. The trial design was a randomised block design with seeds of each genotype sown in two adjacent rows. The trial was replicated. Soil samples were collected during the flowering stage from the rhizosphere of 10 random sorghum plants of each replicate. The rhizosphere soil samples from the random plants were homogenised and the soil samples were stored at 4 ˚C for FDA analysis.

Fluorescein diacetate analysis (FDA): This procedure was adapted and modified from Schnürer and Roswell (1982). A stock solution was prepared by dissolving FDA in acetone (2 mg/ml) and stored at -20 ºC. A buffer solution of 60 mM sodium phosphate was prepared, adjusted to pH 7.6 and stored at 4 ºC. Soil samples (2 g)

were added to 20 ml of the buffer followed by 0.2 ml of the FDA solution in a 50 ml tube. The mixture was then shaken by hand, incubated at 28 ºC for 20 min, and placed in a Multi Reax shaker (Heidolph, Labotec) at 200 rpm for 10 min. After shaking, 15 ml of chloroform - methanol (2:1 v/v) solution was added to the soil solutions to stop the FDA from being hydrolysed further. The mixture was then stored at 4 ºC until the sediment settled and the clear supernatant was placed in a 2 ml eppendorf tube. The eppendorf tubes were then centrifuged at 10000 rpm for 5 min to remove excess debris whereafter the supernatant was measured at 490 nm using a spectrophotometer (CARY Bio 100 UV-Visible Sectrophotometer). A GLM-ANOVA was conducted to determine differences in FDA content between treatments using statistical software NCSS2007 (Hintze, 2007). The means were compared using Fisher's (LSD) and significant differences were determined at ($P < 0.05$).

Phospholipid fatty acid analysis (PLFA): This procedure uses phospholipid fatty acids (PLFA) as biomarkers to determine total microbial composition in soil and differentiate them into different functional groups by means of their respective fatty acid profiles (Table 3). The procedure used was adapted and modified from White *et al.* (1979) as described by Marschner (2007). Soil samples (2 g) were placed in 25 ml centrifuge tubes and methanol (3.8 ml), citrate buffer (1.5 ml), chloroform (1.9 ml) and Blight and Dyer reagent (2 ml) were added. The tubes were then shaken for 2 hr and centrifuged at 3500 g for 10 min. The supernatant was transferred into a new centrifuge tube. The pellet in the old centrifuge tube was washed with Blight and Dyer reagent (2.5 ml), shaken and centrifuged (as in the previous step) and the supernatant was then added to the supernatant in the new centrifuge tube. The pooled supernatants were diluted with chloroform (3.1 ml) and citrate buffer (3.1 ml),

vortexed and centrifuged at 3500 g for 10 min. The lower phase (3 ml) was then transferred into a 10 ml glass tube and the content was dried at 40 ˚C under a flow of N_2. The dried samples were dissolved in chloroform (300 µl) and pipetted onto a conditioned (2 x 1 ml chloroform) silica-bonded column. Neutral lipids were eluted with chloroform (5 ml) and glycolipids were eluted with acetone (10 ml). Both were then discarded. Phospholipids were eluted with methanol (5 ml), transferred to a 10 ml centrifuge tube and the content was again dried at 40 ˚C under a flow of N_2. An internal standard (C19:0; 30 µl) was then added to the dried samples, followed by a methanol - toluol solution (1:1, v/v; 1 ml) and 0.2 M KOH-MeOH (1 ml). The suspension was then vortexed and incubated for 15 min at 37 ˚C. A hexane-chloroform solution (2 ml), 1 M acetic acid (0.3 ml) and deionised water (2 ml) were added to the suspension. The suspension was vortexed for 1 min, centrifuged for 5 min at 3500 g and the supernatant was transferred into a 10 ml glass tube. Another aliquot of hexane-chloroform solution (2 ml) was added to the pellet, the solution was vortexed and centrifuged as described previously and the supernatant was added to the previous supernatant. The supernatant was dried at 40 ˚C under a flow of N_2.

The dried supernatant was dissolved in iso-octane (130 µl). Fatty acid methyl esters (FAME) were quantified by using a Varian 430 flame ionization gas chromatograph (GC) with a fused silica capillary column (Chrompack CPSIL 88; 100 m length, 0.25 µm ID, 0.2 µm film thickness) at 40 ~ 230 ˚C (hold 2 min; 4 ˚C/ min; hold 10 min). Fatty acid methyl esters suspended in iso-octane (1 µl) were injected into the column by using a Varian CP 8400 autosampler with a split ratio of 100:1. The injection port and detector were both maintained at 250 ˚C. Hydrogen (45 psi), functioned as the carrier gas, while nitrogen was used as the makeup gas. Varian Star Chromatography Software was used to record the chromatograms. FAME samples

were identified by comparing the relative retention times of FAME peaks of samples with standards (SIGMA, 189-19). Peak identification of the fatty acids and the fatty acid profile were done by using external standards (Supelco 37 component FAME mix; Sigma BAME mix). Calculations were done according to the methods by Marschner (2007), and the data were classified into different bacterial acid methyl esters (BAME) according to Whalen and Sampredo (2009). The BAME data were then grouped into their respective microbial groups and principal component analysis (PCA) was performed.

Ergosterol analysis: This procedure was adapted and modified from the method of Jambunathan, Kherdekar, and Vaidya (1991). Ground sorghum root powder (5 g) was placed in 50 ml centrifuge tubes and methanol (25 ml) was added before tubes were shaken vigorously with a Multi Reax shaker (Heidolph, Labotec) for 30 min and the sediment was allowed to settle. The supernatant was transferred into a new 50 ml centrifuge tube containing KOH pellets (1.5 g) and shaken until the KOH dissolved. N-hexane (5 ml) was added to the mixture that subsequently was incubated for 30 min in a water bath at 75 ˚C. The mixture was cooled to room temperature and distilled water (2.5 ml) was added. The mixture was vortexed, allowed to settle and the upper hexane layer was transferred into a new 50 ml centrifuge tube. This process was repeated. The hexane extracts were evaporated in a water bath at 75 ˚C and the residue was resuspended in methanol (2.5 ml) and filtered through a 0.45 µl syringe filter. The filtrate was analysed using a Perkin Elmer PDA-UHPLC with a SIL-20A auto sampler. The extract was loaded onto a reverse phase column (C18 125 A 10 µm particle size, 150 x 4.6 mm) at 50 ˚C. The mobile phase consists of methanol: water (96: 4) at a flow rate of 1.2 ml/ min. Ergosterol content was determined by using a

standard (Sigma) at a retention time of approximately 7 min. A GLM-ANOVA was conducted to determine differences in ergosterol content between treatments using statistical software NCSS2007 (Hintze, 2007). The means were compared using Fisher's (LSD) and significant differences were determined ($P < 0.05$).

Results

Effect of phenolics extracts and sorgoleone on soil microorganisms

FDA analysis: No significant differences ($P > 0.05$) were found between the microbial activities of soils treated with sorghum extracts compared to the untreated control (Figure 1).

PLFA analysis: General bacteria content of the treated soil samples ranged from 90.61 nmol/g (sorgoleone 0.5x) to 135.01 nmol/g (RTx436 0.5x) (Figure 2). Gram positive bacteria content of the treated soil samples ranged from 7.09 nmol/g (SCAY 1x) to 13.26 nmol/g (sorgoleone 1x) (Figure 2). Gram-negative bacteria content of the treated soil samples ranged from 1.95 nmol/g (sorgoleone 0.5x) to 31.81 nmol/g (RTx430 0.5x) (Figure 2). Fungal content of the treated soil samples ranged from 19.29 nmol/g (sorgoleone 0.5x) to 42.41 nmol/g (RTx 436 0.5x) (Figure 2). Total PLFA content revealed that the highest microbial biomass was found in the soils of the untreated control. The lowest microbial biomass was found in the sorgoleone 0.5x treated soils. The microbial biomass of all four phenolic treated soils showed higher microbial biomass in all 0.5x treatments compared to the 1x treatments. The microbial biomass of sorgoleone treated soils showed a higher microbial biomass in the 1x treatment compared to the 0.5x treatment.

Cluster analysis of PLFA content clearly showed differences (< 55 % similarity) between the functional diversity of soils treated with phenolics and

sorgoleone compared to the untreated control with the exception of soils treated with extracts from RTx 430 0.5x and RTx 436 1x (Figure 3).

Effect of sorghum genotypes on soil microorganisms

FDA analysis: Significant differences ($P < 0.05$) in soil microbial activity in the rhizosphere were found between the 22 grain sorghum genotypes (Figure 4). The lowest activity was found in the rhizosphere soils of SC 103-12E (purple plant), SCAY 21 (tan plant) and SCAY 14 (tan plant) genotypes, while the highest was found in the rhizosphere soils of SC 109-14E (purple plant), SC 630-11EII (purple plant) and SC 630-11Eii (purple plant) genotypes. No clear relationship was observed between phenolic and sorgoleone content of the different genotypes and microbial activity in rhizosphere soils.

Effects of phenolic extracts and sorgoleone on fungal pathogens

Significant differences ($P < 0.05$) were evident between leaf length, root length and root rot severity of the extract treated plants inoculated with soil-borne pathogens compared to the untreated control (Figures 5, 6 and 7). No significant differences ($P > 0.05$) were found between plant fresh weight of treated plants inoculated with soil-borne pathogens compared to the untreated control. Significant extract x pathogen interactions ($P < 0.05$) were found between the leaf length, root length, root rot severity and plant fresh weight.

Significantly shorter ($P < 0.05$) average leaf lengths ranging from 171.33 mm to 309.42 mm were observed in sorghum plants treated with phenolics/sorgoleone and inoculated with *A. strictum, C. capsici, C. trifolii, F. equiseti, F. oxysporum, F. thapsinum* and *P. macrostoma* compared to leaf lengths ranging from 256.89 mm to

328.72 mm of the untreated control (Figure 5). On average, the leaf length of sorghum plants inoculated with *C. trifolii* and *F. thapsinum* were found to be the most inhibited by the sorghum extracts, while sorghum plants inoculated with *A. alternata* were found to be the least inhibited. On average, the leaf length of sorghum plants treated with both concentrations of RTam 428 and sorgoleone extracts were found to be the most inhibited, while sorghum plants treated with both concentrations of SCAY 21 extracts were found to be the least inhibited.

Significantly longer ($P < 0.05$) average root lengths ranging from 330.67 mm to 562 mm were observed in sorghum plants treated with phenolics/sorgoleone and inoculated with *A. strictum, A. alternata, C. capsici, C. trifolii, F. oxysporum* and *P. macrostoma* compared to root lengths ranging from 268.94 mm to 333.39 mm of the untreated control (Figure 6). Significantly shorter root lengths were observed in sorghum plants treated with phenolics/sorgoleone and inoculated with *F. equiseti* compared to the untreated control. On average, the root length of sorghum plants inoculated with *C. trifolii* and *P. macrostoma* were found to be the most stimulated by the phenolics/sorgoleone extracts, while sorghum plants inoculated with *F. oxysporum* were found to be least stimulated. On average, the root lengths of sorghum plants inoculated with *F. equiseti* and *F. thapsinum* were found to be the most inhibited by phenolics/sorgoleone extracts compared to the untreated control. On average, the root length of sorghum plants treated with RTx 436 1x and sorgoleone 0.5x extracts were found to be the most stimulated, while sorghum plants treated with RTam 428 0.5x and sorgoleone 1x extracts were found to be the least stimulated.

Significantly lower ($P < 0.05$) average root rot severity ranging from 7.22 % to 29.31 % was observed in sorghum plants treated with phenolics/sorgoleone and

inoculated with *A. alternata, C. capsici, C. trifolii, F. equiseti, F. oxysporum, F. thapsinum* and *P. macrostoma* compared to root rot severity ranging from 19.44 % to 41.11 % of the untreated control (Figure 7). On average, the root rot severity of sorghum plants inoculated with *A. alternata, C. trifolii* and *F. thapsinum* were found to be the most inhibited by the extracts, while plants inoculated with *A. strictum* and *C. capsici* were found to be the least inhibited.

Significant differences ($P < 0.05$) in leaf length, root length and root rot severity of diseased plants were found between the different concentrations of phenolics and sorgoleone (Figure 5, 6, 7). On average, the root rot severity of sorghum plants treated with SCAY 21 0.5x, RTam 428 1x and sorgoleone 1x extracts were the most significantly different, while sorghum plants treated with RTx 436 0.5x and SCAY 21 1x extracts were the least significantly different. No significant differences ($P > 0.05$) were found in the ergosterol content of diseased sorghum roots treated with phenolic extracts and sorgoleone compared to the untreated control.

Discussion

The objective of the present study was to determine the direct effects of sorghum phenolic extracts and sorgoleone on soil-borne organisms in the rhizosphere and soil-borne pathogens. The results of the field trial indicated that sorghum genotype directly influences soil microbial activity. This effect of sorghum genotypes on microbial activity is consistent with the findings of Aira *et al.* (2010) who demonstrated significant differences in microbial activity in rhizosphere soils of two different maize genotypes. This effect on soil microbial activity could be the result of different phenolic or allelopathic compounds within the root exudates of the different genotypes. Phenolic and other allelochemical extracts of allelopathic plants are

known for their effect on soil microbial organisms. Qu and Wang (2007) observed significant increases of up to 185 mg C/Kg soil and significant decreases of up to 69 mg C/Kg soil in C utilising soil microbial biomass by 2,4-di-tert-butylphenol and vannilic acid extracts respectively compared to 115 mg C/Kg soil of the untreated control. Similarly, Gopal *et al.* (2006) found significantly reduced soil bacterial and fungal diversity of up to ± 0.008 Shannon Weaver index value by different concentrations (1x, 2x and 5x recommended dosage) of azadirachtin extracted from neem compared to the untreated control.

The present study indicated distinct differences in functional diversity between rhizosphere soils treated with sorghum extracts compared to untreated control soils. This suggests that sorghum phenolics and sorgoleone directly influence the diversity and structure of soil microbial organisms. *Sorghum bicolor* contains several phenolics known to affect soil microbial organisms such as benzoic and cinnamic acids. Results in this study are consistent with the findings of Liu *et al.* (2016), who investigated the effect of benzoic acid present within root exudates of peanuts (*Arachis hypogaea* L.) on soil microbes. The authors found significant increases in relative abundance of up to 1.55 % in *Fusarium* and 1.65 % in *Trichoderma* and significant decreases in relative abundance of up to 1.08 % in *Mortierella* in treated soils compared to the untreated control. Wu, Wang and Xue (2009) investigated the effect of different concentrations (25, 50, 100 and 200 mg/kg soil) of cinnamic acid present within root exudates of cucumber (*Cucumis sativus*) on soil microbial communities. They showed that cinnamic acid promoted soil microbial diversity that can utilise carbon sources such as cyclodextrin, glycogen and 2-hydroxy benzoic acid and inhibited soil microbial diversity which can utilise hydrobutyric acid, L-asparagine and D-galacturonic acid. These changes in functional diversity and

biomass may ultimately affect microbial ecological processes, the health and wellbeing of adjacent or succeeding plants and the pathogenicity of soil-borne pathogens.

Significantly shorter leaf length, longer root length and lower root rot were observed in sorghum plants treated with phenolics and sorgoleone compared to the untreated control. Results in this study confirm that sorghum phenolics and sorgoleone can effectively inhibit soil-borne pathogens such as *A. alternata, C. trifolii, F. oxysporum, F. equiseti* and *F. thapsinum*. The inhibitory effect was less effective on sorghum plants inoculated with *A. strictum* and *C. capsici*. This inhibition of soil-borne pathogens by plant allelopathic extracts, allelopathic plant parts or purified allelochemicals are also found in other plants against similar pathogens. Wu *et al.* (2008) showed significant inhibition of up to 5.2 cm in hyphal growth and 100 % suppressed germination of *F. oxysporum* f.sp. *niveum* (Fon) by salicylic acid (800 mg/L agar). Gomez-Rodriguez *et al.* (2003) showed significant inhibition of up to 20 % in conidia germination and up to 24 % in number of germ tubes per conidium of inoculated *Alternaria solani* on tomato leaflets by marigold (*Tagetes erecta* L.) leaves. Studies have also shown inhibition of other important plant pathogens by allelopathic plants. Zhang *et al.* (2008) who investigated the effect of Canada goldenrod (*Solidago Canadensis* L.) root extracts on *Pythium ultimum* Trow and *Rhizoconia solani* Kühn in microcosms. The authors found significant inhibitions of up to 91 % and 84 % in the growth and reductions of up to 81 % and 50.34 % in damping-off rates of *P. ultimum* and *R. solani* respectively by the root extracts compared to the untreated control.

Significant differences in leaf length, root length and root rot severity were also found between different concentrations of phenolic extracts. These differences in

inhibitory effects by different concentrations of plant extracts are similar to the findings of Qi, Zhen and Li (2015) who investigated the effect of plant phenolics such as *p*-hydroxybenzoic, vanillic, syringic and salicylic acids in decomposing maize straw against *Rhizoctonia cerealis* Murray & Burpee and *Gaeumannomyces graminis* Sacc. in wheat (*Triticum aestivum* L.). The authors found reduced occurrence of sharp eyespot by lower concentrations (0.03 and 0.06 g/ml water) of the extracts while higher concentration (0.12, 0.24 and 0.48 g/ml water) of the extracts promoted the occurrence of sharp eyespot. They related this to growth promoting effects of wheat roots by allelopathic compounds that reduced pathogens populations.

Sorghum plant extracts are well known for their herbicidal properties and are utilised as biological control agents against several small seed weeds (Weston, Alsaadawi and Baerson, 2013). Our findings suggest the possible utilization of sorghum phenolics and sorgoleone extracts as biological control agents against soil-borne fungal pathogens. Since the production of sorghum phenolics and sorgoleone are the highest in seedlings, sorghum could also be utilised as a cover crop not only to reduce weeds but also improve soil health. Modern weed and pathogen control methods are focusing on searching for natural alternatives to reduce environmental damage and reduce health concerns. Manipulating allelopathic crops has been identified as a possible alternative for applying synthetic pesticides and allelochemicals have shown potential for being developed into commercial pesticides. Several studies have shown profound suppressive effects of allelopathic plant extracts against fungal and bacterial plant pathogens (Chérif, Arfaoui and Rhaiem, 2007; Shafique *et al.*, 2007). Rhouma *et al.* (2009) investigated the effects of leaf extracts from pepper trees (*Pistacia* L. spp., *Schinus* Raddi spp.) against *Agrobacterium tumefaciens* Smith & Townsend, *Pseudomonas savastanoi* pv. *savastanoi* (Janse)

Gardan, *Fusarium solani* Sacc. and *Rhizoctonia solani*. The authors found significant inhibitions of up to 68 % in mycelial growth and inhibition zones of up to 29 mm against the pathogens by the extracts. Similarly, Larçin *et al.* (2015) investigated the effects of flower extracts from pot marigold (*Calendula officinalis* L.) on 11 bacterial plant pathogens such as *Pseudomonas tomato*, *Xanthomonas vesicatoria* (Pammel) Dowson, *Erwinia amylovora* (Burr.) Winslow, *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis and *A. tumefaciens*. The authors demonstrated successful antibacterial activity with minimal concentrations of 256 µg/ml agar against *E. amylovara* and 512 µg/ml agar against *C. michiganensis* by the marigold flower extracts.

In conclusion, the results of the present study indicate direct effects of sorghum phenolics and sorgoleone on general soil microbial activity and diversity. A significant inhibitory effect of sorghum allelopathic extracts on certain soil-borne pathogens was also observed. The extracts with the most effective inhibitory effect on the soil-borne pathogens were SCAY 21 0.5x phenolic extracts and sorgoleone 1x extracts. These results suggest that sorghum phenolics and sorgoleone have the potential to be used as a biological control agent against soil-borne pathogens. Further research should be conducted to clarify and understand the mechanisms of these inhibitory effects and determine the potential benefits of using grain sorghum extracts and cover crop to inhibit soil-borne pathogens.

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Table 1. Grain sorghum genotypes used in the field experiment with regards to plant and seed colour.

Line No	Line	Pl. Col.	Pericarp	Mesocarp	Testa	Spread.	Plant	Seed colour
1	BTx378	PPQQ	RRYYII	zz	b1b1B2B2	SS	purple	red, chalky
2	BTx3197	PPQQ	RRyyii	zz	b1b1B2B2	SS	purple	white, chalky
3	RTx430	PPQQ	RRyyll	ZZ	b1b1b2b2	SS	purple	white, pearly, yellow endosperm
4	RTam428	PPqq	rryyll	ZZ	b1b1B2B2	ss	red	white, pearly
5	RTx436	ppQQ	RRyyii	ZZ	B1B1b2b2	--	tan	white
6	BTx635	ppQQ	RRyyii	ZZ	--	--	tan	white pearly food grad sorghum
7	BTX ARG-1	ppQQ	RRyyii	ZZ	--	--	tan	waxy endosperm
8	RTx2917						tan	red
9	SC630-11EII	PPQQ	RRYYII	ZZ	b1b1B2B2	SS	purple	dark red, pearly
10	SC630-11 Eii	PPQQ	RRYYii	ZZ	b1b1B2B2	SS	purple	light red, pearly
11	SC748-5	PPQQ	rrYYIl	ZZ	b1b1B2B2	ss	purple	lemon yellow, pearly
12	SC109-14E	PPQQ	RRyy--	zz	B1B1B2B2	ss	purple	white pearly, purple testa
13	S5C719-11E	PPQQ	RRYYii	zz	B1B1B2B2	ss	purple	red, chalky,
14	SC103-12E	PPQQ	RRYYII	zz	B1B1B2B2	SS	purple	dark brown-red, chalky
15	Dobbs	PPQQ	RRyyii	zz	B1B1B2B2	SS	purple	brown, chalky
16	Hegari	PPQQ	RRyyii	zz	B1B1B2B2	ss	purple	white, chalky
17	RTam2566	PPQQ	RRYYII	ZZ	B1B1B2B2	ss	purple	dark brown-red, pearly
18	Tx2911						red	red, chalky
19	SCAY13						tan	lemon-yellow
20	SCAY16						tan	lemon-yellow
21	SCAY21						tan	red
22	SCAY14						tan	lemon-yellow

Table 2. Sorghum phenolic extract and sorgoleone concentrations applied to glasshouse experiments.

Sorghum genotype	**Type of extract**	**Concentration**	**Concentration detail**
RTx 430	Phenolic extract	0.5x	0.5 mg (dry plant material)/ ml, 1000 L/ Ha
RTx 430	Phenolic extract	1x	1 mg (dry plant material)/ ml, 1000L/ Ha
RTx 436	Phenolic extract	0.5x	0.5 mg (dry plant material)/ ml, 1000 L/ Ha
RTx 436	Phenolic extract	1x	1 mg (dry plant material)/ ml, 1000L/ Ha
Rtam 428	Phenolic extract	0.5x	0.5 mg (dry plant material)/ ml, 1000 L/ Ha
Rtam 428	Phenolic extract	1x	1 mg (dry plant material)/ ml, 1000L/ Ha
SCAY 21	Phenolic extract	0.5x	0.5 mg (dry plant material)/ ml, 1000 L/ Ha
SCAY 21	Phenolic extract	1x	1 mg (dry plant material)/ ml, 1000L/ Ha
BTx 3197	Sorgoleone extract	0.5x	0.3 kg (dried sorgoleone)/ Ha
BTx 3197	Sorgoleone extract	1x	0.6 kg (dried sorgoleone)/ Ha

Table 3. List of fatty acids and signature lipids used to determine soil microbial functional groups.

Bacterial Acid Methyl Esters (BAME's)		
Component Name	**Formula**	**Signature lipid**
Methyl octanoate	C8:0	
Methyl decanoate	C10:0	
Methyl 2-hydroxydecanoate	2-OH-C10:0	
Methyl undecanoate	C11:0	
Methyl dodecanoate	C12:0	
Methyl 3-hydroxydodecanoate	3-OH-C12:0	Gram negative bacteria
Methyl tridecanoate	C13:0	
Methyl tetradecanoate	C14:0	General bacteria
Methyl 2-hydroxytetradecanoate	2-OH-C14:0	Gram negative bacteria
Methyl 3-hydroxytetradecanoate	3-OH-C14:0	Gram negative bacteria
Methyl 13-methyltetradecanoate	i-C15:0	Gram positive bacteria
Methyl 12-methyltetradecanoate	a-C15:0	Gram positive bacteria
Methyl cis-10 pentadecanoate	$C15:1^{10}$	
Methyl hexadecanoate	C16:0	General bacteria
Methyl 14-methylpentadecanoate	i-C16:0	Gram positive bacteria
Methyl cis-9-hexadecanoate	$C16:1^{9}$	Gram negative bacteria
Methyl octadecanoate	C18:0	General bacteria
Methyl trans-9-octadecenoate	$C18:1^{9}$	
Methyl cis-9-octadecenoate	$C18:1^{9}$	Fungi
Methyl cis-7-octadecenoate	$C18:1^{7}$	
Methyl cis-9,12-octadecadienoate	$C18:2^{9,12}$	Fungi
Methyl cis-9,12,15 octadecatrienoate	$C18:3^{9,12,15}$	Fungi
Methyl cis-9,10-methyleneoctadecanoate	$C19:0^{\Delta}$	Gram negative bacteria
Methyl cis-11 eicosenoate	$C20:1^{11}$	
Methyl cis-11,14,17 eicosatrienoic	$C20:3^{11,14,17}$	
Methyl docosanoate	C22:0	
Methyl cis-13 docosadienoate	$C22:1^{13}$	
Methyl tetracosanoate	C24:0	
Methyl cis-15 tetracosanoate	C24:1c15	

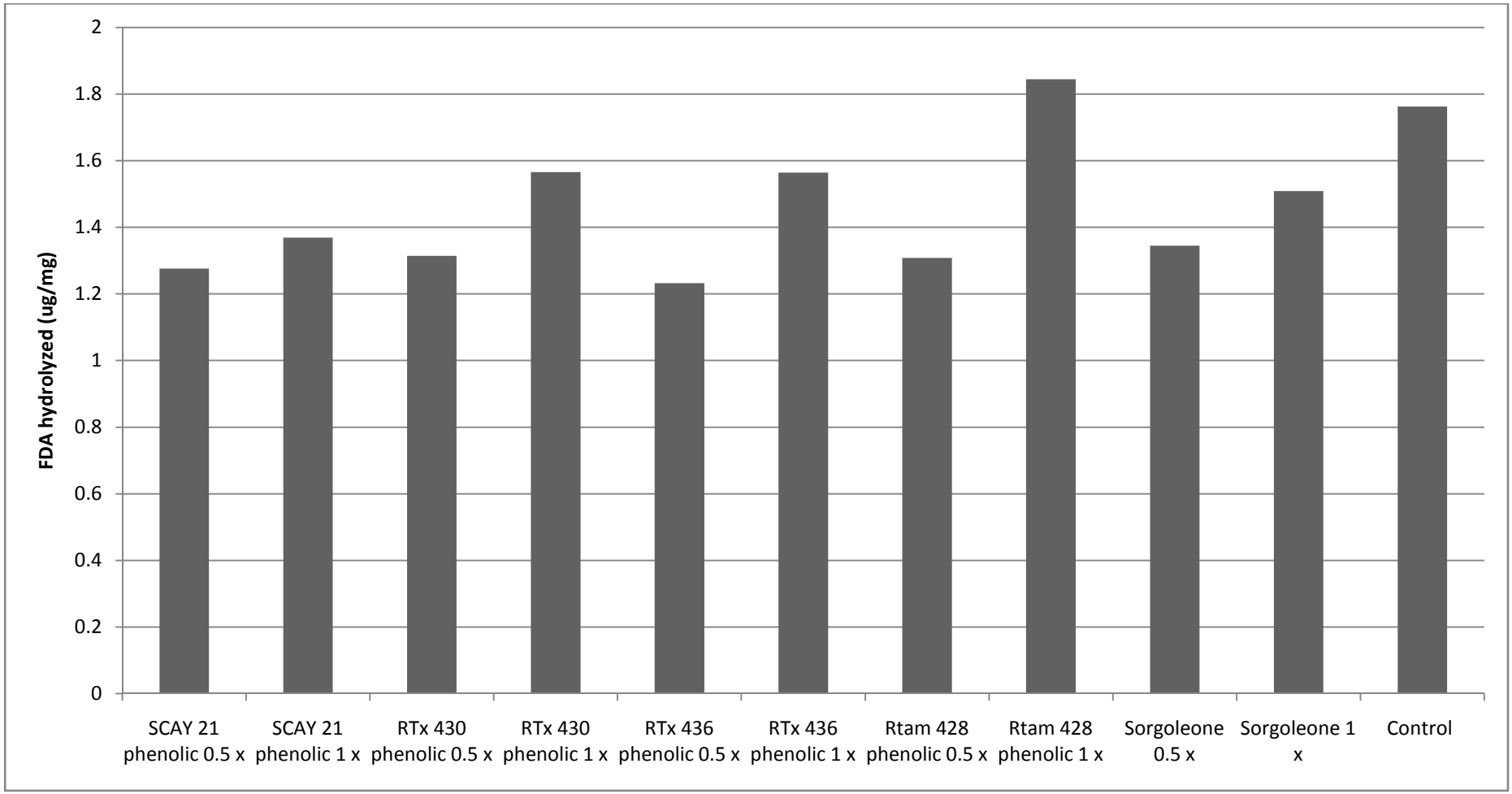

Figure 1. Average FDA hydrolysis of soils treated with sorghum extracts.

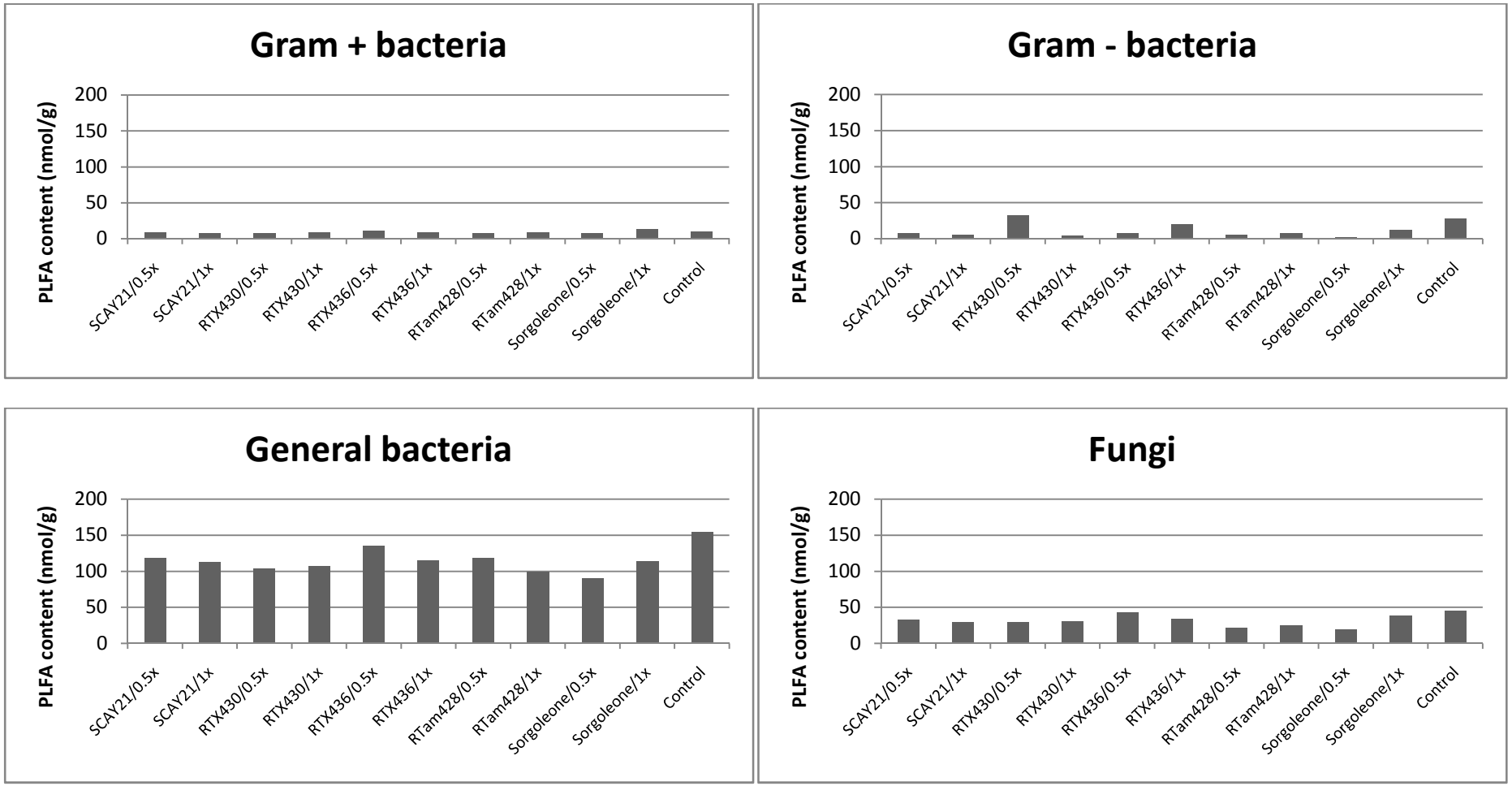

Figure 2. PLFA content expressing soil microbial functional groups of soils treated with sorghum extracts.

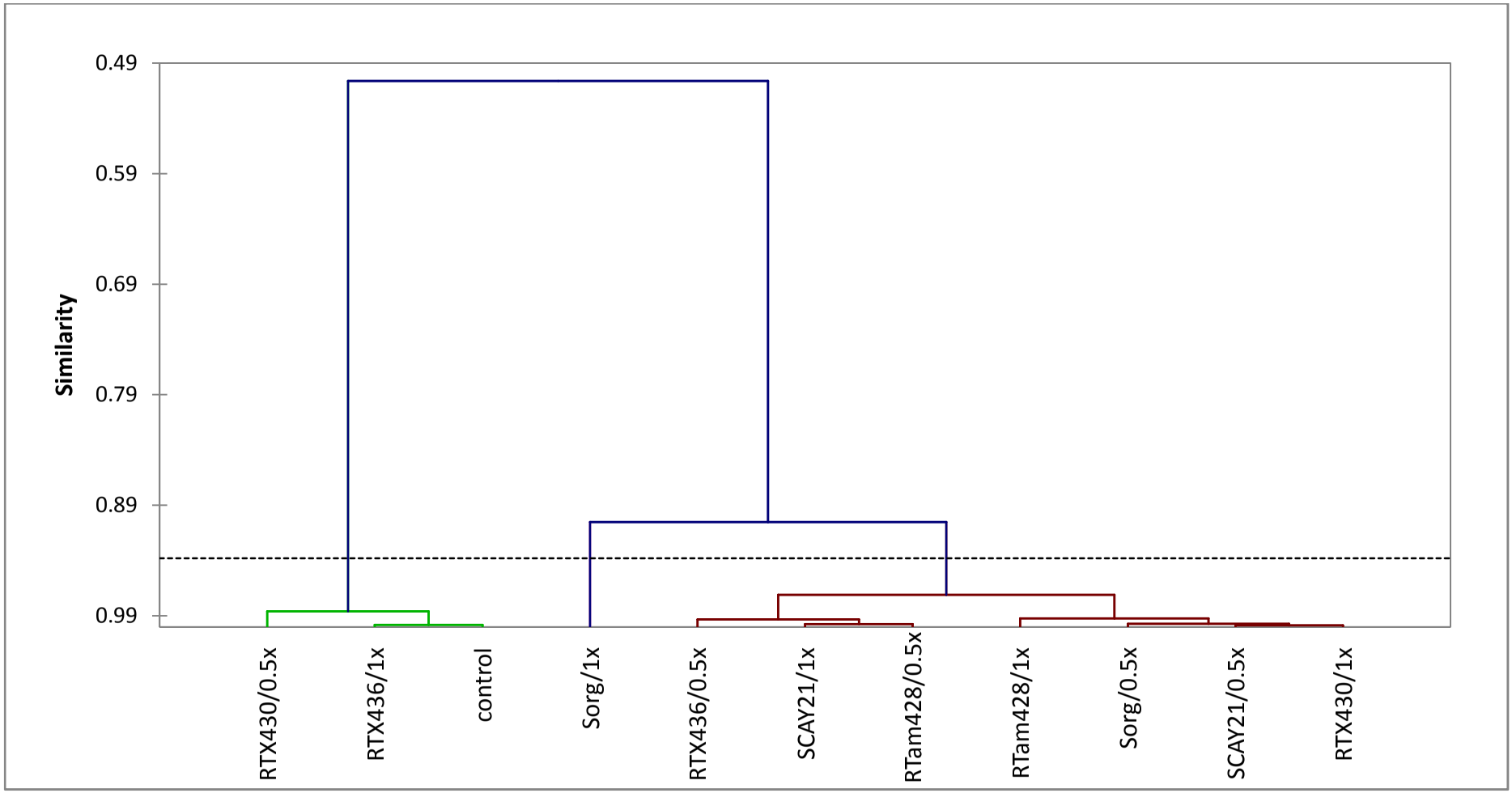

Figure 3. Average PLFA content of soils treated with sorghum extracts.

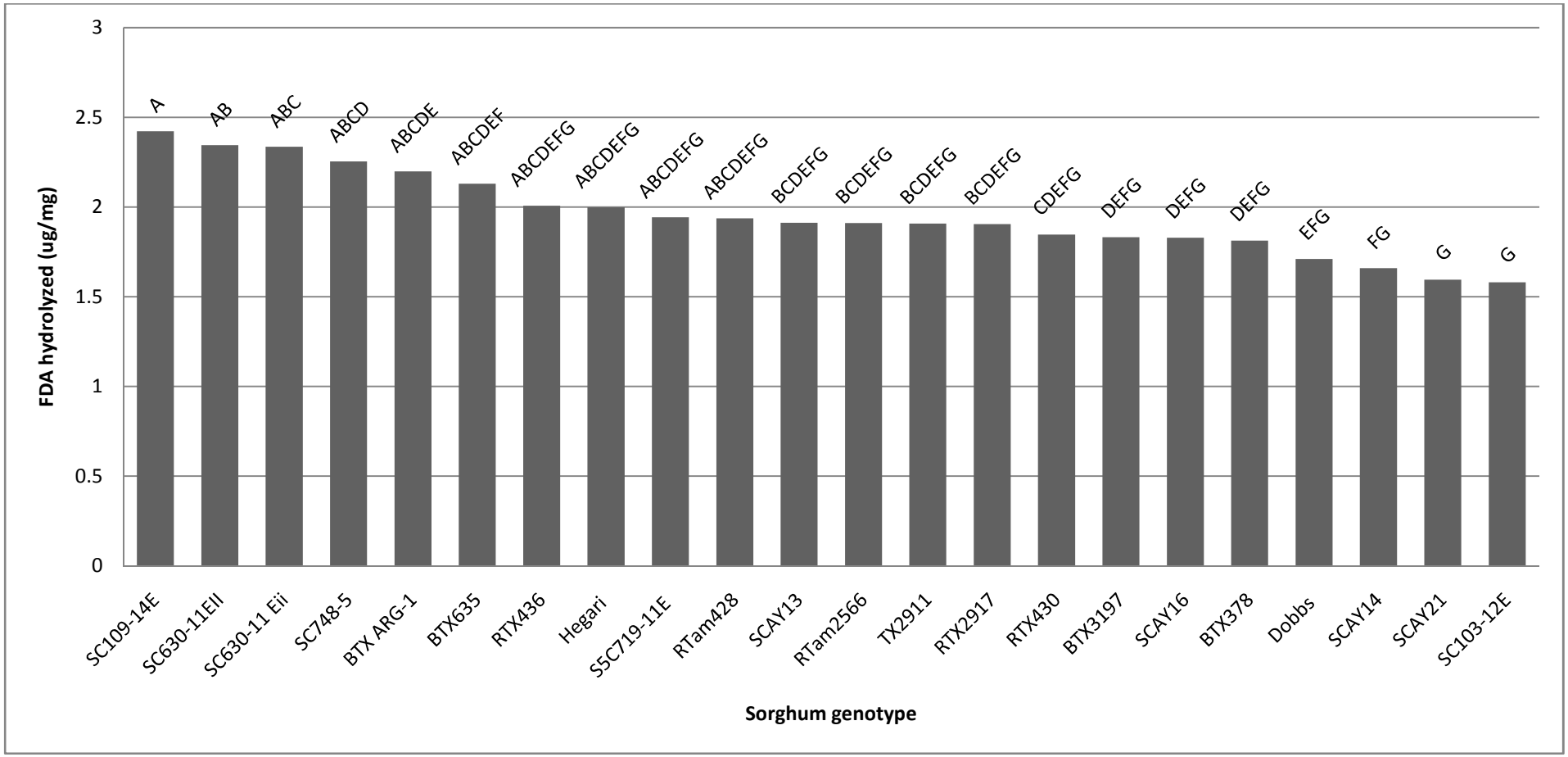

Figure 4. FDA hydrolysis of rhizosphere soil from 22 grain sorghum genotypes. Bars with the same uppercase letter/s are not significantly different ($P > 0.05$).

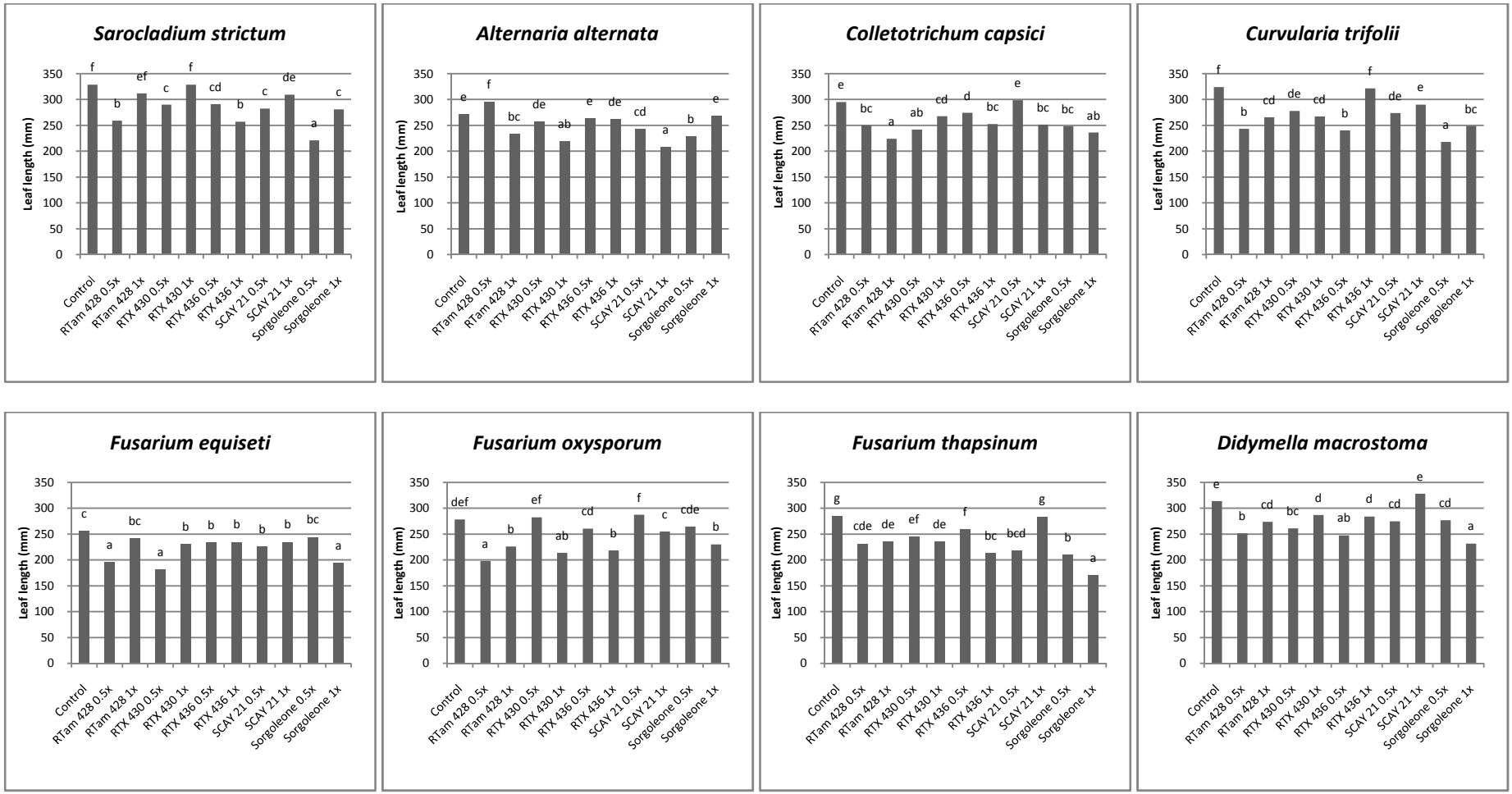

Figure 5. Leaf length (mm) of sorghum plants challenged with soil-borne pathogens. Bars with the same lowercase letter/s are not significantly different (P > 0.05).

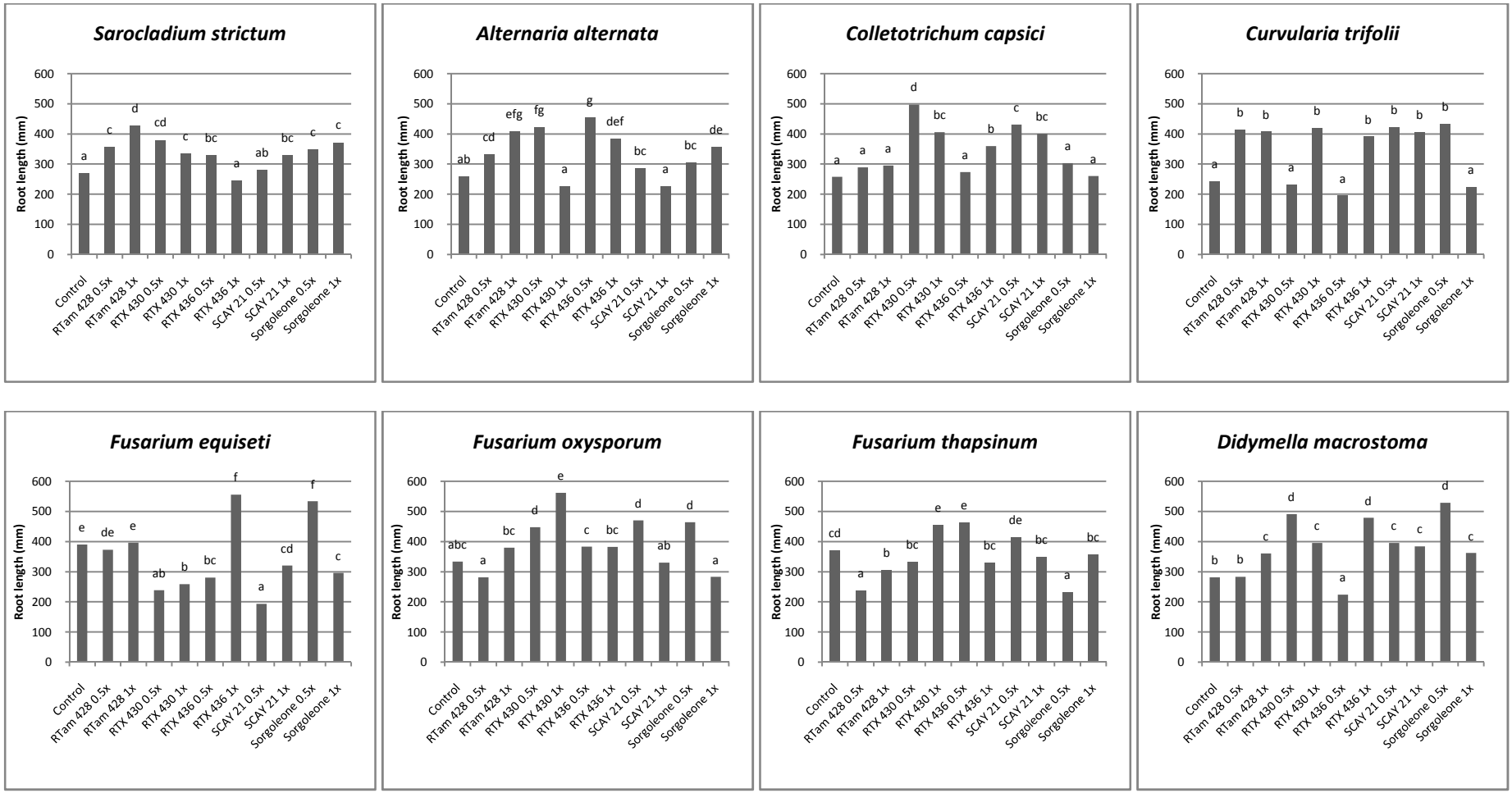

Figure 6. Root length (mm) of sorghum plants challenged with soil-borne pathogens. Bars with the same lowercase letter/s are not significantly different (P > 0.05).

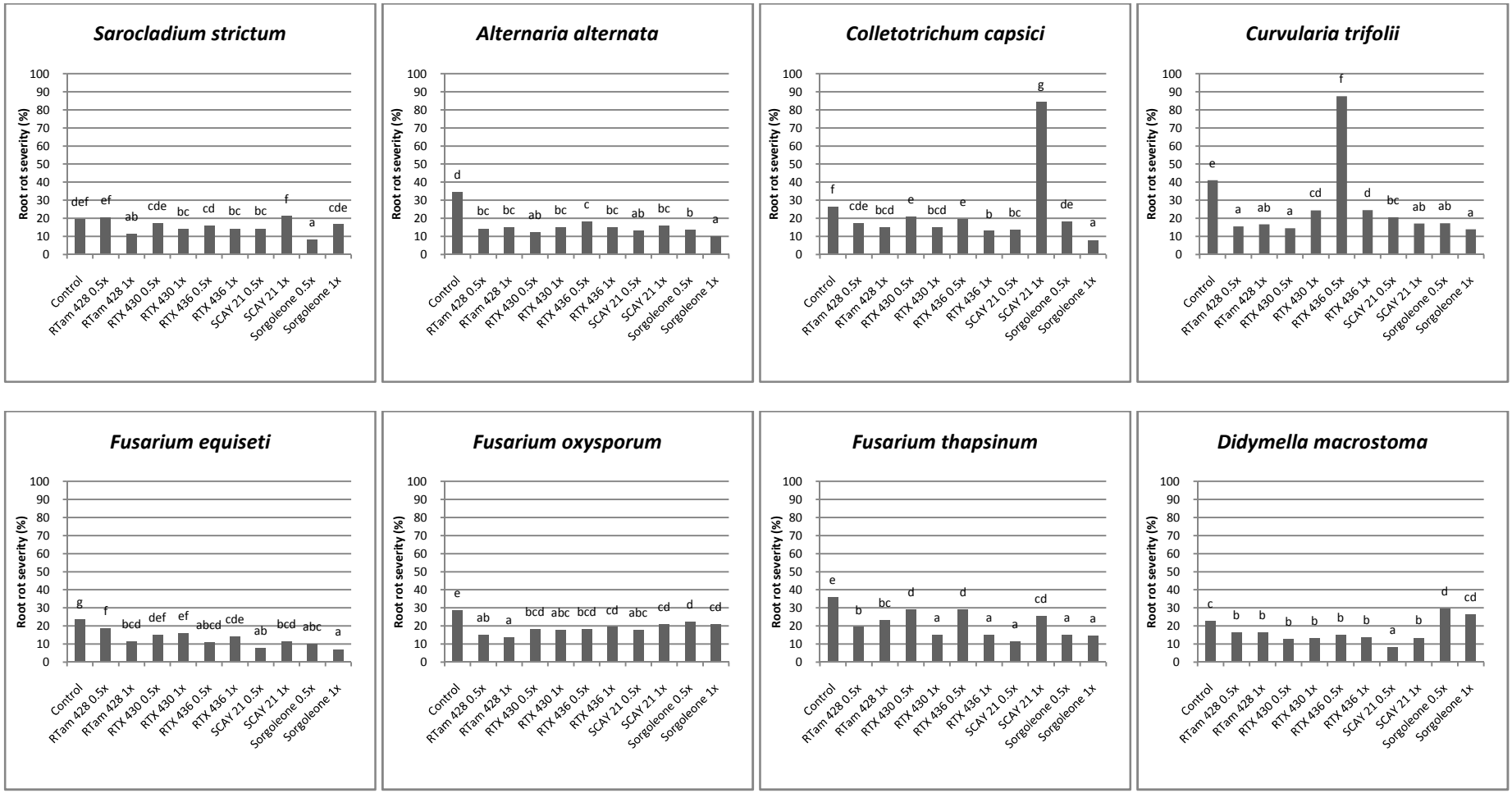

Figure 7. Root rot severity (%) of sorghum plants challenged with soil-borne pathogens. Bars with the same lowercase letter/s are not significantly different (P > 0.05).

CHAPTER FOUR

INDIRECT EFFECT OF SORGHUM PHENOLIC EXTRACTS AND SORGOLEONE ON SOIL-BORNE PATHOGENS

Abstract

Plants produce a wide variety of phenolic and other compounds during secondary metabolism known as allelochemicals which they exude into their surrounding environment. Allelochemicals are known for their direct and indirect effects on surrounding soil organisms and provide plants with an advantage during competition with other plants, as well as against pests and pathogens. This study investigated the possible indirect effects of allelochemicals produced by various grain sorghum genotypes on soil-borne plant pathogens in the field as well as in the greenhouse. The microbial functional diversity and biomass of rhizosphere soils treated with sorghum phenolics and sorgoleone extracts were analysed using PLFA. The PLFA content showed little similarity (< 55 %) between the bacterial and fungal content in soils treated with phenolics and sorgoleone compared to the untreated control. The phenolics and sorgoleone treated rhizosphere soils were then inoculated with eight soil-borne pathogens and planted with sorghum seedlings (PAN 8806), to investigate indirect effects of the sorghum allelopathic extracts on the pathogens. Rhizosphere soils previously treated with phenolics and sorgoleone extracts were most effective against suppression of *Alternaria alternata* and *Fusarium thapsinum* and least effective against *Didymella macrostoma*. Rhizosphere soils previously treated with RTx 436 0.5x and SCAY 21 0.5x extracts were most effective in suppressing the eight pathogens. These results could clarify the potential residual effects of sorghum allelochemicals on the suppression of soil-borne plant pathogens.

Introduction

Plants produce a wide variety of chemical and organic compounds such as vitamins, amino acids, proteins during secondary metabolism which they exude into their surrounding above- and below-ground environment (Benizri and Amaiud, 2005; de Albuquerque *et al.*, 2010). This phenomenon is referred to as allelopathy and the compounds, known as allelochemicals, are well known for their direct and indirect effects on organisms in their adjoining environment. They provide plants with an advantage during competition with other surrounding plants as well as protecting them against insect pests and plant pathogenic microorganisms (Huang and Chou, 2005; de Albuquerque *et al.*, 2010; Rattan 2010; Won *et al.*, 2013; Li *et al.*, 2014). Initial studies on allelochemicals focused mainly on their phytotoxic properties, however recent studies have focused more on their effect towards microbes. It has therefore been established that allelochemicals play a vital role in determining the biomass and the community diversity of surrounding microbial organisms both above- and below-ground (Kong *et al.*, 2008; Gimsing *et al.*, 2009).

Microbial organisms are a vital constituent of soils and facilitate several vital ecological processes (Beare *et al*., 1996; Gunapala and Scow, 1997; Foissner, 1999; Bin-Ru *et al*., 2005) that ultimately affect the growth and health of plants (Liu, Glenn and Buckley, 2008). Organisms that occur specifically in the rhizosphere can promote the growth of adjacent plants (Tang *et al*., 2014; Liu *et al*., 2017) and also protect the host plant from attack by soil-borne pathogens (Udai *et al.*, 2016). Low diversity of rhizosphere fungi and bacteria can however lead to an increase in pathogen inoculum in surrounding soil and ultimately result in a disease outbreak (Qiu *et al.*, 2012; Manikandan, Karthikeyan and Raguchander, 2017; van Agtmaal *et al.*, 2018). Pathogenic bacteria and fungi multiply rapidly in soils with low microbial

diversity and can cause losses in crop yield of up to 80 % or more (Huang and Chou, 2005; Wu *et al.*, 2008; Hooks *et al.*, 2010; Wu *et al.*, 2013).

Allelochemicals can affect soil microbes by either promoting or inhibiting their growth and diversity (Kong *et al.*, 2008; Gimsing *et al.*, 2009). Several *in vitro* studies have demonstrated the influence of allelochemicals improving soil microbial biomass and community diversity and the inhibition of soil-borne plant pathogens (Kong *et al.*, 2004; Wu *et al*., 2008; Zhang *et al.*, 2008; Zhang *et al.*, 2011; Qi, Zhen and Li, 2015). The genotype of the donor plant can influence the overall effect of associated allelochemicals on soil microbes. Different genotypes of crops such as maize, rice, tomato and blueberry were shown to have significantly different rhizobiomes (Lin *et al*., 2007; Aira *et al*. 2010; Poli *et al.,* 2016; Jiang *et al.*, 2017). Although several recent studies have shown direct promotion or inhibition of allelopathy on soil microbial diversity, the indirect effect of allelopathic plants on soil-borne plant pathogens is not well understood.

The objectives of the present study were firstly, to investigate possible indirect effects of grain sorghum genotypes on soil-borne plant pathogens in the field and secondly, to investigate possible indirect effects of phenolic extracts from sorghum and its specific allelochemical, sorgoleone on eight soil-borne plant pathogens in the greenhouse.

Materials and methods

Influence of sorghum genotype on soil-borne pathogens

A total of 22 genotypes of *Sorghum bicolor,* representing a range of phenotypic characteristics associated with grain sorghum, including variation in plant and grain colours were selected (Table 1).

The study was conducted on a farm situated in the Alma district, in the Limpopo province of South Africa (-24°28'59.99"S, 28°03'60.00"E) during 2014 and 2015. Seed was sown in October 2014 and sorghum plants were harvested in April 2015. The trial design was a randomised block design with seeds of each genotype sown in two adjacent rows. The trial was replicated. Sorghum plants were harvested by carefully uprooting them so as to maintain as much of the root volume as possible. Vegetative disease assessment was performed on the harvested sorghum plants with root weight and percentage visual root-rot being recorded. The roots of each sorghum plant were removed from the plant and dried at room temperature. The dried sorghum roots were then ground using liquid nitrogen and stored for ergosterol analysis. A GLM-ANOVA was conducted to determine differences in root weight and root-rot rating between samples using statistical software NCSS2007 (Hintze, 2007). The means were compared using Fisher's least significant difference (LSD) and significant differences were determined ($P < 0.05$).

Influence of sorghum extracts on soil microbial diversity

Phenolics were extracted from four sorghum genotypes: RTx 430 (purple plant), RTx 436 (tan plant), Rtam 428 (red plant), SCAY 21 (tan plant) and sorgoleone was extracted from genotype BTx 3197. Phenolic extraction was adapted from the method of Won *et al*. (2013). Sorghum seeds of each genotype were grown

separately in rectangular plastic containers (500 ml) containing vermiculite in an incubator set at ± 28 ºC and 12 hr day/night cycles. Seedlings were watered daily to field water capacity. After two weeks, seedlings were removed from the containers, roots were excised and the remaining vegetative material was air dried at room temperature for 7 days before being ground into a fine powder using liquid nitrogen. Powder of each genotype was placed in 50 ml tubes and immersed in methanol (1:50 w/v) for 24 hr at room temperature.

Sorghum seeds of BTx 3197 were germinated in a closed rectangular plastic container (500 ml) between two sheets of sterilised tissue paper. The tissue paper were wetted with sterilized water until holding capacity and sprayed with sterilized water daily during the incubation period. The container was incubated at ± 25ºC for 7 days in the dark. Seedlings were removed from the tissue paper and roots were retained for sorgoleone extraction according to the methods of Netzly and Butler (1986) and Uddin *et al*. (2010). Freshly cut seedling roots were weighed and placed in a 50 ml tube and immersed in (1:20 w/v) methanol for 30 sec after which the extract was filtered using filter paper (Selecta faltenfilter nr. 595½, Schleicher & Schüll). The filtered extracts were evaporated under a fume hood at room temperature. The amount of root exudate containing sorgoleone was determined by physically weighing the exudate powder according to the method of Czarnota, Rimando and Weston (2003). The exudate powder was weighed with a scale (RADWAD Wagi Elektroniczne, Lasec) and determined proportionally to the weight of the original roots.

Microcosms were prepared using 18 cm plastic pots filled with sieved natural loam soil purchased in Bloemfontein. Phenolic extracts and sorgoleone was applied at two different concentrations prior to the planting of the sorghum seedlings (Table

2). Each extract was applied as a methanol/water mixture (< 3 % methanol extract/ L water) directly onto the soil. A control treatment was prepared without sorghum extracts and each treatment was replicated three times. Sorghum seeds (PAN 8806) were pre-germinated and 6 seedlings were planted in each microcosm. Seedlings were cultivated at a mean temperature of 24 ˚C and watered daily. After 6 weeks cultivation, the sorghum plants were removed and soil samples were collected directly from the rhizosphere of each sorghum plant. The soil sample was stored at -80 ˚C for phospholipid fatty acid analysis (PLFA) in order to determine microbial diversity.

Influence of previous sorghum extracts on soil-borne pathogens

Eight soil-borne fungal pathogens *(Sarocladium strictum* Gams*, Alternaria alternata* Keissl.*, Curvularia trifolii* Boedijn*, Didymella macrostoma* Mont.*, Colletotrichum capsici* Butler & Bisby*, Fusarium thapsinum* Klittich*, Fusarium equiseti* Corda*, Fusarium oxysporum* Schlecht.) previously isolated from diseased sorghum roots were used for this study (van Rooyen, 2012). A medium for growing inoculum was prepared by placing sorghum grain (100 g) in glass beakers and soaking them in deionised water (100 ml) for 24 hr. The excess water was then decanted and the seeds were autoclaved twice for 25 min at 121 ˚C. Agar plugs of each fungal isolate were then placed in the beaker and incubated under UV light at room temperature for 3~4 weeks. The beakers were shaken every two days to promote fungal growth. The inoculum was then removed from the beaker, dried at 25 °C for 72 hours, ground into a fine powder and stored in sterile rectangular plastic containers (500 ml) at room temperature.

Microcosms were designed using 12 cm plastic pots filled with soil collected from the previous experiment. A control treatment was prepared with untreated

sieved natural loam soil. Inoculum powder equal to 0.3 % of the soil mass was mixed into the pots 2 weeks prior to the planting of sorghum seedlings. Sorghum seeds (PAN 8806) were pre-germinated and six seedlings were planted in each microcosm. Seedlings were cultivated at a mean day and night temperature of 24 ˚C and watered daily. After 6 weeks, the sorghum plants were carefully removed from the soil and washed before leaf length, root length, plant fresh weight and the visual root disease severity was assessed. The roots were then dried at room temperature before being ground into powder using liquid nitrogen for ergosterol analysis. A GLM-ANOVA was conducted to determine differences in leaf length, root length, plant weight and the severity of root disease between samples using statistical software NCSS2007 (Hintze, 2007). The means were compared using Fisher's (LSD) and significant differences were determined ($P < 0.05$).

Phospholipid fatty acid analysis: This procedure uses phospholipid fatty acids (PLFA) as biomarkers to determine total microbial composition in soil and differentiate them into different functional groups by means of their respective fatty acid profiles (Table 3). The procedure used was adapted and modified from White *et al.* (1979) as described by Marschner (2007). Soil samples (2 g) were placed in 25 ml centrifuge tubes and methanol (3.8 ml), citrate buffer (1.5 ml), chloroform (1.9 ml) and Blight and Dyer reagent (2 ml) were added. The tubes were then shaken for 2 hr and centrifuged at 3500 g for 10 min. The supernatant was transferred to a new centrifuge tube and the pellet in the old centrifuge tube was washed with Blight and Dyer reagent (2.5 ml), shaken and centrifuged (as in the previous step) and the supernatant was added to the supernatant in the new centrifuge tube. The pooled supernatants were diluted with chloroform (3.1 ml) and citrate buffer (3.1 ml),

vortexed and centrifuged at 3500 g for 10 min. The lower phase (3 ml) was then transferred into a 10 ml glass tube and the content was dried at 40 ˚C under a flow of N_2. The dried samples were dissolved in chloroform (300 µl) and pipetted onto a conditioned (2 x 1 ml chloroform) silica-bonded column. Neutral lipids were eluted with chloroform (5 ml) and glycolipids were eluted with acetone (10 ml). Both elutants were then discarded. Phospholipids were eluted with methanol (5 ml), and the elutant was transferred to a 10 ml centrifuge tube and the content was again dried at 40 ˚C under a steady flow of N_2. An internal standard (C19:0; 30 µl) was then added to the dried samples, followed by a methanol - toluol solution (1:1, v/v; 1 ml) and 0.2 M KOH-MeOH (1 ml). The suspension was vortexed and incubated for 15 min at 37 ˚C. A hexane-chloroform solution (2 ml), 1 M acetic acid (0.3 ml) and deionised water (2 ml) were added to the suspension. The suspension was vortexed for 1 min, centrifuged for 5 min at 3500 g and the supernatant was transferred into a 10 ml glass tube. Another aliquot of hexane-chloroform solution (2 ml) was added to the pellet, the solution was vortexed and centrifuged as described previously and the supernatant was added to the previous supernatant. The supernatant was dried at 40 ˚C under a flow of N_2.

The dried supernatant was dissolved in iso-octane (130 µl). Fatty acid methyl esters (FAME) were quantified using a Varian 430 flame ionization gas chromatograph (GC) with a fused silica capillary column (Chrompack CPSIL 88; 100 m length, 0.25 µm ID, 0.2 µm film thickness) at 40 ~ 230 ˚C (hold 2 min; 4 ˚C/min; hold 10 min). Fatty acid methyl esters suspended in iso-octane (1 µl) were injected into the column using a Varian CP 8400 autosampler with a split ratio of 100:1. The injection port and detector were both maintained at 250 ˚C. Hydrogen (45 psi), functioned as the carrier gas, while nitrogen was used as the makeup gas. Varian Star

Chromatography Software was used to record the chromatograms. FAME samples were identified by comparing the relative retention times of FAME peaks of samples with standards (SIGMA, 189-19). Peak identification of the fatty acids and the fatty acid profile were done by using external standards (Supelco 37 component FAME mix; Sigma BAME mix). Calculations were done according to the methods by Marschner (2007), and the data were classified into different bacterial acid methyl esters (BAME) according to Whalen and Sampredo (2009). The BAME data were then grouped into their respective microbial groups and principal component analysis (PCA) was performed.

Ergosterol analysis: This procedure determines the total fungal biomass in plant material by measuring ergosterol content. This procedure was adapted and modified from the method of Jambunathan, Kherdekar and Vaidya (1991). Ground sorghum root powder (5 g) was placed in 50 ml centrifuge tubes and methanol (25 ml) was added before tubes were shaken vigorously with a Multi Reax shaker (Heidolph, Labotec) for 30 min. The sediment was allowed to settle. The supernatant was transferred into a new 50 ml centrifuge tube containing KOH pellets (1.5 g) and shaken until the KOH dissolved. N-hexane (5 ml) was added to the mixture which subsequently was incubated for 30 min in a water bath at 75 ˚C. The mixture was cooled to room temperature and distilled water (2.5 ml) was added. The mixture was vortexed, allowed to settle and the upper hexane layer was transferred to a new 50 ml centrifuge tube. Hexane (5 ml) was added to the residue and subsequent to being shaken, the upper layer was transferred to the previous aliquot. This process was repeated. The hexane extracts were evaporated in a water bath at 75 ˚C and the residue was resuspended in methanol (2.5 ml) and filtered through a 0.45 µl syringe

filter. The filtrate was analysed using a Perkin Elmer PDA-UHPLC with a SIL-20A auto sampler. The extract was loaded onto a reverse phase column (C18 125 A 10 µm particle size, 150 x 4.6 mm) at 50 ˚C. The mobile phase consists of methanol: water (96:4) at a flow rate of 1.2 ml/min. Ergosterol content was determined by using a standard (Sigma) at a retention time of approximately 7 min. A GLM-ANOVA was conducted to determine differences in ergosterol content between treatments using statistical software NCSS2007 (Hintze, 2007). The means were compared using Fisher's (LSD) and significant differences were determined at ($P < 0.05$).

Results

Influence of sorghum genotype on soil-borne pathogens

No significant differences ($P > 0.05$) in root mass or root rot rating were observed between the different sorghum genotypes (Figure 1, 2). The ergosterol content of the sorghum genotypes also did not differ significantly ($P > 0.05$) (Figure 3).

Influence of sorghum extracts on soil microbial diversity

PLFA analysis: General bacteria PLFA content of the treated soil samples ranged from 90.61 nmol/g (sorgoleone 0.5x) to 135.01 nmol/g (RTx436 0.5x) (Figure 4). Gram positive bacteria PLFA content of the treated soil samples ranged from 7.09 nmol/g (SCAY 1x) to 13.26 nmol/g (sorgoleone 1x) (Figure 4). Gram-negative bacteria PLFA content of the treated soil samples ranged from 1.95 nmol/g (sorgoleone 0.5x) to 31.81 nmol/g (RTx430 0.5x) (Figure 4). Fungal PLFA content of the treated soil samples ranged from 19.29 nmol/g (sorgoleone 0.5x) to 42.41 nmol/g (RTx 436 0.5x) (Figure 4). Total PLFA content revealed that the highest

microbial biomass was found in the soils of the untreated control. The lowest microbial biomass was found in the sorgoleone 0.5x treated soils. The microbial biomass of all four phenolic treated soils showed higher microbial biomass in all 0.5x treatments compared to the 1x treatments. The microbial biomass of sorgoleone treated soils showed a higher microbial biomass in the 1x treatment compared to the 0.5x treatment.

Cluster analysis of PLFA content clearly showed differences (< 55 % similarity) between the functional diversity of soils treated with phenolics and sorgoleone compared to the untreated control with the exception of soils treated with extracts from RTx 430 0.5x and RTx 436 1x (Figure 5).

Influence of previous sorghum extracts on soil-borne pathogens

Significant differences ($P < 0.05$) were evident between root rot severity of the diseased sorghum plants that were planted in soil previously treated with sorghum phenolic and sorgoleone extracts compared to the untreated control. No significant differences ($P > 0.05$) were found between the leaf length, root length and plant fresh weight of these plants. No significant extract x pathogen interactions ($P < 0.05$) were evident for leaf length, root length and root rot severity.

Significantly lower ($P < 0.05$) average root rot severity ranging from 15.31 % to 23.68 % was observed in sorghum plants planted in soil previously treated with phenolic extracts compared to 29.1 % of the untreated control (Figure 6). Significantly higher ($P < 0.05$) root rot severity ranging from 27.22 % to 42.78 % was observed in sorghum plants planted in soil previously treated with phenolic and sorgoleone extracts that were inoculated with *A. strictum* compared to 19.44 % of the untreated control. Average root rot severity of sorghum plants inoculated with *A.*

alternata and *F. thapsinum* was most suppressed by extracts, while sorghum plants inoculated with *P. macrostoma* were least suppressed. Average root rot severity in soils previously treated with extracts RTx 436 0.5x and SCAY 21 0.5x was most suppressed, while sorgoleone 0.5x had the least influence. No significant differences ($P > 0.05$) were found in ergosterol content of diseased sorghum roots planted in soils previously treated with phenolics and sorgoleone extracts compared to the untreated control.

Discussion

In the present study, the objective was to determine the possible indirect effects of sorghum phenolics and sorgoleone on soil-borne pathogens. Our results showed distinct variations in microbial functional diversity between rhizosphere soils treated with sorghum extracts compared to untreated controls. This indicates direct effects of phenolic and sorgoleone extracts on the diversity and structure of soil microbial organisms. *Sorghum bicolor* produces a wide range of phenolics such as ferulic, *p*-coumaric, *p*-hydroxybenzoic, vanillic, syringic, caffeic, *trans*-cinnamic and gentisic acids (Sene, Dore, and Pellissier, 1999; Won *et al.,* 2013). Several of these phenolic acids such as *p*-hydroxybenzoic, *p*-coumaric, cinnamic, caffeic and syringic acids have been shown to significantly stimulate or inhibit soil microbial diversity. Results in this study are consistent with Wu, Wang and Xue (2009) who observed significant differences in soil microbial composition and genetic diversity between soils treated with different concentrations (25, 50, 100 and 200 mg/kg soil) of cinnamic acid from cucumber (*Cucumis sativus*) root exudates. The authors showed that cinnamic acid promoted soil microbial diversity that can utilise carbon sources such as cyclodextrin, glycogen and 2-hydroxy benzoic acid and inhibited soil

microbial diversity that can utilise hydrobutyric acid, L-asparagine and D-galacturonic acid. Similarly, Qu and Wang (2007) demonstrated significant differences between the soil microbial biomass and community diversity in soils treated with different concentrations of vanillic acid (0.05, 0.1, 0.2 mg/g soil). Soil microbial biomass was inhibited by 0.05 mg/g vanillic acid and promoted by 0.1 and 0.2 mg/g vanillic acid. The authors also showed increases in the microbial DNA profiles in soils treated with 0.1 and 0.2 mg/g vanillic acid compared to untreated controls. Plant phenolics and root exudates thus ultimately stimulate or inhibit microbial diversity in surrounding plants.

The results of the microcosm experiment indicated significantly suppressed root rot severity in sorghum planted in soils previously treated with sorghum phenolics and sorgoleone compared to the untreated control. Several plant phenolics have shown significant direct inhibitory effects on soil bacteria and fungi (Kong *et al.*, 2008; Gimsing *et al.*, 2009; Heleno *et al.*, 2013; Sadeghi *et al.*, 2013; Lima *et al.*, 2016; Mishra *et al.*, 2017). For example cinnamic and *p*-hydroxybenzoic acids which are also produced by *S. bicolor* suppresses several plant pathogens. Suppressed root rot by sorghum extracts which we observed is consistent with the findings of Sadeghi *et al.* (2013) who demonstrated significant inhibition in fungal growth of up to 30 % against *Penicillium italicum* Wehmer, up to 30 % against *Aspergillus niger* van Tieghem and up to 40 % against *Botrytis cinerea* Pers. by cinnamic acid extracts of Persian leek (*Allium ampeloprasum* Subsp. *Persicum* L.). Similarly, Heleno *et al.* (2013) demonstrated the antifungal activity (99.5 % mortality) of *p*-hydroxybenzoic (HA) and cinnamic acid (CA) extracts of a medicinal mushroom (*Ganoderma lucidum* (Curtis) Karst) with minimum fungicidal concentrations of between 0.25 mg (HA)/ml agar and 0.06 mg (CA)/ml agar against *Aspergillus fumigates* Fresenius,

Aspergillus versicolor (Vuillemin) Tiraboschi, *Aspergillus ochraceus* Wilhelm, *Aspergillus niger, Penicillium funiculosum* Thom., *Penicillium ochrochloron* Biourge and *Penicillium verrucosum* Dierckx.

The possible indirect inhibitory effect on soil-borne pathogens could be related to the changes in the rhizobiome by sorghum extracts. Several studies have indicated that plants use root exudates and allelochemicals to determine the diversity of their rhizobiome (Sturz and Christie, 2003; Rasmann and Turlings, 2016; Finkel *et al.*, 2017; Zhang, Vivanco and Shen, 2017). These selected microbial organisms often contain beneficial and antagonist organisms that not only promote the growth of the plant but also protect the plant from plant pathogens (Sturz and Christie, 2003; Finkel *et al.*, 2017). Several studies have shown significant suppressive effects of the rhizobiome on plant pathogens such as *Pseudomonas syringae* pv. *tomato* van Hall, *Xanthomonas vesicatoria* (Pammel) Dowson, *Fusarium* spp., *Rhizoctonia solani* Kühn, *Pythium* spp. and plant-parasitic nematodes (Akhtar and Malik, 1999; Ji *et al.*, 2005; Zhang *et al.*, 2008; Susi *et al.*, 2011; Qiu *et al.*, 2012; van Agtmaal *et al.*, 2018). The suppression of soil-borne pathogens in this study could be related to changes in the rhizobiome caused by the phenolics and sorgoleone treatments. The suppressed root rot of sorghum plants planted in soils previously treated with phenolics and sorgoleone extracts are consistent with Qi, Zhen and Li (2015). The authors found reduced occurrence of sharp eyespot caused by *Rhizoctonia cerealis* Murray & Burpee and *Gaeumannomyces graminis* Sacc. in wheat plants planted in soils treated with decomposed maize straw products (0.03 and 0.06 g/ml water) which contained several plant phenolics such as *p*-hydroxybenzoic, vanillic, syringic and salicylic acids.

In conclusion, the results of the present study suggest that some sorghum phenolics and sorgoleone have strong indirect inhibitory effects on some soil-borne plant pathogens. The possible explanation of this indirect inhibitory effect could be due to changes in the rhizobiome caused by phenolics and sorgoleone treatments or differences between sorghum genotypes. These results suggest that sorghum has the potential to be manipulated as a biological control agent against soil-borne plant pathogens. Further research should be conducted to investigate the effect of sorghum phenolics and sorgoleone on the fungal and bacterial microbiome of sorghum genotypes using next generation sequencing.

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Table 1. Grain sorghum genotypes used in the field experiment with regards to plant and seed colour.

Line No	Line	Pl. Col.	Pericarp	Mesocarp	Testa	Spread.	Plant	Seed colour
1	BTx378	PPQQ	RRYYII	zz	b1b1B2B2	SS	purple	red, chalky
2	BTx3197	PPQQ	RRyyii	zz	b1b1B2B2	SS	purple	white, chalky
3	RTx430	PPQQ	RRyyll	ZZ	b1b1b2b2	SS	purple	white, pearly, yellow endosperm
4	RTam428	PPqq	rryyll	ZZ	b1b1B2B2	ss	red	white, pearly
5	RTx436	ppQQ	RRyyii	ZZ	B1B1b2b2	--	tan	white
6	BTx635	ppQQ	RRyyii	ZZ	--	--	tan	white pearly food grad sorghum
7	BTX ARG-1	ppQQ	RRyyii	ZZ	--	--	tan	waxy endosperm
8	RTx2917						tan	red
9	SC630-11EII	PPQQ	RRYYII	ZZ	b1b1B2B2	SS	purple	dark red, pearly
10	SC630-11 Eii	PPQQ	RRYYii	ZZ	b1b1B2B2	SS	purple	light red, pearly
11	SC748-5	PPQQ	rrYYIl	ZZ	b1b1B2B2	ss	purple	lemon yellow, pearly
12	SC109-14E	PPQQ	RRyy--	zz	B1B1B2B2	ss	purple	white pearly, purple testa
13	S5C719-11E	PPQQ	RRYYii	zz	B1B1B2B2	ss	purple	red, chalky,
14	SC103-12E	PPQQ	RRYYII	zz	B1B1B2B2	SS	purple	dark brown-red, chalky
15	Dobbs	PPQQ	RRyyii	zz	B1B1B2B2	SS	purple	brown, chalky
16	Hegari	PPQQ	RRyyii	zz	B1B1B2B2	ss	purple	white, chalky
17	RTam2566	PPQQ	RRYYII	ZZ	B1B1B2B2	ss	purple	dark brown-red, pearly
18	Tx2911						red	red, chalky
19	SCAY13						tan	lemon-yellow
20	SCAY16						tan	lemon-yellow
21	SCAY21						tan	red
22	SCAY14						tan	lemon-yellow

Table 2. Sorghum phenolic extract and sorgoleone concentrations applied to glasshouse experiment.

Sorghum genotype	**Type of extract**	**Concentration**	**Concentration detail**
RTx 430	Phenolic extract	0.5x	0.5 mg (dry plant material)/ ml, 1000 L/ Ha
RTx 430	Phenolic extract	1x	1 mg (dry plant material)/ ml, 1000L/ Ha
RTx 436	Phenolic extract	0.5x	0.5 mg (dry plant material)/ ml, 1000 L/ Ha
RTx 436	Phenolic extract	1x	1 mg (dry plant material)/ ml, 1000L/ Ha
Rtam 428	Phenolic extract	0.5x	0.5 mg (dry plant material)/ ml, 1000 L/ Ha
Rtam 428	Phenolic extract	1x	1 mg (dry plant material)/ ml, 1000L/ Ha
SCAY 21	Phenolic extract	0.5x	0.5 mg (dry plant material)/ ml, 1000 L/ Ha
SCAY 21	Phenolic extract	1x	1 mg (dry plant material)/ ml, 1000L/ Ha
BTx 3197	Sorgoleone extract	0.5x	0.3 kg (dried sorgoleone)/ Ha
BTx 3197	Sorgoleone extract	1x	0.6 kg (dried sorgoleone)/ Ha

Table 3. List of fatty acids and signature lipids used to determine soil microbial functional groups.

Bacterial Acid Methyl Esters (BAME's)		
Component Name	**Formula**	**Signature lipid**
Methyl octanoate	C8:0	
Methyl decanoate	C10:0	
Methyl 2-hydroxydecanoate	2-OH-C10:0	
Methyl undecanoate	C11:0	
Methyl dodecanoate	C12:0	
Methyl 3-hydroxydodecanoate	3-OH-C12:0	Gram negative bacteria
Methyl tridecanoate	C13:0	
Methyl tetradecanoate	C14:0	General bacteria
Methyl 2-hydroxytetradecanoate	2-OH-C14:0	Gram negative bacteria
Methyl 3-hydroxytetradecanoate	3-OH-C14:0	Gram negative bacteria
Methyl 13-methyltetradecanoate	i-C15:0	Gram positive bacteria
Methyl 12-methyltetradecanoate	a-C15:0	Gram positive bacteria
Methyl cis-10 pentadecanoate	$C15:1^{10}$	
Methyl hexadecanoate	C16:0	General bacteria
Methyl 14-methylpentadecanoate	i-C16:0	Gram positive bacteria
Methyl cis-9-hexadecanoate	$C16:1^{9}$	Gram negative bacteria
Methyl octadecanoate	C18:0	General bacteria
Methyl trans-9-octadecenoate	$C18:1^{9}$	
Methyl cis-9-octadecenoate	$C18:1^{9}$	Fungi
Methyl cis-7-octadecenoate	$C18:1^{7}$	
Methyl cis-9,12-octadecadienoate	$C18:2^{9,12}$	Fungi
Methyl cis-9,12,15 octadecatrienoate	$C18:3^{9,12,15}$	Fungi
Methyl cis-9,10-methyleneoctadecanoate	$C19:0^{\Delta}$	Gram negative bacteria
Methyl cis-11 eicosenoate	$C20:1^{11}$	
Methyl cis-11,14,17 eicosatrienoic	$C20:3^{11,14,17}$	
Methyl docosanoate	C22:0	
Methyl cis-13 docosadienoate	$C22:1^{13}$	
Methyl tetracosanoate	C24:0	
Methyl cis-15 tetracosanoate	C24:1c15	

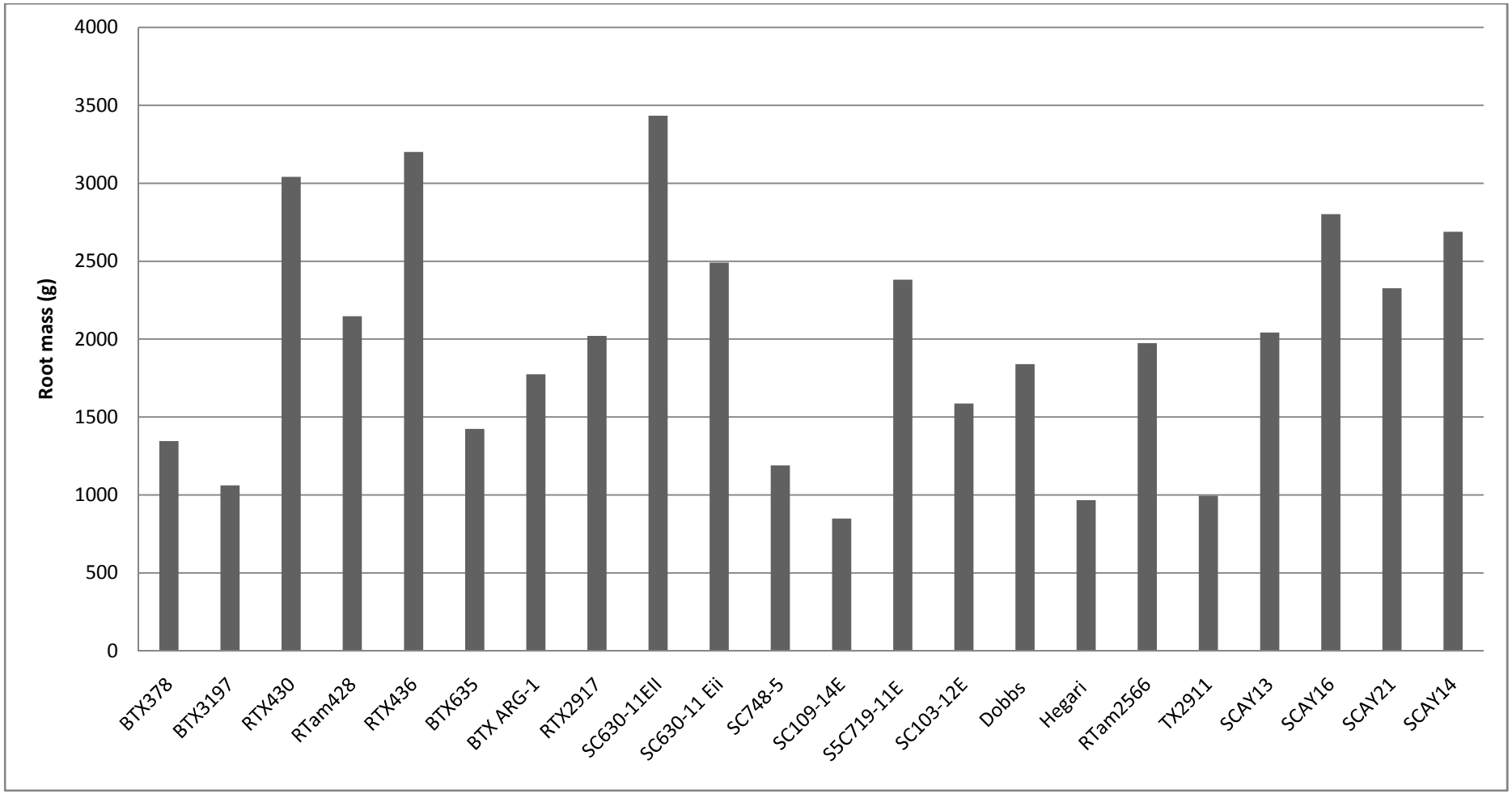

Figure 1.Root mass (g/ 50 plants) of sorghum genotypes in the field trial.

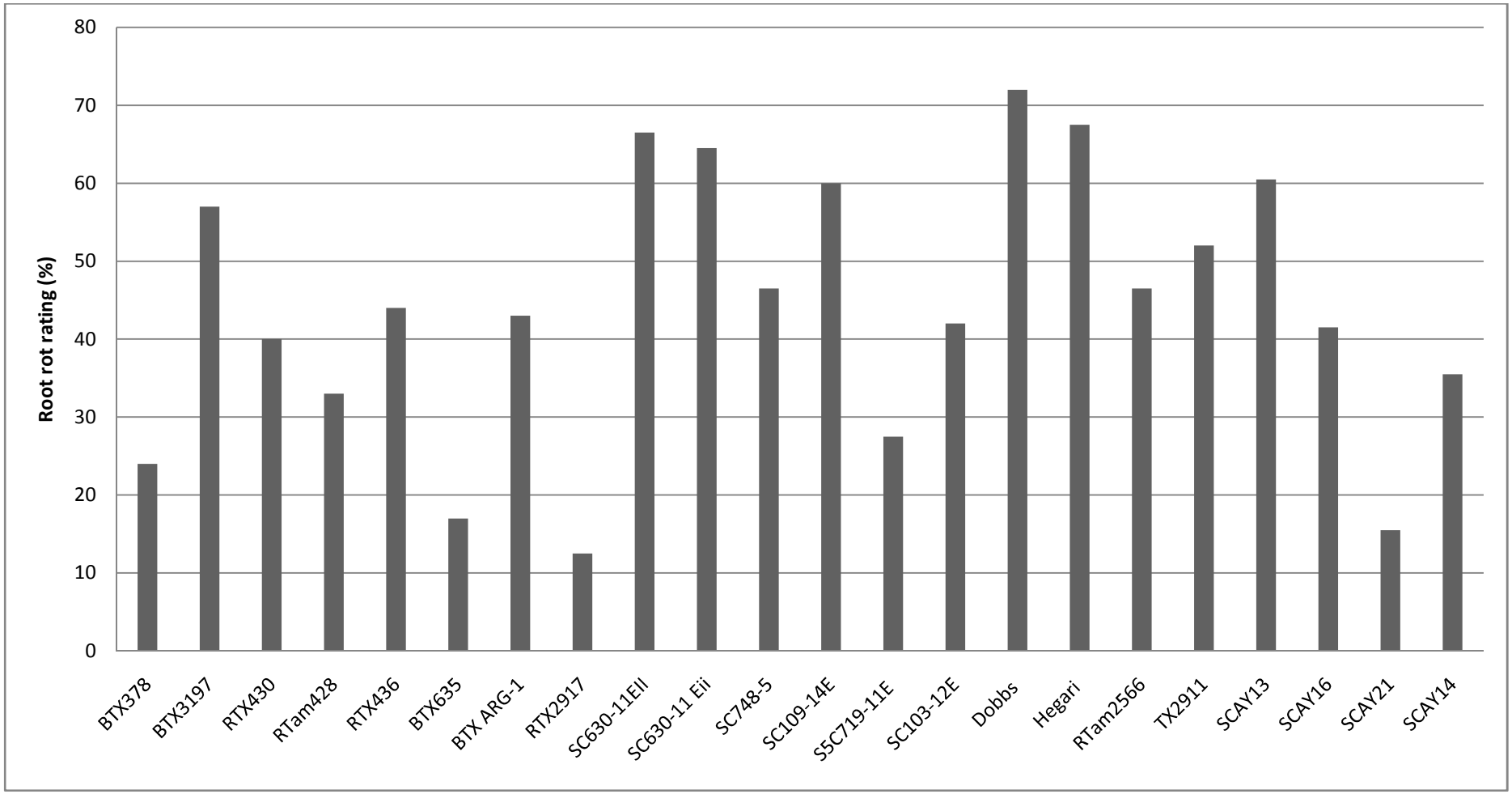

Figure 2. Root rot rating (%) of sorghum genotypes in the field trial.

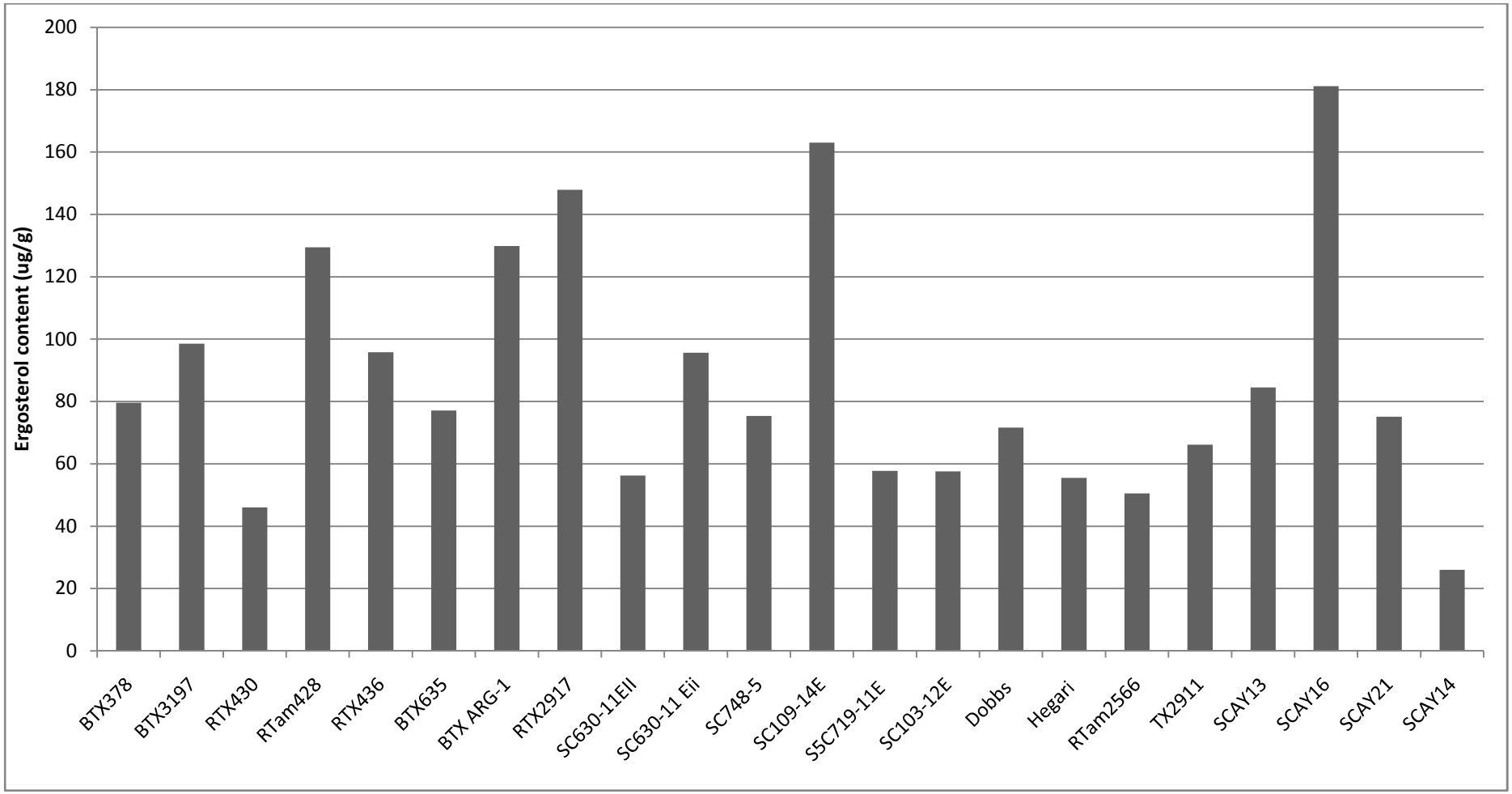

Figure 3. Ergosterol content (ug/g) of sorghum genotypes in the field trial.

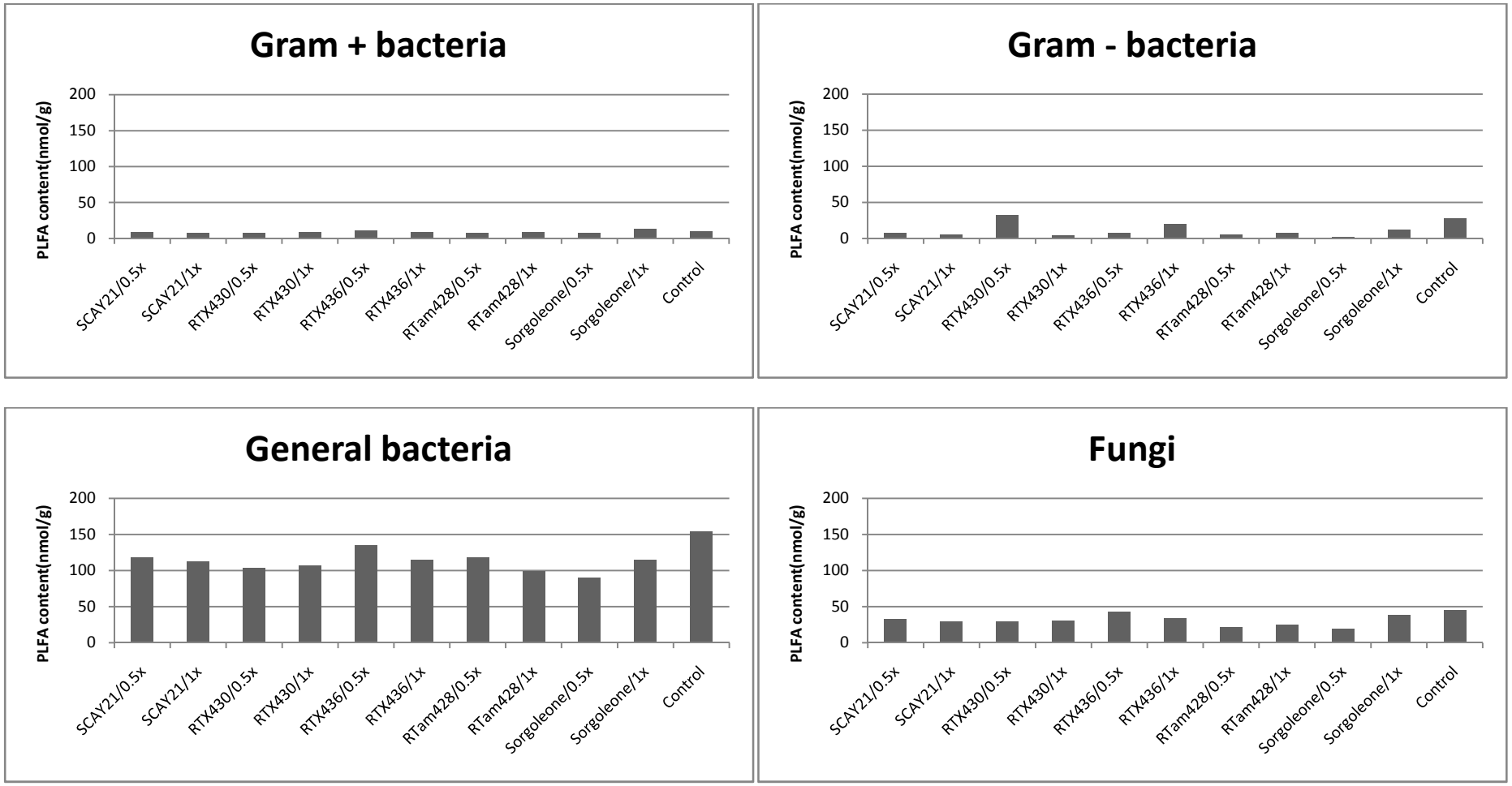

Figure 4. PLFA content expressing soil microbial functional groups of soils treated with sorghum extracts.

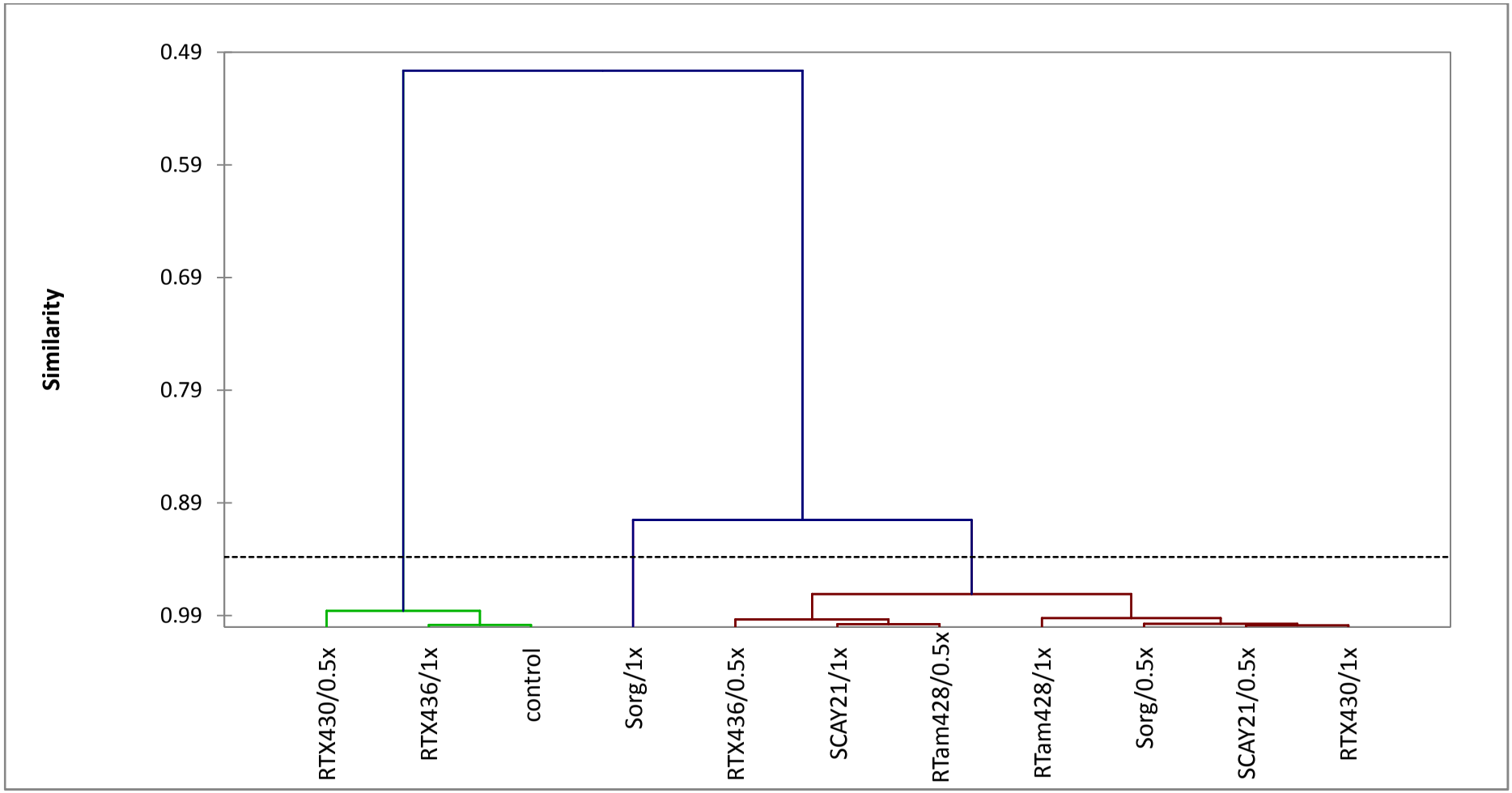

Figure 5. Average PLFA content of soils treated with sorghum extracts.

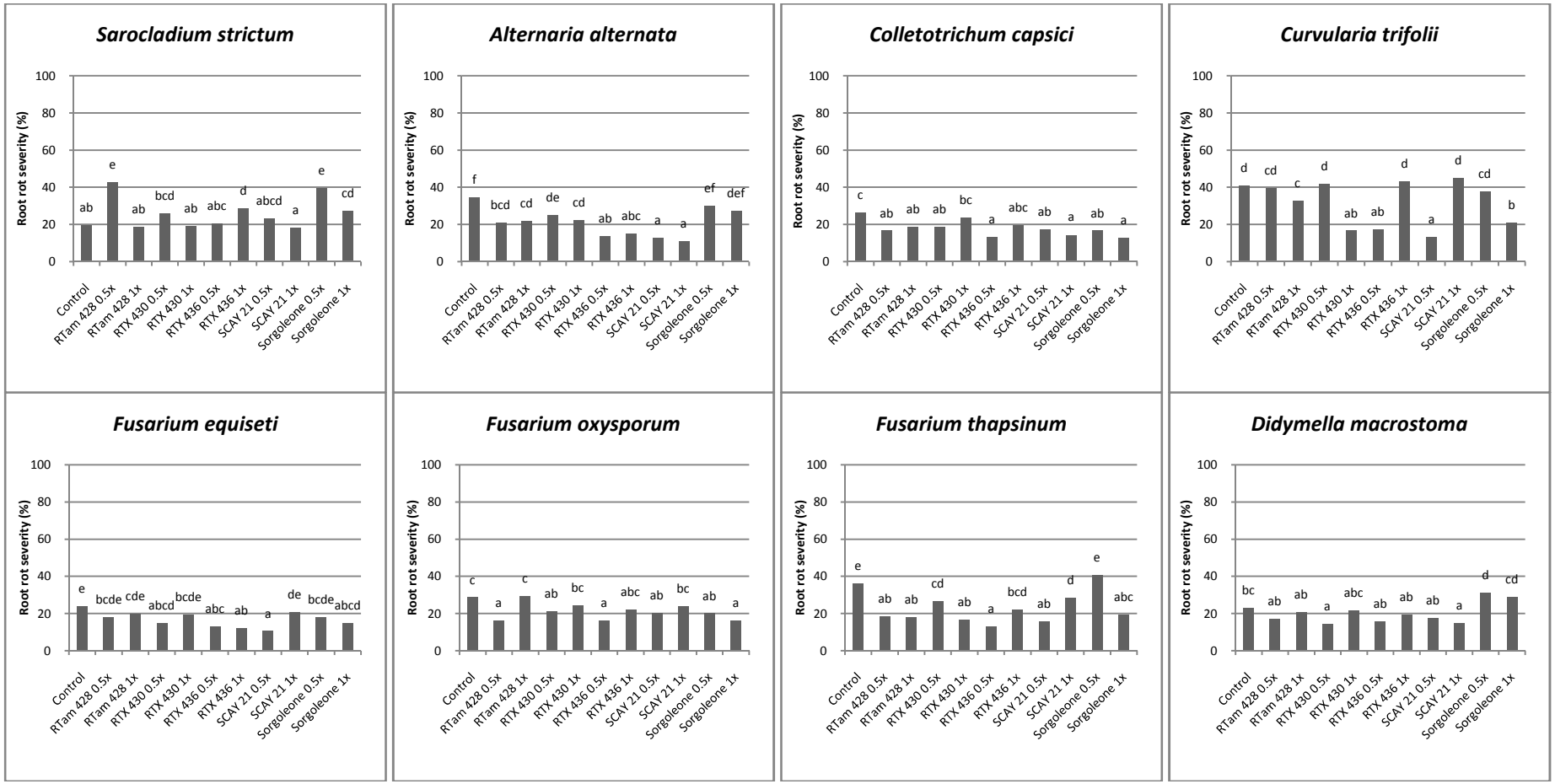

Figure 6. Root rot severity (%) of sorghum plants challenged with soil-borne pathogens. Bars with the same lowercase letter/s are not significantly different (P > 0.05).

CHAPTER FIVE

EFFECT OF CROP ROTATION WITH TWO SORGHUM GENOTYPES ON SOIL MICROBIAL POPULATIONS, SORGHUM ROOT ROT AND SORGHUM YIELD

Abstract

Grain sorghum is an important cereal crop in Africa and Asia but long-term cultivation of sorghum is known to cause "soil sickness" that reduces crop yield and increases the incidence of root disease. One solution is crop rotation that can reduce weeds, improve soil health, fertility and microbial diversity. This study investigated the effects of crop rotation of two sorghum genotypes: NS5511 (red plant, brown seed) and PAN8706W (tan plant, white seed) with soybean (*Glycine max*), cowpea (*Vigna unguiculata*), dry bean (*Phaseolus vulgaris*), mono crop sorghum (*S. bicolor*) and a fallow treatment on soil microbial properties, root disease and sorghum yield. Significantly higher ($P < 0.05$) microbial activity was observed in soils associated with legume rotation systems compared to sorghum monoculture and fallow treatments. Distinct variation in gram-negative, gram-positive, general bacterial and fungal PLFA content was found between sorghum in rotation with soybean compared to cowpea and dry bean systems. During the 1st rotation, significantly higher ($P < 0.05$) root mass was recorded in sorghum following cowpea and soybean compared to sorghum monoculture while in the 2nd rotation significantly higher root mass was recorded in sorghum subsequent to soybean and dry bean compared to fallow. A significantly lower ($P < 0.05$) yield was recorded in sorghum plants undergoing fallow and monoculture treatments compared to the legume rotation systems. Results also indicated that sorghum genotype significantly affected soil microbial populations as well as growth and yield. A significantly higher yield was recorded in sorghum cultivar NS5511 in soybean and dry bean rotation systems compared to sorghum cultivar PAN8706W.

Introduction

Grain sorghum (*Sorghum bicolor* L.) is the world's fifth most important cereal crop. It originated in Africa, flourishes in semi-arid regions and is produced as a major food source in parts of Africa and Asia (Dykes, Rooney and Rooney, 2013). Grain sorghum is known for its allelopathic properties on surrounding plants and continuous monoculture is known to cause "soil sickness" (de Albuquerque *et al.*, 2011; Weston, Alsaadawi and Baerson, 2013). Previous studies have related this "sickness" to the direct effect of accumulated allelochemicals that severely affect subsequent crops. However, recent studies have related this "sickness" to indirect effects of allelochemicals on soil microbial communities and the ecological processes they facilitate (Inderjit and Weiner, 2001; van der Heijden, Bardgett and Straalen, 2008). Early studies on allelochemicals mainly focused on their phytotoxic properties, whereas more recent studies have focused on their effects on soil microbes. Allelopathic plants affect soil microbes by either inhibiting them or promoting their growth ultimately influencing community structure and diversity either positively or negatively (Kong *et al.*, 2008; Gimsing *et al.*, 2009).

Soil microbes are extremely sensitive to their surrounding environment and their community structure and diversity can be influenced by adjacent plant species (Kong *et al.*, 2008). Nutrients and allelochemicals present in root exudates are the key factors in influencing soil microbes which can either promote or inhibit their activity and diversity (Kong *et al.*, 2008; Gimsing *et al.*, 2009). Soil microbes play vital roles in soil fertility by facilitating several ecological processes that can affect soil carbon and nitrogen content which ultimately promotes or inhibits plant growth (Beare *et al.*, 1996; Gunapala and Scow, 1997; Foissner, 1999; Bin-Ru *et al.*, 2005). Soil microbes can also benefit plants by promoting their growth through

improvements in their nutrient uptake capabilities and by improving their resistance towards soil-borne pathogens (Liu, Glenn and Buckley, 2008; Tang *et al.*, 2014; Liu *et al.*, 2017). They can also improve resistance towards soil-borne pathogens by competing for nutrients and living space or via direct predation (Udai *et al.*, 2016).

Various crop management strategies are employed to reduce "soil sickness". Crop rotation in particular, is designed to improve soil fertility, reduce pests and diseases and ultimately improve crop yield (Mhlanga *et al.*, 2014; Mhlanga *et al.*, 2015; Bakhshandeh *et al.*, 2017). Crop rotation not only improves soil nutrients and yield compared to monoculture systems. Recent studies also showed that it can improve rhizosphere microbial diversity, specifically the biomass of beneficial soil microbes (Govaerts *et al.*, 2005; Govaerts *et al.*, 2007; Jiang *et al.*, 2016; Liu *et al.*, 2017). Crop rotation not only changes physical structure, temperature, moisture, nutrients and organic matter in soil but can also introduce allelochemicals depending on their specific design (Jabran *et al.*, 2015). These changes can directly and indirectly determine soil microbial biomass and diversity. A well designed crop rotation system can promote microbial biomass and diversity while simultaneously reducing the incidence of soil-borne pathogens (Venter, Jacobs and Hawkins, 2016; Ashworth *et al.*, 2017; Bakhshandeh *et al.*, 2017; Zhao *et al.*, 2017).

The objectives of this study were to investigate the effects of rotation systems with two sorghum genotypes and three legume crops on: (i) the diversity of soil microbial populations in the sorghum rhizosphere and (ii) sorghum root disease and yield.

Materials and Methods

Trial design and sampling

The study was conducted on a farm situated in the Alma district, in the Limpopo province of South Africa (-24°28'59.99"S, 28°03'60.00"E) from 2013 to 2016. Two sorghum genotypes NS5511 (red plant, brown seed) and PAN8706W (tan plant, white seed) were planted in soil previously planted with soybean (*Glycine max* L.), cowpea (*Vigna unguiculata* L.), dry bean (*Phaseolus vulgaris* L.), sorghum (*S. bicolor*) as well as a fallow treatment. Each rotation treatment was separated into six blocks. The rotation system was initiated in 2013 and was followed by a rotation with sorghum subsequent to single legume/fallow rotation in 2014. This was followed by a rotation with sorghum subsequent to a double legume/fallow system and double sorghum subsequent to a single legume rotation/fallow in 2015. The trial was replicated three times. Sorghum seeds were sown in October and plants were harvested in April of the next year.

Soil samples were collected for all three seasons to determine differences in microbial populations between the rotation systems during flowering (between January and March). Soil was collected from the rhizosphere of five random sorghum plants in each block and the soils were homogenised and separated into two sub-samples for analysis. One sub-sample was immediately refrigerated at 4 °C for fluorescein diacetate (FDA) analysis. The second sub-sample was stored at -80 °C for phospholipid fatty acid (PLFA) analysis. PLFA analysis of soil samples collected from the 1st rotation trial was done to determine differences in microbial populations between rotation treatments only, while soil samples collected from the 2nd rotation trial was done to determine differences in microbial populations between rotation treatments and sorghum genotypes.

Sorghum plants were harvested in the 1st and 2nd rotation trials to determine root rot severity and yield. Whole sorghum plants were randomly collected within each block from each rotation system. The roots from each plant were removed from the plant at the crown and root mass and root rot severity was recorded. Root mass was expressed as mass/50 plants. The roots were air dried, ground into a powder using liquid nitrogen and stored for ergosterol analysis. Sorghum grain was used to determine yield with mass expressed as the mean mass/50 plants. A GLM-ANOVA was conducted to determine differences in root mass, root-rot rating and grain mass between samples using statistical software NCSS2007 (Hintze, 2007). The means were compared using Fisher's least significant difference (LSD) and significant differences were determined ($P < 0.05$).

Fluorescein diacetate analysis: This procedure utilises fluorescein diacetate (FDA), which is hydrolysed by microbial organisms, to determine microbial enzyme activity in soil as a reflection of total microbial biomass. This procedure was adapted and modified from Schnürer and Roswell (1982). A stock solution was prepared by dissolving FDA in acetone (2 mg/ml) and stored at -20 ºC. A buffer solution of 60 mM sodium phosphate was prepared, adjusted to pH 7.6 and stored at 4 ºC. Soil samples (2 g) were added to 20 ml of the buffer followed by 0.2 ml of the FDA solution in a 50 ml tube. The mixture was then shaken by hand, incubated at 28 ºC for 20 min, and placed in a Multi Reax shaker (Heidolph, Labotec) at 200 rpm for 10 min. After shaking, 15 ml of chloroform: methanol (2:1 v/v) solution was added to the soil solutions to stop the FDA from further hydrolysing. The mixture was stored at 4 ºC until the sediment settled and the clear supernatant was placed in a 2 ml eppendorf tube. Eppendorf tubes were then centrifuged at 10000 rpm for 5 min to

remove excess debris whereafter the supernatant was measured at 490 nm using a spectrophotometer (CARY Bio 100 UV-Visible Sectrophotometer). A GLM-ANOVA was conducted to determine differences in FDA content between treatments using statistical software NCSS2007 (Hintze, 2007). The means were compared using Fisher's (LSD) and significant differences were determined ($P < 0.05$).

Phospholipid fatty acid analysis: This procedure uses phospholipid fatty acids (PLFA) as biomarkers to determine total microbial composition in soil and differentiate them into different functional groups by means of their respective fatty acid profiles (Table 3). The procedure used was adapted and modified from White *et al.* (1979) as described by Marschner (2007). Soil samples (2 g) were placed in 25 ml centrifuge tubes and methanol (3.8 ml), citrate buffer (1.5 ml), chloroform (1.9 ml) and Blight and Dyer reagent (2 ml) were added. The tubes were then shaken for 2 hr and centrifuged at 3500 g for 10 min. The supernatant was transferred into a new centrifuge tube. The pellet in the original centrifuge tube was washed with Blight and Dyer reagent (2.5 ml), shaken and again centrifuged (as in the previous step) whereafter the supernatant was added to the supernatant in the new centrifuge tube. The pooled supernatants were diluted with chloroform (3.1 ml) and citrate buffer (3.1 ml), vortexed and centrifuged at 3500 g for 10 min. The lower phase (3 ml) was then transferred into a 10 ml glass tube and the content was dried at 40 ˚C under a flow of N_2. The dried samples were dissolved in chloroform (300 µl) and pipetted onto a conditioned (2 x 1 ml chloroform) silica-bonded column. Neutral lipids were eluted with chloroform (5 ml) and glycolipids were eluted with acetone (10 ml). Both elutants were then discarded. Phospholipids were eluted with methanol (5 ml), and the elutant was transferred to a 10 ml centrifuge tube. The content was again dried at

40 ˚C under a steady flow of N_2. An internal standard (C19:0; 30 µl) was then added to the dried samples, followed by a methanol - toluol solution (1:1, v/v; 1 ml) and 0.2 M KOH-MeOH (1 ml). The suspension was vortexed and incubated for 15 min at 37 ˚C. A hexane-chloroform solution (2 ml), 1 M acetic acid (0.3 ml) and deionised water (2 ml) were added to the suspension. The suspension was vortexed for 1 min, centrifuged for 5 min at 3500 g and the supernatant was transferred into a 10 ml glass tube. Another aliquot of hexane-chloroform solution (2 ml) was added to the pellet, the solution was vortexed and centrifuged as described previously and the supernatant was added to the previous supernatant. The supernatant was dried at 40 ˚C under a flow of N_2.

The dried supernatant was dissolved in iso-octane (130 µl). Fatty acid methyl esters (FAME) were quantified using a Varian 430 flame ionization gas chromatograph (GC) with a fused silica capillary column (Chrompack CPSIL 88; 100 m length, 0.25 µm ID, 0.2 µm film thickness) at 40 ~ 230 ˚C (hold 2 min; 4 ˚C/min; hold 10 min). Fatty acid methyl esters suspended in iso-octane (1 µl) were injected into the column using a Varian CP 8400 autosampler with a split ratio of 100:1. The injection port and detector were both maintained at 250 ˚C. Hydrogen (45 psi), functioned as the carrier gas, while nitrogen was used as the makeup gas. Varian Star Chromatography Software was used to record the chromatograms. FAME samples were identified by comparing the relative retention times of FAME peaks of samples with standards (SIGMA, 189-19). Peak identification of the fatty acids and the fatty acid profile were done by using external standards (Supelco 37 component FAME mix; Sigma BAME mix). Calculations were done according to the methods by Marschner (2007), and the data were classified into different bacterial acid methyl esters (BAME) according to Whalen and Sampredo (2009). The BAME data were

then grouped into their respective microbial groups and principal component analysis (PCA) was performed.

Ergosterol analysis: This procedure determines the total fungal biomass in plant material by measuring ergosterol content. This procedure was adapted and modified from the method of Jambunathan, Kherdekar, and Vaidya (1991). Ground sorghum root powder (5 g) was placed in 50 ml centrifuge tubes and methanol (25 ml) was added before tubes were shaken vigorously with a Multi Reax shaker (Heidolph, Labotec) for 30 min. The sediment was allowed to settle. The supernatant was transferred into a new 50 ml centrifuge tube containing KOH pellets (1.5 g) and shaken until the KOH dissolved. N-hexane (5 ml) was added to the mixture which subsequently was incubated for 30 min in a water bath at 75 ˚C. The mixture was cooled to room temperature and distilled water (2.5 ml) was added. The mixture was vortexed, allowed to settle and the upper hexane layer was transferred to a new 50 ml centrifuge tube. Hexane (5 ml) was added to the residue and subsequent to being shaken, the upper layer was transferred to the previous aliquot. This process was repeated. The hexane extracts were evaporated in a water bath at 75 ˚C and the residue was resuspended in methanol (2.5 ml) and filtered through a 0.45 µl syringe filter. The filtrate was analysed using a Perkin Elmer PDA-UHPLC with a SIL-20A auto sampler. The extract was loaded onto a reverse phase column (C18 125 A 10 µm particle size, 150 x 4.6 mm) at 50 ˚C. The mobile phase consists of methanol: water (96:4) at a flow rate of 1.2 ml/min. Ergosterol content was determined by using a standard (Sigma) at a retention time of approximately 7 min. A GLM-ANOVA was conducted to determine differences in ergosterol content between treatments using

statistical software NCSS2007 (Hintze, 2007). The means were compared using Fisher's (LSD) and significant differences were determined ($P < 0.05$).

Results

Effect of crop rotation on microbial populations

FDA analysis: Significant differences ($P < 0.05$) were evident between the microbial activities of soils undergoing rotation treatments over all three seasons although there were inconsistencies between seasons (Figure 1). A lower microbial activity was recorded in soils of the 2nd and 3rd rotation compared to soils of the 1st rotation (Figure 1). The highest microbial activity of the 1st rotation trial was recorded in soil previously planted with soybean while the lowest was in previously fallowed soil. In the 2nd season, the highest microbial activity was recorded in soil previously planted with cowpea and the lowest in soil previously planted with soybean. The 3rd season showed the highest microbial activity in soil previously planted with sorghum and the lowest in soil previously planted with soybean.

PLFA analysis: General bacteria PLFA content in the soil samples ranged from 86.87 nmol/g (cowpea) to 102.58 nmol/g (fallow) (Table 1). Gram-positive bacteria PLFA in the soil samples ranged from 6.38 nmol/g (cowpea) to 11.02 nmol/g (soybean) (Table 1). Gram-negative bacteria PLFA in the soil samples ranged from 2.50 nmol/g (cowpea) to 11.52 nmol/g (fallow) (Table 1). Fungal PLFA of the soil samples ranged from 18.63 nmol/g (fallow) to 28.08 nmol/g (soybean) (Table 1). The highest total PLFA content was found in soil previously planted with soybean and in soil undergoing fallow treatments while the lowest was in soils previously planted with cowpea.

Cluster analysis of PLFA contents of the different microbial functional groups clearly showed differences (< 84 % similarity) between soils previously planted with soybean compared to other rotation treatments (Figure 2).

Effect of sorghum genotype on microbial properties

FDA analysis: No significant differences in overall microbial activity were found between the soils planted with genotypes PAN8706W or NS5511 in both 1st and 2nd rotation trials (Figure 3). The highest microbial activity of the 1st rotation trial was observed in soil planted with NS5511 undergoing cowpea rotation while the lowest was soil planted with NS5511 undergoing fallowed treatment. In the 2nd season, the highest microbial activity was in soil planted with PAN8706W undergoing cowpea rotation and the lowest was in soil planted with PAN8706W undergoing soybean rotation.

PLFA analysis: General bacteria PLFA content in the soil samples ranged from 74.27 nmol/g (dry bean/NS5511) to 151.78 nmol/g (cowpea/PAN8706W) (Table 2). Gram positive bacteria PLFA in the soil samples ranged from 3.17 nmol/g (monocrop/NS5511) to 8.25 nmol/g (fallow/PAN8706W) (Table 2). Gram-negative bacteria PLFA in the soil samples ranged from 1.06 nmol/g (dry bean/NS5511) to 8.61 nmol/g (dry bean/PAN8706W) (Table 2). Fungal PLFA in the soil samples ranged from 11.62 nmol/g (dry bean/NS5511) to 67.70 nmol/g (cowpea/PAN8706W) (Table 2). The highest total PLFA content was found in soil previously planted with cowpea while the lowest was in soils previously planted with monocropped sorghum. A higher average PLFA content was found in soils planted with genotype PAN8706W compared to genotype NS5511.

Cluster analysis of PLFA contents of the different microbial functional groups displayed differences (< 93 % similarity) between soils planted with genotype PAN8706W undergoing cowpea and fallow treatments compared to the other rotation treatments (Figure 4). The PLFA contents also showed differences (< 95 % similarity) between soils planted with genotype NS5511 undergoing soybean and cowpea rotation compared to the other rotation treatments (Figure 4).

Effect of crop rotation on sorghum root disease and yield

Root disease: Significant differences ($P < 0.05$) were evident between the root mass of harvested sorghum plants from the 1st and 2nd seasons (Figures 5 and 7). However, no significant differences ($P > 0.05$) in root rot rating were recorded between sorghum plants from the 1st and 2nd rotation trials. Significant differences ($P < 0.05$) were also evident between the ergosterol content of sorghum plants from the 2nd season (Figure 8). The highest root mass in the 1st season was observed in sorghum undergoing cowpea rotation while the lowest was in sorghum undergoing monoculture (Figure 5). In the 2nd season, the highest root mass was recorded in sorghum undergoing dry bean rotation and the lowest was in sorghum undergoing fallow treatment (Figure 7). The results of the 2nd season also showed that sorghum undergoing cowpea rotation had the highest ergosterol content while sorghum undergoing fallow treatment had the lowest (Figure 8).

Yield: Significant differences ($P < 0.05$) were evident between the grain mass of the harvested sorghum plants from the 1st and 2nd seasons (Figures 6 and 9). The highest grain mass in the 1st season was recorded in sorghum undergoing dry bean and soybean rotations while the lowest was found in sorghum undergoing monoculture (Figure 6). In the 2nd season, sorghum undergoing dry bean rotation had the highest

grain mass while the lowest was in sorghum undergoing monoculture and fallow treatment (Figure 9). A significantly lower ($P < 0.05$) grain mass was observed in sorghum plants undergoing fallow and monoculture treatments compared to the other rotation treatments in the 1st season. Similarly a significantly lower grain mass was recorded in sorghum plants undergoing fallow and monoculture treatments compared to sorghum plants undergoing soybean and dry bean rotation in the 2nd season.

Effect of sorghum genotype on root disease and yield

Root disease: A significant difference ($P < 0.05$) was evident between the root mass of sorghum genotypes NS5511 and PAN8706W undergoing cowpea rotation in the 1st season (Figure 5). However, no significant difference was observed between the root mass of genotypes NS5511 and PAN8706W in the 2nd season (Figure 7). Ergosterol content also showed no significant differences between NS5511 and PAN8706W in the 2nd season (Figure 8).

Yield: A significant difference ($P < 0.05$) was evident between the grain mass of NS5511 and PAN8706W sorghum plants undergoing monocropping in the 1st season (Figure 6). In the 2nd season, the grain mass of sorghum genotypes undergoing soybean and dry bean rotation was found to be significantly different ($P < 0.05$) (Figure 9).

Discussion

The objective of the present study was to investigate the effect of rotation with two sorghum genotypes and various legumes on microbial populations in the sorghum rhizosphere, sorghum root disease and yield. Significantly higher microbial activity was observed in soils undergoing rotation compared to monoculture and fallow

treatments. Venter, Jacobs and Hawkins (2016) also found differences of up to 15 % soil microbial biomass (Shannon's diversity index) in soils undergoing crop rotation compared to those from a monoculture system. Crop rotation systems are known to improve soil health, soil fertility and soil microbial populations (Jiang *et al.*, 2016; Liu *et al.*, 2017). Zhao *et al.* (2017) reported significant improvements in soil microbial activity (FDA analysis) of up to 36.37 µg/g h^{-1} in soils undergoing ginseng/maize rotation compared to 14.18 µg/g h^{-1} in soils undergoing ginseng monoculture and related this occurrence to root exudates released by the respective crop plants. The authors suggested that nutrients present within root exudates not only improved microbial activity but also improved nutrient cycling and N content. The low microbial activity in soils undergoing monoculture was related to microbial dormancy caused by limited nutrients.

Results in this study showed a similar decrease in soil microbial activity in the 2nd and 3rd seasons where sorghum was planted repeatedly. This decrease in soil microbial activity could be caused by the limited availability in types of nutrients released by root exudates or a build-up of sorghum allelochemicals. Sorghum produces a wide range of phenolics such as ferulic, *p*-coumaric, *p*-hydroxybenzoic, vanillic, syringic, caffeic, *trans*-cinnamic and gentisic acids (Won *et al.,* 2013). Several of these phenolic acids such as cinnamic, *p*-hydroxybenzoic, *p*-coumaric, caffeic and syringic acids have been shown to significantly stimulate or inhibit soil microbial diversity (Kong *et al.*, 2008; Heleno *et al.*, 2013; Sadeghi *et al.*, 2013; Lima *et al.*, 2016; Mishra *et al.*, 2017). Li *et al.* (2017) similarly observed decreases of up to 67.12 % in microbial enzyme activity in rhizosphere soils of a 20-year-old tea bush monoculture compared to a 10-year-old monoculture. The authors related this to toxic levels of phenolic acids due to build-up in the longer monoculture which not

only affects nutrient cycling but also the incidence of soil-borne pathogens (Qiu *et al.*, 2012; Manikandan, Karthikeyan and Raguchander 2017; van Agtmaal *et al.*, 2018).

Results of this study indicated differences of less than 84 % similarity in bacterial and fungal diversity between soils undergoing soybean rotation compared to the other treatments. This is consistent to the work of Ashworth *et al.* (2017), who found significant differences in soil microbial DNA profiles between soils undergoing maize monoculture, soybean monoculture and maize/soybean rotation. The authors related this to the positive response of soil microbes to legume species. However, results of this study showed no differences in bacterial and fungal diversity between soils undergoing cowpea and dry bean rotation compared to fallow and monoculture. Another possible explanation of this variance in microbial diversity is the presence of different forms of root exudates in the various rotation treatments. Liu *et al.* (2017) reported differences in soil bacterial communities of up to 70.1 % in community variation in the Betaproteobacteria, Acidobacteria, Actinobacteria and Alphaproteobacteria taxa between soils undergoing soybean monoculture compared to soybean/maize rotation. The authors related this to microbial reactions towards the various types of root exudates. Similarly, our results suggest that compounds such as nutrients and allelochemicals present in root exudates of soybean compared to cowpea and dry bean can drastically affect soil microbial diversity.

The results of the field trials showed no significant differences between the visual root rot rating of sorghum plants undergoing rotation compared to fallow and monoculture. However a significantly lower ($P < 0.05$) sorghum root mass was found in fallow and monoculture soils compared to crop rotation. Linh *et al.* (2015) found increased plant height of up to 74.5 cm and root mass density of up to 156.15 g/m² in rice plants grown in rice/mung bean/maize rotation compared to plant height of up to

65.4 cm and root mass density of up to 91.11 g/m² in rice plants grown in monoculture. The authors also found increases in yield of up to 6.52 t/ha in rice plants grown in rotation compared to yield of up to 4.8 t/ha in rice plants grown in monoculture. Results in this study showed similar increases in yield between sorghum grown in all rotations compared to monoculture and fallow treatments. This is also similar with Nevens and Reheul (2001) who found higher yield of up to 15.8 t/ha in maize plants grown in crop rotation systems compared to 13 t/ha in maize plants grown in monoculture.

Results in this study showed significant differences in soil microbial activity between the rotation treatments planted with PAN8706W and NS5511. Cluster analysis revealed that there was less than 93 % similarity between the bacterial and fungal diversity of soil planted with sorghum genotypes PAN8706W and NS5511 which indicates that genotype has a significant impact on soil microbial activity and diversity. These differences in microbial activity and diversity could be the result of different nutrients and allelopathic compounds within the root exudates of the different sorghum genotypes since the composition of phenolics/sorgoleone in the root exudates are determined by their respective genotype (Sene, Dore, and Pellissier, 1999; Won *et al.*, 2013). Aira *et al.* (2010) reported similar differences in soil microbial activity and community diversity in a field trial planted with two different maize genotypes. The authors related this difference in microbial diversity to differences in the composition of root exudates between genotypes. As previously mentioned, sorghum produces various phenolics such as cinnamic, *p*-hydroxybenzoic, *p*-coumaric, caffeic and syringic acids which are known for their effect on soil microbial organisms. Wu, Wang and Xue (2009) reported significant differences in soil microbial composition and genetic diversity between soils treated with different

concentrations (25, 50, 100 and 200 mg/kg soil) of cucumber (*Cucumis sativus*) root exudate extracts. The authors found that cinnamic acid within the root exudates promoted soil microbial diversity that can utilise carbon sources such as cyclodextrin, glycogen and 2-hydroxy benzoic acid and inhibited soil microbial diversity that can utilise hydrobutyric acid, L-asparagine and D-galacturonic acid.

The present study demonstrated distinct differences between the microbial properties of soil undergoing fallow and monoculture compared to crop rotation. Vegetative assessment showed improved plant growth and yield of sorghum undergoing all three rotation treatments compared to fallow and monoculture treatments. In terms of yield, sorghum was most stimulated by soybean and dry bean rotation. Results of this study also indicated that sorghum genotype significantly affects microbial activity and diversity in the rhizosphere. Further research should be conducted to clarify these effects of crop rotation and sorghum genotype on soil microbial organisms and soil-borne diseases by using molecular techniques such as next generation sequencing (NGS). This information could be used to develop rotation systems that have the potential of not only reducing plant disease, improving plant growth and plant yield but also maintaining a diverse soil microbial community.

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Table 1. Microbial biomass of different functional groups indicated by total PLFAs (nmol/g soil) in rhizosphere soil collected from the 1st season.

Treatment	Fallow	Monocrop	Cowpea	Dry bean	Soybean
Gram + bacteria PLFA	8.55	9.68	6.38	9.84	11.02
Gram - bacteria PLFA	11.52	5.67	2.50	5.22	9.19
General bacteria PLFA	102.58	95.12	86.87	89.79	92.81
Fungal PLFA	18.63	26.02	23.41	21.81	28.08
Total PLFA	141.28	136.49	119.17	126.66	141.10

Table 2. Microbial biomass of different functional groups indicated by total PLFAs (nmol/g soil) in rhizosphere soil collected from the 2nd season.

Treatment	**Fallow**		**Monocrop**		**Cowpea**		**Dry bean**		**Soybean**	
Sorghum genotype	PAN8706W	NS5511	PAN8706W	NS5511	PAN8706W	NS5511	PAN8706W	NS5511	PAN8706W	NS5511
Gram + bacteria PLFA	8.25	4.28	4.73	3.17	5.46	4.18	5.50	3.89	4.65	3.64
Gram - bacteria PLFA	4.93	7.81	7.20	2.03	4.26	4.74	8.61	1.06	1.47	2.29
General bacteria PLFA	81.16	83.93	95.75	76.42	151.78	76.80	127.79	74.27	89.21	85.19
Fungal PLFA	27.21	13.80	18.73	13.69	67.70	14.79	19.33	11.62	16.20	24.12
Total PLFA	121.55	109.82	126.41	95.32	229.19	100.51	161.24	90.84	111.52	115.25

Table 3. List of fatty acids and signature lipids used to determine soil microbial functional groups.

Bacterial Acid Methyl Esters (BAME's)		
Component Name	**Formula**	**Signature lipid**
Methyl octanoate	C8:0	
Methyl decanoate	C10:0	
Methyl 2-hydroxydecanoate	2-OH-C10:0	
Methyl undecanoate	C11:0	
Methyl dodecanoate	C12:0	
Methyl 3-hydroxydodecanoate	3-OH-C12:0	Gram negative bacteria
Methyl tridecanoate	C13:0	
Methyl tetradecanoate	C14:0	General bacteria
Methyl 2-hydroxytetradecanoate	2-OH-C14:0	Gram negative bacteria
Methyl 3-hydroxytetradecanoate	3-OH-C14:0	Gram negative bacteria
Methyl 13-methyltetradecanoate	i-C15:0	Gram positive bacteria
Methyl 12-methyltetradecanoate	a-C15:0	Gram positive bacteria
Methyl cis-10 pentadecanoate	$C15:1^{10}$	
Methyl hexadecanoate	C16:0	General bacteria
Methyl 14-methylpentadecanoate	i-C16:0	Gram positive bacteria
Methyl cis-9-hexadecanoate	$C16:1^{9}$	Gram negative bacteria
Methyl octadecanoate	C18:0	General bacteria
Methyl trans-9-octadecenoate	$C18:1^{9}$	
Methyl cis-9-octadecenoate	$C18:1^{9}$	Fungi
Methyl cis-7-octadecenoate	$C18:1^{7}$	
Methyl cis-9,12-octadecadienoate	$C18:2^{9,12}$	Fungi
Methyl cis-9,12,15 octadecatrienoate	$C18:3^{9,12,15}$	Fungi
Methyl cis-9,10-methyleneoctadecanoate	$C19:0^{\Delta}$	Gram negative bacteria
Methyl cis-11 eicosenoate	$C20:1^{11}$	
Methyl cis-11,14,17 eicosatrienoic	$C20:3^{11,14,17}$	
Methyl docosanoate	C22:0	
Methyl cis-13 docosadienoate	$C22:1^{13}$	
Methyl tetracosanoate	C24:0	
Methyl cis-15 tetracosanoate	C24:1c15	

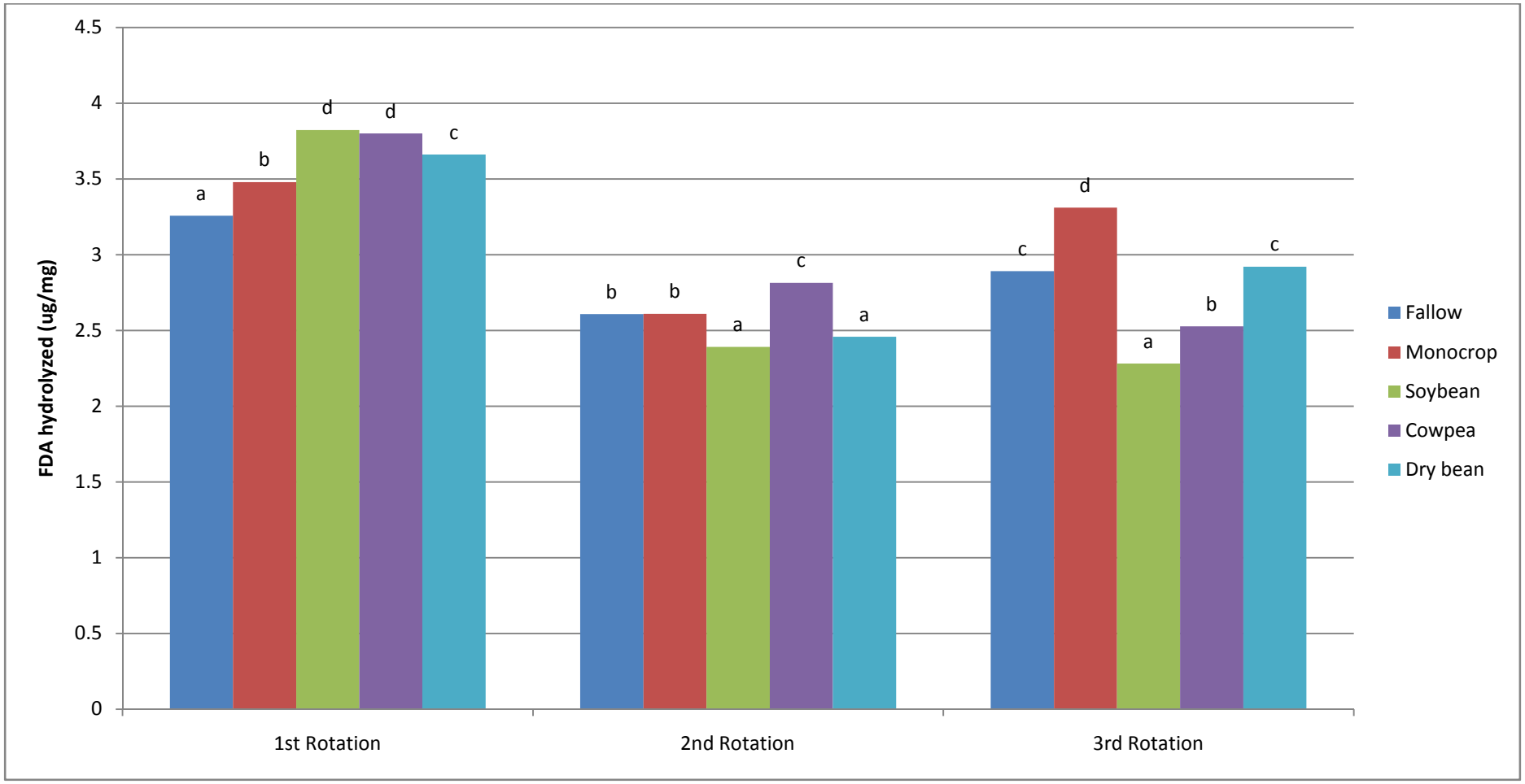


Figure 1. FDA hydrolysis (ug/mg soil) of rhizosphere soil collected from 3 seasons. Bars with the same uppercase letter/s are not significantly different ($P > 0.05$).

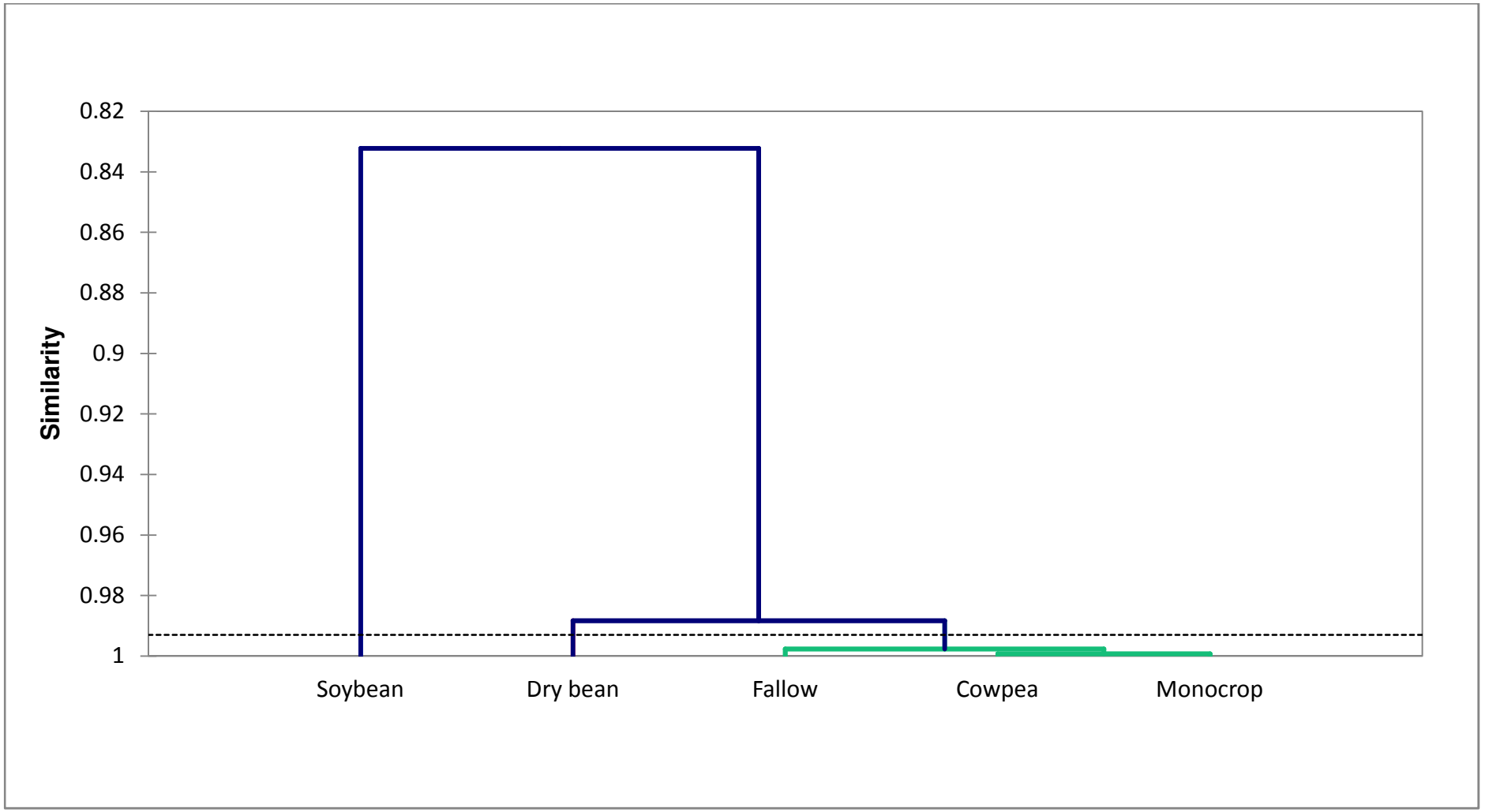


Figure 2. Microbial diversity indicated by total PLFAs of rhizosphere soils collected from the 1st season.

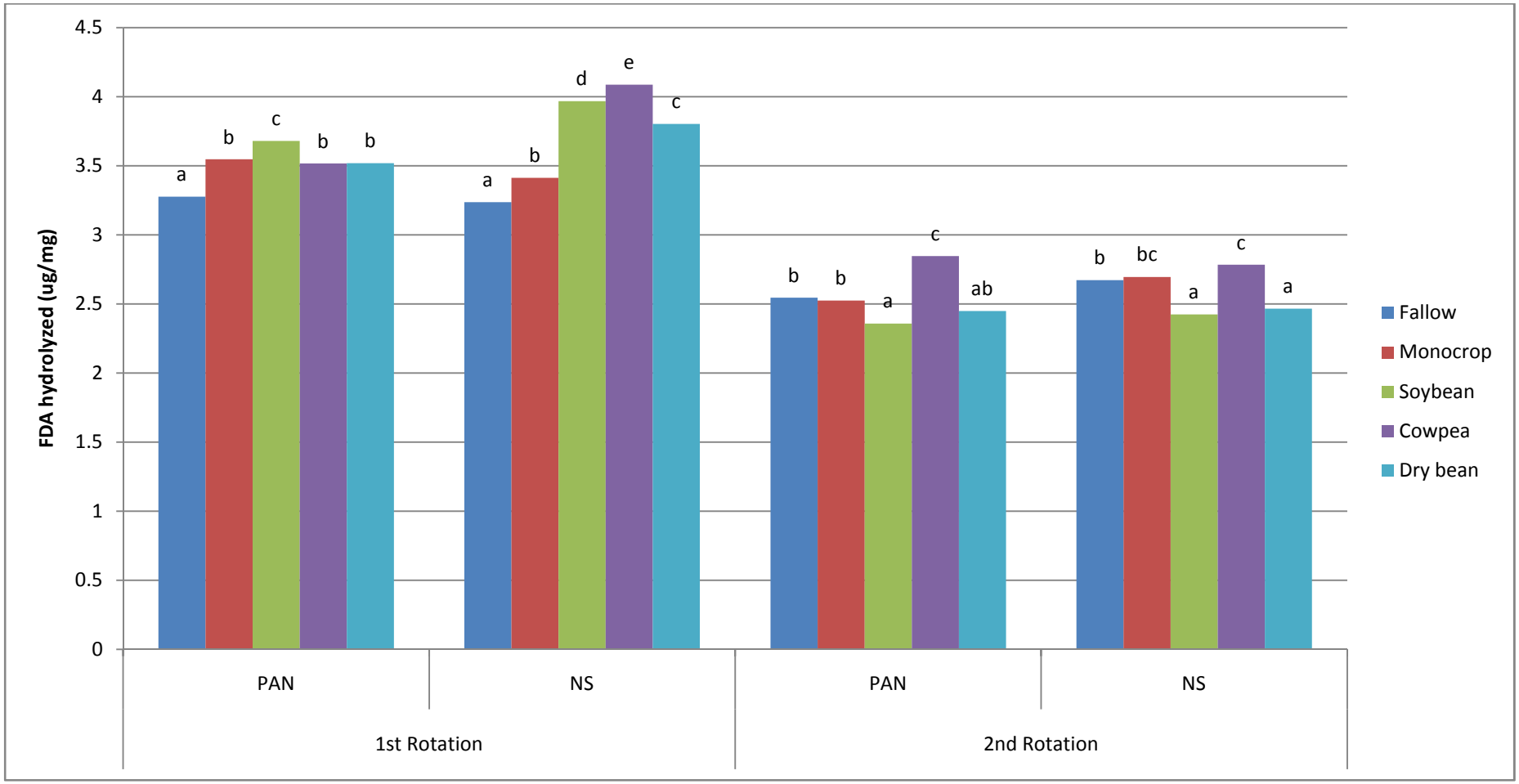


Figure 3. FDA hydrolysis (ug/mg soil) of rhizosphere soil collected from 2 seasons. Bars with the same uppercase letter/s are not significantly different ($P > 0.05$).

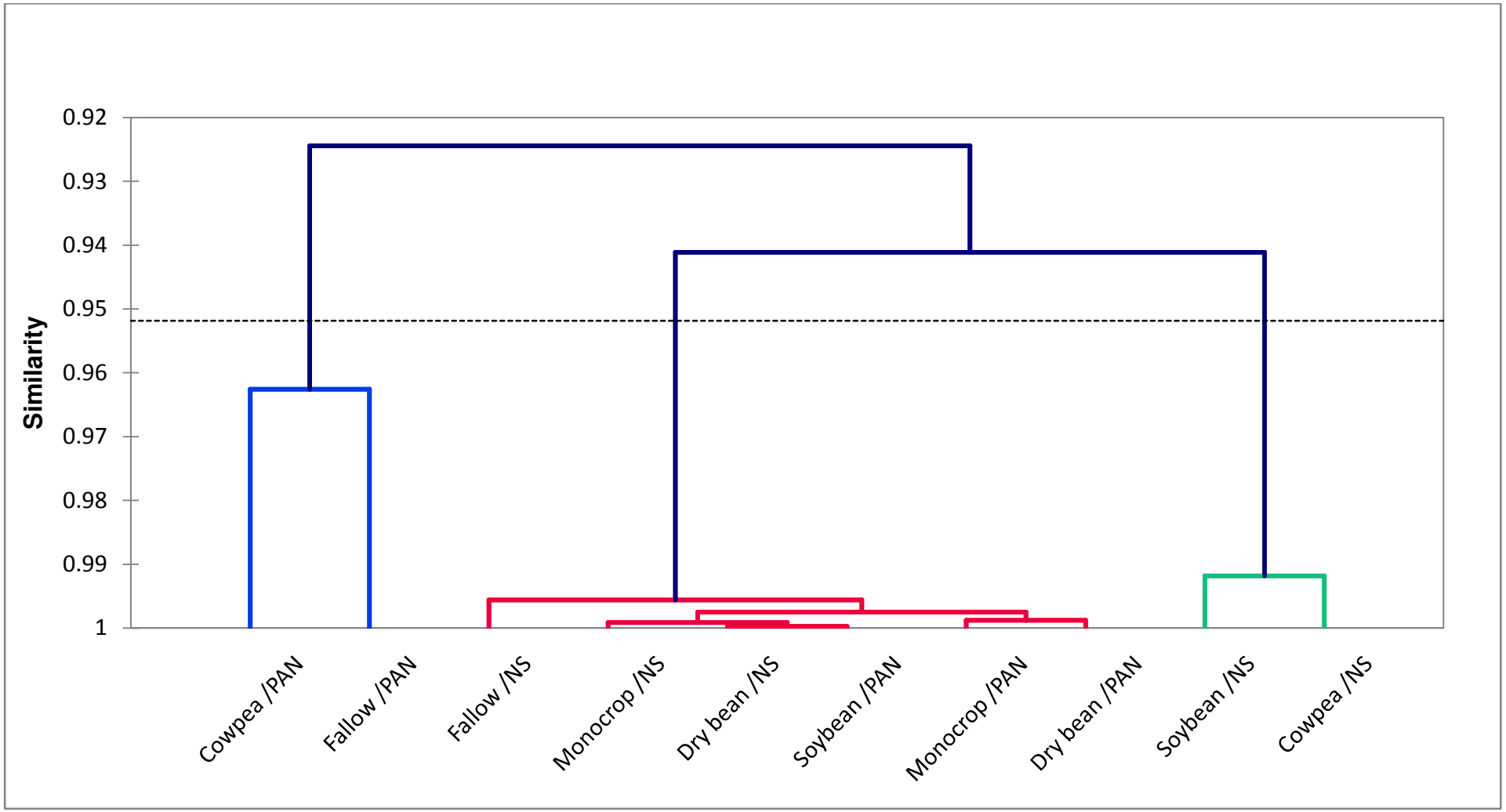
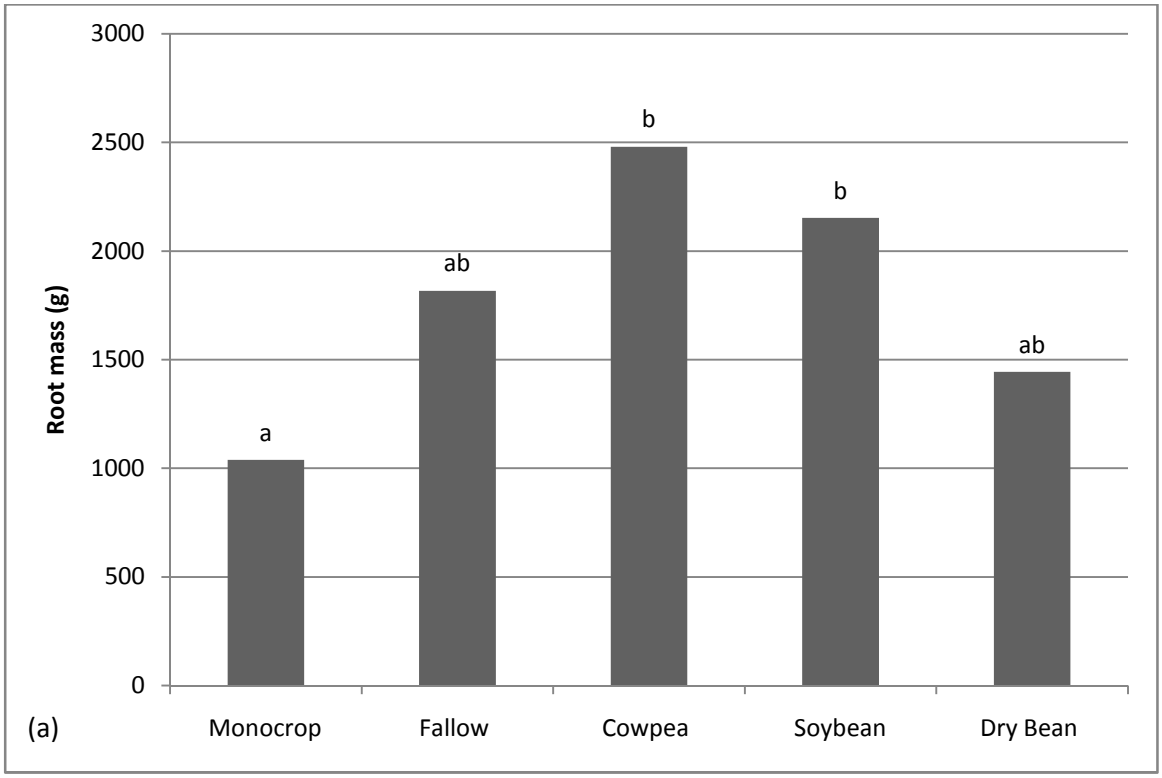


Figure 4. Microbial diversity indicated by total PLFAs of rhizosphere soils collected from the 2nd season.



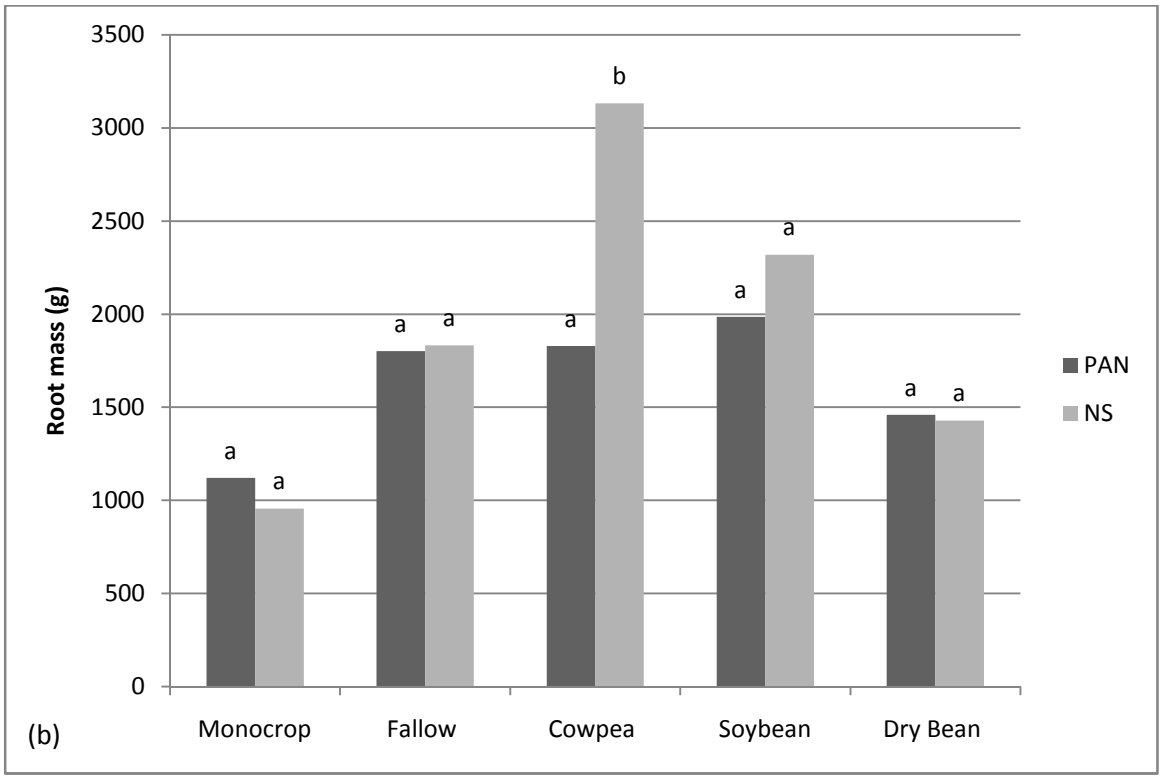
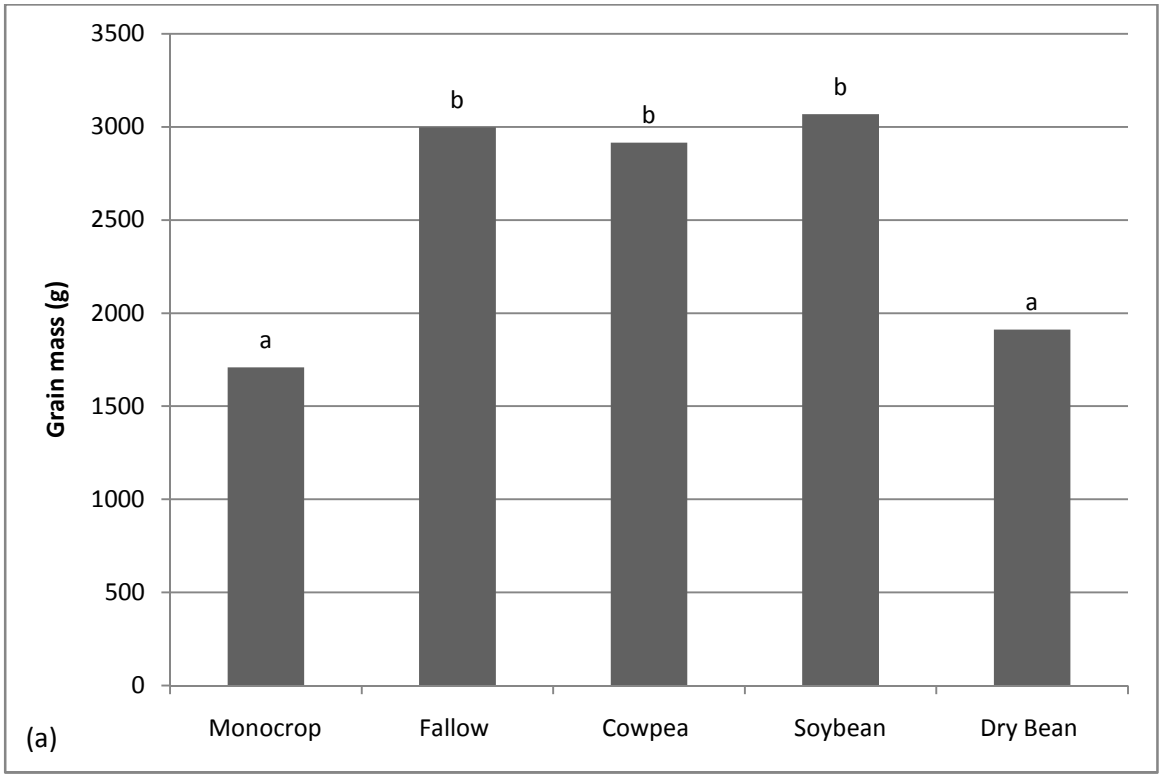


Figure 5. Average root mass (g/50 plants) comparing rotation treatment (a) and genotype difference (b) of sorghum plants harvested in the 1st season. Bars with the same lowercase letter/s are not significantly different (P > 0.05).



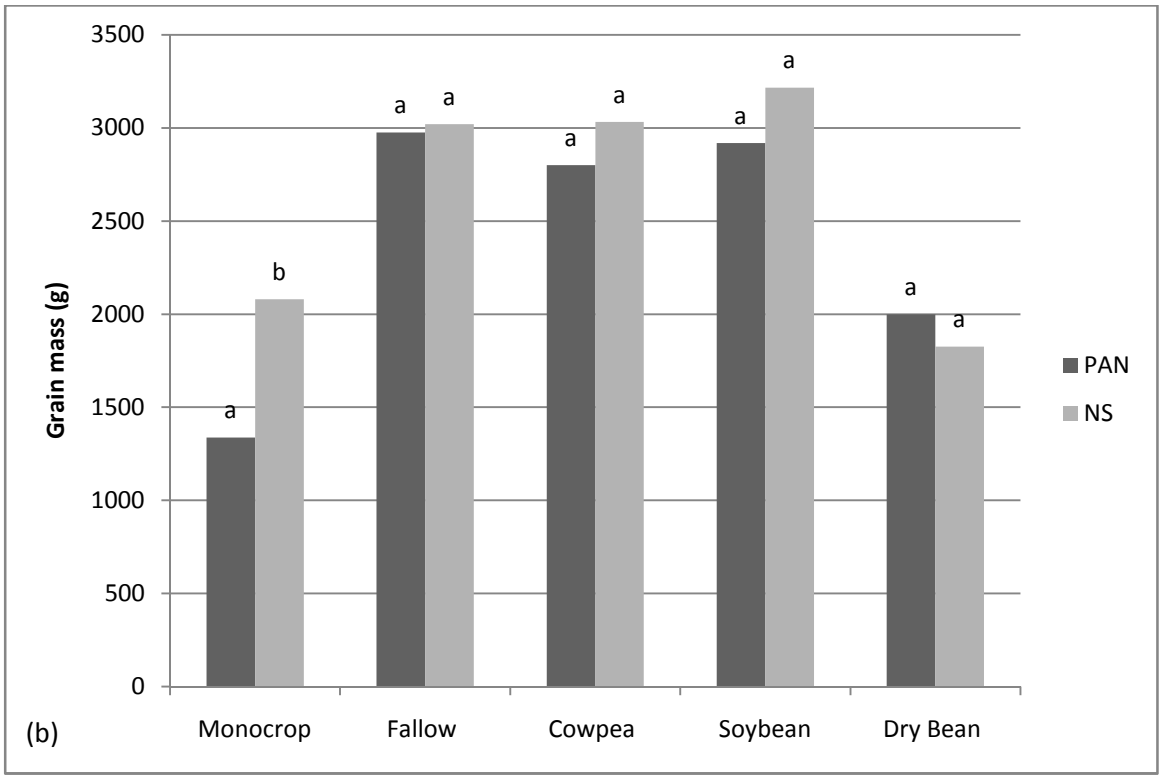
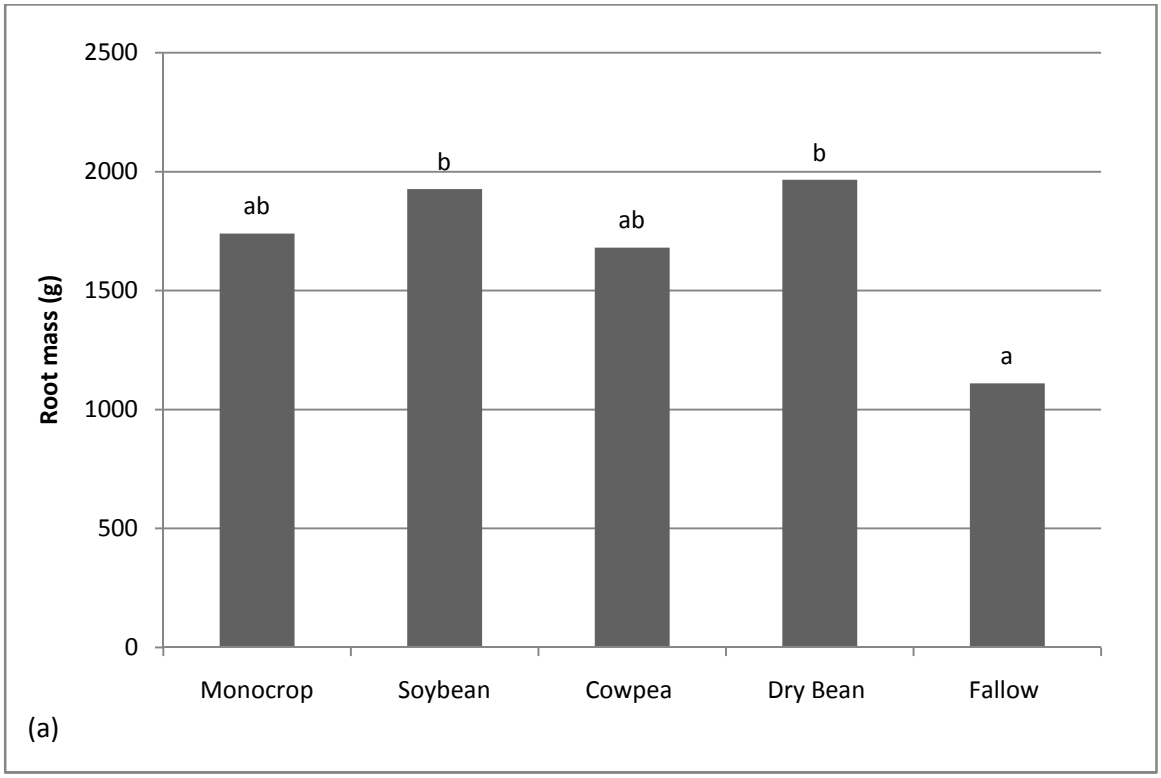


Figure 6. Average grain mass (g/50 plants) comparing rotation treatment (a) and genotype difference (b) of sorghum plants harvested in the 1st season. Bars with the same lowercase letter/s are not significantly different (P > 0.05).



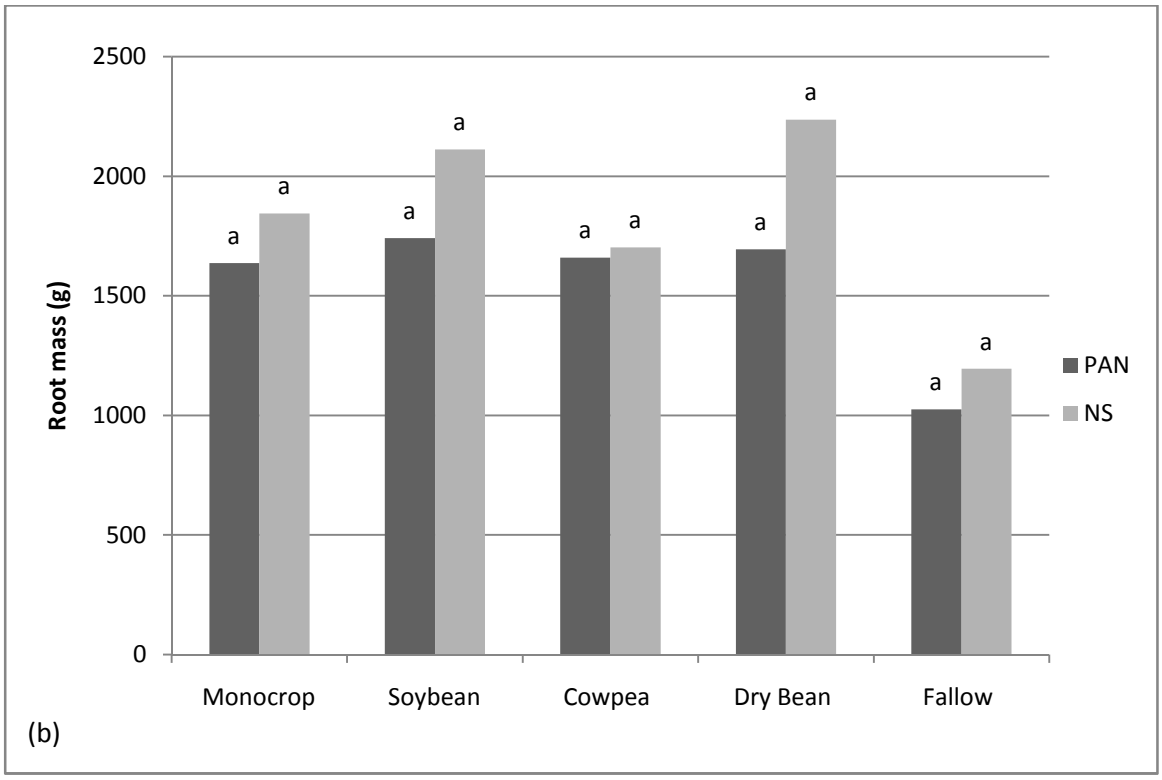
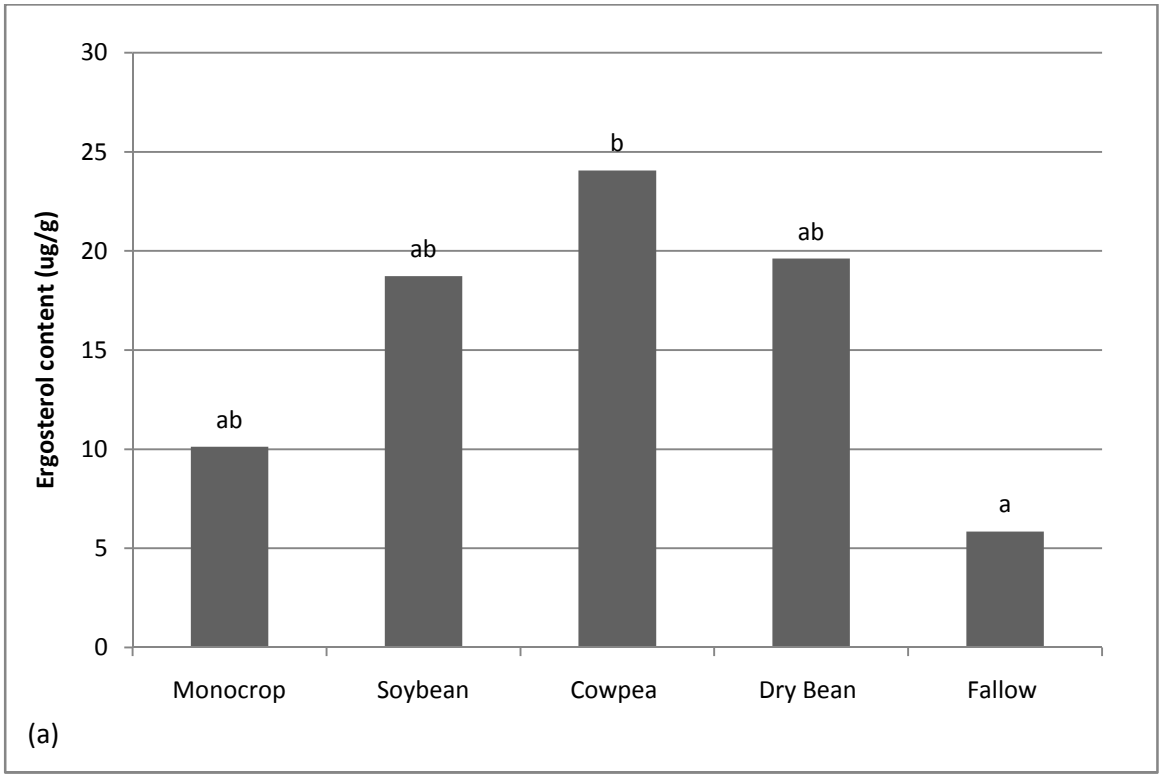


Figure 7. Average root mass (g/50 plants) comparing rotation treatment (a) and genotype difference (b) of sorghum plants harvested in the 2nd season. Bars with the same lowercase letter/s are not significantly different (P > 0.05).



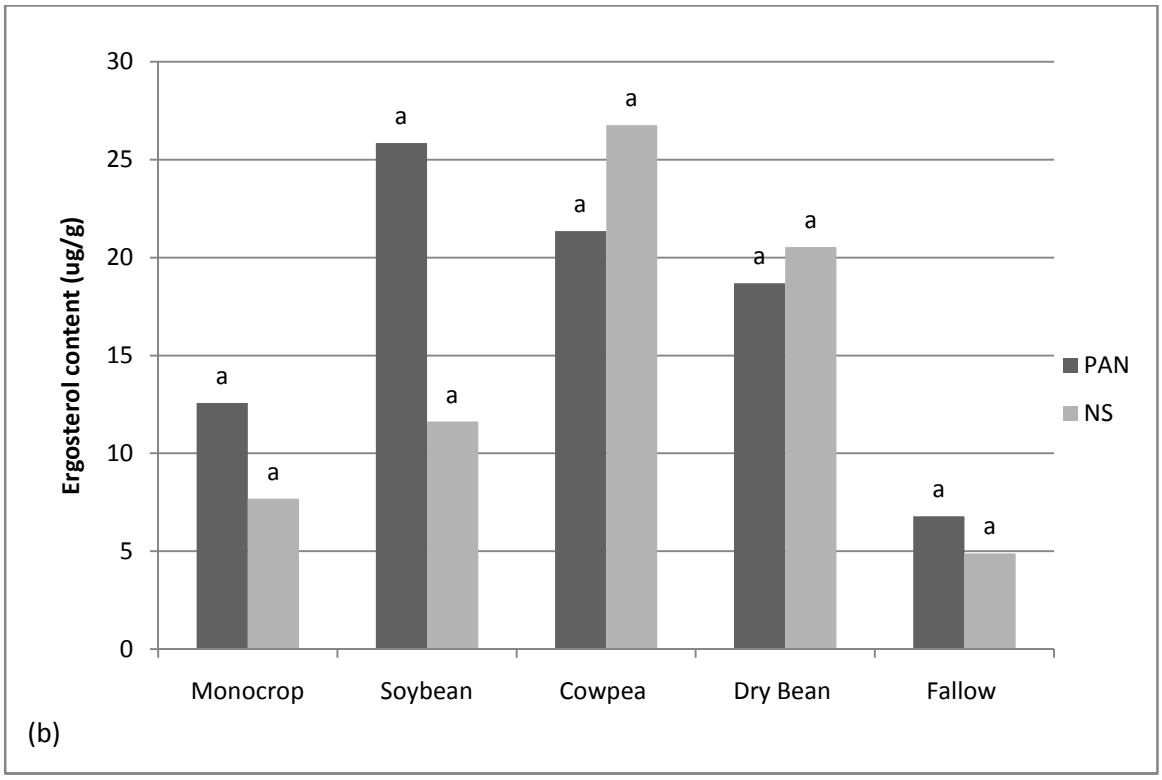
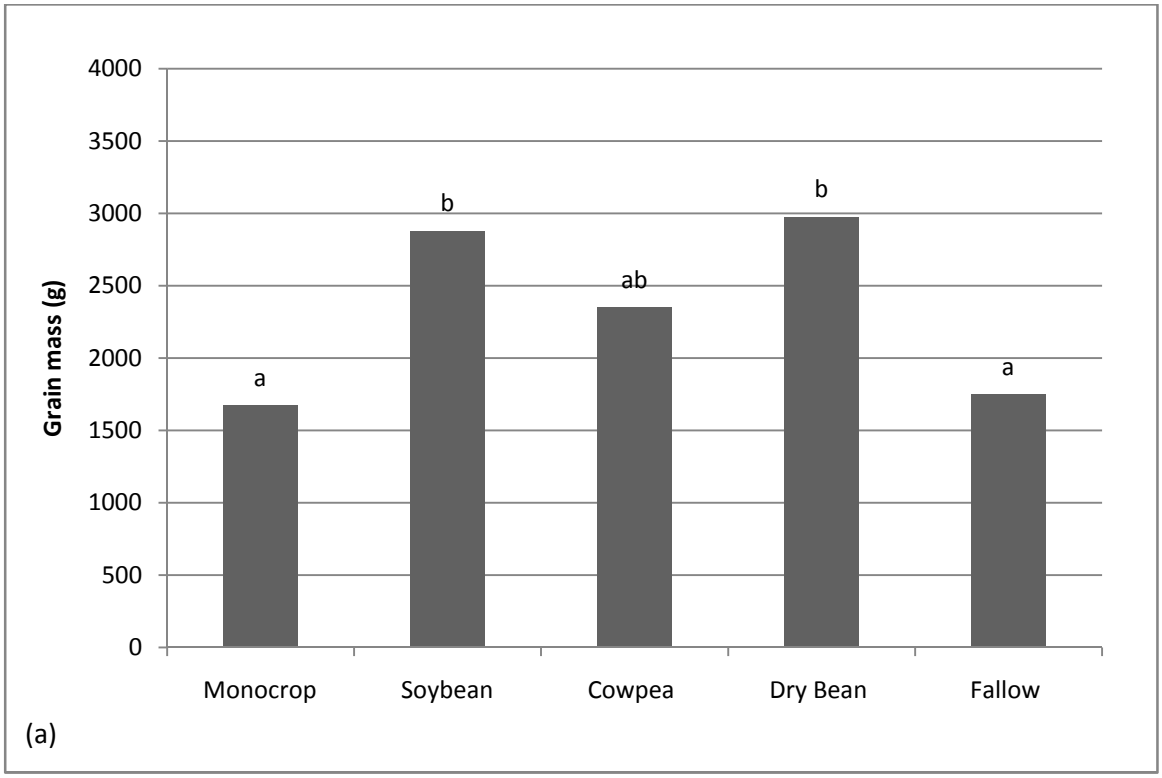


Figure 8. Average ergosterol content comparing rotation treatment (a) and genotype difference (b) of sorghum roots harvested in the 2nd season. Bars with the same lowercase letter/s are not significantly different (P > 0.05).



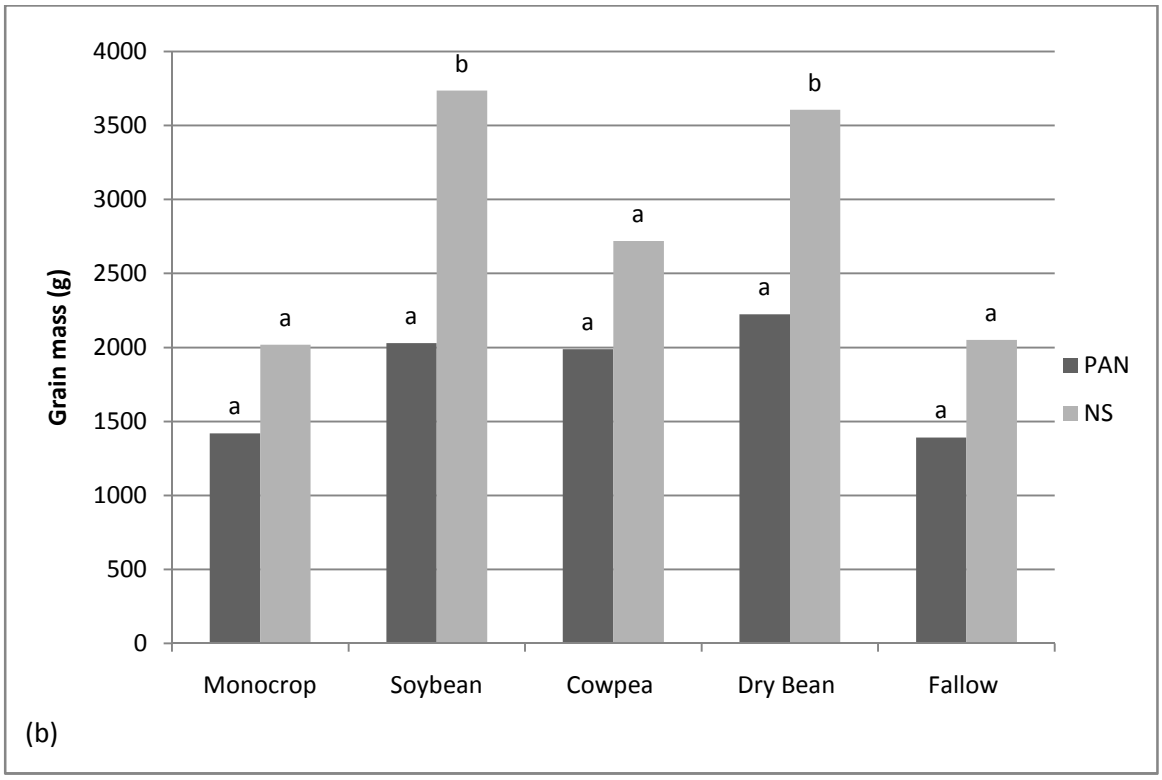


Figure 9. Average grain mass (g/50 plants) comparing rotation treatment (a) and genotype difference (b) of sorghum plants harvested in the 2nd season. Bars with the same lowercase letter/s are not significantly different (P > 0.05).

Summary

This study evaluated and investigated sorghum allelopathy and its various effects on microbial populations in the rhizosphere and effect of soil-borne pathogens. The effect of sorghum allelopathic extracts on rhizosphere soil microbial activity and diversity as well as the growth and incidence of soil-borne pathogens was investigated. The effect of different sorghum genotypes on the rhizobiome, root rot and yield were also investigated.

Evaluation of the allelochemical contents of 22 sorghum genotypes showed variation between the concentration of their total phenolics and sorgoleone. The highest phenolic content was genotypes BTX ARG-1 (tan plant), RTam 2566 (purple plant), RTx 436 (tan plant) and BTx 635 (tan plant) and the lowest phenolic content was genotypes S5C719-11E (purple plant) and SCAY 21 (tan plant). The highest amount of sorgoleone was produced by genotype BTx 3197 (purple plant) and the lowest was genotype Tx 2911 (red plant). Phenolic extracts of RTx 430, RTx 436, Rtam 428, SCAY 21 and sorgoleone extracts of genotype BTx 3197 were selected for further experiments.

The effect of sorghum phenolic and sorgoleone extracts on eight soil-borne pathogens *in vitro* showed that the extracts significantly stimulated the colony diameter of most of the pathogens. Phenolic extracts of RTx 430 and RTx 436 was found to be the most stimulating against the eight soil-borne pathogens. Sorgoleone extracts was found to significantly inhibit the colony diameter of *C. capsici* and *F. equiseti* compared to the control.

Microcosms investigating the direct effects of sorghum phenolic and sorgoleone extracts on soil microbial populations showed differences (< 55 % similarity) between the functional diversity of rhizosphere soils treated with phenolics

and sorgoleone compared to the untreated control. Microcosms investigating effect of the extracts on soil-borne pathogens showed significant differences in leaf length, root length and root rot rating of the treated plants compared to the control. The root rot of sorghum plants inoculated with *A. alternata, C. trifolii* and *F. thapsinum* were found to be the most inhibited by the extracts and plants inoculated with *A. strictum* and *C. capsici* were found to be the least inhibited. Extracts of SCAY 21 0.5x, RTam 428 1x and sorgoleone 1x were the most inhibiting and extracts of RTx 436 0.5x and SCAY 21 1x, the least. Microcosms investigating the indirect effects of the extracts on soil-borne pathogens showed that soils previously treated with extracts were most suppressive against *A. alternata* and *F. thapsinum* and least against *P. macrostoma*. Soils previously treated with RTx 436 0.5x and SCAY 21 0.5x extracts were most effective in suppressing the pathogens.

The rotation trial with two sorghum genotypes showed significantly higher microbial activity in soils undergoing rotation compared to monoculture and fallow. The trial also showed differences (< 84 % similarity) between soils previously planted with soybean compared to other treatments. In the 1st season, a significantly higher root mass was recorded in sorghum undergoing cowpea and dry bean rotation compared to monoculture. In the 2nd season, significantly higher root mass was recorded in sorghum undergoing soybean and dry bean rotation compared to fallow. A significantly lower ($P < 0.05$) yield was recorded in sorghum plants undergoing fallow and monoculture compared to other rotations.

The results of this study revealed various effects of sorghum allelochemical extracts on microbial populations in the rhizosphere and soil-borne pathogens. Further research should be conducted to clarify these effects by using molecular techniques such as next generation sequencing (NGS).