Fusarium spp. and associated mycotoxins in South African maize

by

Sonia-Mari Joubert

A thesis submitted in accordance with the requirements for the degree of
Philosophiae Doctor

Faculty of Natural and Agricultural Sciences
Department of Plant Sciences: Plant Pathology
University of the Free State
Bloemfontein, South Africa

Promoter
Prof. N. W. McLaren

Co-promoters
Prof. B. C. Flett
Dr. A. Schoeman

January 2020

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Declaration

I, Sonia-Mari Joubert, declare that the PhD thesis that I submit for the PhD degree qualification at the University of the Free State is my independent work, and I have not previously submitted it for a qualification at another institution of higher education.

I, Sonia-Mari Joubert, hereby declare that all royalties as regards intellectual property that was developed during the course of and/or in connection with the study at the University of the Free State, will accrue to the University.

I, Sonia-Mari Joubert, hereby declare that I am aware that the research may only be published with the dean's approval.

27 January 2020

Acknowledgements

Gordon B. Hinkley said “You will come to know that what appears today to be a sacrifice will prove instead to be the greatest investment you will ever make”. Without the investment of the following entities I would not have been able to complete my PhD.

- The Agricultural Research Council – for inviting me to be part of the PDP program and for funding the project.
- The Maize Trust – for personal and research funding.
- GWK – for inviting me into your maize fields.
- University of Stellenbosch – for supplying me with my fungi of choice.
- My promoter Prof. McLaren and my co-promoters Prof. Flett and Dr. Schoeman – for your support, guidance and patience, without which I would not have been able to reach the finish line.

“Go forth in life to achieve your dreams but don't forget the reasons and the people who make the journey worth the trouble” – Anonymous.

- The journey towards my PhD would not have been possible if not for my parents. Their caring, encouragement and endless support sustained me through my years in academia.
- I might have started the PhD journey alone, but I ended it with my best friend for life. Thank you, husband of mine for your love, support and drying more tears than I care to remember.
- To those who have cheered for me along the way: my brother and his family, my in-laws, my family and all my friends – you are all a-maize-ing.
- My ARC colleagues and mentors – you were my guides and inspiration, and for that I am truly grateful.
- With an exceedingly grateful heart, a huge thank you my Heavenly Father: “He only is my rock and my salvation: he is my defence; I shall not be moved.” – Psalm 62:6.

Preface

This thesis is a compilation of five independent manuscripts that focuses on *Fusarium* pathogens/pathogen complexes of maize including the *Fusarium graminearum* species complex (FGSC), *F. verticillioides* and *F. oxysporum* species complex (FOSC). **Chapter 1** consist of a literature overview that includes a discussion on the importance of maize and the respective *Fusarium* spp. as plant pathogens. The epidemiology and mycotoxin production of these pathogens/pathogen complexes is discussed. The potential for mycotoxin translocation is discussed and the current knowledge of maize defence responses to these pathogen/pathogen complexes is reviewed.

Chapter 2 presents a survey that was conducted on maize roots, crowns, stalks and grain to determine the distribution of FGSC, *F. verticillioides* and FOSC throughout South Africa. Various agricultural management practices were scrutinised to determine their role in the infection of maize by these pathogens/pathogen complexes, by determining the *Fusarium* spp. target DNA concentrations in various tissues. The concentration of deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA) and fumonisin (FUM) were quantified in maize grain to determine the extent of mycotoxin contamination in maize grain throughout South Africa.

Chapter 3 was conceptualised after the Agricultural Research Council-Grain Crops received an enquiry from Northern Cape Provinces' farmers about prematurely senescing areas (PSA) within irrigated maize fields. Initial diagnosis pointed to FGSC infection. During the first season a variety of abiotic factors were monitored together with FGSC target DNA. During the subsequent two seasons, fields were monitored for infection, however the PSA's were not observed again. During the third season sequential sampling was conducted over four growth stages to determine the growth stage that posed the highest risk.

In **Chapter 4**, the translocation of DON, NIV, ZEA and FUM from the roots and stalks to the grain in two maize cultivars was evaluated. This study provided insight into the risk of root and stalk rots to human and animal health.

In **Chapter 5**, maize stalk defence responses were evaluated against *F. boothii*, a member of the FGSC. At present there is no knowledge of maize stalk defence responses against FGSC

infection. Thus, this study aids in understanding FGSC stalk rot in maize and the maize response to this pathogen complex.

Sun Tzu in the art of war stated that “if you know the enemy and know yourself, you need not fear the result of a hundred battles”. Each of the five chapters presented in this thesis focused on a different aspect of the *Fusarium*/maize interaction, however they complement each other to further our understanding of three *Fusarium* spp./spp. complexes. This knowledge can then be used in various other disciplines such as agricultural practices, epidemiological prediction models and resistance breeding to produce heathier plants.

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Chapter 1: Pathogenicity, toxigenicity and epidemiology of *Fusarium* spp. infecting maize

1.1 Introduction

Agriculture is an essential part of South Africa's economy because it creates employment, provides the country with foreign exchange and feeds a population that is estimated to grow by 2 % per year (Goldblatt, 2010). The most common agricultural commodities are grain crops such as maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), millet (*Pennisetum glaucum* L.) and rye (*Secale cereal*e L.) (Anonymous, 2015). Maize is the most widely cultivated grain crop in South Africa. Advanced cultivation practices such as improved cultivars, effective crop rotations and enhanced fertilisation have increased the yield and number of crops per year (Goldblatt, 2010). However, this increased cropping intensity has also resulted in an increase in disease prevalence. Bacteria, viruses and fungi all have members that are pathogenic on maize and contribute to a reduction in grain yield and quality (Shurtleff *et al.*, 1993). There are many species in the genus *Fusarium* that are destructive pathogens on a wide range of crops. The most important *Fusarium* spp. that affect maize are *F. avenaceum* (Fr.) Sacc., *F. cerealis* (Cooke) Sacc, *F. culmorum* (W.G. Sm.) Sacc., *F. equiseti* (Corda) Sacc., *F. graminearum* Schwabe species complex (FGSC), *F. poae* (Peck) Wollenw, *F. proliferatum* (Matsush.) Nirenberg, *F. sporotrichioides* Sherb., *F. subglutinans* (Wollenw. and Reinking) P.E. Nelson, Toussoun and Marasas. and *F. verticillioides* (Sacc.) Nirenberg, while *F. oxysporum* Schlectend.: Fr. (*F. oxysporum* species complex or FOSC), *F. semitectum* Berk. and Ravenel. and *F. solani* (Mart.) Sacc (*F. solani* species complex or FSSC) are found to a lesser extent (Nicolaisen *et al.*, 2009). *Fusarium* infection is of importance because many species produce various mycotoxins in different maize tissues. The mycotoxin classes that cause the most harm are deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA) and fumonosins (FUM). Trichothecenes, such as DON and nivalenol NIV cause flu like symptoms in humans, ZEA causes reproduction problems in animals and FUM may cause cancer in humans (Brera *et al.*, 2008). The FGSC has been shown to produce DON, NIV and ZEA, *F. verticillioides* is a producer of FUM whereas *F. oxysporum* is a producer of T-2, HT-2 and ZEA (Leslie and Summerell, 2006; Foroud and Eudes, 2009; Boutigny *et al.*, 2011). In this study we will aim to improve our understanding of these pathogens on various maize tissues.

1.2 Maize

Maize is a member of the Poaceae family which consists of 600 genera and 8500 species (Simpson and Ogorzaly, 2001). Historically there are six different maize types namely pod, dent, flint, pop, flour, and sweet (Simpson and Ogorzaly, 2001). Maize was first domesticated in Mesoamerica and Teosinte is considered its progenitor (Doebley, 2004). Several modifications occurred over the 10 000-year domestication period to obtain the maize cultivars seen today. The first modification was the feminisation of male spikes which led to nutrient relocation and resulted in the production of large ears (Iltis, 1983; Simpson and Ogorzaly, 2001). Through human selection, all grain produced were viable. The ears also became fully sheathed with leaves in order for whole harvesting to occur (Simpson and Ogorzaly, 2001). Maize became insensitive to day length because it was preferentially cultivated in temperate regions (Simpson and Ogorzaly, 2001).

Maize is grown widely around the world because of its variability and adaptability (Du Plessis, 2003). It is the most important food crop in South Africa with approximately 12.1 million tons produced during the 2011/12 season and 11.7 million tons during the 2013/14 season (Anonymous, 2015). Both the vegetative and reproductive organs of the maize plant can be utilised to produce a variety of products (Simpson and Ogorzaly, 2001; Oladejo and Adetunji, 2012). These products include breakfast cereals, which are produced from ears and biofuels such as ethanol and starch, which are produced from stalks and grain, respectively (Du Plessis, 2003). Maize is however, considered a second-cycle crop, with 80 to 90 % of the crop utilised as animal feed in developed countries (Simpson and Ogorzaly, 2001; Du Plessis, 2003). In developing countries it serves as a direct food source to 200 million people (Du Plessis, 2003). It was estimated that between 2001 and 2003, 9 % of the world's maize crops were lost due to bacterial and fungal diseases. These losses differed from region to region e.g. Europe only experienced a 4 % loss whereas West Africa had a 14 % loss. The differences could be due to first world countries being able to invest in resistant germplasm and pesticides, whereas this is not always an option for third world countries (Balint-Kurti and Johal, 2009).

1.3 The taxonomy of the genus *Fusarium*

Members of the genus *Fusarium* belongs to the phylum Ascomycota, class Ascomycetes and order Hypocreales (Moretti, 2009). The genus was first described in 1809 by Link using the Morphological species concepts, which was largely based on the shape of the conidia (Leslie and Summerell, 2006, Leslie and Bowden, 2008; Cai *et al.*, 2011). As this concept is not restricted to *Fusarium* spp. this was not a definitive way to separate species. Until 1935 many *Fusarium* spp. were named according to the pathogens' preference for different hosts and habitats. This type of naming system is referred to as the ecological species concept (Leslie and Bowden, 2008). Wollenweber and Reinking (1935) consolidated all the *Fusarium* spp. known at that time and approximately 1000 isolates were placed into 16 sections, 65 species, 55 varieties and 22 forms, based on the presence or absence of microconidia and chlamydospores, as well as the shape of micro- and macroconidia (Nelson, 1991; Burgess *et al.*, 1994). These findings were published in *Die Fusarien* which was used as a stepping stone for subsequent research on the taxonomy of the *Fusarium* genus (Wollenweber and Reinking, 1935; Nelson, 1991). This period was dominated by two groups of *Fusarium* taxonomists namely the splitters of which Wollenweber and Reinking were a part and lumpers which included taxonomists such as Snyder and Hansen (1940) (Snyder and Hansen, 1940; Nelson, 1991). Gordon and his co-workers (1944) tried to find an intermediary path between the lumpers and the splitters by using conidiogenous cells, particularly those producing macroconidia, as a primary taxonomic character (Gordon, 1944; Leslie and Bowden, 2008). However, this was still not yet recognised as the standardised method of Fusarium taxonomy. Booth (1971) took Gordon's techniques and added mono- and polyphialides as splitting characters for *Fusarium* spp. (Nelson, 1991; Burgess *et al.*, 1994). These characteristics were subsequently used by Nelson (1983), to further define the Morphological species concept of *Fusarium* spp.. Leslie and Summerall (2006) used Nelson (1983) and Burgess' (1994) work, together with the phylogenetic species criterion to establish The *Fusarium* laboratory manual that is used by many, as the standard for *Fusarium* taxonomy (Nelson *et al.*, 1983; Burgess *et al.*, 1994; Leslie and Summerell, 2006). In 2013 there was a change in the International Code of Nomenclature for algae, fungi and plants. A concept was proposed to abandon the use of teleomorph names. However, many plant pathologists still apply the teleomorph name as the disease name (Geiser *et al.*, 2013).

Currently, there are two schools of thought when it comes to the taxonomy of *Fusarium* spp. There are the taxonomists that relay on theoretical species concepts, with the main concept being an Evolutionary species concept, whereas other relay on an operational species concepts including the Morphological species concept, Biological species concept and Phylogenetic species concept (Taylor *et al.*, 2000). Although each concept has their advantages and limitation, this study will touch on each concept only as much as it pertains to the pathology of each species.

1.4 The genus *Fusarium* as plant pathogen

Recently 495 fungal pathologists voted for the top 10 most scientifically and economically important plant pathogens. Two members of the genus *Fusarium* got fourth (FGSC) and fifth (FOSC) place, highlighting the significance of these groups as major pathogens on many important crop plants (Dean *et al.*, 2012). This genus of fungi is found globally, on many different crops and various other substrates (Moretti, 2009). In South Africa *F. culmorum* was the first species to be described. It was isolated from wheat stalks and roots, grown near Stellenbosch, Western Cape in the 1930's. Doidge (1938) described 26 *Fusarium* spp. commonly found in South Africa (Doidge, 1938; Marasas *et al.*, 1987). Marasas (1987) revised the list to 28 *Fusarium* spp.. Those most commonly found on maize were *F. chlamydosporum, F. equiseti,* FGSC*, F. verticillioides,* FOSC*, F. poae, F. scirpi* Lambotte and Fautrey, *F. solani* and *F. subglutinans* (Marasas *et al.*, 1987).

1.4.1 The *Fusarium graminearum* species complex (FGSC)

The FGSC has not changed as much from its initial identification as other *Fusarium* spp. has. Originally *F. graminearum* together with *F. graminum* Coda and *F. sambucinum* Fuckel fell under *F. roseum* (Wollenweber and Reinking, 1935; Aoki *et al.*, 2012). Gray (1821) determined that the haplotype of *F. roseum* matched that of *F. sambucinum* and thus, the latter was designated as the type specimen and the name was kept. *F. graminearum* was separated from *F. roseum* and changed little until the advent of phylogenetic species recognition (Gams *et al.*, 1997; Greuter and Hawksworth, 1999; Aoki *et al.*, 2012). During the 1980's *F. cerealis* and *F. pseudograminearum* O'Donnell, T. Aoki was separated from *F. graminearum* because they were found to be ecologically and phylogenetically distinct (Nirenberg, 1990; Aoki and O'Donnell, 1999). *F. pseudograminearum* was known as group 1 and is heterothallic, and *F.*

graminearum was known as group 2 and is homothallic (Leslie and Bowden, 2008). It was subsequently discovered that members within the species *F. graminearum* also had host, climatic and regional preferences (Table 1.1). In 2000, 7 distinct lineages were identified which demonstrated allopatric speciation, through vicariance (O'Donnell *et al.*, 2000). Although, O'Donnell et al. (2000) did suggest at the time, that introgression between lineages were possible, as agricultural and horticultural plants and plant tissue are moved globally. The movement of plants together with the agricultural practice of crop monoculture forces these distinct lineages in the same environment and may give rise to novel genotypes. Taylor *et al.* (2000) then reviewed multiple methods of species identification, advocating the use of a method known as genealogical concordance phylogenetic species recognition, which investigated species limits. In 2002, Ward *et al.* used the genealogical concordance phylogenetic species recognition method and correlated it with the trichothocene chemotype. This was done by sequencing the tri-cluster, a set of 8 genes responsible for trichothecene production. Using this technique, eight lineages were resolved. In 2004 the lineage designation was finally abandoned in favour of distinct species within a complex. The move towards multiple species within a complex not only facilitates various role players such as plant pathologist, mycotoxicologists and quarantine specialist, in reporting the movement of various trichothecene chemotypes. It also assisted plant breeders to incorporate multiple species during their programmes to ensure that more robust resistance is achieved (O'Donnell *et al.*, 2004). Epithets were given to each of the eight previous lineages, with a ninth not given a lineage number (O'Donnell *et al*., 2004). The first eight species were 1] *F. austroamericanum*, [2] *F. meridionale*, [3] *F. boothii*, [4] *F. mesoamericanum*, [5] *F. acaciae-mearnsii*, [6] *F. asiaticum*, [7] *F. graminearum*, and [8] *F. cortaderiae* and *F. brasilicum*. In 2007 Starkey *et al*. described two new species, namely *F. vorosii* and *F. gerlachii*. Although the type specimen of *F. vorosii* was isolated in Hungary (FgHF012), it is more closely related to *F. asiaticum* than *F. graminearum*. Also, many of the early specimens of this species were isolated in Japan. Ward *et al.* (2008) developed a multilocus genotyping (MLGT) assay that enables rapid, simultaneous species identification. From this point forward, this technique was used for the identification of new species. A twelfth species, *F. aethiopicum* was described in 2008 and was isolated from wheat in Ethiopia (O'Donnell *et al.* 2008). *F. ussurianum* was described for the first time in 2009, when it was isolated from an oat seed in Russia (Yli-Mattila *et al*., 2009). In 2011, two new species were described, *F. nepalense* and *F. louisianense*. The type specimen of *F. nepalense* was isolated from rice seed in 1997 in Nepal, whereas the type specimen for *F. louisianense* was isolated in Louisiana in 2007 from wheat seed (Sarver *et al.,* 2011).

Fusarium sp. NRRL 34461 was isolated from South African soil and although it was first thought to be part of the diverse species *F. acaciae-mearnsii* (Starkey *et al.*, 2007), it has since been described as an unresolved independent species (Sarver *et al.,* 2011).

The four species within the complex that have been found to be pathogenic to maize in South Africa are *F. graminearum* s.s., *F. meridionale*, *F. boothii*, and *F. acacia-mearnsii*. These species have been found to differ in virulence with *F. boothii* the most virulent member on maize grain and *F. graminearum* s.s. the most virulent on wheat grain. This is based on the species ability and rate of colonisation and the ability to deposit mycotoxins (Lamprecht *et al.*, 2011; Beukes, 2015). The FGSC species are strong saprophytes and survive on debris of a vast number of plant species, but especially on the stalks of cereals and ears of maize (Sutton, 1982).

The morphology of FGSC is as follows: they produce macroconidia that are relatively slender, thick walled and can be straight to slightly curved. The macroconidia can be 5 to 6 septate with distinct septa. Unlike other *Fusarium* spp., the presence of macroconidia is rare. No microconidia are present. Chlamydospores may form, but this happens over an extended period. The absence of chlamydospores is not a diagnostic character (Leslie and Summerell, 2006).

1.4.2 The *Fusarium oxysporum* species complex (FOSC)

Kistler (1997) best described *F. oxysporum* as a large taxonomic unit. In "Die Fusarien" Wollenweber and Reinking, placed *F. oxysporum* into the section *Elegans* together with 9 other species (Wollenweber and Reinking 1935; Kistler, 1997). Section *Elegans* was segregated into three sub-sections based upon whether or not the conidia are borne on sporodochia and upon the width of the microconidia (Wollenweber and Reinking 1935; Kistler, 1997). Snyder and Hanson (1940) decided to combine all these species into one species under the name *F. oxysporum* because the differences in morphological characters that separated the species within *Elegans* were small and dependant on environmental factors (Snyder and Hanson, 1940; Kistler, 1997).

Table 1.1: The 16 members of the FGSC with their lineage number, geographic distribution and potential chemotypes (O'Donnell *et al.*, 2000; Aoki *et al.*, 2012; Lamprecht *et al.*, 2011; O'Donnell *et al.*, 2008; Starkey *et al.*, 2007).

Species	Distribution	Known hosts	Chemotype
F. austroamericanum	South America	Herbaceous vine and maize	NIV, 3ADON, ZEA
F. meridionale	Asia, Australia, South America and South Africa	Orange twig, barley stalks, maize, wheat and soil	NIV and ZEA
F. boothii	North America, South Africa, South America, Central America	Maize	15ADON, ZEA
F. mesoamericanum	Central America, North America	Banana and grape ivy	NIV, 3ADON and ZEA
F. acacia-mearnsii	Australia and South Africa	Black wattle and soil	NIV and 3ADON
F. asiaticum	Asia, North America, South America	Barley, maize, oat, rice and wheat	NIV, 3ADON, 15ADON and ZEA
F. graminearum s.s.	Globally	Fern, leather leaf, maize, millet, various cereals and wheat	NIV, 3ADON, 15ADON and ZEA
F. cortaderiae	Oceania, South America	Pampas grass, maize carnation, barley, wheat and soil	NIV and 3ADON
F. brasilicum	North America and South America	Barley and oat	NIV and 3ADON
F. aethiopicum	Ethiopia	Wheat	15ADON
F. gerlachii	North America	Wheat and giant cane	NIV
F. vorosii	Hungary and Asia	Wheat	3ADON and 15ADON
F. ussurianum	Russia and Asia	Wheat and oat	3ADON
F. louisianense	North America	Wheat	NIV
F. nepalense	Asia	Rice	15ADON
Fusarium sp. NRRL 34461	South Africa	Soil	-

This naming process did not, however take host specificity into consideration and thus, the use of specialised forms (*formae specialis*) for strains with different host preferences came into practice (Armstrong and Armstrong, 1981; Kistler 1997). In 1985 Puhalla described an approach and procedure known as the vegetative-compatibility grouping (VCG) method to further characterise *formae specialis.* This method classified strains of *F. oxyporum* based on the isolates ability to anastomose and form heterkaryons (Puhalla, 1985; O'Donnell *et al.* 2009). Thus, the VCG is based on genetic differences rather than morphological or host-range differences. The VCG method was an important advancement in *F. oxysporum* taxonomy, as it showed that strains related by clonal decent should fall in the same group. However, *F. oxysporum's* parasexuality, which is the ability of distinct, vegetative incompatible lineages to exchange genetic information such as pathogenicity, makes it difficult to distinguish between VCG. (Baayen et al. 2000). Nevertheless, an attempt was made to standardise the VCG's and each group was given a four- or five-digit number (Kistler, 1997; O'Donnell *et al.*, 2009). Some *formae specialis* may have multiple VCG's whereas others have only one (Baayen *et al.,* 2000). VCG may then be further subdivided into races. The term race can be used to describe the isolates preference to a specific cultivar, but it can also be used to describe an isolates preference to a specific host species (Stall and Walter, 1965; Armstrong and Armstrong, 1981; Kistler, 1997). Another train of thought separates races based on isolates specificity to certain genotypes (Ramirez-Villupadua *et al*., 1985; Kistler, 1997). Because of this inconsistent use of the term race, its use should be limited (Kistler, 1997). The term *Fusarium oxysporum* species complex is now used as blanket term to incorporate all the species, lineages, VCG's and races (Leslie and Summerell, 2006; Leslie and Bowden, 2008).

The FOSC consists of isolates that may be pathogenic or non-pathogenic. Pathogenic strains, the better defined group of the two, cannot be separated from non-pathogenic strains based on morphology or phylogeny (Sutherland *et al.*, 2013). They are only separated from the non-pathogenic group based on their ability to cause disease. Although no *formae specialis* or VCG has been described for FOSC species of maize, it has been found to cause both Fusarium root (Munkvold and Leslie, 1999) and seedling rot (Selwet, 2011) on maize plants. Non-pathogenic FOSC may be endophytic in the cortex of plant roots or active saprophytes that are found abundantly in soil and degrading plant tissue (Munkvold and Leslie, 1999). A pathogenic strain may broaden its host range or change non-pathogenic strains to pathogenic strains through horizontal gene transfer of small supernumerary chromosomes (Ma *et al.*, 2010).

The FOSC may either produce abundant or sparse aerial mycelium with white, pink, salmon or purple pigmentation on the bottom of the colony. The complex mainly reproduces asexually through microconidia, macroconidia and chlamydospores. The microconidia are abundant, one- or two-celled, and can be oval, elliptical or kidney shaped. Macroconidia can be short to medium in size and is usually straight or has a slight curve with foot shaped basal attenuated apical cells. The macroconidia are usually thin walled and can have 4-8 septa. They can be sparse in some strains but can be abundant in sporodochia. Areal mycelia may be present as false heads and the conidiogenous cells are short monophialides. Chlamydospores form quickly and are abundant (Leslie and Summerell, 2006).

1.4.3 *Fusarium verticillioides*

F. verticillioides was first discovered in 1904 in Nebraska (USA). Wollenweber and Reinking (1935) established the section *Liseola* based on the morphology of *F. moniliforme* Sheldon, *F. lactis* Pirotta and Riboni and *F. neoceras* Wollenw. and Reinking (Kvas *et al.*, 2009). *Liseola* consists of species that do not form clamydospores (O'Donnell *et al.*, 1998). Snyder and Hansen (1945) combined the three species under the name *F. moniliforme* as they felt that the characters Wollenweber and Reinking used were too unstable to separate the species (Snyder and Hansen, 1945; Kvas *et al.*, 2009). Booth (1971) then separated *F. subglutinans* from *F. moniliforme* based on the morphology of conidiogenous cells (Booth, 1971; Kvas *et al.*, 2009). Nelson *et al*. (1983) used Booth's model of two species and further divided *Liseola* into *F. anthophilum* (A. Braun) Wollenw and *F. proliferatum* (Nelson *et al.*, 1983; Kvas *et al.*, 2009). After this split molecular, morphological and biological traits were used on a variety of *Fusarium* spp. and thus, the *Gibberella fujikuroi* complex was established, which was later changed to the *Fusarium fujikuroi* species complex (FFSC) (Seifert *et al.*, 2003; Kvas *et al.*, 2009). Although there are more than 50 species in the FFSC, the focus of this study was on *F. verticillioides* and thus the other members of this complex will not be discussed. *F. verticillioides* was separated from *F. moniliforme* because it is heterothallic. *F. verticillioides* was chosen as the species name because *F. moniliforme* is a broad species concept, which cover multiple species and *F. verticillioides* is the older name (Seifert *et al.*, 2003; Guo *et al.*, 2015). Before 2013, *F. verticillioides* was also known as *Gibberella moniliforme* Wineland which corresponds with the mating population A of *Gibberella fujikuroi* (Meyer and Jensen, 1998). However, since the "One fungus, one name" policy, only *F. verticillioides* is used (Geiser *et al.*, 2013).

F. verticillioides may be endophytic in which case, the fungus may grow asymptomatically. Sometimes *F. verticillioides* may reduce the severity of other diseases such as corn smut caused by *Ustilago maydis* (DC.) Corda (Lee *et al.*, 2009). *F. verticillioides* may also grow saprophytically on cereal residue as both normal and thickened hyphae. This growth is dependent on temperature and co-habitation with certain bacteria (Manzo and Claflin, 1984).

The morphology of *F. verticillioides* is as follows: they have relatively long and slender macroconidia that are slightly falcate or straight. They have thin walls with 3 to 5 septa and may be abundant, however this is strain dependent. The microconidia are oval to club shape and abundant. The conidiogenous cells are monophialidic. No chlamydospores are produced (Leslie and Summerell, 2006). *F. verticillioides* are morphological indistinguishable from *F. thapsinum,* if the latter does not produce its diagnostic yellow pigment. *F. proliferatum* and *F. verticillioides* only differ in that the first forms chains of microconidia from polyphialides. *F. verticillioides* is very similar to *F. andiyazi* except that the latter produces pseudochlamydospores. *F. nygamai* L.W. Burgess and Trimboli is somewhat similar to *F. verticillioides* with the latter producing microconidia in short chains or false heads from monophialides (Leslie and Summerell, 2006).

1.5 Epidemiology of *Fusarium* spp.

1.5.1 Life cycle of *Fusarium* spp.

Fusarium spp. that survive saprophytically on previous season's residues may produce sexual (ascospores) and asexual (macro- and microconidia) spores which act as primary dispersal units (Trail, 2009). The spores may become airborne by means of environmental factors such as rain splash and wind or by insect vectors (Oren *et al.*, 2003). Insect vectors only aid the dispersal of certain *Fusarium* spp.. FGSC has no notable insect vectors whereas *F. verticillioides* have a variety of insect vectors that assist its dispersal (Trail, 2009). In South Africa the most notable *F. verticillioides* insect vectors include the spotted stalk borer (*Chilo partellus* (Swinhoe)) and the maize stalk borer *Busseola fusca* (Fuller) (Kfir, 1997). It was shown that shore flies (Diptera: *Ephydridae*) and sciarid flies (Diptera: *Sciaridae*) are vectors of *F. oxysporum* f.sp *cucumerinum* (Scarlett *et al.*, 2014). FOSC may be dispersed between fields as airborne pathogens but are more commonly soilborne pathogens (Perez-Nadales *et al.*, 2014). Air- or soilborne spores germinate once in contact with their host plant. The hyphae may either find

natural openings to penetrate the plant or may produce appressoria which penetrate the epidermis and cuticle through which the hyphae can enter the plant (Trail, 2009; Perez-Nadales *et al.*, 2014). The spores may also land on the silks of ears and grow down towards the grain (Oren *et al.*, 2003). Once within the plant tissue it spreads inter- and intracellularly through the epidermis into the xylem and pith as a biotrophic pathogen, later becoming a necrotrophic pathogen. The symptoms that present themselves are dependent on the tissue that is infected and will be discussed below (Trail, 2009; Perez-Nadales *et al.*, 2014).

1.5.2 Root rots

Root rots are caused by a combination of organisms including fungi, bacteria, nematodes and insects (Munkvold and Leslie, 1999). Although they occur in maize plants in every field and season, they do not commonly cause economic losses, unless the soil moisture is conducive to increased disease severity (Munkvold and Leslie, 1999). However, once disease sets in, 1.8 ton/ha maize can be lost for every 25% disease severity increase (Lamprecht *et al.*, 2006). The combination of organisms associated with root rot is dependent on the host, genotype, environmental conditions and crop residues (Munkvold and Leslie, 1999). Symptoms of root rot may include roots that are pink, slightly brown to black and can be limited to a small part of a single root to the rotting of the entire root system (Figure 1.1) (Munkvold and Leslie, 1999). Badly infected roots may also be hollow because of the breakdown of cortical tissue. Root rot symptoms have been found on roots as deep as 90 cm below ground (Sumner and Hook, 1985). It is difficult to predict the symptoms of root rots due to the complexity of the disease and pathogens (Liu *et al.*, 2012). It is also difficult to determine whether *Fusarium* is the primary pathogen or a secondary saprophyte. FOSC and *F. solani* are most often associated with *Fusarium* root rot, however *F. verticillioides* has also been isolated (Munkvold and Leslie, 1999). *F. graminearum* s.s., *F. meridionale* and *F. boothii* have also been isolated from maize roots locally (Lamprecht *et al.*, 2011; Boutigny *et al.*, 2012). Root rots are more prevalent when the soil moisture is too high or too low, especially when the plants are under additional stress such as nutrient deficiencies (Munkvold, 2003). An increase in disease severity was observed for FOSC infected roots at temperatures >29 °C, especially when the roots were wounded (Warren and Kommedahl, 1973). Water uptake becomes problematic for plants with advanced root rot. This often leads to premature plant senescence. It is important to fully understand root rot pathogens' epidemiology, because they are often associated with crown and stalk rots (Dodd, 1980).

Figure 1.1: Root and crown rots caused by *Fusarium* spp. may discolour the tissue pink (Photo: S.-M. Joubert).

1.5.3 Stalk rots

Stalk rot, like root rot, is caused by a variety of organisms with Gibberella stalk rot, caused by the FGSC and Fusarium stalk rot caused by *F. verticillioides, F. proliferatum* and *F. subglutinans* being the most prevalent (White, 1999; Khokhar *et al.*, 2014). FOSC has been shown to be a stalk rot pathogen of cucumber (Cerkauskas, 2001), however no reference could be found associating FOSC with stalk rot of maize. Stalk rot is present every year, in every field to varying degrees and usually occurs 55 - 65 days after sowing (White, 1999; Khokhar *et al.*, 2014). Global stalk rot yield losses usually range from 5 to 40 %, however in some cases 100 % yield loss has been recorded (Khokhar *et al.*, 2014). Yield losses from stalk rots can be attributed to lodging that hinders harvesting, as well as lightweight ears that are often missed by harvesters (Figure 1.2a) (White, 1999; Singh *et al.*, 2012). Stalk rots seem to be more prevalent later during the season, especially when the stalks have been subjected to mechanical damage, when the maize planting densities are high, when foliar diseases are present or in the presence of insect infestation (Sumner and Hook, 1985; Ahmad *et al.*, 1997). Symptoms of stalk rot include permanent leaf wilting, grey/drooping ears and green-yellow to yellow-brown stalks that are soft when pressed. The results of stalk rot include stalk breakage, stalk lodging and premature plant death (Jackson *et al.*, 2009). Gibberella stalk rot produces a pink to red discoloration with black perithecia on the stalk surface (Figure 1.2b). Fusarium stalk rot

presents as white to pink discoloration within the pith with no sexual reproductive organs present on the stalks (Liu *et al.*, 2012).

Figure 1.2: Stalk rots often results in (a) lodged plants. Gibberella and Fusarium stalk rot present as (b) pink discolouration on the stalk with degraded stalk tissue. (Photo: S-M. Joubert).

1.5.4 Ear rots

Ear rot was first described by Bisby and Bailey (1923) in Canada (Mesterházy *et al.*, 2012). Ear rot is primarily caused *Fusarium* spp. including Gibberella ear rot caused primarily by the FGSC and Fusarium ear rot caused by *F. verticillioides, F. proliferatum* and *F. subglutinans* (Payne, 1999). No publications could be found to indicate that the FOSC may play a role as ear rot pathogens. Gibberella ear rot, caused by the FGSC, starts as white mycelial growth at the tip of the ear and spreads towards the base. The mycelia of older infections turn a distinctive red colour (Figure 1.3a). If the infection occurs early in the season, the red/pink mycelia cover the whole ear which causes the maize husks to adhere to the ears. Perithecia that are blue/black in colour may form on the husks (Payne, 1999). Perithecia contain spores which then act as inoculum for future infestations (Jackson *et al.*, 2009). In order for the FGSC to infect the maize ears they need to be <8 to 10 days old (Reid *et al.*, 1999). The optimum temperature for Gibberella ear rot is between 26 and 28 °C, followed by persistent precipitation (Reid *et al.*, 1999). Infections are worse where maize is grown in monoculture or when maize is rotated with wheat (Woloshuk and Shim, 2013).

F. verticillioides colonises the ears more readily when the silks have senesced. Fusarium ear rot, caused by *F. verticillioides,* starts as individual or clustered infected kernels that are

distributed randomly on the ear (Figure 1.3b). The mycelia can be white, light pink or lavender in colour or infections may show no visible symptoms. Fusarium ear rot occurs in hot dry weather during or just after flowering (Payne, 1999).

Ear rot caused infection by *Fusarium* spp. not only results in economic losses but also poses a health risk to consumers because of potential mycotoxin contamination (Payne, 1999). Understanding infection patterns associated with *Fusarium* spp. occurring on maize ears will help to implement better management strategies. These management strategies include the breeding of resistant cultivars and adapting current farming practices by implementing crop rotation systems and limiting over- or under irrigation. Through this understanding, fungal infection may be reduced or prevented (Czembor *et al.*, 2010).

a b

Figure 1.3: a) Gibberella ear rot starts at the tip of the maize ear and grows to the opposite end whereas b) Fusarium ear rot is often associated with insects and is randomly infected kernels (Photos: S.-M. Joubert).

1.5.5 Control measures for fungal infection

Currently there are no resistant cultivars or chemicals that can effectively control infection of maize by *Fusarium* spp. (Czembor *et al.*, 2010; Khokhar *et al.*, 2014). Thus, a multifaceted approach, which includes progressive farming practices together with tolerant cultivars, is required to reduce disease incidence and severity (Kulkarni and Anahosur, 2011). Reducing plants stress levels, such as nutrient deficiencies, high planting densities and water stress reduce the chance and severity of infections (Khokhar *et al.*, 2014). Crop rotation is important as the planting of cereals in succession results in higher disease incidence. Rotation with a non-host crop such as canola, alfalfa and peas reduces inoculum build-up (Czembor *et al.*, 2010). This

is especially important in fields in which no or limited tillage practices are applied (Khokhar *et al.*, 2014). Removal of the previous season's crop residues reduces the available plant material that the pathogen can use to over winter. Less material means less inoculum available for subsequent infections (Czembor *et al.*, 2010).

1.6 Mycotoxins

It is estimated that 25 % of world crops are affected by unacceptably high levels of mycotoxin contamination (Iheshiulor *et al.*, 2011). Mycotoxins are low molecular weight, secondary metabolites that are produced by the mycelia of certain fungi (Bennett, 1987; Brera *et al.*, 2008). These secondary metabolites are toxic to humans and animals even at low concentrations (Bennett, 1987; Hussein and Brasel, 2001). There are two classes of mycotoxins, the major and minor classes. The major class include the mycotoxins that pose the biggest threat to human and animal health. These are aflotoxins, ochratoxins, trichothecenes, fumonisins (FUM), patulin and zearalenone (ZEA). The minor classes include ergot alkaloids, citrinin, cyclopiazonic acid, sterigmatocystin, moniliformin, gliotoxin, citreoviridin, tremorgenic mycotoxins, penicillic acid, roquefortine, 3-nitropropionic acid and fusaproliferin (Brera *et al.*, 2008). Although more than 300 mycotoxins are currently known, only 10 are the focus of most studies because of their adverse effect on humans. Some fungi are able to produce more than one mycotoxin and one type of mycotoxin may be produced by different genera and species (Brera *et al.*, 2008).

Environmental factors such as temperature, humidity, climate change and water activity may all influence mycotoxin production (Brera *et al.*, 2008). Similarly, certain plant physiological traits may influence mycotoxin production. Mycotoxin contamination is more likely to occur when kernels are mechanically damaged or damaged by pest attack, as *Fusarium* infection is more likely to occur (Brera *et al.*, 2008). pH also plays an important role as a lower pH induces mycotoxin production whereas a higher pH suppresses it (Woloshuk and Shim, 2013). It has been shown that the addition of reactive oxygen species such as hydrogen peroxide and diamide, triggers the production of mycotoxins whereas antioxidants reduce mycotoxin levels (Reverberi *et al.*, 2010). Mycotoxin production usually take places before harvest but may occur post-harvest and during storage (Zinedine *et al.*, 2007).

Mycotoxin contamination causes a variety of downstream effects. When humans and animals consume food contaminated with mycotoxins it may lead to an increase in health care needs and costs. Food and feed contamination require additional costs involved in sorting, handling and disposal of these products. These financial losses are additional to the loss of product that is not marketable (Hussein and Brasel, 2001). Secondary carry-over of mycotoxins can occur when animals eat contaminated feed. Mycotoxins may then appear in milk, eggs and to a lesser extent in meat which in turn are consumed by humans (Brera *et al.*, 2008). Most mycotoxins are extremely stable and can withstand temperatures of up to 180 ºC which means that they can also withstand cooking and a variety of industrial processes (Brera *et al.*, 2008).

1.6.1 Trichothecenes

Trichothecenes consist of 170 related compounds which makes this the largest class of mycotoxins. A commonality between all the members is their sesquiterpenoid 12,13-epoxytrichothec-9-ene ring system (Binder, 2007). Their C12 epoxide functionality is essential for their toxicity (Foroud and Eudes, 2009). Trichothecenes are non-volatile compounds that also act as potent protein and DNA synthesis inhibitors. Protein synthesis is inhibited by trichothecenes interacting with ribosomal peptidyltransferase sites of eukaryotic ribosomes. It affects all the major organs in humans and animals, especially the digestive tract (Harris *et al.*, 1999; Trail, 2009). It has been shown that, unlike other mycotoxins, trichothecenes are not carcinogenic (Hussein and Brasel, 2001).

Tricothecenes have arbitrarily been divided into four types namely A, B, C and D. Type A tricothecenes, such as T-2 and HT-2 mycotoxins are produced by *F. oxysporum* and *F. sporotrichiodies* and *F. poae*. Type B trichothecenes, such as DON and NIV are produced by FGSC and *F. colmorum*. Both type C and D are not produced by *Fusarium* spp. (Foroud and Eudes, 2009).

Most research on trichothecene toxicity has been focused on animals. Toxicity in animals can present as weight loss, decreased feed conversion, feed refusal, vomiting, bloody diarrhoea, severe dermatitis, haemorrhaging, decreased egg production, abortion, and death (Bennett and Klich, 2003; Yazar and Omurtag, 2008). Less is known about the influence of trichothecenes on human health, however it has been shown to cause inhibition of protein synthesis and immunomodulatory effects (Sudakin, 2003).

1.6.1.1 Type B trichothecenes

Type B trichothecenes are one of the mycotoxin groups produced by FGSC. The primary mycotoxins within this type are DON and its acetylated derivatives, 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON), and NIV and its acetylated derivatives, 4-acetylnivalenol or Fusarenone X (Figure 1.4) (Boutigny *et al.*, 2011). It has been shown that 3-ADON is more phytotoxic and has a higher pathogenic potential than 15-ADON. DON levels are regulated throughout the world with a maximum tolerable level (MTL) or tolerable daily intake (TDI) of 1 $\mu g.g^{-1}$ body weight (bw) DON in the EU and in the USA (Trail, 2009, Anonymous, 2016). In South Africa, new legislation was instated during 2016 that set the MTL of DON in cereal grains intended for further processing at 2 $\mu g.g^{-1}$ bw and for processed maize at 1 $\mu g.g^{-1}$ (Anonymous, 2016). Nivalenol has a MTL of 2 $\mu g.g^{-1}$ bw in the EU (Anonymous, 2016)

Mycotoxin	R1		R2	R3	R4
DON	–OH	–H	–OH	–OH	
3–ADON	–OAc	–H	–OH	–OH	
15–ADON	–OH	–H	–OAc	–OH	
NIV	–OH	–OH	–OH	–OH	

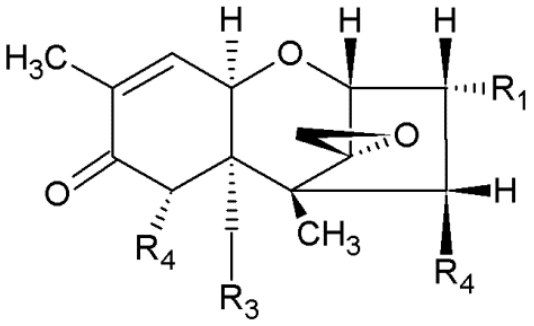

Figure 1.4: A basic type B tricothecene structure indicating the differences within the type (Foroud and Eudes, 2009).

1.6.2 Zearalenone

ZEA is produced by species of both the FGSC and FOSC (Beev *et al.*, 2013). It is structurally very similar to oestrogens such as 17 β-oestradiol (Binder, 2007). Chemically it is known as 6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid lactone (Figure 1.5) (Zinedine *et al.*, 2007). The structural similarity enables these mycotoxins to bind with oestrogen receptors. After animal ingestion, the C-8 keto group is reduced (Zinedine *et al.*, 2007). This results in the production of the derivates, α- and β-zearalenol (ZOL) and α- and β-zearalanol (ZAL) (Figure 1.5). However, Bottalico *et al.* (1985) found that these derivatives are also present in maize stalks during infection by *Fusarium* spp. in lower concentrations than ZEA (Zinedine *et al.*, 2007; Minervini and Dell'Aquila, 2008). ZEA and its derivatives bind with different affinities, with α-ZAL>α-ZOL>β-ZAL>ZEA>β-ZOL (Minervini and Dell'Aquila, 2008). Oral uptake of ZEA accounts for 80 – 85 % of all ZEA contamination methods. After ingestion the mycotoxin is quickly absorbed into the blood stream. The metabolites of ZEA can be detected as little as 30 minutes after ingestion (Minervini and Dell'Aquila, 2008). Resulting symptoms are an enlarged uterus, swelling of the vulva and vagina, enlarged mammary glands, anoestrus (periods of infertility) and abortion (Minervini and Dell'Aquila, 2008). Contamination usually ranges from 0.004 – 8 $\mu g.g^{-1}$ (Minervini and Dell'Aquila, 2008). The European standard states that the acceptable levels of ZEA contamination should be <2 $\mu g.g^{-1}$ bw (Queiroz *et al.*, 2012). There are no such regulations for ZEA in South Africa but it is suggested that it should be <0.02 – 0.03 $\mu g.g^{-1}$ bw (Burger *et al.*, 2014). ZEA is produced in higher concentrations when *Fusarium* spp. infects the plants at temperatures <25 °C and at 16 % humidity (Milani, 2013).

1.6.3 Fumonisins

FUM are a diester of propane-1,2,3-tricarboxylic acid and either 2-acetylamino- or 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane, in both cases the C_{14} and C_{15} hydroxy groups are esterified with the terminal carboxy groups propane-1,2,3-tricarboxylic acid (Bezuidenhout *et al.*, 1988). The most notable producers of FUM are *F. verticillioides, F. proliferatum*, *F. anthophilum, F. nygamai*, as well as *Alternia alternata* f. sp. *lycopersici* (Fr.) Keissl.. The twenty-eight known FUM are divided into series namely A, B, C and P.

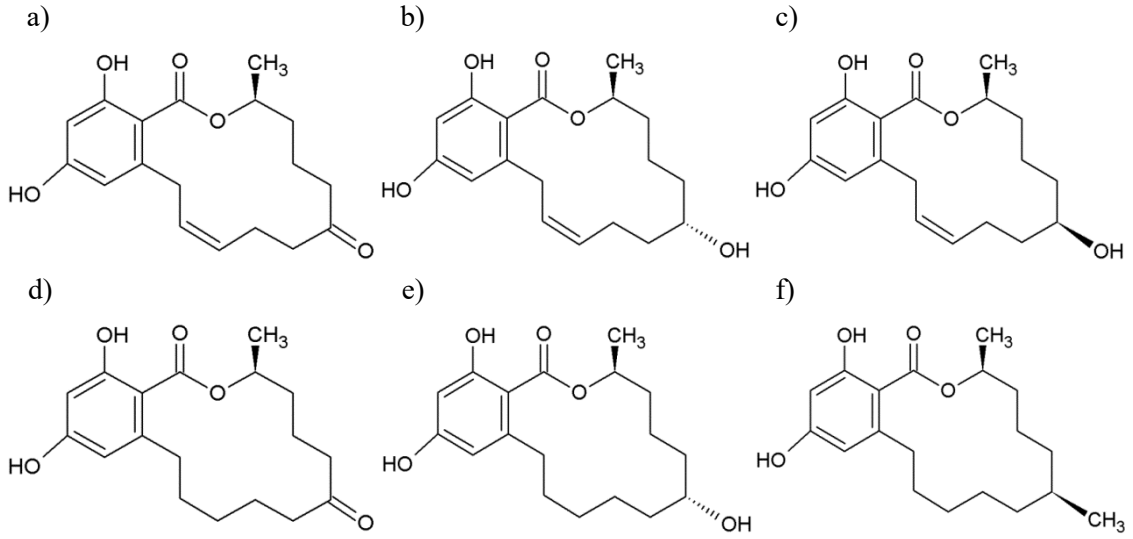

Figure 1.5: Basic chemical structure of a) ZEA and its derivatives (b-f). b) α-ZOL, c) β-ZOL, d) zearalanone (ZAN), e) α-ZAL, f) β-ZAL (Minervini and Dell'Aquila, 2008).

The three analogues of FUM most commonly recorded are FB_1, FB_2 and FB_3 (Figure 1.6) (Colhoun, 1973; Lanubile *et al.*, 2013). *F. verticillioides* mainly produces FB_1 (Mudge *et al.*, 2006). FUM are often found in high quantities coinciding with *F. verticillioides* isolation in grain. It is thus suggested, that FUM may be involved in the pathogen's ability to infect the host plant as it cannot form appressoria or produce cell wall degrading enzymes (Munkvold and Desjardins, 1997). They also play a role in cell division by changing cell regulators (Gelderblom *et al.*, 1991).

FUM are responsible for various diseases in humans and animals. In humans, FUM causes both liver and oesophageal cancer. Horses are very sensitive to FUM. Ingesting FUM may result in the disease equine leucoencephalomalacia (Munkvold and Desjardins, 1997). FUM ingestion may also lead to pulmonary edema and hydrothorax in swine, cardiac failure in baboons, atherogenic effects in vervet monkeys, brain haemorrhaging in rabbits, renal cancer and hepatocarcinogenicity in rats and some birth defects in animals and humans (especially neural tube defects) (Yazar and Omurtag, 2008). Strict guidelines have been set to minimise the risk of FUM, because of the high health risk they pose. The maximum allowable concentration of FUM in milled maize products in European countries is 2 $\mu g.g^{-1}$ bw (Bennett and Klich, 2003). The MTL set for FUM in South Africa is 4 $\mu g.g^{-1}$ bw for raw maize material and 2 $\mu g.g^{-1}$ bw for processed maize products (Anonymous, 2016).

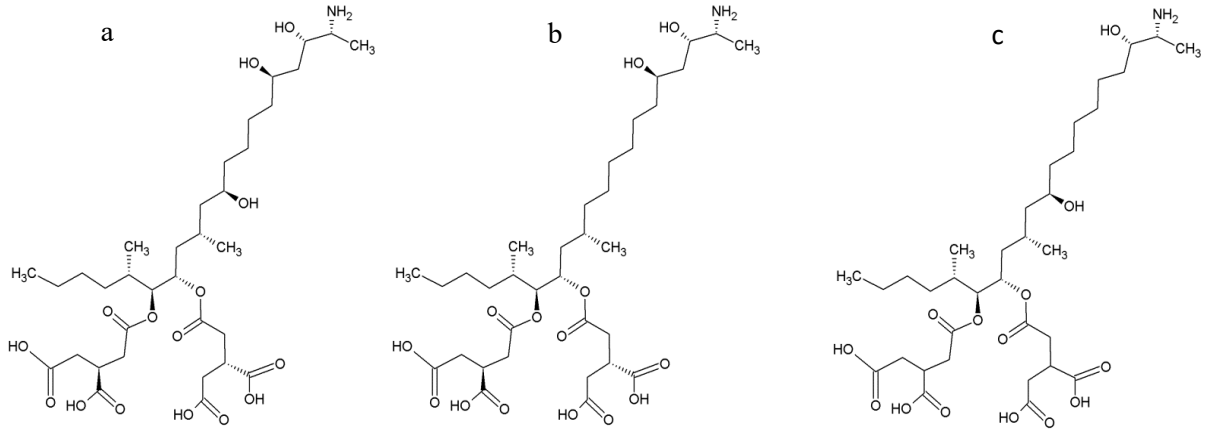

Figure 1.6: The chemical structure of a) FB_1, b) FB_2 and c) FB_3 (Anonymous 2, 2016).

1.6.4 Probable daily intake of mycotoxins by the South African population

In South Africa high levels of mycotoxins consumption have been identified in all the provinces. It was shown that when super maize milling fraction was consumed, there were no difference in mycotoxin consumption between the different provinces (Burger *et al.*, 2014). The concentration of mycotoxins was also far less in the super maize milling fraction compared to the special maize milling fraction (Burger *et al.*, 2014). The probable daily intake (PDI) of FB_1 in the special maize fraction was the highest in the Northern Cape (87.3 $ng.kg^{-1}$ $bw.day^{-1}$) and Mpumalanga (86.0 $ng.kg^{-1}$ $bw.day^{-1}$) provinces, with the North West province (64.3 $ng.kg^{-1}$ $bw.day^{-1}$) and the Western Cape (47.0 $ng.kg^{-1}$ $bw.day^{-1}$) having the lowest PDI (Burger *et al.*, 2014). DON PDI in the special maize fraction was the lowest in the North West province (24.3 $ng.kg^{-1}$ $bw.day^{-1}$) and the Western Cape (18.0 $ng.kg^{-1}$ $bw.day^{-1}$), with the other provinces having similar intake levels (Burger *et al.*, 2014). ZEA followed a similar pattern to FB_1 with the highest PDI in the special maize fraction, in the Northern Cape (8.0 $ng.kg^{-1}$ $bw.day^{-1}$) and Mpumalanga (8.0 $ng.kg^{-1}$ $bw.day^{-1}$) provinces, and the lowest PDI in the North West province (6.0 $ng.kg^{-1}$ $bw.day^{-1}$) and the Western Cape (4.3 $ng.kg^{-1}$ $bw.day^{-1}$) (Burger *et al.*, 2014). South Africans consume maize in large quantities, especially people living in rural areas, and are therefore more likely to consume high doses of multiple mycotoxins (Pray *et al.*, 2013).

1.6.5 Control measures for mycotoxin contamination

Mycotoxin contamination can be controlled pre- and post-harvest. Pre-harvest includes limiting fungal infection as mentioned in section 1.5.5 (Kulkarni and Anahosur, 2011; Khokhar *et al.*, 2014). During post-harvest prevention, the regulation of moisture content is critical as high moisture in grain is conducive to fungal infection (Choudhary and Kumari, 2010). The grain should also be stored at low temperatures. Fungicides, insecticides and preservatives may prolong the longevity of the grain (Czembor *et al.*, 2010; Choudhary and Kumari, 2010). Secondary prevention can also be implemented which includes re-drying of products, removal of contaminated grain and inactivation of mycotoxins (Bozoğlu, 2009).

1.7 Mycotoxin translocation

The photosynthetic stress-translocation balance states that a plant under stress will translocate carbohydrates from lower regions of the plant i.e. the roots and stalks, to the fruiting body of the plant (Dodd, 1980). The plant is unable to compensate for this loss through photosynthesis, resulting in carbohydrate deficiency and ultimately, cellular senescence. Certain defence systems are dependent on carbohydrates and cannot function without a constant supply. This creates the perfect environment for toxigenic fungal growth and the mycotoxins produced may be translocated with the carbohydrates through the phloem to the fruiting body (Dodd, 1980).

A recent study showed that *F. culmorum*, a causal agent of crown rot, was able to infect wheat from roots to the third internode (Covarelli *et al.*, 2012). DON however was found in the upper nodes as well as the kernels (Covarelli *et al.*, 2011; Covarelli *et al.*, 2012). Winter *et al.* (2013) found that in wheat the concentration of DON reached 10 $\mu g.g^{-1}$ dry weight (DW) in the lower stalk and after translocation 1.9 $\mu g.g^{-1}$ DW in the ear and husks. However, no DON or its derivative DON-3-glucoside was found in the mature grain. They concluded that translocation is interrupted between the rachilla and grain. Sutton *et al.* (1976) showed that ZEA can be translocated from the stalks of maize plants to the ears but not from the ears to the stalks (Sutton *et al.*, 1976). Most *Fusarium* spp./maize studies have been focused on the grain, and rightly so because grain is the main product. However, the possibility of translocation of mycotoxins from roots and stalks to maize grain highlights the importance of understanding and studying root and stalk rots in greater detail.

1.8 Plant-Pathogen interactions

Plants are sessile organisms and have developed an array of chemical and physical defence mechanisms to protect themselves against pathogen attack. When a pathogen is capable of infection and the environmental conditions are optimum, the host may either be compatible in which case disease will occur or incompatible in which case little or no disease will be present. An incompatible reaction can occur because a) the plant acts as a non-host and does not support pathogen infection b) the plant has constitutively expressed defence mechanisms which include physical and chemical barriers also known as non-host resistance or c) the plant has induced resistance that actively defends the plant against infection also known as host resistance (Hammond-Kosack and Jones, 1996).

1.8.1 Systemic acquired resistance and induced systemic resistance

Inducible defence mechanisms such as systemic acquired resistance (SAR) and induced systemic resistance (ISR) are the plants final defence strategy. These mechanisms are induced in response to effectors/elicitors, which can be defined as molecules that induce physiological and biochemical responses associated with resistance (Thomma *et al.*, 2011). SAR and ISR work on the premise that the plant has been preconditioned by prior infection or treatment that result in resistance against subsequent challenges by a pathogen (Choudhary *et al.*, 2007). Both mechanisms offer the plant protection against a wide range of organisms including bacteria, fungi and viruses (Dong, 2004; Ferreira *et al.*, 2006). SAR may be induced locally by salicylic acid and oxidative stress which are usually released in the presence of necrotic lesions (Dong, 2004; Balint-Kurti and Johal, 2009). Methyl salicylate has been suggested to be a mobile signal that induces SAR systematically. This is achieved by the compound being produced at the site of infection, transported through the phloem and turned into salicylic acid where it is needed. Nonexpressor of PR genes 1 (NPR1) has been shown to be a key regulator of SAR (Balint-Kurti and Johal, 2009). ISR are induced by the presence of plant growth-promoting rhizobacteria (Choudhary *et al.*, 2007). Unlike SAR, ISR is induced by jasmonate and ethylene and does not end in the accumulation of PR proteins (Balint-Kurti and Johal, 2009).

1.8.2 Hypersensitive response

The hypersensitive response, unlike SAR and ISR are induced (in some cases) twice during infection. The first time is in reaction to pathogen recognition, during which an oxidative burst takes place. An oxidative burst is the release of reactive oxygen species (ROS) such as superoxide anions (O_2^-), perhydroxyl radicals (HO_2), hydroxyl (OH) radicals, and peroxide (H_2O_2) (Prell and Day, 2000). This causes cytological changes to occur that includes the movement of the nucleus and cytoplasm towards the site of pathogen penetration. The cells become more permeable which allows the exchange of potassium ions out of the cells and hydrogen ions into the cells (Prell and Day, 2000). The second stage occurs hours after infection and is likely only to occur in cultivars that are resistant to pathogen infection (Yang *et al.*, 1997). ROS may either act directly against attacking pathogens or they may play a part in reinforcing the plant cell wall against penetration (Prell and Day, 2000).

1.8.3 Pathogenesis related proteins

Pathogenesis related (PR) proteins are produced in reaction to a variety of biotic stresses (Dong, 2004). All PR proteins have certain characteristic properties which define them. They are extractable at low pH, predominantly localised in intercellular spaces, have relatively low molecular weights and are resistant to proteolytic enzymes (Legrand *et al.*, 1987). PR proteins are divided into different classes according to their biological properties (Prell and Day, 2000). There are 17 different PR protein classes with chitinase being the most common protein (van Loon and Pieterse, 1999).

Chitin and β-1,3-D-glucan are components in the construction of cell walls and septa of many fungi, including *Fusarium* spp. (Lopez-Romero and Ruiz-Herrera, 1986; Douglas, 2001). Four classes of PR proteins, PR-1, PR-3, PR-4, and PR-8, have chitinase properties whereas PR-2 has β-1,3-glucanase activity. The hydrolysis of fungal cell walls by these proteins inhibits fungal growth. Oligomers that are released from the hydrolysis of chitin and β-1,3-D-glucan may then act as elicitors. These elicitors are recognised and reactive oxygen species are released by the plant (Shetty *et al.*, 2009). Peroxidase is a member of a large multigenic family of enzymes that plays a role in many physiological processes during the plant's life cycle (Almagro *et al.*, 2009). PR-9 has peroxidase activity and has been shown to limit the spread of infection by either creating structural barriers and/or producing a highly toxic environment

through the production of reactive oxygen species (Almagro *et al.*, 2009). To date no PR protein studies have been done on maize during FGSC infection.

1.9 Objectives of this study

This study was conceptualised because our understanding of *Fusarium* spp.-maize interactions is often limited to ear rots. During this study the concentration of FGSC, *F. verticillioides* and FOSC target DNA was determined in maize roots, crown, stalks and grain in South Africa. This is the first time the target DNA of these three fungi was determined in the vegetative organs of maize plants. This survey will give us an insight into the effectiveness of certain farming practices on controlling diseases caused by *Fusarium* spp., the organs which the *Fusarium* spp. target and also the extent of mycotoxin contamination in South African grain. The Northern Cape is not the largest producer of maize however interesting patterns of infection were observed within their fields. Thus, the second part of the study was to evaluate FGSC infection in maize plants in infected fields over three seasons and at different growth stages in the Northern Cape Province. This will give us insight in two aspects; we wanted to determine if FGSC was responsible for the infection that was noted in the field. We also wanted to determine if there was a maize growth stage at which the fungi were more likely to infect. Previous studies have shown that mycotoxin translocation is possible. The third part of this study was performed to determine the possibility of mycotoxins translocation from maize roots and stalks into the grain. Unlike previous studies that determined the translocation of one mycotoxin, this study will incorporate DON, NIV, FUM and ZEA. Lastly, it will be determined if PR protein genes are expressed within the internodes of two maize cultivars in response to FGSC infection. It will be the first time that focus is placed on stalk rots with regard to PR proteins and not grain. This study will broaden our knowledge of the *Fusarium* spp.-maize interaction and hopefully, aid in disease prediction and future prevention.

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Chapter 2: Impact of agricultural practices on colonization of maize using *Fusarium* spp. target DNA

2.1 Abstract

Good agricultural practice is a system that is implemented to mitigate the danger of fungicide overuse and overcome the limited availability of resistant cultivars. However, a thorough understanding of the impact of agricultural practices on fungal pathogens is required to differentiate good agricultural practices from those that may have detrimental effects. The maize pathogens, *Fusarium graminearum* species complex (FGSC), *F. oxysporum* species complex (FOSC) and *F. verticillioides* were selected to evaluate the impact of agricultural practices on a) tissue preference of fungal pathogens, b) the accumulation of fungal target DNA as an indication of colonization and c) mycotoxin production. To achieve this aim, maize was collected throughout the maize production regions of South Africa, over three seasons (2012-2015) using a survey to determine the agricultural practices used. The roots, crowns, internode 1 (In1), internode 2 (In2) and grain of ten plants were sampled and processed. The concentration of FGSC, FOSC and *F. verticillioides* target DNA was determined using quantitative real time PCR. The three mycotoxins that were evaluated were deoxynivalenol (DON), zearalenone (ZEA) and fumonisins (FUM). Mycotoxin concentrations were determined in grain samples using a 70:30 methanol:H_2O extraction method in conjunction with liquid chromatography-tandem mass spectrometry (LC-MS/MS). As crop rotation x province interactions were observed for all three pathogens / pathogenic complexes, it could be concluded that crop rotation only has an impact on *Fusarium* spp. when provinces, and thus regional conditions, are also taken into consideration. This phenomenon was limited to the tissue closest to the ground, namely roots and crowns. However, in most instances the crop rotation systems within a province did not differ significantly from one another, indicating the limited effect maize, wheat, soybean and sunflower rotations have on the control of *Fusarium* spp.. Fields in which conservation agriculture was practiced, i.e. fields with limited or no tillage, showed a significant increase in FGSC and FOSC target DNA ($P = 0.02$ and $P = 0.003$ respectively). This increase was seen with *F. verticillioides* as well ($P < 0.001$) but was limited to the North West province alone. Furthermore, only certain tissues, i.e. roots and crowns were affected by the type of field preparation. An increase in all three pathogens / pathogenic complexes' fungal target DNA was observed in dryland fields, with all tissues affected.

Agricultural practices had a limited effect on the mycotoxin concentrations. Only FUM were found at higher concentrations in maize grain, in fields that practiced maize/wheat rotations. The majority of grain samples contained ZEA and FUM. FUM did not exceed the maximum tolerable level (MTL) set by the government, during the three seasons. The highest FUM concentration (1.26 $\mu g.g^{-1}$) was observed during the 2012/13 season. ZEA exceeded the suggested levels for maize in South Africa, with a maximum concentration of 1.77 $\mu g.g^{-1}$, during the 2014/15 season. It did not however exceed the EU suggested levels of 2 $\mu g.g^{-1}$ bw. DON was less abundant but greatly exceeded the MTL during the second season (18.04 $\mu g.g^{-1}$). Although the FGSC and *F. verticillioides* are seen as mainly grain pathogens, higher fungal target DNA was observed in the stalks than in the grain. FOSC causes root rot in multiple crops, and this was confirmed in this study with the greatest concentration of fungal target DNA found in the roots. However, this FOSC was also found in crown, stalk and grain tissues which has not been previously observed. This study highlights the importance of establishing the pathogen/s affecting a field and determining the appropriate agricultural practices in response.

Keywords: FGSC, FOSC, *F. verticillioides,* maize, mycotoxins, agricultural practices

2.2 Introduction

Maize (*Zea mays* L.) is an important food crop globally. Numerous *Fusarium* species have been shown to cause disease with effects on yield and grain quality (Shurtleff *et al.*, 1993). Both the FGSC and *F. verticillioides* are well-documented pathogens of maize and cause diseases such as Gibberella stalk and ear rot, and Fusarium stalk and ear rots respectively (Payne, 1999; White, 1999). The FOSC had not been shown conclusively to be a maize pathogen, with only one study in Poland showing that FOSC causes maize ear rot (Selwet, 2011). The FOSC has been shown to cause crown and root rot in soybeans and peppers (Pérez-Hernández *et al.*, 2014), as well as wilting in bananas (García-Bastidas *et al.*, 2014) and cucumbers (Cerkauskas, 2001). These pathogens do not only cause disease, but also produce mycotoxins that pose a health risk to consumers (Brera *et al.*, 2008). The mycotoxins most commonly produced by FGSC are deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA). The mycotoxin produced by FOSC of importance to this study is ZEA and the mycotoxin produced by *F. verticillioides* is FUM (Colhoun, 1973; Brera *et al.*, 2008; Boutigny *et al.*, 2011; Beev *et al.*, 2013). Correct farm management practices are essential if disease severity is to be limited.

Maize is generally grown in monoculture due to high demands. Monocultures have only been practiced for the last 100 years. This practice involves annual planting of a single crop on an extensive scale. Monoculture was primarily implemented to ease agricultural practices such as harvesting, fertilisation and irrigation (Finckh and Wolfe, 2006; Reddy, 2013). Although monocultures can deliver high yields, the cost of this agricultural practice is that pathogens are more likely to overcome the plants' defence responses and cause large-scale losses (Reddy, 2013). To this end, maize is often rotated with other crops. However, crops that can be used effectively in rotation systems with maize to reduce infection by *Fusarium* spp. are limited due to the wide *Fusarium* spp. host range, which includes all cereals, soybean and canola (Marburger *et al.*, 2015).

The act of burying plant debris is an ancient practice for controlling pathogens (Sumner *et al.*, 1981). In recent year's conservation agriculture, which includes reduced tillage practices and no-till, has become more applied in order to limit soil erosion, conserve energy, increase soil moisture and increase crop yields (Lori *et al.*, 2009). A reduction in tillage leads to higher levels of plant debris on the soil surface, resulting in the ideal environment for pathogen to

proliferate (Dill-Macky and Jones, 2000). This is especially true for *Fusarium* spp. that are strong saprophytes (Akinsanmi *et al.*, 2007). Under certain circumstances a reduction in tillage may indirectly influence pathogen activity. This happens for instance when reduced tillage is associated with a soil temperature decrease, which in turn may retard seed germination and seedling development, predisposing the plant to diseases (Sumner *et al.*, 1981). Although a reduction in tillage may result in higher residue levels, the colonisation of these residues is also dependent on temperature, physio-chemical factors such as pH and water activity (Leplat *et al.*, 2013).

Irrigation is used in agriculture to increase food production (Hong and Moorman, 2005). However, an increase in moisture for the host plant also results in an increase in moisture for the respective pathogen activities (Rotem and Palti, 1969). Controlling factors such as the water quality, quantity and timing of irrigation may limit infection. Water quality includes the type of water sources used for irrigation. Rivers and ponds may be more contaminated with pathogens than closed systems such as wells (Yun, 2003). As water becomes scarcer it is often recycled, increasing the inoculum potential (Hong and Moorman, 2005). The quantity of water must be optimised as too little water may subject the plant to drought stress and too much will lead to the germination of fungal spores or water stress. The time of day during which irrigation takes place is of importance. Irrigation in the afternoon may result in higher humidity during the cooler evenings, providing more favourable conditions for fungal germination (Ogle and Dale, 1997).

The initial purpose of this study was to determine the tissue preference of FGSC, FOSC and *F. verticillioides*. The hypothesis was that the concentrations of target fungal DNA would indicate the levels of tissue colonization and that these would confirm that FGSC and *F. verticillioides* are primarily ear rot pathogens, whereas FOSC causes wilting and root rots as stated in literature (Munkvold and Leslie, 1999). In addition, the effect of farming practices on tissue preference was also evaluated by quantifying the respective concentration of FGSC, FOSC and *F. verticillioides* target DNA in maize roots, crowns, stalks and grain at selected localities in 5 provinces, to determine the impact of location crop rotation tillage practices and water regimes on tissue colonization. Mycotoxins in grain harvested from the targeted fields were also quantified.

2.3 Materials and Methods

2.3.1 Collection of samples

A survey was conducted across South Africa to determine the concentrations of FGSC, FOSC and *F. verticillioides* in maize root, stalk and ear tissues. A questionnaire was compiled to collect information including whether the crops were under irrigation or dryland (water regimes), tillage practices applied crop rotations (Table 2.1). Ten maize plants were sampled from 44 fields at 17 localities during 2012/13, 36 fields at 16 localities during 2013/14 and 29 fields at 16 localities during 2014/15 and the respective crop practice information was collected. The localities were spread over five provinces namely Northern Cape, Free State, Mpumalanga, North West and KwaZulu-Natal provinces. The number of fields, sampled over the three seasons, included 31 maize monoculture fields, 45 maize/wheat rotation fields and 32 maize/soybean rotation fields and two maize/sunflower fields were sampled. These included samples from 58 tilled and 53 no-tilled fields as well as 61 irrigated and 49 dryland fields sampled over three seasons, across the different provinces were unevenly distributed with eight fields in Mpumalanga, 40 in KwaZulu-Natal, 14 in the North West, 34 in the Northern Cape and 14 in the Free State provinces. The ten plants were removed so as to maximize root integrity and included at least one ear. The plants were sampled at physiological maturity, from BBCH 87 to 89 according to the BBCH monograph (Meier, 2001). The roots, crowns, Internode (In) 1 and In2 and the first ear were removed from each of the ten plants. The respective tissues were pooled and stored at 4 ºC until analysis.

2.3.2 Total DNA isolation from infected maize material

A modified CTAB method was employed to extract DNA (Saghai-Maroof *et al.*, 1984). The kernels were milled with a Cyclotech sample mill (Foss Tecator, Hoganas, Sweden) and the roots, crowns and stalks were ground with liquid nitrogen (Afrox, South Africa) until a fine powder was formed. Approximately 250 mg of ground tissue, from the ten pooled plants, was added to 900 µl of CTAB buffer which contained β-mercaptoethanol (Merck KGaA, Darmstadt, Germany). The CTAB buffer consisted of 2 % CTAB (Sigma-Aldrich, Steinheim, Germany), 5 M NaCl (Merch KGa, Darmstadt, Germany), 1 M Tris (Merch KGa, Darmstadt, Germany), 0.5 M EDTA (Sigma-Aldrich, Steinheim, Germany) and 0.2 % β-mercaptoethanol at pH 8.0. The samples were frozen in liquid nitrogen and heat shocked in 95 °C water for 5

minutes. After cooling the samples, 2 µl of RNase A/T1 Mix (Fermentas, Hanover, USA) was added to the mixture. The samples were incubated for an hour at 37 °C. After incubation, 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) (phenol - Sigma-Aldrich, Steinheim, Germany; Chloroform - Merch KGa, Darmstadt, Germany; isoamyl alcohol – Merck, KGaA, Darmstadt Germany) was added to each sample. The samples were vortexed and centrifuged for 10 minutes at 15294 x g. The supernatant was transferred to another tube containing 500 µl of phenol:chloroform:isoamyl alcohol and centrifuged again. The supernatant was transferred to a clean tube and 550 µl ice cold isopropanol (Merch KGa, Darmstadt, Germany) was added. The samples were incubated for 30 minutes at - 20 °C followed by a 20-minute centrifugation step at 20817 x g (4 °C). The supernatant was discarded, and the pellet was washed with 70 % ethanol (Merch KGa, Darmstadt, Germany) by centrifuging the samples for 5 minutes at 20817 x g. The supernatant was removed and air-dried. The pellet was re-suspended in 1 x TE buffer. The samples were stored at - 20 °C until used. The DNA was quantified with a NanoDrop spectrophotometer and the quality determined by an A260/A280 ratio of 1.8 to 2. All DNA was then diluted to 10 ng/µl for further analysis.

2.3.3 Total DNA extraction of pure fungal cultures

Pure fungal cultures were used to create standard curves, in order to ensure correct identification and quantification of tissue colonization. A representative species of each complex was chosen. The sensitivity of the primers is such that they do not differentiate between the different species within the complex, and thus the entire complex is quantified. *F. graminearum* (isolate: PPRI 7723) and *F. oxysporum* (isolate: PPRI 7729) cultures were obtained from the Agricultural Research Council – Plant Protection Research Institute. A *F. verticillioides* (isolate: MRC 826) culture was obtained from the Agricultural Research Council – Grain Crops Institute. These cultures were plated onto half strength potato dextrose agar (PDA) (Merck, Whitehouse Station, USA) and allowed to grow for 7 days. Genomic DNA was extracted using the above methodology. The DNA concentration was determined using a Nanodrop spectrophotometer and the quality was determined by an A260/A280 ratio of 1.8 to 2. The DNA was stored undiluted at - 20 ºC until used.

2.3.4 qPCR

Quantitative PCR primers, for the quantification of FGSC, FOSC and *F. verticillioides* target DNA were obtained from Nicolaisen *et al.* (2009) and Schoeman *et al.* (2016). The primer sequences specific to FGSC are FgramB379 (CCA TTC CCT GGG CGT) and FgramB411 (CCT ATT GAC AGG TGG TTA GTG ACT GG). Those for FOSC are FoxyASCF (CTC TCC TCG ACA ATG AGC AT) and FoxyASCR (GGT CTG TGA AAC GAT GTC AGT A) and for *F. verticillioides,* Fver356 (CGT TTC TGC CCT CTC CCA) and Fver412 (TGC TTG ACA CGT GAC GAT GA) (IDT) (Nicolaisen *et al.*, 2009; Schoeman, 2016). A standard curve of known DNA concentration was created by diluting the DNA of the three selected fungal isolates by 4-, 16-, 64-, 256-, 1024-, and 4096 times in maize DNA that was free of pathogenic fungal contamination according to a method used by Boutigney *et al.* (2012). The maize DNA was obtained by growing maize grain on a sterile water agar medium. After two weeks growth, the fungus-free maize seedling was ground in liquid nitrogen and DNA was extracted using the methodology described in 2.3.2. Maize seedling DNA was deemed to be the best source since it also provided the cleanest DNA. The qPCR reaction mix consisted of 1 x iTaq Universal SYBR Green supermix (Biorad, Hercules, USA), 0.2 µM of each primer (IDT) and 0.8 ng DNA. FGSC, FOSC and *F. verticillioides* target DNA concentrations were determined in the various tissues by completing a real time qPCR assay for each sample in triplicate. The Cq value of the target DNA was compared to the Cq value of the standard curve to obtain the starting target DNA concentration. The reaction conditions for the real time qPCR were 95 °C for 10 minutes and 40 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 15 seconds. A melt curve analysis was performed by heating the products to 95 °C for 30 seconds then cooling to 40 °C for 30 seconds followed by gradual heating of the product from 60 °C to 95 °C with a 1 °C increment every 10 seconds. This was done to identify different amplicons which include non-specific products. To ensure that the unknown samples are compared to a standard curve of quality the efficiency (optimum 90-10 %), R^2 (optimum >0.9) and slope (optimum -3.1 and -3.6) was determined and falls within the acceptable range (Figure 2.1 – 1a-3a). The melt curve was determined to occur at 77-78 ºC. If a sample fell beyond these temperatures, they were discarded (Figure 2.1 – 1b-3b).

2.3.5 Liquid chromatography/Mass spectrophotometry

Maize kernels from the respective samples were ground using a Cyclotec sample mill. Maize flour (5 g) was added to a 20 ml, 70:30 Methanol (Romil Ltd, Cambridge, UK):H_2O solution. After vortexing, the slurry was placed into a Labotec orbital shaker for 30 minutes at 200 rpm. The samples were centrifuged at 500 x g for 10 minutes at 4 °C. The supernatant was filtered through a 0.25 µm syringe filter and placed in the fridge overnight. The samples were subsequently centrifuged again at 20817 x g for 10 minutes and sent to the University of Stellenbosch (South Africa) for analysis of DON, NIV, 3-ADON (3-acetylDON), 15-ADON, ZEA and FUM using LC-MS/MS.

2.3.6 Statistical analysis

A T-test assuming unequal variance was used to determine whether the mean fungal target DNA concentrations were significantly different between the tissues (Microsoft Office, Excel 2013). A combined analysis of variance (ANOVA) for unreplicated data was performed for the survey, using the localities and years as respective replicates. Water regime, tillage practices and previous year's crops were used as factors for comparison purposes (Anonymous, 2004). The standardized residuals were acceptable for normal distributed data (Shapiro-Wilks test) and therefore the means of the significant effects were separated using Fisher's unprotected t-test (least significant difference) tested at 5 % level of significance (Shapiro and Wilk, 1965; Montgomery, 1984).

2.4 Results

2.4.1 FGSC

The FGSC appeared to colonise In2 more aggressively than the other tissues, with the highest concentration of target DNA at 2992.0 $pg.\mu l^{-1}$, although this did not differ significantly from In1 (2219.0 $pg.\mu l^{-1}$) (Figure 2.2). Mean FGSC target DNA concentrations in crowns (1051.7 $pg.\mu l^{-1}$), In1 and In2 were significantly (P=0.05) higher than in the roots (102.9 $pg.\mu l^{-1}$) and grain (70.2 $pg.\mu l^{-1}$). In2 had significantly (P=0.05) higher FGSC target DNA concentrations when compared to the crowns. In1 and the crowns did not differ significantly from each other.

A significant (P=0.04) province x crop rotation interaction was observed for the mean FGSC target DNA concentration measured in maize crowns over three seasons (Figure 2.3). The FGSC were more aggressive colonisers in the crowns of maize grown in rotation with soybeans in the North West province with a target DNA concentration of 9245.0 pg.µl^{-1}, whereas maize/wheat, maize/sunflower and maize monoculture all had significantly (P=0.04) lower FGSC target DNA concentrations across the four provinces (0-2867.0 pg.µl^{-1}). A significant (P=0.02) tillage practice effect was observed for the mean FGSC target DNA concentration in grain (Figure 2.4). The FGSC colonised grain in no-till fields more aggressively, which resulted in significantly higher FGSC target DNA concentrations (84.8 pg.µl^{-1}) than the tilled fields (33.6 pg.µl^{-1}). A significant (P=0.05) water regime effect was observed for the mean FGSC target DNA concentrations in the crowns, In1 and grain (Figure 2.5). FGSC target DNA concentrations in crowns (651.2 pg.µl^{-1}), In1 (974.8 pg.µl^{-1}) and grain (42.5 pg.µl^{-1}) of maize grown under irrigation were lower compared to crowns (1573.0 pg.µl^{-1}), In1 (2278.9 pg.µl^{-1}) and grain (81.0 pg.µl^{-1}) of maize grown under dryland conditions.

2.4.2 FOSC

The FOSC colonised the roots significantly (P=0.05) more aggressively than the other tissues, as indicated by the high concentration of target DNA at 14438.1 pg.µl^{-1} (Figure 2.6). FOSC target DNA concentration in crowns (7668.3 pg.µl^{-1}) was significantly higher than in In1 (3779.3 pg.µl^{-1}), In2 (2494.5 pg.µl^{-1}) and grain (267.3 pg.µl^{-1}). The target DNA concentrations of FOSC in maize In1 did not differ significantly from the target DNA concentration in In2, however both were significantly (P=0.05) higher than in grain.

A significant (P=0.004) tillage practice x province interaction was observed for the mean FOSC target DNA concentration measured in maize roots over three seasons (Figure 2.7). This interaction indicated that the roots of maize in rotation with sunflowers, in the North West province had significantly higher FOSC concentrations (72094.0 pg.µl^{-1}) than any other province or crop rotation system. There was one exception; the roots of maize grown in monoculture in the Free State province (39440.0 pg.µl^{-1}). The FOSC target DNA concentration in roots of monoculture maize in the Free State province were significantly higher than the maize/wheat rotation in the Northern Cape (1969.0 pg.µl^{-1}) and the Free State (2668.0 pg.µl^{-1}), the maize/soybean rotation in Mpumalanga (2771.0 pg.µl^{-1}) and the maize/sunflower rotation in the Free State provinces (1419.0 pg.µl^{-1}).

A significant (P=0.003) tillage practice effect was observed on the mean FOSC target DNA concentration in the roots (Figure 2.8). The FOSC target DNA concentrations were significantly higher in maize roots grown in fields that were no-till (23583.0 pg.µl^{-1}) compared to the roots of maize planted in tilled fields (6397.0 pg.µl^{-1}). A significant (P=0.003) tillage practice x province interaction was observed on the mean FOSC target DNA concentration measured in maize In2 over three seasons (Figure 2.9). The FOSC target DNA concentration was significantly higher in In2 of maize grown in no-till fields in the Free State province (10218.0 pg.µl^{-1}) compared to the tilled fields in the Free State province (920.0 pg.µl^{-1}). It was also significantly higher than the maize grown under both tillage practices in the Northern Cape province (no-till - 1515.0 pg.µl^{-1} and tilled fields - 584.0 pg.µl^{-1}) as well as the tilled fields in KwaZulu-Natal (1509.0 pg.µl^{-1}) and Mpumalanga provinces (482.0 pg.µl^{-1}), and no-till fields in North West province (367.0 pg.µl^{-1}). A significant (P=0.05) water regime effect was observed for the mean FOSC target DNA concentration in roots, crowns, In1 and In2 (Figure 2.10). The FOSC target DNA concentrations in the roots (24411.0 pg.µl^{-1}), crowns (13042.0 pg.µl^{-1}), In1 (6261.0 pg.µl^{-1}) and In2 (4740.0 pg.µl^{-1}) were all significantly higher in the maize grown in dryland fields compared to the FOSC target DNA concentrations in the roots (6591.0 pg.µl^{-1}), crowns (2801.0 pg.µl^{-1}), In1 (1659.0 pg.µl^{-1}) and In2 (770.0 pg.µl^{-1}) of maize grown in the irrigated fields. No maize was sampled in Mpumalanga province in no-till fields.

2.4.3 *F. verticillioides*

F. verticillioides colonised In1 more aggressively than the other tissue, with a target DNA concentration of 1263.8 pg.µl^{-1} (Figure 2.11). *F. verticillioides* target DNA concentrations were significantly (P=0.05) higher in In1 than in the roots (131.6 pg.µl^{-1}) and grain (330.5 pg.µl^{-1}) but did not differ significantly from the crowns (803.7 pg.µl^{-1}) and In2 (770.0 pg.µl^{-1}). The target DNA concentration of *F. verticillioides* was significantly (P=0.05) higher in the crowns than in the roots and grain but did not differ significantly from In2. In2 had significantly (P=0.05) higher *F. verticillioides* target DNA concentrations than the roots but did not differ significantly from the grain. The grain had significantly (P=0.05) higher *F. verticillioides* target DNA than the roots.

A significant (P<0.0001) province x crop rotation interaction was observed for the mean *F. verticillioides* target DNA concentration measured in maize crowns (Figure 2.12). The maize/soybean rotation (10459.0 pg.µl^{-1}) grown in the North West province had significantly

higher *F. verticillioides* target DNA concentration compared with the crop rotation systems in the other provinces. A significant (P=0.03) province effect was observed for the mean *F. verticillioides* target DNA concentration measured in maize roots (Figure 2.13). The roots of maize grown in North West province had significantly higher *F. verticillioides* target DNA concentrations (518.5 pg.µl^{-1}) compared to the maize grown in Mpumalanga (12.1 pg.µl^{-1}), KwaZulu-Natal (51.0 pg.µl^{-1}) and Northern Cape (56.5 pg.µl^{-1}) provinces. The *F. verticillioides* target DNA concentrations in the roots of maize grown in the North West province did not differ significantly from those grown in the Free State. A significant (P=0.0003) tillage practice x province interaction was observed for the mean *F. verticillioides* target DNA concentration measured in maize crowns (Figure 2.14). The crowns of maize grown in no-till fields, in the North West province had significantly higher *F. verticillioides* target DNA concentrations (4677.9 pg.µl^{-1}) than the fields that were tilled in the North West province (432.5 pg.µl^{-1}). The *F. verticillioides* target DNA concentrations in the crowns in maize grown in no-till fields, in the North West province, were also significantly higher than in crowns in maize grown in till and no-till fields in the KwaZulu-Natal, Northern Cape and Free State provinces, as well as the till fields in Mpumalanga (range of 145.0 – 1015.9 pg.µl^{-1}). No maize samples were collected from no-till fields in Mpumalanga province.

2.4.4 Mycotoxin analysis

The mycotoxin data were analysed in the same manner as the *Fusarium* spp. target DNA concentrations, to determine whether agricultural practices had an influence on mycotoxin concentration. NIV and 3-ADON were found in certain fields throughout South Africa, however this was limited to 8.3 % of the samples analysed for NIV and 4.6 % of the samples analysed for 3-ADON. NIV concentrations ranged from 0.07 µg.g^{-1} to 0.31 µg.g^{-1}, whereas 3-ADON concentrations ranged from 0.04 µg.g^{-1} to 0.25 µg.g^{-1}. ZEA, DON and FUM were more frequently detected with 78.8 % of the samples containing ZEA, 14.8 % DON and 95.4 % FUM. ZEA concentrations ranged from 0.002 µg.g^{-1} to 18.46 µg.g^{-1}.DON concentrations ranged from 0.29 µg.g^{-1} and 52.83 µg.g^{-1}. FUM concentrations ranged from 0.0002 µg.g^{-1} to29.61 µg.g^{-1}. The concentration of ZEA was significantly (P≤0.05) higher in the North West province (1.26 µg.g^{-1}) and KwaZulu-Natal (0.62 µg.g^{-1}) province compared to Mpumalanga (0.09 µg.g^{-1}) province (Figure 2.15). The concentration of ZEA was significantly (P≤0.05) higher in the grain of maize grown in rotation with wheat (1.75 µg.g^{-1}) compared to grain of maize grown in rotation with sunflowers (0.26 µg.g^{-1}) (Figure 2.16). The concentration of DON

was very high during the third season (18.04 $\mu g.g^{-1}$), with significantly (P=0.04) lower concentrations during the second season (0.75 $\mu g.g^{-1}$) (Figure 2.17). The total FUM concentrations were determined in maize grain across all three seasons, although only seasons 2013/14 and 2014/15 showed significant differences in concentrations (Figure 2.18). FUM concentrations during the third season (1.19 $\mu g.g^{-1}$) were significantly (P=0.02) higher than during the second season (0.29 $\mu g.g^{-1}$) (Figure 2.19). The maize grown in monoculture (0.82 $\mu g.g^{-1}$) and maize/wheat rotations (1.71 $\mu g.g^{-1}$) had significantly ($P \leq 0.05$) higher concentrations of total FUM in their grain compared to maize/sunflower rotations (0.07 $\mu g.g^{-1}$). The grain of maize grown in rotation with wheat also had significantly (P=0.03) higher FUM concentrations than the grain of maize grown in rotation with soybean (0.31 $\mu g.g^{-1}$).

2.5 Discussion

As farming is becoming increasingly more competitive and input costs are becoming higher, it is important that farmers gain knowledge on the impact of agricultural practices on crop diseases. This study was the first analysis of FGSC, *F. verticillioides* and *F. oxysporum* that that has taken key agricultural practices into account. The FGSC and *F. verticillioides* have previously been recorded as pathogens of maize in South Africa (Marasas *et al.*, 1979; Rheeder *et al.*, 1993; Boutigny *et al.*, 2012). Although these pathogens/pathogen complexes are known to cause root, crown, and stalk rots they have been more frequently associated with ear rots (Payne, 1999; White, 1999). It was thus expected that the highest concentrations of these fungi should be found in maize grain. However, the high concentrations of both FGSC and *F. verticillioides* in the stalks (highest in In2 and In1, respectively), indicated a preference for stalk tissue. The FOSC is a soilborne pathogen complex and the high concentrations of FOSC in maize roots were expected (Roncero *et al.*, 2003).

The recommendation to farmers will always be to rotate maize with non-host plants. However, crop profitability plays a major role in the decision of crops to incorporate into the rotation system (Gebremedhin and Schwab, 1998). Thus, the crops most often used in rotation with maize, locally, are wheat, soybeans, and sunflowers. The majority of graminaceous crops are vulnerable to FGSC and *F. verticillioides* infection (Trail, 2009; Boutigny *et al.*, 2012). Based on previous studies it was expected that the highest target DNA concentrations of these species would be found in maize monoculture or maize/wheat rotations (Dill-Macky and Jones, 2000; Landschoot *et al.*, 2013; Spolti *et al.*, 2015). In contrast, crowns of maize/soybean rotation

fields in the North West province had the highest FGSC and *F. verticillioides* concentrations. Studies have indicated that species of the FGSC are responsible for pre- and post-emergence damping off, seed and root rot, and pod blight of soybean (Nelson, 1999; Ellis *et al.*, 2011). The FOSC's target DNA concentration were highest in the roots of maize grown in rotation with sunflowers in the North West province, whereas the maize/wheat rotations had lower concentrations of FOSC. Nahar and Mushtaq (2007) showed that *F. oxysporum* causes diseases such as seedling rot and stunting in sunflowers (Nahar and Mushtaq, 2007). Indications are that FGSC, *F. verticillioides* and FOSC may have been pathogens or secondary colonizers in sunflower and soybean tissue, which provided a large inoculum source for the maize during subsequent seasons. Thus, farmers using soybeans and sunflower in rotation with maize, with the aim of breaking the *Fusarium* spp. disease cycle should be advised that these rotations may be more harmful than beneficial. However, this trend needs to be verified as the sample size of the maize/sunflower rotation was small.

The effect of crop rotation was limited to the North West province. Boutigny *et al.* (2012) showed that FGSC and *F. verticillioides* infection of grain shifted from the eastern to the western maize production areas. From the late 1970's to the early 1990's, FGSC in grain was limited to KwaZulu-Natal, the Eastern Free State and Mpumalanga provinces (Marasas *et al.*, 1979; Rheeder *et al.*, 1993). In 2012 it was noted that higher concentrations of FGSC was recorded in the western Free State and North West provinces, which is in accordance with this study, although limited to crowns and roots (Boutigny *et al.*, 2012). This change could be attributed to climate change and anthropogenic activity (Das *et al.*, 2016).

Tillage practices often come under scrutiny as they constitute a major strategy for disease control (Sumner *et al.*, 1981). The argument between no-till and till continues, as no-till reduces soil erosion and enhances soil condition but leaves stubble within the fields resulting in inoculum reservoirs (Govaerts *et. al.*, 2006). In this study, the three pathogens/pathogen complexes were found in higher concentrations in no-till fields compared with tilled fields. This effect was limited to certain provinces for target DNA concentrations of FOSC in In2 and *F. verticillioides* in crowns, whereas target DNA concentrations for FGSC in grain and FOSC in roots did not depend on the province. This indicates that no-till may have a negative impact on the grain and roots regardless of environmental conditions, whereas the impact of no-till on stalk tissue is environment dependent. A previous study reported that stalk rot is environment

dependant, thus if the environmental conditions are conducive for stalk rot, then only will no-till have an impact on the severity (Mehl, 2014).

Previous studies have found that a crop with identical irrigation treatments, under different weather conditions, will not show the same disease development pattern (Rotem and Palti, 1969). In this study, the maize crowns, stalks (In1) and grain had higher FGSC target DNA concentrations in the dryland fields despite reports that Gibberella stalk and ear rot are favoured by warm, wet conditions during infection (White, 1999; Leplat *et al.*, 2013). FOSC infected all maize plant tissues more readily in dryland conditions. FOSC is not a known stalk rot pathogen, however as a root rot pathogen it is widely adapted and may infect maize plants regardless of the weather conditions (White, 1999). Other effects of irrigation must also be taken into consideration. Irrigation may increase the plants vigour, rate of growth and length of the crop's life span. This results in fuller shoots, more shade, lower micro-climate temperatures and longer periods of high moisture (Rotem and Palti, 1969). Thus, by the time the maize plants were sampled in irrigated fields, although similar in age to the maize in the dryland fields, were still green whereas the maize plants in the dryland fields had started to senesce. This indirect irrigation effect could explain the higher fungal concentrations in dryland fields.

In this study there were no differences between the mycotoxins in dryland and irrigated fields or between the tilled fields compared to no-till fields, indicating that although these practices may influence fungal growth and infection, they do not influence ZEA, DON and FUM concentrations. There were too few grain samples that contained NIV and 3-ADON to do a comparison of agricultural practices. Crop rotation affected mycotoxin concentrations in this study, with the maize/wheat rotations having the highest FUM and ZEA concentrations. The maximum tolerable level (MTL) of mycotoxin is a limit set by the local government as a measure to ensure the safety of food (Trail, 2009). In South Africa, no legislative MTL has been set for ZEA, but Burger *et al.* (2014) suggested MTL's for maize in South Africa (Burger *et al.*, 2014) but according to the European Union this is 1.25 $\mu g.g^{-1}$ for unprocessed maize (Anonymous, 2016). This limit was exceeded during the third season with the Mpumalanga province having the lowest ZEA concentrations. The MTL for DON was accepted into legislation in South Africa in 2016 and is set at 2 $\mu g.g^{-1}$ for unprocessed cereal grains and 1 $\mu g.g^{-1}$ for processed maize (Anonymous, 2016). The MTL for DON was not exceeded during the second season but was exceeded during the third season. The MTL of FUM was also accepted into legislation in South Africa in 2016 and is set at 4 $\mu g.g^{-1}$ for unprocessed maize

and 2 $\mu g.g^{-1}$ for processed maize. The total FUM concentrations were not exceeded in any of the seasons. ZEA exceeded the suggested levels set by Burger *et al.* (2014) for maize in South Africa, with a maximum concentration of 1.77 $\mu g.g^{-1}$, during the 2014/15 season. It did not however exceed the EU suggested levels of 2 $\mu g.g^{-1}$ bw. DON was less abundant but greatly exceeded the MTL during the second season (18.04 $\mu g.g^{-1}$).

2.6 Conclusion

The control of disease through the adaptation of cultural practices is as old as agriculture itself, however our understanding of the influence of these practices on individual pathogens must be honed. In this study the North West province, in conjunction with, crop rotations, had the highest concentration of FGSC, FOSC and *F. verticillioides.* Unexpectedly, maize/wheat and maize monoculture did not contain the highest concentration of fungi. FGSC and *F. verticillioides* favours stalk tissue whereas FOSC favours roots. As expected, no-till fields favours pathogen/pathogenic complex disease but this is limited to certain tissues. Dryland fields favoured pathogen/pathogenic complex infection. ZEA and FUM were found in the majority of samples but FUM did not exceed the MTL set by the government whereas ZEA did. DON was less abundant but greatly exceeded the MTL during the third season. This suggests that although mycotoxins are season dependent there is a possibility that levels that could cause harm to humans and animals may be exceeded. This study enhances our knowledge of the influence of agricultural practices on these three *Fusarium* spp.. It also clearly indicates the importance of appropriate agricultural practices in reducing the risk of significant infection levels.

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Table 2.1: Questionnaire posed to farmers, to elucidate the farming practices used, including the type of crop rotation, water activity and tillage practices.

Province	Locality	GPS coordinates	Crop rotation			Water activity	Tillage practices
			2012/2013	2013/14	2014/15	Dryland/Irrigation	Till/No-till
Free State	Koppies	27°18'33.08 S 27°19'38.08 E	Su/M	M/M	M/M	D	NT
	Viljoenskroon 1	27°19'08.05 S 27°08'33.07 E	So/M	M/M	M/M	D	T
	Vijoenskroon 2	27°18'27.08 S 27°06'12.30 E	M/M	So/M	M/M	D	NT
	Jacobsdal 1	29°07'46.59 S 24°40'50.81 E	W/M	W/M	W/M	I	T
	Jacobsdal 2	29°06'12.99 S 24°38'57.54 E	W/M	W/M	W/M	I	T
	Jacobsdal 3	29°22'59.02 S 24°37'14.08 E	W/M	W/M	W/M	I	NT
Northern Cape Province	Richie/Modderdam	29°19'58.40 S 24°53'09.93 E	W/M	W/M	W/M	I	T
	Richie/Riet Rivier	29°05'49.45 S 24°35'38.94 E	W/M	W/M	W/M	I	T
	Douglas 1	29°05'57.3S 23°37'53.6 E	W/M	W/M	W/M	I	NT
	Douglas 2	29°14'28.3 S 23°47'09.2 E	W/M	W/M	W/M	I	T
	Douglas 3	29°06'26.7 S 23°46'33.0 E	W/M	W/M	W/M	I	T
	Douglas 4	29°05'59.2 S 23°46'35.4 E	W/M	W/M	X	I	T
	Douglas 5	29°07'24.1 S 23°44'11.4 E	W/M	W/M	X	I	T
	Prieska 1	29°31'55.9 S 23°01'05.4 E	W/M	W/M	W/M	I	T
	Prieska 2	29°39'10.8 S 22°52'47.0 E	W/M	W/M	W/M	I	T
	Prieska 3	29°39'50.2 S 23°51'17.2 E	W/M	W/M	W/M	I	T
KwaZulu Natal	Paulpietersburg 1	27°22'53.8 S 30°41'04.6 E	So/M	So/M	So/M	D	NT
	Paulpietersburg 2	27°22'42.62 S 30°40'54.74 E	So/M	So/M	So/M	I	T
	Paulpietersburg 3	27°20'29.71 S 30°38'21.78 E	So/M	X	So/M	I	NT
	Paulspietersburg 4	27°25'41.2 S 30°36'29.9 E	O/M	M/M	So/M	D	NT
	Paulspietersburg 5	27°25'42.8 S 30°36'58.5 E	B/M	So/M	X	D	NT

	Paulpietersburg 6	27°21’47.8 S 30°43’02.4 E	B/M	So/M	X	D	NT
	Paulpietersburg 7	27°27’01.9 S 30°31’03.1 E	So/M	So/M	So/M	D	NT
	Paulpietersburg 8	27°27’45.0 S 30°35’25.3 E	So/M	M/M	X	D	NT
	Paulpietersburg 9	27°27’35.95 S 30°32’29.80 E	M/M	M/M	X	D	NT
	Paulpietersburg 10	27°27’35.0 S 30°32’48.1 E	So/M	X	X	D	NT
	Dundee 1	28°11’11.9 S 29°57’43.9 E	M/M	M/M	X	D	T
	Dundee 2	28°10’46.9 S 29°57’11.2 E	R/M	M/M	M/M	I	T
	Bergville 1	28°42’38.80 S 29°26’56.20 E	So/M	So/M	X	I	NT
	Bergville 2	28°42’36.58 S 29°27’25.20 E	W/M	X	So/M	I	T
	Bergville 3	28°42’14.46 S 29°27’03.11 E	So/M	W/M	W/M	I	NT
	Bergville 4	28°43’32.21 S 29°26’38.04 E	W/M	W/M	W/M	I	NT
	Winterton 1	28°53’22.8 S 29°32’02.6 E	W/M	So/M	X	I	NT
	Winterton 2	28°47’26.88 S 29°31’20.76 E	W/M	So/M	So/M	I	NT
North West Province	Lichtenburg1	26°35’10.9 S 26°9’9.2 E	So/M	M/M	X	D	NT
	Lichtenburg 2	26°34’59.8 S 26°7’43.0 E	Su/M	M/M	M/M	D	NT
	Lichtenburg 3	26°34’46.06 S 26°06’28.67 E	Su/M	M/M	M/M	D	NT
	Lichtenburg 4	26°33’33.59 S 26°08’42.99 E	M/M	M/M	M/M	D	T
	Carletonville 1	26°10’57.9 S 27°27’07.70 E	M/M	M/M	M/M	D	T
	Carletonville 2	26°10’43.4 S 27°26’57.1 E	M/M	M/M	M/M	I	T
Mpumalanga	Middelburg 1	25°46’26.9 S 29°56’48.8 E	So/M	M/M	So/M	D	T
	Delmas 1	26°9’20.82 S 28°40’47.99 E	M/M	M/M	M/M	D	T
	Ogies 1	26°3’51.2 S 29°0’28.1 E	So/M	M/M	X	D	NT

M – maize monoculture, W – wheat/maize rotation, So – soybean/maize rotation, Su – sunflower/maize rotation and X – no maize samples.

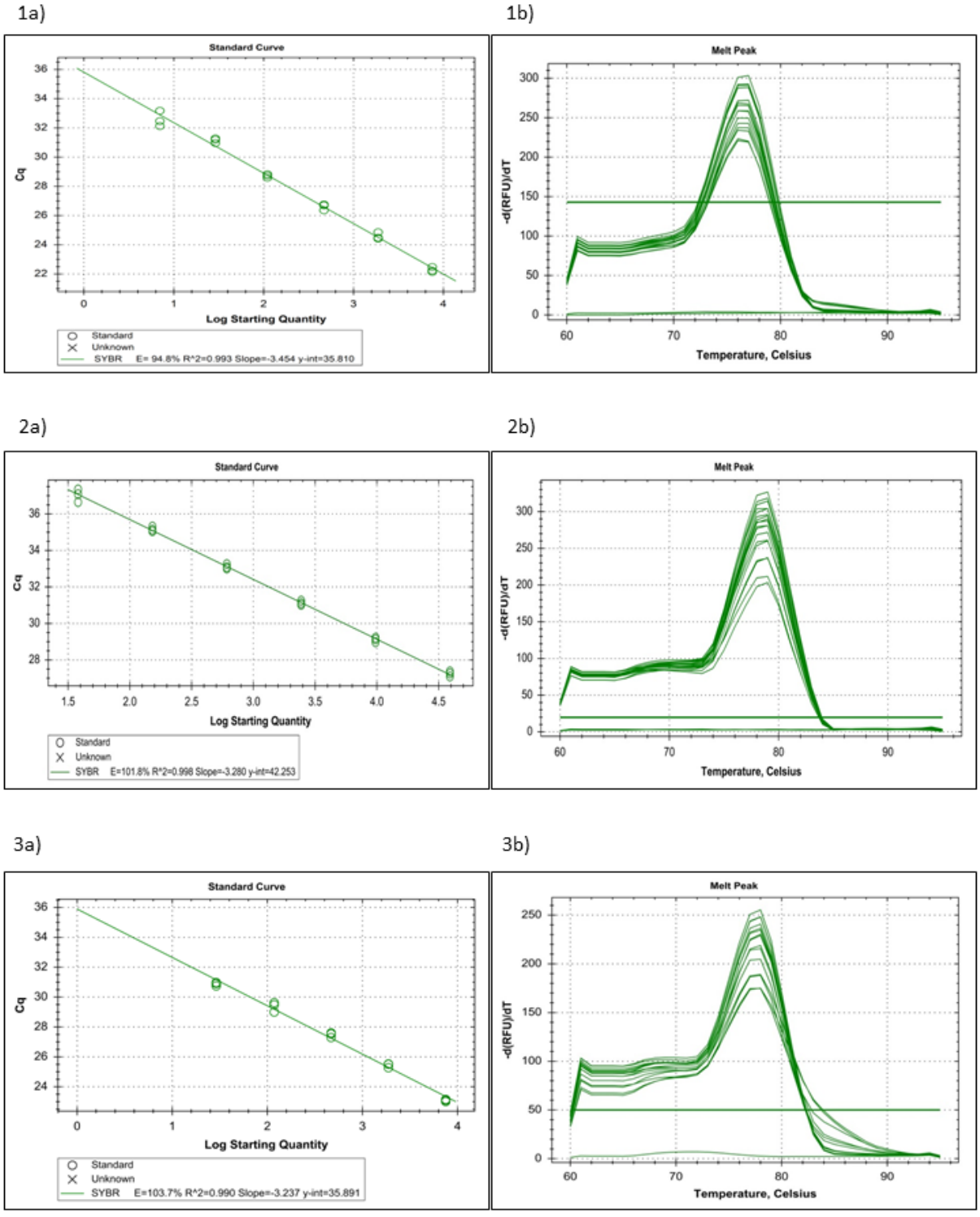
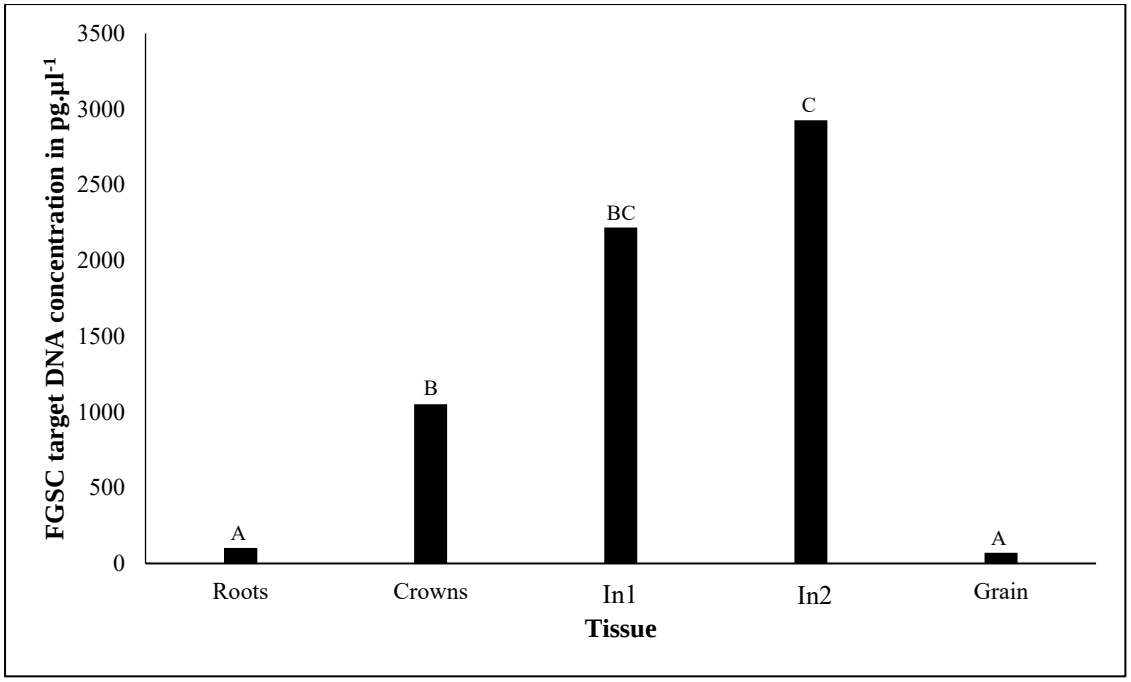


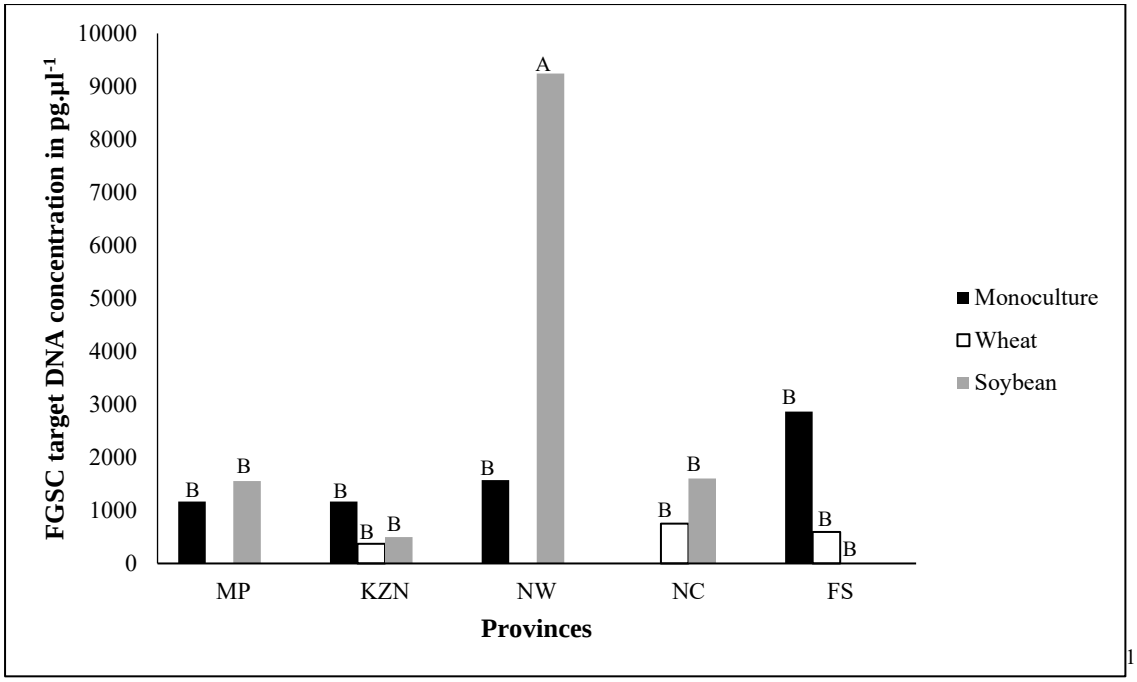
Figure 2.1: To ensure that the samples are measured against a good quality standard, the standard curves (a) and melt curves (b) of 1) FGSC, 2) FOSC and 3) *F. verticillioides* were shown. The PCR efficiency, slope (M) and R^2 values are indicated for each of the fungal species.



[1]; [2]

Figure 2.2: Mean FGSC target DNA concentrations in maize roots, crowns, stalks and grains sampled over the period 2012-2015. [3]

[1] pg.µl^{-1}-picogram per microliter
[2] In1-internode 1; In2-internode 2
[3] A different letter denotes significance at P≤0.05.



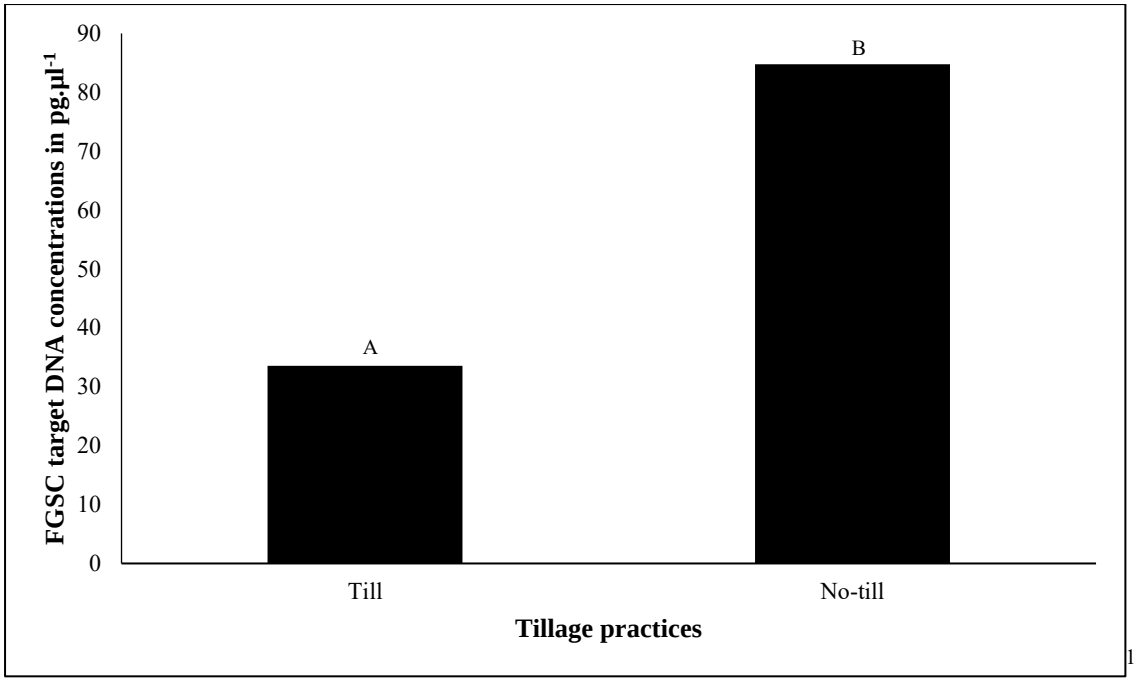
[1], [2]

Figure 2.3: Mean FGSC target DNA concentration in maize crowns over the period 2012-2015 indicating crop rotation x province interactions. [3]

[1] pg.µl^{-1}-picogram per microliter

[2] Mpumalanga-MP, KwaZulu-Natal-KZN, North West province-NW, Northern Cape-NC, Free State-FS

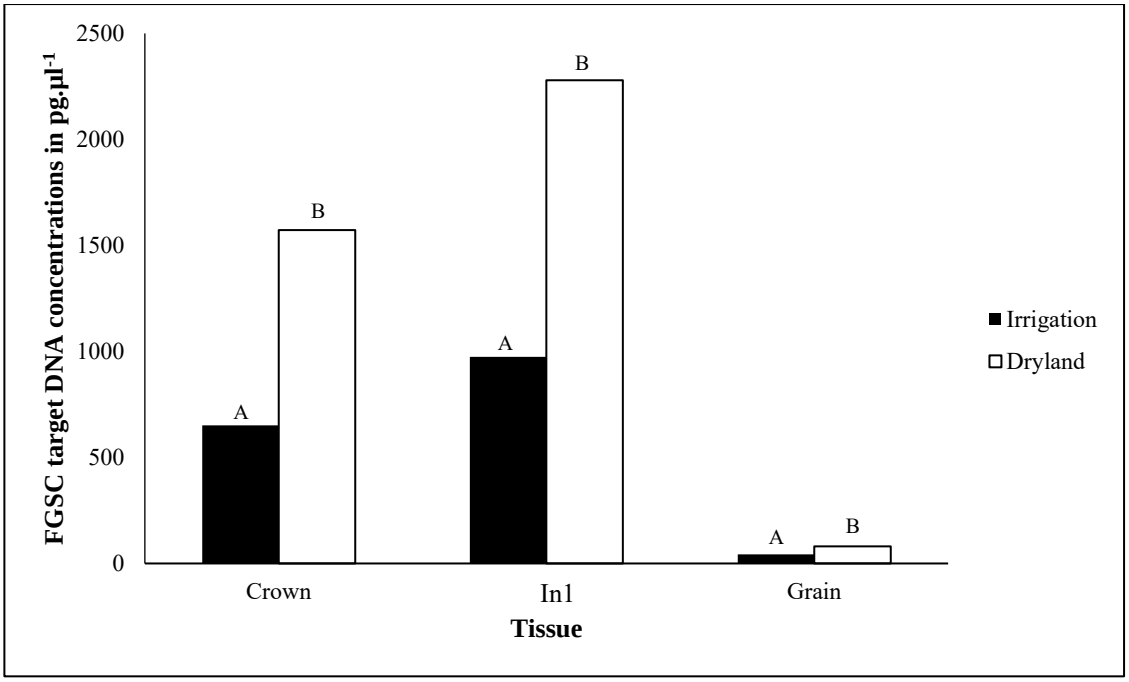
[3] A different letter denotes a significant difference at P=0.0427.



[1]

Figure 2.4: Mean FGSC target DNA concentration in grain over the period 2012-2015 in tilled and no-till fields. [2]

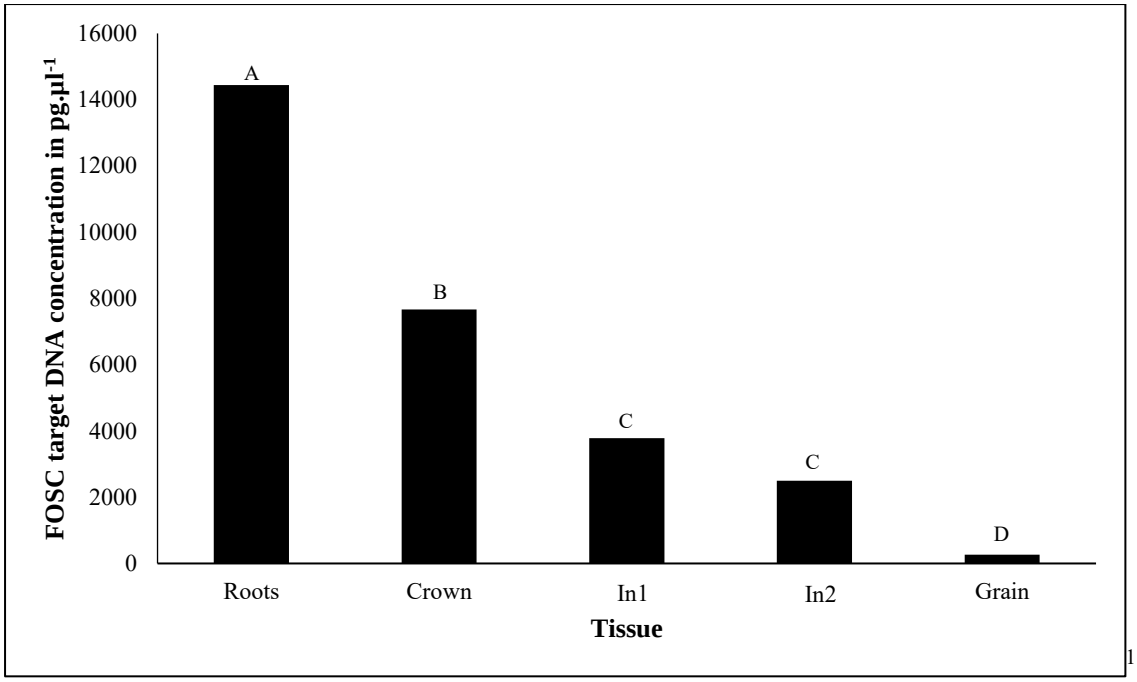
[1] pg.µl^{-1}-picogram per microliter
[2] A different letter denotes a significant at P=0.0247.



[1]; [2]

Figure 2.5: Mean FGSC target DNA concentration in crowns, In1 and grain, associated with irrigated and dryland fields, over the period 2012-2015. [3]

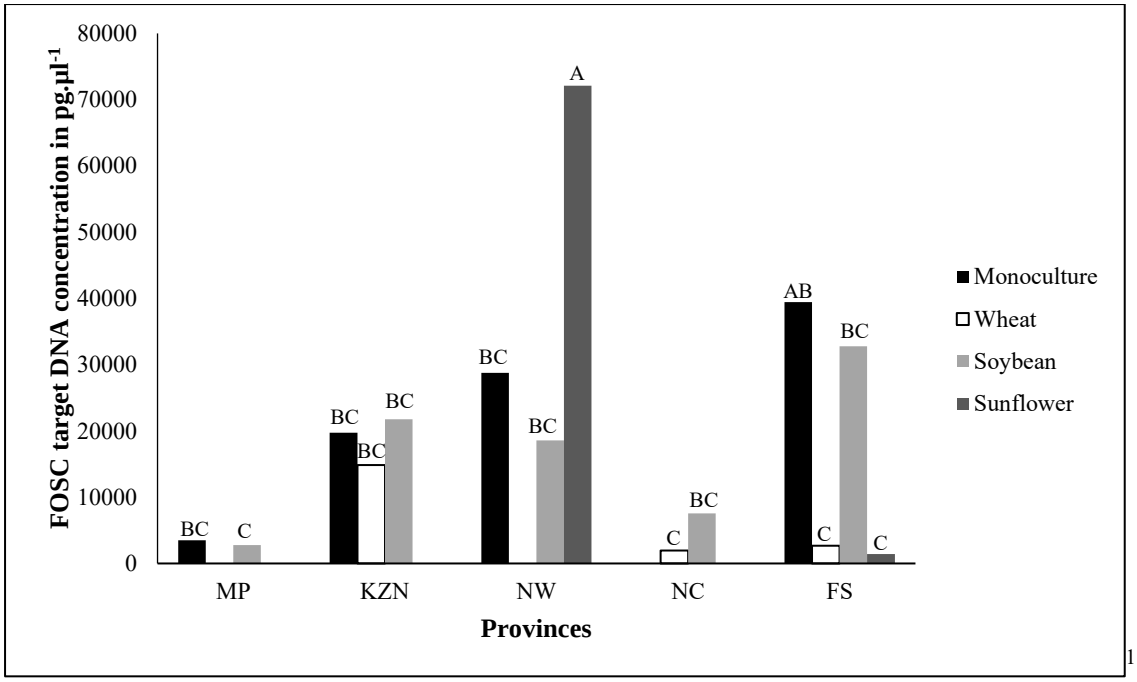
[1] pg.µl^{-1}-picogram per microliter
[2] In1-internode 1; In2-internode 2
[3] A different letter denotes a significant difference at $P<0.05$.



[1], [2]

Figure 2.6: Mean FOSC target DNA concentrations in maize roots, crowns, stalks and grains over the period 2012-2015. [3]

[1] pg.µl^{-1}-picogram per microliter
[2] In1-internode 1; In2-internode 2
[3] A different letter denotes a significant difference at $P<0.05$.



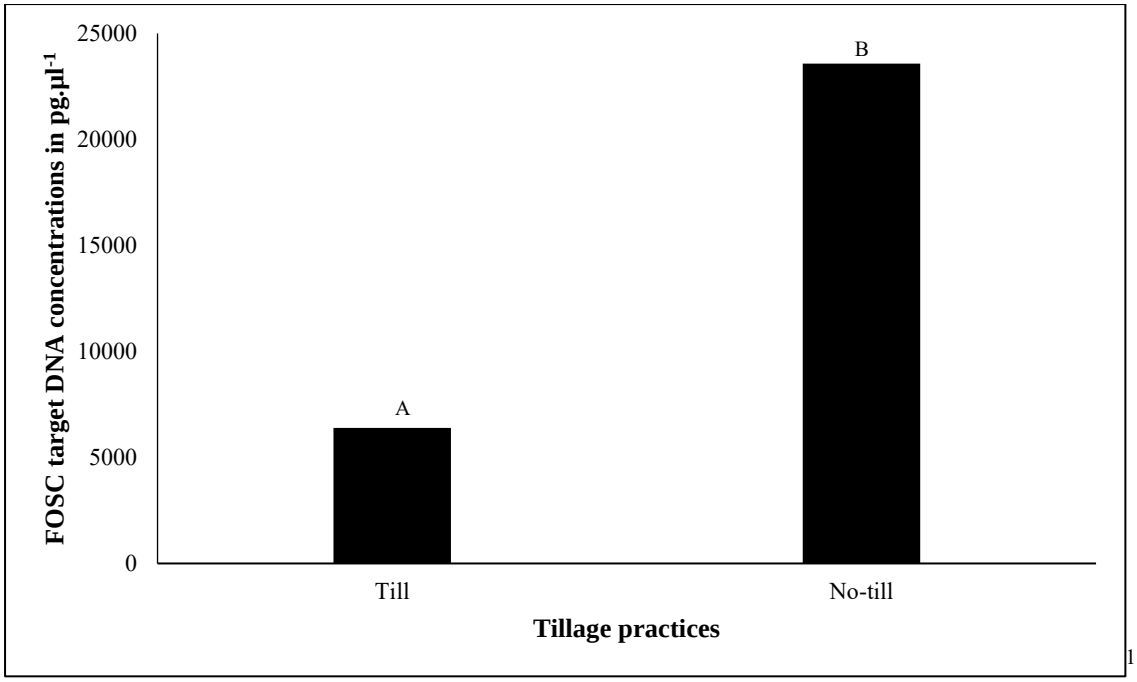
[1], [2]

Figure 2.7: Province x crop rotation interaction for the mean FOSC target DNA concentration measured in maize roots over the period 2012-2015. [3]

[1] pg.µl^{-1}-picogram per microliter

[2] Mpumalanga-MP, KwaZulu-Natal-KZN, North West province-NW, Northern Cape-NC, Free State-FS

[3] A different letter denotes a significant difference at P=0.0427.



[1]

Figure 2.8: Tillage practice main effect for the mean FOSC target DNA concentration in the roots over the period 2012-2015. [2]

[1] pg.µl^{-1}-picogram per microliter
[2] A different letter denotes a significant difference at P=0.0032.

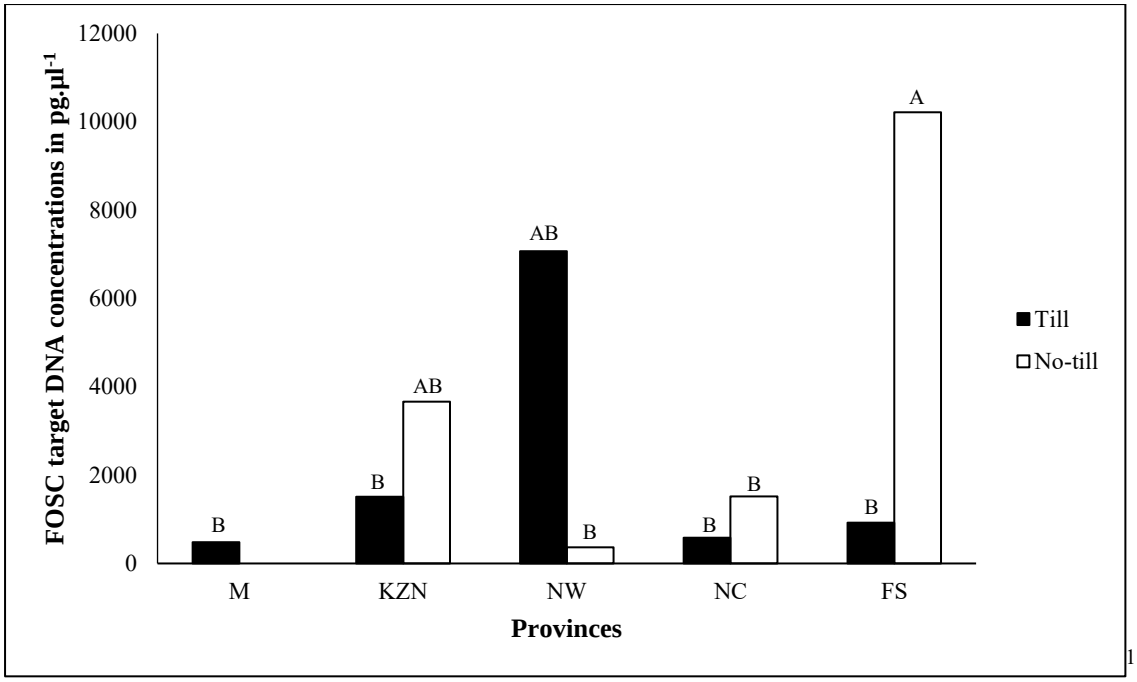
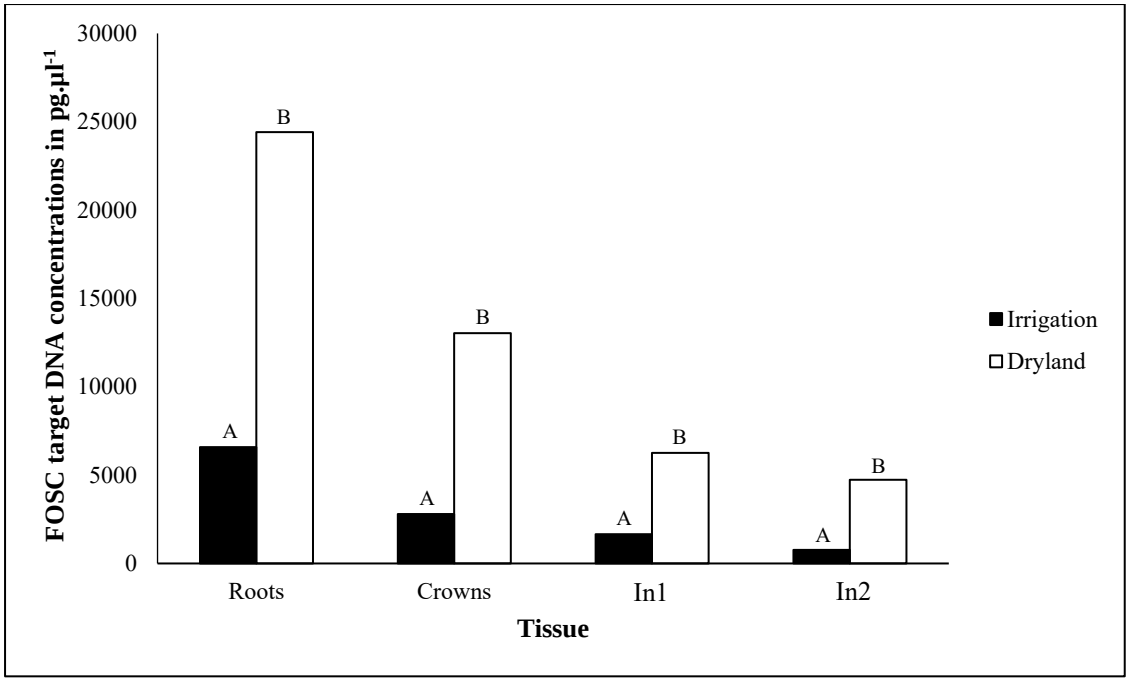


Figure 2.9: Tillage practice x province interaction for the mean FOSC target DNA concentration measured in maize In2 over the period 2012-2015. [3]

[1] pg.µl^{-1}-picogram per microliter

[2] Mpumalanga-MP, KwaZulu-Natal-KZN, North West province-NW, Northern Cape-NC, Free State-FS

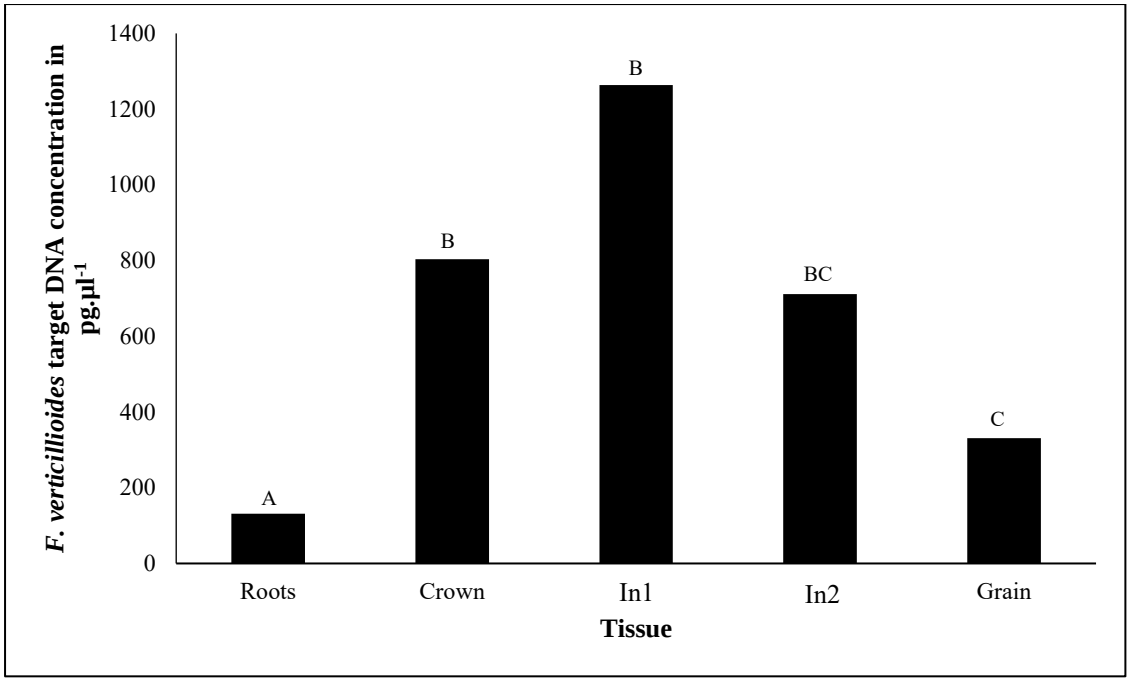
[3] A different letter denotes a significant difference at P=0.0029.



[1]; [2]

Figure 2.10: Irrigation practice main effect for the mean FOSC target DNA concentration in roots, crowns, In1 and In2 over the period 2012-2015. [3]

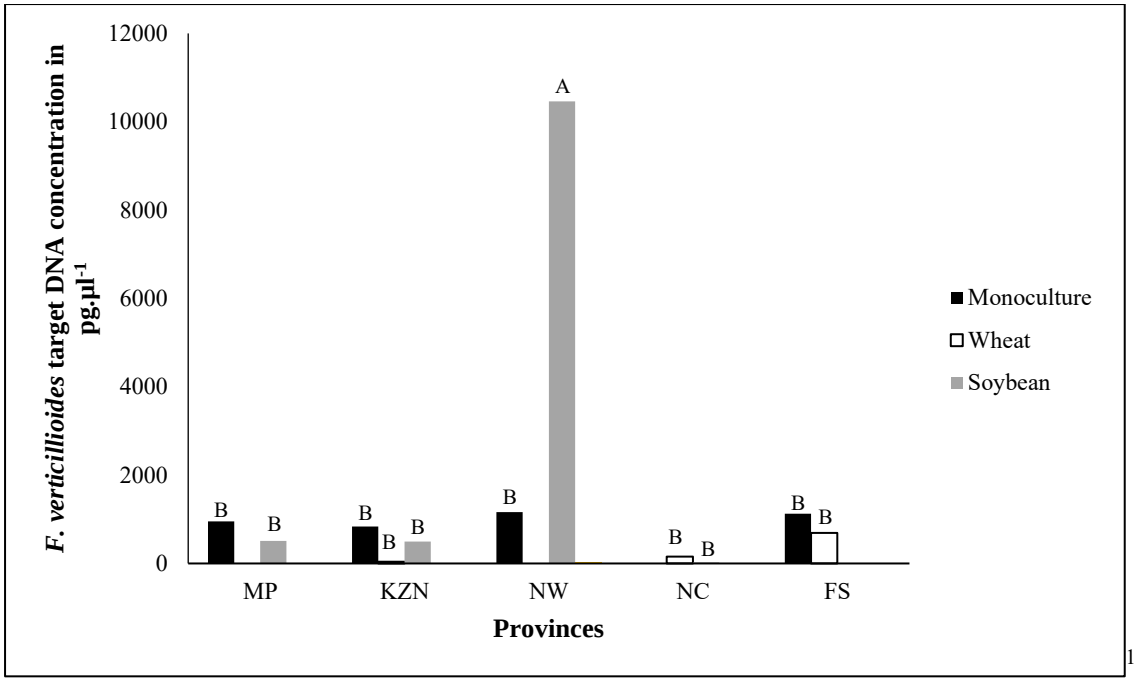
[1] pg.µl^{-1}-picogram per microliter
[2] In1-internode 1; In2-internode 2
[3] A different letter denotes a significant difference at $P<0.05$.



[1]; [2]

Figure 2.11: Mean *F. verticillioides* target DNA concentrations in maize roots, crowns, stalks and grains over the period 2012-2015. [3]

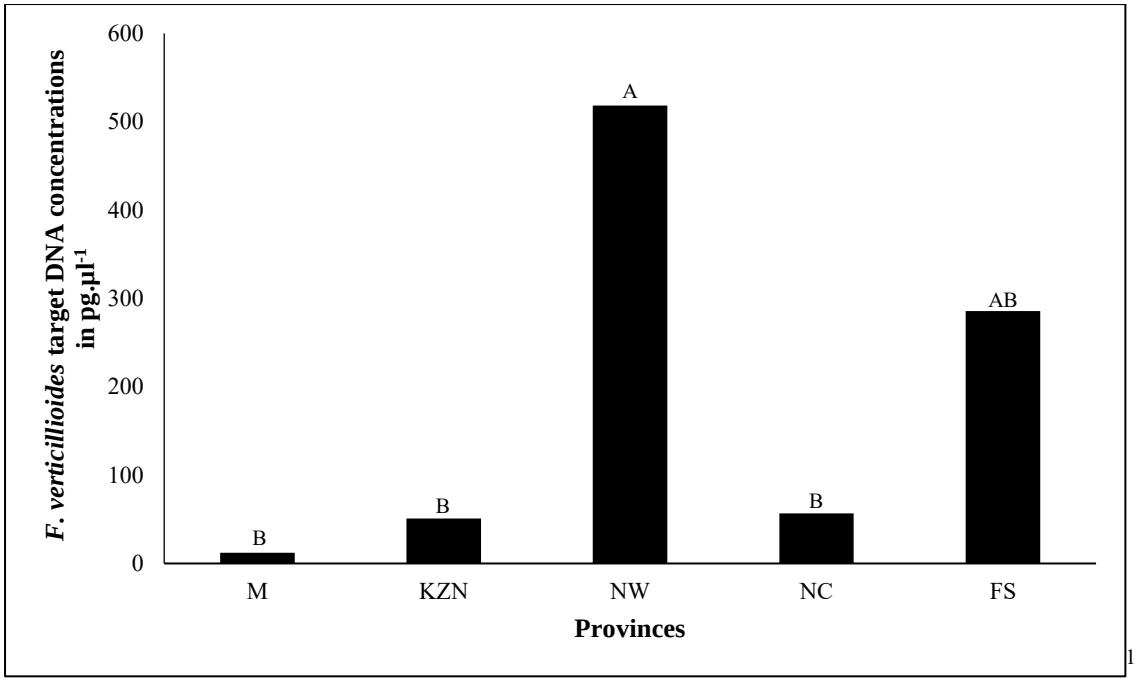
[1] pg.µl^{-1}-picogram per microliter
[2] In1-internode 1; In2-internode 2
[3] A different letter denotes a significant difference at P≤0.05.



[1], [2]

Figure 2.12: Province x crop rotation interaction for the mean *F. verticillioides* target DNA concentration measured in maize crowns over the period 2012-2015. [3]

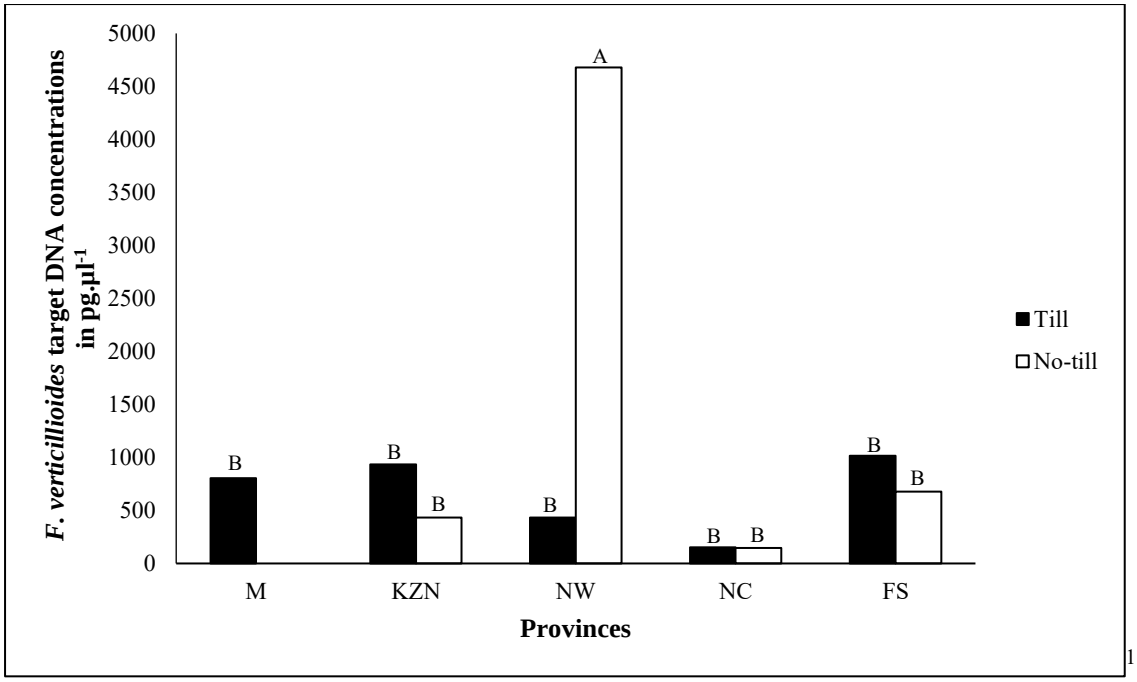
[1] pg.µl^{-1}-picogram per microliter
[2] Mpumalanga-MP, KwaZulu-Natal-KZN, North West province-NW, Northern Cape-NC, Free State-FS
[3] A different letter denotes a significant difference at P <0.0001.



[1], [2]

Figure 2.13: Province main effect for the mean *F. verticillioides* target DNA concentration measured in maize roots over the period 2012-2015. [3]

[1] pg.µl^{-1}-picogram per microliter
[2] Mpumalanga-MP, KwaZulu-Natal-KZN, North West province-NW, Northern Cape-NC, Free State-FS
[3] A different letter denotes a significant difference at P=0.0275.



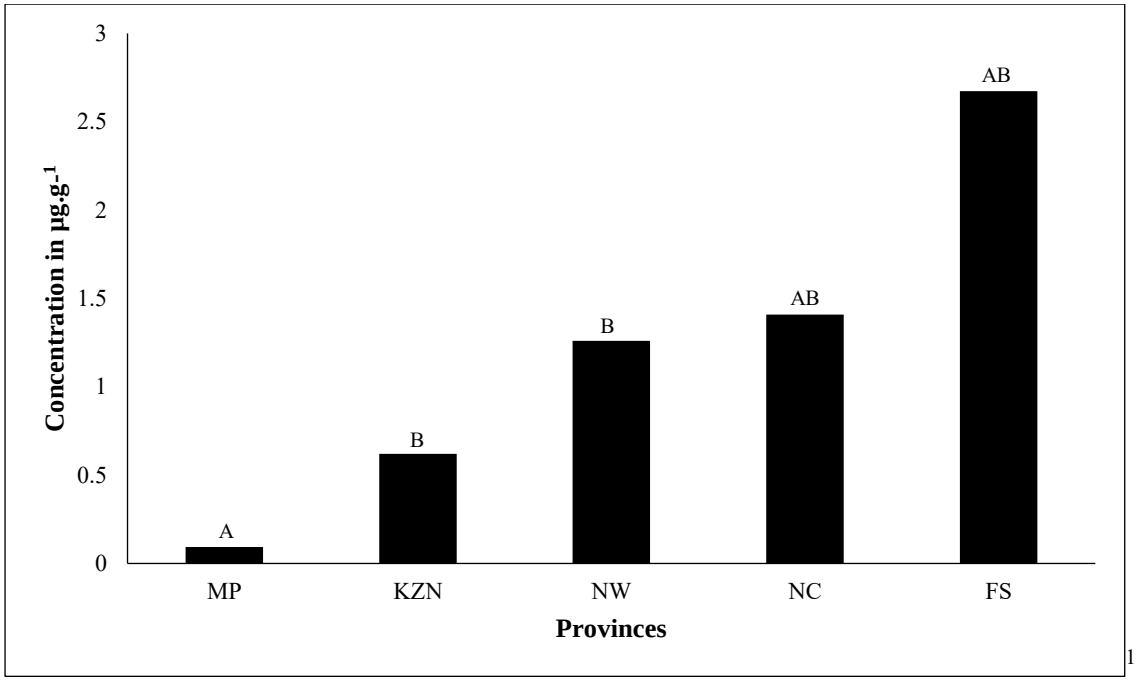
[1]; [2]

Figure 2.14: Tillage practice x province interaction for the mean *F. verticillioides* target DNA concentration measured in maize crowns over the period 2012-2015.[3]

[1] pg.µl^{-1}-picogram per microliter

[2] Mpumalanga-MP, KwaZulu-Natal-KZN, North West province-NW, Northern Cape-NC, Free State-FS

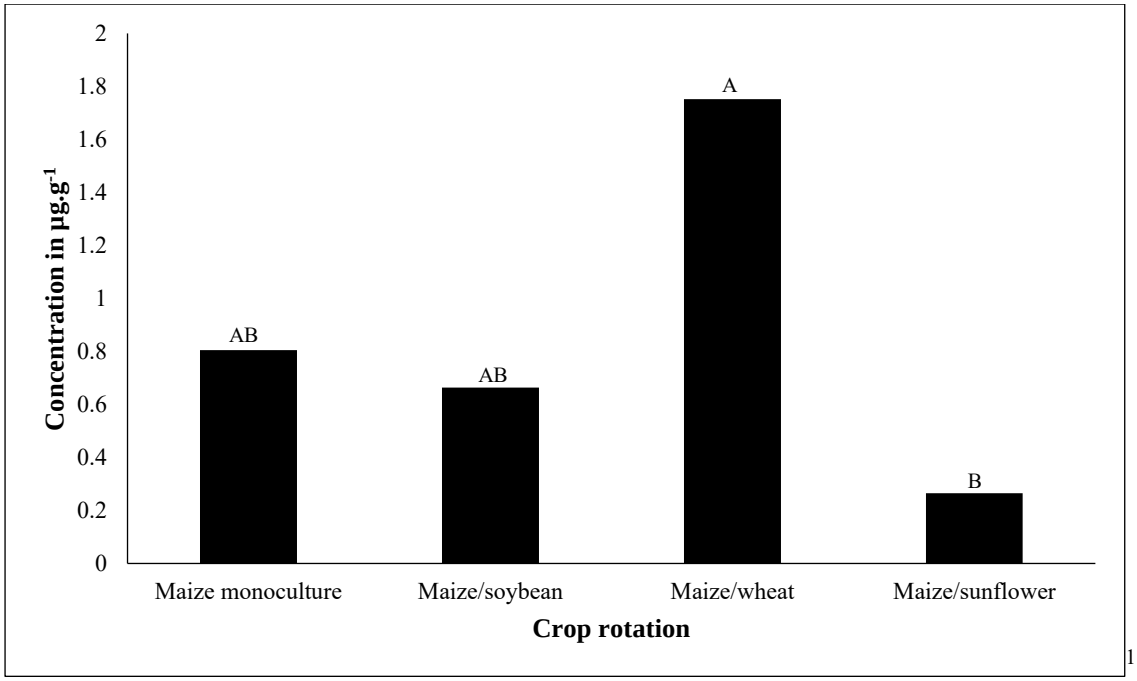
[3] A different letter denotes a significant difference at P=0.0003.



[1], [2]

Figure 2.15: Concentration of ZEA[3] in grain over the period 2012-2015. [4]

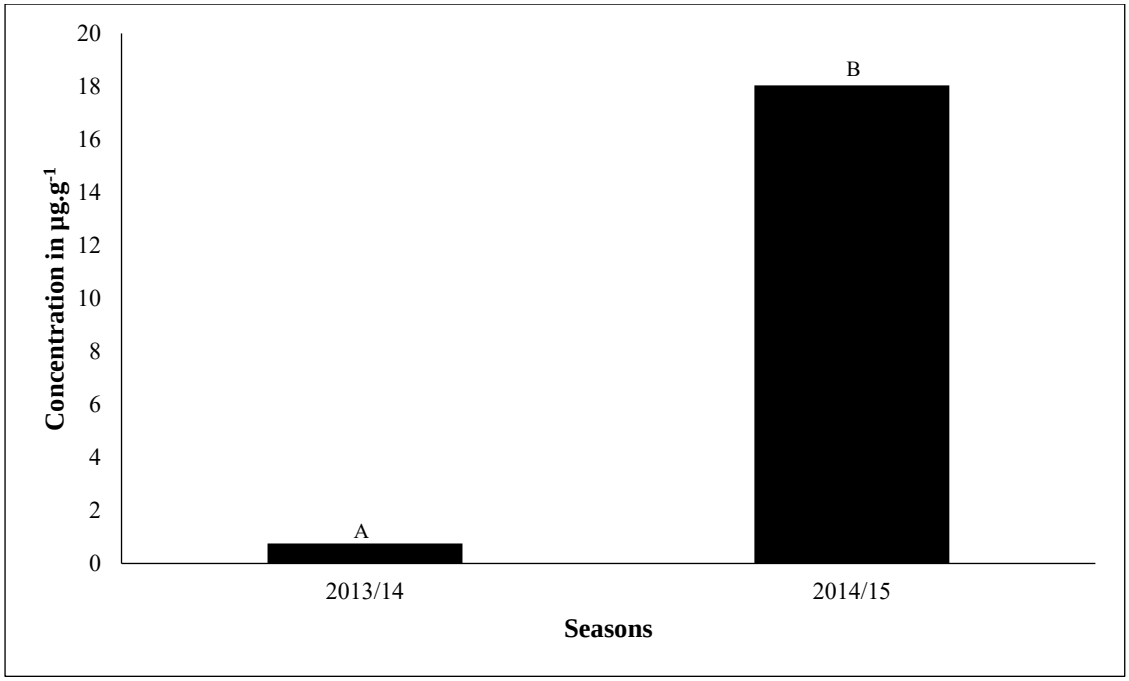
[1] µg.g^{-1}-microgram per gram
[2] Mpumalanga-MP, KwaZulu-Natal-KZN, North West province-NW, Northern Cape-NC, Free State-FS
[3] Zearalenone
[4] A different letter denotes a significant difference at P≤0.05.



[1]

Figure 2.16: Concentration of ZEA[2] in grain of maize monoculture, maize/soybean, maize/wheat and maize/sunflower rotations over the period 2012-2015. [3]

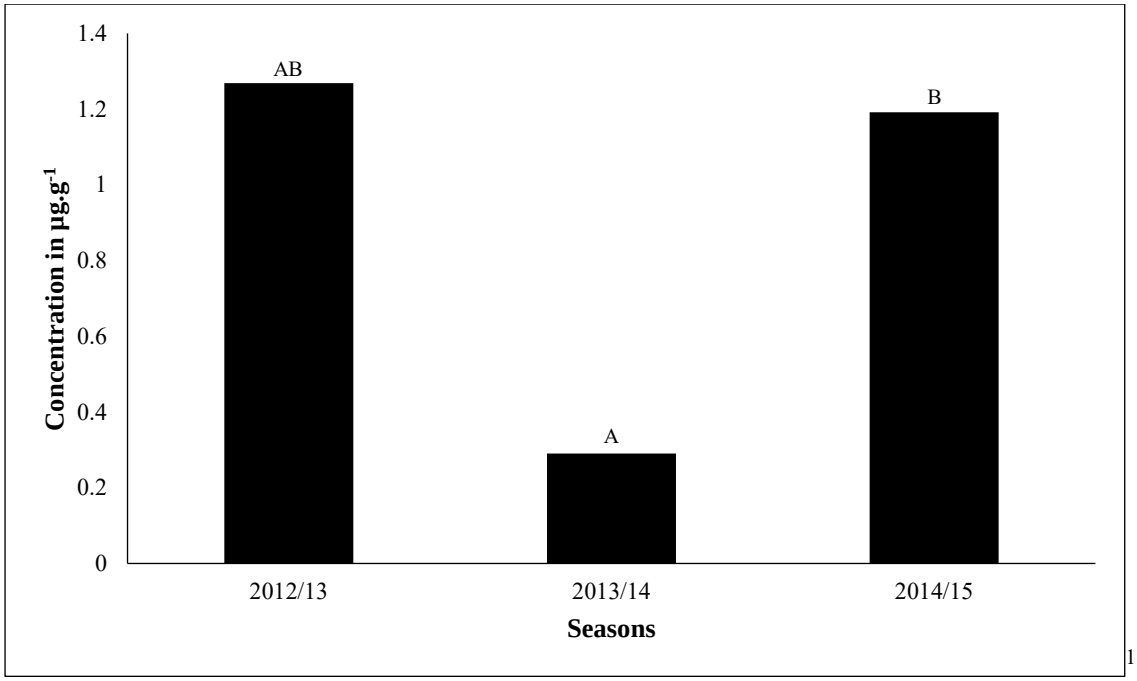
[1] µg.g^{-1}-microgram per gram
[2] Zearalenone
[3] A different letter denotes a significant difference at P≤0.05.



[1]

Figure 2.17: Concentration of DON[2] in the grain during 2013/14 and 2014/15 seasons, with no DON detected in grain, during the 2012/13 season. [3]

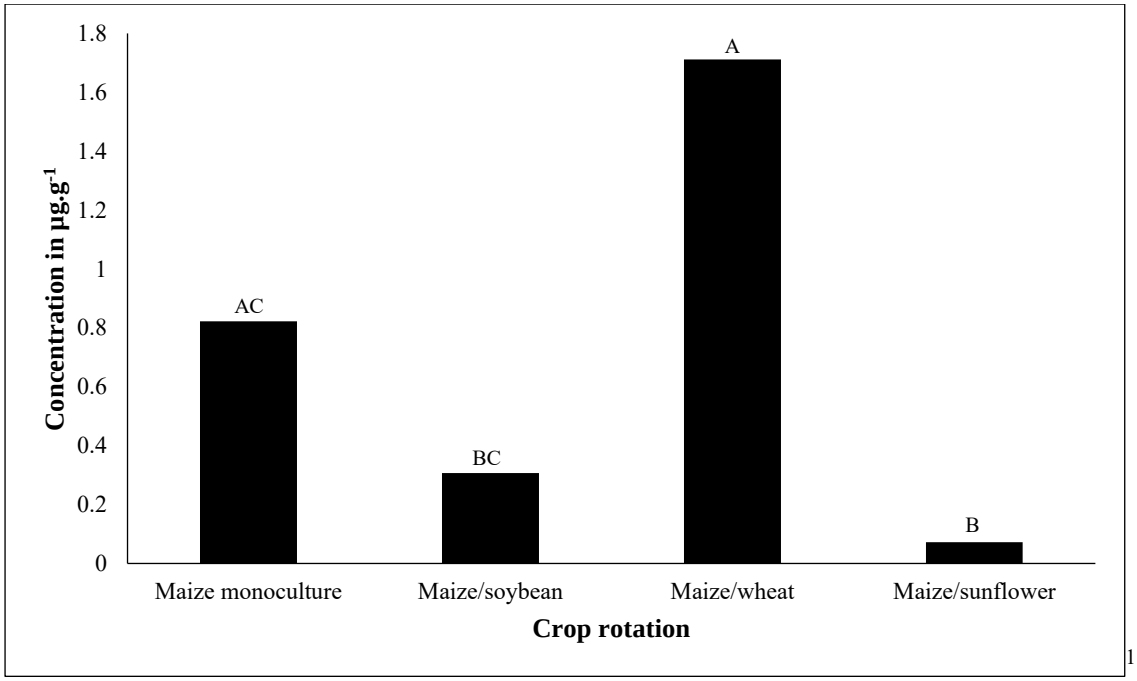
[1] µg.g^{-1}-microgram per gram
[2] Deoxynivalenol
[3] A different letter denotes a significant difference at P≤0.05.



[1]

Figure 2.18: Total FUM ($FB_1 + FB_2 + FB_3$)[2] in maize grain over three seasons. [3]

[1] µg.g^{-1}-microgram per gram
[2] FUM-Fumonisin; FB_1-Fumonisin group B_1; FB_2 Fumonisin group B_2; FB_3-Fumonisin group B_3
[3] A different letter denotes significance at $P \leq 0.05$.



[1]

Figure 2.19: Total FUM (FB1 + FB2 + FB3) in grain of maize monoculture, maize/soybean, maize/wheat and maize/sunflower rotations over the period 2012 to 2015.[2]

[1] µg.g^{-1}-microgram per gram
[2] A different letter denotes significance at P≤0.05.

Chapter 3: Analysis of fields infected with FGSC in the Douglas (Northern Cape) area

3.1 Abstract

Maize is a major food source in South Africa with an estimated consumption rate of between 475 to 690 g maize and maize-based products per person, per day. The Northern Cape region is a high potential maize production region, with high yields obtained under irrigation. During the 2012/13 maize season, areas with prematurely senescing plants were observed in seven maize fields. Although visual symptoms indicated Gibberella stalk rot, multiple tests were conducted to determine the etiology of the prematurely senescing plants. Maize samples were collected from three prematurely senescing areas (PSA) and three visibly green areas (VGA) over three seasons (2012/13, 2013/14 and 2014/15) in multiple fields. The *Fusarium graminearum* species complex (FGSC) was quantified in the roots, crowns, internode 1 (In1), internode 2 (In2) and grain using species-specific primers with quantitative PCR (qPCR). The mycotoxins deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) were quantified in maize grain using liquid chromatography-tandem mass spectrometry (LC-MS/MS). During the first season the concentration of micro- and macro-nutrients were determined in the soil and leaves. Soil penetrometer readings as well as physiological characters were observed during all three seasons. During the last season a sequential sampling was carried out, where the same tissues as mentioned were sampled at the 6-week, flowering, soft dough and physiologically mature growth stages. The roots, crowns, stalks and grain of both the VGA and PSA plants, had high concentrations of FGSC, with no trend being observed over three seasons. It should be noted, however, that during the first season the concentrations of FGSC in In1 and In2 were significantly higher than that quantified in the subsequent seasons. This indicates that the incidence of FGSC were higher during first season in maize stalks compared to the second and third seasons. The penetrometer and physiological characters did not differ between the PSA and VGA plants. Elements that differed significantly between VGA and PSA's were calcium (Ca), magnesium (Mg) and sodium (Na) in soils, and boron (B) and potassium (K) in leaves. Soil pH also differed between these areas with a slightly lower pH in VGAs. It was shown that these five nutrients impacted FGSC target DNA concentrations and increased (PSA) or decreased (VGA) the plants susceptibility. Ca increased disease incidence with a trend towards higher stalk rot incidence, whereas Mg, Na, K and B decreased disease incidence

by increasing overall plant health and decreasing disease incidence and severity by the various role they play in plant defence responses. It was also shown that during the soft dough stage, maize is the most sensitive towards plant disease. Thus, it could be seen that poor soil nutrition (environment), coupled with a susceptible plant (soft dough stage) and significantly high FGSC target DNA concentrations in In1 and In2 of season 2012/13 (virulent pathogens) resulted in the PSAs in maize fields.

Keywords: Disease triangle, FGSC, prematurely senescing, maize

3.2 Introduction

The annual yield loss of crops due to disease is difficult to determine because there is always some level of disease in fields (White and Carson, 1999). The incidence or severity of a disease is determined by three major elements including the susceptibility of the host, the virulence of the pathogen/pathogenic complex and optimum environmental conditions. This is known as the disease triangle, which is unique to plant disease since animal and humans can escape inhospitable environments (Agrios, 2005; Francl, 2007). Three other factors that influence disease are vectors, time and human activity (Francl, 2007).

Maize (*Zea mays* L.) was first introduced into Africa around 1500 (Miracle, 1965). Since then it has become a major food source throughout the continent. Maize is the largest summer field crop in South Africa followed by sunflower seeds, soybeans and groundnuts (DAFF, 2019). The South African maize consumption rate is estimated to be between 475 – 690 g of maize and maize-based products per person per day. Although the Northern Cape only accounts for approximately 700 000 tons of maize, which accounts for 6 % of maize production in South Africa, it is a high potential maize production system due to the availability of irrigation. During the 2012/13 season, yield was 12,5 $t.ha^{-1}$, in 2013/14, 13.1 $t.ha^{-1}$ and during 2014/15 and 13.4 $t.ha^{-1}$ (Anonymous, 2016)

Members of the *Fusarium graminearum* species complex (FGSC) cause disease in maize roots, crowns, stalks and ears (Payne, 1999; White, 1999). The complex consists of 16 species, three of which have been recorded on South African maize namely, *F. boothii*, *F. graminearum* s.s. and *F. meridionale*. *F. boothii* occurs in maize grain, whereas *F. graminearum* s.s. and *F. meridionale* are more likely to infect roots (Boutigny *et al.*, 2012). Infection by FGSC results in a loss of grain quality and yield if the ears become infected. Lodging of maize plants occurs if the stalks are infected. Another consequence of infection by FGSC is the potential of mycotoxins such as deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) (Popovski and Celar, 2013). Mycotoxins can cause acute or chronic toxicity in humans and animals (Tan *et al.*, 2012).

Environmental factors may have a direct or indirect influence on infection of host crops by FGSC (Popovski and Celar, 2013). Temperature has a direct effect on infection with an optimum between 26 – 28 °C (Mesterházy *et al.*, 2012). Water may influence pathogenic

activity directly by increasing the humidity within the crop canopy or indirectly by distributing the spores throughout the field (Trail, 2009). Environmental factors can be manipulated by farming practices e.g. irrigation can increase humidity and tillage practices may alter inoculum levels, both of which could lead to an increase in disease if not closely monitored (Schaafsma *et al.*, 2005). The final element for disease to occur is time. All these abovementioned factors must be present for some duration for disease to occur (Francl, 2007).

Quantitative PCR is the preferred method to quantify the concentration of FGSC species within host tissues. Since members of the FGSC are notorious for their slow growth the risk of contamination by fungi that could overgrow a petri dish and confound quantification of the role of FGSC in the infection process and tissue colonization is high. Furthermore, it has been shown that pathogen identification through molecular techniques are rapid, less laborious, more sensitive, specific and efficient (Adzitey *et al*., 2012). This method was therefore applied to quantify disease intensity in the current study.

The purpose of this study was to 1) compare FGSC target DNA concentrations in the roots, crowns, stalks and grain in maize from prematurely senescing areas within commercial maize production fields with those from visibly green areas (VGA) over three seasons (2012/13-2014/15), 2) quantify the abiotic factors that influenced the colonisation of PSA plants during the first season (2012/13) 3) quantify DON, NIV and ZEA in grain from PSA and VGA plants 4) quantify FGSC target DNA concentrations in the roots, crowns, stalks and grain in maize from PSA and VGA during four growth stages i.e. six week old plants, flowering, soft dough stage and before harvest 5) determine differences in growth rate between the maize in PSA and VGA.

3.3 Materials and methods

3.3.1. Collection and analysis of sample material during the 2012/13 season

During the 2012/13 season, seven fields in the Douglas area, Northern Cape, were selected for investigation due to visible disease outbreak (Table 3.1). Three PSA's were identified within each field based on plants showing symptoms and signs of infection by FGSC. The symptoms included pink discolouration of the stalks, stalks that was soft due to pith disintegration which resulted in lodging and degenerated root systems with pink discolorations. VGA's were

selected adjacent to PSA for comparison and to determine those factors influencing premature death or survival, respectively. Ten plants from both PSA and VGA were randomly selected at physiological maturity. The roots, crown, internode 1 (In1) and 2 (In2) and the first ear were removed from each plant. The average mass of ears before threshing, ear length, threshed grain mass, moisture content, 1000 kernel weight and threshing percentage were determined. The Griekwa Stad Cooperative (GWK) performed soil and leaf nutrient analyses as well as soil penetrometer readings. The nutrient analysis was only performed during the season (2012/13) that the symptoms were observed. Nutrient analysis would have been performed if symptoms occurred in the subsequent seasons (2013/14 and 2014/15), however as PSA's did not appear, no nutrient analysis were performed. Samples were collected during the 2013/14 and 2014/15 seasons at the same locations within the fields as the first season. This was done to evaluate if the pathogens are present even in the absence of symptoms (2013/14 season) and at which growth stage the plant is most susceptible (2014/15 season).

3.3.2 Total DNA isolation from infected maize material

The respective tissues from each of the ten plants collected were pooled. The roots, crowns and stalks, were ground in liquid nitrogen (Afrox, South Africa) with a mortar and pestle. The grain was milled using a Cyclotec sample mill (Foss Tecator, Hoganas, Sweden).

DNA was extracted from 250 mg of the respective ground tissues using an optimised CTAB method (Saghai-Maroof *et al.*, 1984). A 900 µl aliquot of CTAB buffer (containing β-mercaptoethanol (Merck KGaA, Darmstadt, Germany) (2 % CTAB (Sigma-Aldrich, Steinheim, Germany) 5 M NaCl (Merch KGa, Darmstadt, Germany), 1 M Tris (Merch KGa, Darmstadt, Germany), 0.5 M EDTA (Sigma-Aldrich, Steinheim, Germany), 0.2 % β-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany) pH 8.0) was added to the plant tissues in an Eppendorf tube. Cells were disrupted to facilitate extraction using the heat shock method which involves freezing the sample, using liquid nitrogen, followed by adding the sample to boiling water (95 °C) for 5 minutes. After the samples were cooled to room temperature, an RNase A/T1 mix (2 µl) (Fermentas, Hanover, USA) was added. The samples were incubated for an hour at 37 °C, after which 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) (phenol - Sigma-Aldrich, Steinheim, Germany; Chloroform - Merch KGa, Darmstadt, Germany; isoamyl alcohol – Merck, KGaA, Darmstadt Germany) were added. The samples

were vortexed for a minute to ensure even mixing of the phenol:chloroform:isoamyl alcohol mixture. This was followed by a centrifugation step at 15294 x g for 10 minutes. The supernatant was transferred to a fresh tube and another 500 µl phenol:chloroform:isoamyl alcohol was added. After the sample was vortexed and centrifuged at 15294 x g for 10 minutes, the supernatant was transferred to a fresh tube containing 550 µl ice cold isopropanol (Merch KGa, Darmstadt, Germany). The DNA were precipitated at 20 °C for 30 minutes, followed by centrifugation at 4 °C for 20 minutes. The supernatant was discarded. The pellet was washed with 70 % ethanol (Merch KGa, Darmstadt, Germany) by centrifuging the samples for 5 minutes at 20 817 x g. The excess liquid was removed, and the residual liquid was left to evaporate. A 1X TE buffer (AppliChem GmbH, Darmstadt, Germany) was used to resuspend the dried pellet, after which the DNA was quantified and the quality evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc, Wilmington, USA). If the samples were below the acceptable A260/A280 ratio of 1.8 – 2, they were discarded and re-extracted. The DNA was diluted to 10 ng for qPCR analysis and stored at -20 °C until used.

3.3.3 qPCR

Quantitative PCR analyses were performed, to determine the concentration of FGSC target DNA within each of the respective tissues using the primer pair FgramB379 (CCA TTC CCT GGG CGT) and FgramB411 (CCT ATT GAC AGG TGG TTA GTG ACT GG) (IDT, Iowa, USA) (Nicolaisen *et al.*, 2009; Schoeman, 2016). The reaction mixture consisted of 0.8 ng DNA, 1X iTaq Universal Sybr Green supermix and 0.2 µM of each primer. Three technical replicates were included for each sample. The samples were amplified in a CFX96 (Bio-Rad) using the following conditions: 95 °C for 10 minutes; 40 cycles of 95 °C for 15 seconds; 60 °C for 15 seconds and 72 °C for 15 seconds. A melt curve was performed to determine if non-target products were amplified. This was done by heating the products to 95 °C for 30 seconds then allowing them to cool to 40 °C for 30 seconds followed by gradually heating the products from 60 °C to 95 °C with a 1 °C increment every 10 seconds. Any samples that showed non-target amplification were disqualified. The amplified samples were then compared to a standard curve.

A standard curve was generated by diluting FGSC DNA of known concentration 4x, 16x, 64x, 256x, 1024x, and 4096x in maize DNA that was free of fungal contamination. This maize DNA was obtained by growing maize grain on a sterile Water Agar medium. The maize kernel was

sterilised with a 1 % Sodium hypochloride solution before it was placed on the sterile Water Agar medium. After two weeks growth a maize seedling from cultures free of fungal growth was ground using liquid nitrogen and DNA was extracted using the above protocol.

3.3.4 LC-MS/MS

Milled maize grain from the respective samples flour was used to make 1:4 (m/v) slurry with 70:30 Methanol:H_2O solution in a 50 ml NEST centrifugation tube (Wuxi NEST biotechnology Co., Ltd, Jiangsu, China). The tube was vortexed to ensure even mixing after which it was placed on a Labotec orbital shaker for 30 minutes at 200 rpm. The NEST centrifugation tube was centrifuged at 500 x g for 10 minutes at 4 °C. The supernatant was removed with a syringe and filtered through a 0.25 µm Acrodisc® syringe filter with Supor® Membrane (Pall Corporation, New York, USA). To ensure thorough precipitation of unwanted particles, the supernatant was incubated overnight at 4 °C. The filtrate was then centrifuged at 14 000 rpm for 10 minutes. The supernatant (2 ml) was transferred to a clean tube and sent for LC-MS/MS analysis at the Central Analytical Facility (CAF) at the Stellenbosch University.

3.3.5 Statistical analysis

A factorial analysis of variance (ANOVA) was performed for the FGSC target DNA concentrations in the respective tissues, grain characters as well as nutrient concentrations in soil and leaves. Localities, seasons, and areas-within-fields (PSA/VGA) were used as factors for comparison purposes. The means of the significant effects were separated using Fisher's unprotected t-test (Least Significant Difference) at 5 % level of significance (Shapiro and Wilk, 1965; Montgomery, 1984). Simple and non-linear regression (NCSS 2007) was performed to determine the relationship between mean nutrient concentrations in soil and leaves, and FGSC target DNA concentrations in various tissues. Relationship between grain characteristics and FGSC target DNA concentrations in various tissues were also determined. T-tests were performed on nutrient concentrations between the PGA and VGA soil and leaves. The mycotoxins could not be analysed due to insufficient data points.

3.3.6 Collection and analysis of sample material during the 2013/14 season

During the 2013/14 season, only four of the seven original fields were planted with maize. The protocol as outlined above was followed except that nutrients and penetrometer readings were not included. Regression analyses were performed to determine the relationship between grain characteristics and FGSC target DNA concentrations in various tissues as well between FGSC target DNA concentrations in various tissues and mycotoxin concentrations in grain.

3.3.7 Collection and analysis of sample material during the 2014/15 season

During the 2014/15 season six of the original seven fields were planted with maize. Maize was sampled at four growth stages namely 6 weeks, flowering, soft dough stage and physiological maturity. FGSC target DNA was determined in all the tissues to establish a timeline of infection. Plant length and leaf surface areas were measured at the 6 weeks growth stage and physiological maturity. Ear mass prior to threshing, threshed grain mass, 1000 kernel weight, root discolouration and root volume were determined at physiological maturity. The protocol as outlined above was followed except that nutrients and penetrometer readings were not performed.

ANOVA was performed for the FGSC target DNA concentrations, at physiological maturity and across the four growth stages, grain characters and phenotypic characters. Regression analyses as described above were conducted. T-tests were performed on leaf surface area between the PGA and VGA soil and leaves.

3.3.8 Analysis of sample material over three seasons

Analysis of variance was conducted using combined data from the localities common to all seasons i.e. fields, A, E and F. Regressions analyses were performed as described above.

3.4 Results

3.4.1 Evaluation of PGA and VGA plants during 2012/13

3.4.1.1 General observations

PSA's (Figure 3.1) occurred after heavy rainfalls, approximately four months after planting and were typified by large areas of lodging (Figure 3.2). Examination of stalk symptoms typified by shredded stalk tissues and pink discoloration of the stalk suggested infection by FGSC associated with high inoculum levels and optimum weather conditions.

3.4.1.2 FGSC target DNA

A significant difference in FGSC target DNA concentrations in maize crowns (P=0.006) and In1 (P=0.03) were observed, but this observation was attributed to high concentrations in Field E (Figures 3.3 and 3.4). The remaining localities did not differ significantly from each either, neither did the PSA and VGA plants. FGSC target DNA in roots, In2 and grain did not differ significantly between localities or areas-within-fields. Although, when the mean of the PSA FGSC target DNA concentrations in In2 (1664.76 pg/µl) and the grain (44.78 pg/µl) were compared to that of VGA FGSC target DNA concentrations in In2 (1183.51 pg/µl) and grain (28.19 pg/µl), there was a trend were the mean across all localities were higher in the PSA plants compared to the VGA plants.

3.4.1.3 Physiological character comparison

During the 2012/13 season the weight before and after threshing of field A differed significantly (P<0.001) from that of field B but no significant differences were noted between these two localities and the remaining five localities or between the remaining five localities (Figure 3.5a and c). The mean weight before and after threshing of the seven localities in the VGA did not differ significantly from the mean weight before and after threshing of the seven localities in the PSA (Figure 3.5b and d). Average ear length was significantly higher in PSA plants compared to VGA plants (P=0.003) (Figure 3.5e), while the 1000 kernel weight (P=0.001) (Figure 3.5f) was significantly higher in the VGA plants compared to the PSA plants. The grain moisture (P=0.01) (Figure 3.5g) of plants in field E were the highest and were

significantly higher than fields A, B, C, D and G but did not differ significantly from field F. The grain moisture of field F were significantly higher than that of field A. There was no significant difference between the grain moisture of fields B, C, D, F and G. Within a field, the grain moisture of the PSA plants did not differ from that of the VGA plants. The grain moisture of the VGA plants was significantly higher (P=0.001) than that of the PSA plants (Figure 3.5h). The threshing percentage between differed significantly (P=0.001) between localities (Figure 3.5i). There were no significant differences between fields A, E and F. Fields A and E had a higher threshing percentage than fields C, D and G (Figure 3.5j). All localities had a significantly higher threshing percentage than field B. Within a field, the threshing percentage of the PSA plants did not differ from that of the VGA plants. The penetrometer analysis done on the PSA soil showed that it did not differ significantly from the VGA soil (data not shown).

3.4.1.4 Nutrient evaluation in soil and leaves

A comparison of the concentration of nutrients in soil showed that sodium (Na) (P<0.003) (Figure 3.6a) was significantly higher in field C compared to the rest of the localities. The Na concentration did not differ significantly between the PSA and VGA soil, even when the mean Na concentrations were considered (Figure 3.6b). The concentration of potassium (K) in the soil (P<0.001) (Figure 3.6c) were significantly higher in fields B, C and F compared to the rest of the localities. Field E had significantly the lowest K concentration compared to the rest of the localities. However, there were no significant differences between the K concentrations of the VGA and PSA soils, within the localities or when the mean of all the localities are combined (Figure 3.6d). A significant locality x areas-within-field interaction effect (P=0.016) were recorded for magnesium (Mg) ($mg.kg^{-1}$) in soil (Figure 3.6e). This interaction showed that the VGA soil within field D had significantly higher concentrations of Mg than the PSA soil of field D. Although the remaining localities had various concentrations of Mg in the soils, the Mg concentrations of PSA and VGA soils within localities did not differ significantly from each other. The mean concentration of manganese (Mn) in the soil of PSA did not differ significantly from that of the VGA soil when the localities values are combined. There is however a tendency for the concentration of Mn to be higher in VGA soil compared to PSA soil (Figure 3.6f). The concentration of zinc (Zn) (P<0.001) (Figure 3.7g) were significantly higher in field F's soil compared to the other localities with field A having the lowest concentration. However, the concentration of Zn did not differ significantly between the PSA and VGA soil, even when the mean Zn concentrations of all localities were considered (Figure

3.6h). The concentration of Mn (P=0.001) (Figure 3.6i) in the soil were significantly higher in fields B, C and F compared to fields A, D, E and G. The PSA and VGA concentration of Mn did not differ significantly from one another, even when the mean Mn concentrations of all localities were considered (Figure 3.6j). The concentration of copper (Cu) (P=0.003) (Figure 3.6k) in the soil of field B, E and F were significantly higher than that of fields A and D. However, the concentration of Cu did not differ significantly between the PSA and VGA soils, even when the mean Cu concentrations of all localities were considered (Figure 3.6l).

There was no significant difference in the pH of the different localities PSA and VGA soils (Figure 3.6m), however the pH (P=0.04) of the soil was significantly higher in the PSA compared to the VGA when the mean of all the localities were considered (Figure 3.6n). The percentage calcium (Ca) (P=0.003) in the soil of field B was significantly the lowest when compared to the other localities. However, the VGA and PSA soils of the individual localities did not differ from each other (Figure 3.6o). When the mean percentage of Ca of all the localities PSA soil was compared to the VGA soil, a significant difference was observed (Figure 3.6p). The percentage Ca in the PSA soil were significantly higher compared to that of the VGA soil. The percentage Mg (P<0.001) in the soil of field C was significantly higher than fields A, B, D and G (Figure 3.6q). Although the percentage Mg in the PSA soil did not differ significantly from the VGA soil, a significant difference was observed when the mean of all the localities PSA soil was compared to that of the VGA soil. It showed that the percentage Mg was significantly higher in the VGA soil compare to the PSA soil (Figure 3.6r). The percentage K (P<0.001), in the soil of fields B and C were significantly higher than fields A, D, E and G. However, the VGA and PSA soils of the individual localities did not differ from each other (Figure 3.6s). The mean of all the localities PSA soil was compared to that of the VGA soil and they did not differ significantly from each other (Figure 3.6t). The percentage Na (P=0.003), in the soil of field C was significantly higher than the rest of the localities, with field F's soil being significantly the lowest (Figure 3.6u). The percentage Na of the individual localities PSA and VGA soil did not differ significantly from each other, however when the mean PSA and VGA Na percentage of the soil was compared a significant difference was observed. The VGA soil had significantly higher percentage Na compared to the PSA soil. (Figure 3.6v).

The percentage of iron (Fe) (P=0.003) in the leaves differed significantly between the localities, but only if the mean of the VGA and PSA percentages of that field was considered (Figure

3.7a). The leaves of fields F, G and A had significantly the highest Fe percentage compared to field D and E. When the mean Fe percentage of the PSA and VGA leaves was considered across all localities, they did not differ significantly from each other (Figure 3.7b). The percentage of Mn (P=0.01) in the leaves differed significantly between the localities, but only if the mean of the VGA and PSA of that field was considered (Figure 3.7c). The leaves of field E contained the highest percentage Mn when compared to the rest of the localities. When the mean Fe percentage of the PSA and VGA leaves was considered across all localities, they did not differ significantly from each other (Figure 3.7d). A significant locality x areas-within-field interaction effect (P=0.03) were recorded for the percentage Zn in leaves (Figure 3.7e). Two localities showed a significant difference between the percentage Zn in PSA leaves compared to the VGA leaves. Field E showed significantly higher percentage of Zn in VGA leaves compared to PSA leaves, whereas field G showed significantly higher percentage Zn in PSA leaves compared to VGA leaves. The rest of the localities had various Zn percentages, but the percentage Zn in the PSA leaves did not differ significantly from the VGA leaves. When the mean Zn percentage of the PSA and VGA leaves was considered across all localities, they did not differ significantly from each other (Figure 3.7f). The percentage nitrogen (N) (P=0.001) in the leaves varied between localities but only if the mean of the PSA and VGA leaves were considered (Figure 3.7g). Fields C, E, F and G had significantly higher percentage N compared to the other localities, with field B having statistically the lowest percentage N. When the mean N percentage of the PSA and VGA leaves was considered across all localities, they did not differ significantly from each other (Figure 3.7h). The percentage molybdenum (Mo) (P=0.002) were significantly higher in fields A and E compared to the other localities but only if the mean of the VGA and PSA percentages were considered (Figure 3.7i). When the mean Mo percentage of the PSA and VGA leaves was considered across all localities, they did not differ significantly from each other (Figure 3.7j). The percentage Ca in fields D and F were significantly the highest compared to fields B, C, and G, and field E was significantly higher than field G but only if the mean of the VGA and PSA percentages were considered (Figure 3.7k). When the mean Ca percentage of the PSA and VGA leaves was considered across all localities, they did not differ significantly from each other (Figure 3.7l). The percentage Mg (P=0.001) the leaves of field E was significantly the highest compared to the rest of the localities and field G the lowest, but only if the mean PSA and VGA percentages were considered (Figure 3.7m). When the mean Mg percentage of the PSA and VGA leaves was considered across all localities, they did not differ significantly from each other (Figure 3.7n). The percentage K (P=0.001) in field E was significantly lower than the other localities, but

only if the mean PSA and VGA percentages were considered (Figure 3.7o). However, when the mean of all the localities percentage K of PSA leaves were compared to the VGA leaves a significant difference was observed. The VGA leaves had significantly higher K percentages than the PSA leaves (Figure 3.7p). The percentage of Na (P=0.001) were significantly higher in localities C compared to the other localities but only if the mean PSA and VGA percentages were considered (Figure 3.7q). When the mean Na percentage of the PSA and VGA leaves was considered across all localities, they did not differ significantly from each other (Figure 3.7r). A significant locality x areas-within-field interaction effect (P=0.02) were recorded for sulphur (S) percentage in soil (Figure 3.7s). Only fields E and G showed a difference between the percentage S in PSA leaves compared to VGA leaves. In field E the VGA leaves had a higher percentage S compared to the PSA leaves, whereas in field G the PSA leaves had a higher percentage S compared to the VGA leaves. When the mean S percentage of the PSA and VGA leaves was considered across all localities, they did not differ significantly from each other (Figure 3.7t).The percentage phosphorus (P) in the leaves were significantly higher in field E compared to the other fields (Figure 3.7u). Field G had significantly higher P in the PSA leaves compared to the VGA leaves. When the mean P percentage of the PSA and VGA leaves was considered across all localities, they did not differ significantly from each other (Figure 3.7v). The percentage boron (B) (P=0.001) in the VGA leaves were significantly higher than that of the PSA when the mean of all the localities was considered (Figure 3.7w).

Highly significant relationships were recorded between the K (R^2 =0.99) and Mn ($R^{2=}$0.94) concentration in the soil and the FGSC target DNA concentration in the crowns (Figure 3.8). A similar significant relationship was recorded between nitrogen (R^2=0.86), Na (R^2=0.72), Mg (R^2=0.72), Mn (R^2=0.98) and Zn (R^2=0.93) in the leaves and the FGSC target DNA concentration in the crowns (Figure 3.9). Significant relationships were also recorded between K concentrations (R^2=0.80) and K percentages (R^2=0.69), as well as Mn (R^2=0.68) in soil and FGSC target DNA concentration in maize In1 as well as between Ca (R^2=0.53) in soil and FGSC target DNA concentration in In2 (Figures 3.10 and 3.11). A significant relationship was evident between soil density (R^2=0.93), Na (R^2=0.77) and Zn (R^2=0.83) in the soil, and FGSC target DNA concentrations in maize grain (Figure 3.12).

Mycotoxin levels were low during the 2012/13 season in all seven localities (data not shown). ZEA was detected in field F in a single PSA at 0.15 $\mu g.ml^{-1}$. DON was detected at the same point at 0.93 $\mu g.ml^{-1}$ and in field G in a VGA at 0.06 $\mu g.ml^{-1}$. 15-acetyl-DON (ADON) was

detected in field A at two VGA's at an average of 0.02 µg.ml^{-1}, in field B in a single VGA at 0.02 µg.ml^{-1} and in field F at two PSA's at 0.08 and 0.02 µg.ml^{-1}.

3.4.2 Evaluation of PSA and VGA plants during 2013/14

During season 2013/14 four localities were planted with maize. Although FGSC was quantified in the roots (9.98 pg.µl^{-1}), crowns (243.93 pg.µl^{-1}), In1 (283.52 pg.µl^{-1}), In2 (1216.27 pg.µl^{-1}) and grain (52.94 pg.µl^{-1}), there were no significant differences between localities or areas-within-fields.

Ear mass before threshing (P=0.01) were significantly affected by locality (Figure 3.13a). Field E had significantly higher ear mass before threshing compared to fields F and G but only if the mean of the PSA and VGA ear mass before threshing was considered. There was no significant difference between VGA and PSA ear mass before threshing when the mean of all localities was considered (Figure 3.13b). The same was observed for ear mass after threshing (Figure 3.13c and d). The grain moisture (P=0.02) of the PSA were significantly higher than that of the VGA, when the mean of all the localities are combined (Figure 3.13e). The 1000 kernel weight (P=0.03) of the VGA localities were significantly higher than the weight of the PSA localities, when the mean of all the localities are considered (Figure 3.13f).

A highly significant relationship between ear mass before threshing (R^2=0.80) and threshed grain mass (R^2=0.85) with FGSC target DNA concentration in maize roots was recorded (Figure 3.14). A significant relationship (R^2=0.77) was observed between the threshing percentage and the FGSC target DNA concentration in maize In2 (Figure 3.15). Although DON (0.04 – 2.05 µg.ml^{-1}) and ZEA (0.10 – 5.82 µg.ml^{-1}) were detected during the 2013/14 season, the concentration did not differ significantly between the VGA and PSA plants or between localities. Significant relationships between DON and FGSC target DNA concentration in the crowns (R^2=0.74) and In1 (R^2=0.76) were recorded (Figure 3.16).

3.4.3 Evaluation of PSA and VGA plants during 2014/15

During the 2014/15 season six localities were planted with maize. A significant locality x areas-within-field interaction (P=0.02) was observed for FGSC target DNA in In1 (Figure 3.17). The concentration of FGSC target DNA in In1 of the VGA plants of field B was significantly higher

than the concentration of FGSC target DNA in In1 of all the other localities PSA and VGA plants. When the mean concentration of FGSC target DNA in the crowns of the PSA and VGA plants were combine, it was found that field B had significantly higher target DNA concentrations compared to the rest of the fields (Figure 3.18). The mean concentration of FGSC target DNA in In2 of PSA and VGA plants were determined (Figure 3.19). It was found that the FGSC target DNA concentrations in In2 were significantly (P=0.01) higher in field C compared to fields A, D, E and F. Field B had significantly higher concentrations than field D and F with not differing significantly from each other. FGSC target DNA concentrations in the roots and grain did not differ significantly between PSA and VGA plants or between localities

Ear mass before threshing (P<0.001) showed significant difference between localities but only if the mean PSA and VGA ear mass was considered (Figure 3.20a). Field C's ear mass before threshing were significantly higher than fields A, B, D and F. Field E's ear mass before threshing was also significantly higher than fields A, B and D. The ear mass before threshing of the PSA plants did not differ significantly from the VGA plants when the mean ear mass before threshing across all localities was considered (Figure 3.20b). Ear mass after threshing (P<0.001), also showed a significant difference between localities but only if the mean PSA and VGA ear mass was considered (Figure 3.20c). Fields C and E had significantly higher ear mass after threshing compared to fields A, B, D and F, whereas field F were significantly higher than fields A and B. The ear mass after threshing of the PSA plants did not differ significantly from the VGA plants when the mean ear mass after threshing across all localities was considered (Figure 3.20d). The 1000 kernel weight (P=0.003) of fields C and E were significantly higher than fields A, B, and D with field C also being significantly higher than field F (Figure 3.20e). Field F's 1000 kernel weight was significantly more than field B. This is however only true when the mean 1000 kernel weight of the PSA and VGA plants are considered. The 1000 kernel weight of the PSA plants did not differ significantly from the VGA plants when the mean 1000 kernel mass across all localities was considered (Figure 3.20f). The threshing percentage (P<0.001) of fields A and B were significantly higher compared to fields C, E and F but only when the mean of the PSA and VGA plants were considered (Figure 3.20g). When the mean threshing percentage of VGA and PSA plants were considered, they did not differ significantly from each other (Figure 3.20h). The average ear length (P<0.001) of fields C and E were significantly higher than fields A, B and D with field C also being significantly higher than field F (Figure 3.20i). Field F's average ear length was

significantly higher than field B. When the mean of average ear length of PSA and VGA plants were considered, they did not differ significantly from each other (Figure 3.20j)

The root volume of physiologically mature plants (P=0.003) were significantly higher in field E compared to the rest of the fields, but this is only true if the mean of the PSA and VGA plants were considered (Figure 3.21a). Field D had statistically the lowest volume of all the localities. The mean root volume of physiologically mature plants did not differ between PSA and VGA plants when the mean across all six fields were considered (Figure 3.21b). The root length of physiologically mature plants (P=0.02) in field B were statistically the longest compared to fields A, D, E and F, with fields A, C, F being statistically longer than fields D and E (Figure 3.21c). However, this only applied if the mean PSA and VGA of each locality was considered. The root length of physiologically mature PSA and VGA plants did not differ significantly from each other when the mean over six localities was considered (Figure 3.21d). The effective root volume (P<0.001), of physiologically mature plants of field E were significantly the highest compared to the other localities, with fields A and F being significantly higher than field D (Figure 3.21e). The effective root volume of physiologically mature plants of VGA and PSA plants did not differ significantly from each other when the mean across the six localities were considered (Figure 3.21f). The effective root length of physiologically mature plants (P=0.02) were significantly higher in field B and C compared to fields D, E and F (Figure 3.21g). This only applied when the mean PSA and VGA of each locality was considered. The PSA and VGA's effective root length of physiologically mature plants did not differ significantly from each other when all six localities were considered (Figure 3.21h). The plant length of 6-week-old plants (P<0.001) were significantly longer in fields D, E and A compared to the rest of the localities with field C having statistically the shortest (Figure 3.21i). This only applied when the mean PSA and VGA of each locality was considered. The PSA and VGA's plant length of 6-week-old plants did not differ significantly from each other when all six localities were considered (Figure 3.21j). The plant length at physiological maturity (P<0.001) showed the same pattern as was found during the 6-week measurement (Figure 3.21k and l).

The third-, fifth- and eight leaf surface area, determined at 6 weeks and physiological maturity of both the VGA and PSA (Figure 3.22) indicated significant differences between PSA and VGA leaves at certain localities, although no consistent pattern emerged. At 6 weeks the PSA plants' leaf surface area of the third leaf was significantly higher than the VGA plants' leaf surface area of the third leaf in field A but in field B the opposite is true (Figure 3.22a). The

PSA plants' leaf surface area of the fifth leaf was also significantly higher than that of the VGA plants' leaf surface area of the fifth leaf of fields A and F but the opposite is true for field B (Figure 3.22c). At physiological maturity the PSA plants' leaf surface area of the third leaf was significantly higher than the VGA plants' leaf surface area of the third leaf in field D but not in the rest of the localities (Figure 3.22b). Whereas the VGA plants' leaf surface area of the fifth leaf was significantly higher than that of the PSA plants' leaf surface area of the fifth leaf of field D (Figure 3.22d).

A significant locality x areas-within-fields interaction was observed when the FGSC target DNA concentrations were quantified in the crowns ($P \leq 0.05$) (Figure 3.23). However, only two localities, namely fields C and E, showed a significant difference in FGSC target DNA concentration in the crowns. Field C had significantly higher FGSC target DNA concentrations in the VGA's crowns than in the PSA's crowns however in field E the opposite is true. A significant growth stage x locality x areas-within-field interaction ($P \leq 0.05$) was observed when the FGSC target DNA concentrations were quantified in In1 (Figure 3.24). The FGSC target DNA concentration was significantly higher in the PSA's In1 of Field B compared to the VGA's In1 of field B at the soft dough stage. However, at the physiologically mature stage the opposite is true. In field F the VGA's In1 had significantly higher concentrations of FGS target DNA at the soft dough stage compared to that quantified in the PSA's In1. A trend was observed where there was a sharp increase in both PSA and VGA's FGSC target DNA concentrations in In1 during the soft dough stage. In fields A and C these sharp increases were significantly higher than the concentrations were at 6 week and flowering growth stages. A significant locality x areas-within-fields interaction ($P \leq 0.05$) was observed when the FGSC target DNA concentrations were quantified in grain (Figure 3.25). Fields B, C, D, E and F all had significantly higher FGSC target DNA concentrations in the grain of the PSA plants compared to the grain in the VGA plants. A significant ($P \leq 0.0001$) growth stage effect was observed for FGSC target DNA concentrations in In2 (Figure 3.26). The soft dough stage had significantly the highest mean (PSA and VGA) FGSC target DNA concentration in In2 compared to the 6-week-old, flowering and physiologically mature plants. Significant relationships were recorded between ZEA (R^2=0.73), DON (R^2=0.99) and 3-ADON (R^2=0.73) and FGSC target DNA concentration (Figure 3.27).

3.4.4 Evaluation of PSA and VGA plots common to three seasons

Respective data of mean FGSC target DNA concentration from three localities common to the three seasons, together with the areas within the localities, were combined and subjected to ANOVA. A significant (P=0.05) areas-within-field effect on the mean FGSC target DNA concentrations in roots was recorded (Figure 3.28). The FGSC target DNA was significantly higher in the VGA's roots compared to the PSA's roots when the mean of all three seasons were evaluated. A significant (P=0.03) locality x season interaction was observed for FGSC target DNA concentrations in maize crowns (Figure 3.29). Field E during the 2012/13 season and field A during the 2013/14 season had significantly higher FGSC target DNA concentrations in the crowns than fields A and F during the 2012/13 season, fields E and F during the 2013/14 season and all three fields during the 2014/15 season. However, the target DNA concentrations did not differ significantly between the PSA and VGA crown in any of the localities during all three seasons. The FGSC target DNA concentration in In1 (P=0.02) differed significantly between seasons but only if the mean VGA and PSA FGSC target DNA concentration were considered (Figure 3.30). The 2012/13 season had significantly higher FGSC target DNA concentrations in In1 during the 2012/13 season compared to the 2013/14 and 2014/15 seasons. Similarly, the FGSC target DNA concentrations of In2 (P=0.04) were significantly higher during season 2012/13 compared to the other seasons but only if the mean VGA and PSA FGSC target DNA was considered (Figure 3.31).

The ear mass, both weight before (P=0.002) and after threshing (P=0.04) showed significant (P=0.05) locality x season interactions (Figure 3.32 and 3.33 respectively). During the 2012/13 season field A, E and F had significantly higher ear mass, before and after threshing than the same fields had during the 2013/14 and 2014/15 seasons. However, this was only true if the mean PSA and VGA ear mass was evaluated. The PSA and VGA ear mass before and after threshing did not differ significantly from each other. The ear mass, both weight before and after threshing showed significant (P=0.05) locality x season interactions when the mean of the three seasons were considered (Figure 3.34). The ear mass before threshing were significantly higher in VGA plants in field F compared to the PSA plants in field F (Figure 3.34a). The same was seen with the threshed grain mass (Figure 3.34b). The average ear length (P=0.001) was significantly higher during the 2012/13 season compared to the other two seasons however the average ear length did not differ between PSA and VGA plants (Figure 3.35a). The threshing

percentage (P=0.002) was significantly higher during the 2012/13 and 2014/15 seasons compared to the 2013/14 season however the threshing percentage did not differ between PSA and VGA plants (Figure 3.35b). Grain moisture was significantly affected by a locality x season interaction (P=0.04) as well as a locality x areas-within-field interaction (P=0.008) (Figure 3.36). During the 2013/14 the PSA plants' grain moisture was significantly higher than the VGA plants' grain moisture (Figure 3.37a). Field E had significantly higher grain moisture in the PSA plants compared to the VGA plants (Figure 3.36b). Regression analysis indicated a significant relationship between FGSC target DNA concentration in grain and ear mass before threshing (R^2=0.66) and threshed grain mass (R^2=0.67) as well as average ear length (R^2=0.74) (Figure 3.37).

DON and ZEA was quantified in all three seasons, however the first season did not have sufficient data points and only the second and third season's data was used for analysis. No relationships were recorded with DON or ZEA.

3.5 Discussion

Premature senescence in plants can be caused by a variety of stresses including drought stress, water logging, nutrient deficiencies and disease (Can and Amasino, 1997; Byamukama, 2013). Maize is grown under irrigation in the Douglas region and the PSA occurred after a heavy rainstorm. Drought was thus ruled out as the cause of the prematurely senesced plants. However, slow drainage of soils subsequent to excessive moisture, increases the risk of premature senescence (Ciampitti *et al.*, 2014). Penetrometer readings are an indication of soil compactness and thus, waterlogging or drainage (Duiker, 2004). The absence of significant differences in penetrometer readings between PSA and VGA would suggest that water would have run off, in the tested areas, at more or less the same rate and would not account for the differences in plant growth observed in the studied localities.

Lodged plants showed distinct signs of *Fusarium* spp. infection. The roots, crowns, stalks and grain of both the VGA and PSA plants, had high concentrations of FGSC, although no trend was observed over three seasons to suggest that the cause of premature senescence in the PSA's could be attributed solely to FGSC. Although it should be noted that during the first season the concentrations of FGSC in In1 and In2 were significantly higher than that quantified in the subsequent seasons. This indicates that the incidence of FGSC were higher during first season

in maize stalks compared to the second and third seasons. Dodd (1980) stated that, after flowering and during grain fill, maize plants become more susceptible to stalk rot, as carbohydrates in the stalks are relocated to the grain. As a result, plants lose their carbohydrate mediated disease responses, leaving them vulnerable to pathogens (Dodd and White, 1999). The sequential sampling implemented during the 2014/15 season confirmed these reports in that the plants had higher FGSC target DNA concentrations during the soft dough stage than during the early growth stages. However, it failed to show differences between VGA and PSA's, in the rate at which the onset of the susceptible stage occurred.

Plants require 14 nutrients, excluding carbon, hydrogen and oxygen, to function optimally. These nutrients are divided into macronutrients which includes N, P, K, Ca, Mg, Na and S, and micronutrients which includes B, chlorine (Cl), Cu, Fe, Mn, Mo and Zn (McKenzie, 2001). It was hypothesised that a nutrient deficiency during the first season was a contributing factor for the maize's early senescence (PSA's). Elements that differed significantly between VGA and PSA's were Ca, Mg and Na in soils, and B and K in leaves. Soil pH also differed between these areas with a slightly lower pH in VGAs. Ca has been well documented for its ability to suppress plant disease (Rahman and Punja, 2007), although excessive Ca in soil can limit the uptake of other nutrients, leading to an increased predisposition to disease (Hosier and Bradley, 1999). The latter observation may be a contributing factor to the PSAs. The concentration of Ca was significantly high in the PSA soil. Also, as the Ca concentrations increased so did the FGSC target DNA concentrations in In2, leading to a higher incidence of stalk rot. This results in the top part of the maize plant to be cut off from water. Although, statistically there were no higher incidence of stalk rot in In2 in the PSAs compared to the VGAs, there was a trend towards higher stalk rot in the PSA. Mg in soil may affect plant health positively by increasing plant vigour or by playing an active role in plant defence responses (Huber and Jones, 2012). It has been shown to decrease *F. oxysporum* wilt in carnations and cotton (Lyakh, 1986; Huber and Jones, 2012), although it may cause an increase in certain plant diseases such as peanut pod rot (Halleck and Garren, 1968; Huber and Jones, 2012) caused by *Fusarium* spp.. In this study the Mg concentrations in the VGA soil was significantly high and thus it shows that Mg assisted with overall plant health. A study performed by Deliopoulos *et al.* (2010) indicated that Na salts, applied to the soil, have been shown to suppress diseases such as smuts, powdery mildew and rusts. This was observed as the concentration of Na were significantly high in the VGA soil (Deliopoulos *et al.,* 2010). Also, it was shown that higher concentrations of Na in the soil suppressed disease in the crowns because as the Na concentrations in the soil increased, the

FGSC target DNA concentration decreased. The micronutrient B has been shown to play a role in cell wall structure and plant metabolism (Spann and Schumann, 2009). In addition, it has also been shown to be toxic to pathogenic fungi (Pratt, 2000; Spann and Schumann, 2009). Higher B concentrations in tomatoes has even been shown to decrease the severity of Fusarium wilt (Edginton and Walker, 1958; Stangoulis and Graham, 2007). Thus, the longevity of the VGA plants might be attributed to stronger cell walls, which aids primary plant defences and structural integrity. Like B, K has been shown to play an important role in cell wall structure by constructing cellulose (Spann and Schumann, 2009; Prabhu *et al.*, 2007). This was observed in this study, as the mean K concentration in VGA leaves was significantly high. Also, as the K concentration increased in the soil, the FGSC target DNA concentrations decreased in the crowns and In1. Thus, K may have contributed to the increased longevity of the VGA plants. Maize grows optimally at pH levels between 5.5 - 6.5 (Anonymous, 2017). In this study, soil pH ranged from 5.5 - 6.3, thus in the ideal range for maize cultivation. However, the PSA soil's pH was significantly high. It has been shown that *Fusarium* spp. can grow and sporulate in a pH ranging from 4.0 to 8.0 (Panwar *et al*., 2016). This indicate that although the pH of the soil may have played a role in the PSA, it did not increase the incidence of disease or cause the plant to lose vigour. The exact mechanism by which the pH affected the PSA plants remains unclear.

Since the weight before and after threshing did not differ significantly between that of the PSA plants and that of the VGA plants it could be assumed that the significant differences seen between the moisture, 1000 kernel weight and threshing percentage is physiological in nature. In other words, the plants stay greener for longer, this in turn resulted in the VGA plants supplying water and nutrients to the ears for longer, resulting in grain with a higher moisture content and heavier kernels.

Overall, the concentration of mycotoxins did not differ significantly between VGA and PSA. However, in both the 2013/14 and 2014/15 seasons a relationship between mycotoxin concentrations and the concentration FGSC target DNA was recorded. At low FGSC target DNA concentrations, DON, ADON and ZEA concentrations were higher. Studies have shown that trichothecenes are virulence factors (Harris *et al.*, 1999; Ortega *et al.*, 2013). At low FGSC target DNA, DON and ADON are produced to aid infection while after establishment, less trichothecenes are needed to maintain infection. ZEA is not a virulence factor but prepares and protects the substrate for infection by FGSC (Gaffoor *et al.*, 2005; Utermark and Karlovsky,

2007; Ahmed, 2010). The maximum tolerant level (MTL) for DON (2 $\mu g.g^{-1}$), NIV (2 $\mu g.g^{-1}$) and ZEA (1.25 $\mu g.g^{-1}$) set by South African legislation and the European Union was exceeded only during the third season (Anonymous 2, 2016).

3.6 Conclusion

The disease triangle states that when the environmental conditions are optimal, virulent pathogens are present and the host plant is susceptible, disease will occur (Agrios, 2005). In this study it was observed that five nutrients impacted FGSC target DNA concentrations and increased (PSA) or decreased (VGA) the plants susceptibility. Ca increased disease incidence with a trend towards higher stalk rot incidence, whereas Mg, Na, K and B decreased disease incidence by increasing overall plant health and decreasing disease incidence and severity by the various role they play in plant defence responses. It was also shown that during the soft dough stage, maize is the most sensitive towards plant disease. Thus, it could be seen that poor soil nutrition (environment), coupled with a susceptible plant (soft dough stage) and significantly high FGSC target DNA concentrations in In1 and In2 of season 2012/13 (virulent pathogens) resulted in the PSAs in maize fields.

3.7 References

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Table 3.1: Background information on the seven fields that were surveyed in Douglas. All fields were rotated with wheat during the winter season.

Locality	GPS	Cultivar	2011/12	2012/13	2013/14	2014/15	Plants/ha
Field A	29°06'55.62"S 23°44'58.31"E	DKC62-80BR	C	M	M	M	88 000
Field B	29°06'03.70"S 23°46'25.67"E	DKC64-78BRGEN	M	M	C	M	88 000
Field C	29°05'59.50"S 23°46'20.81"E	DKC62-80BR	M	M	C	M	88 000
Field D	29°05'52.15"S 23°37'54.47"E	DKC62-80BR/DKC64-78BRGEN	C	M	C	M	88 000
Field E	29°11'08.74"S 23°43'32.93"E	PAN736BR	L	M	M	M	90 000
Field F	29°05'47.92"S 23°38'20.45"E	PAN736BR	M	M	M	M	92 000
Field G	29°07'29.51"S 23°42'30.10"E	DKC62-80BR	M	M	M	C	95 000

[1]

[1] M – maize; C – cotton; L – lucerne

Figure 3.1: One of seven fields in the Douglas region during the 2012/13 season. Symptoms such as lodging and pink discolouration were recorded in PSA[1] (Photo courtesy of GWK).

[1] PSA = prematurely senescing areas

Figure 3.2: PSA[1] plants had a high incidence of plant lodging (Photo: S.-M. Joubert).

[1] PSA = prematurely senescing plants

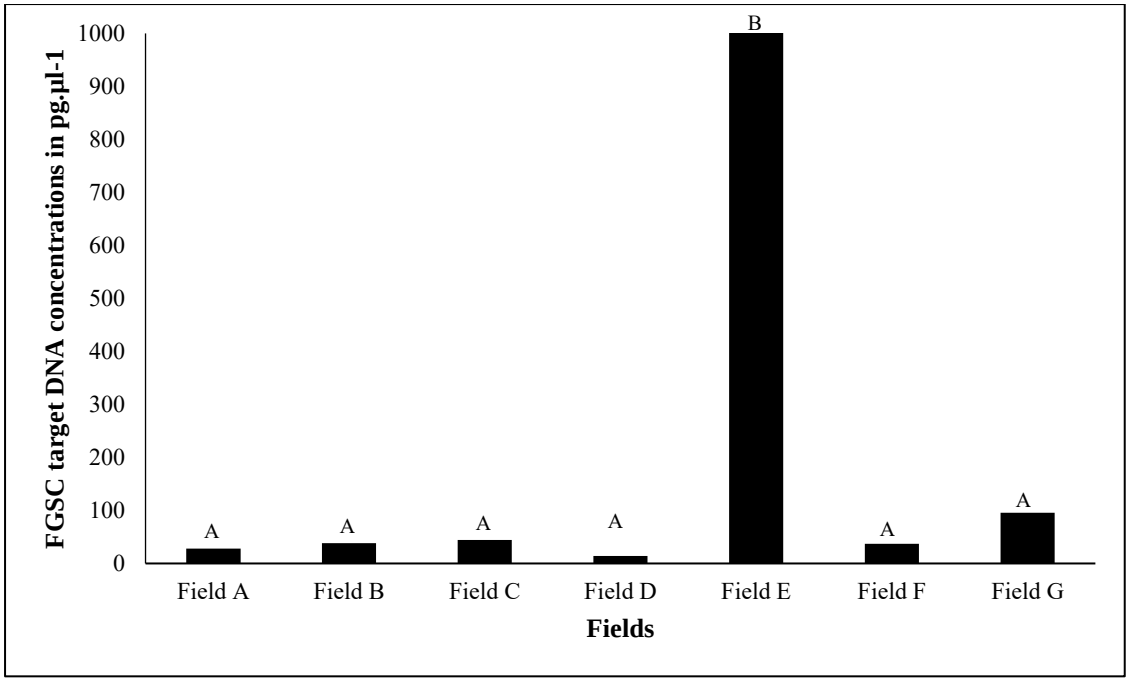

Figure 3.3: Mean FGSC[1] target DNA concentrations in maize crowns during the 2012/13 season indicating significant differences between localities. [2]

[1] FGSC = *Fusarium graminearum* species complex
[2] A different letter denotes significance at P=0.0059.

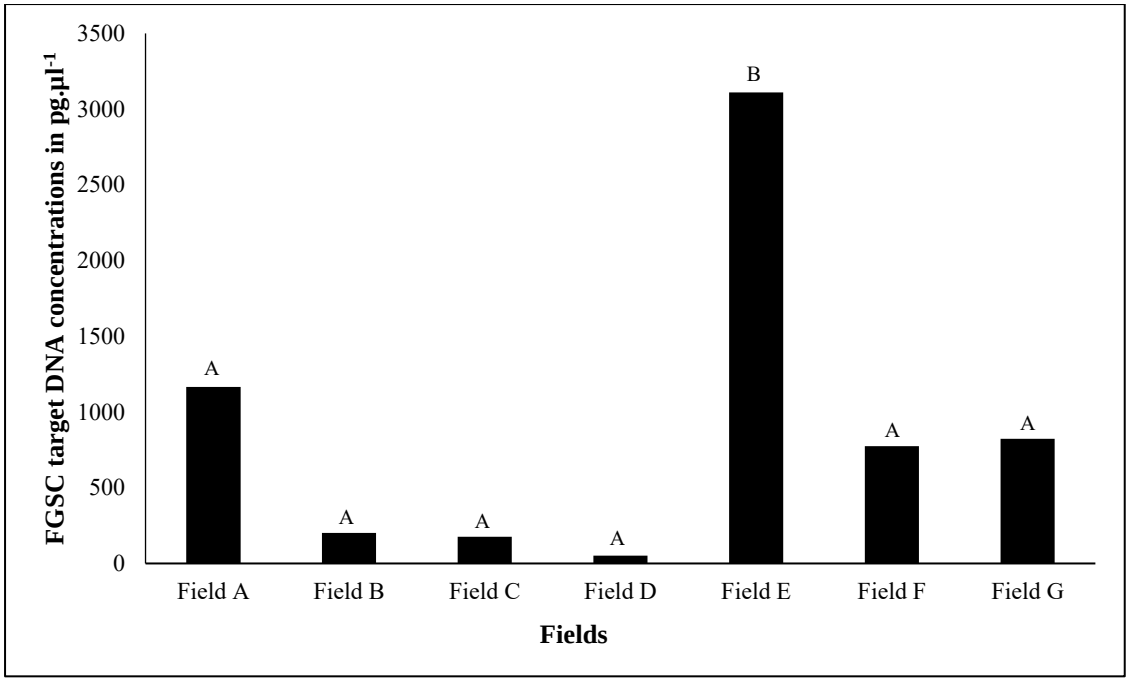

Figure 3.4: Mean FGSC[1] target DNA concentration in maize internode 1 during the 2012/13 season indicating significant differences between localities. [2]

[1] FGSC = *Fusarium graminearum* species complex
[2] A different letter denotes significance at P=0.0334.

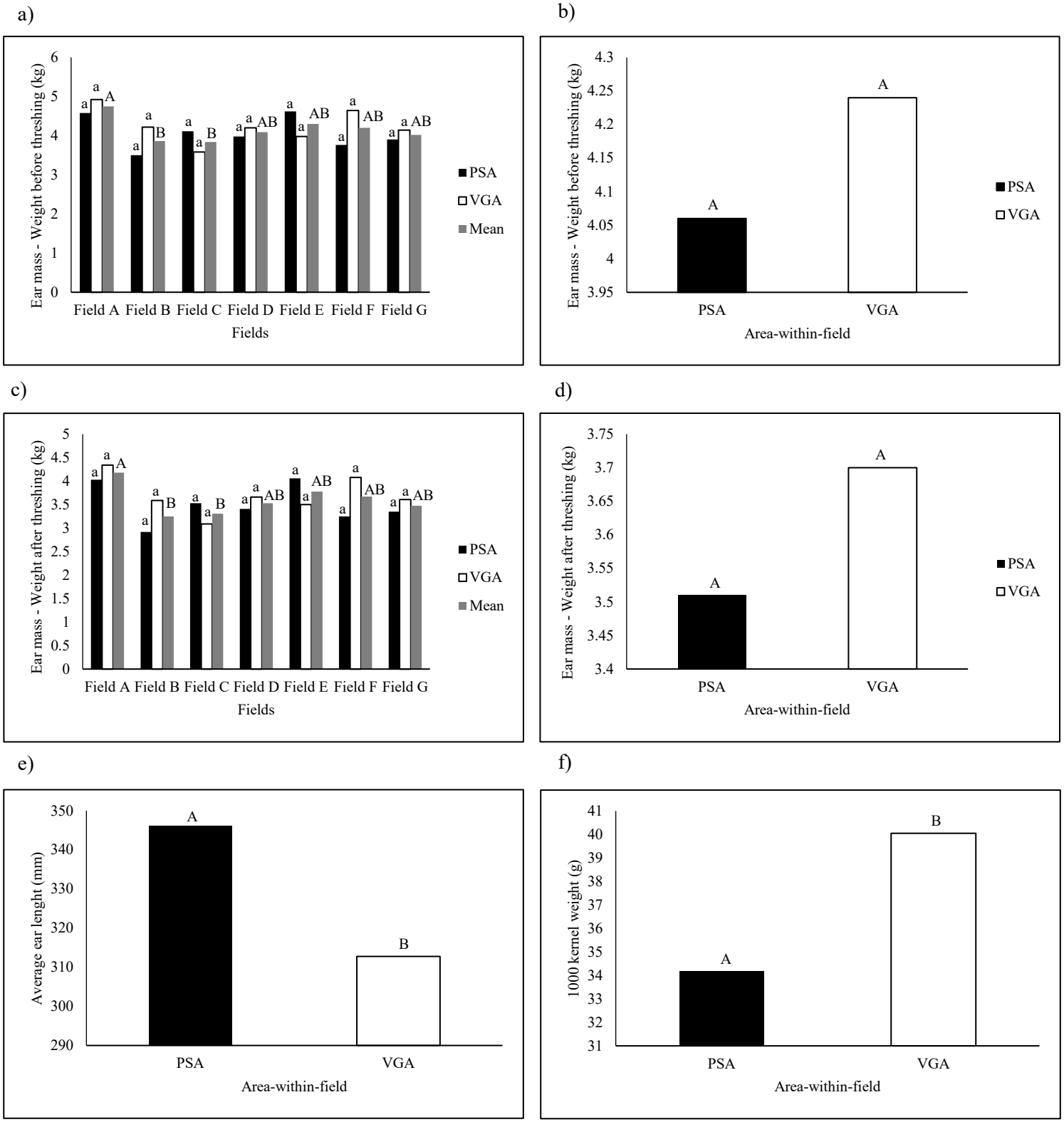

Figure 3.5: Ear and grain characters determined in PSA and VGA[1] maize leaves over seven localities during 2012/13. [2]

[1] PGA = prematurely senescing areas; VGA = visibly green areas
[2] A different letter denotes significance at $P \leq 0.05$.

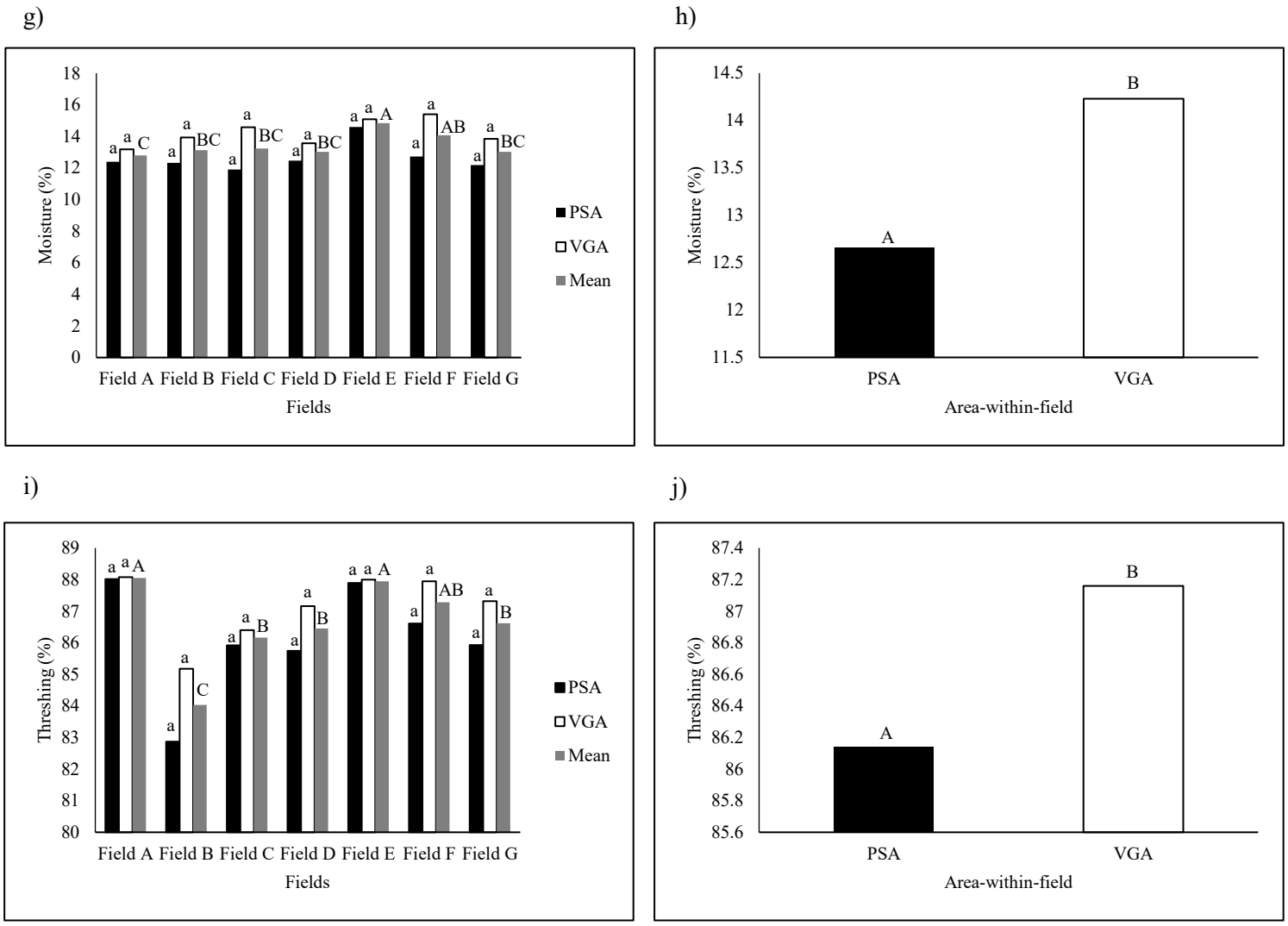

Figure 3.5 (cont.): Ear and grain characters determined in PSA and VGA[1] maize leaves over seven localities during 2012/13. [2]

[1] PGA = prematurely senescing areas; VGA = visibly green areas

[2] A different letter denotes significance at $P \leq 0.05$.

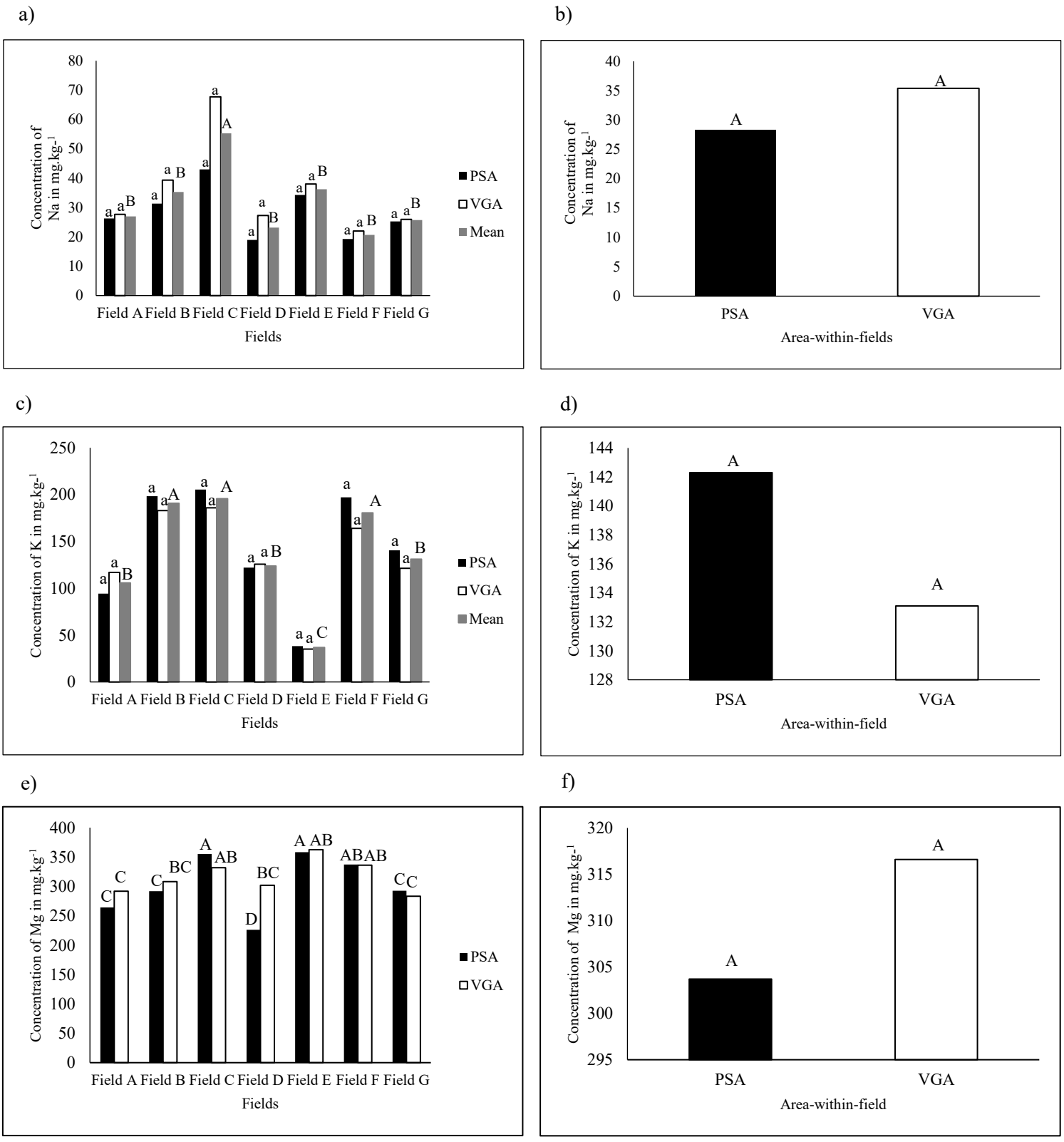

Figure 3.6: Nutrient concentrations and soil characters determined in PSA and VGA[1] soil over seven localities during 2012/13. [2]

[1] PGA = prematurely senescing areas; VGA = visibly green areas
[2] A different letter denotes significance at $P \leq 0.05$.

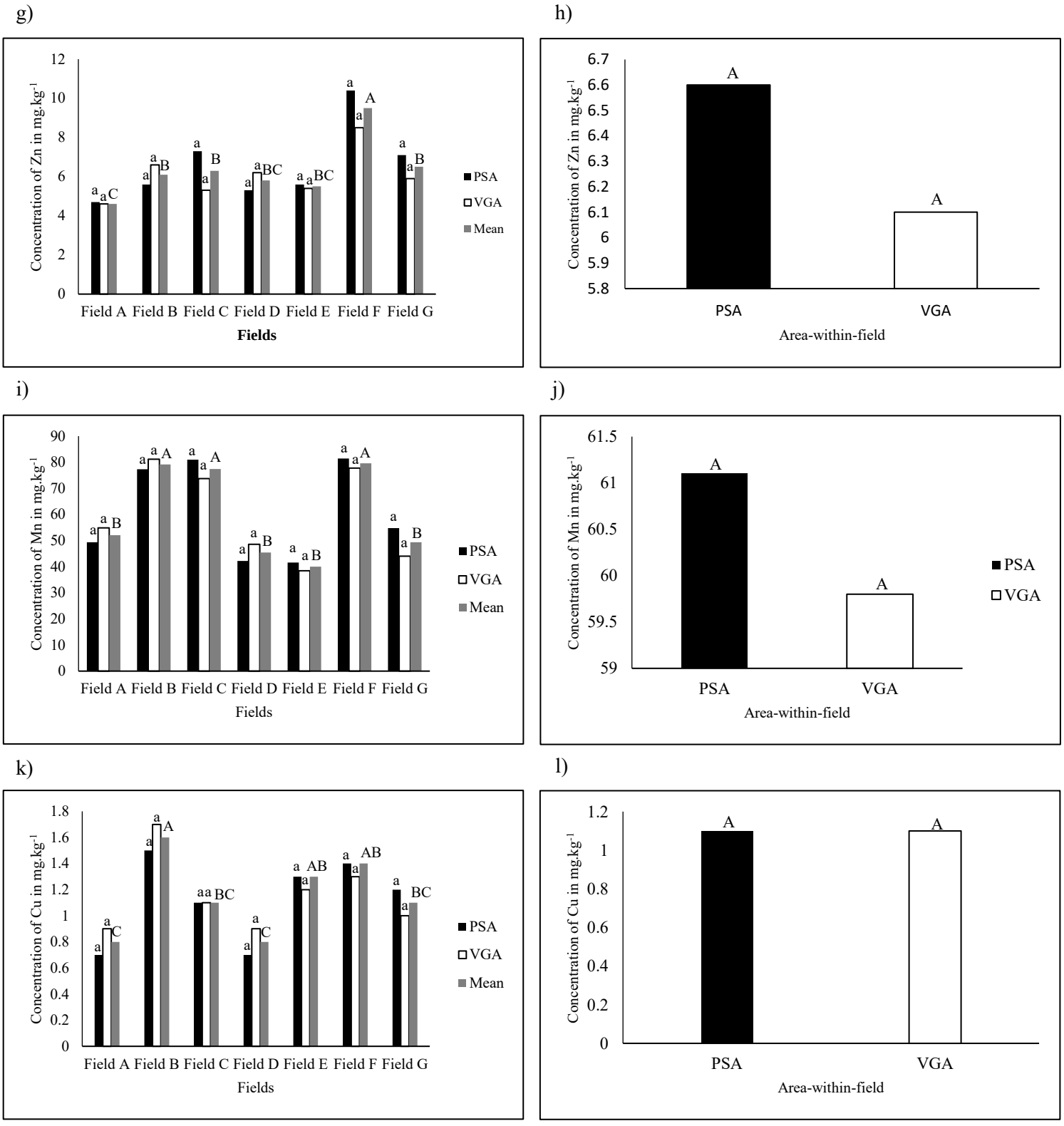

Figure 3.6 (cont.): Nutrient concentrations and soil characters determined in PSA and VGA[1] soil over seven localities during 2012/13. [2]

[1] PGA = prematurely senescing areas; VGA = visibly green areas
[2] A different letter denotes significance at $P \leq 0.05$.

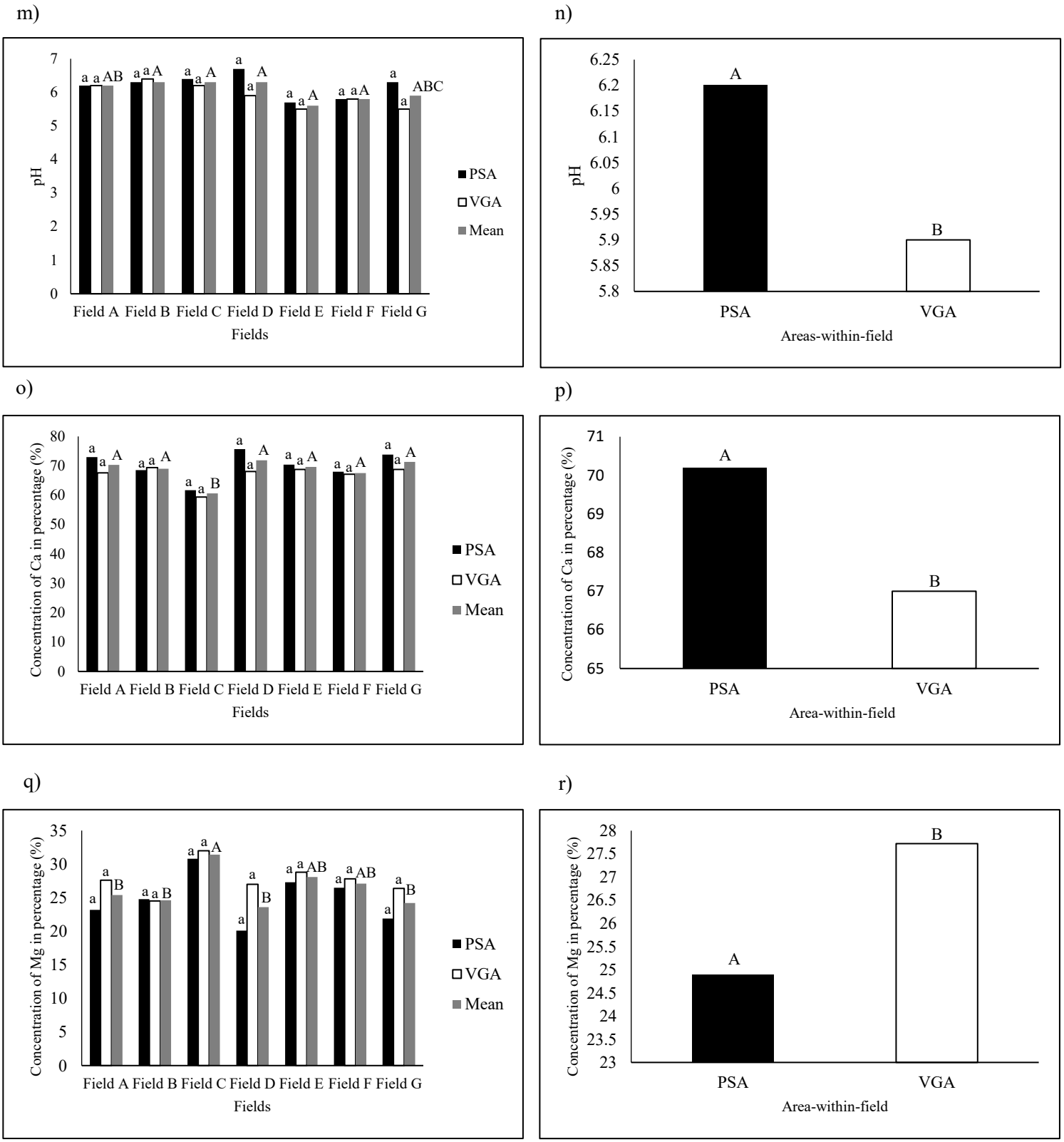

Figure 3.6 (cont.): Nutrient concentrations and soil characters determined in PSA and VGA[1] soil over seven localities during 2012/13. [2]

[1] PGA = prematurely senescing areas; VGA = visibly green areas

[2] A different letter denotes significance at $P \leq 0.05$.

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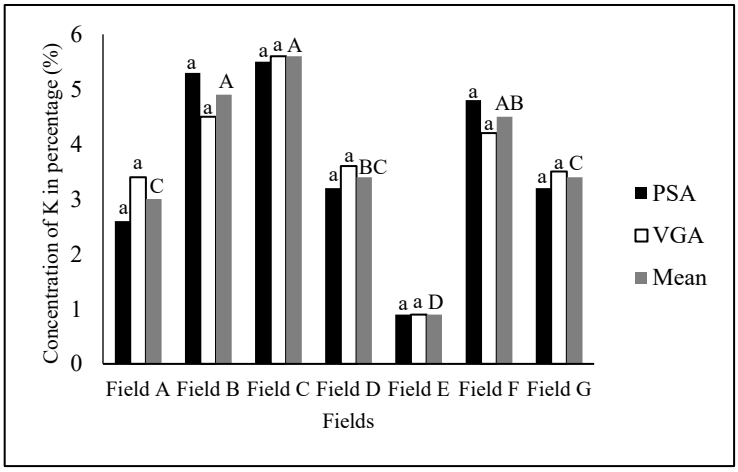

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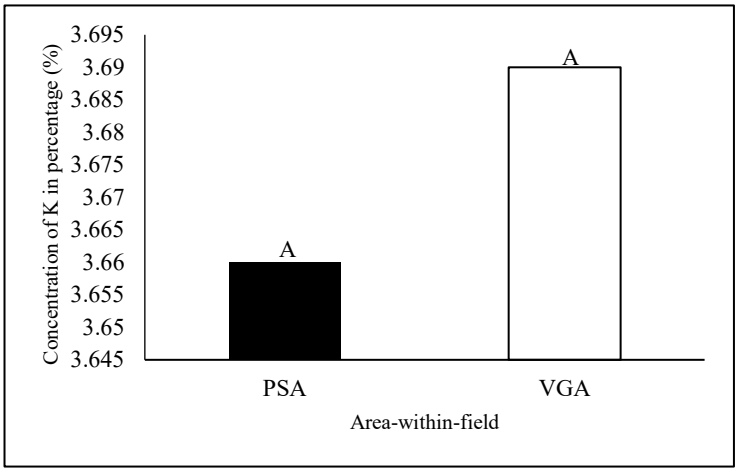

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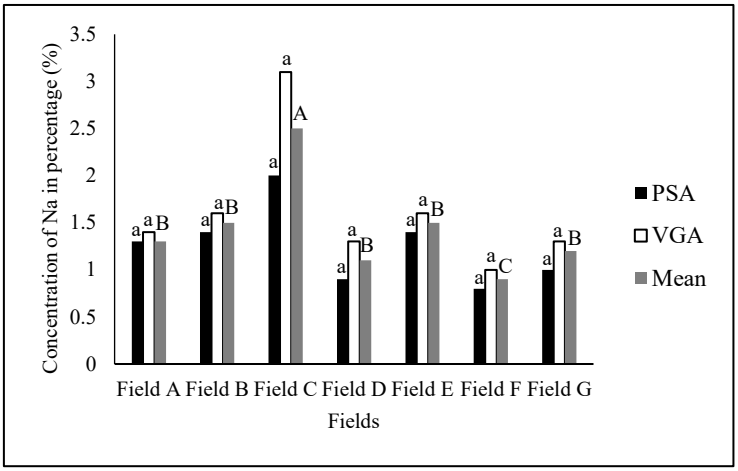


v)

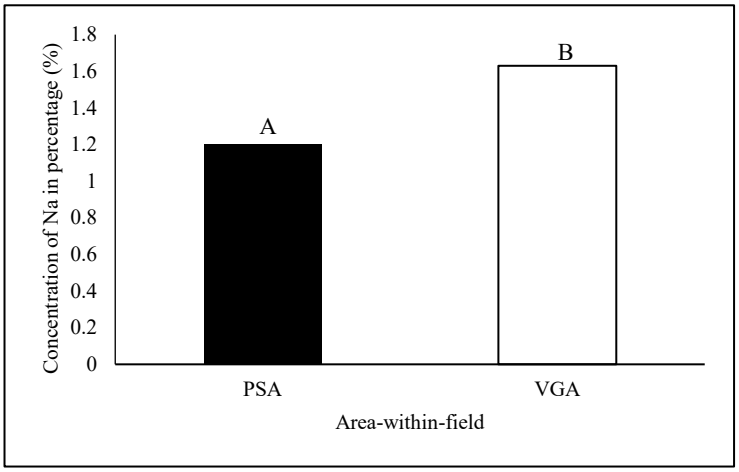


Figure 3.6 (cont.): Nutrient concentrations and soil characters determined in PSA and VGA[1] soil over seven localities during 2012/13. [2]

[1] PGA = prematurely senescing areas; VGA = visibly green areas
[2] A different letter denotes significance at $P \leq 0.05$.

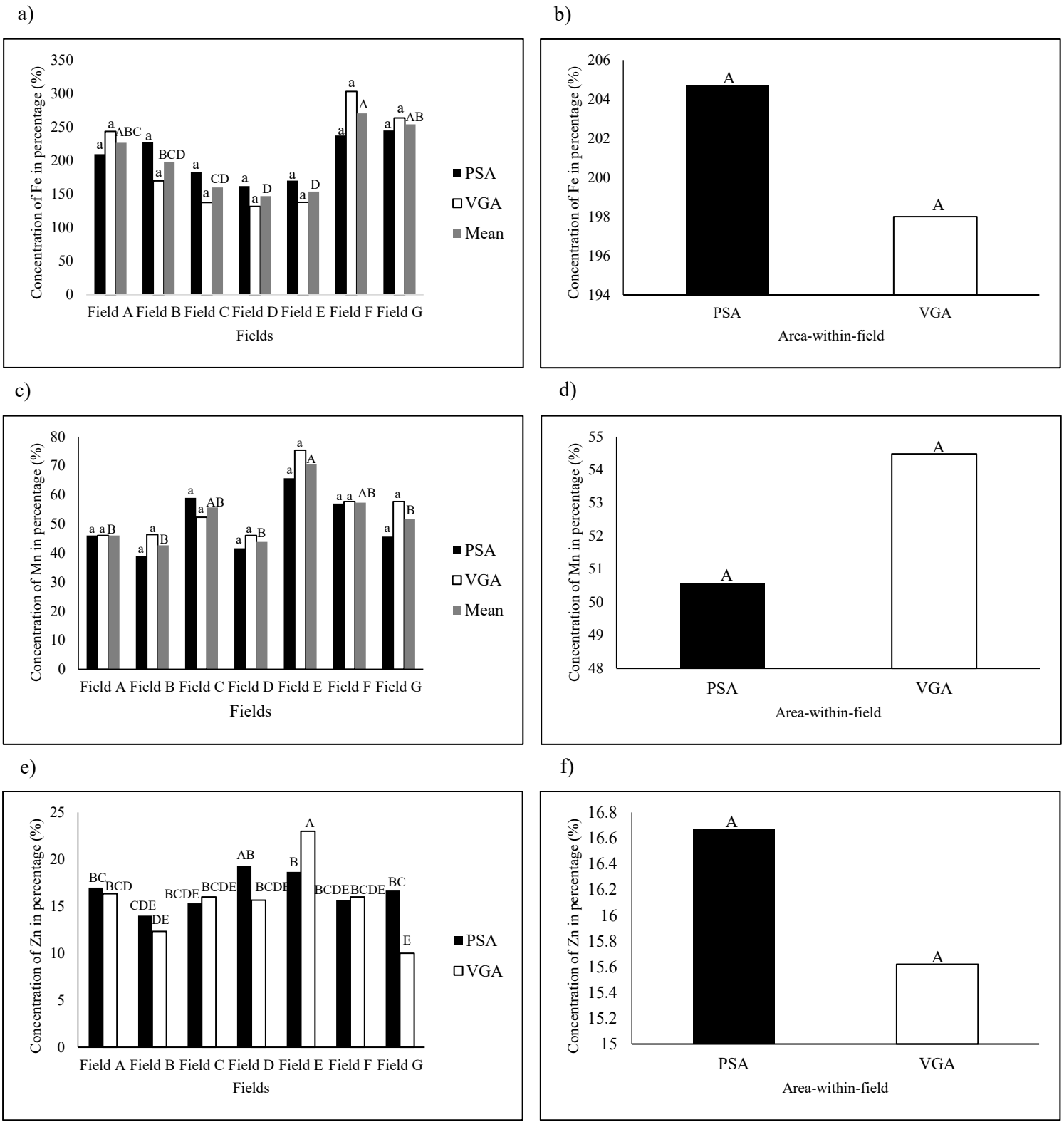


Figure 3.7: Nutrient concentrations determined in PSA and VGA[1] maize leaves over seven localities during 2012/13.

[1] PGA = prematurely senescing areas; VGA = visibly green areas

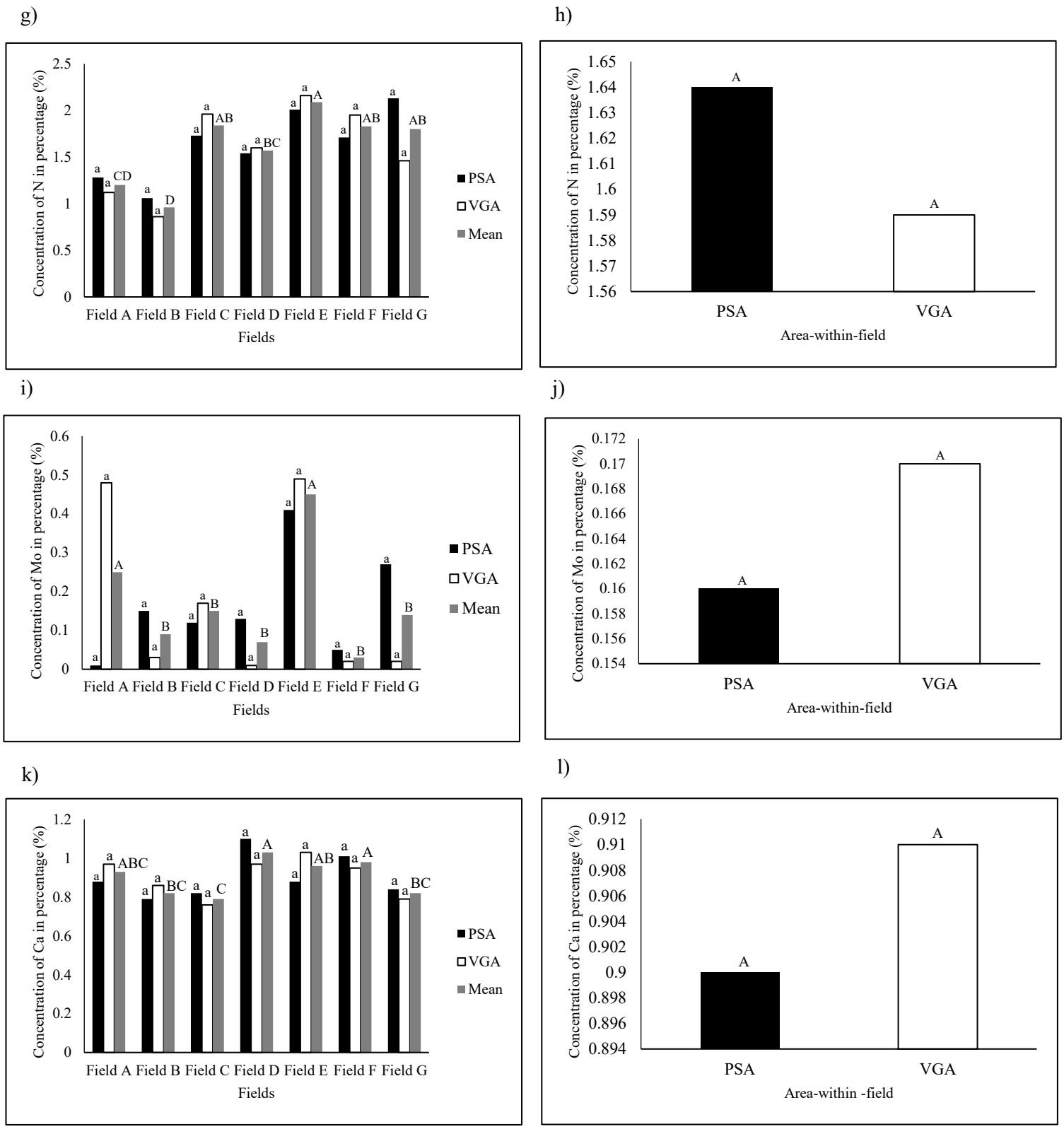


Figure 3.7 (cont.): Nutrient concentrations determined in PSA and VGA[1] maize leaves over seven localities during 2012/13.

[1] PGA = prematurely senescing areas; VGA = visibly green areas

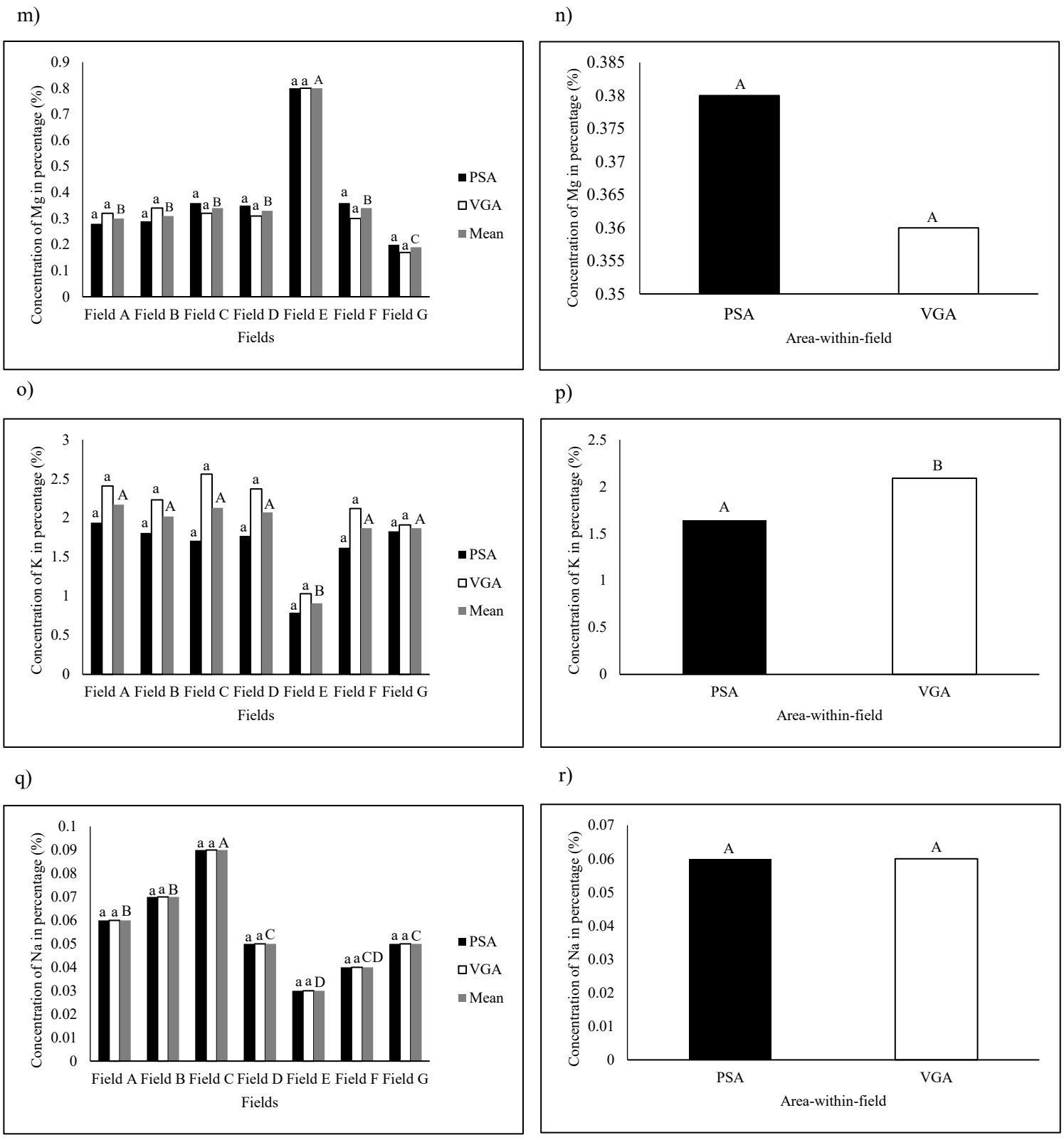


Figure 3.7 (cont.): Nutrient concentrations determined in PSA and VGA[1] maize leaves over seven localities during 2012/13.

[1] PGA = prematurely senescing areas; VGA = visibly green areas

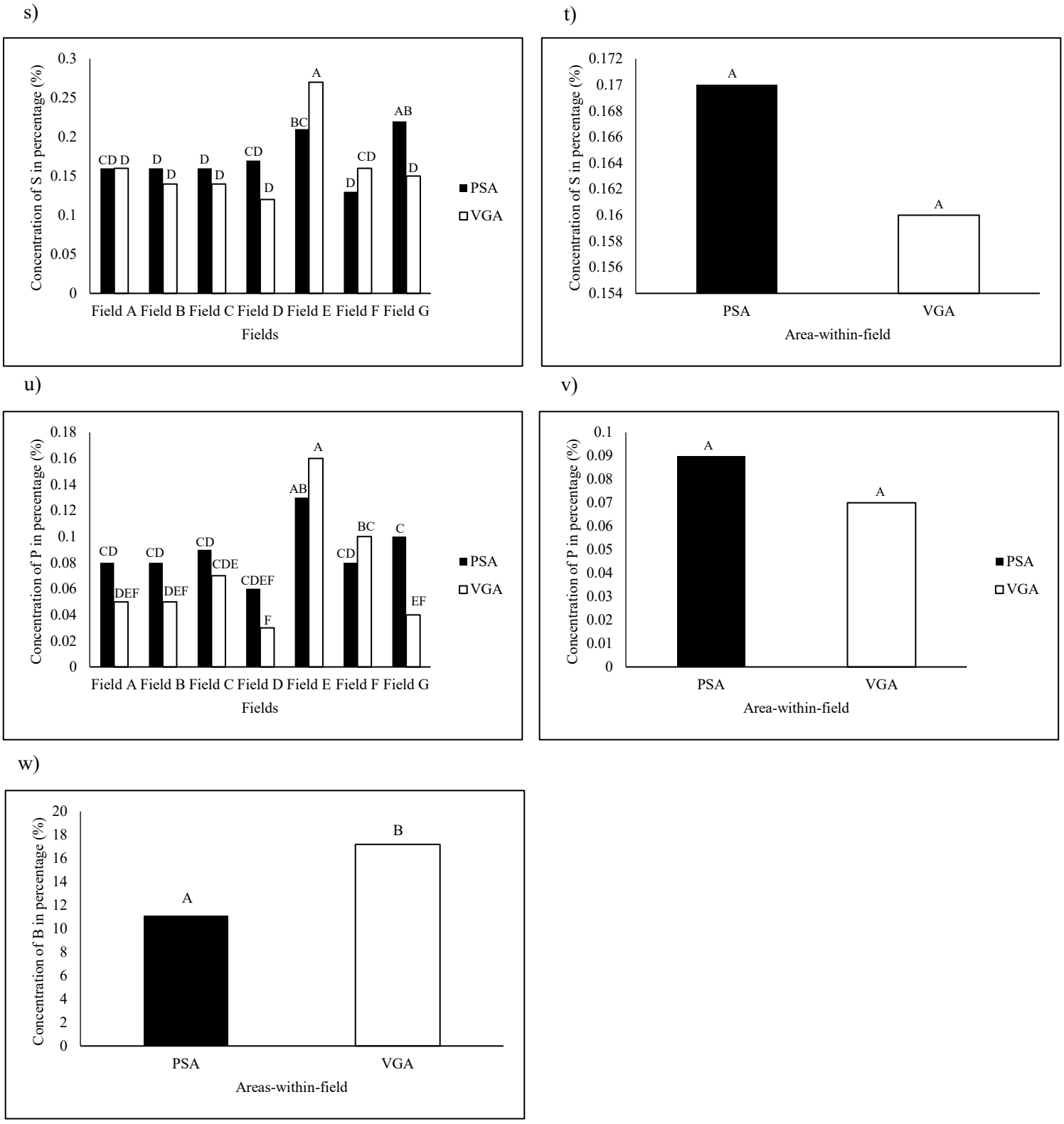


Figure 3.7 (cont.): Nutrient concentrations determined in PSA and VGA[1] maize leaves over seven localities during 2012/13.

[1] PGA = prematurely senescing areas; VGA = visibly green areas

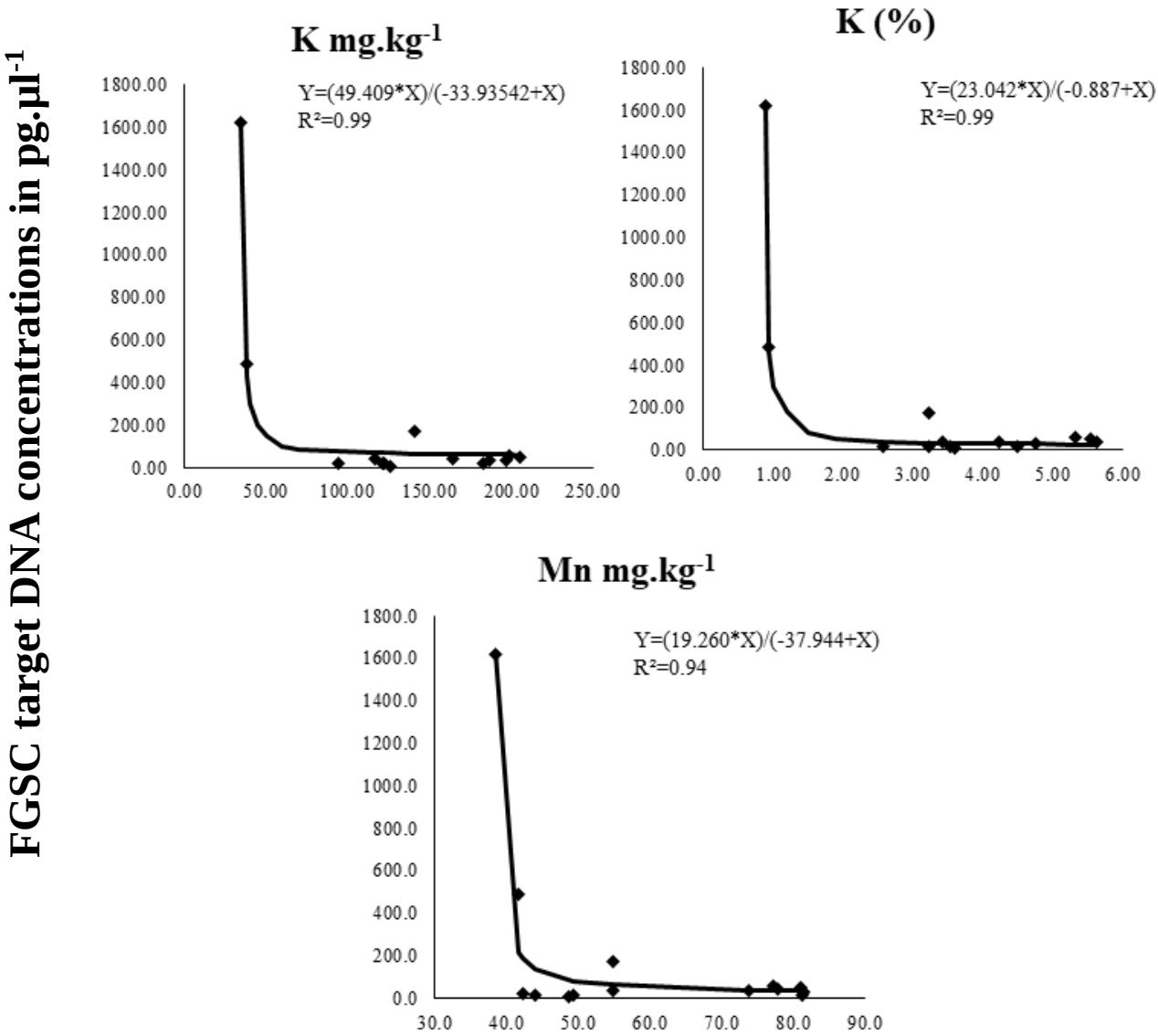


Figure 3.8: Relationships between nutrients tested in soil and FGSC[1] target DNA concentration in maize crowns over seven localities during 2012/13.

[1] FGSC = *Fusarium graminearum* species complex

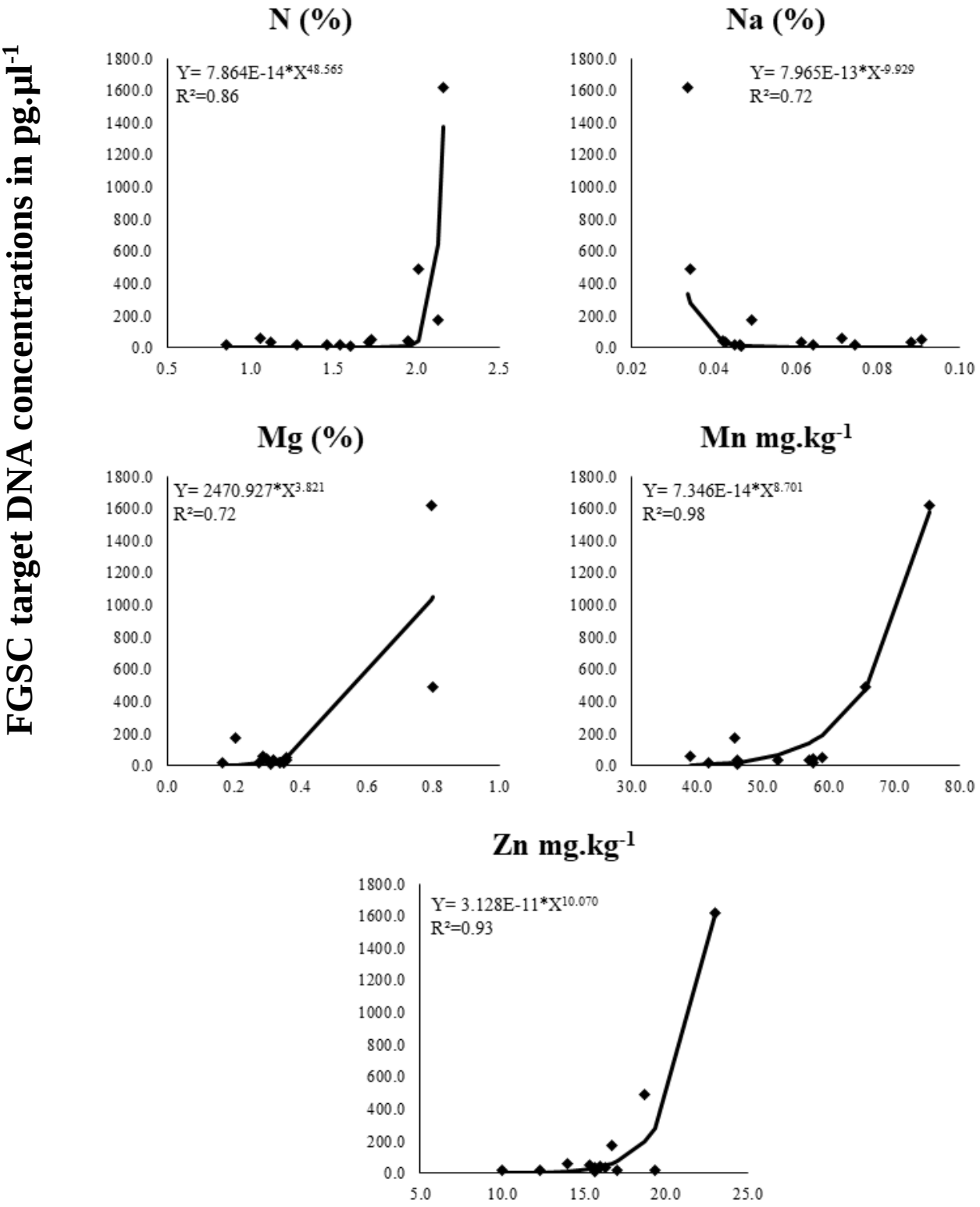


Figure 3.9: Relationships between nutrients tested in leaves and FGSC[1] target DNA concentration in maize crowns over seven localities during 2012/13.

[1] FGSC = *Fusarium graminearum* species complex

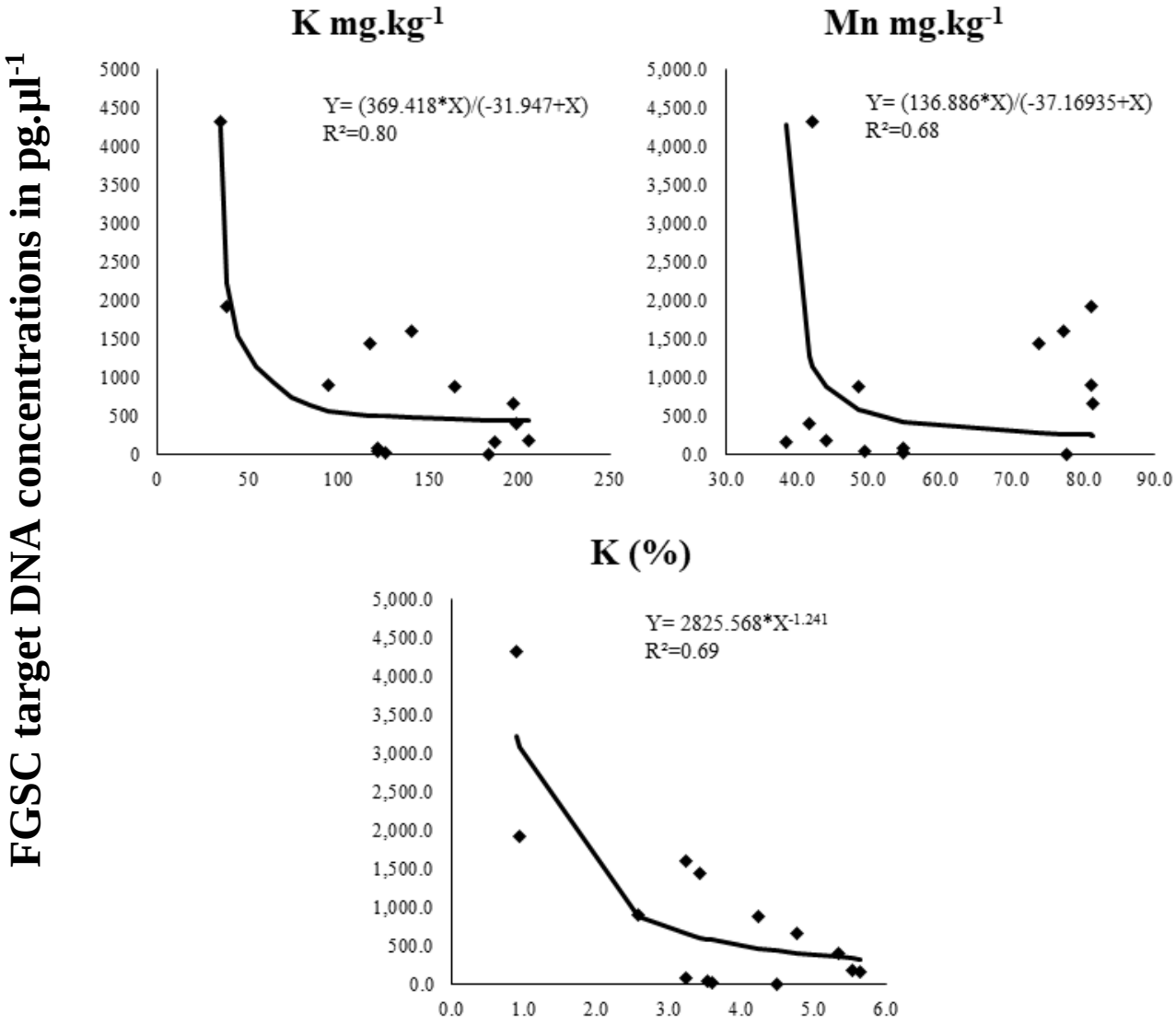


Figure 3.10: Relationships between nutrients tested in soil and FGSC[1] target DNA concentration in maize In1 over seven localities during 2012/13.

[1] FGSC = *Fusarium graminearum* species complex

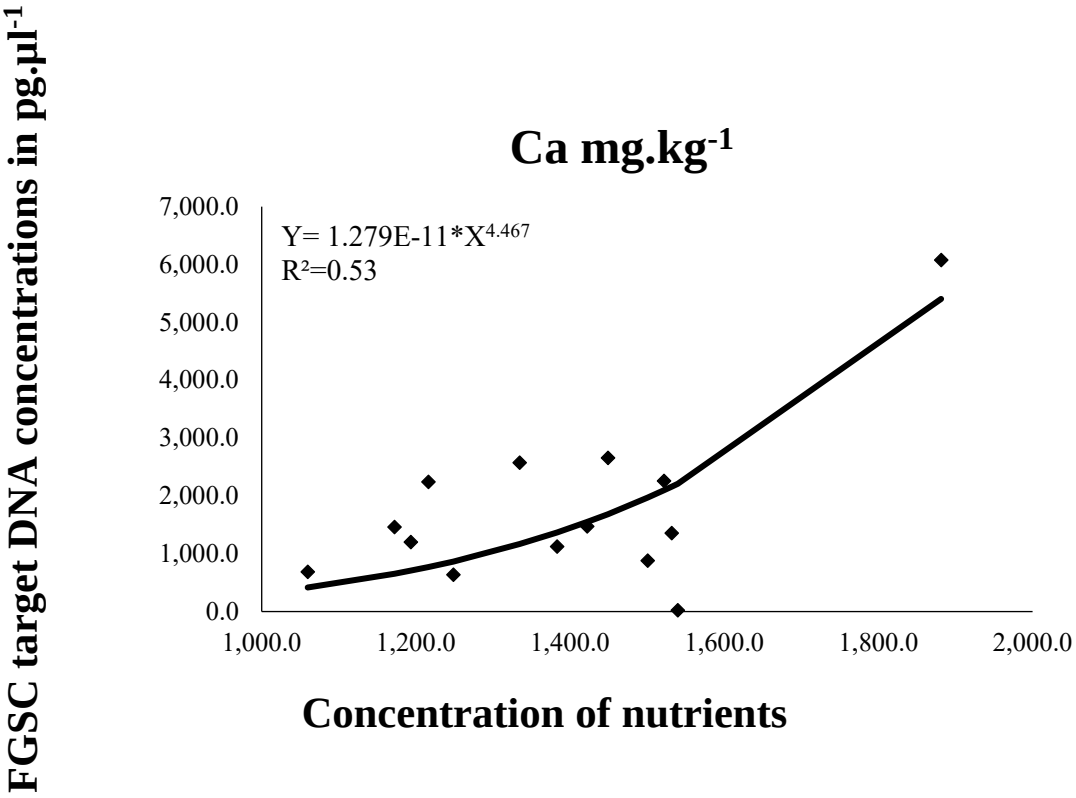


Figure 3.11: Relationships between nutrients tested in soil and FGSC[1] target DNA concentration in maize internode 2 over seven localities during 2012/13.

[1] FGSC = *Fusarium graminearum* species complex

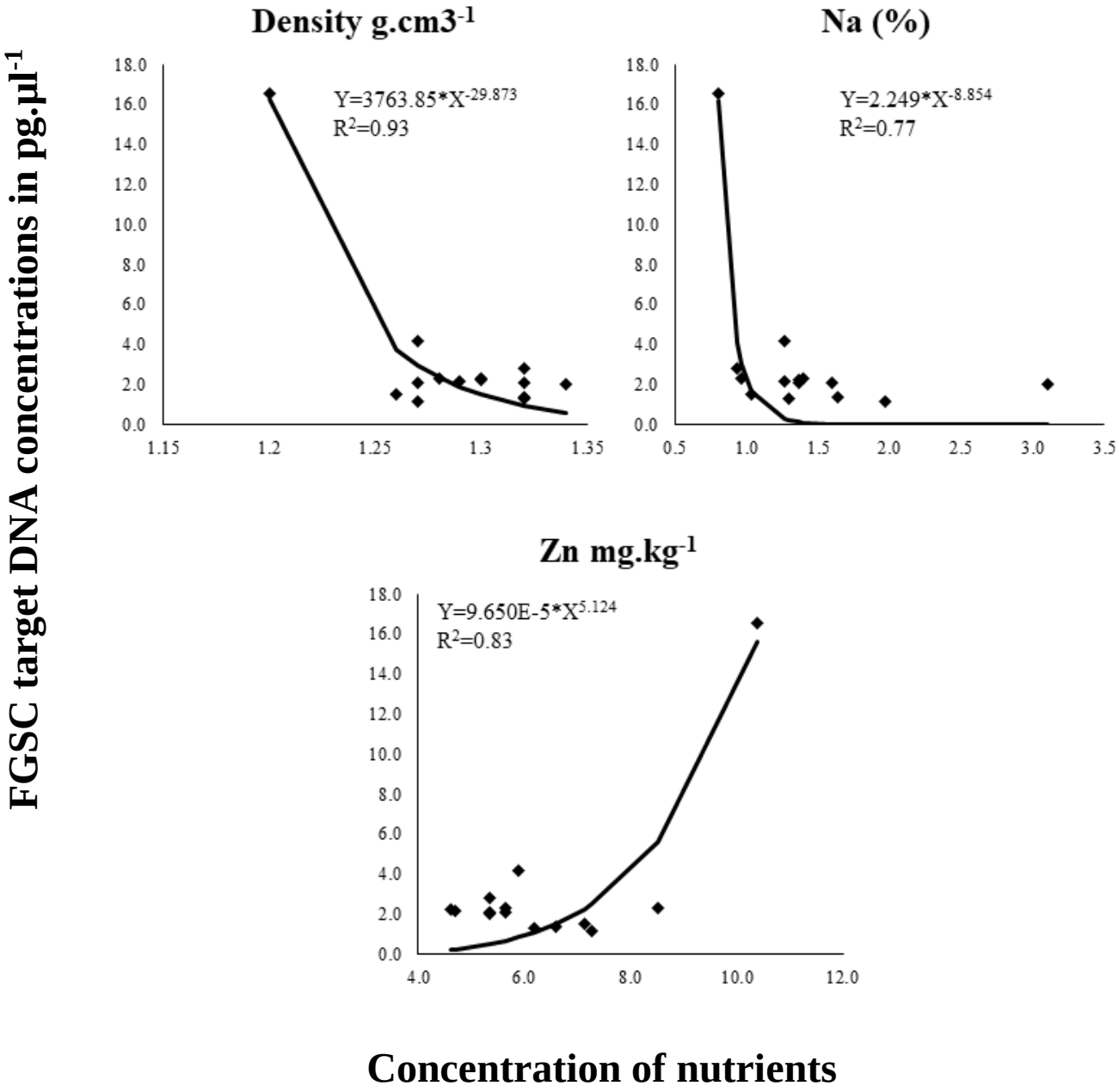


Figure 3.12: Relationships between nutrients tested in soil and FGSC[1] target DNA concentration in maize grain over seven localities during 2012/13.

[1] FGSC = *Fusarium graminearum* species complex

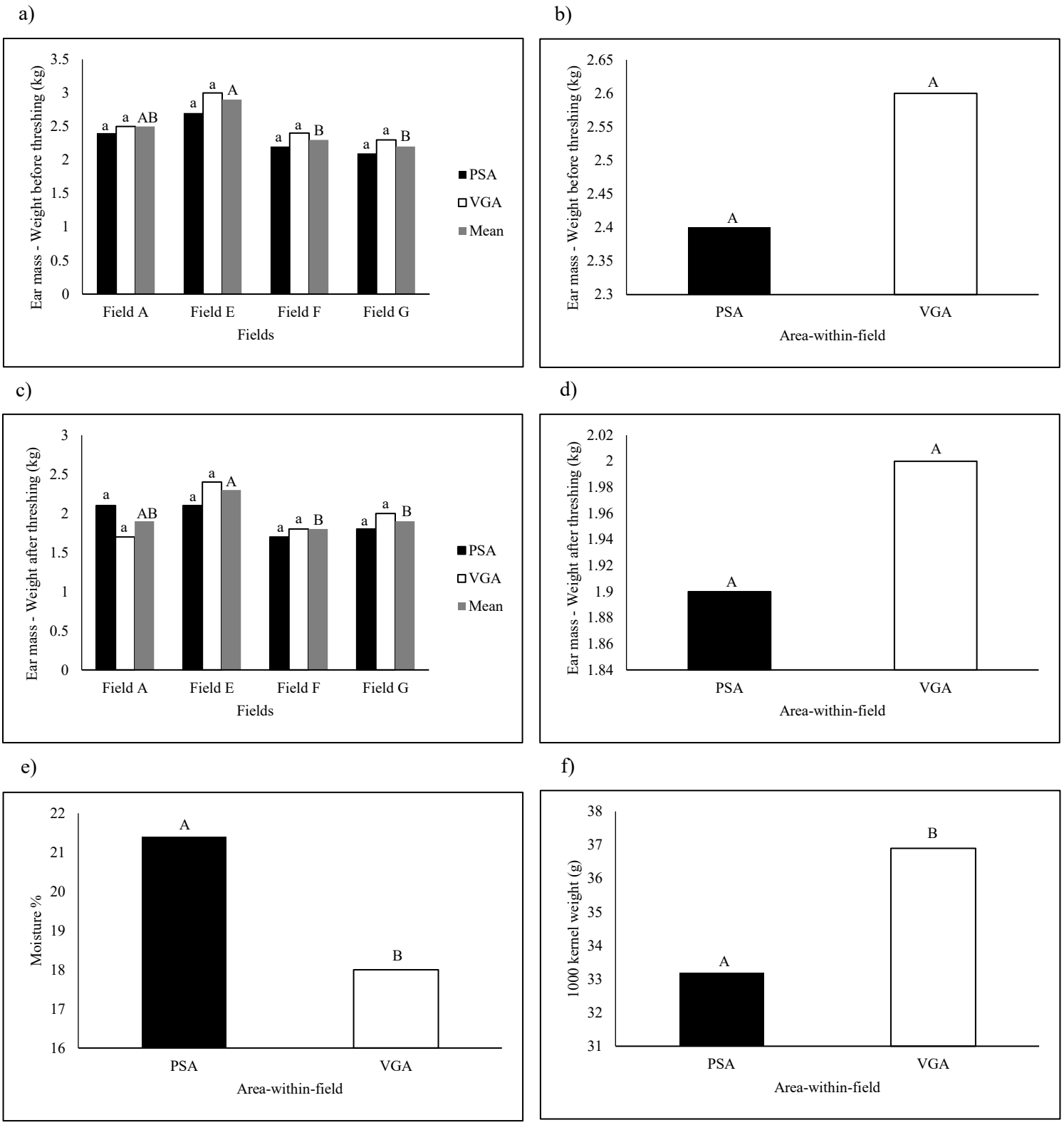


Figure 3.13: Ear and grain characters determined in PSA and VGA[1] maize plants over four localities during 2013/14. [2]

[1] PGA = prematurely senescing areas; VGA = visibly green areas
[2] A different letter denotes significance at $P \leq 0.05$.

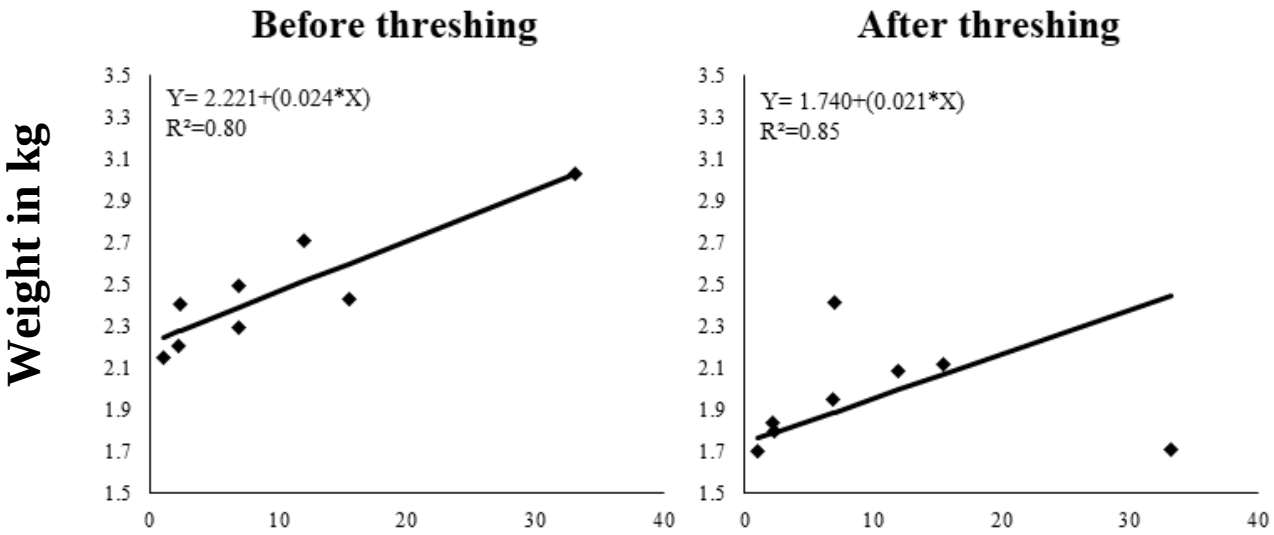


Figure 3.14: Relationships between the FGSC[1] target DNA concentration in roots and the ear mass before threshing and threshed grain mass over four localities during 2013/14.

[1] FGSC = *Fusarium graminearum* species complex

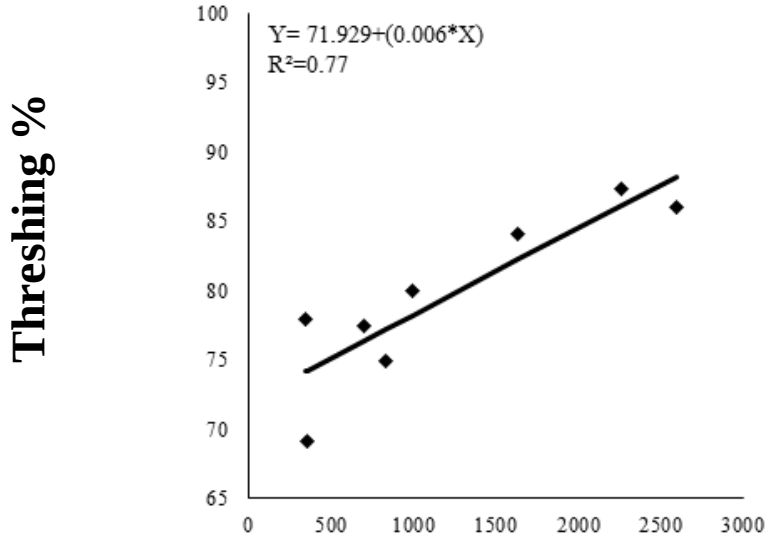


Figure 3.15: Relationship between the FGSC[1] target DNA concentration in internode 2 and threshing percentage of grain over four localities during 2013/14.

[1] FGSC = *Fusarium graminearum* species complex

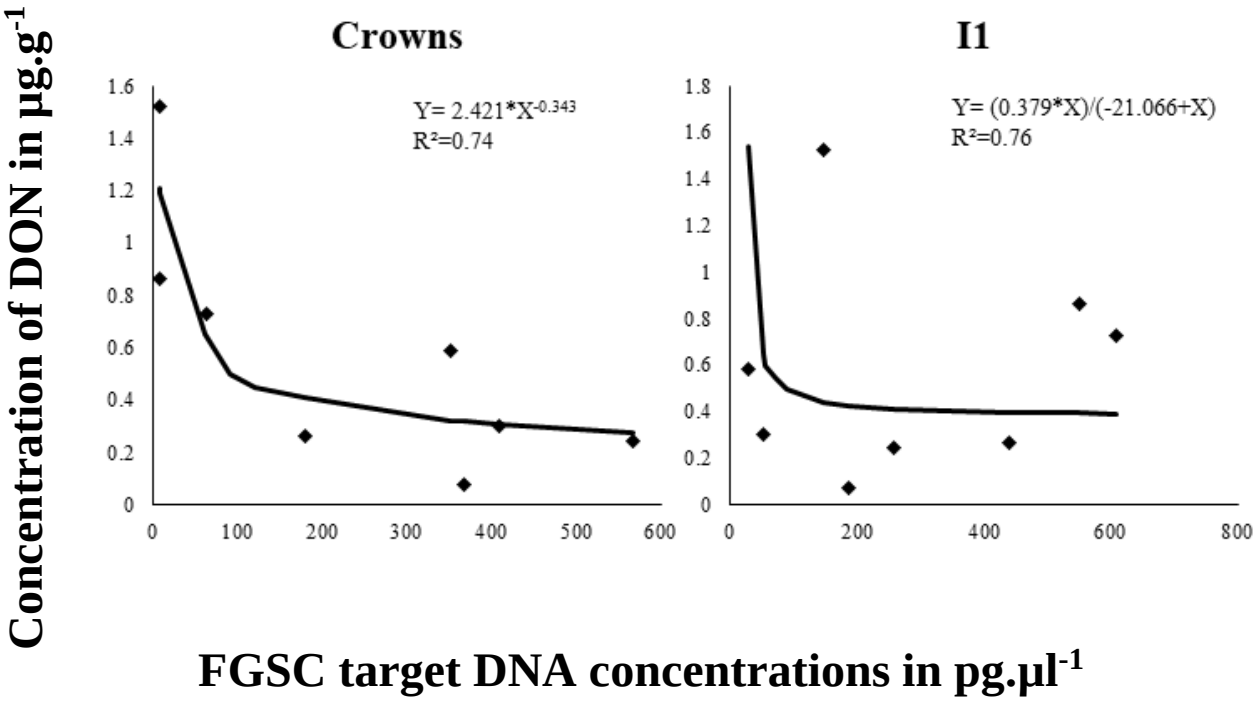
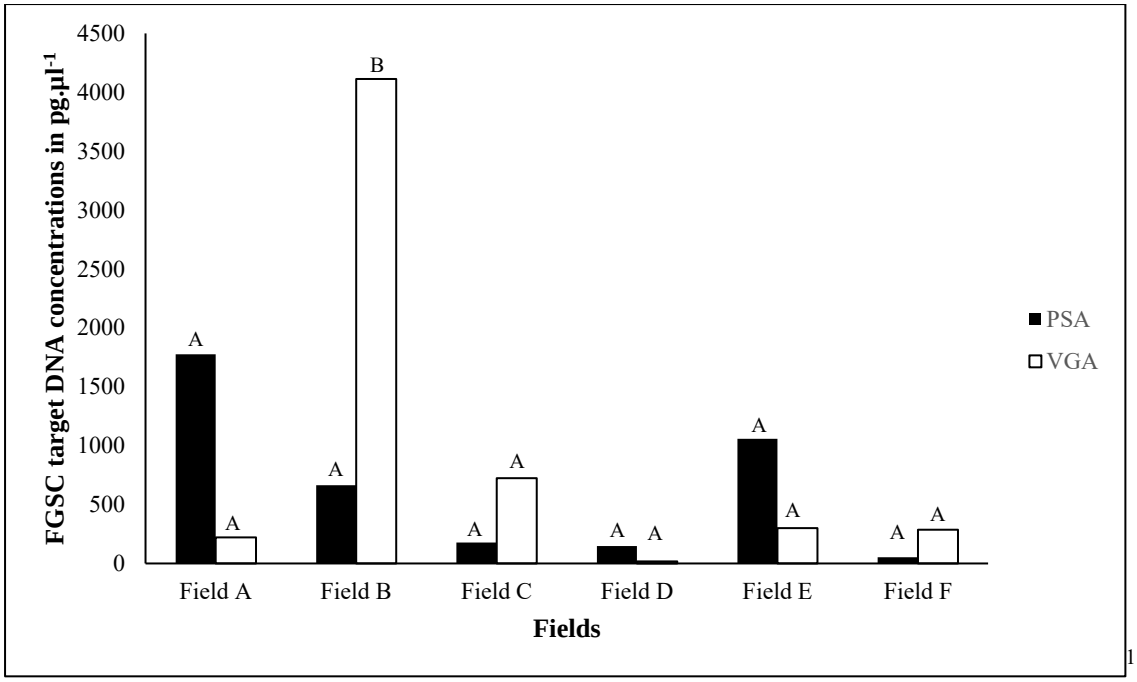


Figure 3.16: Relationships between DON[1] quantified in maize grain and FGSC target DNA in maize crowns and internode 1 over four localities during 2013/14.

[1] DON = deoxynivalenol; FGSC = *Fusarium graminearum* species complex



[1]

Figure 3.17: Mean FGSC[2] target DNA concentration in maize internode 1 during the 2014/15 season indicating a significant locality x season interaction. [3]

[1] PSA - prematurely senescing area; VGA – visibly green area
[2] FGSC = *Fusarium graminearum* species complex
[3] A different letter denotes significance at P=0.0215.

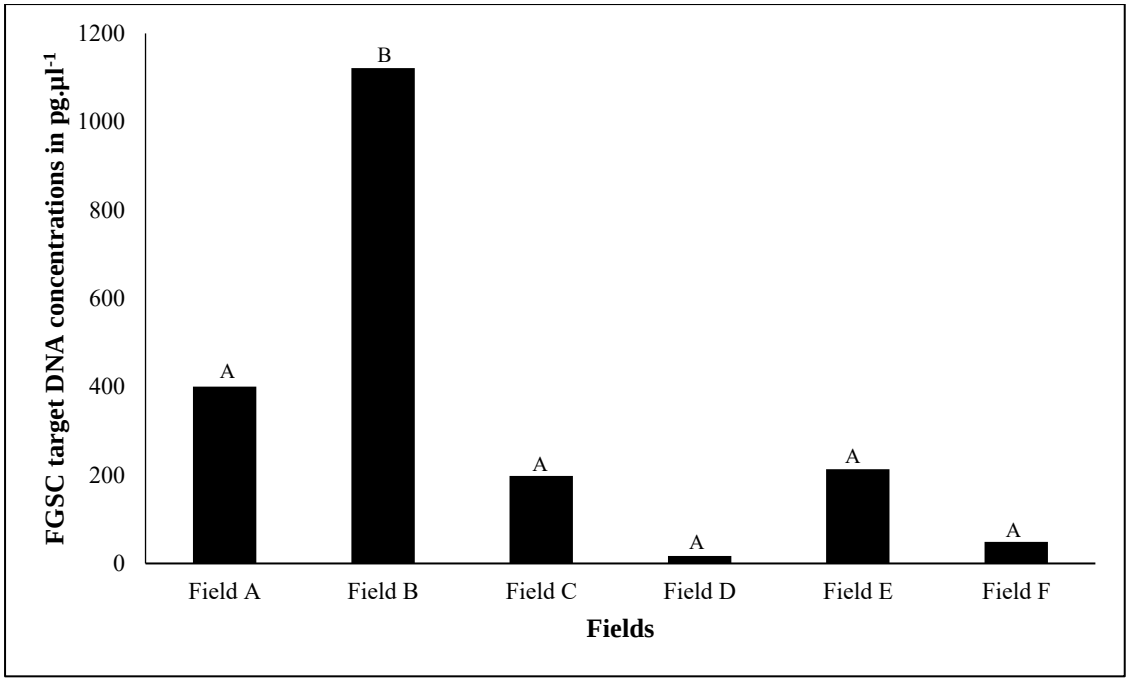


Figure 3.18: Mean FGSC[1] target DNA concentrations in maize crowns during the 2014/15 season indicating significant differences between localities. [2]

[1] FGSC = *Fusarium graminearum* species complex
[2] A different letter denotes significance at P=0.0052.

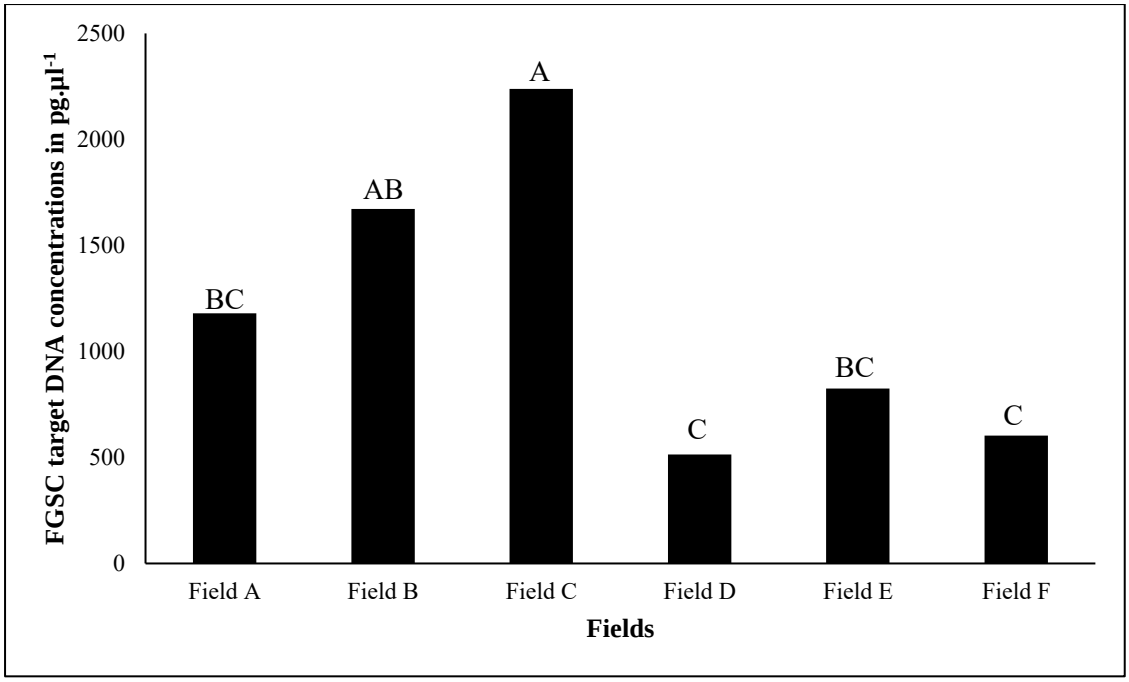


Figure 3.19: Mean FGSC[1] target DNA concentration in maize internode 2 during the 2014/15 season indicating significant differences between localities. [2]

[1] FGSC = *Fusarium graminearum* species complex
[2] A different letter denotes significance at P=0.0123.

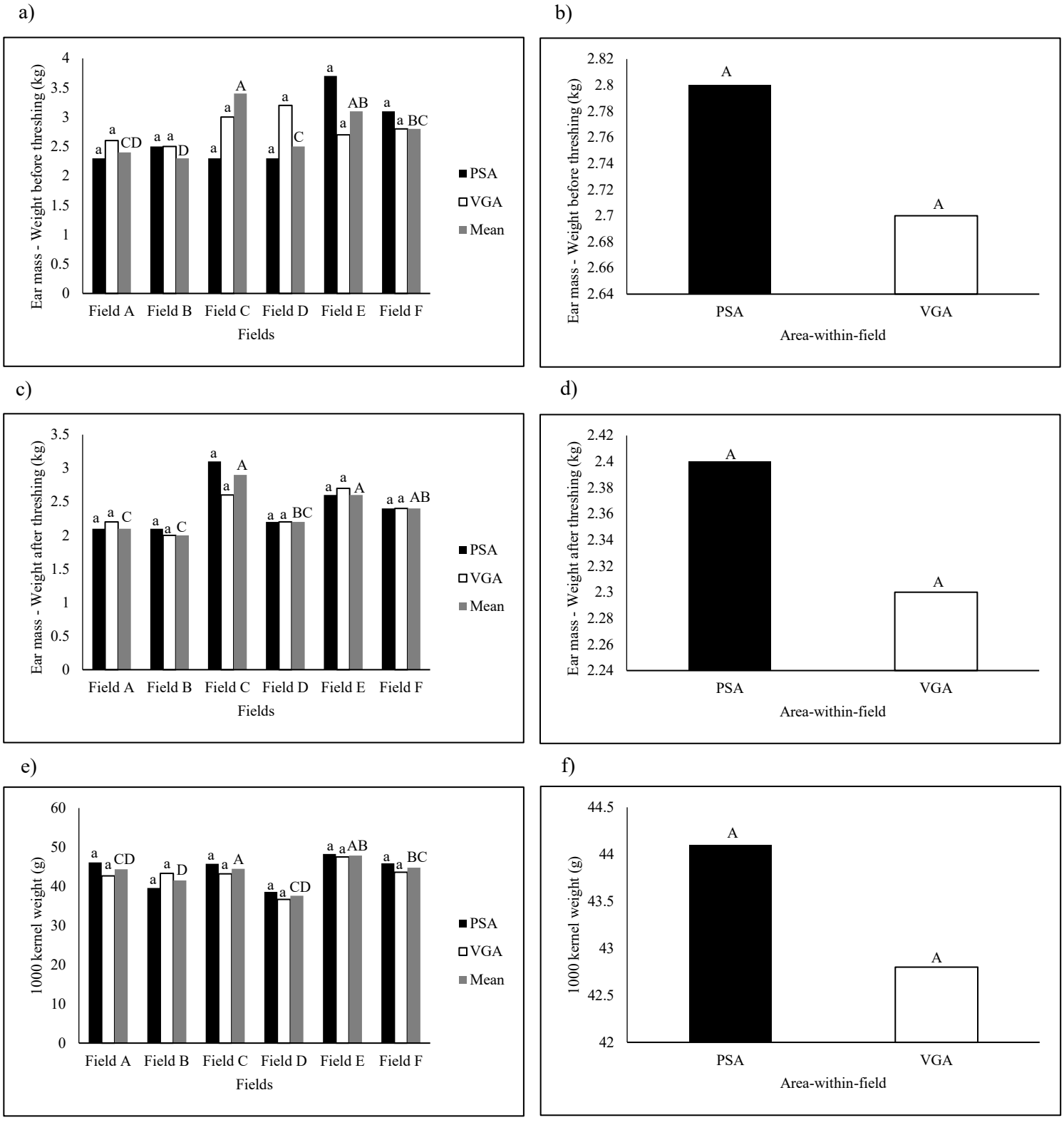


Figure 3.20: Ear and grain characters determined in PSA and VGA[1] maize plants over six localities during 2014/15. [2]

[1] PGA = prematurely senescing areas; VGA = visibly green areas
[2] A different letter denotes significance at $P \leq 0.05$.

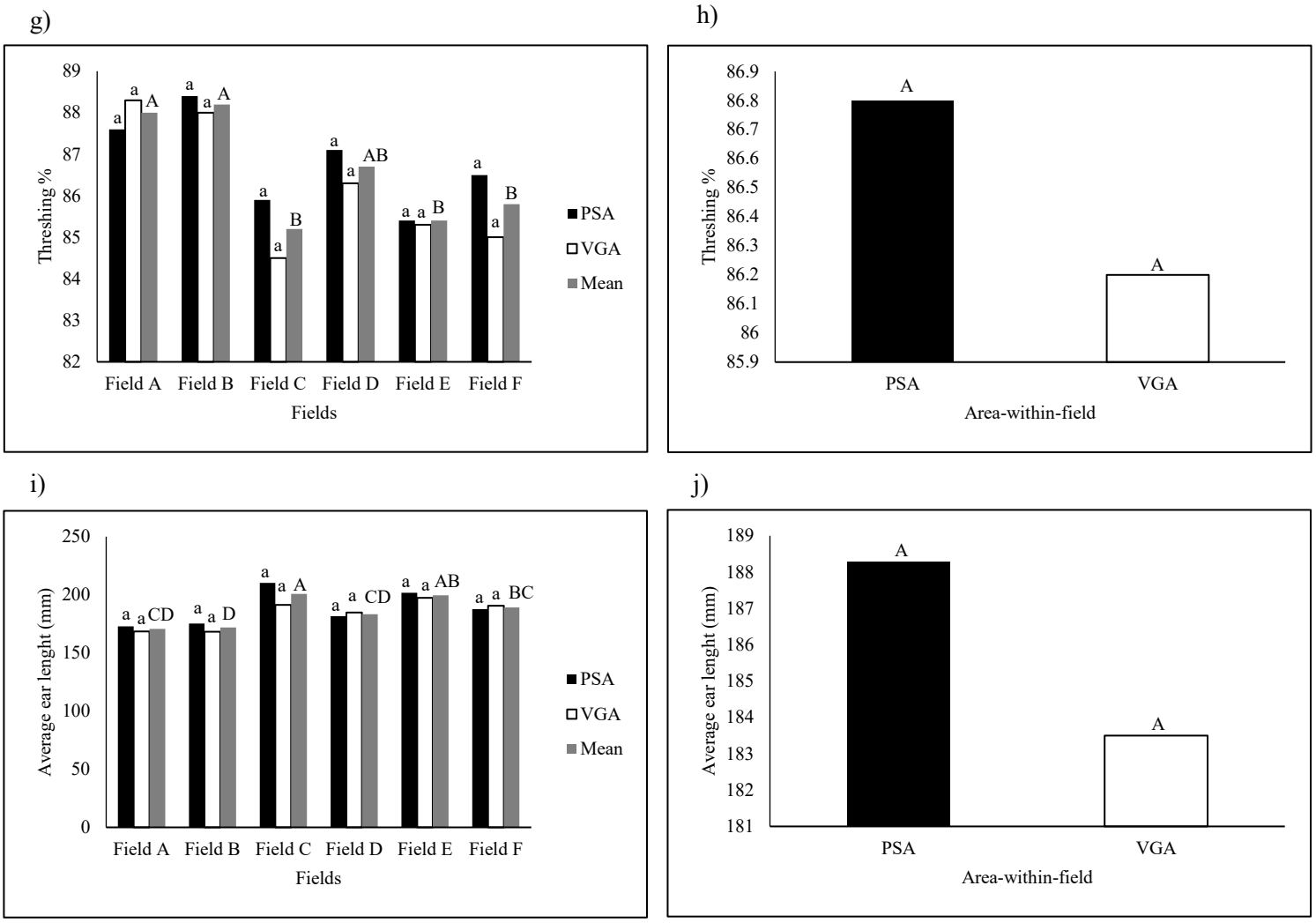


Figure 3.20 (cont.): Ear and grain characters determined in PSA and VGA[1] maize plants over six localities during 2014/15. [2]

[1] PGA = prematurely senescing areas; VGA = visibly green areas

[2] A different letter denotes significance at $P \leq 0.05$.

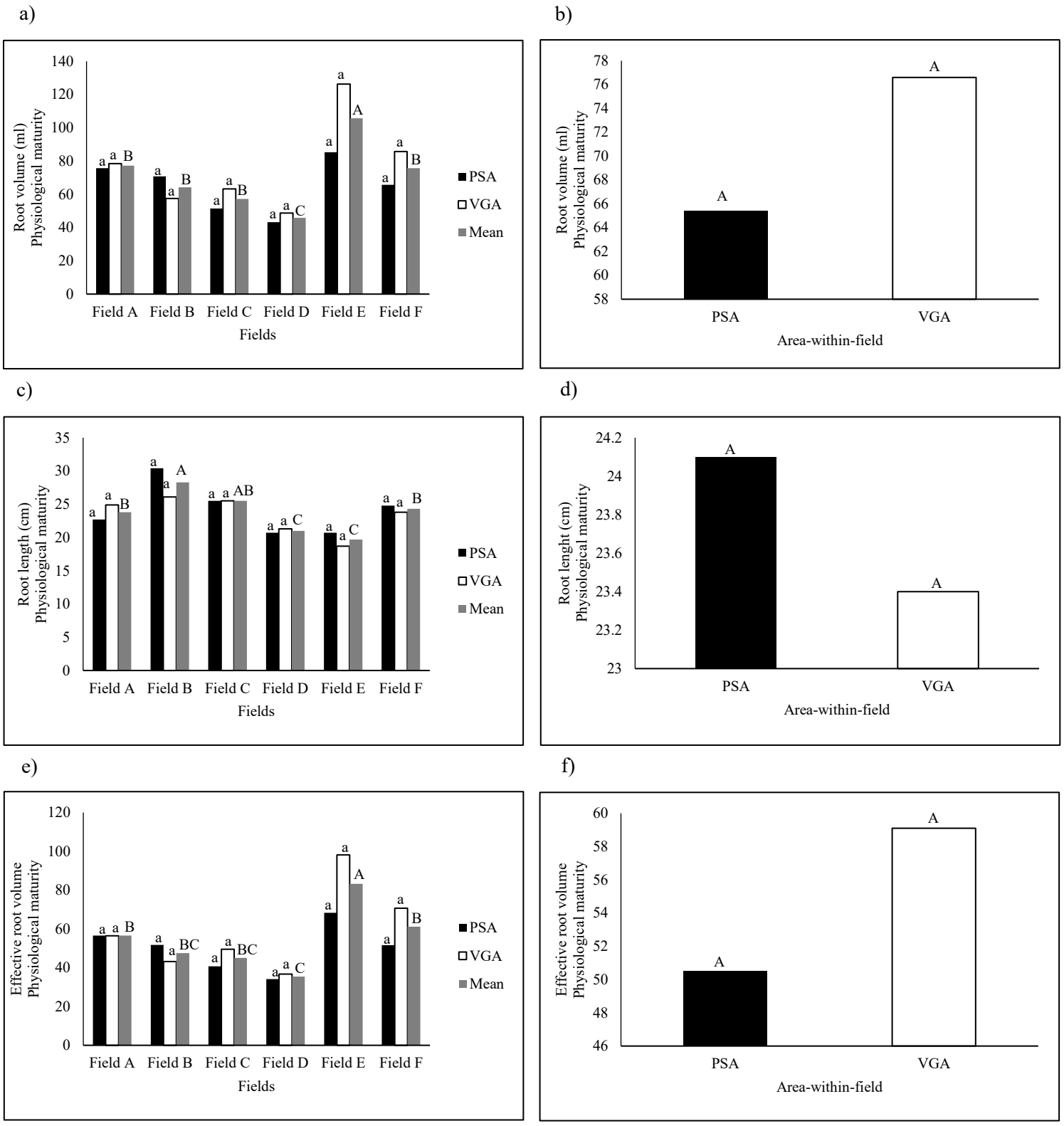


Figure 3.21: Phenotypic measurements of PSA and VGA[1] maize plants over six localities during 2014/15. [2]

[1] PGA = prematurely senescing areas; VGA = visibly green areas

[2] A different letter denotes significance at $P \leq 0.05$.

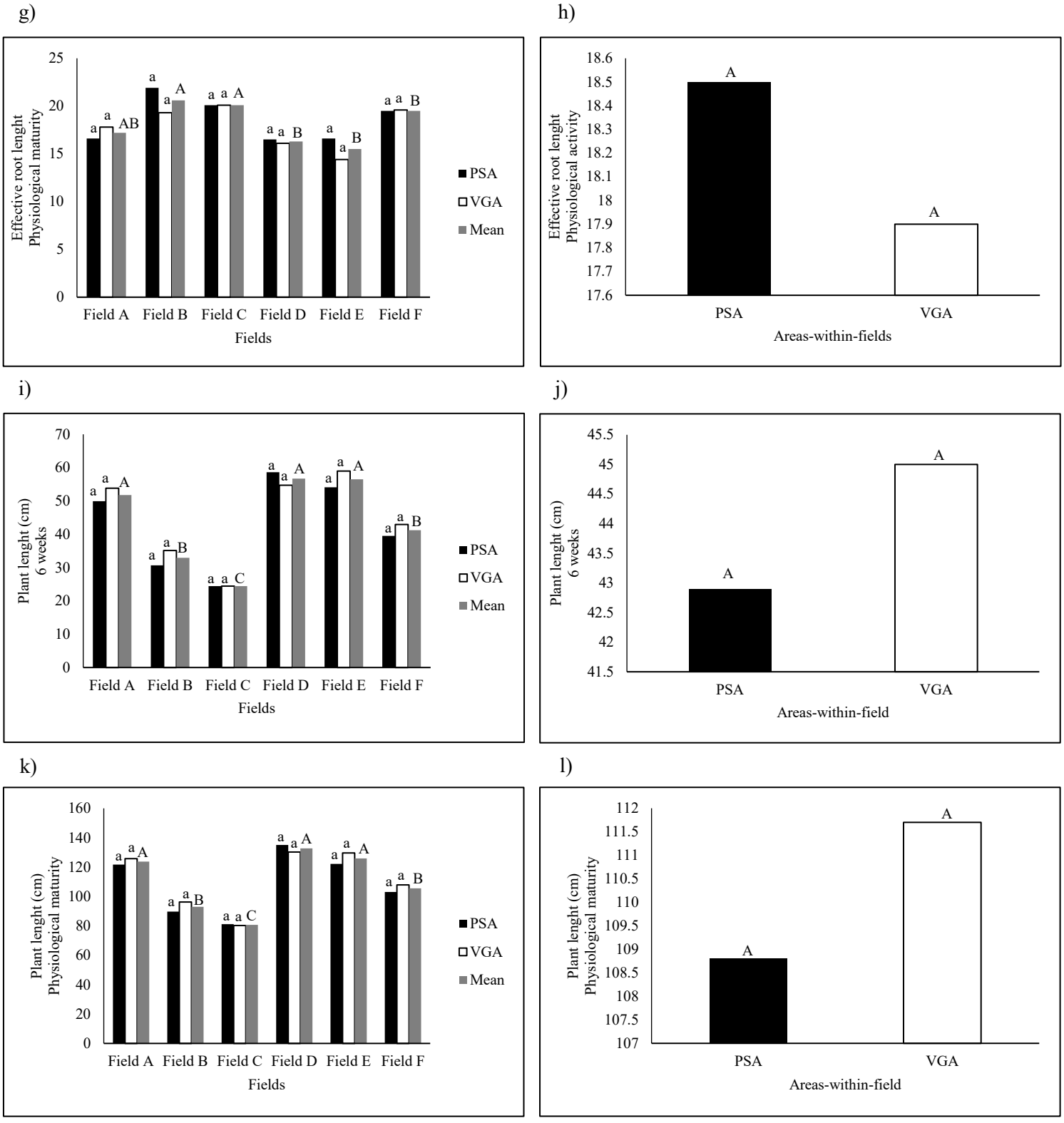


Figure 3.21 (cont.): Phenotypic measurements of PSA and VGA[1] maize plants over six localities during 2014/15. [2]

[1] PGA = prematurely senescing areas; VGA = visibly green areas

[2] A different letter denotes significance at $P \leq 0.05$.

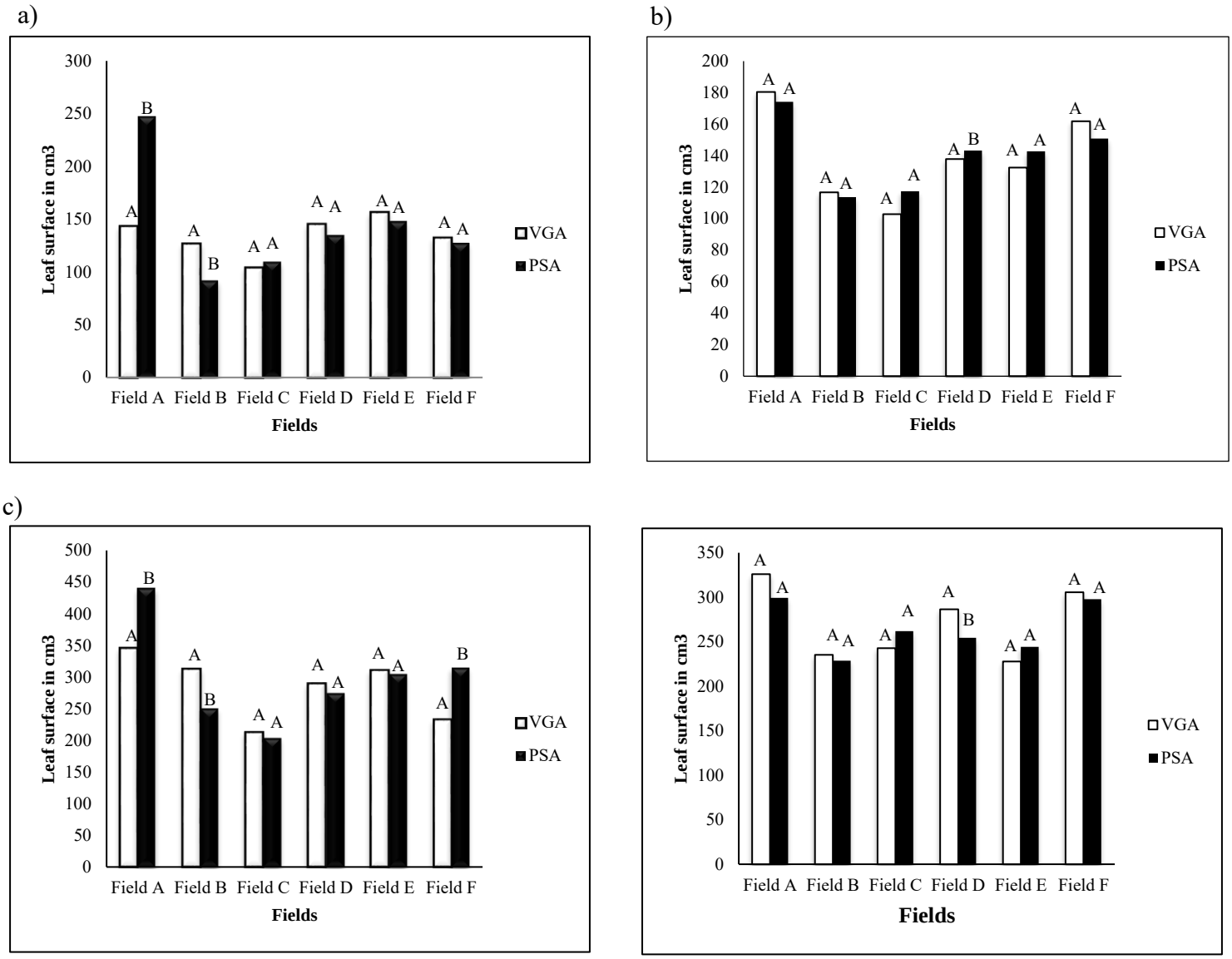


Figure 3.22: The leaf surface area of the a) third and c) fifth leaf determined at 6 weeks growth stage. The leaf surface area of the b) third and d) fifth leaf was determined at physiological maturity. [1]

[1] A different letter only denotes significance between different growth stages at $p \leq 0.05$ but not between visibly green plants and prematurely senescing plants.

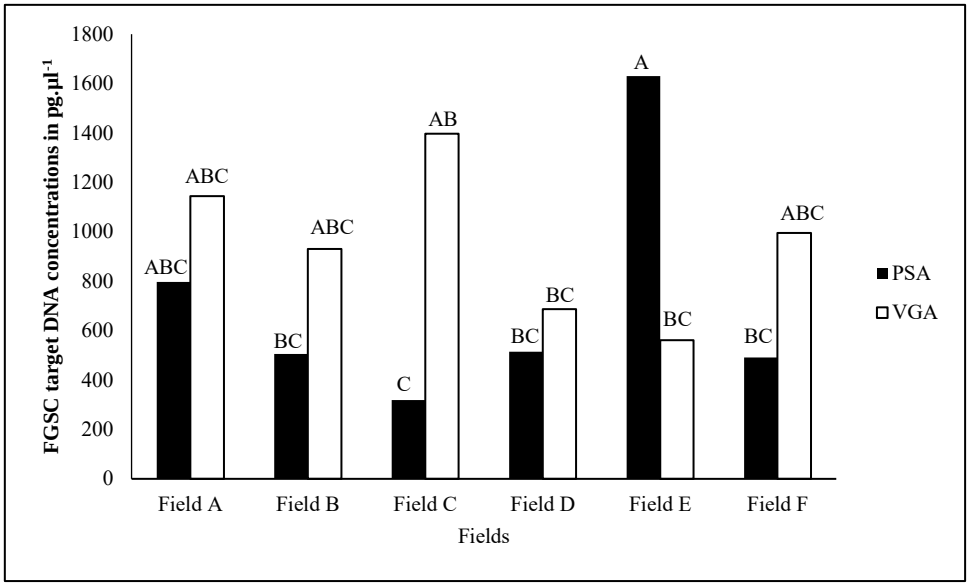


Figure 3.23: FGSC[1] target DNA quantification at all four growth stages in crowns over six localities during 2014/15. [2]

[1] FGSC = *Fusarium graminearum* species complex
[2] A different letter denotes significance at $P \leq 0.05$.

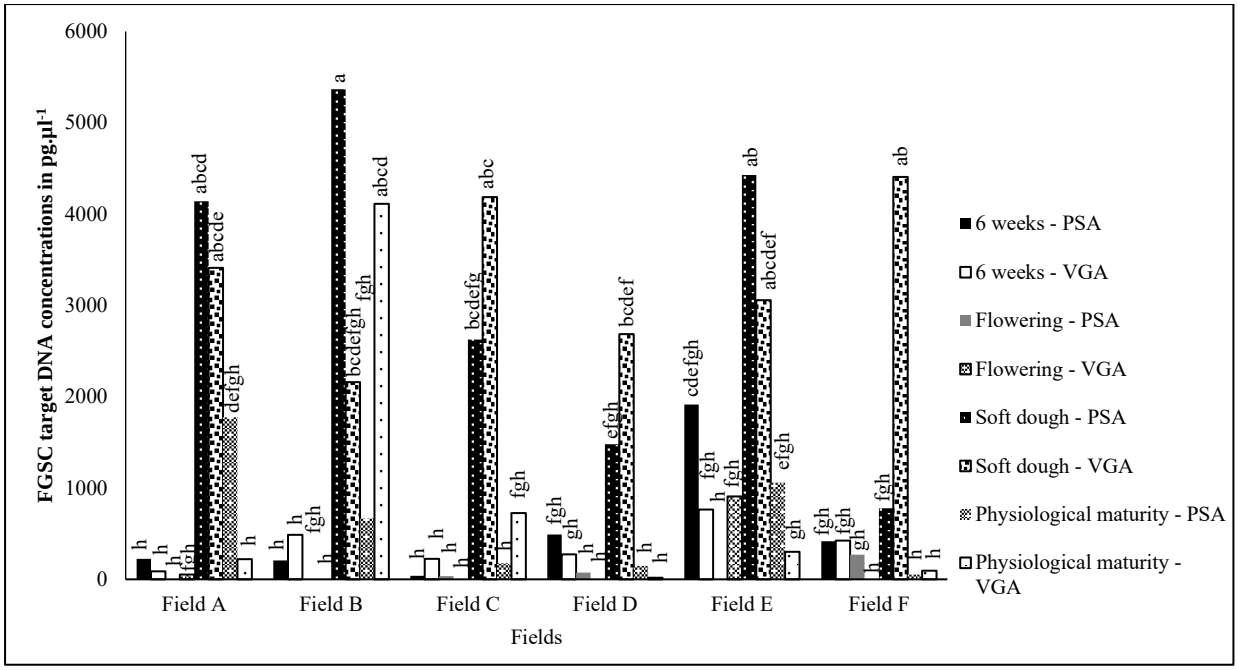


Figure 3.24: FGSC[1] target DNA quantification at four growth stages in internode 1 over six localities during 2014/15. [2]

[1] FGSC = *Fusarium graminearum* species complex
[2] A different letter denotes significance at $P \leq 0.05$.

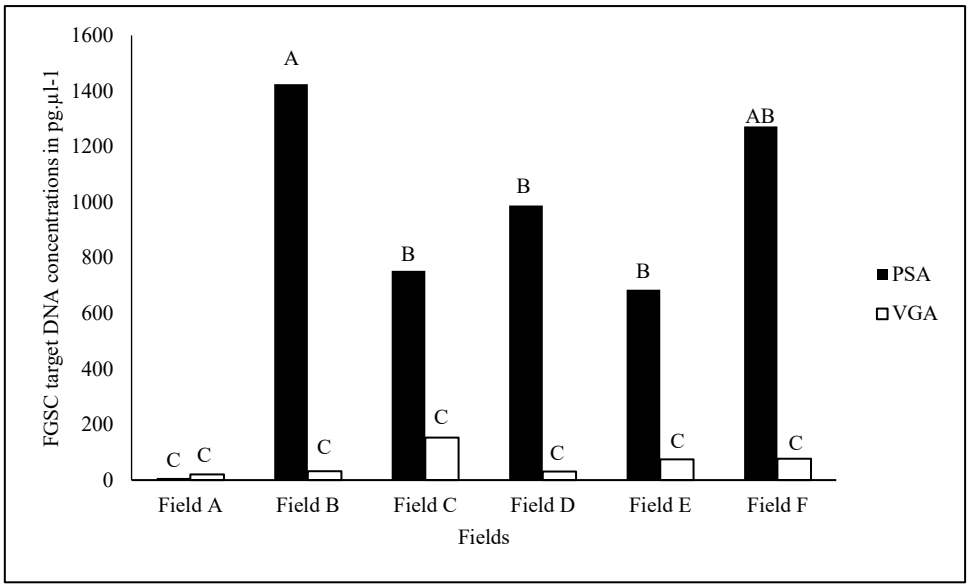


Figure 3.25: FGSC[1] target DNA quantification at all four growth stages in grain over six localities during 2014/15.

[1] FGSC = *Fusarium graminearum* species complex

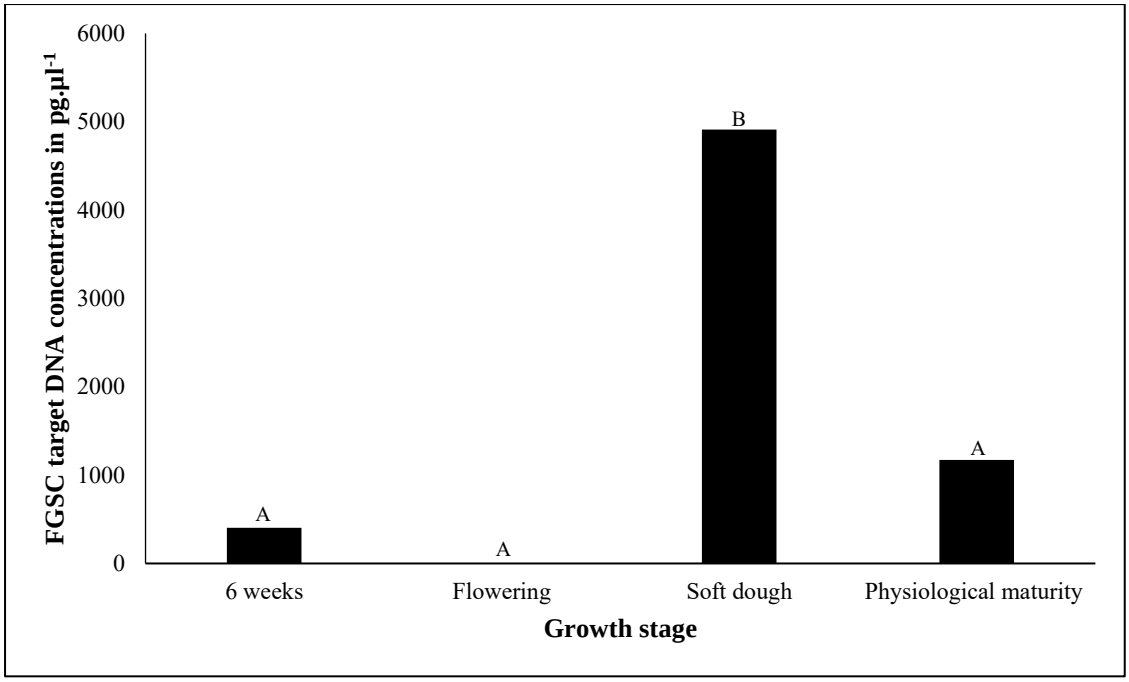


Figure 3.26: FGSC[1] target DNA concentrations in internode 2 at different growth stages during 2014/15. [2]

[1] FGSC = *Fusarium graminearum* species complex
[2] A different letter only denotes significance between different growth stages at $P \leq 0.0001$.

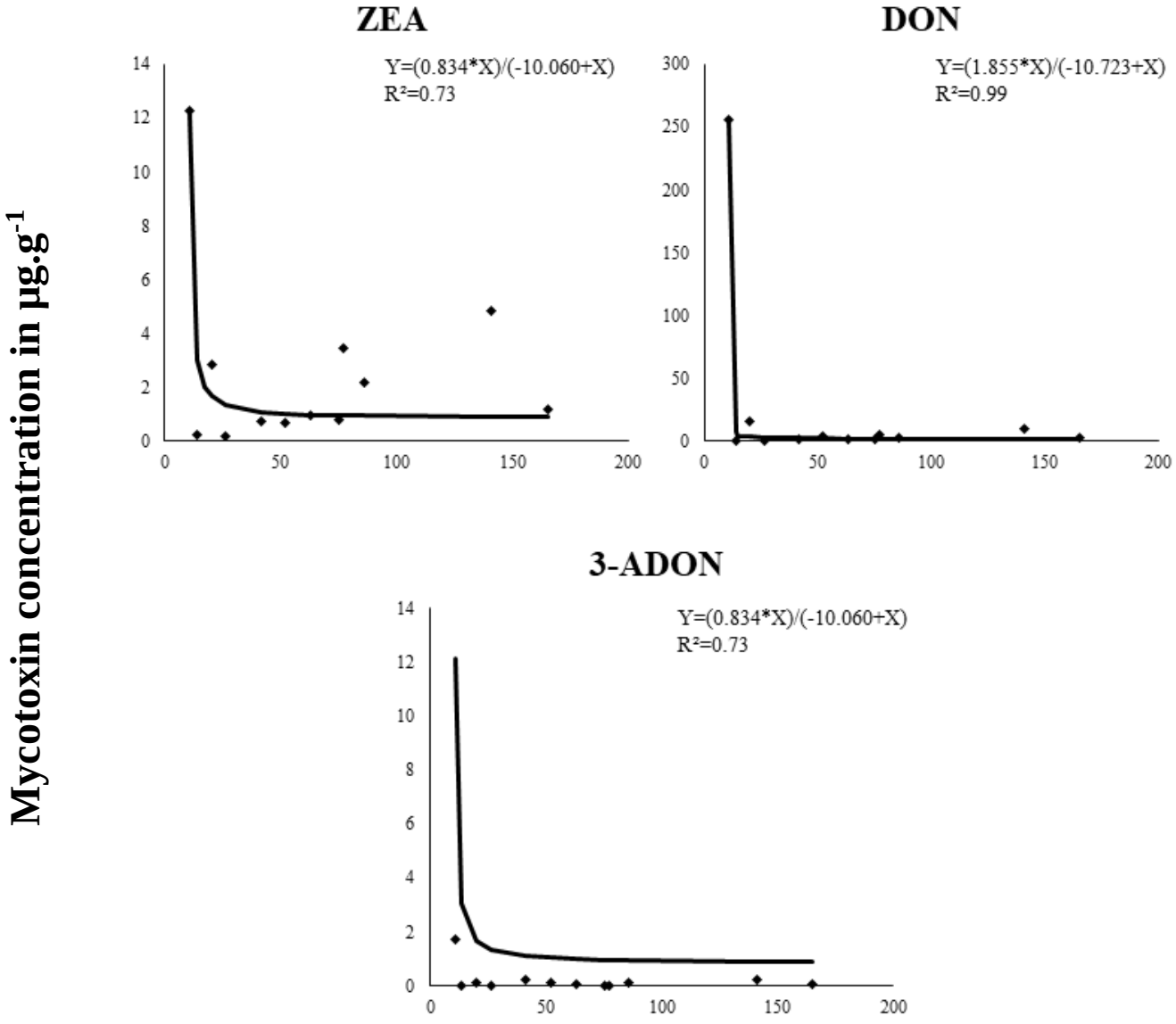
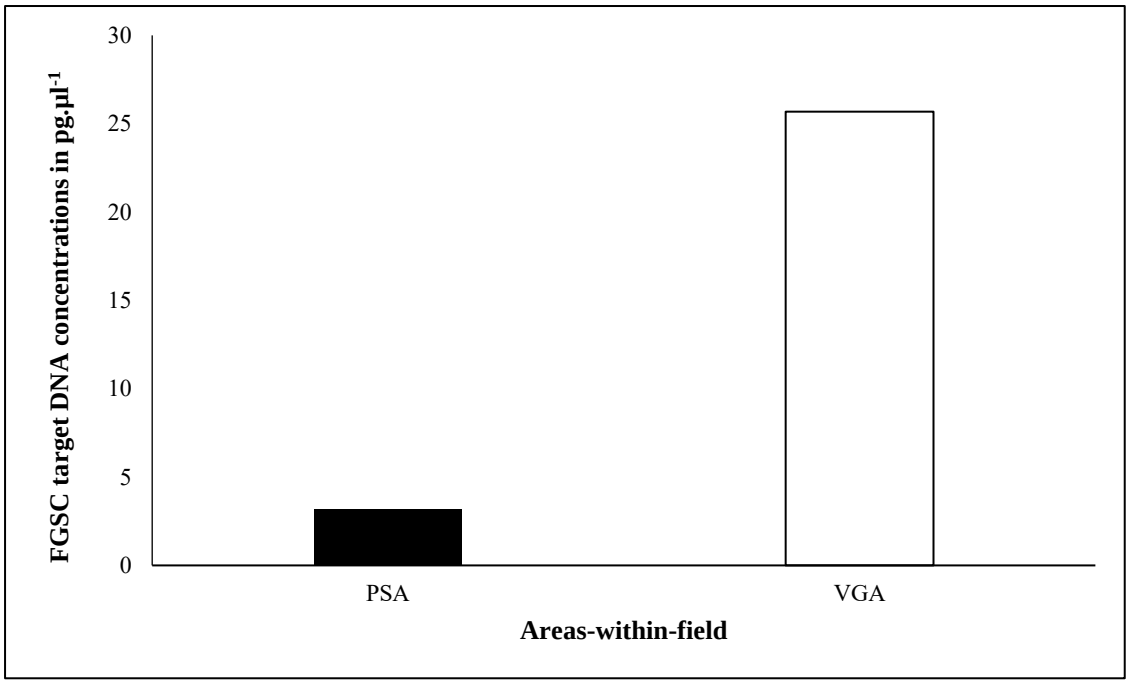


Figure 3.27: Relationships between FGSC[1] target DNA concentration in maize grain and mycotoxins determined in maize grain during 2014/15.

[1] FGSC = *Fusarium graminearum* species complex



[1]

Figure 3.28: Mean FGSC[2] target DNA concentrations in maize roots over three seasons indicating areas-within-field main effects. [3]

[1] PSA - prematurely senescing area; VGA – visibly green area
[2] FGSC = *Fusarium graminearum* species complex
[3] A different letter denotes significance at P=0.052.

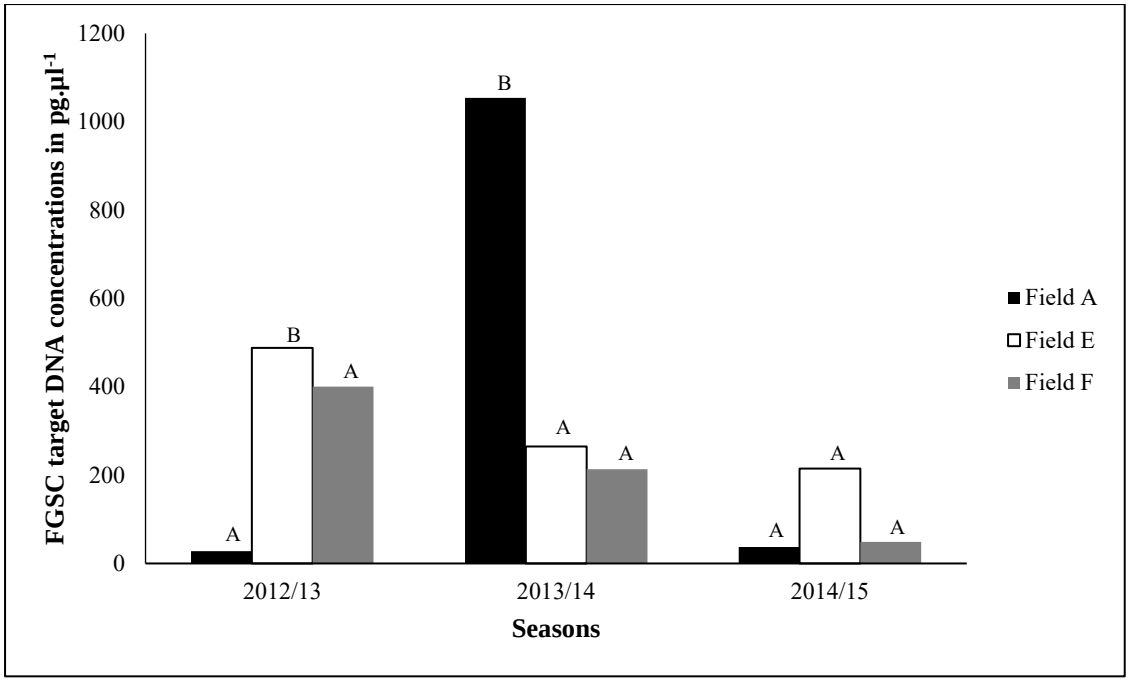


Figure 3.29: Mean FGSC[1] target DNA concentrations in maize crowns over three seasons indicating significant locality x season interactions. [2]

[1] FGSC = *Fusarium graminearum* species complex
[2] A different letter denotes significance at P=0.0250.

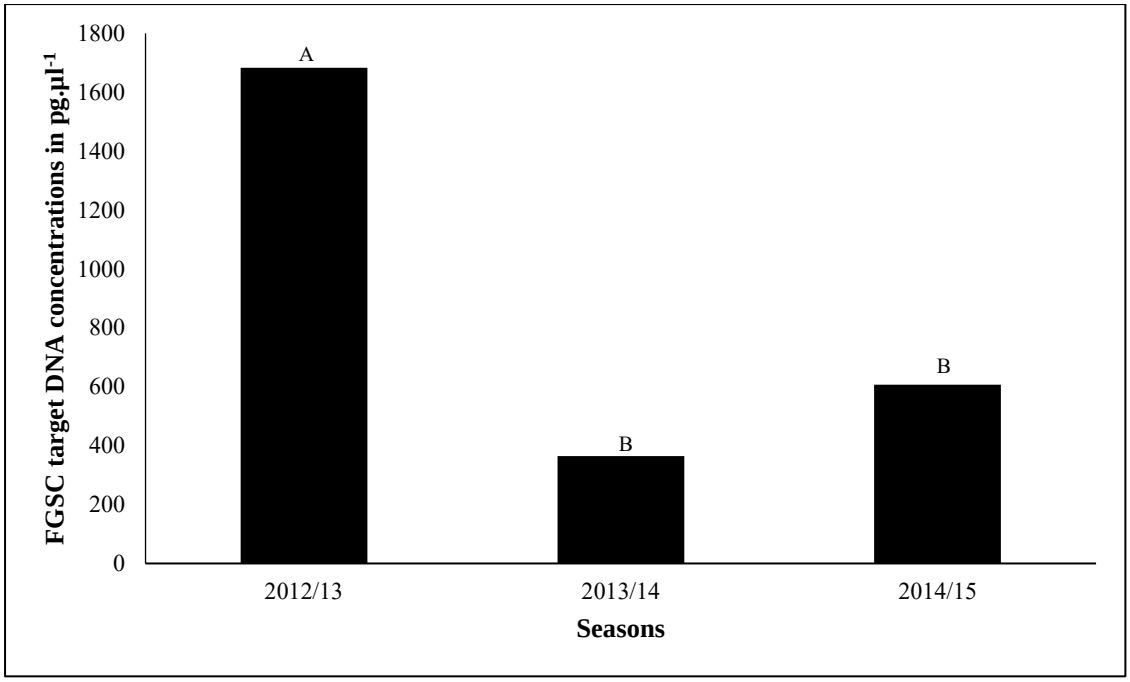


Figure 3.30: Mean FGSC[1] target DNA concentration in maize internode 1 associated with seasons when the localities and areas-within-fields are pooled. [2]

[1] FGSC = *Fusarium graminearum* species complex
[2] A different letter denotes significance at P=0.0184.

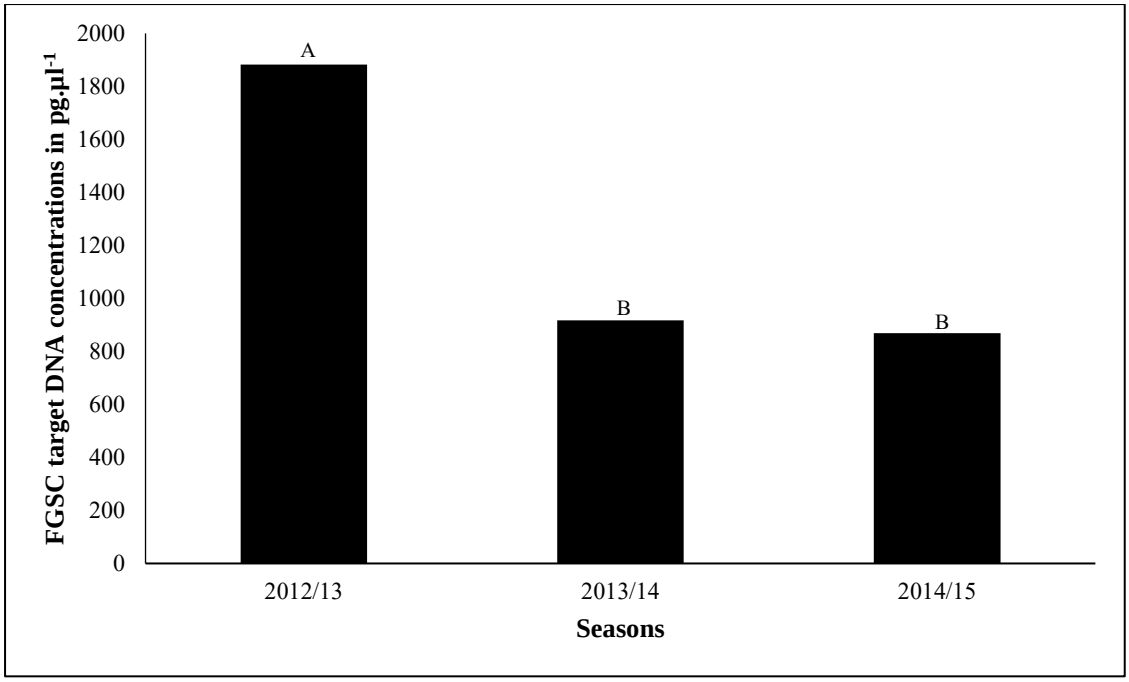


Figure 3.31: Mean FGSC target DNA concentration in maize internode 2 associated with seasons when the localities and areas-within-fields are pooled. [1]

[1] A different letter denotes significance at P=0.0372.

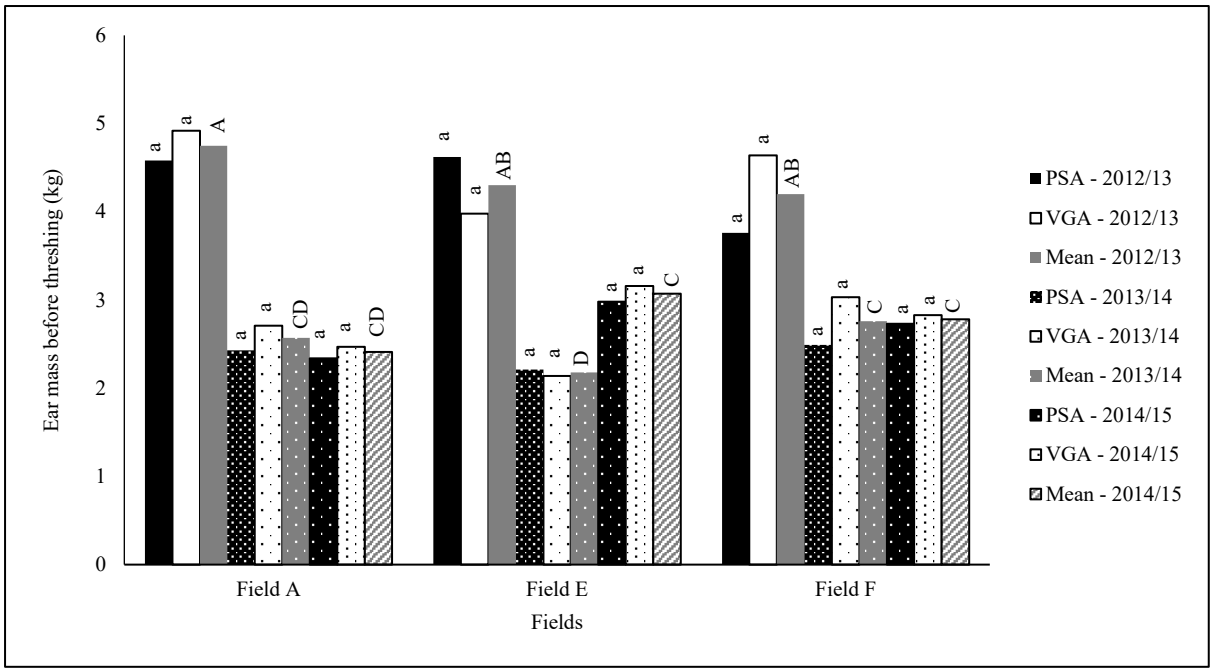


Figure 3.32: Locality x season interaction for ear mass before threshing over three seasons.[1]

[1] A different letter denotes significance at $P \leq 0.05$.

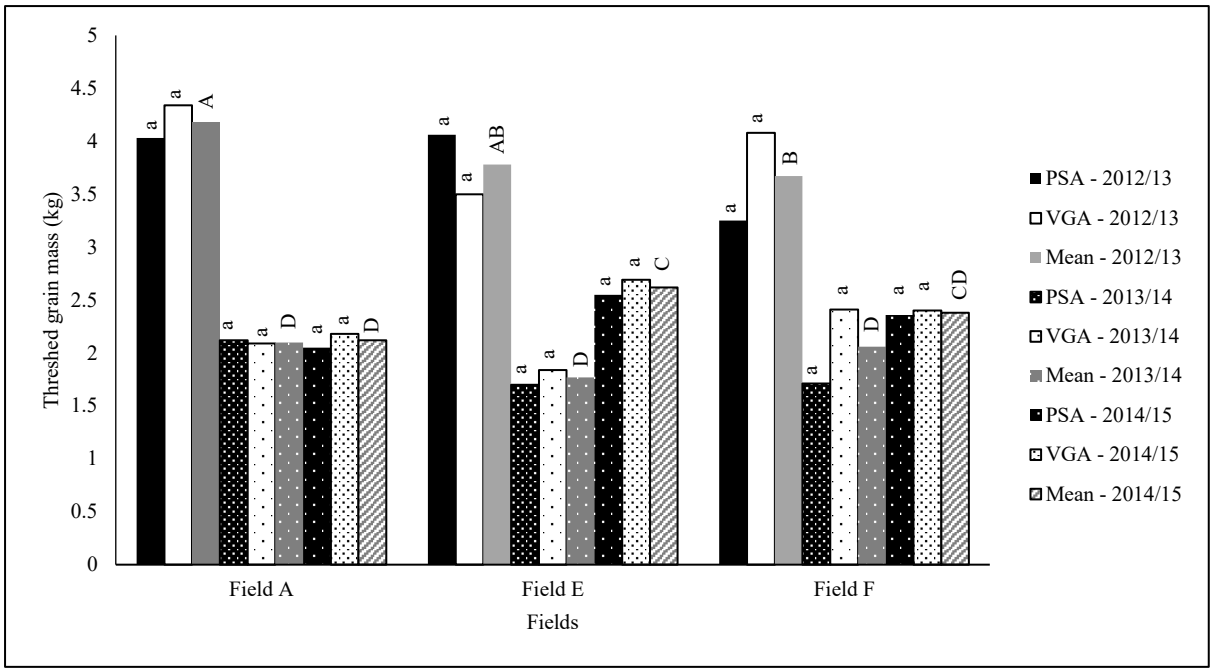
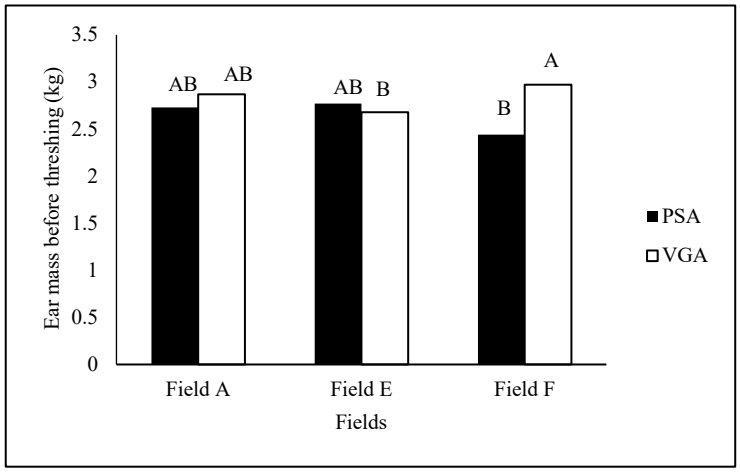


Figure 3.33: Locality x season interaction for threshed grain mass over three seasons.[1]

[1] A different letter denotes significance at $P \leq 0.05$.

a)



b)

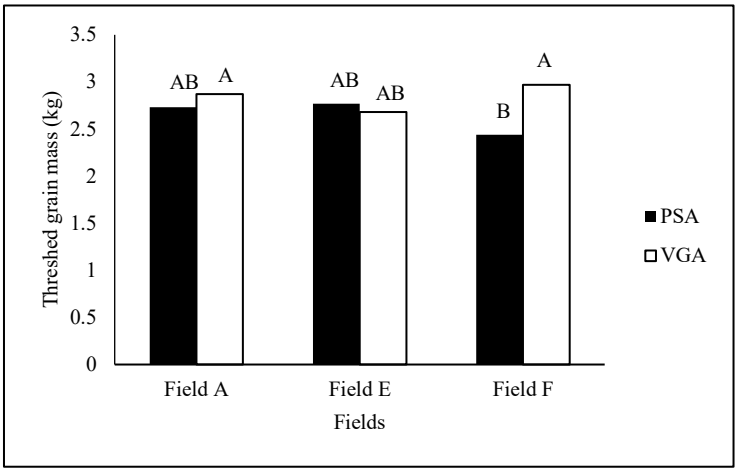


Figure 3.34: Locality x areas-within-field interaction on the three seasons' mean ear mass before threshing and threshed grain mass.

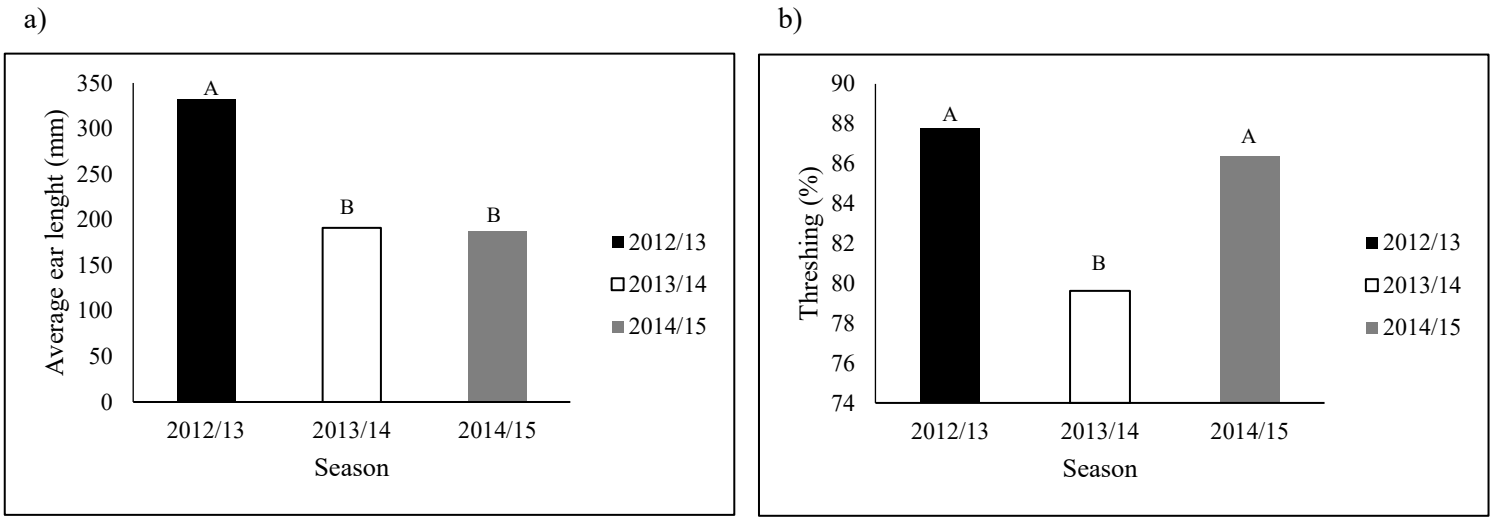


Figure 3.35: Season main effect on average ear length and threshing percentage during over three seasons. [1]

[1] A different letter denotes significance at $P \leq 0.05$.

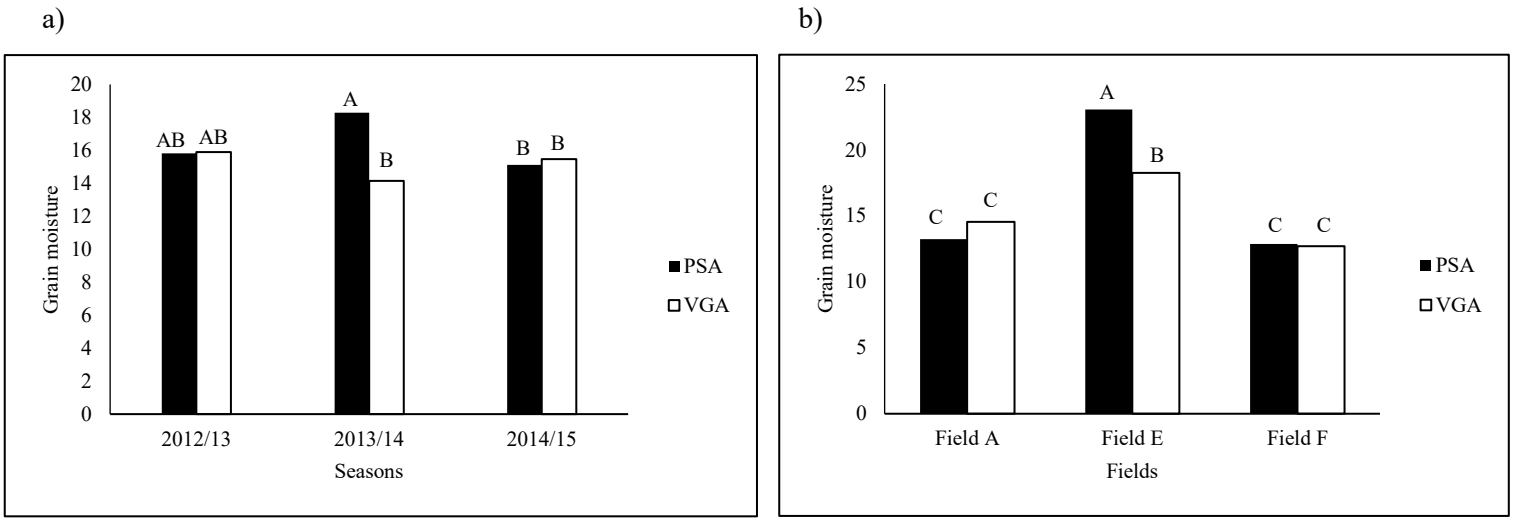


Figure 3.36: Locality x season and locality x areas-within-field interactions on grain moisture across three seasons. [1]

[1] A different letter denotes significance at $P \leq 0.05$.

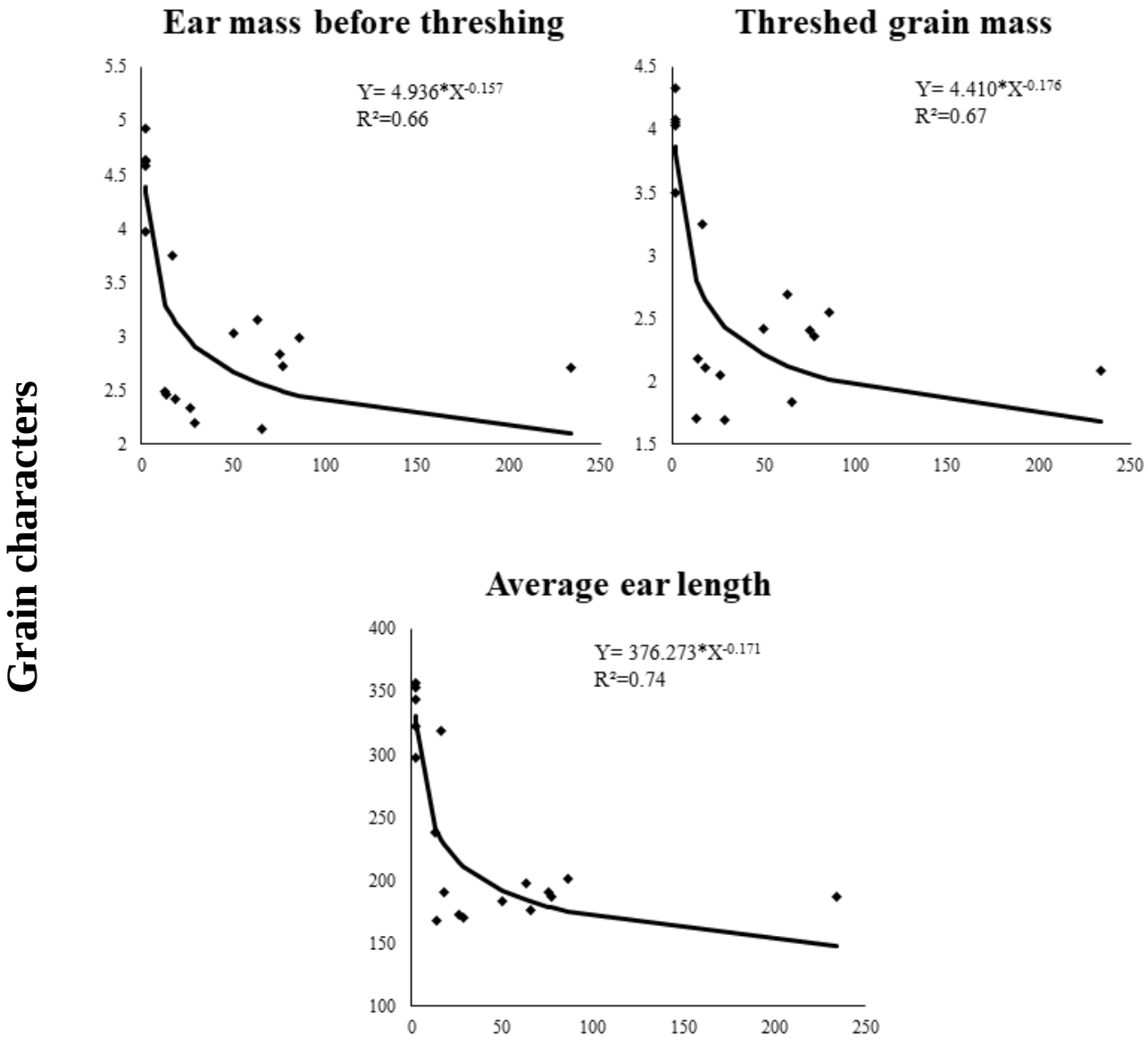


Figure 3.37: Relationship between FGSC target DNA concentration quantified in maize grain and the grain characteristics ear mass before threshing, threshed grain mass[1] and average ear length[2].

[1] Grain mass in kilogram - kg
[2] Ear length in millimetre - mm

Chapter 4: Evaluation of the possible systemic movement of fumonisins, deoxynivalenol, nivalenol and zearalenone via vascular bundles in maize

4.1 Abstract

It is estimated that approximately 25 – 50 % of the world's crops are contaminated with mycotoxins that can be produced pre-, during- or post-harvest. Important mycotoxins in maize in South Africa are deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) which are products of the *Fusarium graminearum* species complex (FGSC) and fumonisin (FUM) which is a product of *F. verticillioides.* The aim of this study was to determine whether DON, NIV, ZEA and FUM are translocated from maize roots and crowns to maize ears. Sterile soil was inoculated with DON-producing *F. graminearum sensu strictu* and F. *boothii*, NIV producing *F. acacia-mearnsii*, *F. meridionale* and *F. cortidariae* and FUM producing *F. verticillioides*. The five FGSC species are also ZEA producers. The concentration of the FGSC's total target DNA and each of the four mycotoxin concentrations were determined in the roots, crowns, internode (In) 2, In4, In6, In8, flowers and grain, using qPCR and LC-MS/MS respectively. Although NIV and ZEA were detected in the roots and crowns, they were not found in the upper tissues of the maize plants. DON co-occurred with FGSC species in the In8, flowers and grain. FUM was only detected in trace amounts, but the possibility of FUM translocation was demonstrated. Thus, mycotoxin translocation of DON, NIV and ZEA could not be confirmed but the possibility of FUM translocation was shown.

Keywords: mycotoxin translocation, FGSC, *F. verticillioides*, systemic infection

4.2 Introduction

Mould damage of grain and subsequent mycotoxin contamination has been regarded as an increasing consumption hazard (Bhat and Miller, 1991). Mycotoxins have been intensely studied since 1962, with the outbreak of Turkey-X disease, caused by aflatoxins (Reverberi *et al.*, 2010). Today it is estimated that approximately 25 – 50 % of the world's crops are contaminated with mycotoxins, which can be produced pre-, during- or post-harvest (Fanelli and Logrieco, 2014; Storm *et al.*, 2014). Mycotoxins pose a considerable risk to human and animal health, thus the allowable mycotoxin concentrations in food and feed is regulated globally (Escrivá *et al.*, 2015). The main risk of mycotoxin exposure is not acute doses but rather an ongoing exposure to low concentration, which may have long term immunologic, biologic and physiologic effects (Escrivá et al., 2015). Symptoms may present themselves even at doses that cannot be detected by modern technology (Kanora and Maes, 2009; Escrivá et al., 2015). Although 400 mycotoxins have been discovered to date, some of the most important mycotoxins on maize include the trichothecenes, deoxynivalenol (DON) and nivalenol (NIV) as well as zearalenone (ZEA). These mycotoxins are produced by specific members of the *Fusarium graminearum* species complex (FGSC). Another important and destructive mycotoxin is fumonisin (FUM) which is produced by *F. verticillioides* (Placinta *et al.*, 1999; Aoki *et al.*, 2012). These mycotoxins may co-occur as they are produced by the same species or species that co-infect maize plants (Streit *et al.*, 2012).

The mycotoxin that occur on local maize can cause disease in humans and animals (Placinta *et al.*, 1999). DON for example can cause feed refusal and a decrease in weight gain in swine. It also has immunosuppressant properties, which results in an increase in other diseases and may mask toxicoses (Bennett and Klich, 2003). ZEA may cause reproductive problems such as a reduction in litter size, swine estrogenic syndrome, and male infertility (Minervini and Dell'Aquila, 2008). FUM has been linked to a variety of diseases including equine leukoencephalomalacia, porcine pulmonary edema, liver cancer in rats and human oesophageal cancer (Reid *et al.*, 1999).

Mycotoxins may cause phytotoxicity in plants. Trichothecene production can result in necrosis, chlorosis and mortality of plants, which explains the wide range of symptoms observed during colonization by *Fusarium* spp. (Ismaiel and Papenbrock, 2015). Maize that is susceptible to Gibberella ear rot was shown to be more sensitive to DON phytotoxicity than resistant cultivars

(Cossette and Miller, 1995). FUM has been shown to have an inhibitory effect on root elongation when sprayed into germinating seedlings although the phytotoxic effect was overcome within a few days (Hamed and Abbas, 1992).

A question has arisen as to whether mycotoxins can be translocated from roots and lower plant tissues to maize kernels, as this could have a serious impact on mycotoxin-related food safety. According to the world health organisation (WHO) mycotoxins can be avoided by a) visually inspecting grain and removing any insect damaged or mouldy grain and b) by properly drying and storing grain. However, both rely on controlling mycotoxins post-harvest and assume that the mycotoxins would always co-occur with mycelial growth. It also fails to take into consideration mycotoxins that are released during milling practices. Trichothecenes have been found to be more toxic when inhaled than ingested (Peraica *et al.*, 1999). ZEA translocation has been shown to be possible in maize (Sutton *et al.*, 1976). However, this study used outdated techniques to determine the presence of *F. graminearum s.l.* and thus, the presence of ZEA could be attributed to asymptomatic infection of the fungus rather than mycotoxin translocation. DON translocation has been indicated in wheat infected with *F. culmorum* and FUM translocation has been shown to be possible from maize seedling roots to leaves (Zitomer *et al.*, 2008; Covarelli *et al.*, 2012). The aim of this study was to determine whether DON, NIV, ZEA and FUM can be translocated from mature maize roots and crowns to maize ears and grain.

4.3 Materials and Methods

4.3.1 Greenhouse trials

Drakensberg budgie bird seed mix (100 g) were added to 150 ml ddH_2O in Erlenmeyer flasks and sterilised in the autoclave for 30 minutes. Upon cooling, each flask was inoculated with 5-day old cultures of either *Fusarium graminearum* s.s., *F. boothii*, *F. acacia-mearnsii*, *F. cortidariae*, *F. meridionale* or *F. verticillioides* (Table 4.1) that had been cultivated on Potato Dextrose Agar. The FGSC species were obtained from the University of Stellenbosch and *F. verticillioides* from the Agricultural Research Council – Grain Crops Institute. The inoculated flasks were incubated at room temperature for 2 weeks on a Labotec (South Africa) orbital shaker at 200 rpm.

Trials were conducted in the greenhouse. Three treatments were prepared by separating the inoculum into DON producers (*F. graminearum* and F. *boothii*), NIV producers (*F. acacia-mearnsii*, *F. meridionale* and *F. cortidariae*) and *F. verticillioides*. One isolate of each represented species was used. The pooled inoculum was mixed with steam sterilised potting soil. Approximately 750 ml of inoculum was mixed with 40 L sterile soil. This mixture was placed in 80 L plant-bags, after which the remaining space was filled with sterile potting soil. An untreated control, which contained sterile soil only, was added as a fourth treatment.

Two maize cultivars, PANNAR 6Q-245 and PANNAR 6479, pre-treated with Celest® 100 FS (Fludioxonil 100 g/l, L 6173) and Poncho® 600 FS (Clothianidin Neonicotinoid 600 g/l, L 8581), were obtained from PANNAR Seed Company (Pty) Ltd. Seeds were surface sterilised with 1 % sodium hypochlorite for one minute, followed by three rinsing steps with ddH_2O. Seeds were plated onto Water Agar and incubated at room temperature for 4 days. Those seeds that were visually free from fungal and bacterial growth were planted in the respective soils and grown under greenhouse conditions at a 27/20 °C day/night temperature regime and 14/10 h day/night photoperiod. Plants were watered daily. The experiment consisted of four plants per replicate and three biological replicates.

The plants were sampled at two growth stages namely flowering (BBCH principle growth stage 5) and soft dough (BBCH principle growth stage 8, 83 – 85). The roots, crowns, internodes 1 to 10 (In1 – In10), flowers and grain were cut, bagged individually and stored at -20 °C until used. The frozen samples were subsequently ground using an Ika A11 analytical grinding mill. A 5 g milled fraction was placed in a 50 ml Falcon tube and 250 mg into 2 ml Eppendorf tubes (Eppendorf Scientific, Hamburg, Germany).

4.3.2 Mycotoxin analysis with LC-MS/MS

DON, NIV, ZEA and FUM concentrations in roots, crowns, In2, In4, In6, In8, flowers and grain were determined using LC-MS/MS. A 20 ml aliquot of 70 % methanol was added to the 5 g tissue fraction in falcon tubes. The mixture was shaken for 30 minutes at 200 rpm at room temperature using “The Rock-it” platform rocker (United Scientific, South Africa). The samples were centrifuged for 10 minutes at 500 x g at 4 °C. The supernatant was filtered using a 0.25 µm Acrodisc® syringe filter with a Supor® Membrane (Pall corporation, New York, USA). The filtrate was kept at 4 °C overnight and centrifuged at maximum speed the following

day. The supernatant was transferred to a new 2 ml Eppendorf tube and sent to the Central Analytical Facility (CAF) at the University of Stellenbosch for analysis.

4.3.3 Total DNA isolation

The 250 mg of ground tissue from section 4.3.1 were added to 900 µl of CTAB buffer (Saghai-Maroof *et al*., 1984) which contained β-mercaptoethanol (Merck KGaA, Darmstadt, Germany), 2 % CTAB (Sigma-Aldrich, Steinheim, Germany), 5 M NaCl (Merch KGa, Darmstadt, Germany), 1 M Tris (Merch KGa, Darmstadt, Germany), 0.5 M EDTA (Sigma-Aldrich, Steinheim, Germany), 0.2 % β-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany) at pH 8.0. The samples were flash frozen and incubated in boiling water for 5 minutes. After the 5-minute incubation, 2 µl of RNase A/T1 Mix (Fermentas, Hanover, USA) was added to break down the RNA and incubated for an hour at 37 °C. To deactivate the RNase and clean the samples from phenols, 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) (phenol - Sigma-Aldrich, Steinheim, Germany; Chloroform - Merch KGa, Darmstadt, Germany; isoamyl alcohol – Merck, KGaA, Darmstadt Germany) was added. The samples were vortexed and centrifuged for 10 minutes at 15294 x g. This cleaning step was repeated, after which the supernatant was transferred to another tube. The DNA was precipitated by adding 550 µl ice cold isopropanol (Merch KGa, Darmstadt, Germany) and incubating the samples at – 20 °C for 30 minutes. The samples were centrifuged for 20 minutes, after which the supernatant was discarded. The pellet was further cleaned by adding 70 % ethanol (Merch KGa, Darmstadt, Germany) and centrifugation at 20817 x g for 5 minutes. The ethanol was removed, and the pellet was air-dried. 1x TE buffer was added to the pellet and the sample was vortexed. The DNA was quantified with a NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc, Wilmington, USA). Samples that did not have an A260/A280 ratio of 1.8 – 2 were discarded. Those that met the correct standards were diluted to 10 ng/µl for further analysis.

4.3.4 Total DNA extraction of pure fungal cultures

The *F. graminearum* s.s., *F. boothii*, *F. acacia-mearnsii*, *F. cortaderiae*, *F. meridionale* or *F. verticillioides* cultures were plated onto half strength potato dextrose agar (PDA) (Merck, Whitehouse Station, USA) and allowed to grow for 7 days. Genomic DNA was extracted using the above methodology. The DNA concentration was determined using a Nanodrop

spectrophotometer and the quality was determined by an A260/A280 ratio of 1.8 – 2. The DNA was stored undiluted at - 20 ºC until used.

4.3.5 Quantification of Target DNA

A standard curve was set up for the DON, NIV and FUM producers. This was done by diluting the pure cultures DNA mentioned in 4.3.1, 4-, 16-, 64-, 256-, 1024-, and 4096 times in maize DNA that was free of pathogenic fungal contamination. The maize DNA was prepared by surface sterilising a kernel and plating it onto a sterile Water Agar medium. The kernel germinated and grew on the sterile medium for 14 days and was inspected for fungal and bacterial contamination. The whole seedling, including the roots was frozen in liquid nitrogen and DNA was extracted as described above. FGSC DNA was quantified using the primer sequences FgramB379 (CCA TTC CCT GGG CGT) and FgramB411 (CCT ATT GAC AGG TGG TTA GTG ACT GG). These primers are specific to the complex as a whole, but do not differentiate between species within the complex. *F. verticillioides* DNA was quantified using Fver356 (CGT TTC TGC CCT CTC CCA) and Fver412 (TGC TTG ACA CGT GAC GAT GA) (IDT) (Nicolaisen *et al.*, 2009; Schoeman, 2016). A reaction mixture was set up with 0.2 µM of each primer, 1x iTaq Universal SYBR Green supermix and 0.8 ng DNA. The qPCR was performed on a CFX96 (Bio-Rad, Hercules, USA) and the protocol was as follows: 95 °C for 10 minutes and 40 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 15 seconds. A melt curve analysis was performed by heating the products to 95 °C for 30 seconds then cooling to 40 °C for 30 seconds followed by a gradual heating from 60 °C to 95 °C with a 1 °C increment every 10 seconds.

4.4 Results

4.4.1 FGSC target DNA and mycotoxin translocation

No FGSC target DNA was detected at flowering stage in all tissues in PAN 6Q-245 inoculated with *F. boothii* and *F. graminearum* s.s.. DON was however detected at low concentrations in roots (0.3 µg.g^{-1}) and crowns (0.11 µg.g^{-1}) at flowering stage. FGSC target DNA, from the *F. boothii* and *F. graminearum* s.s. treatment, could be detected in the roots, crowns, In2, In4, In6, In8, flowers and grain of PAN 6Q-245 at soft dough stage (Figure 4.1). DON was detected

in In8, flowers and grain of this cultivar at soft dough but not in other plant organs (Figure 4.1). NIV was detected in the roots while ZEA was not detected.

FGSC target DNA, particularly in the treatment containing *F. boothii* and *F. graminearum* s.s., was detected in the roots, crowns, In2, In4 and flowers of PAN 6479 at flowering stage (Figure 4.2). DON was detected in the roots and crowns whereas ZEA was only detected in the flowers (Figure 4.2). FGSC target DNA could be detected in the roots, crowns, In2, In4, In6, In8 and grain of PAN 6479 at the soft dough stage (Figure 4.3). DON and ZEA was detected in the roots and crowns but ZEA was found at lower concentrations (Figure 4.3).

FGSC target DNA, particularly in treatments including *F. acacia-mearnsii*, *F. cortidariae* and *F. meridionale* was detected in the roots (0.19 $pg.\mu l^{-1}$), In2 (0.25 $pg.\mu l^{-1}$) and In6 (14.67 $pg.\mu l^{-1}$) of PAN 6Q-245 at the flowering stage, but no mycotoxins were detected in any of the tissue (data not shown). Very low concentrations of FGSC target DNA were detected in PAN 6Q-245 roots, crowns, In2, In6, In8 and flowers at the soft dough stage (Figure 4.4). NIV was detected in the roots of PAN 6Q-245 at the soft dough stage (Figure 4.4). FGSC target DNA, for this treatment was detected in PAN 6479 roots (0.76 $pg.\mu l^{-1}$), crowns (3.21 $pg.\mu l^{-1}$), In2 (0.59 $pg.\mu l^{-1}$), In4 (0.64 $pg.\mu l^{-1}$), In6 (0.52 $pg.\mu l^{-1}$), In8 (0.42 $pg.\mu l^{-1}$) and flowers (0.79 $pg.\mu l^{-1}$) however no mycotoxins were detected in any tissue at the flowering stage (data not shown). No FGSC target DNA or mycotoxins were detected at the soft dough stage of PAN 6479 infected with *F. acacia-mearnsii*, *F. cortidariae* and *F. meridionale.* The control samples did not contain any mycotoxins or FGSC target DNA.

4.4.2 *F. verticillioides* target DNA and mycotoxin translocation

F. verticillioides target DNA was detected in PAN 6Q-245 roots, crowns, In4 and In6 at the flowering stage (Figure 4.5). FUM was detected in the roots, crowns, In2, In4, In6, In8 and flowers (Figure 4.5). No *F. verticillioides* target DNA or FUM was detected in PAN 6Q-245 at the soft dough stage. *F. verticillioides* target DNA was detected in PAN 6479 roots, crowns, In2, In4 and In6 at the flowering stage (Figure 4.6). FUM was detected in the roots, crowns, In2, In4, In6, In8, flowers and grain (Figure 4.6). *F. verticillioides* target DNA was detected in PAN 6479 roots, crowns, In2, In6 and In8 at the soft dough stage (Figure 4.7). FUM was detected in the roots, crowns, In2, In4, In6, In8, flowers and grain (Figure 4.7). The control plants did not contain *F. verticillioides* target DNA or FUM concentrations.

4.5 Discussion

The concept of mycotoxin translocation from lower infected tissues to upper plant parts, is not new and is often linked with Dodd's (1980) theory of photosynthetic stress-translocation balance (Sutton *et al.*, 1976; Dodd and White, 1999). This theory states that the roots and stalks of maize plants become more susceptible to infection by pathogens during grain fill as most carbohydrates are translocated from the roots and stalks to the grain. Carbohydrates are used in a variety of processes including plant defence responses, which leads to higher infection rates of both the roots and stalks as the levels decrease. In certain instances, the mycotoxins produced by these pathogens, may be translocated with the carbohydrates from the lower plant tissues to the grain (Dodd, 1980; Dodd and White, 1999). The hypothesis was proposed that tricothecenes and FUM are hydrophilic compounds (Tibola *et al*., 2015) and could therefore be transported from the roots to the flower tissues via the xylem. ZEA is hydrophobic (Tibola *et al*., 2015) and is consequently less mobile in the plant.

Two studies, using qPCR and LC-MS/MS analysis, indicated that DON could be translocated from the roots of wheat plants into the upper plant tissues (Covarelli *et al.*, 2012; Winter *et al.*, 2013). The current study seems to indicate this not to be the case with maize as no translocation was recorded at flowering or at the soft dough stage despite the pathogen being recorded in the lower tissues. Although DON was not detected in the lower region of PAN 6Q-245 it was detected in In8, flowers and grain. It has been shown in previous studies that a *F. graminearum* isolate, mutated to not produce DON, could infect wheat plants and cause crown rot (Mudge *et al.*, 2006; Quesada-Ocampo *et al.*, 2016). A study performed by Janse van Rensburg *et al.* (2014) also showed that although there could be a positive relationship between *F. verticillioides* and FUM, there were situations where low mycotoxin concentrations were quantified at high target DNA concentrations, and vice versa. A similar conclusion was reached in this study as FGSC target DNA was quantified in the absence of DON and low FGSC target DNA was quantified with high concentration of DON. Thus, in this study it is suggested that DON was produced as an added virulence factor when infecting PAN 6Q-245 ears (Mudge *et al.*, 2006; Quesada-Ocampo *et al.*, 2016).

Previous studies have found that ZEA could be translocated from inoculated maize stalks to ears. Although ZEA was found in uninoculated ears, no ZEA was recorded in the stalks (Sutton *et al.*, 1976) raising uncertainty about the observation. ZEA can be produced by all five FGSC

species used in the current study (Beukes, 2015, Beukes *et al*., 2017). However, it was only detected in PAN 6479 roots and crowns of maize inoculated with *F. boothii* and *F. graminearum* s.s.. Sutton *et al.*'s (1979) study estimated that a 5-10 times dilution occurs during translocation and estimated that the roots contained 9 -15 $\mu g.g^{-1}$ ZEA that translocated 1.8 $\mu g.g^{-1}$ to the grain. If this is applied to our study using the lowest root ZEA concentration of 0.02 $\mu g.g^{-1}$, then a concentration of 0.002 $\mu g.g^{-1}$ should have been recorded in the grain, which is higher than ZEA's level of detection. If translocation was taking place, ZEA should have been detected in higher concentrations throughout the stalk, which was not observed. This could be related to different growth conditions and genotypes compared to those used in previous studies and warrants further investigation.

This was the first study to determine the possibility of NIV translocation. Although high concentrations of NIV were detected in the roots, it was not found in the upper regions of the plant. Even when FGSC target DNA was present in the grain, there was no detectable NIV in the upper plant tissues. Thus, there was no evidence of NIV translocation. This could be attributed to either NIV's inability to translocate or the conditions were not favourable for NIV translocation.

Zitomer *et al.* (2008) and Winter *et al*. (2013) showed that FUM can be translocated from the roots to the leaves of *F. verticillioides* infected maize seedlings. Similarly, the FUM concentration in this study was too low (lower than the LOD and thus not reliable) FUM was quantified in the roots, crowns, stalks and certain instances grains and flowers, whereas *F. verticillioides* target DNA could only be detected to In6/In8. This indicates the possibility of FUM translocation.

4.6 Conclusion

DON and ZEA translocation throughout maize plants has in the past been shown to be possible, however in this study translocation of DON, NIV and ZEA was not shown. This was attributed to either the inability of these mycotoxins to be translocated in maize, the conditions were not ideal for translocation, or the maize genotypes were not conducive to translocation. The possibility of FUM translocation has only been evaluated in maize seedlings from roots to the leaves. In this study it was shown that there is a possibility of FUM translocation but because of the trace amounts of FUM found this possibility could not be confirmed. Thus, mycotoxin

translocation of DON, NIV and ZEA was not confirmed but the possibility of FUM translocation was shown.

4.7 References

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Table 4.1: FGSC and *F. verticillioides* isolates collected from wheat and maize throughout South Africa, used to determine the possibility of mycotoxin translocation in maize.

Fungal species	Isolate ID	Geographical origin in South Africa	Original host	Chemotype
F. verticillioides	GCI340	Kwazulu-Natal	Maize	FUM
F. boothii	2.881	Free State	Wheat	15-ADON
F. acacia-mearnsii	2.889	Kwa-Zulu Natal	Wheat	NIV
F. meridionale	2.853	Kwa-Zulu Natal	Wheat	NIV
F. graminearum s.s.	2.574	Mpumalanga	Wheat	15-ADON
F. cortaderiae	2.551	Western Cape	Wheat	NIV

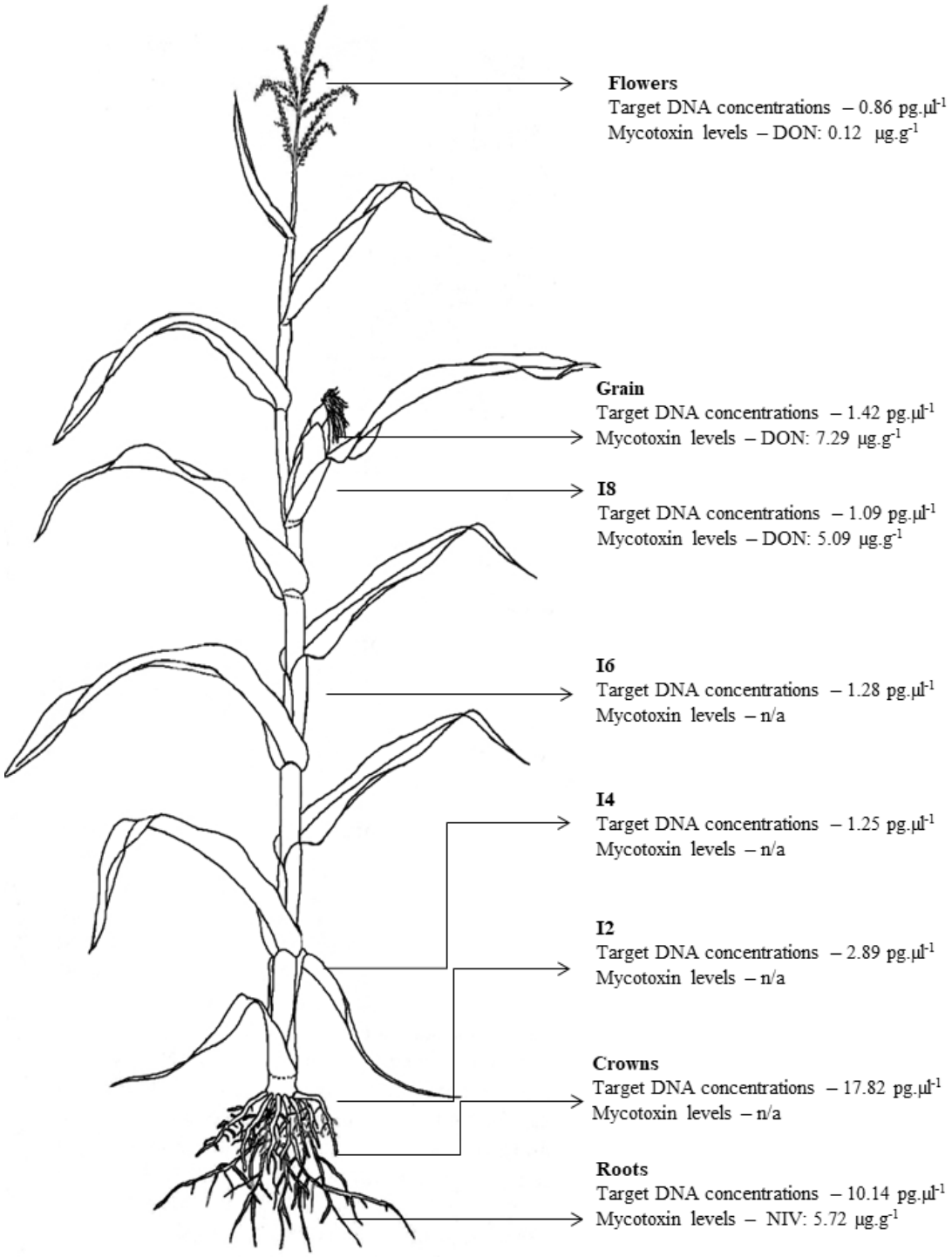

Figure 4.1: Mycotoxin translocation determined in PAN 6Q-245 when inoculated with both *F. boothii* and *F. graminearum* s.s. and harvested at the soft dough stage. [1]

[1] Target DNA concentrations = FGSC target DNA; n/a = not applicable as there were no mycotoxins present

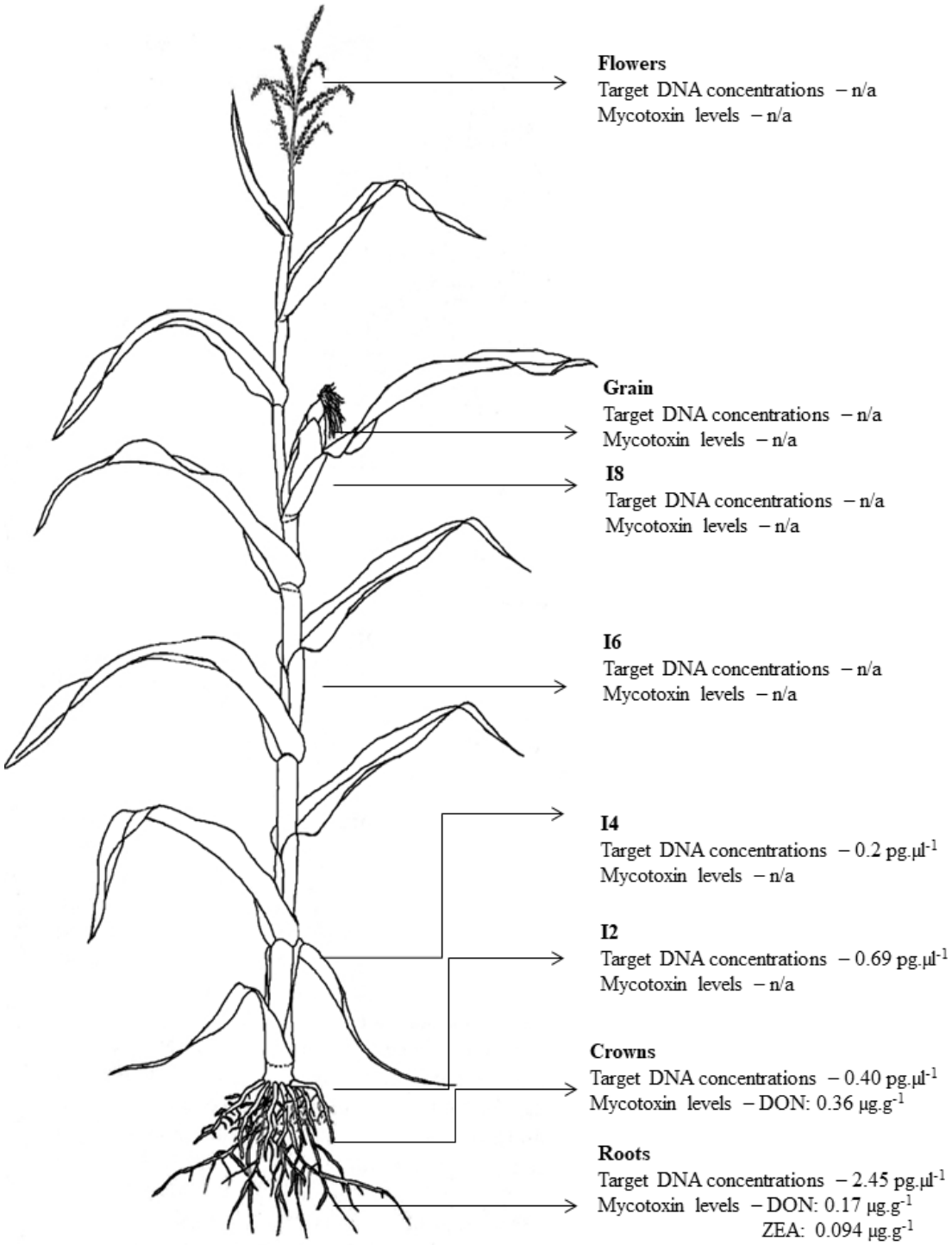

Figure 4.2: Mycotoxin translocation determined in PAN 6479 when inoculated with both *F. boothii* and *F. graminearum* s.s. and harvested at the flowering stage.[1]

[1] Target DNA concentrations = FGSC target DNA; n/a = not applicable as there were no mycotoxins present

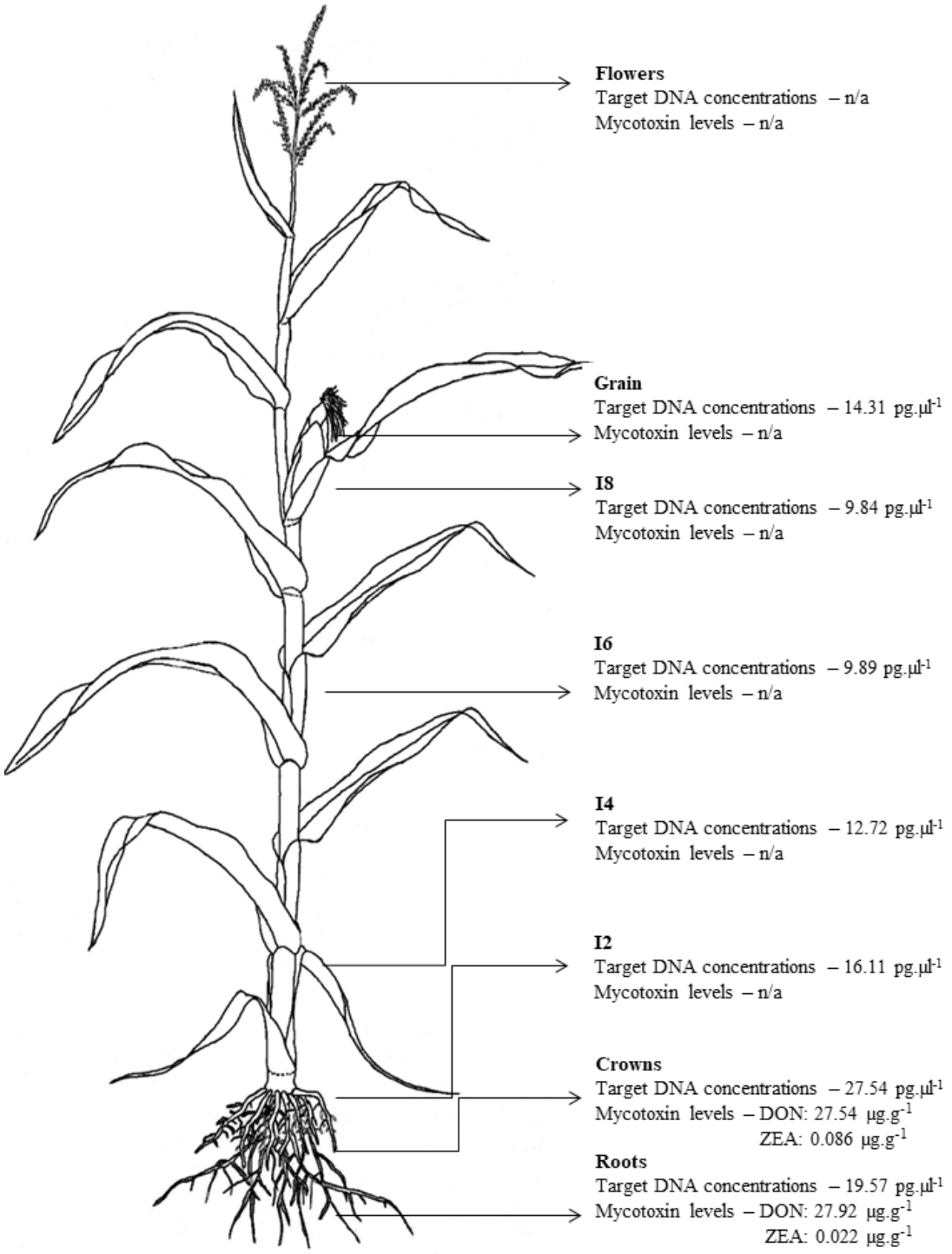

Figure 4.3: Mycotoxin translocation determined in PAN 6479 inoculated with both *F. boothii* and *F. graminearum* s.s. and harvested at the soft dough stage. [1]

[1] Target DNA concentrations = FGSC target DNA; n/a = not applicable as there were no mycotoxins or target DNA present

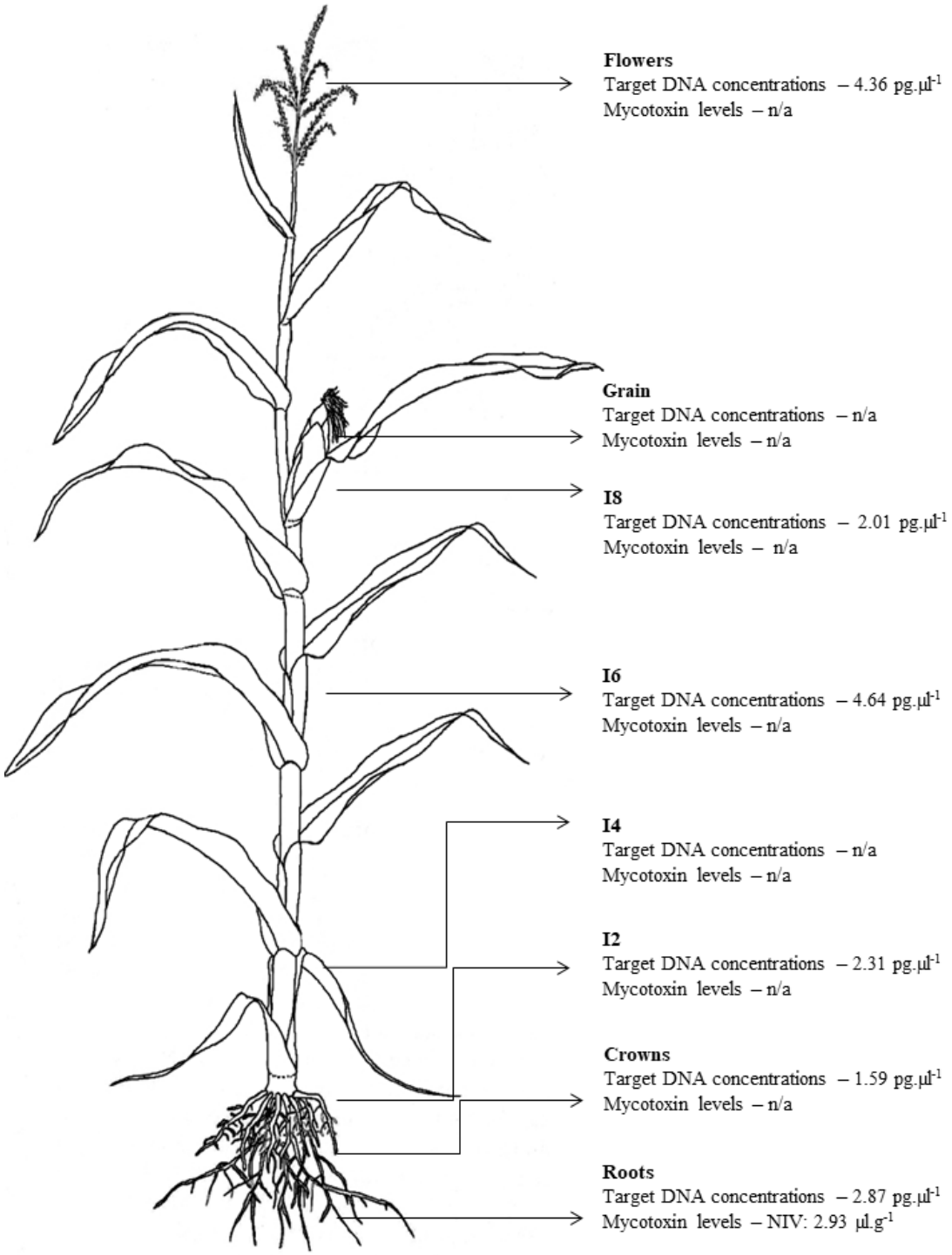

Figure 4.4: Mycotoxin translocation determined in PAN 6Q-245 inoculated with both *F. acacia-mearnsii*, *F. cortaderiae*, *F. meridionale* and harvested at the soft dough stage.[1]

[1] Target DNA concentrations = *F. verticillioides* target DNA; n/a = not applicable as there were no mycotoxins or target DNA present

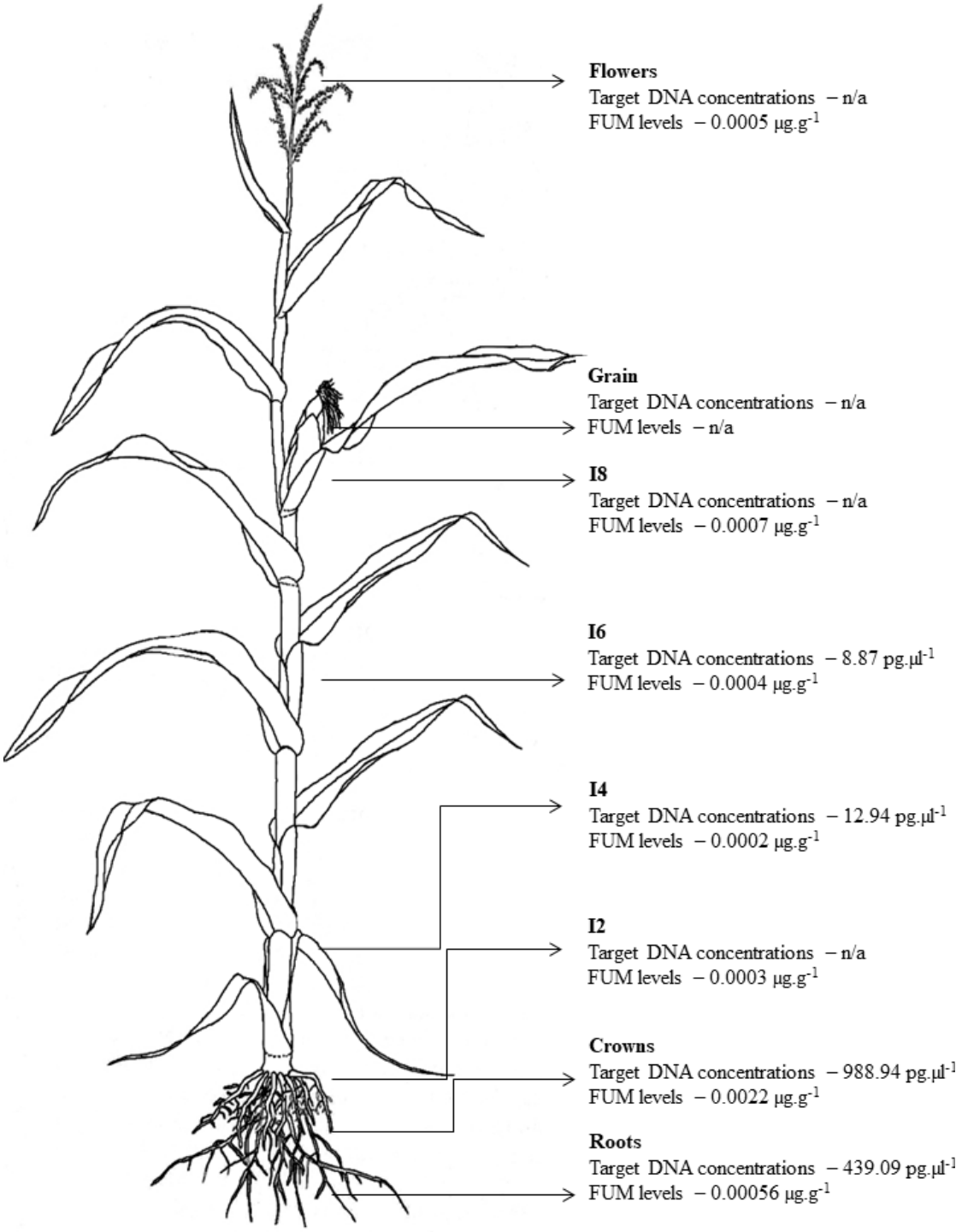

Figure 4.5: Mycotoxin translocation determined in PAN 6Q-245 inoculated *F. verticillioides* and harvested at the flowering stage. [1]

[1] Target DNA concentrations = FGSC target DNA; n/a = not applicable as there were no mycotoxins or target DNA present

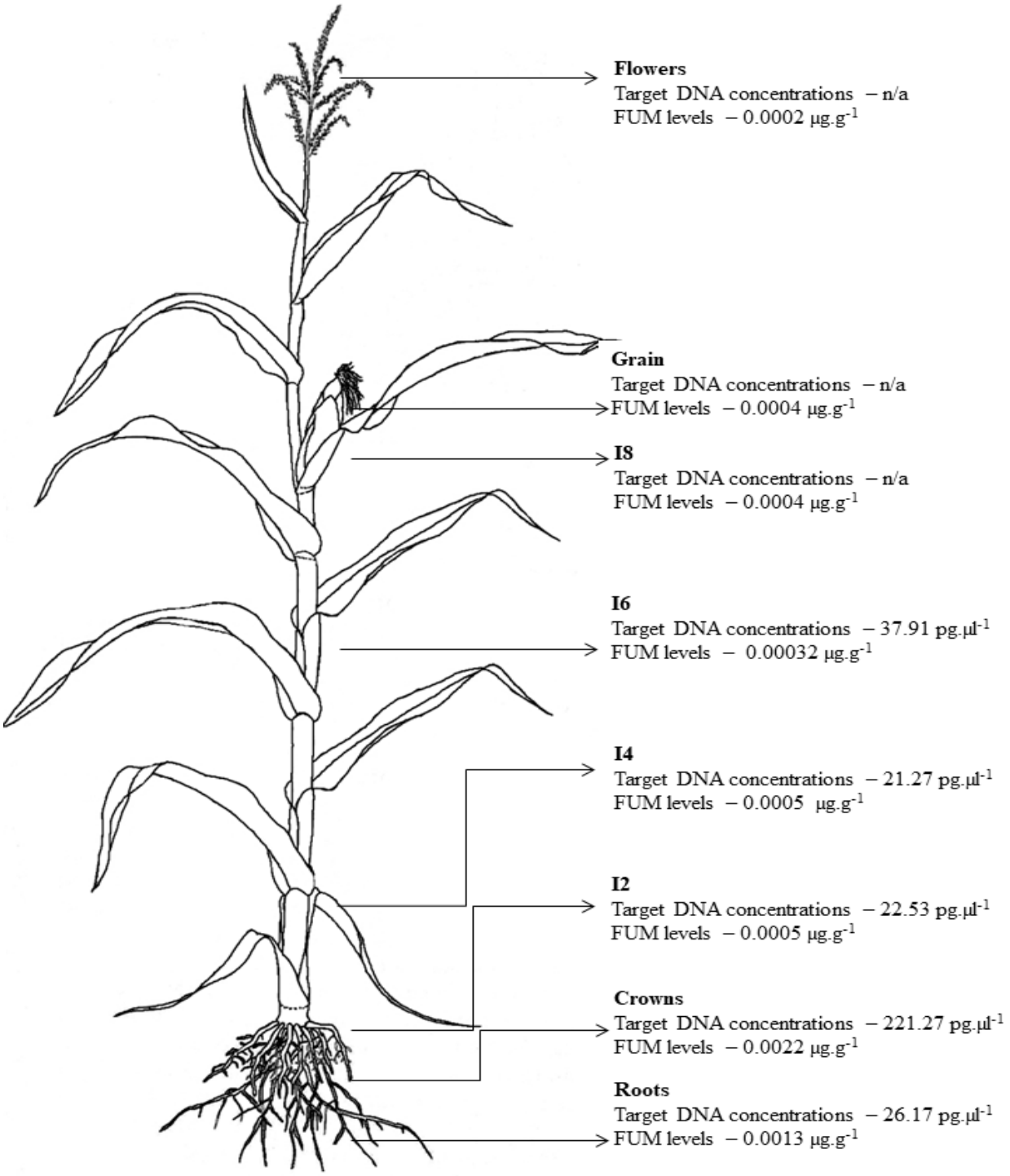

Figure 4.6: Mycotoxin translocation determined in PAN 6479 inoculated with *F. verticillioides* and harvested at the flowering stage. [1]

[1] Target DNA concentrations = *F. verticillioides* target DNA; n/a = not applicable as there was no target DNA present

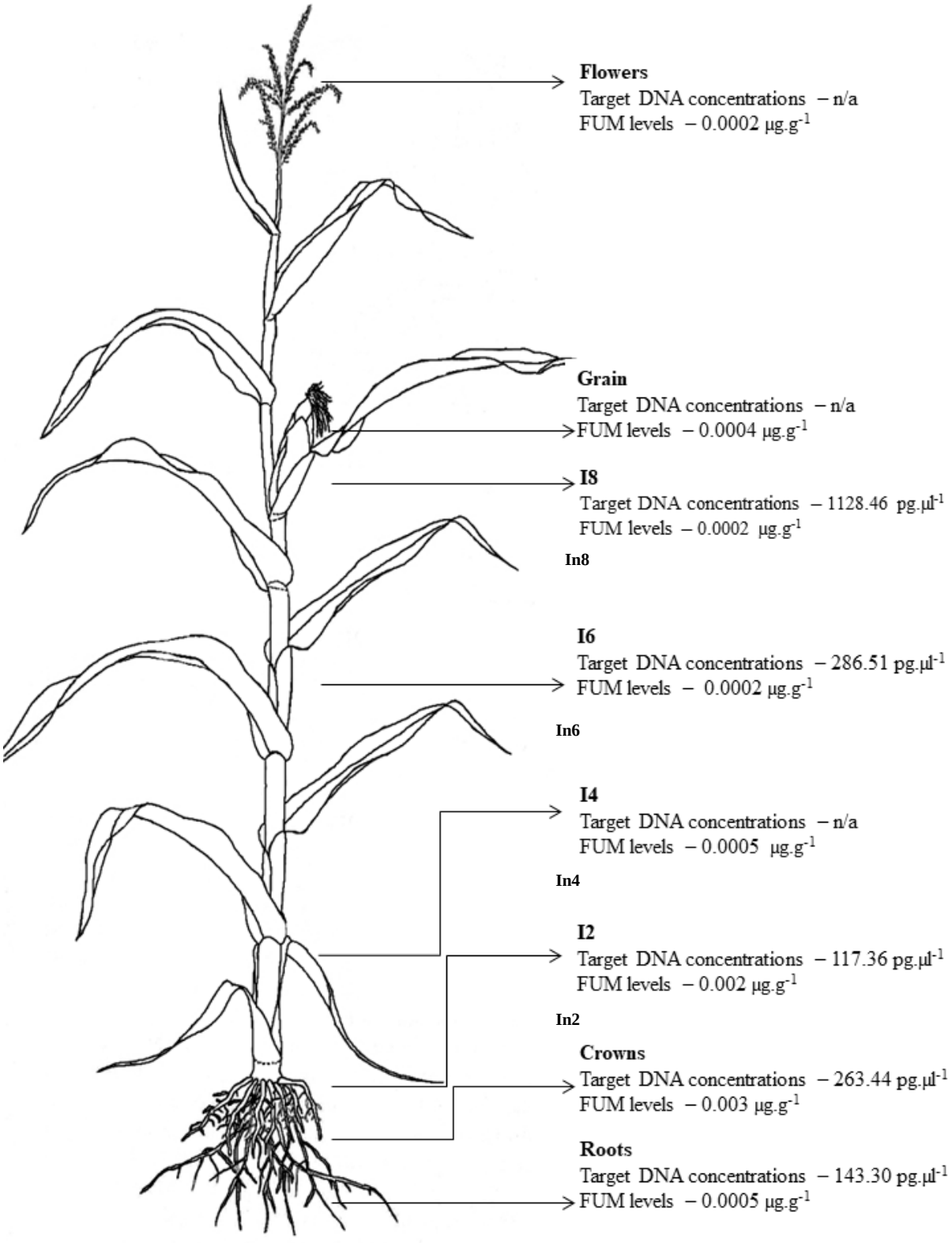

Figure 4.7: Mycotoxin translocation determined in PAN 6479 inoculated with *F. verticillioides* and harvested at the soft dough stage. [1]

[1] Target DNA concentrations = *F. verticillioides* target DNA; n/a = not applicable as there were no mycotoxins or target DNA present

Chapter 5: The expression of defence genes during maize stalk infection by FGSC

5.1 Abstract

Plants are attacked by a variety of pathogens daily. The genus *Fusarium* boasts many pathogens that infect all crop plants to varying degrees. Three species of the *Fusarium graminearum* species complex (FGSC) namely *F. graminearum* s.s., *F. meridionale* and *F. boothii* have been identified as maize pathogens in South Africa. Observations indicated that *F. boothii* produced spores more prolifically and could be a more prevalent pathogen. Defence responses in maize have primarily been studied in maize ears but not in maize stalks. In this study six-week-old stalks of two maize cultivars (PAN-6Q-245 – resistant; PAN 6479 – susceptible) were inoculated in triplicate, by injecting the stalks with *F. boothii* spores. After 24, 48, 72 and 96 hours after inoculation (hai), with one set sampled 12 days after inoculation (dai), the stalks were cut into 10 cm sections, starting just above the soil surface and placed immediately in liquid nitrogen. Ten constitutively expressed genes were tested for their stability, these genes were the Ubiquitin-conjugating enzyme, Folylpolyglutamate synthase, Leunig, Cullin, Phosphoglucomutase, cytoplasmic 1, Membrane protein PB1A10.07c, Actin 1, Elongation Factor 1 alpha, Cyclophilin and Tubulin Beta. Of these ten genes, two genes namely the membrane protein PB1A10.07c (MEP) and tubulin (TUB) served as reference genes for downstream analysis. Five genes of interest namely Pathogenesis-related protein 1 (PR-1), 1,3-β-glucanase – PRm6, Chitinase – PRm3, Putative peroxidase and WRKYGQK domain 1 (WRKY1) were identified. These genes have previously been identified as defence genes in maize against *F. verticillioides*. The five genes were examined for their role in maize defence responses against *F. boothii*. Only two of the five genes could be amplified efficiently and stably with RT-qPCR. PR-1 and WRKY1 were used for further analysis. PR-1 was significantly suppressed 48 hai in PAN 6Q-245 and significantly induced 96 hai in both cultivars. The pathogen induced PR-1 more readily in PAN 6Q-245 compared to PAN 6479, after the biotroph/necrotroph switch. This might indicate PR-1's role in maize stalk resistance to *F. boothii*. WRKY1 was significantly induced at 72 hai in PAN 6479, whereas the gene expression of WRKY1 in the *F. boothii* inoculated PAN 6Q-245 maize stalks did not differ significantly at any time from the water inoculated maize stalks. WRKY1 may be responsible for jasmonic acid (JA)-suppression and it is thus hypothesised that WRKY1 does not play a role in *F. boothii*-maize stalk rot resistance but may be responsible for the susceptibility of

PAN 6479. This study presents an indication of defence responses in maize stalks and two genes were identified as potential role players in the defence against infection by the FGSC.

Keywords: Maize, defence genes, RT-qPCR WRKY1, PR-1

5.2 Introduction

Plants are a source of nutrients to many organisms including bacteria, viruses, fungi and nematodes (Xie *et al.*, 2010). Numerous forms of active and passive defence mechanisms to these pathogens have been identified (Agrios, 2005). Physical barriers are the plants first line of defence, which if left intact, may be sufficient to prevent non-host pathogens from infecting (Ferreira *et al.*, 2006). A plant-pathogen interaction starts once the outer barriers are overcome. The first step is the recognition of the pathogen's elicitors or pathogen-associated molecular patterns (PAMPs) by the plant's pathogen recognition receptors (Dangl and Jones, 2001). This recognition results in the activation of the plants basal defence responses, which lead to signal transduction and the amplification of kinase cascades (Chen *et al.*, 2014). This triggers the activation of reactive oxygen species (ROS), pathogenesis related (PR) proteins and the production of a wide variety of secondary metabolites that limit further pathogen infection (Hammond-Kosack and Jones, 1996; Chen *et al.*, 2014). ROS triggers hormone-controlled defence pathways such as systemic acquired resistance (SAR). The SAR also activates the production of PR proteins. Currently, 17 different PR protein families have been divided according to their structures and biological activities (Xie *et al.*, 2010). WRKY is a stress responsive transcriptional regulator that activates or represses plant stress responses and may also be involved in developmental processes within the plant (Eulgem *et al.*, 2000; Ferreira *et al.*, 2006; Oh *et al.*, 2007; Golldack *et al.*, 2011). Defence responses against *Fusarium* spp. are complicated because they are hemi-biotrophic pathogens that start as biotrophs but become necrotrophs during the latter stage of infection (Trail, 2009; Chen *et al.*, 2014). Thus, defence responses such as programmed cell death are not effective against *Fusarium* spp. as necrotic lesions aid colonization (Trail, 2009).

The FGSC is a mycotoxigenic pathogenic complex. It consists of 16 species, three of which have been consistently isolated from maize in South Africa i.e. *F. graminearum* s.s., *F. meridionale* and *F. boothii* (Boutigny *et al.*, 2011). One of the diseases caused by the FGSC is Gibberella stalk rot in maize, which is characterised by pith disintegration (White and Carson, 1999). This results in softened stalks that break easily (Afolabi *et al.*, 2008). Stalk softening results in yield loss due to premature plant death, which prevents normal grain fill and/or cause plant lodging (White, 1999; Afolabi *et al.*, 2008). Conventional plant breeding has produced cultivars resistant to Fusarium stalk rot and Fusarium and Gibberella ear rot (Afolabi *et al.*, 2008; Chen *et al.*, 2014). However, this resistance is highly variable and is dependent on

locality, weather conditions, agricultural practices, moisture availability, grain fill and kernel number, leaf disease severity, cloud cover and insect damage (White, 1999). Maize cultivars that are high yielders and produce larger ears tend to be more susceptible to stalk rots, as they draw large quantities of carbohydrates from the stalks to fill grain, leaving a carbohydrate-sink (Dodd, 1980). The carbohydrate mediated defence responses in the stalks are then unable to function. To date the mechanisms that confer resistance to maize stalks caused by FGSC infection are still unclear (Afolabi *et al.*, 2008).

Lanubile *et al*., (2010) performed a microarray analysis to determine differential gene expression of defence proteins in maize grain against *F. verticillioides* infection (Lanubile *et al*., 2010). During this study, 20 defence genes were identified that were further evaluated using reverse transcription quantitative real-time PCR (RT-qPCR). In the current study five genes, namely 1,3-β-glucosidase PRm6, chitinase PRm3, putative peroxidase, PR-1 and WKRY1 were selected to determine whether they were differentially expressed during infection of maize stalks by *F. boothii*. The activation of defence genes in maize stalks have not been investigated and could lead to a better understanding of the mechanisms that convey FGSC resistance to certain maize cultivars.

5.3 Materials and methods

5.3.1 Inoculation of maize stalks and sampling

Seed of two maize cultivars, PAN 6Q-245 and PAN 6479, were obtained from PANNAR Seed Company (Pty) Ltd. PAN 6Q-245 is regarded as resistant to stalk rots whereas PAN 6479 is known to be more susceptible. Seeds were planted into sterile potting soil, in 20 L bags and grown under greenhouse conditions at a 27/20 ºC day/night temperature regime with a 14/10 h day/night photoperiod. Eighteen plants were planted of each cultivar, per replicate, with a total of three replicates. *F. boothii* (isolate: 2.881) was obtained from the University of Stellenbosch. This culture was grown in Armstrong *Fusarium* medium for optimum spore production (Leslie and Summerell, 2006). The plants were grown for six weeks, after which the second internode of each cultivar was inoculated with 2 ml of a *F. boothii* spore suspension containing 2 x 10^6 spores.ml^{-1} using a 2 ml syringe. Control plants were inoculated with sterile ddH_2O, also using a 2 ml syringe. After 24, 48, 72 and 96 hours (hai), with one set sampled 12 days after inoculation (dai), the stalks were cut into 10 cm sections, starting just above the soil surface

and placed immediately in liquid nitrogen (Afrox, South Africa). Visual confirmation of infection was not possible during the early infection period. The tissue was stored at -80 ºC.

5.3.2 RNA extraction

RNA was extracted using a CTAB/Polyvinylpyrrolidone (PVPP) extraction method (Rubio-Piña and Zapata-Pérez, 2011). The 10 cm stalk was ground in liquid nitrogen and one gram tissue, was mixed with 1.6 ml RNA extraction buffer (2 % (w/v) CTAB (Sigma-Aldrich, Steinheim, Germany); 0.1 M Tris-HCl (Merch KGa, Darmstadt, Germany) (pH 8); 1.4 M NaCl (Merch KGa, Darmstadt, Germany); 20 mM EDTA (Sigma-Aldrich, Steinheim, Germany) (pH 8); 2 % (w/v) PVPP (Sigma-Aldrich, Chemie, GmbH; Steinheim, Germany) 10 % β-mercaptoethanol (Merck KGaA, Darmstadt, Germany)). The sample was homogenized, after which it was frozen in liquid nitrogen for 2 minutes and incubated at 95 °C for 5 minutes. After cooling, the sample was placed on ice and 800 µl chloroform/phenol (1:1) (phenol - Sigma-Aldrich, Steinheim, Germany; Chloroform - Merch KGa, Darmstadt, Germany) was added. The mixture was vortexed and centrifuged at 15294 x g for 20 min at 4 °C. The supernatant was transferred to a new tube and based on the supernatants volume, a third volume of 8 M lithium chloride (Sigma-Aldrich, Steinheim, Germany) was added and kept at -20 °C overnight. The samples were centrifuged at 15294 x g for 20 min at 4 °C. The supernatant was removed, and the pellet was washed with 100 % ethanol (Merck KGaA, Darmstadt, Germany), by adding 500 µl 100 % ethanol to the pellet, and centrifuging it at 15294 x for 20 minutes. This was followed by adding 500 µl 70 % ethanol and repeating the centrifugation process. The pellet was dried and resuspended in 250 µl molecular water. One microliter of DNase (Promega Corporation, Madison, USA) and 1 µl of DNase buffer were added to the sample. An equal volume chloroform/phenol (1:1) were added to the samples, vortexed and centrifuged as before. The supernatant was removed, and 0.1 X 3 M sodium acetate and 2 X 100 % ethanol were added. This was left at -20 °C for an hour after which the samples were centrifuged for 30 minutes at 20817 x g. The pellet was washed with 70 % ethanol as mentioned above. After removing the ethanol, the pellet was air-dried and resuspended in 30 µl ddH_2O.

5.3.3 RT-qPCR

The quantification of defence gene expression was performed using RT-qPCR. cDNA was synthesised using an iScript cDNA synthesis kit according to the manufacturer's specifications (Biorad, Hercules, USA). Ten reference genes were selected from Manoli *et al.* (2012) and Lin *et al.* (2014) to determine the most stably expressed genes using qBase+ geNorm (Biogazelle) (Table 5.1) (Manoli *et al.*, 2012; Lin *et al.*, 2014). The ten genes were tested on both cultivars to determine their stability and the number of genes to be used to obtain stable expression. Of these ten reference genes, two were selected (Figure 5.1 and 5.2). The two selected genes that served as reference genes were membrane protein PB1A10.07c (MEP) and tubulin (TUB). They served as a baseline and were compared to five genes of interest chosen for evaluation. These included the PR proteins chitinase (CHIT), 1,3-β-glucanase (GLUC), peroxidase (POX) and PR-1, as well as the transcription factor WRKY1 (Table 5.2) (Lanubile *et al.*, 2013).

A 2x, 4x, 8x, 16x, 32x and 64x serial dilution of cDNA was used to generate a standard curve for each of the defence genes and the two reference genes. The optimum primer and temperature conditions were determined using the Taguchi method (Thanakiatkrai and Welch, 2012). Each RT-qPCR run contained a non-template control to regulate contamination and all PCRs were performed with three technical repetitions. A sample containing only RNA (no reverse transcriptase (RT) control) was used as control, to exclude the possibility of genomic DNA contamination.

RT-qPCR reactions were performed using Universal SYBR Green Supermix (Bio-Rad, Hercules, USA) according to the manufacturer's specifications. The RT-qPCR was performed in a reaction containing 0.5 x Sensimix, 0.5 µM forward and reverse primer (Tables 5.1 and 5.2) and 1-2 µl of template cDNA to a total volume of 25 µl on a CFX96 qPCR machine (Bio-Rad, Hercules, USA). Three technical replicates were performed for each stalk piece over three biological replicates with 3 plants per replicate. This resulted in 27 reactions per time point. The reaction conditions for the qPCR differed between the reference gene and the gene of interest. The reaction conditions for the reference genes were as follows: 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 30 s, 60 ºC for 30 s and 72 ºC for 30 s. For melting curve analysis, the samples were denatured at 95 ºC and cooled to 65 ºC at 20 ºC per second. The fluorescence signals were collected between 65 ºC and 95 ºC at 0.2 ºC per second. The genes of interest had the following reaction conditions namely 95 ºC for 10 min, followed by 50

cycles of 95 ºC for 45 s, 55 ºC for 45 s and 72 ºC for 1 min. The melt curve analysis was the same for both genes.

5.3.4 Data analysis

The Biogazelle qBase+ program was used to determine the fold change of the genes of interest by normalising to the two reference genes using a modified version of the delta-delta Cq method (Hellemans *et al.*, 2007). The nine values obtained from the RT-qPCR analysis (three technical replicates x three biological replicates) were analysed simultaneously. This program evaluates the repeatability of the RT-qPCR and calculates one gene expression value from the nine values obtained. The stability of the reference genes was once again determined after injection damage was inflicted on the stalks. To ensure that the fold change was attributed to the fungi and not the injection damage, the fold change of the *F. boothii* inoculated plants was compared to the fold change of water inoculated plants. If there were no significant difference between the fold change of the water inoculated plant and that of the infected plant, the data was discarded. The fold change observed when no significant differences are observed is attributed to plant wounding (by injection) and not to the pathogen. Student t-tests, assuming unequal variance, were performed on all data sets.

5.4 Results

5.4.1 GeNorm analysis

All ten reference genes had a geNorm M value <0.5 (Figure 5.1). The four lowest M values, that were <0.075, were folylpolyglutamate synthase (FPGS), MEP, TUB and actin (ACT). According to the geNorm V analysis, it was determined that two reference genes would be sufficient to use (Figure 5.2) and thus, MEP and TUB were selected. A standard curve was set up for both reference genes. The efficacy of MEP ranged from 85 – 89.9 %, the R^2 was 0.990 – 0.995 and the slope were between -3.5 – -3.7 (Figure 5.3.1). The efficacy of TUB ranged from 90 – 104 %, the R^2 was between 0.995 – 0.999 and the slope were between -3.2 – -3.6 (Figure 5.3.2).

5.4.2 Genes of interest

All five genes of interest were examined. Only those genes amplified optimally were selected for further investigation. The gene of interest, POX did not amplify and were discarded. Two genes of interest, CHIT and GLUC amplified, but only at cycle 35 to 38 and with the melt curve was unstable. Only PR-1 and WRKY1 were further examined, because amplification of these genes resulted in trusted data. A standard curve was set up for both genes. The efficiency of PR-1 ranged from 89 – 95.4 %, the R^2 was between 0.940 – 0.994 and the slopes was between -3.2 – -3.8 (Figure 5.4.1). The efficiency of WRKY1 ranged from 93 – 103 %, the R^2 was between 0.936 – 0.970 and the slopes were between -3.2 – -3.8 (Figure 5.4.2).

5.4.3 Gene expression analysis

There was a significant difference between the gene expression of PR-1 of the water inoculated and *F. boothii* inoculated plants at 48 hai and 96 hai for PAN 6479 (Figure 5.5). A significant difference was observed between water and *F. boothii* inoculated plants for PR-1 expression in PAN 6Q-245 at 48 hai (Figure 5.6). The gene expression of WRKY1 in PAN 6Q-245 did not differ significantly between the water inoculated control and the *F. boothii* inoculated samples. Thus, the gene expression observed could not be attributed to the pathogen and the data was discarded. Significant differences in the expression of WRKY1 in water and *F. boothii* plants were recorded 72 hai in PAN 6479 (Figure 5.7). The gene expression of PR-1 of the *F. boothii* inoculated plants did not differ significantly at 48 hai from the water inoculated plants of PAN 6479. This data point was only included to illustrate that PAN 6Q-245 was significantly suppressed at 48 hai with a fold change of -0.56 compared to PAN 6479 (Figure 5.8). During 96 hai both cultivar's PR-1 gene was induced with a fold change of 0.42 for PAN 6Q-245 and 0.07 for PAN 6479. The gene expression of WRKY1 in the *F. boothii* inoculated plants did not differ significantly at 72 hai from the water inoculated plants of PAN 6Q-245. This data point was only included to illustrate that PAN 6479 significantly induced WRKY1 at 72 hai and had a fold change of 0.26 as compared to PAN 6Q-245 (Figure 5.9).

5.5 Discussion

Studies of defence responses against *Fusarium* spp. have mainly focused on *F. verticillioides* on maize ears and FGSC on wheat ears (Lanubile *et al*., 2010; Lanubile *et al.*, 2013; Maschietto

et al., 2016). Limited information is available on maize stalk defence responses and none against FGSC. This study was performed to elucidate the defence responses employed by maize stalks against *F. boothii* infection.

When studying plant defence responses against *Fusarium* spp. it is important to note that these pathogens are hemibiotrophs (Bushnell *et al.*, 2003). Schlink (2010) reported that *Fagus sylvatica* L. would express different genes during the biotrophic phase of *Phytophtora citricola* Sawada infection compared with the necrotrophic phase. During the biotrophic stage, between 12 – 24 hai, limited defence responses are triggered in the roots because perception of the pathogen and penetration prevention is prioritised (Bushnell *et al.*, 2003; Attard *et al.*, 2010; Lanubile *et al*., 2010). A similar result was seen in this study where the gene expression of PR-1 and WRKY1 did not differ significantly between the water and the *F. boothii* inoculated plants at 24 hai. The change from biotroph to necrotroph occurs between 48 and 72 hai (Lanubile *et al*., 2010). Changes in defence responses were only observed subsequent to 48 hai in this study.

PR-1 is an antifungal protein and it was expected that this gene would be upregulated after pathogen recognition (Prell and Day, 2000). However, in PAN 6Q-245, the cultivar that is more resistant to stalk rot, PR-1 was significantly supressed 48 hai. A similar result was observed by Chen *et al.* (2014) which indicated that 48 h after *F. oxysporum* inoculation, *Arabidopsis thaliana* (L.) Heynh. roots showed many more down- than up-regulated genes. Similarly, Fountain *et al.* (2015) also reported that PR-1 was suppressed during early time points in the susceptible maize cultivar when inoculated with *Aspergillus flavus*. However, when they examined the resistant maize cultivar, PR-1 gene expression did not show any difference over the inoculated time-points (Fountain *et al.*, 2015). It would appear that PR-1 was induced (96 hai) in the resistant cultivar only after the switch from biotroph to necrotroph. The susceptible cultivar showed limited induction of PR-1 at 96 hai. Although Sekhon *et al.* (2006) found no correlation between PR-1 expression and Fusarium ear rot resistance, it would seem in this study that PR-1 expression contributed to maize resistance against *F. boothii* infection (Sekhon *et al.*, 2006).

The WRKY group is a large group of transcription factors that not only play a role in abiotic and biotic stresses, but also in seed development, seed dormancy, plant development and plant senescence (Eulgem *et al.*, 2000). WRKY can also be artificially induced by signalling substances such as salicylic acid (Eulgem *et al.*, 2000). In this study WRKY1 was only

significantly induced in PAN 6479 at 72 hai, during the biotroph/necrotroph switch. A similar induction was observed for WRKY67 in two susceptible cultivars namely B37 (Burden Research centre inbred line) and N85-N5 (Syngeta), which reached a peak at 96 hai (Fountain *et al.*, 2015). They suggested that this induction occurred in order to suppress jasmonic acid (JA)-based defence responses and promote salicylic acid (SA)-based defence responses (Fountain *et al.*, 2015). Rushton *et al.* (2010) also reported that WRKY1 in barley acts as a negative regulator for pathogen triggered immunity (PTI) against the biotrophic pathogen, powdery mildew (*Blumeria graminis* f. sp. *hordei*). Thus, it would seem that the resistant cultivar does not induce nor suppress WRKY1 during this infection period and may thus not play a role in maize stalk resistance against *F. boothii* infection. The role of WRKY1 in the susceptible cultivar is more complicated. Should WRKY1 function in the same manner as WRKY67, by suppressing JA-defence responses, or act as a negative regulator for PTI, then WRKY1 could be partly responsible for PAN 6479's susceptibility (Tamaoki *et al.*, 2013).

Although the role of PR-1 and WKRY1 in stalk rot has become clearer there are still limitations. PR-1 and WKRY1 cannot be classified as differentially expressed genes as the cut-off point for selecting such a gene is a 1.5 fold change (Bustin *et al.*, 2009; Chen *et al.*, 2014). Also, the MIQE standard for the R^2 value should be between 0.990 – 1. This could not be obtained, even after multiple optimisation attempts (Bustin *et al.*, 2009). Thus, using primers designed for maize grain for other maize tissue is not ideal and PR-1 and WKRY1 gene expression in maize should be further evaluated.

5.6 Conclusion

This study is the first to examine maize stalk defence responses. It was expected that PR-1 would be highly upregulated at each time-point in the resistant cultivar with limited induction in the susceptible cultivar. Instead PR-1 was initially supressed, between 0 – 48 hai, then induced 96 hai. This was attributed to the biotroph/necrotoph switch. It is hypothesised that PR-1 plays a role in *F. boothii*-maize stalk rot resistance. WRKY1 was only induced in the susceptible cultivar at 72 hai. It is hypothesised that WRKY1 does not play a role in *F. boothii*-maize stalk rot resistance but may be responsible for the susceptibility of PAN 6479. Although some assumptions could be made from this study, further analysis is required to gain a definitive understanding of maize defence responses toward FGSC.

5.7 References

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Table 5.1: Ten reference genes selected from Manoli *et al.* (2012) and Lin *et al.* (2014) to determine gene expression stability in stalks of the maize cultivars, PAN6Q-245 and -6479.

Annotation	Gene name	**Sequence**	**References**
Ubiquitin-conjugating enzyme	UBCE-F	TGCGTTAATCACGAGACAGG	Manoli *et al.* 2012
	UBCE-R	AATCACAAAGACAGGCAGGG	
Folylpolyglutamate synthase	FPGS-F	ATCTCGTTGGGGATGTCTTG	Manoli *et al.* 2012
	FPGS-R	AGCACCGTTCAAATGTCTCC	
Leunig	LUG-F	TCCAGTGCTACAGGGAAGGT	Manoli *et al.* 2012
	LUG-R	GTTAGTTCTTGAGCCCACGC	
Cullin	CUL-F	GAAGAGCCGCAAAGTTATGG	Manoli *et al.* 2012
	CUL-R	ATGGTAGAAGTGGACGCACC	
Phosphoglucomutase, cytoplasmic 1	PGM-F	GCTCAAGCTCTCCAAGATGC	Manoli *et al.* 2012
	PGM-R	AACGCTCCCAATTACAGCAC	
Membrane protein PB1A10.07c	MEP-F	TGTACTCGGCAATGCTCTTG	Manoli *et al.* 2012
	MEP-R	TTTGATGCTCCAGGCTTACC	
Actin 1	ACT-F	GATTCCTGGGATTGCCGAT	Manoli *et al.* 2012
	ACT-R	TCTGCTGCTGAAAAGTGCTGAG	
Elongation Factor 1 alpha	EF1-F	TGGGCCTACTGGTCTTACTACTGA	Lin *et al.* 2014
	EF-R	ACATACCCACGCTTCAGATCCT	
Cyclophilin	CYP-F	CTGAGTGGTGGTCTTAGT	Lin *et al.* 2014
	CYP-R	AACACGAATCAAGCAGAG	
Tubulin Beta	β-TUB-F	CTACCTCACGGCATCTGCTATGT	Lin *et al.* 2014
	β-TUB-R	GTCACACACACTCGACTTCACG	

Table 5.2: Five genes of interest were selected from Lanubile (2010).

Annotation	Gene name	Sequence
Pathogenesis-related protein 1	PR-1-F	GAACTCGCCGCAGGACTAC
	PR-1-R	GAGCCCCAGAAGAGGTTCTC
1,3-β-glucanase – PRm6	GLUG-F	GCGCAGACCTACAACCAGA
	GLUG-R	GGAGAAATTGATGGGGTACG
Chitinase – PRm3	CHIT-F	GGCTCTACGCCTACGTCAAC
	CHIT-R	GATGGAGAGGAGCACCTTGA
Putative peroxidase	POX-F	GCACAAGGTCCTGTTCGTCT
	POX-R	TTTCCCTGATCTCTCCCTCA
WRKYGQK domain	WRKY1-F	GAGGAGCAATCTTGGGATCA
	WRKY1-R	CAAGACTCCCTTCCATGCTC

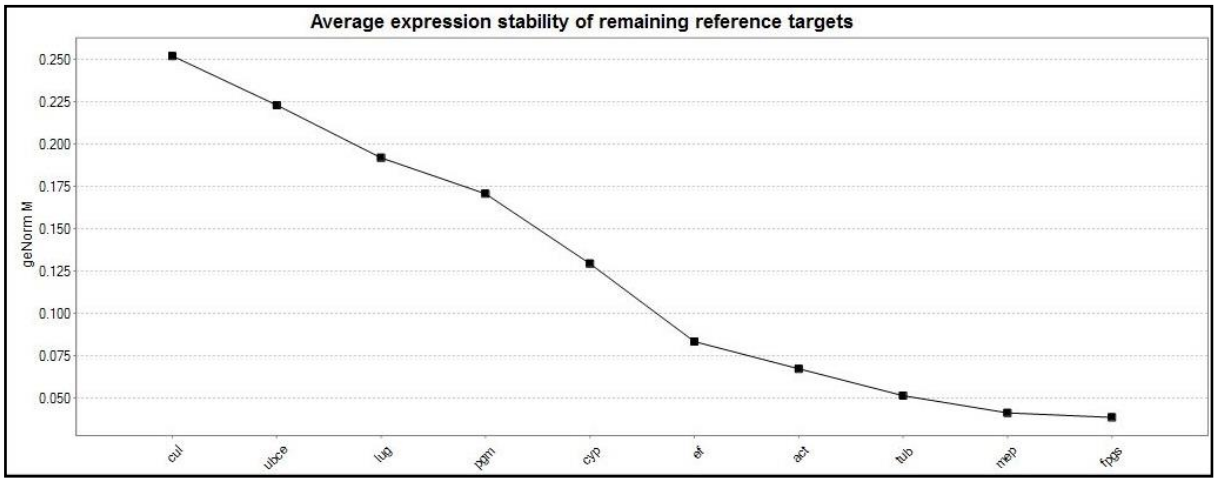


Figure 5.1: Expression stability and relative ranking of 10 reference genes in maize stalks.

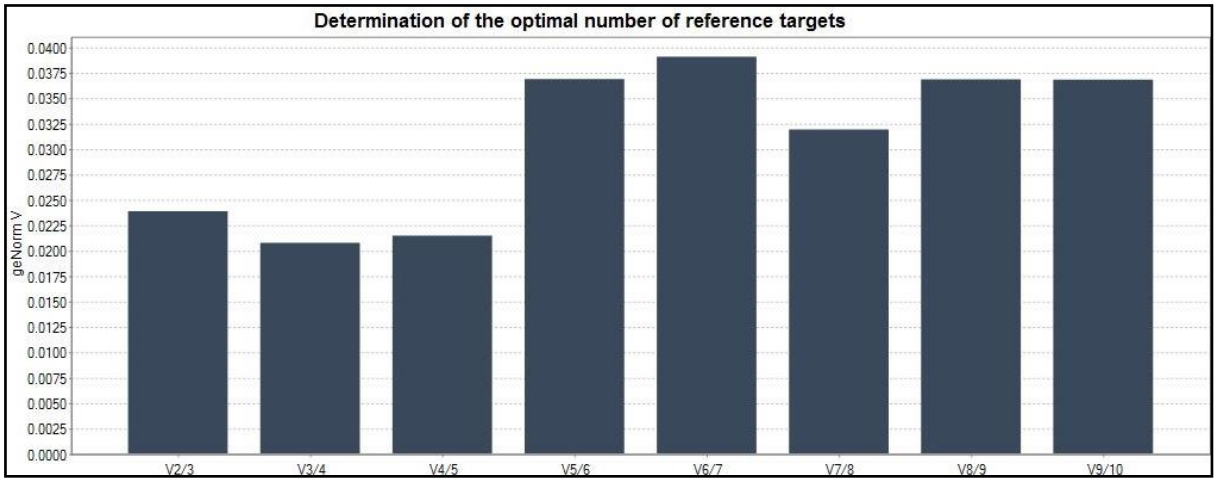
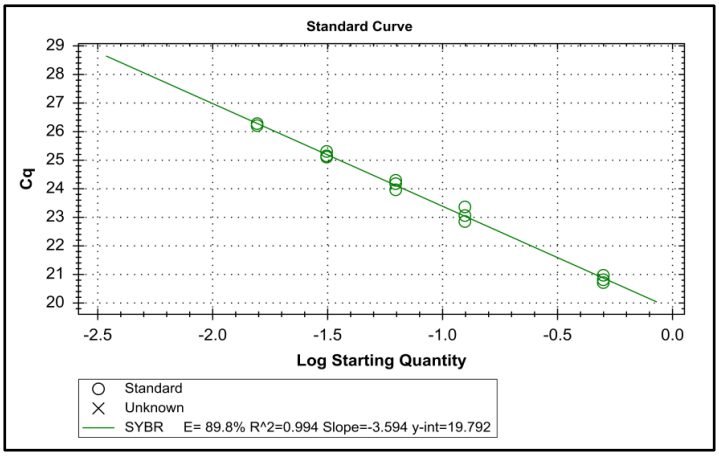


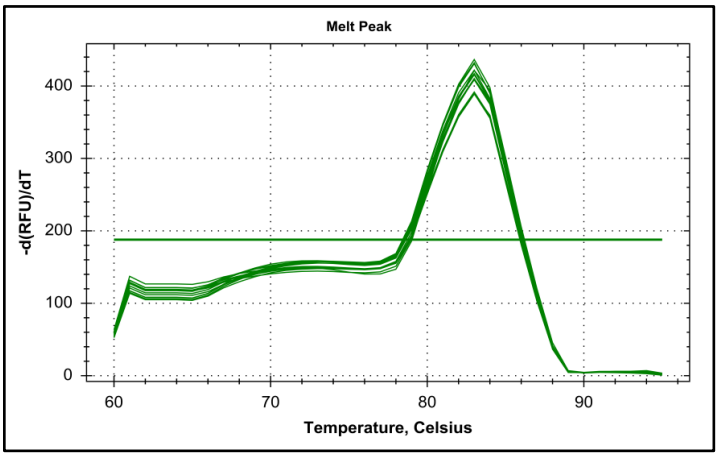
Figure 5.2: The optimum number of reference genes needed for RT-qPCR data normalization.[1]

[1] V = pairwise variation; e.g. V2/3 pairwise normalization factor between 2 and 3 genes, where a large variation indicates that an additional gene would significantly affect the outcome.

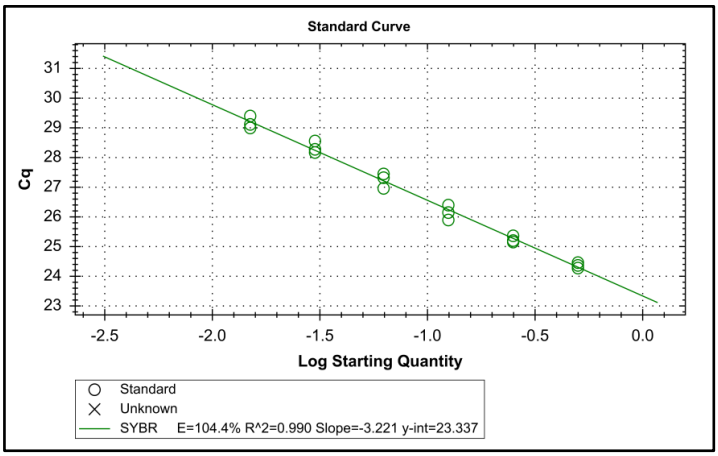
1a)



1b)



2a)



2b)

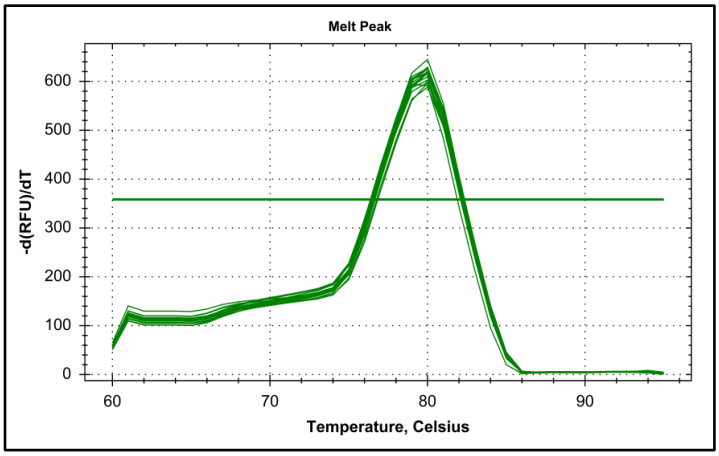
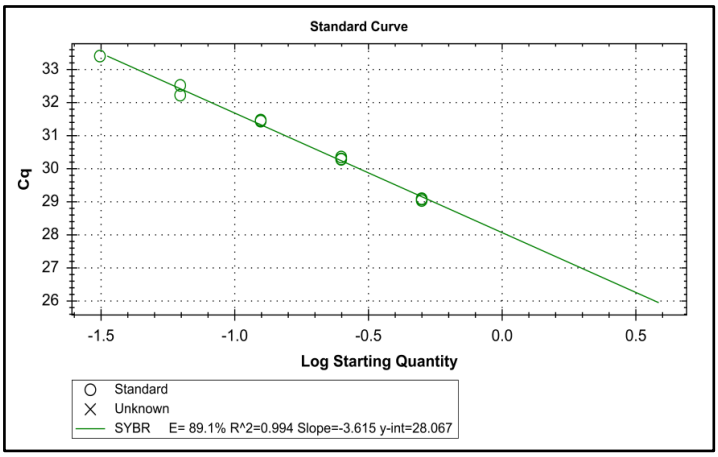


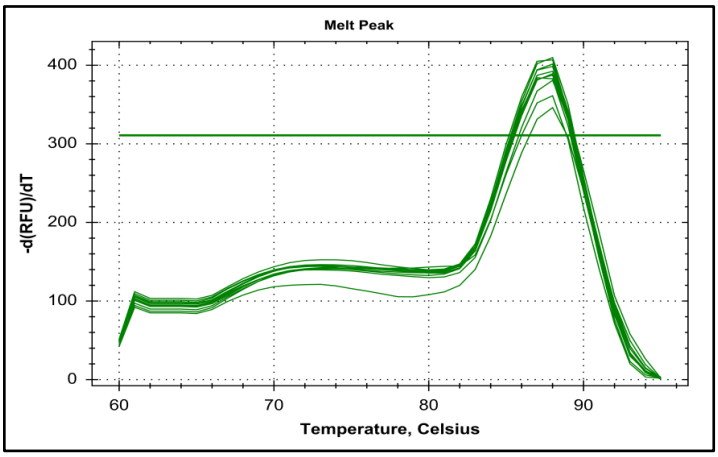
Figure 5.3: Two reference genes, MEP (1) [1]and β-Tubulin (2), selected from the list of ten as the most stable. The standard curve (a) and melt curve (b) was incorporated to establish the qPCR stability and quality.

[1] MEP = Membrane protein PB1A10.07c

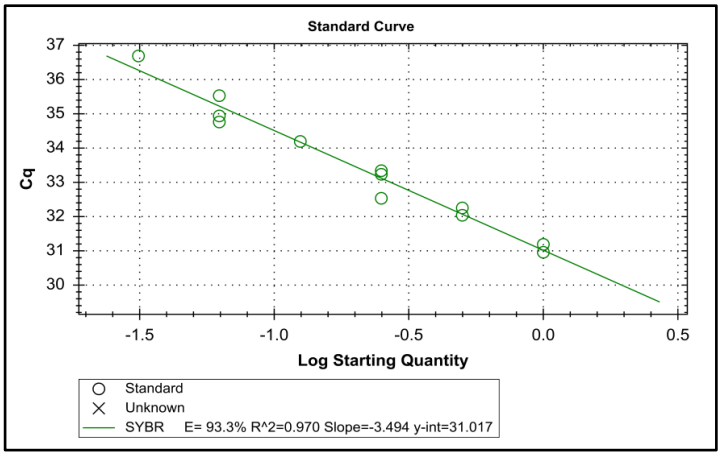
1a)



1b)



2a)



2b)

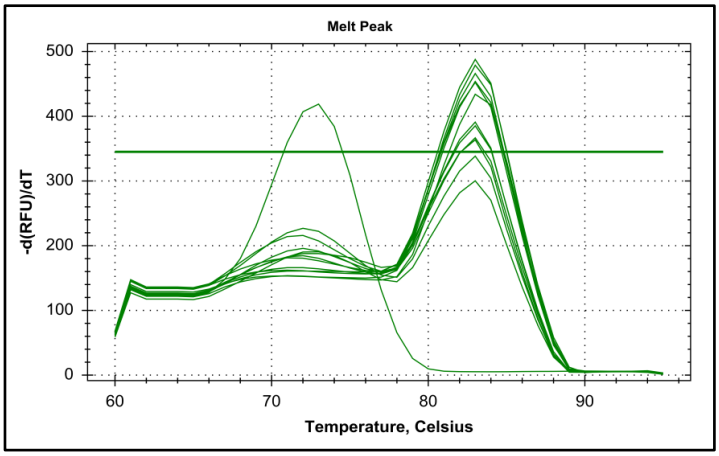


Figure 5.4: Two reference genes, PR-1 (1) and WRKY1 (2), selected from five target genes. The standard curve (a) and melt curve (b) was incorporated to show the quality of the qPCR assays.

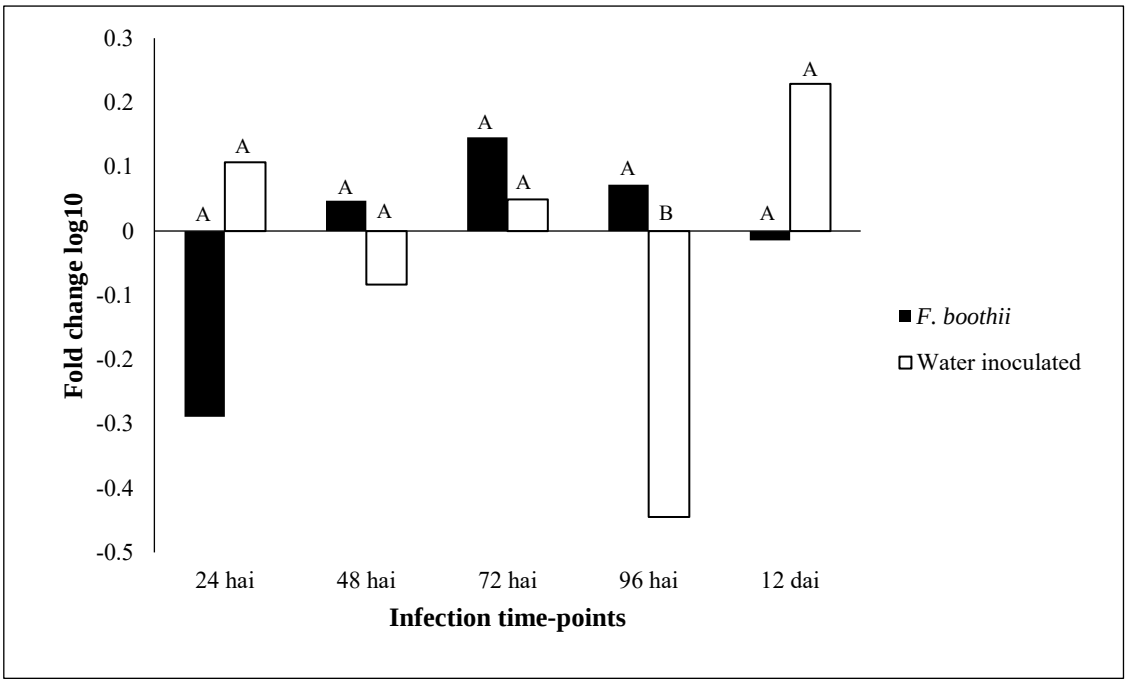


Figure 5.5: The gene expression of PR-1[1] determined in the maize stalks of cultivar PAN 6479, infected with *F. boothii*, at five different time-points. [2]

[1] hai – hours after infection

[2] A different letter denotes significant differences at P=0.052 between infected and water inoculated plants but not between time-point.

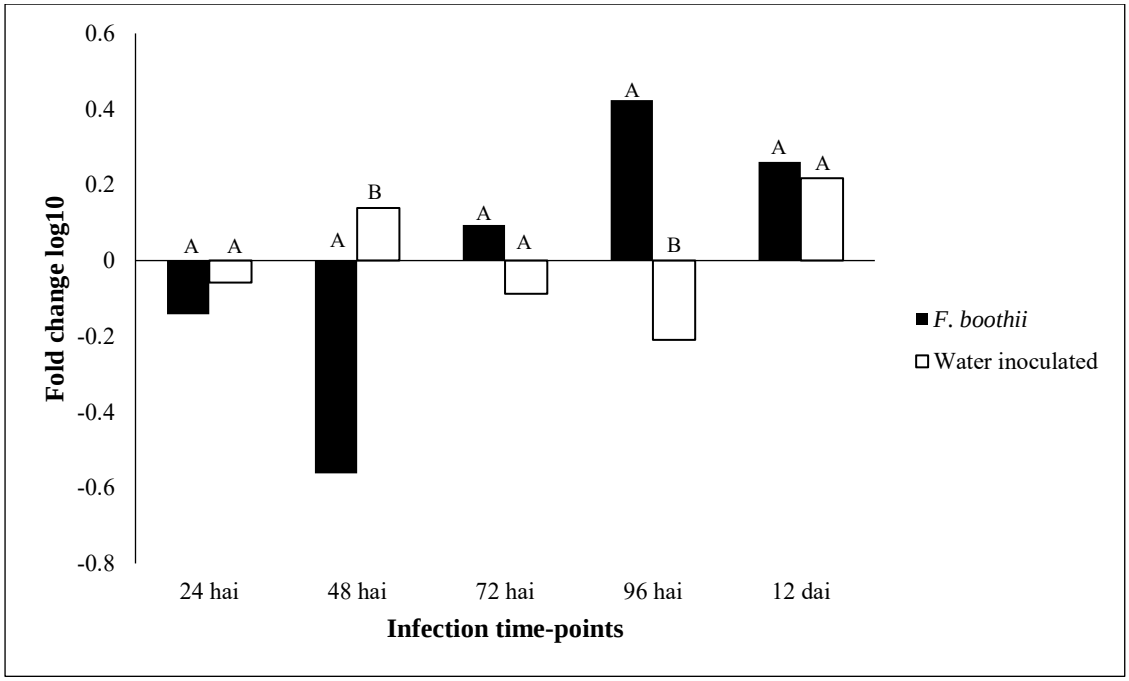


Figure 5.6: The gene expression of PR-1 [1] determined in the maize stalks of cultivar PAN 6Q-245, infected with *F. boothii*, at five different time-points. [2]

[1] hai – hours after infection

[2] A different letter denotes significant differences at P≤0.05 between infected and water inoculated plants but not between time-point.

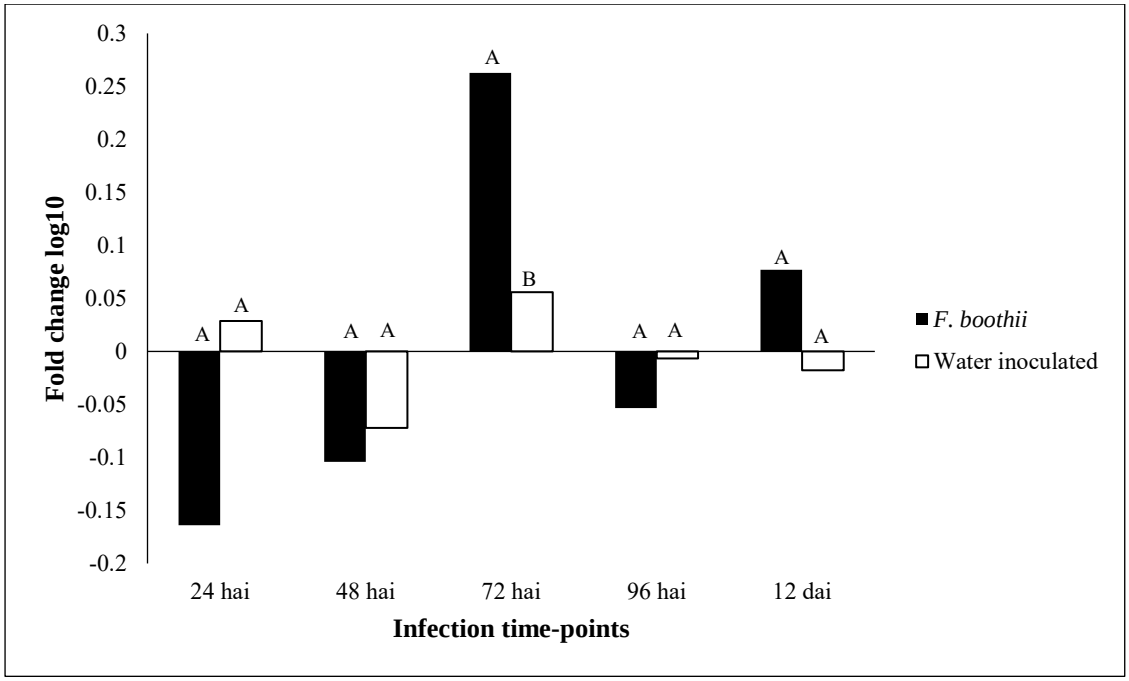


Figure 5.7: The gene expression of WKRY1 [1] determined in the maize stalks of cultivar PAN 6479, infected with *F. boothii*, at five different time-points.[2]

[1] hai – hours after infection

[2] A different letter denotes significant differences at P=0.041 between infected and water inoculated plants but not between time-point.

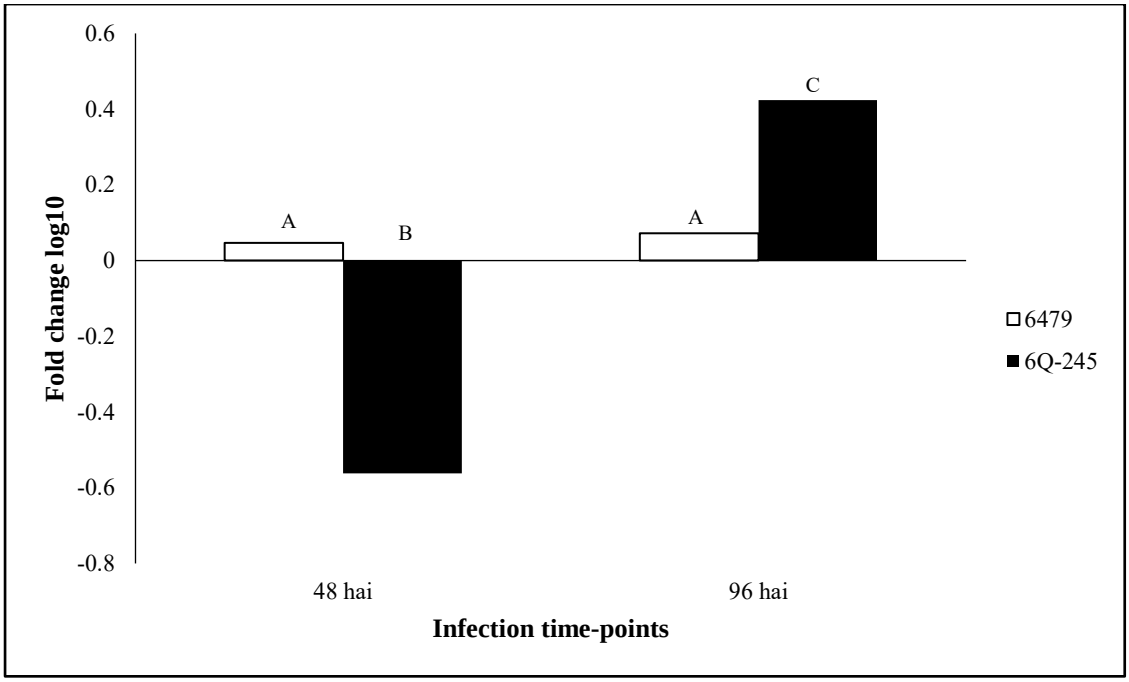


Figure 5.8: Fold change observed for the expression of PR-1 between cultivar PAN 6479 and PAN 6Q-245 at 48 hai[1] and 96 hai. [2]

[1] hai – hours after infection
[2] A different letter denotes significance at $P \leq 0.045$.

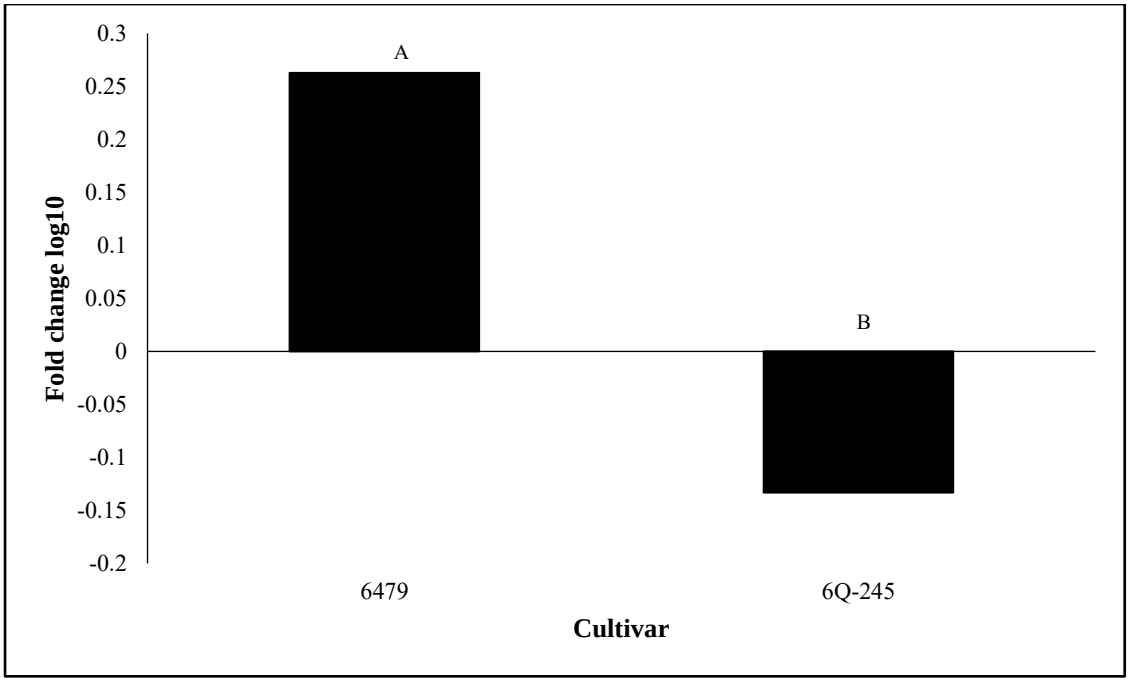


Figure 5.9: Fold change observed for the gene expression of WRKY1 between cultivar PAN 6479 and PAN 6Q-245 at 48 hai[1] and 96 hai. [2]

[1] hai – hours after infection
[2] A different letter denotes significance at P=0.041.

Summary

Maize is an important staple food and feed crop in South Africa. This crop is however threatened by pathogens/pathogen complexes such as the *Fusarium graminearum* species complex (FGSC), the *F. oxysporum* species complex (FOSC) and *F. verticillioides*. These pathogens not only cause a decrease in grain yield and quality but may also produce mycotoxins, the most prevalent being deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA) and fumonisins (FUM). This study was conceptualised because our understanding of *Fusarium* spp.-maize interactions is often limited to ear rots. Thus, this study consisted of five diverse chapters, which together gives a better understanding of the interaction between maize and *Fusarium* spp..

In Chapter 2 the North West province, in conjunction with, crop rotations, had the highest concentration of FGSC, FOSC and *F. verticillioides*. Unexpectedly, maize/wheat and maize monoculture did not contain the highest concentration of fungi. FGSC and *F. verticillioides* favours stalk tissue whereas FOSC favours roots. As expected, no-till fields favours pathogen/pathogenic complex disease but this is limited to certain tissues. Dryland fields favoured pathogen/pathogenic complex infection. ZEA and FUM were found in the majority of samples but FUM did not exceed the MTL set by the government whereas ZEA did. DON was less abundant but greatly exceeded the MTL during the third season. This suggests that although mycotoxins are season dependent there is a possibility that levels that could cause harm to humans and animals may be exceeded. This study enhances our knowledge of the influence of agricultural practices on these three Fusarium spp.. It also clearly indicates the importance of appropriate agricultural practices in reducing the risk of significant infection levels.

In Chapter 3 the effect of abiotic and biotic factors on prematurely senescing plants in the Northern Cape was determined. It was observed that five nutrients impacted FGSC target DNA concentrations and increased (PSA) or decreased (VGA) the plants susceptibility. Ca increased disease severity with a trend towards higher stem rot incidence, whereas Mg, Na, K and B decreased disease incidence by increasing overall plant health and decreasing disease incidence and severity by the various role they play in plant defence responses. It was also shown that during the soft dough stage maize is the most sensitive towards plant disease. Thus, it could be seen that poor soil nutrition (environment), coupled with a susceptible plant (soft dough stage)

and significantly high FGSC target DNA concentrations in In1 and In2 of season 2012/13 (virulent pathogens) resulted in the PSAs in maize fields.

In Chapter 4 mycotoxin translocation throughout maize plants has in the past been shown to be possible, however in this study translocation of DON, NIV and ZEA was not shown. This was attributed to either the inability of these mycotoxins to be translocated in maize, the conditions were not ideal for translocation, or the maize genotypes were not conducive to translocation. The possibility of FUM translocation has only been evaluated in maize seedlings from roots to the leaves. In this study it was shown that there is a possibility of FUM translocation but because of the trace amounts of FUM found this possibility could not be confirmed. Thus, mycotoxin translocation of DON, NIV and ZEA was not confirmed but the possibility of FUM translocation was shown.

Chapter 5 the first to examine maize stalk defence responses. It was expected that PR-1 would be highly upregulated at each time-point in the resistant cultivar with limited induction in the susceptible cultivar. Instead PR-1 was initially supressed, between 0 – 48 hai, then induced 96 hai. This was attributed to the biotroph/necrotroph switch. It is hypothesised that PR-1 plays a role in F. b*oothii*-maize stalk rot resistance. WRKY1 was only induced in the susceptible cultivar at 72 hai. It is hypothesised that WRKY1 does not play a role in F. boothii-maize stalk rot resistance but may be responsible for the susceptibility of PAN 6479. Although some assumptions could be made from this study, further analysis is required to gain a definitive understanding of maize defence responses toward FGSC.

Controlling pathogens or pathogen complexes such as *Fusarium* spp. is extremely difficult as they are highly adaptive, complex and diverse. However, as our understanding of these fungi increases so do the strategies in containing infections. This study focused on the interaction between *Fusarium* spp. and maize. The findings of this study could contribute to the prediction of *Fusarium* spp. infection on maize, the reduction in Fusarium disease on maize, the adaption of farming practices, and crops with less mycotoxin contamination.