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Evaluation of the capacity of hydrogen sulfide to reduce infection of maize by *Aspergillus flavus*.

By

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Keywords

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Biotic stress

Oxidative stress

Lipid peroxidation

Cell death

Abstract

Maize (*Zea mays* L.) is grown globally as an important grain crop in South Africa, United States, China and Brazil and plays a major role in the worldwide economy. In South Africa, the grain is utilised for food consumption, livestock feed, for malting purposes and bioethanol production. Maize contains approximately 72% starch, 10% protein, 4% fat and supplying an energy density of 365 Kcal/100 g. The production of grain crops in South Africa is restricted by various factors such as abiotic and biotic stresses. The fungal genus Aspergillus is one of the most important biotic stresses affecting maize in the country. *Aspergillus flavus* can contaminate a wide range of agricultural commodities either in storage or field. Hydrogen sulfide appears to have a potential in the mechanism of resistance against pathogen attack by *Aspergillus flavus*.

Hydrogen sulfide (H_2S) generated from sodium hydrosulfide (NaHS) was investigated for its potential to reduce growth of the phytopathogen *A. flavus*. Various concentrations of NaHS were tested using an agar media plating assay for their ability to inhibit *A. flavus* growth. The utilisation of 1 mM of sodium hydrosulfide inhibited *Aspergillus flavus* mycelial growth. Physiological and biochemical parameters were determined in maize plants treated with *A. flavus* and 1 mM of NaHS, such as shoot length, root length, cell death, fresh weight, hydrogen peroxide (H_2O_2) concentration, malondialdehyde (MDA) content and superoxide (O_2^-) content. In leaves and roots of maize, changes in enzymatic activity of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) were examined.

Significant increase of biomass, root length and shoot length were observed in plants treated with NaHS when compared to untreated plants. The physiological analysis in plants treated with *A. flavus* showed significant reduction in shoot length and biomass, which may be associated with high levels of reactive oxygen species (ROS). The excessive ROS accumulation resulted in elevated levels of lipid peroxidation and cell death in maize treated with *A. flavus*. There were no significant changes in enzymatic activities of SOD. However, changes in enzymatic activities of APX and CAT were observed.

Abbreviations

ABA: Abscisic acid

APX: Ascorbate peroxidase

AsA: Ascorbate

A.flavus: *Aspergillus flavus*

CAT: Catalase

CO_2: Carbon dioxide

Cu/Zn-SOD: Copper/zinc superoxide dismutase

DHAR: Dehydroascorbate reductase

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

Fe-SOD: Ion superoxide dismutase

GSH: Glutathione

GSSG: Glutathione disulfide

GPX: Glutathione peroxidase

GR: Glutathione reductase

H_2S: Hydrogen sulfide

IPP: Isopentenyl pyrophosphate

MDA: Malondialdehyde

MDHAR: Monodehydroascorbate reductase (MDHAR)

Mn-SOD: Manganese superoxide dismutase

NAD(P)H: Reduced nicotinamide adenine dinucleotide phosphate

TEMED: N, N, N′, N′-Tetramethylethylenediamine

$OH^{\cdot}$: Hydroxyl radical

$^1O_2$: Singlet oxygen

O_2^-: Superoxide

PAGE: Polyacrylamide gel electrophoresis

PCD: Programmed cell death

PCR: Polymerase chain reaction

PVP: Polyvinylpyrolidone

RWC: Relative water content

ROS: Reactive oxygen species

SOD: Super oxide dismutase

SA: South Africa

TCA: Trichloroacetic acid

RNS: Reactive nitrogen species

Table of Contents

Declaration

I declare that **Evaluation of the capacity of hydrogen sulfide to reduce infection of maize by *Aspergillus flavus*** is my own work, that has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

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List of figures

List of tables

1 Chapter One

Literature Review

1.1 Introduction

Crops experience an increased number of abiotic and biotic stress combinations, due to climate change, which adversely affect their growth and yield (Pandey *et al.*, 2017). Biotic stress, resulting from interactions with other organisms, are mainly infection or mechanical damage by herbivory or trampling, as well as effects of parasitism (Cable *et al*., 2017). Abiotic environmental factors include extreme temperature (chilling, freezing, and heat), salinity, heavy metals (ion toxicity), light intensity, the supply of water, minerals and CO_2 (Ramakrishna and Ravishankar, 2011).

Maize (*Zea mays L.*) is negatively affected by both abiotic and biotic stresses. It is a widely grown staple food, feed and industrial crop which plays a critical role in supporting the growing world population. In South Africa, maize is the most important grain crop and is produced in various parts of the country under diverse environments (du Plessis, 2003). Maize is one of the most important cereal crops in terms of its production and consumption (Ranum *et al*, 2014). The usage of maize varies in different countries. In Asia, maize is mainly used for both food and animal feed while it is mainly used for food in Latin America and Africa (de Lange *et al.*, 2014). In Ethiopia approximately 47 diseases were verified in maize and 25 more diseases were recorded in 1984, which are a major limiting factor to its production (Tsedaley and Adugna, 2016). The production process of maize is highly dependent on suitable environmental factors such as warm temperatures accompanied by moderate rain-fall to maintain good levels of soil moisture (Gong *et al*., 2014; Li *et al.*, 2017). Fungi are the most dominant pathogens of maize and are the principal cause of crop loss in the field and during storage (Tsedaley and Adugna, 2016).

The world population obtains more than 36% of its calories from maize (Cassidy *et al.*, 2013). With maize being one of the most widely produced cereal in the world, the global world trade of the commodity is large (Kodamaya *et al.*, 2007; Cassidy *et al.*, 2013). This chapter aims to present an overview of maize and its interaction with *Aspergillus flavus* - the causative pathogen of Aspergillus ear rot.

1.2 Maize (*Zea mays L.*) as an important crop

Maize (*Zea mays L.*) is the most important grain crop in South Africa and is produced throughout the country under diverse environments (Tandzi and Mutengwa, 2019). Successful maize production depends on the correct application of production inputs that will sustain the environment as well as agricultural production. In developed countries, maize is used mainly as second-cycle produce, in the form of meat, eggs and dairy products. In developing countries, maize is consumed directly and serves as staple diet for approximately 200 million people (Cassidy *et al.*, 2013). Approximately 8, 0 million tons of maize grain are produced in South Africa annually on approximately 3, 1 million ha of land (Staller, 2010). Half of the production consists of white maize for human food consumption. Maize is a warm weather crop and is not grown in areas where the mean daily temperature is less than 19 ºC or where the mean of the summer months is less than 23 ºC. Although the minimum temperature for germination is 10 ºC, germination will be faster and less variable at soil temperatures of 16 to 18 ºC (Caverzan *et al*., 2016). At 20 ºC, maize should emerge within five to six days. The critical high temperature detrimentally affecting yield is approximately 32 ºC. Frost can damage maize at all growth stages and a frost-free period of 120 to 140 days is required to prevent damage.

1.3 Maize production in South Africa

Maize is of major importance for the South African economy. In 2016, maize yielded over 15% of the gross value of all agricultural products, while accounting for about 40% of the entire cultivated area in the country (Kodamaya *et al.*, 2007; Ranum *et al*., 2014, Beukes *et al*., 2016). South Africa meets its annual maize consumption requirements entirely from domestic production, which has been steadily increasing over the years. Local consumption of maize is about 7.5 metric tonnes per year, but the country often produces surpluses that are exported, mainly to neighbouring countries in the South African Development Community (SADC) (Staller, 2010). Interestingly, despite the growth in production, the entire area used for maize farming has in fact declined from 3.8 million hectares in the mid-eighties to approximately 3 million hectares between 1996 and 1997 (Staller, 2010; Cassidy *et al.*, 2013). For the past decade, an average of approximately 8 million tons of maize has been produced every year. Although the area planted has declined during the same period, the relative stability of production can mainly be attributed to the fact that the yield has increased over the years as

production technologies have improved (Ranum *et al*., 2014). Yellow maize yield were normally higher than white maize yield, with yellow maize being at its lowest drought year from 1991 to1992 and its highest production in the year of 1993 and1994 (Rose *et al.*, 2017). The highest yield of white maize has been achieved from 1994 to 1996. This can be attributed primarily to the fact that white maize is used mainly for human consumption and yellow mainly for animal feed and thus a lot of focus get placed on white maize (Kodamaya *et al.*, 2007).

During the drought years (2016 and 2017), even though there were brief declines in the yield, commercial maize producers were able to recover quickly due to government subsidies and grants instituted through the Maize Board and through other government policies. These policies existed until 2016-2018, after which the Maize Control Board ceased to operate. This was significant, as "free agricultural trade policies" were introduced at the time (Kodamaya *et al.*, 2007; Rose *et al.*, 2017).

1.4 Plant responses to various stresses

Plant productivity and growth is adversely affected by various stresses. These include cold, heat, salinity, drought, pathogens and herbivores. Such stresses have negative impact on crop yield and quality (Singh *et al.*, 2013; Das and Roychoudhury, 2014). Plant responses to these stresses can either take an active or passive form. An active defense response involves a suitable and rapid response when a stress is detected while a passive response is more delayed and takes more time to be activated (Showler, 2016). When plants perceive a stress, a response is initiated to activate specific signal transduction pathways which facilitate the generation of appropriate physical and physiological responses. Various signalling pathways are involved in different stress responses with multiple cross-talks occurring, resulting in a highly complex myriad of networks (de Lange *et al.*, 2014), as illustrated in Figure 1.1.

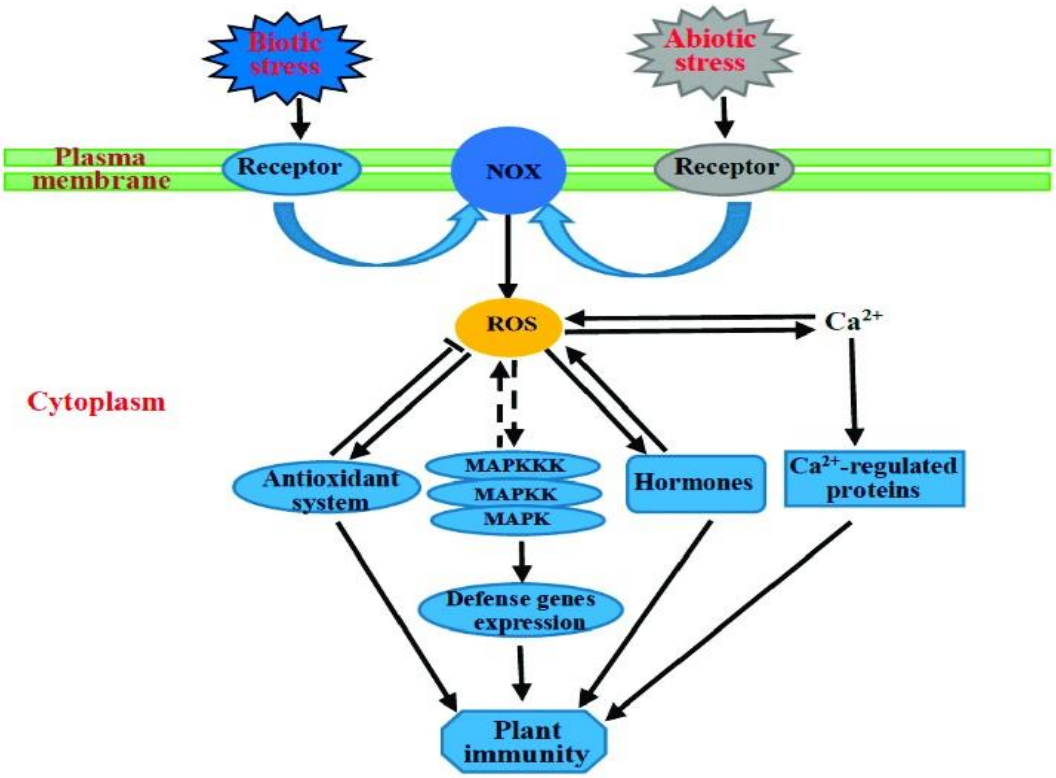


Figure 1. 1. Networks triggers during plant responses to abiotic and biotic stress (figure adapted from Zhu and Lafayette, 2017).

1.4.1 Effect of abiotic stress on plant growth

Abiotic stress refers to any type of physical environmental condition that adversely affects plant productivity, development and survival. These adverse environmental conditions include drought, high and low temperatures, soil nutrient deficiency, excess salt or toxic metals like aluminium and arsenate, and extremes in soil pH. Effects of these abiotic stresses are worsened by climate change, which has been predicted to result in an increased frequently of extreme weather (Zhu, 2016). Events that occur in plant tissue in response to abiotic stress are shown in Figure 1.2.

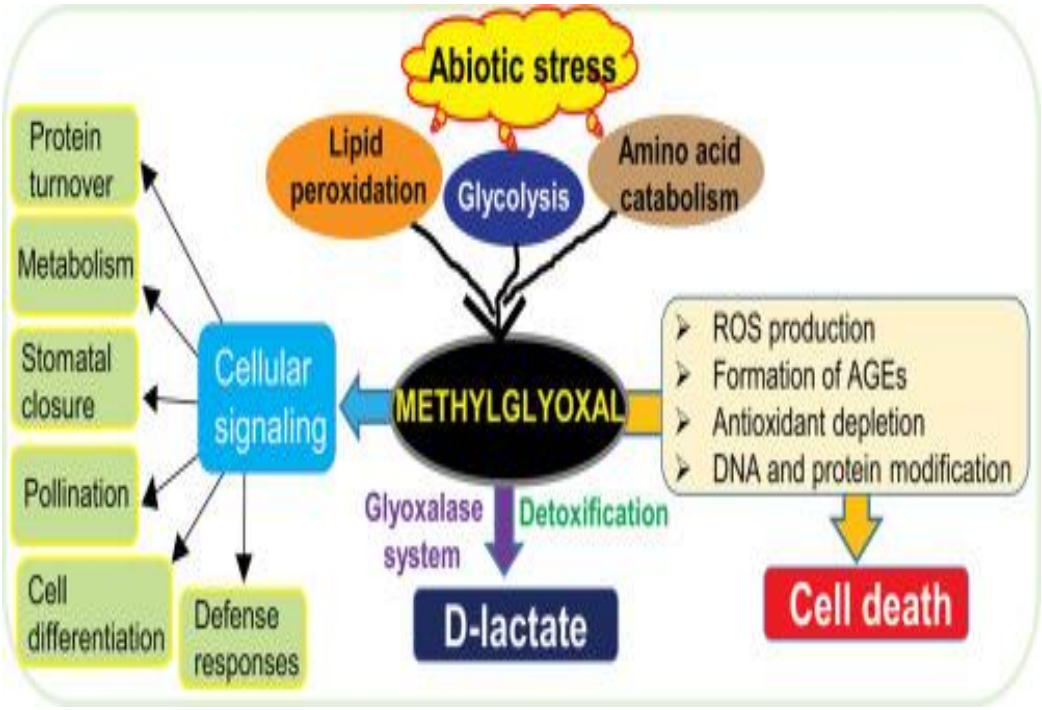


Figure 1. 2: Plant signalling and responses to abiotic stress (figure adapted from Showler, 2016).

1.4.1.1 Drought

Drought limits maize yields in most maize production regions worldwide (Gong *et al.*, 2014). Responses to drought are regulated by a complex network driven by multigenic mechanisms. Many processes involved in plant growth and development including osmotic adjustment, antioxidant capacity, photosynthetic rate regulation, and abscisic acid (ABA) accumulation are affected by drought (Cramer *et al.*, 2011). Crop failure in drought-stressed plants infested with herbivorous pests is pronounced because drought can lead to more successful colonization of the plants by the pests (Showler, 2016).

1.4.1.2 High and low temperatures

Global climate change associated with what may seem to be small annual increases in average global temperature have the potential to change the decomposition of organic matter and could

in turn affect the bioavailability of carbon (Santoyo *et al.*, 2017). This global warming can lead to either extreme temperature highs and extreme temperature lows and other extreme weather events in a given season (Herring *et al*., 2018). Production, germination and seedling growth of plants can be affected by both high and low temperatures.

1.4.1.3 Soil nutrient deficiency

In various regions worldwide, the effect of nutrients in the soil and their impacts on plants has been studied broadly. One of the major restrictions to production is infertility soil in agricultural soil, which is determined by the three main nutrients nitrogen (N), carbon (C), and phosphorus (P), and micronutrients (Kibblewhite *et al*, 2008; Rao *et al*., 2016). Nutrient deficiency are generally resolved immediately with the application of chemical fertilizers, which may have negative effects on the environment, human and animal health.

1.4.1.4 Salinity

Salinity is one of abiotic stress that extensively limit crop production. Soil salinity is a global problem and about 20% of all irrigated land is affected by soil salinity, decreasing crop yields significantly (Negr and Schmo, 2017). Plants are affected by salt stress in two main ways: osmotic stress and ionic toxicity. These stresses affect all major plant processes, including photosynthesis, cellular metabolism, and plant nutrition. Soil salinity is commonly the reservoir of a number of soluble salts such as Ca^{2+}, Mg^{2+}, Na^{+} and the anions SO_4^{2-}, Cl^{-}, HCO^{3-} with exceptional amounts of K^{+}, CO_3^{2-}, and NO^{3-} (Newell, 2013; Ghosh *et al*., 2016). The pH of saline soils generally ranges from 7-8.5.

1.4.1.5 Variations in soil pH

The pH is a key factor in many soil science studies and it indicates the concentration of hydronium ions (H_3O^{+}) present in the soil, it also determines the acidity or alkalinity of the soil. Soil pH is one of the main elements that defines the structure of microbiome communities (Lammel *et al.*, 2018). Soil pH varies substantially at the regional and global scale, and therefore can affect microbial communities, as soil microbes show a wide range of pH

tolerance. Soil pH is directly related to the availability of nutrients for plants by controlling the chemical forms of the soil compounds. This has also been suggested to be an indirect limiting factor for microbial soil communities (Garrido-oter and Gonza, 2015; Qi *et al.*, 2018) .

1.4.2 Effect of biotic stress on plant growth

Biotic stresses are usually a result of pathogens such as viruses, bacteria fungi and herbivores or insects. The extent to which biotic stresses cause yield or quality loss depends on the environment and thus varies, depending on the region, agroecology, and country (Pandey *et al.*, 2017). Plants experiencing water stress may also respond differently to pathogen attack than under normal conditions. For instance, they may cause stomatal closure to reduce water loss from infected tissues, thus having a positive effect on plant tolerance against drought stress. Infection with viruses can actually provide protection from drought stress. Furthermore, bacteria and fungi can enhance stress tolerance in a range of crop species by producing antioxidants, suppressing ethylene production, stabilizing soil structure, increasing osmolyte production, and improving abscisic acid (ABA) regulation (Greco *et al.*, 2012).

Biotic stress is first perceived by the receptors present on the membrane of the plant cells. Pathogens produce several pathogen-associated molecular patterns (PAMP's) that are perceived by plant receptors known as pattern recognition receptors (PRR), leading to PAMP triggered immunity (Bigeard *et al.*, 2015). To overcome this response, pathogens produce effector proteins which then enter into the host cell. The host plant in response produces effector specific R-proteins which are encoded by R-genes to resist pathogen invasion. This gives rise to what is known as the hypersensitive response or effector triggered immunity and thus constitutes qualitative resistance (Hell and Mutegi, 2011). Further attack by the pathogen continues, producing enzymes and toxins. Several other signal transduction pathways including induction of phytohormones are also activated, deploying broad biochemical resistance mechanisms in order to minimize or suppress pathogen progress, thus constituting quantitative resistance (Kushalappa and Gunnaiah, 2013). Some of the components of these abiotic stress responses are represented in Figure 1.3.

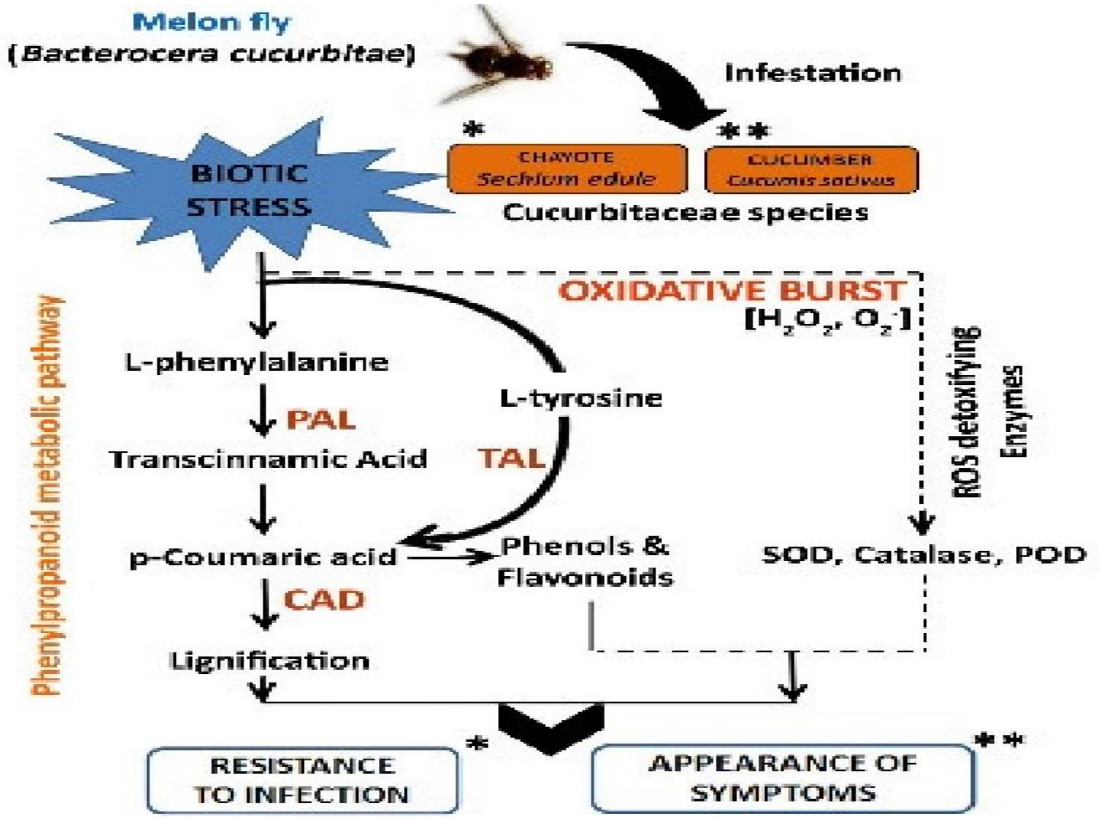


Figure 1. 3: The phenypropanoid metabolic pathway is triggered when plants are affected by biotic stress (figure adapted from Santoyo *et al*., 2017).

1.4.2.1 Insects

Insects are among the most diverse eukaryotic species. Plants can have various types of interactions with insects which can either be beneficial and detrimental to plants, such as antagonistic interactions with herbivores and mutualistic interactions with carnivorous and pollinating insects (Wu and Baldwin, 2010; Nguyen *et al.*, 2016). Plants can respond against insect attack via a complex defense system which includes structural barriers, toxic chemicals, and attraction of natural enemies of the target pests (Belete, 2018). Both direct and indirect defense mechanisms may be present constitutively or induced after damage by the insects. Induced responses have been exploited for regulation of herbivorous insect populations and is one of the important components of insect pest control in agriculture (Donatelli *et al.*, 2017). According to Belete (2018), globally, each year there is a huge crop yield loss caused by different insect pests and it becomes a serious concern. Therefore, understanding the defense systems against insect pests enables development of resistant crops or pest management

systems, reducing the need for hazardous pesticides and supporting safer crop production.

1.4.2.2 Herbivores

Plants respond to herbivore attack through various morphological, biochemical, and molecular mechanisms to counteract the effects of herbivore attack (Machado *et al.*, 2016). These responses can be more extensive than simple modifications of secondary metabolite concentrations. For example, spider mites cause widespread changes in the cytology, histology, and physiology of their host plants, including modifications of photosynthetic and transpiration rates, and they can inject substances that can act as plant growth regulators (Frost *et al.*, 2008). A review by Frost *et al.* (2008) argues that herbivores can influence the plant morphology by causing increased density of prickles, spines, and hairs and causing the return to the juvenile growth form (Karban and Myers, 1989), or by affecting plant phenology through processes such as leaf abscission.

1.4.2.3 Aspergillus species

Aspergillus flavus is a globally occurring fungal pathogen with very toxic metabolites that are produced via secondary metabolism that causes disease in many agricultural crops and contaminates them with aflatoxins. Worldwide, Aspergillus ear rot that is caused by *A. flavus* is a major maize disease. Contamination of maize grain with aflatoxins lead to losses of more than one billion US Dollars lost annually (Mitchell *et al.*, 2016). The disease is most common under high relative humidity, high temperatures (27-38 ˚C), under drought conditions, and during 85% of pollination and grain filling (Aguirre *et al.*, 2006). Maize ears are damaged by insects and birds that attack the kernels and increase the susceptibility of the grains to infection (Pandey *et al.*, 2017)**.**

Circumstances that favour mouldy growth may also favour mycotoxin production, although mould growth can occur with little or no mycotoxin production. *Aspergillus* spores are generated on crop residue, soil surface and discarded kernels. *Aspergillus* can exist in food and feed products, forage, cereal grains, and decaying vegetation. *Aspergillus niger* (Tiegh) appears as a black mould on infected kernels (Amaike and Keller, 2011). On damaged kernels, *A. flavus*

(Link) appears as a greenish-yellow mould and *A. glaucus* (Michele) appears as a greenish mould (Amaike and Keller, 2011). Infection with *A. flavus* in maize results in occurance of ear rot disease that is favoured by hot (>30 ˚C) dry conditions at pollination and during grain filling (Zhu and Lafayette, 2017). Yellow-brown silks are mostly at risk to infection. Fungal spores become airborne and can infect kernels by growing down the silk channel when silks are yellow-brown and still moist. Infection is most common through kernel wounds caused by several types of insects.

1.5 Biotic factors that exacerbate ear rots of maize

The major biotic factors that perpetuate maize ear rot include insects, maize earworms and birds.

1.5.1 Insects

Insects create wounds for rot infections and function as vectors of ear rot pathogens. The insect damage increase the susceptibility of cobs/kernels to ear rot infection. European corn borers, corn ear worms, the beetle complexes and thrips are examples of insects associated with maize ear rots. Thrips cause damage in plant tissues by penetrating, sucking as well as perforating, which harm the epidermal tissue. This disruption of the tissue leads to increase susceptibility to fungal infection.

When earworms attack maize ears, serious damage occurs. They may penetrate down the ear and leave moist castings from their feeding. These castings, frequently visible at the tip of the ear, render the corn unsalable. The association between these insects and maize diseases result from several types of host-insect-pathogen interactions. European maize borer larvae carry spores of *Fusarium* species from the plant surface to the surfaces of damaged kernels or to the interior of stalks, where infections are initiated. Viable spores can be found externally, internally, and in the frass of European maize borer larvae. There is correlation between maize earworm or south western maize borer and *Fusarium* or *Aspergillus* infection incidence in maize (Amaike and Keller, 2011). A second type of interaction is the formation of entry wounds for the fungi when larvae feed on stalks or kernels. Even when the larvae do not directly carry the fungi into the stalks, spores subsequently deposited on the wounded tissue are very

likely to infect the plant.

1.5.2 Birds

According to de Lange *et al.* (2014), the level of blackbird damage to maize, is mainly influenced by kernel maturity (as measured by date of silking). The second most important factor is the peak population level of maize rootworm beetles. The degree within a field to which the husk leaves extended beyond the tip of the ears is strongly correlated with the severity of damage (Amaike and Keller, 2011). Chen *et al.* (2015) estimate that 5,000 birds cause maize ear damage equivalent to 10 % of the total field maize crop. Subsequently, such bird feeding damage increase the susceptibility of the ears to various ear moulds and rots, which may lead to contamination with mycotoxins (Chen *et al.*, 2015)**.**

1.5.3 Occurrence of maize ear rot

Maize is a host to numerous pathogenic species that negatively affect the yield and the quality of the maize crop, imposing serious diseases to its ear and foliage. Worldwide, Ear rots occur wherever maize is grown, reducing yield and quality. In Europe, North and South of America, Asia, South Africa and East Africa, high levels of ear rot infection and mycotoxin accumulation have been reported in pre-harvesting of maize (Miller, 2008). Under favourable condition of grain storage, mycotoxin production from mycotoxigenic fungi in the grain may occur (Ferrigo *et al*., 2016).

1.6 Management of maize ear rots

Many management practices can be used to reduce maize ear rot infection, and the most preferred is growing of resistant varieties (Wolde, 2017). Inbred lines vary in their resistance to the different ear and kernel rot fungi. However, no inbred line or hybrid is completely resistant to all ear rot fungi. Hybrids with poor husk are susceptible to infection by certain ear and kernel rot fungi. Maintenance of balanced soil fertility is recommended based on the results of a soil test. During extended droughts there is a need to irrigate, where possible (Barnard and Preez, 2004). Control of maize earworms and maize borers is recommended by timely

applications of insecticides, where applicable. Maize can be harvested soon as moisture levels permit. Maize ear and shelled grain can be stored at the recommended levels of moisture content below 18 % for ears and 15% for shelled maize (Mutungi *et al*., 2019). The grain can be aerated to maintain a uniform temperature of 4 to 10 ˚C throughout the bulk, where possible. Only properly cleaned grain should be stored in a store that has first been thoroughly cleaned of debris. This practice will limit the development of ear and kernel rot as well as storage rot fungi (Befikadu, 2014).

1.7 Occurrence of aflatoxins in maize

Throughout the maize growing regions of the world, maize is associated with the major mycotoxin problems. The major mycotoxins occurring in maize are aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2). Maize is a staple food for many African countries, but maize in many of these countries is highly colonized by aflatoxin-producing *Aspergillus* species and this is of great concern. In Kenya, where maize is the primary dietary staple, epidemiological investigations determined that the outbreak of acute aflatoxicosis was the result of aflatoxin poisoning from ingestion of contaminated maize (Lewis *et al*., 2005) Infection of maize kernels by toxigenic fungi remains a challenging problem despite decades of research progress. In Nigeria, a study by William *et al*. (2004) showed that the maximum aflatoxin levels found in maize or maize products was 770 ppb, followed by 465 ppb in maize from Mexico.

Initially, aflatoxin contamination was thought to be a postharvest problem. From the beginning of storage to 6 months later, an increasing percentage of samples shows high aflatoxin levels (Kumar, 2017). The means detected at the beginning of storage were between 22 and 190 ppb but between 31 and 221 ppb after 6 months in these aflatoxin positive samples (Hell *et al*., 2000). Resource-poor farmers sometimes leave their maize on the floor in a corner of the room or in the courtyard, with the maize cobs in immediate contact with the floor, which increases the risk of *Aspergillus* development (Kanengoni *et al*., 2015) However, research has indicated that infection with *Aspergillus* and subsequent aflatoxin contamination does also occur prior to harvest in some commodities. Aflatoxin contamination in maize depends on the coincidence of host susceptibility, environmental conditions favourable for infection and, in some cases,

vector activity (Soni *et al*. 2020). In maize, earlier planting dates in temperate areas generally result in a lower risk, but annual fluctuations in weather can jeopardize this advantage (Gornall *et al*., 2010). Aflatoxin development in maize can be affected by several cultural practices, partly because of the relationship between drought stress and susceptibility to *A. flavus* and aflatoxin accumulation (Moral *et al*., 2020). Conditions that tend to expose plants to greater drought stress will lead to higher levels of aflatoxins (Kebede *et al*., 2012). Damage to maize kernels by insects, especially the European corn borer, fall armyworm, and corn earworm has been associated with high aflatoxin levels (Bowen *et al*., 2014).

1.8 Plant responses to fungal invasion

For prevention by fungal invasion, plants use their cell wall through deposition of a sugar polymer called callose. Callose is a component of specialized cell wall or cell wall-associated structures at particular stages of growth and differentiation (Radford *et al*., 1998). At multiple stages of pollen development, callose is involved as a structural component. It is also deposited at cell plates during cytokinesis. Initiation of callose formation in response to wounding of plant cells is a result of a mechanism involving calmodulin (which causes elevated Ca^{2+} ion levels) (Kauss, 1985; Savatin and Varma, 2014**).** The deposition of callose occurs immediately after cell damage by chemical or mechanical injury in biotic stress. GSL5/PMR4/Ca1S12 is responsible for callose deposition in sporophytic tissue in response to wounding or pathogen synthesis (Ellinger and Voigt, 2014). The information mentioned above indicates that callose has a crucial role in plant defense against pathogen infection. The oxidative burst is one of the first events that occur during pathogen recognition (Savatin *et al*., 2014). During oxidative burst, reactive oxygen species (ROS) are released in the form of superoxide ions, hydrogen peroxide, hydroxyl radicals, etc.

Aspergillus flavus (Link ex Fr.) is a plant parasitic pathogen, facultative fungus, which has the potential to colonize a number of common crop species such as corn, cotton, peanuts, and different other crops (Amaike and Keller, 2011). It is a pathogen that is both parasitic and non-parasitic and flourishes abundantly on many organic nutrient sources containing monosaccharides and disaccharides (Chen *et al.*, 2015). The fungus causes aspergillosis diseases in animals that ingest infected crops in which aflatoxins have been produced, with

prevalence increasing in the immunocompromised population (Kumar, 2017). In 2011, Hell and Mutegi reported that *Aspergillus flavus* with the optimal growth temperature ranging from 28 ◦C to 37 ◦C have the ability to survive temperatures ranging from 12 ◦C to 48 ◦C and high humidity of above 80%. Developing maize seedlings are attacked and affected by *A. flavus* in the field before harvest and mature seeds during harvest and in storage, but most are noted as storage mould on plant products (Krishnan *et al*., 2009; de Lange *et al.*, 2014). The colonization of maize with *Aspergillus flavus* results in contamination of their derived agricultural products with aflatoxins. On crops such as maize, peanut, cottonseed and tree nuts, aflatoxins are among the most potent mycotoxins, carcinogenic and teratogenic compounds, produced during infection and growth of fungi like *A. flavus* (Amaike and Keller, 2011; de Lange *et al.*, 2014; Chen *et al.*, 2015). Agricultural commodities contaminated with toxigenic *A. flavus* producing mycotoxins can be harmful to animals and human health.

1.9 Reactive oxygen species (ROS) production and its signal

Reactive oxygen species (ROS) were mostly recognized as toxic by-products of aerobic metabolism. It has become apparent that ROS plays an important signaling role in plants, controlling processes such as growth, development and especially response to biotic and abiotic environmental stimuli in recent years (Das and Roychoudhury, 2014). The major members of the ROS family include free radicals like O_2^- , •OH and non-radicals like H_2O_2 and $^1O_2$. ROS production in plants is mainly localized in the chloroplast, mitochondria and peroxisomes. There are secondary sites as well like the endoplasmic reticulum, cell membrane, cell wall and the apoplast (Kuuzniak and Urbanek, 2005; Torres *et al.*, 2006). ROS have a dual role as they act as secondary messengers in various key physiological phenomena; whereas when the delicate balance between ROS production and elimination is disturbed, they hyper accumulate to induce oxidative damage under several environmental stress conditions such as salinity, drought, cold, heavy metals and UV irradiation (Oa *et al*., 2010).

ROS-induced cell damages can result in cell death due to degradation of biomolecules like pigments, proteins, lipids and DNA (Nita and Gryzbowski, 2015). Redox homeostasis is maintained by two arms of the antioxidant machinery, as shown in Figure 1.4; namely the enzymatic components comprised of superoxide dismutase (SOD), ascorbate peroxidase

(APX), glutathione peroxidase (GPX), glutathione-S-transferase (GST) and catalase (CAT), and the non-enzymatic low molecular compounds like ascorbic acid (AA), reduced glutathione (GSH), α-tocopherol, carotenoids, phenolics, flavonoids and proline (Sharma *et al.*, 2012; Das and Roychoudhury, 2014).

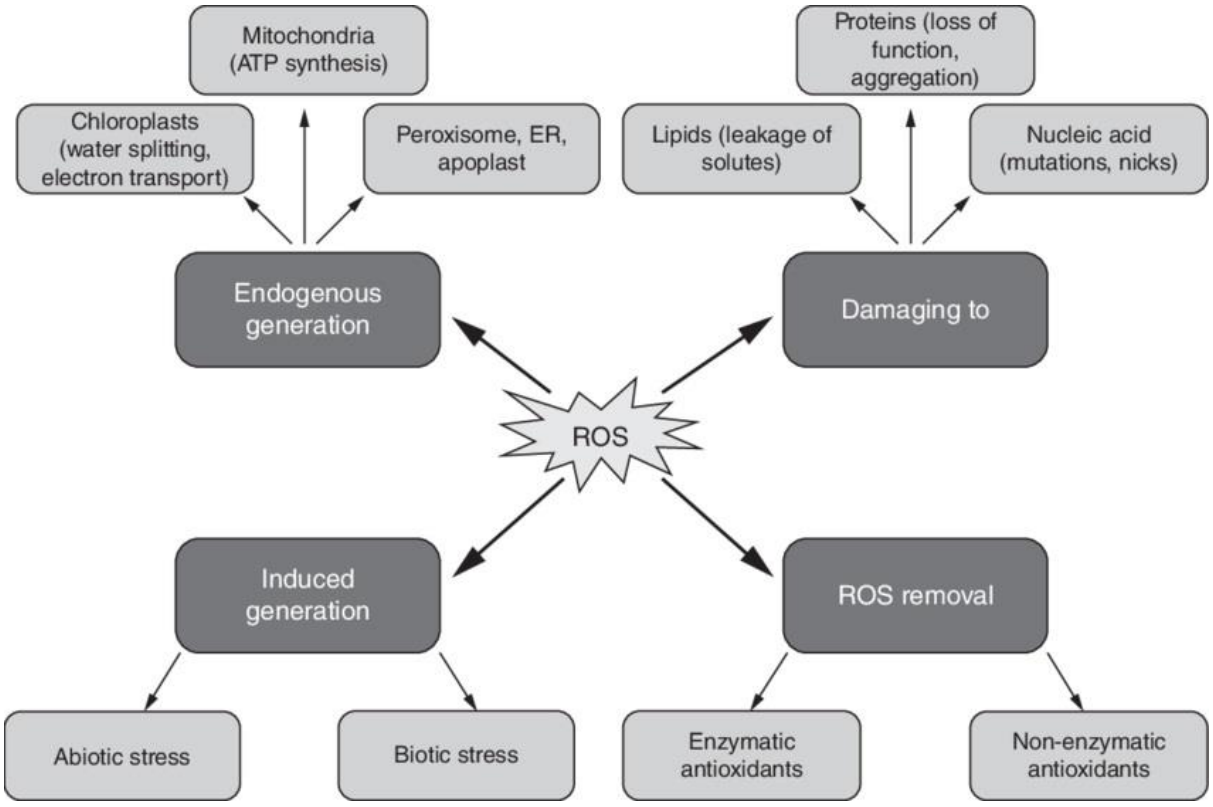


Figure 1. 4: Schematic representation of reactive oxygen species (ROS) production in plants. ROS are generated in different locations such as chloroplasts, mitochondria, plasma membrane, peroxisomes, apoplast, endoplasmic reticulum and cell wall (figure adapted from Tripathy and Oelmuller, 2012).

1.10 Types of ROS and their effects

The most common ROS include $^1O_2$, O_2^-, H_2O_2 and •OH. Singlet oxygen in its ground state it has two unpaired electrons with a parallel spin, which makes it paramagnetic and is unlikely to participate in reactions with organic molecules unless it is activated (Huang *et al.*, 2016). Activation of $^1O_2$ may occur by two different mechanisms that includes absorption of sufficient

energy to reverse the spin on one of the unpaired electrons and stepwise monovalent reduction. In the latter, O_2 is sequentially reduced to O_2^-, H_2O_2 and •OH whereas in the past $^1O_2$ is formed. Electrons in the biradical form of oxygen have parallel spin. Absorption of sufficient energy reverses the spin of one of its unpaired electrons, leading to formation of the singlet state in which the two electrons have opposite spins. This activation overcomes the spin restriction and $^1O_2$ can consequently participate in reactions involving the simultaneous transfer of two electrons (divalent reduction) (Widlansky and Gutterman, 2011). In the light, highly reactive $^1O_2$ can be produced through triplet chlorophyll formation in the antenna system and in the reaction centre of photosystem II (Dreaden and Bridgette, 2012). In the antenna, insufficient energy dissipation during photosynthesis can lead to formation of a chlorophyll triplet state, whereas in the reaction centre it is formed *via* charge recombination of the light-induced charge pair (Schmitt *et al.*, 2014**).**

Further, limited CO_2 availability due to closure of stomata during various environmental stresses such as salinity and drought favours the formation of $^1O_2$.The life time of $^1O_2$ within the cell is probably 3 μs or less (Midge *et al.*, 2010). A fraction of $^1O_2$ has been shown to be able to diffuse over considerable distances of several hundred nanometers (nm). $^1O_2$ can last for 4 μs in water and 100 μs in a nonpolar environment (Helena and Carvalho, 2008). $^1O_2$ reacts with most of the biological molecules at near diffusion-controlled rates (Laloi *et al.*, 2004). It directly oxidizes proteins, polyunsaturated fatty acids and DNA (Nguyen *et al.*, 2016a). It causes nucleic acid modification through selective reaction with deoxyguanosine (Mccarthy-suarez, 2017). It is thought to be the most important species responsible for light-induced loss of photosystem II (PSII) activity which may trigger cell death (Huang *et al.*, 2016). $^1O_2$ can be quenched by β-carotene, α-tocopherol or can react with the D1 protein of photosystem II (Nguyen *et al* , 2016b).

Due to spin restriction, molecular O_2 cannot accept four electrons at a time to produce H_2O. It accepts one electron at a time and hence, during reduction of O_2, intermediates are formed in a step-wise fashion (Schmitt *et al.*, 2014). O_2^- is the primary ROS formed in the cell which initiates a cascade of reactions to generate secondary ROS, either directly or through enzyme- or metal-catalysed processes (Widlansky and Gutterman, 2011) depending on the cell type or cellular compartment. O_2^- is a moderately reactive, short-lived ROS with a half-life of approximately 1 μs. O_2^- is a nucleophilic reactant with both oxidizing and reducing properties (Sharma *et al.*, 2012). The anionic charge of O_2^- inhibits its electrophilic activity toward

electron rich molecules. O_2^- has been shown to oxidize enzymes containing the [4Fe-4S] clusters, such as aconitase or dehydratases (Laloi *et al*., 2004) and reduce cytochrome C (Breusegem and Dat, 2006). O_2^- can accept one electron and two protons to form H_2O_2. It is easily dismutated to H_2O_2 either non-enzymatically or by SOD.

H_2O_2 is generated in the cells under normal as well as a wide range of stressful conditions such as drought, chilling, UV irradiation, exposure to intense light, wounding and intrusion by pathogens. Electron transport chain (ETC) in chloroplast, mitochondria, endoplasmic reticulum and plasma membrane, and *β*-oxidation of fatty acid and photorespiration are major sources of H_2O_2 generation in plant cells. Photooxidation reactions, NADPH oxidase as well as xanthine oxidase (XOD) also contribute to H_2O_2 production in plants. It is also generated as a substrate for lignification and suberization. H_2O_2 is moderately reactive and is a relatively long-lived molecule with a half-life of 1 ms (Mccarthy-suarez, 2017). H_2O_2 has no unpaired electrons, and unlike other oxygen radicals, it can readily cross biological membranes and consequently can cause oxidative damage far from the site of its formation. Because H_2O_2 is the only ROS that can diffuse through aquaporins in the membranes and over larger distances within the cell (Schmitt *et al.*, 2014) and is relatively stable compared to other ROS, it has received particular attention as a signaling molecule involved in the regulation of specific biological processes and triggering tolerance against various environmental stresses such as pathogen infection (Laloi *et al*, 2004; Huang *et al.*, 2016). Both O_2^- and H_2O_2 are only moderately reactive. The formation of •OH is dependent on both H_2O_2 and O_2^- and, thus, its formation is subject to inhibition by both SOD and CAT.

1.11 Reactive Oxygen Species damage lipids

The imbalance between the prooxidant and antioxidant levels in favour of prooxidants is one of the causes of oxidative stress in cells and results in oxidative damage. High levels of reactive oxygen species (ROS) can inflict direct damage to lipids. The primary sources of endogenous ROS production are the mitochondria, plasma membrane, endoplasmic reticulum, and peroxisomes through a variety of mechanisms including enzymatic reactions and/or auto oxidation of several compounds, such as catecholamines and hydroquinone. Different exogenous stimuli, such as the ionizing radiation, ultraviolet rays, tobacco smoke, pathogen infections, environmental toxins, and exposure to herbicide or insecticides, are sources of *in vivo* ROS production.

ROS that can affect lipids are mainly hydroxyl radical (•OH) and hydroperoxyl (HO_2). The hydroxyl radical (•OH) is a small, water-soluble, and chemically most reactive species of activated oxygen. This short-lived molecule can be produced from O_2 in cell metabolism and under a variety of stress conditions. A cell produces around 50 hydroxyl radicals every second (Phaniendra *et al*., 2013). In one day, each cell would generate 4 million hydroxyl radicals, which can be neutralized or attack biomolecules. Hydroxyl radicals cause oxidative damage to cells because they non-specifically attack biomolecules located less than a few nanometres from its site of generation and are involved in cellular disorders such as neurodegeneration, cardiovascular diseases, and cancer (Sharma *et al*., 2012). The hydroperoxyl radical (HO_2) plays a key role in lipid peroxidation. This protonated form of superoxide yields H_2O_2 which can react with redox active metals, including iron or copper. The HO_2 is a much stronger oxidant than superoxide anion radical and could initiate the chain oxidation of polyunsaturated phospholipids, thus leading to impairment of membrane function.

1.12 Products of lipid peroxidation

Lipid peroxidation or reaction of oxygen with unsaturated lipids produces a wide variety of oxidation products. The main primary products of lipid peroxidation are lipid hydroperoxides (LOOH). Malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal are known aldehydes that can be designated as secondary products during lipid peroxidation. MDA appears to be the most mutagenic product of lipid peroxidation, whereas 4-HNE is the most toxic (Ayala *et al*., 2014). MDA has been widely used for many years as a convenient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids because of its reaction with thiobarbituric acid (TBA). The TBA test is predicated upon the reactivity of TBA toward MDA to yield an intensely coloured chromogen red adduct; this test was first used by food chemists to evaluate autoxidative degradation of fats and oils (Guillen-Sans *et al*.,1998).

1.12.1 Biosynthesis of MDA

MDA is an end product generated by decomposition of arachidonic acid and larger polyunsaturated fatty acids (PUFAs), through enzymatic or non-enzymatic processes (Figure 1.5). MDA production by enzymatic processes is well known but its biological have not been

studied. MDA is more chemically stable and membrane-permeable than ROS and less toxic than 4-HNE and methylglyoxal (MG). MDA production by non-enzymatic processes remains poorly understood. It has high capability to react with multiple biomolecules such as proteins or DNA, which leads to the formation of adducts whose excessive production has been associated with different pathological states.

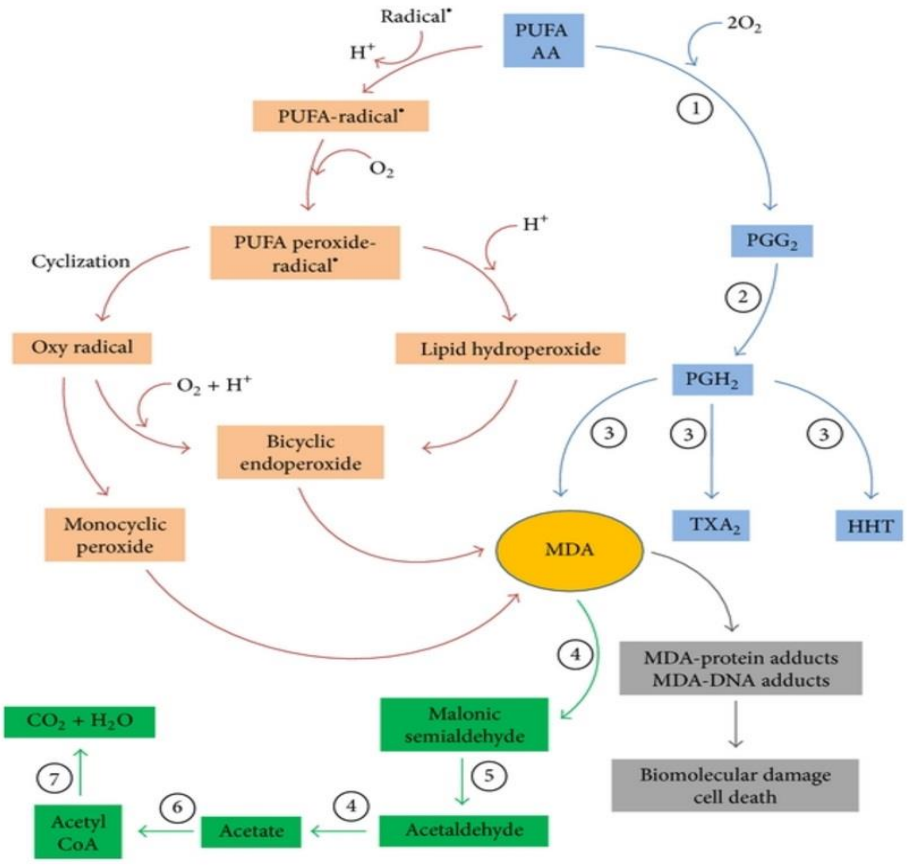


Figure 1. 5: Production and metabolism of MDA. During the biosynthesis of thromboxane A2 (TXA2) and 12-l-hydroxy-5,8,10-heptadecatrienoic acid (HHT) (blue pathway), MDA can be produced in vivo by decomposition of arachidonic acid (AA) and larger PUFAs as a side product, through enzymatic or non-enzymatic processes by bicyclic endoperoxides produced during lipid peroxidation (red pathway). Once formed, MDA can be enzymatically metabolized (green pathway). Key enzymes involved in the formation and metabolism of MDA are cyclooxygenases (1), prostacyclin hydroperoxidase (2), thromboxane synthase (3), aldehyde dehydrogenase (4), decarboxylase (5), acetyl CoAsynthase (6),and tricarboxylicacidcycle (7) (Figure adapted from Das and Roychoudhury, 2014).

1.12.2 Production and Role of Hydrogen Peroxide (H_2O_2) in plants

Hydrogen peroxide (H_2O_2) is the most stable of ROS, it can easily diffuse across cell membranes and reach locations distant from its primary production sites (Cerny *et al*., 2018, Smirnoff and Arnaud, 2019). Therefore, H_2O_2 is well proved as a regulator of a maltitude of physiological processes like acquiring resistance, cell wall strengthening, senescence, phytoalexin production, photosynthesis, stomatal opening and cell cycle (Petrov and Breusegem, 2012). Unlike the charged O_2^-, H_2O_2 has poor reactivity with most organic molecules in the absence of transition metal ions (Kuznniak and Urbanek, 2000). H_2O_2 plays an essential role in plant defence responses against pathogens and also an important signalling molecule in vascular cells (Petrov and Breusegem, 2012). In the normal vasculature, H_2O_2 formation is primarily reliant upon O_2 production and interaction or dismutation of O_2 to form H_2O_2, allowing for cellular signalling (Slesak *et al*., 2007). H_2O_2 is an important signalling molecule in vascular cell apoptosis, proliferation, and modulation of intracellular Ca^{2+} levels (Touyz, 2004). It also upregulates eNOS gene expression and activity, and increases NO production. In addition, H_2O_2 is required for PDGF, EGF, and angiotensin II signalling (Byon *et al*., 2016).

H_2O_2 also mediates the activation and response of important protein kinases including MAP kinase, tyrosine kinase, Src, and PKC in vascular cells (Augusto and Rhian, 2011). H_2O_2 also appears to play a more general role in the phosphorylation and dephosphorylation of serine groups, resulting in modification of protein activity (Petrov and Breusegem, 2012). One example is eNOS, where H_2O_2 stimulates eNOS upregulation and activity resulting in increased NO production (Cai *et al*., 2005). Previous studies by Cai et al. (2005) have demonstrated eNOS upregulation with H_2O_2 exposure via a calcium dependent mechanism. A recent study also suggests that H_2O_2 may contribute to insulin resistance through its inhibition of the Akt pathway in rat aortic smooth muscle cells (Byon *et al*., 2016. In disease states, H_2O_2 is utilized in the formation of additional ROS including •OH and HOCl (Phaniendra *et al*., 2013). H_2O_2 is degraded into H_2O via catalase and glutathione peroxidase (Cerny *et al*., 2018).

1.13 Antioxidative defense system in plants

Plant defence against ROS involves non-enzymatic and enzymatic components. In plant cells, specific ROS producing and scavenging systems are found in different organelles such as chloroplasts, mitochondria, and peroxisomes (Sharma *et al.*, 2012; Das and Roychoudhury, 2014). ROS scavenging pathways from different cellular compartments are co-ordinated. Under normal conditions, potentially toxic oxygen metabolites are generated at a low level and there is an appropriate balance between production and quenching of ROS (Street, 2015). The balance between production and quenching of ROS may be perturbed by a number of adverse environmental factors, giving rise to rapid increases in intracellular ROS levels, which can induce oxidative damage to lipids, proteins, and nucleic acids. In order to avoid the oxidative damage, plants raise the level of endogenous antioxidant defence (Birben *et al.*, 2012). Various components of the defence system involved in ROS scavenging have been manipulated, overexpressed or downregulated to add to the present knowledge and understanding the role of the antioxidant systems.

1.13.1 Enzymatic antioxidant components

The enzymes localized in the different subcellular compartments and comprising the antioxidant machinery include Superoxide Dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX), Monodehydroascorbate reductase (MDHAR), Dehydroascorbate reductase (DHAR), Glutathione Reductase (GR), and Glutathione Peroxidase (GPX).

1.13.1.1 Superoxide Dismutase (SOD)

SOD belongs to the family of metalloenzymes omnipresent in all aerobic organisms. Under environmental stresses, SOD forms the first line of defence against ROS-induced damage. The SOD catalyses the removal of O^{-}_{2} by dismutating it into O_2 and H_2O_2. This removes the possibility of •OH formation by the Haber-Weiss reaction. It is categorized into three isozymes, namely Mn-SOD that is located in the mitochondria, Fe-SOD in chloroplasts, and Cu/Zn-SOD confined in cytosol, peroxisomes, and chloroplasts. The classification is based on the metal ion that SOD uses as a co-factor. SOD activity is upregulated under abiotic stress (Boguszewska *et al*., 2010; Das and Roychoudhury, 2014).

1.13.1.2 Catalase (CAT)

CAT is a tetrameric heme-containing enzyme responsible for catalysing the dismutation of H_2O_2 into H_2O and O_2. It has lesser specificity for organic peroxides (R-O-O-R), but high affinity for H_2O_2 (Das and Roychoudhury, 2014). It has a very high turnover rate (6×10^6 molecules of H_2O_2 to H_2O and O_2 min-1) and is unique amongst antioxidant enzymes in not requiring a reducing equivalent (Sharma *et al*., 2012). Peroxisomes are the hotspots of H_2O_2 production due to β-oxidation of fatty acids, photorespiration, purine catabolism and oxidative stress. However, recent reports suggest that CAT is also found in other subcellular compartments such as the cytosol, chloroplast and the mitochondria. Angiosperms have three *CAT* genes (Das and Roychoudhury, 2014). *CAT1* is expressed in pollen and seeds (localized in peroxisomes and cytosol), *CAT2* predominantly expressed in photosynthetic tissues but also in roots and seeds (localized in peroxisomes and cytosol) and *CAT3* is expressed in leaves and vascular tissues (localized in the mitochondria) (Teixeira and Mota, 1992). Stressful conditions demand greater energy generation and expenditure of the cell. This is fulfilled by increased catabolism which generates H_2O_2. CAT removes the H_2O_2 in an energy efficient manner (Mhamdi *et al.*, 2010).

1.13.1.3 Ascorbate peroxidase (APX)

APX is an integral component of the Ascorbate- Glutathione (ASC-GSH) cycle. While CAT predominantly scavenges H_2O_2 in the peroxisomes, APX performs the same function in the cytosol and the chloroplast. APX reduces H_2O_2 to H_2O and DHA, using ascorbic acid (AA) as a reducing agent (Das and Roychoudhury, 2014).

1.13.1.4 Monodehydroascorbate reductase (MDHAR)

MDHAR is responsible for regenerating AA from the short-lived MDHA, using NADPH as a reducing agent, ultimately replenishing the cellular AA pool. Since it regenerates AA, it is co-localized with the APX in the peroxisomes and mitochondria, where APX scavenges H_2O_2 and oxidizes AA in the process. MDHAR has several isozymes that are localized in chloroplast, mitochondria, peroxisomes, cytosol, and glyoxysomes (Sharma *et al.*, 2012).

1.13.1.5 Dehydroascorbate reductase (DHAR)

DHAR reduces dehydroascorbate (DHA) to AA using reduced Glutathione (GSH) as an electron donor. This makes it another agent, apart from MDHAR, which regenerates the cellular AA pool. It is critical in regulating the AA pool in both symplast and apoplast, thus maintaining the redox state of the plant cell (Oa *et al.*, 2010). DHAR is normally found abundantly in seeds, roots and both green and etiolated shoots.

1.13.1.6 Glutathione reductase (GR)

GR is a flavoprotein oxidoreductase, which uses NADPH as a reductant to reduce glutathione disulphide (GSSG) to GSH. Reduced glutathione (GSH) is used to regenerate AA from MDHA and DHA, and as a result is converted to its oxidized form (GSSG). GR, a crucial enzyme of ASC-GSH cycle catalyses the formation of a disulphide bond in glutathione disulphide to maintain a high cellular GSH/GSSG ratio (Avramova *et al.*, 2016). It is predominantly found in chloroplasts, with small amounts occurring in the mitochondria and cytosol. GSH is a low molecular weight compound which plays the role of a reductant to prevent thiol groups from getting oxidized, and react with detrimental ROS like O_2^- and OH.

1.13.1.7 Glutathione peroxidase (GPX)

GPX is a heme-containing enzyme composed of 40–50 kDa monomers, which eliminates excess H_2O_2 both during normal metabolism as well as during stress. It plays a vital role in the biosynthesis of lignin as well as defends against biotic stress by degrading indole acetic acid (IAA) and utilizing H_2O_2 in the process (Sharma *et al.*, 2012). GPX prefers aromatic compounds like guaiacol and pyragallol as electron donors. Since GPX is active intracellularly (cytosol, vacuole), and extra-cellularly in the cell wall, it is considered as the key enzyme in the removal of H_2O_2.

1.13.2 Non-enzymatic antioxidant

The non-enzymatic antioxidants form the other half of the antioxidant machinery, comprising of AA, GSH, α-tocopherol, carotenoids, phenolics, flavonoids, and the amino acid *cum* osmolyte proline. They not only protect different components of the cell from damage, but also play a vital role in plant growth and development by tweaking cellular process like mitosis, cell elongation, senescence and cell death.

1.13.2.1 Ascorbic Acid (AA)

AA is the most abundant and the most extensively studied antioxidant compound. It is considered powerful as it can donate electrons to a wide range of enzymatic and non-enzymatic reactions (Lobo *et al.*, 2010). The majority of AA in plant cells is the result of the Smirnoff-Wheeler pathway, catalyzed by L-galactano-γ-lactone dehydrogenase in the plant mitochondria, with the remaining being generated from D-galacturonic acid (Kurutas, 2016). Approximately 90% of the AA pool is concentrated not only in the cytosol, but also substantially in apoplast, thus making it the first line of defense against ROS attack (Gallie, 2013). AA is oxidized in two successive steps, starting with oxidation into MDHA, which if not reduced immediately to ascorbate, disproportionate to AA and DHA (Kurutas, 2016). It reacts with H_2O_2, •OH, O_2^-, and regenerates α-tocopherol from the tocopheroxyl radical, thereby protecting the membranes from oxidative damage (Kuivanen *et al.*, 2015). It also protects and preserves the activities of metal binding enzymes. AA in its reduced state acts as the cofactor of violaxanthine de-epoxidase and maintains the dissipation of the excess excitation energy (Kurutas, 2016). AA is also involved in preventing photo-oxidation by pH-mediated modulation of PSII activity and its down regulation, associated with zeaxanthine formation (Lobo *et al.*, 2010; Gallie, 2013).

1.13.2.2 Reduced glutathione (GSH)

Glutathione is a low molecular weight thiol tripeptide (γ-glutamyl-cysteinyl-glycine) abundantly found in almost all cellular compartments like the cytosol, ER, mitochondria, chloroplasts, vacuoles, peroxisomes, and the apoplast (Noctor *et al.*, 2011). It is involved in a wide range of processes like cell differentiation, cell growth/division, regulation of cell death

and senescence, regulation of sulfate transport, detoxification of xenobiotics, conjugation of metabolites, regulation of enzymatic activity, synthesis of proteins and nucleotides, synthesis of phytochelatins and expression of stress responsive genes (Hasanuzzaman *et al*., 2017). This versatility of GSH is due to its high reductive potential. A central cysteine residue with a nucleophilic character is the source of its reducing power. GSH donates its electron to H_2O_2 to reduce it into H_2O and O_2. GSH also plays a vital role in regenerating AA to yield GSSG. The GSSG thus generated is converted back to GSH, either by de novo synthesis or enzymatically by GR. This ultimately replenishes the cellular GSH pool (Koffler *et al*., 2013). GSH also helps in the formation of phytochelatins via phytochelatin synthase which helps to chelate heavy metal ions and thus scavenges another potential source of ROS in plants. Therefore, the delicate balance between GSH and GSSG is necessary for maintaining the redox state of the cell (Rouhier *et al*., 2008).

1.13.2.3 α-Tocopherol

α-Tocopherol belongs to a family of lipophilic antioxidants which are efficient scavengers of ROS and lipid radicals, making them indispensable protectors and essential components of biological membranes. α-Tocopherol has the highest antioxidant capability among the four isomers (α-, β-, γ-, δ-) (Das and Roychoudhury, 2014). Tocopherols (α, β, γ, and δ) are a group of lipophilic antioxidants. α-Tocopherol is the main scavenger of peroxyl radicals in lipid bilayers (Fernandes *et al*., 2013). The α-tocopherol present in the membrane of chloroplasts protects them against photooxidative damage (Havaux *et al*., 2003). The tocopherols are synthesized only by photosynthetic organisms and thus only present in green tissues of plants (Fritsche *et al*., 2017).

1.14 Role of hydrogen sulfide

The biological effects of hydrogen sulfide (H_2S) have received increasing attention in recent years, especially as an important signalling molecule in both aerobic and anaerobic organisms. Hydrogen sulfide has recently been added to nitric oxide (NO) and carbon monoxide (CO) as a newly categorized group of biologically active gases termed gasotransmitters and gasomediators (Li *et al*., 2016).

1.14.1 The impact of hydrogen sulfide on plants undergoing stress

H_2S is a signalling molecule in plants that interacts with reactive oxygen species and nitric oxide for metabolism, also having direct effect on protein activity (Ausma and De Kok, 2019). Plants are able to both make and release measurable amounts of H_2S (Wang, 2012). This enables H_2S to act as an intracellular and an intercellular signal, just as has been proposed for reactive nitrogen species and reactive oxygen species (Meo *et al.*, 2016). Positive effects of H_2S have been shown. Thompson and Kats, (1978) reported that low levels of fumigation with H_2S, at approximately 100 ppb, caused a significant increase in the growth of *Medicago*, lettuce and sugar beets. However, with the sugar beets, the roots had reduced sugar content, which when considered as a sugar crop is not a positive outcome. Interestingly, the beets had less fungal infection following the H_2S treatment, suggesting that the H_2S may inhibit the growth of the fungi. However, Lisjak e*t al.* (2013) showed that fungal infection, particularly with *Pyrenopeziza brassicae*, caused an increase in the enzyme capable of generating H_2S, that is, L-cysteine desulfhydrase, with a possible concomitant high H_2S release from the infected plant. This suggests that the production of H_2S may be part of a defence response of the plant against fungal infection, although it may just be a general toxicity effect of H_2S on microorganisms.

Some parallels exist between H_2S, ROS and RNS in terms of signalling roles, and it should be noted that NO and ROS have anti-pathogenic roles (Xiao *et al.*, 2018). Hasanuzzaman *et al.* (2017) showed that SO_2 treatment of pine needles (*Pinus silvestris* L.) caused H_2S emissions. This suggests that H_2S may be a signal for SO_2 tolerance in the plant. Indeed, Filipovic and Jovanovi (2017) reported similar effects of SO_2 tolerance in young Cucurbitaceae leaves. Recent papers suggest that H_2S has a positive role in stress tolerance in plants. As with the stomatal aperture studies, to undertake such studies the use of H_2S donors is commonly used: either NaSH or GYY4137. Before such compounds as GYY4137 became available, Lisjak *et al.* (2013) showed that in sweet potato the H_2S donor, NaHS, alleviates osmotic stress-induced decrease in chlorophyll concentration. They also measured the levels of antioxidants and showed that spraying NaHS increased the activity of the antioxidant enzymes superoxide dismutase, catalase and ascorbate peroxidase. There was a decrease in the concentration of hydrogen peroxide and lipoxygenase activity in these experiments. Therefore, it was suggested that H_2S may have a role in protecting against oxidative stress in plants.

1.15 Sources of Hydrogen Sulfide for Plant Cells

Hydrogen sulfide has to be present in a defined concentration range to have an effect on plant cells. However, its mere presence within the plant does not necessarily mean that it has a positive role. There are two main sources of in plant cells: from the environment or from cellular biosynthesis. Environmental sources of H_2S may be quite wide ranging, being both natural and man-made. Natural sources include the discharge from volcanoes (Fu *et al.*, 2018), which suggests that plants living downward from a crater will be exposed to significant levels of H_2S to which they must either respond or be resistant if they are to survive. H_2S may be released from coastal marine sediments (Thompson and Kats, 1978) or from anoxic soils such as found in marshlands. Such sources will affect both the aerial parts of plants as well as the roots. It should however be noted that, other sulfur-based compounds will also be present, such as sulfite, as this can be produced by $SO4^{2-}$ reducing bacteria such as *Desulfovibriao* which may be found in waterlogged soils and marshes. In marine soils, sulfite levels are higher than 1 mM (Reno *et al.*, 2015) and again plants surviving in such environments must be resistant or acclimatized to these levels of sulfurous compounds. Man-made sources of H_2S are also common. H_2S is released from waste treatment plants, from many agricultural activities (Furihata *et al*,, 1997) and from geothermal industries. With the release of H_2S from such places into the atmosphere, it will be the aerial parts of the plant which will be exposed to H_2S. A similar situation may also apply to some urban environments which can have surprisingly high levels of H_2S.

One of the sources of the gas has been suggested to be from cars with catalytic converters (Wells and Osborne, 2011) which of course in many countries has been mandatory on new vehicles for several years. Intracellular sources would include the production of H_2S by enzymatic mechanisms. An enzyme responsible for the production of H_2S in animals is cystathionine glyase (CSE) and, for example, this has been studied as part of the mechanism of hypoxic sensing in the carotid body (Filipovic and Jovanovi, 2017). In plants, it appears that the enzymes responsible are desulfhydrases. In Arabidopsis, a cysteine desulfhydrase located to the plastids has been reported (Mhamdi *et al.*, 2010). Other groups have found a similar enzyme which is located in mitochondria (Kopriva *et al.*, 2016). The activities of these enzymes are not constant and have been shown to change under some circumstances. For example, increased activity has been noted following pathogen challenge (Showler, 2016). This would be expected if the enzymes are to perform a role in the creation of a molecule which is to act

as a signal. As a parallel example, the activity of nitric oxide synthase is under the control of signals such as calcium ions, as well as being inducible in some animal cells and therefore it would be expected that an enzyme involved in H_2S production should show similar variations in its activity. Therefore, just as with nitric oxide, there appears to be inducible and regulated enzymes capable of making H_2S. This would mean that H_2S can be generated as and when required and in the location in which the enzyme is placed. This is an important asset if H_2S is to be considered as a cellular signal and not simply a phytotoxin.

1.16 The effects of hydrogen sulfide on stomatal movement

Early work on whether H_2S in the atmosphere has an effect on the transpiration stream of plants, and therefore may alter stomatal apertures, suggested that in the short term there was no effect (Franks and Farquhar, 2001). This included work on several plant species including maize, pumpkins and spinach (Aroca *et al*,. 2018). Several recent studies have taken advantage of the use of H_2S donors. The simple compound sodium hydrosulfide (NaHS) is the most common used H_2S donor (Lisjak *et al*., 2013). NaHS will dissociate rapidly to generate a very short burst of H_2S, which can be relatively high if high concentrations of NaHS are used (Zhao *et al*., 2014). Alternatives have therefore been developed, primarily for use in biomedical research, but these donors are now finding their way into plant science research too. One of the forerunners here is the compound known as GYY4137 (morpholin- 4-ium 4 methoxyphenyl (morpholino) phosphinodithionate (Sies, 2017). GYY4137 will give a lower and much more prolonged concentration of H_2S which will be much more akin to that seen under physiological conditions.

More recent work using H_2S donors has shown that H_2S may have an action although different papers appear to report differing effects on plant stomata. H_2S-mediated stomatal opening was seen using *A. thaliana* as a model system. This was seen in plants treated with NaHS, giving a relatively short burst of H_2S, or with GYY4137 giving a longer more prolonged exposure to H_2S (Jin and Pei, 2015). With leaves in which stomata had not pre-opened in the light, the effects of NaSH and GYY4137 were larger. This work was repeated in *Capsicum annuum* and similar stomatal opening was induced by the treatment with both H_2S donors (Lisjak *et al.*, 2013). However, another group has reported different effects of H_2S on stomatal movements. Lisjak *et al.* (2013) found that using either of the H_2S donors NaHS and GY4137 on several different plant species including *Vicia faba, A. thaliana* and *Impatiens walleriana* caused

stomatal closure. They furthered this by the use of glibenclamide, an ABC transport inhibitor, which reduced the effect. As enzymes such as cystathione g-lyase and L-Cys desulfhydrase may be responsible for H_2S generation, they used an inhibitor of these enzymes, propargylglycine and showed that stomatal movements were reduced, so they concluded that H_2S may help to mediate ABA signalling in guard cells.

Ethylene was shown to mediate auxin-induced stomatal opening (Saber and Ismail, 2013) and to cause stomatal closure (Birben *et al.*, 2012). It may be that the exact environment of the plant, or perhaps the age of the leaves used for the experiments, make a large difference to how the guard cells respond to a specific signal. Guard cell signalling is extremely complex (Huang *et al.*, 2016) and almost certainly a balance between a large number of signals, so treating these cells with H_2S under different conditions may give different overall results. C. *annuum* was grown in the presence of 0 mm or 150 mm NaCl and treated with NaSH or GYY4137, both which caused the stomata to be slightly more open (Diaz-Perez *et al.*, 2020). If the H_2S treatment was given to the salt stressed plants, the stomata were opened even more. Interestingly, some other recent work has showed that stomatal conductance was increased by carbonyl sulfide (Stimler *et al*. 2010). It was suggested here that H_2S mediates this effect which was produced from COS hydrolysis. This would appear to support stomatal opening effects of H_2S. But this being the case, and significant to the discussion below, H_2S seems to be having the opposite effect to NO and ROS, which tend to cause the stomata to close (Kolbert *et al.*, 2019). Therefore, stomata apertures and hence the transpiration streams of plants may be modulated in both directions by the judicial use of either NO or H_2S donors, the former causing closure and the latter allowing further opening.

1.17 Molecular mechanisms which may underlie effects of H_2S in plants

If H_2S is acting as a signal in plants, or indeed if plants are simply responding and adapting to the presence of H_2S, then there must be a molecular mechanism which underpins this. If H_2S is exogenously produced, it may be at the top end of a pathway in which case there needs to be a mechanism by which the cells can respond to it, initiating a signal transduction pathway leading to an effect. However, H_2S may be in the middle of a pathway in which case its production may be an integral part of a pathway and H_2S levels may be altered to get further downstream effects. In a previous study, it was found that soaking *Jatropha curcas* seeds in H_2O_2 increased the germination rate, increased the activity of L-cysteine desulfhydrase and

induced the accumulation of H_2S (Filipovic and Jovanovi, 2017). This suggests that H_2S is in the middle of this signal transduction route and mediates the effects of H_2O_2, which is interesting when it is considered that most groups report that H_2S increases antioxidant levels and lowers oxidative stress. It was also noted (Fu *et al.*, 2014) that H_2S alone could increase germination rates of seeds, which has been seen with NO as well (Bethke *et al*., 2007). Considering some of the work on stomata and some of the work discussed above, there would seem to be a case for H_2S and NO signalling to be interlinked.

Using stomata, when endogenous NO was assessed using a NO sensitive probe in conjunction with using confocal microscopy, it appeared that NO levels were lower in plant tissues treated with either NaSH or GYY4137, suggesting that H_2S does indeed interfere with NO signalling. This effect is perhaps through a scavenging role. This was seen not only in Arabidopsis (Lisjak *et al.*, 2013) but also in *Capsicum* (Lisjak *et al.*, 2013). An interaction between H_2S and NO has been noted before, albeit not in plants. In animal systems, it has been shown that H_2S can inhibit isoforms of nitric oxide synthase (NOS), probably through an interaction between H_2S and the co-factor tetrahydrobiopterin (BH4) (Zhang *et al.*, 2015). However, this mechanism is probably not pertinent here as there is considerable debate about the presence of NOS-like enzymes in plants (Freschi, 2013) so this is unlikely to be a mode of action for H_2S. This does not exclude the interaction of H_2S with any other enzymes which may be involved in NO biosynthesis, such as nitrate reductase.

An alternative mechanism for the interaction of H_2S and NO is that they may react to form novel nitrosothiols, with these compounds also having cellular effects (Whiteman et al. 2006). Although there is evidence that H_2S depletes NO accumulation, others have reported the opposite. Freschi, (2013) used the NO scavenger 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) with H_2S and found that NO removal reversed the effects brought about by H_2S, suggesting that NO may mediate H_2S effects. They further reported that NaSH increased NO generation, in the presence or absence of cadmium stress. Zhang et al., (2015) demonstrated that NaSH increased NO production in alfalfa (*M. sativa*).

If H_2S is interfering with NO signalling, either by decreasing or increasing NO under different circumstances, then this suggests that H_2S is acting as a signalling molecule. This has already

been demonstrated in the case of H_2S metabolism in animals (Saber and Ismail, 2013; Jin and Pei, 2015), so it is sensible to suggest the same for plants, as proposed by Zhang *et al.* (2015). Also, involving NO is a recent paper suggesting that H_2S is involved in ethylene signalling. Here, ethylene caused an increase in H_2S generation and increased desulfhydrase activity in leaves, while inhibitors of H_2S reduced ethylene synthesis. However, ethylene failed to induce H_2S generation in the Atnia1/nia2 mutant, implicating nitrate reductase and NO in the signalling pathway (Lisjak *et al.*, 2013). The authors suggested that H_2S should be placed downstream of NO in the transduction pathway which was used by ethylene to cause stomatal closure in their system. Although this paper strongly supports H_2S as having a signalling role, it appears to be opposite to others (Freschi, 2013; Hu *et al.*, 2014) that suggest that H_2S causes an increase in NO accumulation.

It is likely that the precise cellular localization of these molecules determines their effect on the cell. There are likely to be many other targets for H_2S besides NO metabolism. Over several years, intracellular responses to H_2S have been investigated and it has been found that there are a range of effects in plant cells treated with H_2S. For example, on fumigation of spinach it was found that glutathione levels were increased (Thompson and Kats, 1978). Glutathione is a sulfur rich compound, so this would seem to fit with H_2S metabolism, but it fits well with the notion put forward by Kong et al., (2016) that H_2S can affect oxidative stress responses. In a study by Fu *et al.* (2018), it was demostrated that approximately 40% of the H_2S in leaves was channelled towards glutathione biosynthesis. On stopping the fumigation of the plants, the glutathione levels were reduced, with control levels seen after 48 h of no H_2S. It was also noted that photosynthetic fixation and the photosynthetic electron transport chain were insensitive to the presence of H_2S in these plants.

1. 18 Hypothesis

Sodium hydrosulfide reduces *A. flavus* infection in and regulates ROS accumulation in *Zea mays*.

1. 19 Aims and Objectives

The aim of this study was to explore the potential of using sodium hydrosulfide in the reduction of infection of *Zea mays* by the fungal plant pathogen *Aspergillus flavus*. The objectives of this study included:

(i) To evaluate antifungal activity of NaHS on growth by *A. flavus*.

(ii) To determine the effect of NaHS on maize growth upon infection with *A. flavus*.

(iii) To evaluate the effect of NaHS on H_2O_2 content, lipid peroxidation level and (O_2^-) content in maize upon infection with *A. flavus*.

(iv) To evaluate of the effect of NaHS on enzymatic activity of CAT, APX and SOD applying both in-gel activity staining and spectrophotometric assays in maize infected with *A. flavus*.

2 Chapter Two

Materials and methods

Table 2. 1: List of chemicals and suppliers

Chemicals	Suppliers
Potato Dextrose Agar	Sigma- Aldrich
Petri dishes	Thermo Scientific
6 mm filter papers	Sigma- Aldrich
Promix	Windell Hydroponics
(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazollium Bromide)	Sigma- Aldrich
2- Thiobarbituric acid	Sigma- Aldrich
30 % acrylamide solution	Sigma- Aldrich
L-Ascorbic acid	Sigma- Aldrich
Agarose	Whitehead Scientific
Ammonium persulphate	Sigma- Aldrich
Evans blue	Sigma- Aldrich
Terbinafine	Sigma- Aldrich
Calcium sulphate	Sigma- Aldrich
Sodium dodecyl sulphate (SDS)	Sigma- Aldrich
Hydrogen peroxide	Sigma- Aldrich
NBT	Sigma- Aldrich
TCA	Sigma- Aldrich
Thiobarbituric acid	Sigma- Aldrich
Potassium iodide	Sigma- Aldrich
K_2HPO_4	Sigma- Aldrich
EDTA	Sigma- Aldrich
PVPP	Sigma- Aldrich
Bovin serum albumin	Sigma- Aldrich

Bradford reagent	Sigma- Aldrich
Glycine	Sigma- Aldrich
Tris base	Sigma- Aldrich
TEMED	Bio-Rad
Ferric chloride	Sigma- Aldrich
Potassium ferricynide	Sigma- Aldrich
Riboflavin	Sigma- Aldrich
Potassium cynide	Sigma- Aldrich
KH_2PO_4	Sigma- Aldrich

2.1 Fungal isolate

The toxigenic filamentous fungi that was used in this study was *Aspergillus flavus* and was obtained from the Plant Protection Research Institute (PPRI) at the Agricultural Research Council. Stock cultures were maintained on Potato Dextrose Agar (PDA) at 4 °C. The isolates were subcultured on PDA to prepare inoculum. Sporulating mycelia were removed and inoculated at the centre of the Petri plates containing PDA. The inoculated plates were then incubated at 30˚C for 4 to 6 days until visible fungal growth was observed. Incubation was carried out for a further 24 hours at 25˚C to enable the inoculum to adapt to the final incubation temperature. Spores were gathered by washing the agar surface with a sterile solution containing 0.2% Pure Agar in 1% Tween-80. Spore counts in the resulting suspensions were determined by microscopy with a Neubauer chamber. The isolates were then maintained in 20% glycerol at -20˚C for further studies.

2.2 Morphological identification by phenotypic assays

Aspergillus flavus isolates preserved in the National Collection of Fungi (Agricultural Research Council, Pretoria) were sub-cultured onto 90 mm Petri-dishes containing 3 different media of Czapek Yeast Extract Agar (Lhan *et al.*, 2006), 25% Glycerol Nitrate Agar and Malt Extract Agar (MEA) (Booth, 1971) (Table 2.2-2.4). A replicate of 3 Petri-dishes per media were inoculated in the middle with a 0.2 mm punch of *Aspergillus flavus* strain and incubated simultaneously at different temperatures of 5°C, 25°C and 37°C for seven days (Pitt and Hocking, 1999) to confirm the growth pattern and availability of macro and micro characteristics in the selected strain. To obtain characteristics of *Aspergillus flavus*, isolates were identified by means of colony morphology, including the diameters, colour, texture, – and microscopic characteristics, including conidial heads, stipes, vesicles – and conidia shape, size and texture. Data obtained was compared with valid species descriptions of systematics works (Pitt and Hocking, 1999; Klich, 2002 and Diba *et al.*, 2007).

Table 2. 2: Composition and preparation method of Czapek yeast extract agar (Cz)

Components	Quantities	Method
NaNO3	3.0 g	Components were mixed and autoclaved
$K_2HPO_4.H_2O$	1.3 g	for 15 minutes at 121ºC (15 p.s.i.).
KCI	0.5 g	A preparation of 20 ml was aseptically dispensed into
$MgSO_4.7H_2O$	0.5 g	90 mm petri dishes and allowed to solidify.
Iron solution*	1 ml	
Copper solution*	1 ml	
Zinc solution*	1 ml	
Sucrose	30 g	
Agar	15.0 g	
Distilled water	1 litre	

*** The table shows the constituents and the preparation of the copper solution**

Components	Quantities	Method
$CuSO4.5H_2O$	0.5 g	Mixed components were kept in
Distilled water	100 ml	the freezer and used when needed.

*** The table shows the constituents and the preparation of the zinc solution**

Components	Quantities	Method
$ZnSO4.7H_2O$	0.5 g	Mixed components were kept in
Distilled water	100 ml	the freezer and used when needed.

*** The table shows the constituents and the preparation of the iron solution.**

Components	Quantities	Method
$FeSO_4.7H_2O$	0.5 g	Mixed components were kept in
Distilled water	100 ml	the freezer and used when needed.

Table 2. 3: Composition and preparation method of glycerol nitrate agar (G25N)

Components	Quantities	Method
K_2HPO_4	0.75 g	Mixed components were autoclaved
Czapek concentrate*	7.5 ml	for 15 minutes at 121ºC (15 p.s.i.).
Yeast extract	3.7 g	Using a sterile dispenser, a preparation
Glycerol	250 g	of 20 ml medium was dispensed into 90 mm agar
Agar	12 g	plates and allowed to solidify.
Distilled water	750 ml	

***A table showing Czapek concentration.**

Components	Quantities	Method
$NaNO_3$	30.0 g	Mixed components were kept in
KCl	5.0 g	the freezer and used when needed.
$MgSO_4.7H_2O$	5.0 g	
$FeSO_4.7H_2O$	0.1 g	
Distilled water	100.0 ml	

Table 2. 4: Composition and preparation method of malt extract agar (MEA)

Components	Quantities	Method
MEA	20.0 g	Mixed components were autoclaved
Peptone	5.0 g	for 15 minutes at 121ºC (15 p.s.i.).
Agar	15.0 g	The medium was poured into 90 mm agar plates
Distilled water	1 litre	and allowed to solidify.

2.3 Antifungal activity assay

Antifungal activity was determined by the agar disk diffusion method. A preparation of 3.9% PDA agar was made and autoclaved at 121˚C for 20 minutes. Plates were made by pouring 15 ml of PDA agar onto petri dishes using a 10 ml pipette and plates were kept at room temperature to solidify. After solidifying, an inoculum of 1 ml of the spore suspensions ($37.2x10^4$ conidia/ml) was added on top of 6 mm pure disks and placed on the agar at the centre of each petri dish. The same 6 mm pure disks containing the *A. flavus* spore suspensions or without *A. flavus* were impregnated with different concentrations of NaHS (100 µm, 500 µm and 1 mM) to determine growth inhibition. The study was conducted in triplicates, including a control with

only distilled water instead of NaHS. The cultures were then incubated at 25°C for 3 days. The radial growth of mycelia was measured with a straight ruler on the third day of incubation for evaluation of fungal growth at different concentrations of NaHS (Nguefack *et al.*, 2004).

2.4 Plant growth

Commercial maize (*Zea Mays* cv. Border King) seeds that were used for this study. Maize seedlings were grown in a greenhouse located at University of the Western Cape (day temperature was 25-28°C, night temperatures were 16-19°C, day length was 16 hours with daylight supplemented with high pressure sodium lights to give a light intensity of approximately 600 $\mu mol.m^{-2}.s^{-1}$). Seeds were surface sterilized in 0.35% (v/v) of sodium hypochlorite (bleach) for a maximum of 10 minutes and then washed five times with distilled water. Seeds were then imbibed for 16 hours in aerated 10 mM calcium sulphate ($CaSO_4$). After rinsing with sterile distilled water, the seeds were then incubated inside a plastic container containing a moist paper towel placed at room temperature in the dark and this was done for 3 days to allow most seeds to emerge radicles in a germination chamber. The growth medium (Promix Organic) was mixed with tap water until it was moist enough and transferred into the 20 cm diameter pots. Germinated seeds (four per pot) were sown in each 20 cm diameter plastic pot containing 3 L of Promix Organic under controlled environmental conditions (25-28/16-19˚C) day/night temperature cycle under a 16/8 h light/dark in a green house. Plants were thinned to 1 plant per pot when they had two expanded leaves.

2.5 Treatment of maize plants

The 20 cm diameter plastic pots were arranged in triplicates for each treatment and were prepared as follows (untreated, *Aspergillus flavus*, sodium hydrosulfide and *Aspergillus flavus* + sodium hydrosulfide). The untreated plants were supplied with 200 ml of distilled H_2O every third day until the V2 stage, at which they were harvested. For treatments, the germinated seedlings were inoculated with 1 ml of a suspension *A. flavus* containing 37.2 $x10^4$ conidia/ml inside a sterile petri dishes and were transferred into the plastic pots. The spore suspension (37.2×10^4 conidia/ml) was determined using Neubauer Haemocytometry to count the fungal conidia. After analysis of preliminary results from use of the various NaHS concentrations, 1

mM of sodium hydrosulfide was further used to treat the plants inoculated with *A. flavus* to determine growth inhibition and colonization of the infection, and this experiment was performed in triplicates in three independent experiments. Additionally, each plant was supplied with 200 ml of the solution containing 1 ml of 37.2 x10^4 conodia/ml. The rest of the other plants were only supplied with 200 ml solution that contains *A. flavus* without sodium hydrosulfide treatment and another separate set of plants was treated with 200 ml of NaHS solution with different concentrations (100 µM, 500 µM and 1mM) without inoculation of *A. flavus*. The plants were grown until the V2 stage and observed for growth parameters at harvesting time.

2.6 Growth parameters measurements

Plants were harvested after reaching V2 stage of growth. Four plants from each treatment were carefully removed from the Promix Organic. Shoots and roots were excised and weighed as soon as possible after their excision and the length of the shoot was also measured. The remaining plant material was quickly frozen in a liquid nitrogen and stored at -80˚C.

2.7 Cell viability measurement

A modified method followed for the cell viability assay (Uzuner, 2018) was performed from freshly harvested leaf tissue in samples from all treatments. Fresh leaf tissue from the second youngest leaf was harvested from three different plants of each treatment and stained for 30 min with 0.25% (w/v) Evans blue dye at room temperature. To remove surface bound dye, leaves were washed for 45 min in distilled water, followed by incubation for 1 hour at 55˚C. The Evans blue stain taken by dead leaf cells was extracted using 1% (w/v) SDS. The absorbance, indicative of the level of Evans blue up-take by the leaf tissue, was measured spectrophotometrically at 600 nm.

2.8 Trichloroacetic acid (TCA) extraction

Trichloroacetic acid (TCA) extraction was performed in order to obtain protein-free extracts for use in determining the level of malondialdehyde (MDA) as a measure of lipid peroxidation

and hydrogen peroxide (H_2O_2) content. Plant material (100 mg) was homonegized in 5 volumes of 6% TCA. The mixture was briefly vortexed and centrifuged at 13 000 *g* for 15 minutes at 4˚C. The supernatant was carefully separated from the pellet, transferred to a sterile Eppendorf tube and stored at -20˚C for subsequent experiments (MDA and H_2O_2 assays).

2.9 Malondialdehyde (MDA) content

Malondialdehyde (MDA) is the product of lipid peroxidation which occurs naturally and is used to estimate the degree of lipid peroxidation. The degree of lipid peroxidation was measured on maize leaves and roots using a previously described method (Hosseinzadeh *et al,* 2018). The supernatant from the TCA extraction was used in which 200 µl of the extract was vigorously mixed with 400 µl of 0.5% thiobarbituric acid (TBA). Following boiling at 95˚C for 20 minutes in a water bath, the mixture was then chilled on ice for 10 minutes and centrifuged at 13000 *g* for 5 minutes at 4˚C. After centrifugation, 200 µl of the solution was loaded in triplicates into a microtiter plate to measure the absorbance readings at 532 nm and 600 nm. The value for the non-specific absorption at 600 nm was subtracted from the 532 nm reading and an extinction coefficient of 155 $mM.cm^{-1}$ was used to calculate the concentration of MDA.

2.10 Hydrogen peroxide (H_2O_2) assay

A standard curve with known concentrations of H_2O_2 was prepared in order to determine the amount of hydrogen peroxide in maize leaves and roots. An amount of 10 µM of H_2O_2 was mixed with different quantities of dH_2O, 1 M potassium iodide (KI) and 20 mM potassium phosphate (KH_2PO_4) at pH 5 to a final volume of 200 µl. Both standards and samples were done in triplicates. A master-mix was made for the samples in which 50 µl of TCA extract was mixed with 50 µl KH_2PO_4 and 100 µl KI to make a final volume of 200 µl. The absorbance at 390 nm was measured and H_2O_2 content was determined using the extinction coefficient 39.4 mM^{-1} cm^{1}.

2.11 Superoxide (O_2^-) content

A modified method of Russo et al. (2008) was used to determine superoxide content. Superoxide concentrations were determined by submerging fresh leaf tissue from three different plants in a solution containing either 10 mM KCN (to inhibit Cu/Zn SOD) or 10 mM H_2O_2 (to inhibit Mn and Cu/Zn SOD) or 2% (m/v) SDS (to inhibit Mn and Fe SODs) or no inhibitors; together with 80 mM nitro-blue tetrazolium chloride (NBT) and 50 mM potassium phosphate (pH 7.0). The leaves were incubated for 20 min within the solution after which they were homogenized and centrifuged at 13,000 *g* for 5 min and the supernatant was removed carefully and added to a clean Eppendorf tube. Once the supernatant was free of plant material, it was loaded onto the microtiter plate by adding 200 µl into a well. This process was repeated for untreated as well as treated samples. The samples were spectrophotometrically analysed by reading absorbance at a wavelength of 600 nm. The superoxide concentration was calculated using the NBT extinction coefficient of 12.8 mM cm^{-1}. The intensity of the blue colour produced by the reaction was an indication of superoxide levels.

2.12 Protein extraction

Protein was extracted from maize leaves and roots by homogenizing 400 mg of plant tissue with 0.4 ml of extraction buffer [40 mM K_2HPO_4 at pH 7.4; 1 mM EDTA and 5% (w/v) Polyvinylpolypyrrolidone (PVPP)]. After homogenization, the resulting homogenates were centrifuged at 13000 *g* for 30 minutes at 4˚C. The supernatant was carefully separated from the pellet and stored at -20˚C. Protein concentration of the extracts was quantified in all assays by the Bio-Rad protein assay using bovine serum albumin as a standard.

2.13 Protein quantification (Bradford assay)

The Bradford assay was performed in order to determine the protein concentration of the samples (Bradford, 1976). Protein standards were made using 1 mg/ml of bovine serum albumin (BSA), distilled water and 1X Bio-Rad protein assay dye in order to create a standard curve. A master-mix was prepared composed of 1 µl protein extract for each protein sample, 9

µl dH_2O and 190 µl dye to make up a final volume of 200 µl. The mixture was loaded into a microtiter plate and the absorbance readings were measured at 595 nm after 10 minutes incubation at room temperature. All standards and samples were done in triplicates and the protein concentration of the samples was then determined from the standard curve.

2.14 Catalase activity

Total catalase (CAT) activity was determined according to the method of (Teixeira and Mota, 1992) with minor modifications. The method is based on the consumption of H_2O_2, measuring a decrease in absorbance at 240 nm (ε =39.4 mM^{-1} cm^{-1}). The reaction mixture (1 ml final volume) was made up of 50 mM potassium phosphate (pH 7) buffer containing 50 µg protein extract and 0.5 mM EDTA. The reaction was initiated by adding 1 mM H_2O_2 and the absorbance was immediately measured. CAT activity was expressed as units per mg of protein, one unit being the amount of enzyme which liberates half the peroxide in 100 s at 25˚C. CAT in-gel activity staining was performed in this study as reported by Gara et al., 2000. After electrophoresis, the gels were rinsed extensively with dH_2O, followed by incubation in 0.003% H_2O_2 for 20 minutes in the dark. The gels were then stained in a light box with 2% ferric chloride and 2% potassium ferricyanide until the formation of CAT bands. Proteins representing CAT activity were observed as clear bands on a blue background.

2.15 Ascorbate peroxidase activity

A modified method (Rao *et al.*, 1996) was used in this study to measure total ascorbate activity of maize leaves and roots. The reaction mixture contained 50 µg protein extract, and 2 mM ascorbate which was dissolved in 50 mM potassium phosphate buffer at pH 7 and 0.1 mM EDTA. The reaction was initiated by adding 10 mM H_2O_2 and the absorbance reading was immediately measured at 290 nm for 1 min. APX activity was calculated using the extinction coefficient 2.8 mM^{-1} cm^{-1} and was expressed as units (µmol of ascorbate oxidized per minute) per mg of protein. In-gel APX activity staining was performed using a previously described method (Parida, 2016) in order to determine APX isoforms of maize leaves and roots in response to hydrogen sulfide and *A. flavus* treatments. Native polyacrylamide gel electrophoresis (PAGE) was performed at 4˚C with a running buffer that was composed of 192

mM glycine, 25 mM TRIS and 2 mM ascorbate. Subsequent to electrophoresis, the gel was equilibrated in 50 mM potassium phosphate (KH_2PO_4) buffer at pH 7, containing 2 mM ascorbate, for 20 minutes. The gel was incubated for 20 minutes in a new solution of 50 mM KH_2PO_4 buffer at pH 7.8 containing 4 mM ascorbate and 2 mM H_2O_2. Finally, the gel was stained in a solution containing 50 mM KPO_4 (PH 7.8), 28 mM TEMED and 0.5 mM NBT, with exposure to light. After the formation of achromatic bands, the stain was discarded and the gel was rinsed with dH_2O to stop the reaction.

2.16 Superoxide dismutase activity

Total superoxide dismutase (SOD) activity was measured spectrophotometrically using the method of (Kakkar *et al.*,1984) with slight modifications. The reaction mixture contained 50 µg protein extract, 50 mM potassium phosphate buffer at pH 7.8, 0.1 mM EDTA, 13 mM L-methionine, 2 µM riboflavin and 75 µM nitroblue tetrazolium (NBT). The reaction was initiated by incubating the mixture in a light box for 10 minutes. Absorbance, indicative of the reaction products, was measured at 590 nm and one unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT and the specific activity of plant extracts was expressed as units per mg of protein. Native PAGE for SOD activity staining was performed following the method of Kakkar et al. (1984). Subsequent to electrophoresis, the three gels were incubated in 50 mM potassium phosphate (pH 7.8) buffer containing 2.5 M NBT for 20 minutes in the dark. The gels were then stained on a light box with 50 mM potassium phosphate (pH 7.8) buffer containing 28 mM riboflavin and 28 mM N, N, N, N-tetramethylethylenediamine (TEMED) until SOD isoforms became visible. The different classes of SOD isoforms were identified using H_2O_2 and KCN as inhibitors. The gel was incubated for 20 minutes in 50 mM K-phosphate buffer at pH 7.8, containing either 3 mM KCN or 5 mM H_2O_2 prior to staining for activity.

2.17 Statistical analyses

Four plants from each treatment (Untreated, *A. flavus*, NaHS and *A. flavus* + NaHS) were used to measure growth parameters and each treatment was repeated thrice. Using the GraphPad Prism 5.03 software based on the Tukey–Kramer test at 5% level of significance, analysis of

variance (ANOVA) was used to determine the statistical significance of the differences in the means for the treatments.

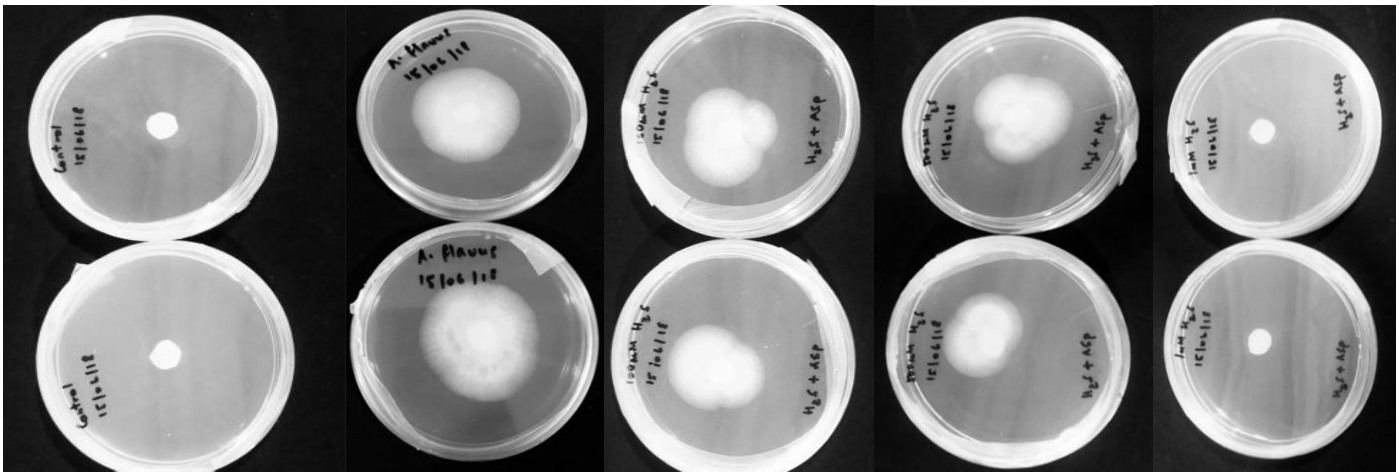
3 Chapter Three

Results

3.1 Inhibition of *Aspegillus flavus* growth by sodium hydrosulfide

The effect of H_2S on *A. flavus* fungal growth was evaluated by preparing three different concentrations of NaHS (100 µM, 500 µM and 1 mM). This experiment was performed to determine the concentration that yielded significant inhibition on the growth of *Aspergillus flavus*. The experiment was carried out on petri-dishes containing PDA agar, at the centre pure (6 mm) disk inoculated with 1ml of the spore suspensions ($37.2x10^4$conidia/ ml) and treated with NaHS concentrations (100 µM, 500 µM and 1 mM). The treatments on petri dishes were prepared and labelled as control with only dH_2O added, *A. flavus* without NaHS, *A. flavus* grown in the presence of either 100 µM of NaHS or 500 µM of NaHS or 1 mM of NaHS; where each treatment was replicated three times (Figure 3.1 and Table 3.1). Treatment with NaHS completely inhibited growth on *A. flavus* at a concentration of 1 mM NaHS (Figure 3.1e). The growth was more reduced under the treatment containing 500 µM NaHS (Figure 3.1d) when compared with 100 µM NaHS (Figure 3.1c) and growth without NaHS treatment (Figure 3.1b). After the evaluation of NaHS treatment on *A. flavus* growth in Petri dishes, the 1 mM concentration of NaHS would further be used to treat maize seedlings inoculated with *A. flavus*, grown on pots in the green house under controlled temperatures.

After three days of incubation at 25˚C from all treatments, the diameter of the area with fungal growth was measured using a ruler to evaluate the effect of NaHS on fungal growth at the various NaHS concentrations. There was no growth that was observed from *A. flavus* treated with 1 mM NaHS and there was marginal difference in growth between 100 µM and 500 µM NaHS treatments. This study reveals that 1 mM NaHS treatment reduces A. *flavus* mycelial growth.



a **b** **c** **d** **e**

Figure 3. 1: Evaluation of sodium hydrosulfide to inhibit *A. flavus* fungal growth. (a) Distilled water added as a control in presence of pure 6mm disc; (b) *Aspergillus flavus* growth without sodium hydrosulfide treatment; (c) *Aspergillus flavus* growth with 100 µM sodium hydrosulfide treatment; (d) *Aspergillus flavus* growth with 500 µM of sodium hydrosulfide treatment and (e) *Aspergillus flavus* growth with 1 mM of sodium hydrosulfide treatment. Growth was evaluated after three days of incubation; where treatments were repeated 3 times.

Table 3. 1: Measurement of *A. flavus* growth at various concentrations of NaHS (100 µM, 500 µM and 1 mM). Treatments were control, *Aspergillus flavus*, *Aspergillus flavus* + sodium hydrosulfide, and were repeated three times

Treatment in triplicates	H_2S applied as various concentrations of NaHS	Average diameter (growth measurement) in cm
Control	Not treated	No growth
Aspergillus flavus	Not treated	8.73 ± 0.56^{a}
Aspergillus flavus + sodium hydrosulfide	100 µM	7.86 ± 0.38^{b}
	500 µM	7.20 ± 0.41^{b}
	1 mM	0^{c}

3.2. Physiological, morphological and biochemical response of maize treated with *Aspergillus flavus* and 1mM of sodium hydrosulfide

3.2.1 Sodium hydrosulfide reduces colonization of maize by *Aspergillus flavus* and enhances maize growth

Maize plants were examined to determine the effect of *A. flavus* and NaHS on plant growth (Figure 3.2). Maize plants that were treated with *A. flavus* showed decreased growth in relation to size and leaf number when compared with uninoculated plants. However, NaHS treatment increased the size of the plants when compared with untreated plants. Treatment of plants with NaHS in the presence of *A. flavus* infection did not result in decreased plant growth (Figure 3.2.). This suggests that NaHS inhibits the pathogen and promotes growth.

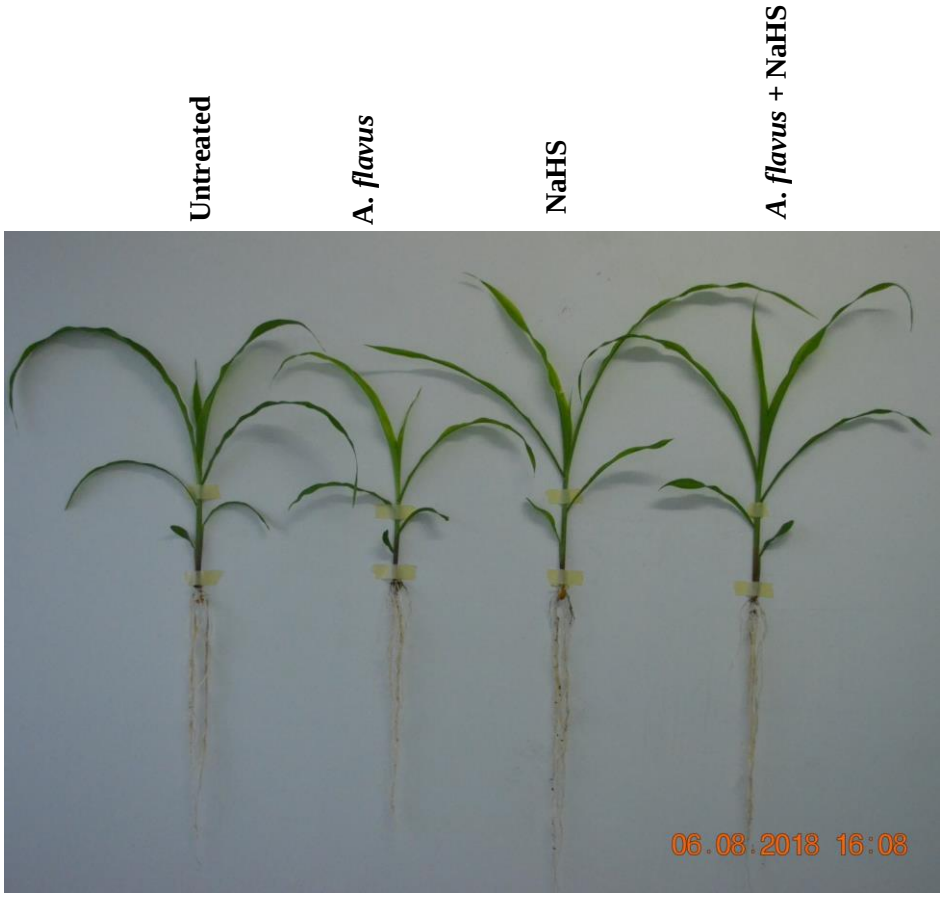


Figure 3. 2: The effect of *A. flavus* and sodium hydrosulfide on maize growth. The treatments were as follows: Untreated (where neither *A. flavus* nor NaHS were applied), *A. flavus*, sodium hydrosulfide at a concentration of 1 mM, *A. flavus* + sodium hydrosulfide (1 mM) at the V2 stage of vegetative growth of maize.

Shoot length, shoot weight and root length from the various maize treatments were examined and measured at the V2 stage. The treatment with sodium hydrosulfide at 1 mM concentration increased shoot length (Figure 3.3 a), shoot weight (Figure 3.3 c) and root length (Figure 3.3 b). Shoot length (Figure 3.3 a) was reduced by approximately 33% by *A. flavus* infection when compared to untreated plants and the same trend was observed for root length (Figure 3.3 b). Shoot length (Figure 3.3 a) only marginally increased (by approximately 28%) in response to treatment with NaHS, and this was also observed for root length (Figure 3.3 b). No significant difference was observed for shoot length in response to the combination of *A. flavus* infection and NaHS (Figure 3.3 a), shoot weight, root length when compared with untreated, plants. No significant difference was also observed on shoot weight when the untreated plants were

compared to NaHS and *A. flavus* + NaHS treatments but shoot weight in the treatment of maize with *A. flavus* was significantly lower than the untreated and that of NaHS and *A. flavus* + NaHS treatments (Figure 3.3 c). These results indicate that H_2S generated from NaHS reduces the negative effects of infection of maize with *A. flavus* and may promote maize growth.

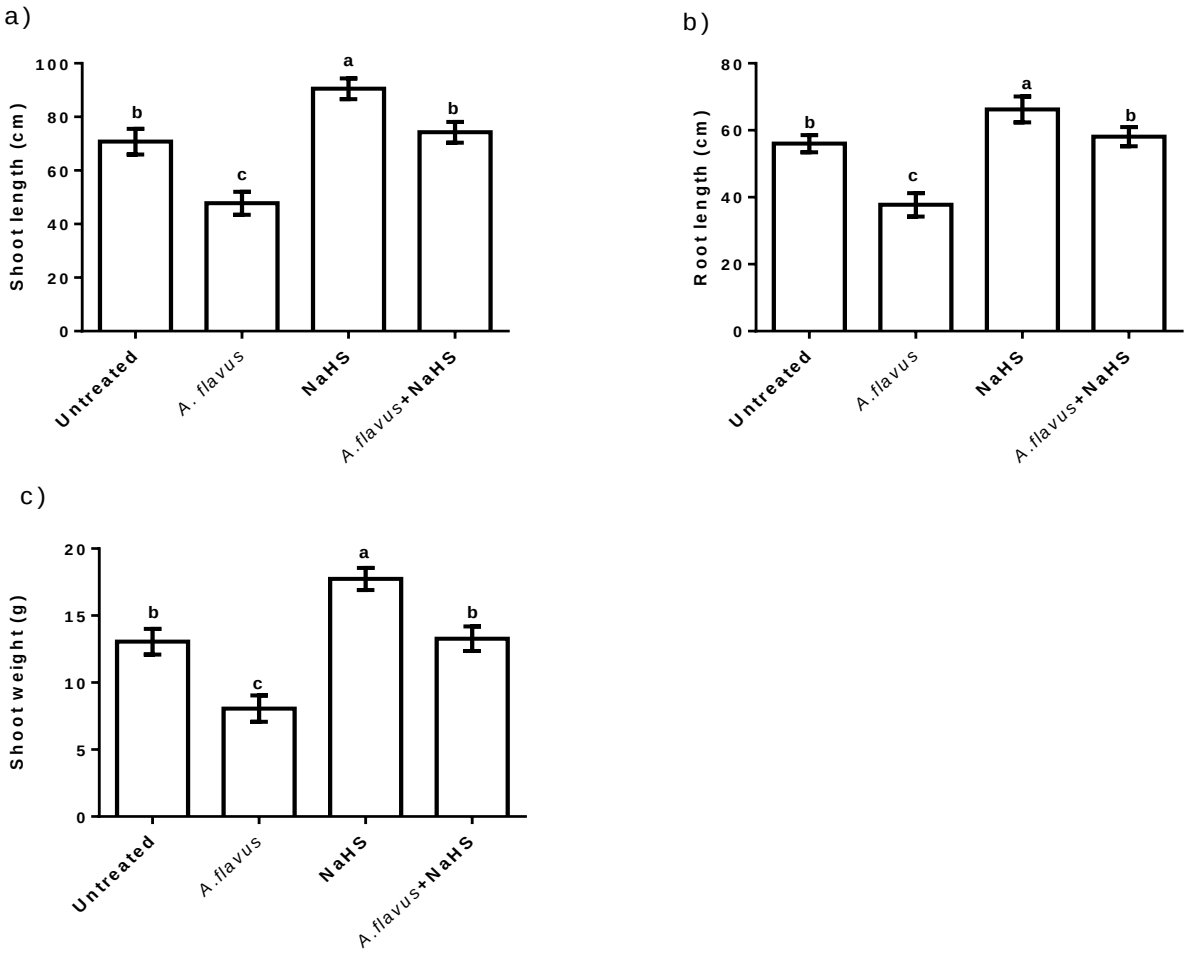


Figure 3. 3: At the V2 stage of vegetation growth, the effect of NaHS and *A. flavus* on maize growth. The treatments evaluated are as follows: Untreated, *A. flavus*, sodium hydrosulfide and *A. flavus* + sodium hydrosulfide. Values are means ± SE of four plants from 4 independent experiments, $p \leq 0.05$.

3.3 The effect of sodium hydrosulfide treatment and *Aspergillus flavus* on H_2O_2, MDA, O_2^- content and cell viability

Results obtained in which changes in H_2O_2, MDA, O_2^- content and cell viability in maize treated with *A. flavus* and sodium hydrosulfide were investigated showed an approximately 57% increase in hydrogen peroxide content in leaves and in roots approximately 98% increase of plants treated with *A. flavus* when compared to untreated plants (Figure 3.4 a & b). However, H_2O_2 levels in plants treated with sodium hydrosulfide alone increased by approximately 82% in the leaves (Figure 3.4 a) compared to untreated and no significant difference was observed when a comparison was made between the sodium hydrosulfide treatment and the combination treatment consisting of *A. flavus* + sodium hydrosulfide. In roots, the level of H_2O_2 (Figure 3.4 b) had a similar trend as leaves for the treatment with *A. flavus* but there was no significant difference observed for the NaHS treatment when compared with untreated plants.

Malondialdehyde (MDA) can be used as an indicator of oxidative stress (Street, 2015). Both leaves and roots under the different treatments showed a similar trend to that of hydrogen peroxide. Leaf lipid peroxidation (Figure 3.4 c) in response to *A. flavus* treatment was higher when compared to other treatments, indicating a high degree of oxidative stress. However, the combination treatment with NaHS and *A. flavus* in both the leaves and roots (Figure 3.4 c and d) on the level of lipid peroxidation shows there is an antagonistic interaction between hydrogen sulfide and *A.flavus.*

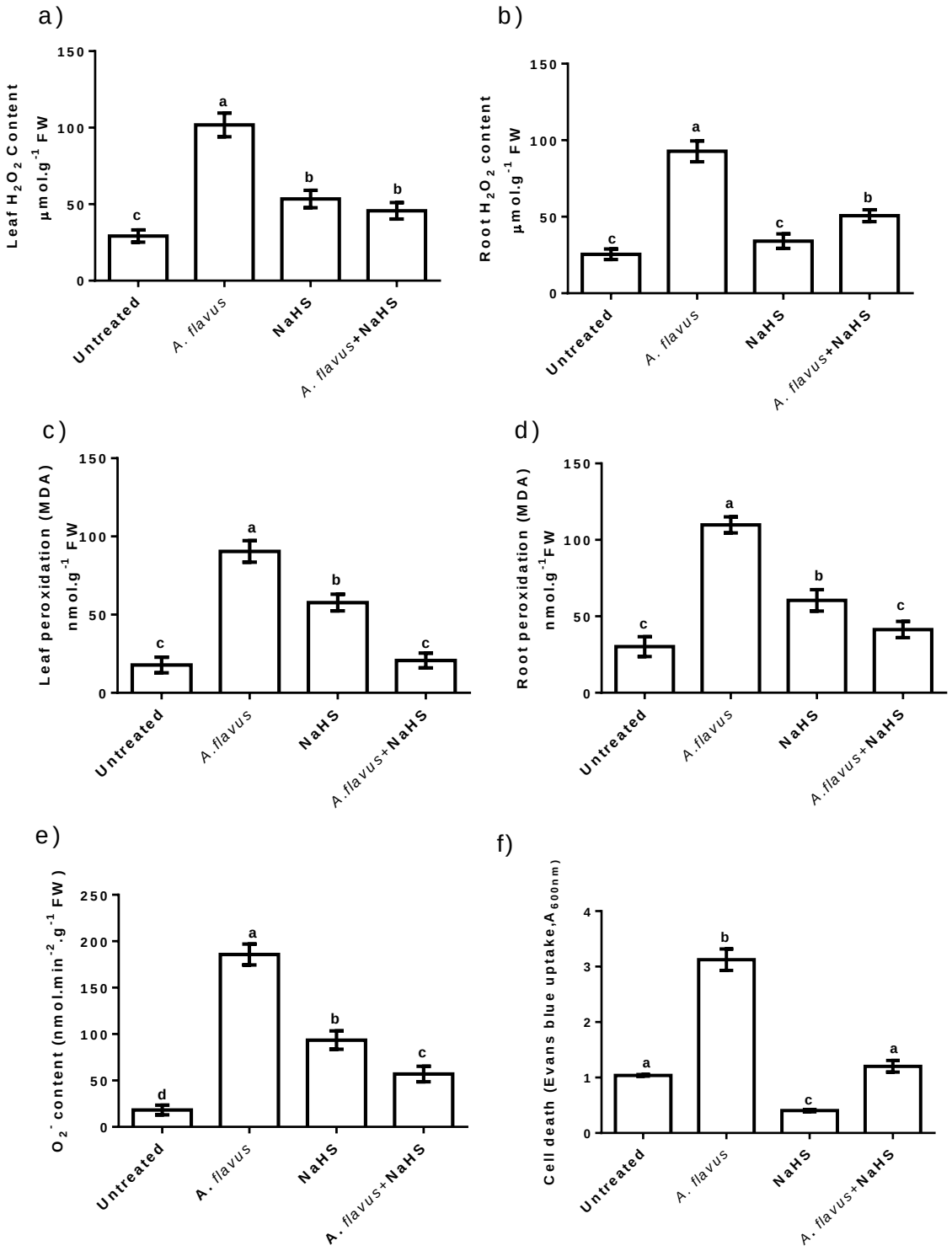


Figure 3. 4: The effect of sodium hydrosulfide and *A. flavus* on H_2O_2, MDA, O_2^- content and cell viability from maize leaves and roots at the V2 stage of vegetative growth. Measurements of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) were done as indication of ROS production; and lipid peroxidation and cell viability as indication of oxidative stress The bars represent the mean of 3 biological

replicates (n = 3), error bars represent the means ± SE and different letters indicate the difference between means at $p<0.05$.

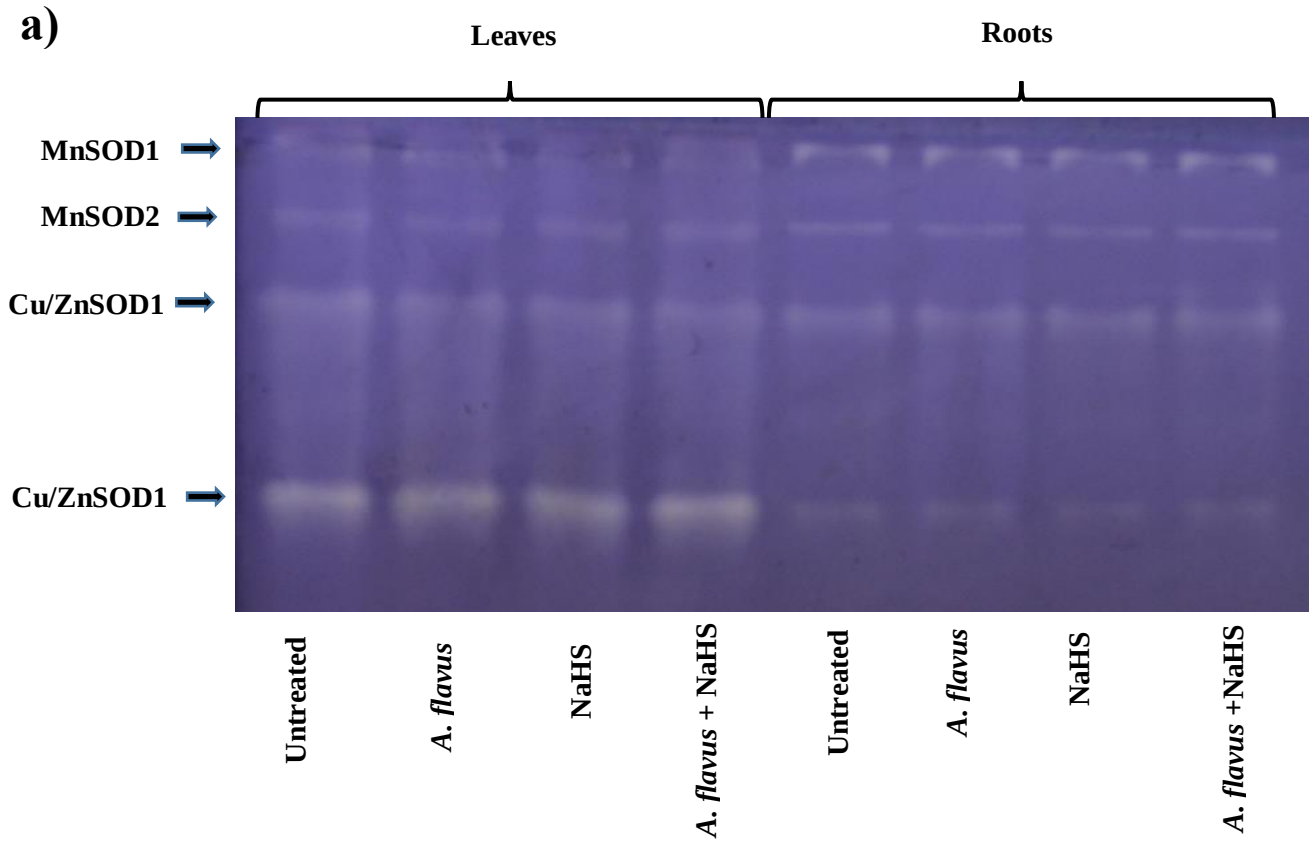
Superoxide content analysis in leaves from plants inoculated with *A. flavus* was 9 times higher in O_2^- content when compared with untreated plants (Figure 3.4 e). Sodium hydrosulphide treatment was also 4 times higher in leaf O_2^- content compared to the untreated plants. The combination of *A. flavus* and sodium hydrosulfide in superoxide content was 2 times higher when compared to the untreated, however, there was reduction of O_2^- accumulation when compared with plants treated with sodium hydrosulfide only.

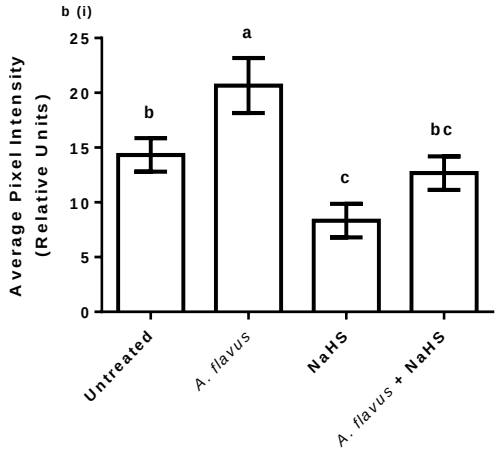
Literature points out that pathogen infection in plants prompts the hyper accumulation of reactive oxygen species that induce oxidative damage, which eventually result in cell death. In this study, the use of Evans blue dye was to determine cell viability since the dye only stains dead cells. The level of cell death on plants treated with *A. flavus* was 2 times higher when compared to untreated plants, but cell death was reduced by approximately 60% in plants treated with sodium hydrosulfide, yet sodium hydrosulfide resulted in a significant increase in O_2^- content (Figure 3.4 e). There was no significant change observed in cell viability when untreated samples were compared to the combination of *A. flavus* and sodium hydrosulfide treatment.

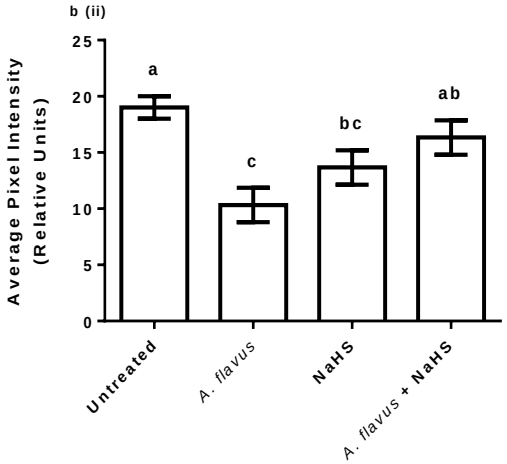
3.4 The effect of sodium hydrosulfide and *Aspergillus flavus* on the activity of antioxidant enzymes in maize

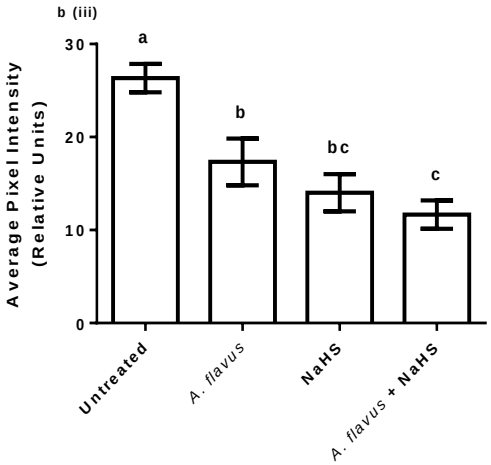
3.4.1 The effect of sodium hydrosulfide and *Aspergillus flavus* on the activity of superoxide dismutase in leaves and roots of maize

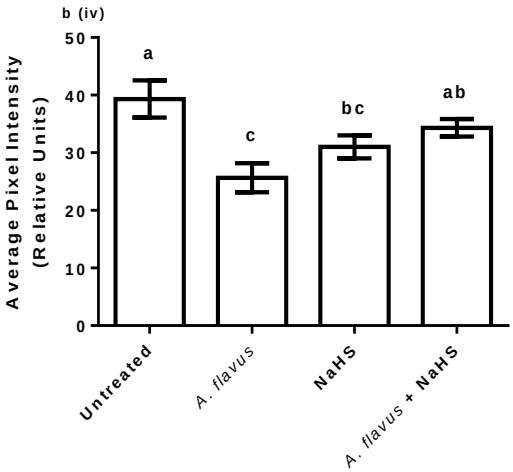
The activity of SOD was investigated in plants in the presence of *A. flavus* and sodium hydrosulfide treatments and evaluation was done on both leaves and roots. The activity of SOD was examined using in-gel activity staining (qualitative) and through a spectrophotometric assay (quantitative). In this study, there were four SOD isozymes identified in leaves and roots. There were two Mn-SOD and two Cu/Zn-SOD. The activity of Mn-SOD 1 and Mn-SOD 2 isoforms in Figure 3.5 b (i) and Figure 3.5 b (ii) showed no significant difference when untreated samples were compared to *A. flavus* + NaHS treatment. However, in leaves Cu/ Zn-SOD 1 activity was reduced by approximately 19% for combination of *A. flavus* and sodium hydrosulfide treatment in Figure 3.5 b (iii). In roots, a similar trend was detected for Mn-SOD activity when treated plants were compared to untreated and same trend for Cu/ Zn- SOD in Figure 3.5 d (ii) and Figure 3.5 d (iii) was observed in roots. The total activity of SOD on both leaves and roots was also examined and there was no significant differences detected between any of the treatments (Figure 3.5 c and Figure 3.5 e) respectively.

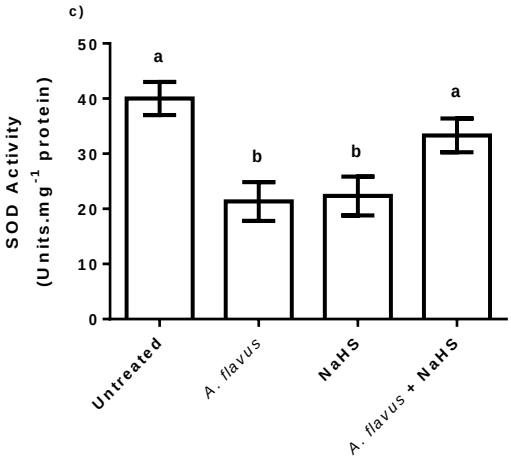












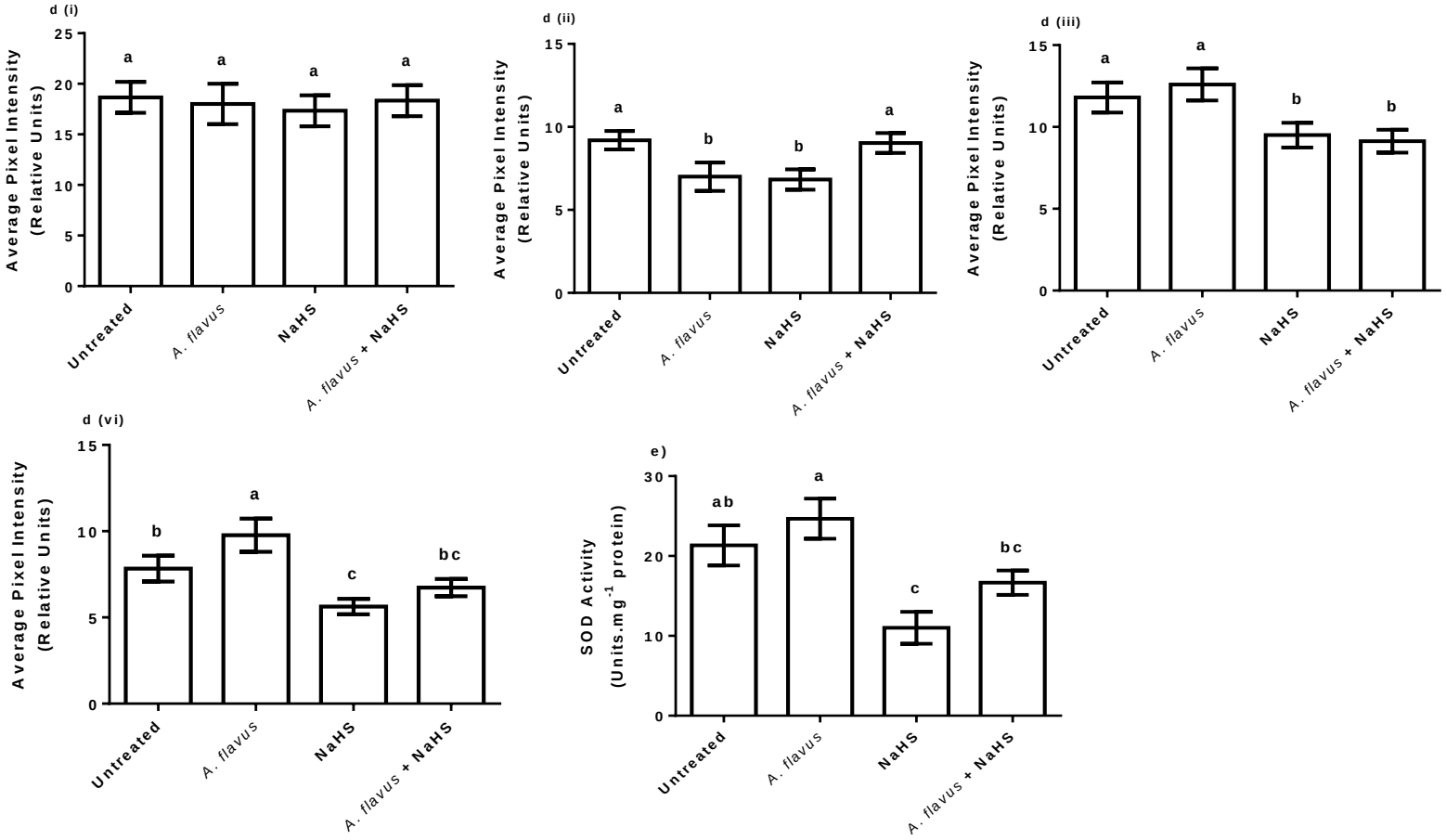
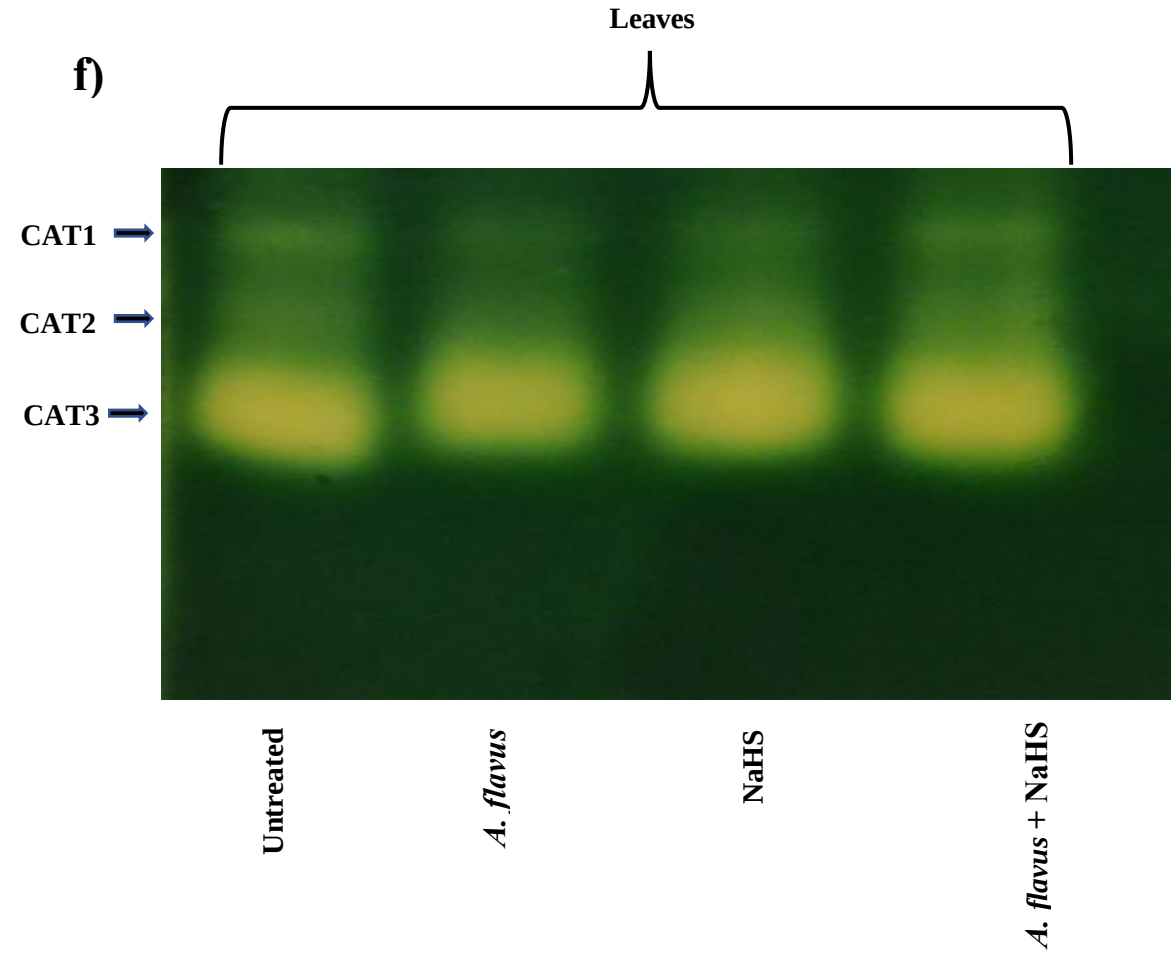


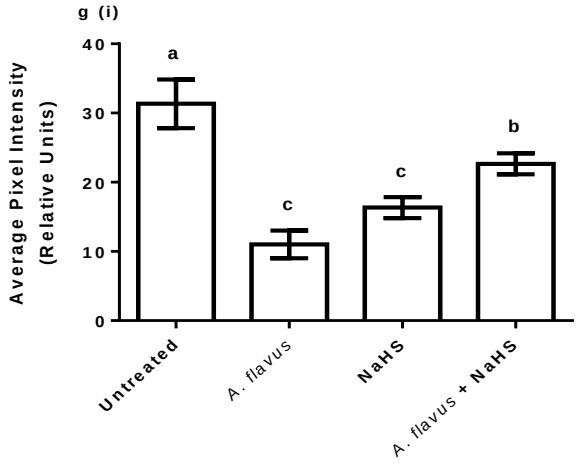
Figure 3. 5: Superoxide dismutase activity in response to leaves and roots of maize treated with sodium hydrosulfide and *A. flavus*. A native PAGE gel for determination of SOD isoforms Figure 3.5 a, while Figure 3.5 b (i-iv) shows pixel intensity ratios in leaves. Total SOD activity is shown in Figure 3.5 c. In roots, Figure 3.5 d (i-iv) represents pixel intensity ratios and Figure 3.5 e shows a total SOD activity in roots. Different letters indicate the differences between means at $p < 0.05$. Values are means ± SE (n = 3).

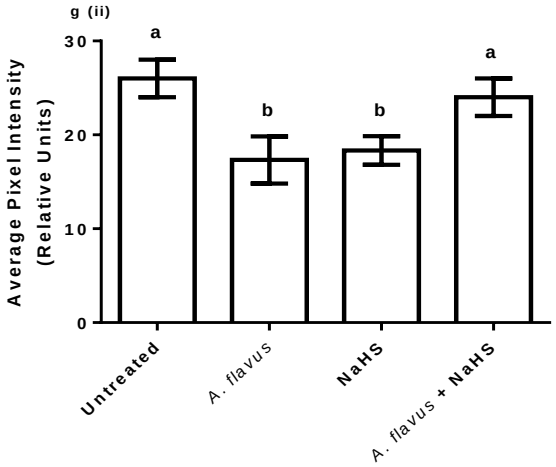
3.4.2 Sodium hydrosulfide and *Aspergillus flavus* affect the activity of catalase (CAT) in both leaves and roots of maize

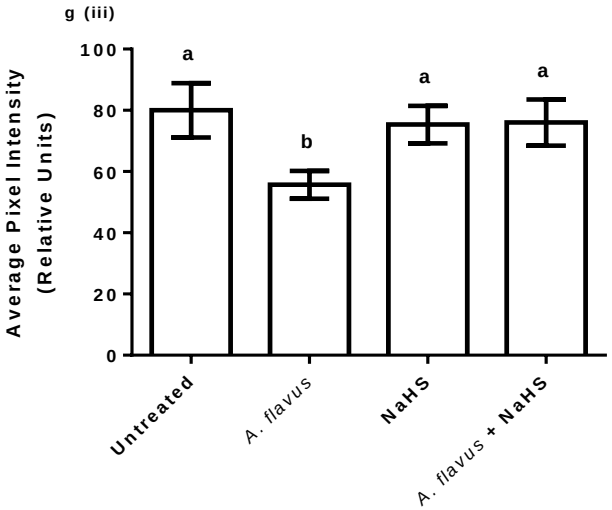
In the present study, we evaluated the effect of *Aspergillus flavus* and sodium hydrosulfide treatments on the activity of catalase isozymes. CAT activity was examined in leaves and roots of maize through an in-gel and spectrophotometric assays. Native PAGE analysis in leaves revealed three CAT isozymes (indicated by a clear zone against a green background) (Figure 3.5 f), with two isozymes detected in roots (Figure 3.5 i). The enzymatic activity of CAT 1 in leaves showed a reduction of approximately 27% from *A. flavus* + sodium hydrosulfide treatment when compared to untreated plants. Furthermore, CAT 1 isoform in plants treated with *A. flavus* showed a decrease of approximately 64% in comparison to untreated samples in Figure 3.5 g (i). There was a similar trend observed when *A. flavus* + NaHS treatment compared to untreated plants in catalase activity of CAT 2.

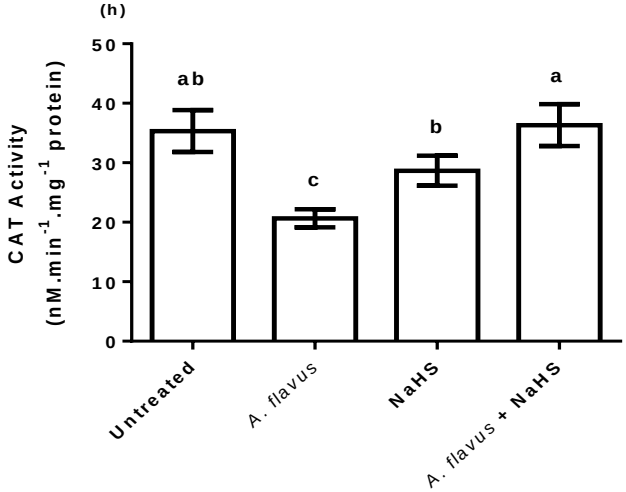
Plants treated with sodium hydrosulfide and *A. flavus* + NaHS showed no significance when compared to untreated in CAT 3 isoform, nonetheless, CAT 3 activity in plants treated by *A. flavus* was decreased by approximately 30% when compared to untreated samples. A similar trend was detected for the total (spectrophotometric) catalase activity in leaves from treatments sodium hydrosulfide and *A. flavus* + sodium hydrosulfide when comparison was made to untreated plants (Figure 3.6 h). The catalase activity for CATa in roots when compared with untreated plants showed a significant reduction of 50% in treatments (*A. flavus*, NaHS and *A. flavus* + NaHS) (figure 3.6 i). The enzymatic activity of CATb was reduced by approximately 37% in NaHS treatment. However, a similar trend was detected for plants treated with *A. flavus* and NaHS + *A. flavus* in comparison with untreated samples. The spectrophotometric catalase activity in roots showed no significant difference between sodium hydrosulfide and untreated samples whereas *A. flavus* and NaHS + *A. flavus* treatments showed a similar trend.

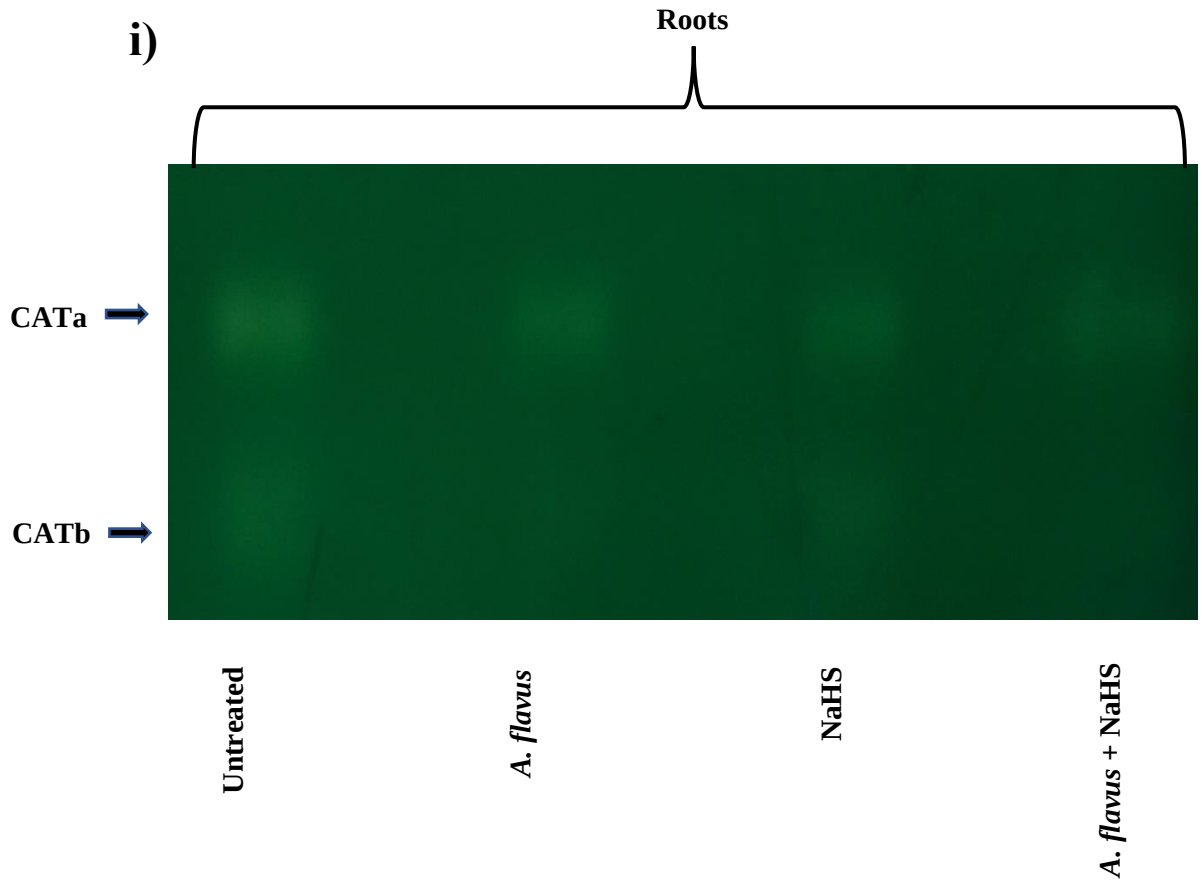












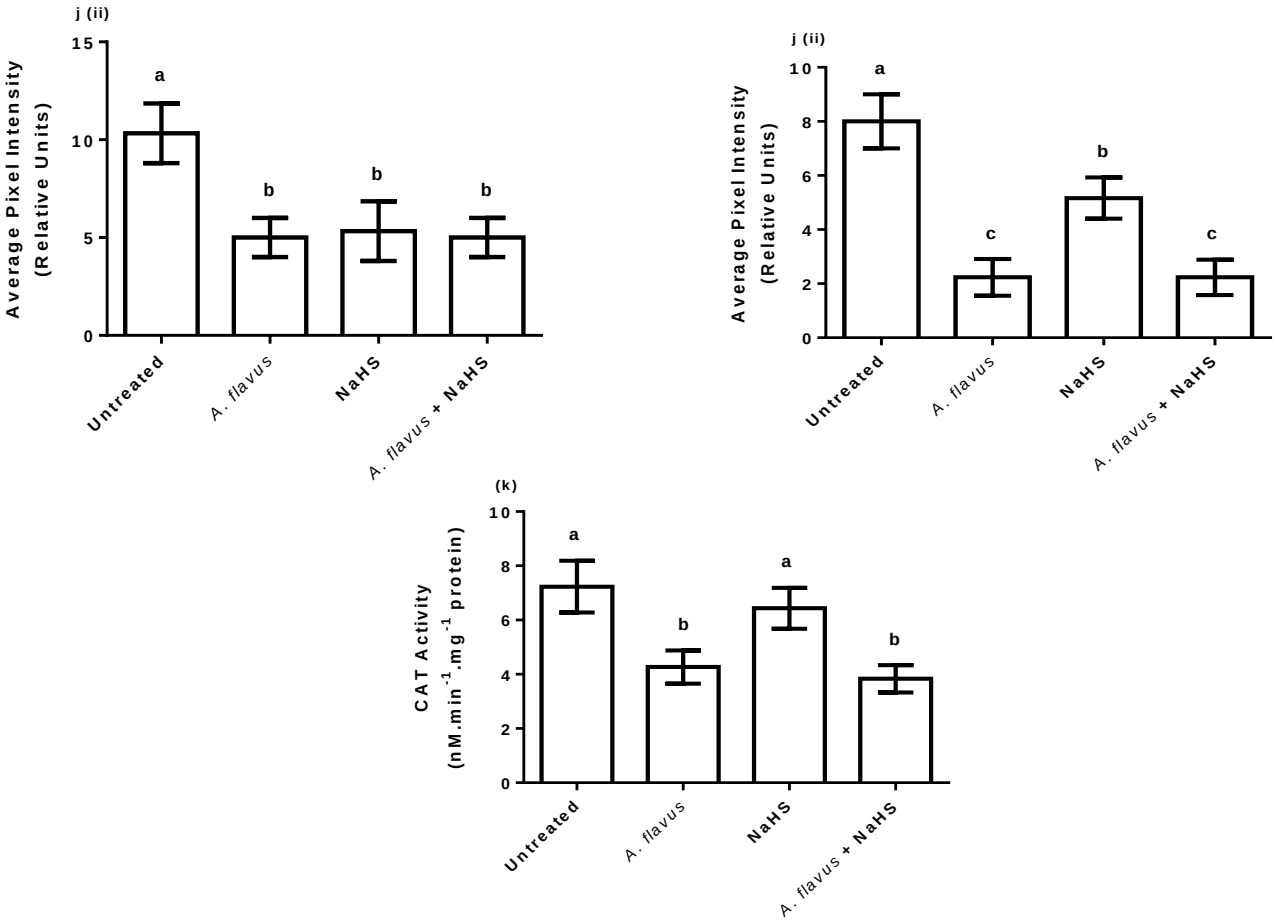


Figure 3. 6: Catalase activity in response to sodium hydrosulfide and *A. flavus* treatment in both leaves and roots of maize. A native PAGE analysis showing a clear zone against a green background representing catalase activity from leaves (Figure 3.6 f) and roots (Figure 3.6 i). The pixel intensity ratios of the generated isoforms is represented for leaves in Figure 3.6 g (i-iii) and for roots in Figure 3.6 j (i-ii). Both in leaves and roots, Figure 3.6 h and 3.6 k show total catalase activity respectively. Data presented are means ± SE of three independent experiments (n = 3). Different letters above error bars denote mean values that are significantly different at $p < 0.05$.

3.4.3 Treatment with sodium hydrosulfide and *Aspergillus flavus* changes the activity of ascorbate peroxide isoforms in leaves and roots of maize

Ascorbate peroxidase (APX) isozymes were determined in the leaves and roots of maize using both in gel-activity staining and spectrophotometric assays. In the leaves, two isozymes (APX 1 and APX6) were detected whereas five isozymes (APX 2, APX 3, APX 4, APX 5 and APX6) were detected in roots (Figure 3.7 l). The expression level of APX 1 isoform in Figure 3.7 m (i) displayed no significant difference in band intensities from treatment of plants in leaves. There was an increase of approximately 17% of APX 6 activity in leaves treated with combination of *A. flavus* + NaHS when compared to untreated samples in Figure 3.7 m (ii). APX total activity in leaves revealed an increase of approximately 66% in treatments (*A. flavus*, NaHS and *A. flavus* + NaHS) when comparison was done to untreated plants (Figure 3.7 n).

The combination of *A. flavus* + sodium hydrosulfide treatment for APX 2 isoform in roots showed a reduction of approximately 27% in Figure o (ii) while APX 3 isoform was decreased by approximately 75% in comparison to untreated samples in Figure 3.7 o (iii). APX 4 activity in roots revealed a decrease of approximately 84% from *A. flavus* + NaHS treatment and for APX 5 isoform, the activity was reduced by approximately 72% when compared to untreated plants, indicating that in roots *A. flavus* + NaHS inhibited the activity of ascorbate peroxidase. A similar trend in roots for APX 6 was observed in plants treated with *A. flavus*, NaHS + *A. flavus* and NaHS when compared to untreated. The total APX activity in roots revealed a reduction of approximately 75% in plants treated with combination of *A. flavus* and sodium hydrosulfide in comparison to untreated plants (Figure 3.7 p).

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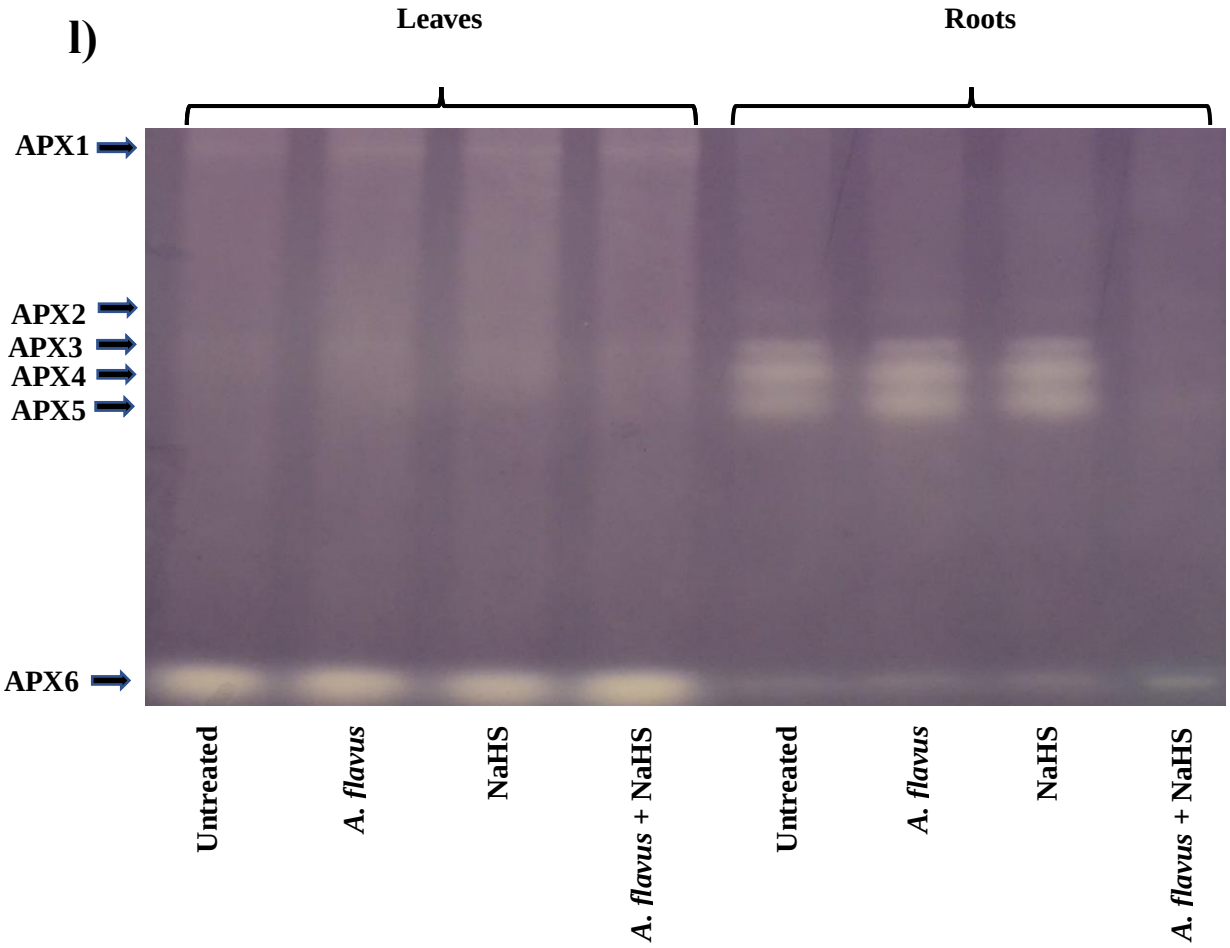

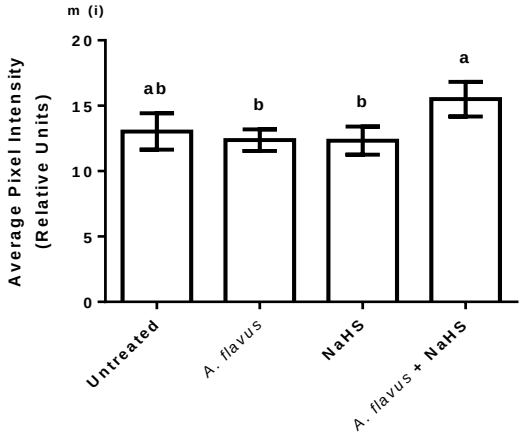

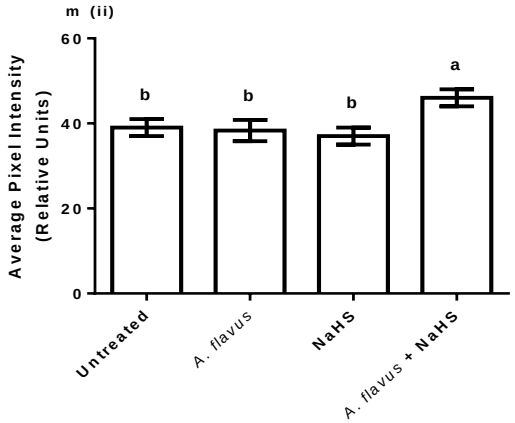

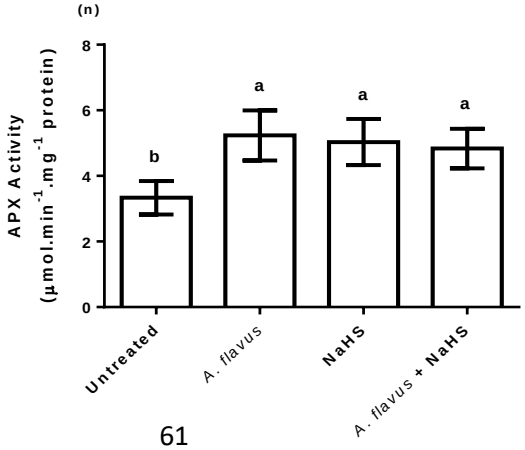

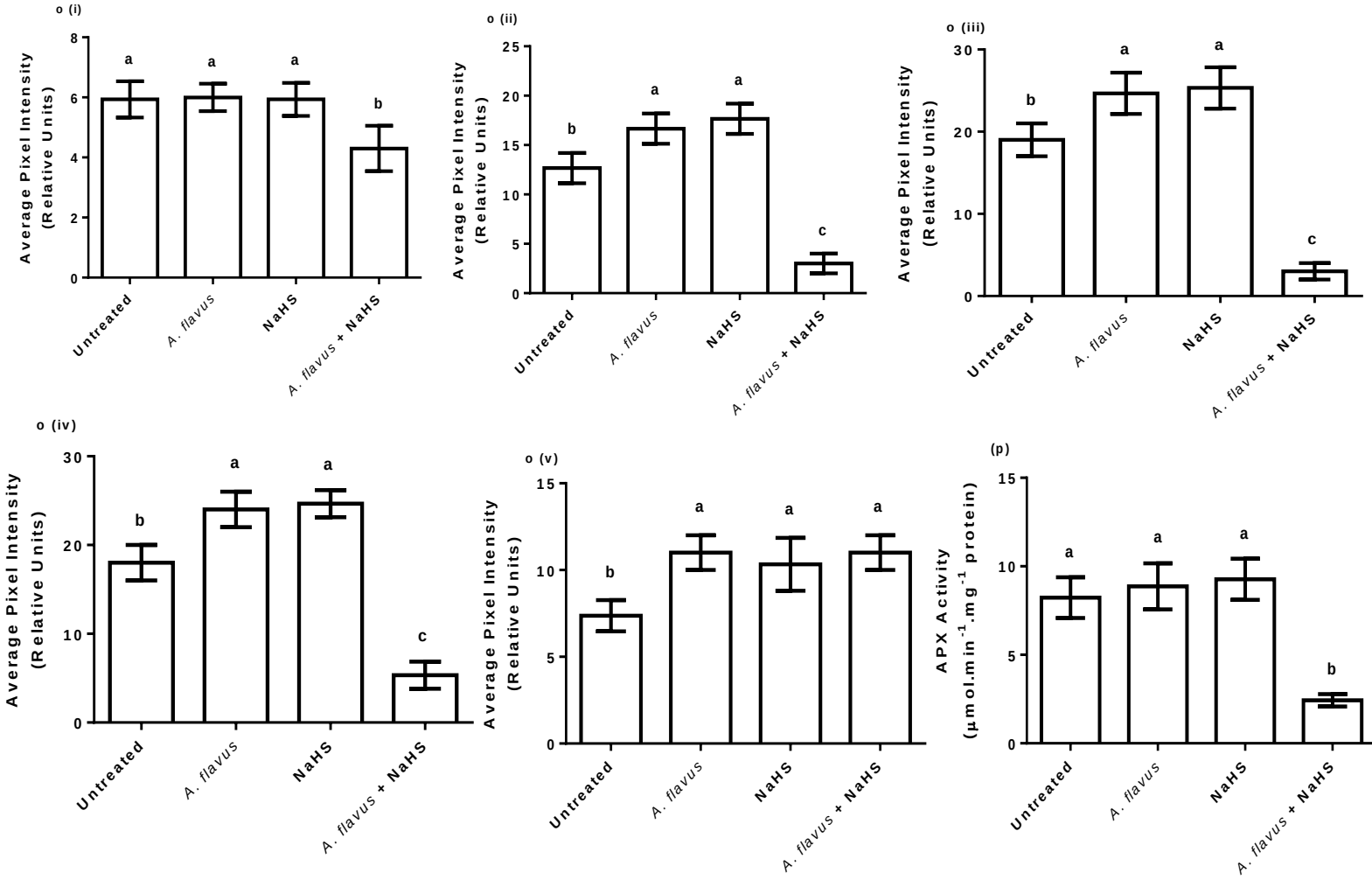

Figure 3. 7: The response of ascorbate peroxidase activity in leaves and roots of maize treated with sodium hydrosulfide and *A. flavus*. APX activity was determined in maize plants subjected to the following treatments: untreated, *A. flavus*, sodium hydrosulfide and *A. flavus* + sodium hydrosulphide. Harvesting was done at the V2 stage of vegetative growth. Figure 3.7 l shows the native PAGE for determination of APX isoforms, while in leaves m (i-ii) represent the relative APX pixel intensity ratio of the detected isoforms and Figure 3.7 n shows total APX activity in leaves. In roots, o (i-iv) represent pixel intensity ratio and Figure 3.7 p shows total APX activity. Different letters indicate the difference between means at $p < 0.05$. Values are means ± S.E (n=3).

4 Chapter four

Discussion

4.1 Antifungal activity of sodium hydrosulfide on *Aspergillus flavus* growth

Recently, hydrogen sulfide has been established as an important gas-signaling molecule with effects on multiple physiological processes in both animal and plant systems. H_2S has regulatory functions in plants that are involved in important processes such as the modulation of defense responses, plant growth and development, and the regulation of senescence and maturation (Aroca *et al.*, 2018). H_2S was shown to increase growth of vegetative parts of plants and to have an impact on disease resistance (Ausma and De Kok, 2019). A study by Thompson and Kats (1978) found that some concentrations of H_2S cause leaf lesions, defoliation, and reduced growth, the dosage and severity of injury being correlated. Sulfur derived from H_2S accumulates in plant tissues, the amount again depending upon the degree of exposure.

In this study, 1 mM sodium hydrosulfide prevented growth of *Aspergillus flavus* in a Petri dish-based assay. Reduction of *A. flavus* growth was also observed in treatments with 500 µM NaHS but was not eliminated to the same extent as seen for 1 mM NaHS. The findings of this study are similar to the results reported by Hu *et .al* (2014), where H_2S reduced growth by *Aspergillus niger* and *Penicillium expansum* isolated from pears. Kumar (2017) showed that inhibition of *Aspergillus flavus* mycelial growth can result from fragmentation of mycelial cytoplasm. The present study proposes that hydrogen sulfide may mediate defense responses against fungal attacks. During ancient times sulfur compounds were used as fungicides (Hu *et al.*, 2014; Fu *et al*., 2018). Thompson and Kats (1978) have also reported that hydrogen sulfide used in the fumigation of alfalfa (*Medicago sativa L*) can have beneficial effects depending on the degree of exposure. Their study reported that high concentrations of hydrogen sulfide cause leaf lesions, defoliation, and reduced growth. This could be due to the plant undergoing oxidative stress since H_2S can trigger ROS production. The findings in this thesis imply that the reduction of fungal growth caused by H_2S liberated from NaHS may be through elevation of ROS production, which would be toxic to the fungus, and by stimulating antioxidant enzyme activity in the plant to counteract pathogen-induced ROS accumulation. The stimulation of the antioxidant enzymes also is in agreement with Fu *et al.*, (2014).

4.2 Evaluation of maize growth in the presence of sodium hydrosulfide and *Aspergillus flavus*

Plants respond to various stresses that are highly complicated and involve changes at the transcriptome, cellular, and physiological levels. Most crop plants grow in suboptimal environments that prevent them from accomplishing their full genetic potential for growth and reproduction (Bruno *et al.*, 2017). *A. flavus* mainly contaminates grain crops and decaying organic matter (Krishnan and Chandrasekar, 2009). *A. flavus* has been reported to cause stalk rot in maize, leading to poor plant development as well as in poor stand, reduced root and shoot weight (Becker *et al.*, 2005; Kumar *et al.*, 2018). In this study, maize colonized by *A. flavus* showed a decrease in biomass.

Maize that was inoculated with *A. flavus* showed a decrease in growth. In the presence of NaHS, there was an increase in growth. Furthermore, shoot length, root length and biomass showed an increase in the presence of NaHS alone. These results suggest that NaHS plays a role in protecting maize against the negative effects of *A. flavus* infection.

The results obtained are similar to findings of Kumar *et al*. (2018) in the case of growth parameter measurements, where NaHS resulted in improved biomass, shoot length and root length in maize despite infection with *Piriformospora indica*. In Uganda's central and eastern regions, *A. flavus* occurrence was reported to spread in some of the important maize (*Zea mays L.*) production areas and this has caused reduction in shoot length, root length and biomass (Sserumaga *et al.*, 2015). The plant cytoskeleton is a highly dynamic subcellular structure that includes microtubules and microfilaments which is associated with plant defense (Yang *et al.*, 2017). It is possible that, in addition to ROS-mediated impact on plant growth in response to NaHS and *A. flavus*, plant growth could be impacted as a result of cytoskeletal changes that influence cell division and cell expansion.

4.3 Sodium hydrosulfide reduces oxidative stress in zea mays treated with *Aspergillus flavus*

Oxidative stress occurs when there is disruption in the balance of antioxidants defences or excessive production of reactive oxygen species. When plants are exposed to biotic stress, such as pathogen infection, accumulation of ROS; which include hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\cdot}$) and superoxide anion radicals (O_2^{-}); occurs (Scandalios, 2005; Zhu and Lafayette, 2017).

The findings of this study revealed high levels of reactive oxygen species (H_2O_2 and O_2^{-}) in plants treated with *A. flavus* from leaves and roots. However, H_2S generated from NaHS caused reduced ROS accumulation in plants exposed to *A. flavus* compared to when no NaHS was applied to *A. flavus*-inoculated plants. This suggests that hydrogen sulfide lowers ROS accumulation (H_2O_2 and O_2^{-}) in leaves and roots of plants infected with *A. flavus*, likely through stimulation of antioxidant enzymes to prevent *A. flavus*-induced oxidative stress to prevent deleterious effects of the infection. H_2S was also reported to have an important role in the regulation of normal plant physiological processes such as pathogen resistance, seed germination, root morphogenesis, photosynthesis and flower senescence (Jin and Pei, 2015). The results in this thesis are in agreement with the findings of Zhang *et al.* (2015), where H_2S treatment reduced accumulation of ROS in *Brassica rapa*.

Enhanced production of ROS can pose a threat to cells by causing peroxidation of lipids as well as oxidation of proteins, damage to nucleic acids, enzyme inhibition, and trigger activation of programmed cell death. Malondialdehyde (MDA) is a marker for lipid peroxidation, responsible for cell membrane damage including changes to the intrinsic properties of the membrane such as fluidity, ion transport and protein cross–linking (Sharma *et al.*, 2012). Plants treated with *A. flavus* had increased leaf and root lipid peroxidation and was associated with enhanced cell death to *zea mays* tissue, which was accompanied by reduced growth. Hydrogen sulfide reduced the levels of lipid peroxidation in leaves and roots of maize, which must be due to reduced ROS production as a result of activation of antioxidant enzymes that scavenge ROS.

4.4 Antioxidant enzyme activities in *zea mays* treated with sodium hydrosulfide and *Aspergillus flavus*

Stress factors such as pathogen infection cause disruption that leads to imbalance between ROS generation and ROS scavenging. Therefore, the survival of the plant depends on many important factors that include their ability to quickly adapt to the change of energy balance, change in growth conditions, intensity and length of stress conditions (Das and Roychoudhury, 2014). Several studies have reported that it is necessary for the cells to control accumulation of ROS because of its multifunctional role to avoid oxidative damage and while allowing for basal levels that are required for essential signal transduction processes (Sharma *et al.*, 2012). In plants, detoxification of excess ROS is attained through enzymatic antioxidants like superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT). In this study, two SOD isozymes were detected in both leaves and roots of maize. However, there was no significant change observed in Mn-SOD activity in any of the treatments, while Cu/Zn- SOD isoform was decreased in plants treated with *A. flavus* + NaHS treatment when compared to untreated, suggesting that SOD is marginally involved in H_2S-mediated responses to *A. flavus* infection in maize.

Catalase is a tetrameric heme-containing enzyme that catalyzes the dismutation of two molecules of H_2O_2 into H_2O and O_2. Catalase has much lower affinity for H_2O_2 than ascorbate peroxidase (APX), although it has a fast turnover rate (Sharma *et al.*, 2012; Mhamdi *et al.*, 2010). In this thesis, three isozymes (CAT 1, CAT 2 and CAT 3) were detected in leaves and two isozymes occurred in roots *(*CATa and CATb*)*. Results reported in this study are in agreement with results of Magbanua *et al.* (2007), who showed that CAT activity was enhanced in maize leaves resistant to *A. flavus* compared to susceptible maize. The finding in the study showing that CAT activity was supressed by infection with *A. flavus* suggests that this suppression allows for excessive H_2O_2 accumulation in response to *A. flavus* infection, which would cause oxidative stress, promote cell death and inhibit growth.

APX is an effective scavenger of H_2O_2 under stressful environmental conditions and has much higher affinity for H_2O_2 than CAT, and is one of the most widely distributed antioxidant enzymes in plants (Das and Roychoudhury, 2014). In this study, two isoforms in leaves were identified (APX 1 and APX 6) and five isoforms in roots (APX 2, APX 3, APX 4, APX 5 and

APX 6). APX 1 band activity in the leaves revealed no significant change amongst the treatments, except that APX 6 was upregulated in the leaves. Total APX activity was significantly inhibited in the roots of *A.flavus* + sodium hydrosulfide treatment. Such inhibition would result in elevated H_2O_2 levels in this treatment in comparison to untreated plants. Similar results were reported by Debona *et al.* (2012) for APX activity when wheat plant was colonized with *Pyricularia oryzae*.

5.0 Conclusion and Perspectives

The outcomes of the present study have demonstrated the effect of *Aspergillus flavus* and sodium hydrosulfide on maize. In this thesis initially, 1 mM of NaHS was observed to yield significant inhibition on growth of *Aspergillus flavus* without any detriment to growth of maize. *Aspergillus flavus* negatively affected maize growth. Plants that were treated with 1 mM of sodium hydrosulfide had enhanced leaf and root growth as well as biomass and this indicates that as much as H_2S suppresses damage of maize by *A. flavus*, it also promotes maize growth.

The *A. flavus* infection resulted in excessive production of ROS (O_2. and H_2O_2). Enhancement of ROS accumulation by the pathogen infection resulted in significant increase in MDA content, indicative of oxidative stress. Cell death was also increased in response to infection by the pathogen. In contrast, low levels of ROS and MDA content were occurred in the presence of H_2S generated from 1 mM NaHS. Based on the results from this study, it is concluded that the regulation of ROS levels in response to the pathogen infection and H_2S is regulated by the H_2S-driven activation of antioxidant enzymes activities. In future, the use of genomics, metabolomics, and proteomics will help in clarifying the molecular networks involved in the regulatory role of H_2S in conferring maize resistance to *A. flavus*. Improved understanding of these aspects will contribute to production of plants with improved levels of resistance to *A. flavus* and reduce aflatoxin contamination in maize.

6.0 References

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