EXPLORING BIOLOGICAL CONTROL AND TRANSGENIC WEED MANAGEMENT APPROACHES AGAINST INFESTATION BY *STRIGA HERMONTHICA* IN MAIZE

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Exploring Biological Control and Transgenic Weed Management Approaches against Infestation by *Striga hermonthica* in Maize

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DECLARATION

This is my original work and has not been presented for degree consideration/a ward in any other university.

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DEDICATION

It takes someone special, a person who cares for you, and aims to help you grow to your fullest potential, to get success in ventures where endurance and patience are necessary virtues and this I found in my beloved wife Mildred Makokha. I dedicate this work to Joseph Neondo, Lincoln Neondo and Tracy Neondo. I also dedicate this milestone to my elder brother, Kennedy Neondo. He overcame many odds and took over the responsibility of educating me after the demise of our late father (Joseph Neondo) eighteen years ago. To me, he is my hero and father. Lastly, I also dedicate this piece of work to my dear mother Sifrosa Neondo, whose enduring love and tender care was and still is my source of comfort.

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ACRONYMS AND ABBREVIATIONS

µg	Microgram
µl	Microliter
2,4-D	2,4-Dichlorophenoxyacetyl monohydrate
ABA	Abscisic acid
Agro	*Agrobacterium tumefaciens*
ANOVA	Analysis of variance
CaMV	Cauliflower mosaic virus
CIM	Callus Induction Media
CIMMYT	Centro Internacional de Mejoramiento de Maízy Trigo
CML	CIMMYT maize line
CMM	Callus maturation media
DAP	Days after Pollination
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ESM	Embry suspension media
GUS	β-glucuronidase
H_2O	Water
IM	Infection Media
KSTP'94	Kakamega *Striga* tolerant population of 1994
LS	Linsmaier and Skoog (1965) media
MS	Murashige and Skoog (1962) media

N6	Chu (1975) media
NCBI	National Center for Biotechnology Information
NaCl	Sodium chloride
°C	Degrees celsius
OD	Optical Density
OPVs	Open pollinated varieties
PAP	Purple acid Phosphatase
PCR	Polymerase Chain Reaction
PRO	Proline
QTL	Quantitative trait locus
RM	Regeneration Media
RNA	Ribonucleic acid
T_0	Zero generation transformants
Vir	Virulent
x-Gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid
YEP	Yeast Extract peptone

ABSTRACT

Striga hermonthica and low soil phosphorus (P) are major contributors to food insecurity in sub Saharan Africa (SSA). Infestation of cereal fields by *S. hermonthica* results in about 30% to 90% yield loss hence emerging as a major constraint in cereal production in SSA. Such great loss is attributed to two aspects of *S. hermonthica* lifecycle namely elevated seed fecundity and its remarkable synchronized lifecycle to that of its host. It is also estimated that crop yield is limited by P availability in 30% to 40% arable land. This is because although the total content of phosphorus in the lithosphere is high, its partial bioavailability is a major limiting factor in crop production. Furthermore, there exists a tripartite relationship among maize, *S. hermonthica* and low P levels, whereby low soil P levels prompt maize roots to produce exudates rich in volatile *S. hermonthica* seed germination stimulants known as strigolactones. The strigolactones are produced as a signal to initiate arbuscular mycorrhiza colonization. Unfortunately these chemical cues are hijacked and used by *Striga* seeds as germination stimulant and hence signal the presence of a susceptible host. To date, there is no integrated, sustainable and effective *S. hermonthica* control strategy that has been widely adopted by small-scale farmers in Africa. This can however be provided by simultaneously addressing poor P access by maize as well as biological control of germinated *S. hermonthica* or piled seed banks in the soil. This therefore calls for the integration of biotechnologies that will shatter the tripartite relationship among low soil P level, stimulation of Arbuscular Mycorrhizal Fungi (AMF) colonization and *S. hermonthica* seed germination.

The aim of this study was to simultaneously explore biocontrol options by bio-prospecting the effectiveness of culturable microbes against *S. hermonthica* as well as enhance P availability to maize by genetic transformation of ecologically adapted maize genotypes with P efficient Purple Acid Phosphatase (*PAP*) genes from *Lupinus albus* (*La*PAP) and *Medicago truncatula* (*Mt*PAP).

To explore the biocontrol frontier, bacterial and fungal isolates from *Striga* suppressive soils were assayed for their ability to produce extra cellular enzymes and antibiotic compounds as well as their ability to induce *S. hermonthica* seed decay and later genotyped using 16S rRNA and 18S rRNA genes, respectively. In order to develop transgenic maize plants expressing target *PAP* genes, a regeneration protocol with an assortment of callus induction and callus maturation/shoot induction media were evaluated. Further, the transformability of target maize varieties was assessed via histochemical analysis of *β*-glucuronidase (GUS) reporter gene. Finally, *Agrobacterium tumefaciens*-mediated transformation of the maize varieties over-expressing *PAP* gene cassette was achieved and transgenic lines evaluated using *S. hermonthica*-host plant infection assays *in vitro* and in potted experiments.

The morphometric analysis of bacterial and fungal descriptors identified bacterial isolates that displayed array of enzymatic and antibiosis properties and also that had ability to cause *Striga* seed decay. For instance isolate SM5ISS (KY041696) with 99% genetic affiliation to *Bacillus* recorded high antibiosis (8cm) and extra cellular enzymatic values (2.5±0.03) and also recorded the highest number of *S. hermonthica*

seed decay (45±0.23%). This bioprospection study summarily identified candidate isolates that caused *S. hermonthica* seed decay.

The regeneration study revealed that Namba nane, KSTP'94 and CML144 varieties recorded a regeneration frequency of 26.1±1.11%, 32.1±1.28% and 35.4±1.24%, respectively, while their corresponding GUS transformability efficiency values were 0.8±0.03%, 1.4±0.19% and 2.1±0.20%, respectively. Transformation of Namba nane with *La*PAP and *Mt*PAP gene construct recorded a transformation efficiency of 0.33±0.03% and 0.36±0.04%, respectively, while the corresponding values for *La*PAP and *Mt*PAP gene constructs in KSTP'94 were 0.69±0.05% and 0.37±0.03%, respectively. Transformation of CML144 with *La*PAP and *Mt*PAP gene construct recorded a transformation efficiency of 0.65±0.03% and 0.34±0.03%, respectively. These results demonstrated that the target maize germplasm was transformable.

Over-expression of *La*PAP and *Mt*PAP in the selected maize genotypes resulted in low number of *S. hermonthica* colonizing transgenic maize in comparison to wild type maize. For instance, in Namba nane the average number of *Striga* plants colonizing individual wild maize plant in both rhizotron and bucket experiments were 9 and 4 while the corresponding numbers for *La*PAP and *Mt*PAP transgenic were 4, 1 and 5, 2, respectively. For KSTP'94 the average number of *Striga* plants colonizing individual wild maize plants in both rhizotron and bucket experiments were 4 and 3 while the corresponding numbers for *La*PAP and *Mt*PAP transgenic was 3, 1 and 3, 1, respectively. In the case of CML144 the average number of *Striga* plants colonizing individual wild maize plant in both rhizotron and bucket experiments were 12 and 7 while the corresponding numbers for *La*PAP and *Mt*PAP transgenic plants was 6, 2 and 8, 3, respectively. Analysis of the ability of root exudate to induce *S. hermonthica* seed germination was higher in wild type than transgenic maize. For instance, the average number of *Striga* seeds stimulated to germinate in Namba nane under treatments; wilt-type, *La*PAP and *Mt*PAP was 7, 4 and 6, respectively. In KSTP'94, the average number of *Striga* seeds stimulated to germinate in Namba nane under treatments; wilt-type, *La*PAP and *Mt*PAP was 5, 2 and 3 respectively. Lastly, in CML144 the average number of *Striga* seeds stimulated to germinate in Namba nane under treatments; wilt-type, *La*PAP and *Mt*PAP was 5, 2 and 3, respectively.

Summarily, this study identified microbes that were potent against *S. hermonthica* and proposes their use in reduction of *S. hermonthica* seed bank in infested soils. Further, it was demonstrated that indeed overexpression of *PAP* genes in maize results in less *S. hermonthica* infestation. The use of the two approaches is therefore recommended in an integrated *S. hermonthica* management package that would be able to impede the parasite in infested and low P soils especially in western Kenya.

CHAPTER ONE
GENERAL INTRODUCTION

1.1 Background of the study

Maize (*Zea mays*) is a crop plant of global importance especially in sub Saharan Africa (Setimela et al., 2017). In Kenya, it is the staple food for over 85% of the population with per capita consumption of approximately 2.7 million metric tons per year (Onono et al., 2013). Small-scale farmers grow the crop for subsistence use which accounts for 70 percent of the overall maize production in Kenya (Onono et al., 2013). Majority of small-scale farmers are poor and prefer planting improved open-pollinated varieties (OPVs) of maize (Warburton et al., 2010). The OPVs tend to out-yield farmers' unimproved landraces and further offer the economic advantage of allowing seed recycling without a significant drop in yield (Warburton et al., 2010). For instance, small-scale farmers in western Kenya opt for their own bulked KSTP'94 and 'Namba nane seed (Wambugu et al., 2012; Omondi et al., 2014). KSTP'94 is a certified OPV that is drought tolerant and partially *Striga hermonthica*-tolerant maize variety while 'Namba nane' is a traditional OPV that is distinct for its eight (nane) rows of kernels per cob (Omondi et al., 2014). These OPVs are preferred because of their early maturity, low input tolerance, culture and good culinary qualities (Anjichi et al., 2005; Gudu et al., 2005; Denning et al., 2009; Macharia et al., 2010; Omondi et al., 2014). However, constraints such as over-reliance on farm-saved seeds, poor farm management practices and heavy *S. hermonthica* infestation have contributed to low maize yields at the farm in the region (Kagot et al., 2014; Wambugu et al., 2012).

S. hermonthica and low soil phosphorus (P) content stand-out as major biotic and abiotic factors limiting maize production in sub Sahara Africa (Vance et al., 2003). Although several *S. hermonthica* management strategies have been proposed and used, very little success has been achieved in this region. This weed produces large amounts of seeds that remain viable for over 14 years in the soil and only germinate in the presence of a suitable host (Mbuvi et al., 2017). *S. hermonthica* infestation alone accounts for about 40% to 100% maize yield loss globally that amounts to about united states dollar (USD) 7 billion annually (AATF, 2006; Khan et al., 2006).

This loss further threatens the fragile food security situation and the lives of the already poor and resource limited small-scale farmers in Kenya and the SSA region (Ejeta, 2007; Jamil et al., 2012). On the other hand, low soil phosphorus (P) content affects 30% to 40% productivity of arable land worldwide by causing poor plant growth and exacerbates *S. hermonthica* infestation rates in fields under cereal farming (Atera et al., 2012; Khan et al., 2008; Vance et al., 2003; Yoneyama et al., 2007). The continued lack of fertilizer application and the heavy infestation of maize fields with *S. hermonthica* have elicited fruitless weed control efforts in the past (Omondi et al., 2014). Even where efforts to reduce *S. hermonthica* infestation has been emphasized, the focus has been short-lived, totally ignoring to explore sustainable *S. hermonthica* control options such bioremediation of inorganic rhizosphere phosphorus (Omondi et al., 2014).

The use of microorganisms as biocontrol agents to reduce the adverse effects of plant diseases and weeds is considered as an important alternative method to minimize the application of chemicals (Pilgeram et al., 2010). A considerable number of plant pathogens have been studied for their possible use as bioherbicides for the control of weeds (Pilgeram et al., 2010). The objective of any biological weed control strategy is not to achieve absolute eradication of the weed but rather reduce establishment of the weed population to a level below the economic threshold (Teka, 2014). Because of the diversity and complexity of reactions and numerous metabolic pathways, microorganisms form an amazing resource for the biological management of crop weeds (Pilgeram et al., 2010). For instance, some plant pathogenic microbes have ability to mitigate plant defense responses by disrupting the plants ability to perceive or respond to the pathogen (Jones & Dangl, 2006). Such microbes have potential of being used as biological control agents against parasitic weeds if the target host is only the weed plant (Watson, 2013).

Although a number of fungal and/or bacterial strains have been reported to have antagonistic effect against weed plants, only a few have been found to be suitable for development of formulation and commercialization as biological weed control products (Pilgeram et al., 2010). This situation may be due to the fact that the biocontrol systems are based on living microorganisms whose activities are

dependent on different biotic and abiotic factors. Knowledge on the mode of action and extent of influence of these factors of biocontrol agents on the weed plant is scanty. In *S. hermonthica*, there have been cases of wilting presumed to be caused by microbes, which could otherwise be used as *S. hermonthica* biocontrol agents (Marley et al., 1999).

Phosphorus is a critical element in plant growth and agricultural sustainability, however it is also the most limiting factor in plant growth due to its rapid immobilization by soil organic and inorganic components (Lambers & Plaxton, 2015; Miller et al., 2001). Despite application of mineral fertilizer and biological phosphorus mobilization processes, most soils are quickly becoming P deficient through oxidation processes (Hassan et al., 2011). Furthermore, few plants can grow in P deficient soils while majority of them rely on symbiotic associations with P-mobilizing microorganisms or artificial supply of phosphorus in form of fertilizers (Wasaki et al., 2003).

Plants respond to rhizosphere P deficiency by developing several adaptive mechanisms such as remodeling of root architecture and Arbuscular mycorrhizal (AM) fungi and rhizobia symbioses with roots to increases the surface area for inorganic phosphorus (Pi) uptake (Guo et al., 2011; López-Arredondo et al., 2014; Tian and Liao, 2015; Younessi-hamzekhanlu et al., 2016). This is important because, approximately 20% of the topsoil is explored by roots during plant growth and therefore, enhancing topsoil foraging which is essential in improving Pi usage by plants (Younessi-hamzekhanlu et al., 2016). Other biochemical/metabolic adaptations by plants to deal with low-P content include induction of intra-cellular and extra-cellular acid phosphatases (APases) to catalyze the hydrolysis of Pi from various monoesters and anhydrides substrates (Tran et al., 2010).

The plant legumes *Lupinus albus* and *Medicago truncatula* are plant species adapted to low rhizosphere P content (Garderner et al., 1985; Tadano and Sakai, 1991; Wasaki et al., 2003). These plants secrete Purple Acid Phosphatase (PAPs) enzymes that mobilize/liberate inorganic phosphate from organic compounds and sparingly inorganic compounds in the lithosphere making it available in forms that is utilizable

by plants (Tadano & Sakai, 1991; Wasaki et al., 2003). Unfortunately plants such as maize have been reported to be inefficient mobilizers of rhizosphere P (Yoneyama et al., 2007). In low P environments, maize also produces PAPs however they are not efficient in P mobilization like those produced by *L. albus* and *M. truncatula*. Furthermore, during P deficiency, maize roots produces exudates that contain chemical compound referred to as Strigolactones, which are meant to signal and initiate *Arbuscular mycorrhiza* fungi colonization. Unfortunately these chemical cues are intercepted by *S. hermonthica* seeds to signal presence of a host by inducing seed germination process of this parasitic weed thereby exacerbating its infestation rates (Yoneyama et al., 2007).

Although maize cannot be crossed with either *L. albus* or *M. truncatula* due to genetic distance, the efficient *PAP* genes of the two legume species can be cloned and introduced into maize via genetic engineering. Transformation of maize with efficient *PAP* genes from low P tolerant species (*L. albus* and *M. truncatula*) has not been reported. This approach holds potential of overcoming the genetic barrier inhibiting introgression of PAP gene of *L. albus* and *M. truncatula* into maize thereby solving the *S. hermonthica* problem and also enhance P availability in farms where it is immobilized.

1.2 Statement of the Problem

Maize yields at the farm level in Sub-Saharan Africa and particularly in Kenya are very low due to elevated *S. hermonthica* infestation rates and low rhizosphere phosphorus content (Jamil et al., 2012). *S. hermonthica* alone accounts for 40 - 100% annual maize yield loss in the region (Khan et al., 2008; Bozkurt et al., 2014). In western Kenya (formerly Nyanza and Western provinces), *S. hermonthica* has infested over 217,000 hectares of arable land, resulting in maize losses of 182,227 tons per year valued at 53million USD (Nzioki et al., 2016). Earlier studies revealed that in Nyanza region of Kenya, farmers expected average maize yield of about 1.5 t/ha in farms free of *S. hermonthica* infestation (Rodenburg et al., 2006). In farms moderately infested by *S. hermonthica*, the expected maize yield was 0.75 t/ha and only about 0.3 t/ha in situations where there was high *S. hermonthica* infestation

(Rodenburg et al., 2006). The corresponding figures for Western region were 1.8 t/ha and 0.9 t/ha and 0.36 t/ha respectively (Rodenburg et al., 2006).

Several agricultural practices have been evaluated as control methods for *S. hermonthica spp*., and these include the use of herbicides and fertilizer (Gworgwor and Weber, 2003), cultural methods (Last, 1960) and resistant crop varieties (Olivier et al., 1991). However none of them have effectively and sustainably yielded satisfactory *S. hermonthica* suppression levels when used alone (Bozkurt et al., 2014; Nzioki et al., 2016; Sunda et al., 2013). This is largely attributed to synchronized *S. hermonthica*-host underground parasitic lifecycle, imperishable *S. hermonthica* soil seed bank and the big disparity between the cost of the control strategies and the farmers' socioeconomic status (Atera et al., 2013; Kagot et al., 2014; Nzioki et al., 2016).

For instance, Crop rotation with non-host crops reduces *S. hermonthica* seed bank but is becoming less feasible with the dwindling farm sizes in the populated rural areas (Sunda et al., 2013; Van Mourik et al., 2011). Intercropping with allelopathic legumes and the push and pull technology using desmodium have also been proposed, though not widely deployed, remains unfeasible especially for non-dairy farmers (Sunda et al., 2013; Van Mourik et al., 2011; Watson, 2013). Use of strigolactone in induction of suicidal *S. hermonthica* seed germination appears promising, however the main limiting factors in the studies and possible practical applications of natural *S. hermonthica* (SLs) is, among others, their availability in very low amounts (Vurro et al., 2011). Similarly, synthetic SL analogues targeting this parasitic plant still have low potential for agricultural applications due to their low stability under alkaline conditions and the high cost of chemical synthesis (Vurro et al., 2011).

Maize varieties such as GAF4 and KSTP'94 that tolerate *S. hermonthica* infection with little yield loss have been identified (Kim, 1991), however none is known to have potential to fully resist the establishment and growth of the parasite or reduce the weed's soil seed bank (Badu-Apraku et al., 2015; Okora et al., 2015; Wambugu et al., 2012). Breeding for *S. hermonthica* resistance is complicated by the ability of

S. hermonthica to overcome host resistance, due to its obligate outcrossing behaviour, which results in high levels of genetic variation within and between populations (Koyama, 2000; Bozkurt et al., 2014). Studies show that genetic variation of virulence genes of this parasite enables it to adapt to new host resistance alleles (Bozkurt et al., 2014).

Numerous species/strains of fungi such as *Fusarium oxysporum* and/or bacteria such as *Pseudomonas* have been reported as potential biocontrol agents against farm weeds. However only few of them have been found to be suitable for making formulations for commercialization as biological weed control products (Avedi et al., 2014). This is possibly due to the fact that the biocontrol systems are based on living microorganisms whose activities are dependent on different biotic and abiotic factors (Musyoki et al., 2015). In scenarios where biological control strategies have been effectively applied, efficacy of the same biocontrol agents have failed to be replicated in other regions due to lack of adaptability of the microbe used (Avedi et al., 2014). Knowledge on the mode of action and extent of influence of these factors of biocontrol agents on the weed plant is scanty (Musyoki et al., 2015).

The total amount of phosphorus in the lithosphere is relatively high, however its limited bioavailability is frequently a major limiting factor in crop production due to low mineralization and fixation processes (Baker et al., 2015). In Kenya, it is estimated that low P affects 30-40% soil productivity/fertility, which impairs proper plant growth and exacerbates *S. hermonthica* infection (Temegne et al., 2015).

S. hermonthica infestation and low rhizosphere phosphorus content all combined cause huge cereal grain losses annually and consequently lead to food insecurity that threatens the lives of the already poor and resource limited small-scale farmers especially in Kenya. This, together with ephemeral food shortages and low maize yields in many parts of Kenya forces hundreds of families in the country to abandon their farms. Although *Lupinus albus* and *Medicago truncatula* are adapted to low soil P level, they can not be crossed with maize through conventional plant breeding program to produce maize adaptable to low soil P due genetic distance between maize and these two plant species.

1.3 Justification

Despite the existence of various *S. hermonthica* control strategies, increased yield loss due to this type of parasitism remains a daunting task in maize production (Stringer et al., 2009; Rodenburg et al., 2011; Jamil et al., 2012). Small-scale farmers particularly in Western region of Kenya need affordable and effective *S. hermonthica* management strategies that can be readily integrated into their production practices. A considerable number of plant pathogens have been studied for their possible use as bioherbicides however, only a few of them have proven to be adequately virulent to control *S. hermonthica* and to compete with commercial chemical herbicides. The relationship between these pathogenic microbes and *S. hermonthica* depends on mutual recognition, signaling, and the expression of pathogenicity and virulence factors of the microbe. The existence of passive, preformed, or inducible defence mechanisms by *S. hermonthica* during attack by these microbes results in compatible (susceptibility) or incompatible (resistance) interactions. Isolation, characterization and utilization of local (region-specific) microbial agents capable of causing *S. hermonthica* seed decay which is critical for designing biocontrol formulation, has not been exploited.

Existing knowledge on induction of *S. hermonthica* seed germination and haustoria development indicate that they can be candidate targets for inhibition if explored. Biotechnological strategies such as genetic transformation aimed at impeding development of this parasitic association holds the potential of reducing the effects of *S. hermonthica* infestation in maize farms.

Using biotechnology, it is possible to down-regulate production of host plant exudates that act as *S. hermonthica* germination stimulants or enhance genetic ability of host plants to resist the penetration and connection of its root vascular system by *S. hermonthica* seedling. For instance, genetic transformation of maize with efficient *PAP* gene cloned from *L. albus* and *M. truncatula* can result in maize varieties with ability to mobilize otherwise unavailable P to readily available Pi. Overexpression of the *PAP* genes in maize is subsequently expected to reduce the production of volatile chemical cues by maize (strigolactone) that induce *S. hermonthica* seed germination thus limiting *S. hermonthica* infestation. Existing scientific findings have revealed

that transgenic plants overexpressing *PAP* genes show altered root architecture under various P levels. This strategy can effectively improve phosphate acquisition ability of maize and at the same time reduce *S. hermonthica* infestation rates. Transformation of KSTP'94 and Namba nane lays foundation for introgressing this trait into existing and future maize hybrids in Kenya.

This study therefore aimed to integrate biocontrol measures with genetic transformation strategies with a view of addressing the *S. hermonthica* menace affecting resource-limited small-scale farmers in sub Sahara African agriculture.

1.4 General objective

To explore biocontrol and genetic transformation options of weed management approaches against *Striga hermonthica* infestation in maize.

1.4.1 Specific objectives

1. To determine and characterize rhizosphere microbes that cause *S. hermonthica* seed decay from *S. hermonthica*-suppressive soils from western Kenya.
2. To develop an *in vitro* regeneration and *Agrobacterium*-mediated *GUS*-transformation protocols for two farmer-preferred maize varieties (KSTP'94 and Namba nane).
3. To determine the transformation efficiency and expression effect of *Purple Acid Phosphatase* (*PAP*) gene in CML144, KSTP'94 and Namba nane varieties on *S. hermonthica* parasitism process.

1.5 Null hypotheses

1. There exists no rhizosphere microbes that cause *S. hermonthica* seed decay in *S. hermonthica* suppressive soils.
2. CML144, KSTP'94 and Namba nane maize varieties are not amenable to *in vitro* regeneration and transformation protocols.
3. CML144, KSTP'94 and Namba nane varieties are not amenable to transformation with *PAP* gene and there exists no *S. hermonthica* suppression effect in transgenic events.

CHAPTER TWO
LITERATURE REVIEW

2.1 Maize domestication and its economic importance

Maize (*Zea mays*) was domesticated from its wild ancestor, teosinte (*Zea mays* sp. *parviglumis*) about 9000 years ago (Prasanna, 2012; Qamar et al., 2015). This domestication event took place in the mid-elevations (~1500 m above sea level) of South Central Mexico starting with the teosinte race Balsas (Matsuoka et al., 2002; Prasanna, 2012). Maize then followed a very complicated pattern of introduction to different continents, including the North and South America, Europe, Africa and Asia (Rebourg et al., 2003; Dubreuil et al., 2006; Prasanna, 2012). Maize landraces with better adaptability were selected by the farmers to the new environments leading to several new derivatives in the process (Prasanna, 2012). This was aided by the fact that maize is a predominantly cross-pollinating species, a feature that makes it to have a broad morphological variability and geographical adaptability (Xu et al., 2009).

In Africa, maize was introduced nearly five centuries ago (McCann, 2005). Since then, the crop expanded in its range from the lowlands to the highlands as well as from the marginal to optimal soil fertility environments with varying success (McCann, 2005; Prasanna, 2012). Some early names for maize like *yabahar mashila*, meaning "sorghum from the sea," in Ethiopia and *Piti*, "sorghum with a hat," and *Mala*, "sorghum that carries a child," in Nigeria attest to the alien nature of this crop in Africa and its resemblance to native sorghum (McCann, 2005; Rich and Ejeta, 2008). In Kenya, maize was introduced in the sixteenth and eighteenth centuries by the Portuguese and English settlers respectively (Anjichi et al., 2005). These pools formed the basis of the first modern maize varieties bred in Kenya and are vital source of genetic diversity for breeding locally adapted varieties (Anjichi et al., 2005).

Depending on the latitude and the climate in which it is grown, maize is classified into three distinct types, tropical, temperate, and subtropical (Xu et al., 2009). Maize can also be classified based on: endosperm and kernel constitution; kernel colour: flint, dent, floury, waxy, sweet, and popcorn; maturity and use (Xu et al., 2009).

Economically, the most important types of maize are grown for grain or fodder and silage production (Xu et al., 2009). However, in the tropics, maize grain is primarily used by human as food (Xu et al., 2009). FAO envisages that an additional 60Mt of maize grain will be needed from the annual global harvest by 2030 (Xu et al., 2009). Globally, maize is the top ranking cereal in terms of productivity and has worldwide significance as human food, animal feed and fodder as well as raw material for a large number of industrial products (Wada et al., 2008; Shiferaw et al., 2011; Sunda et al., 2013; Gui-rong et al., 2013; Ali et al., 2014). It has also become the most popular crop in Africa in terms of cultivated area and total grain production (McCann et al., 2006; Qamar et al., 2015). In sub Sahara Africa, it is the principal staple crop (Cairns et al., 2013). It accounts for 30 % of the total area under cereal production in sub Sahara Africa and more specifically 19 % in West Africa, 61 % in Central Africa, 29 % in Eastern Africa and 65% in Southern Africa (Cairns et al., 2013). Studies show that maize grain constitutes about 9.74 % grain protein, 4.85% grain oil, 9.44% grain crude fibre, 71.97% grain starch, 11.77% embryo while fodder contains 22.99% acid detergent fibre, 51.70% neutral detergent fibre, 28.80% fodder cellulose, 40.18% fodder dry matter, 26.85% fodder crude fibre, 10.35% fodder crude protein and 9.10% fodder moisture (Qamar et al., 2015).

In addition to being an economically important crop, maize is also a classical genetic model for plant research (Xu et al., 2009). It has a number of characteristics that are favourable for an experimental model for crop plants. These characteristics include; a multiple purpose crop with worldwide cultivation which attracts research funding from public and private institutions, outbreeding reproduction system with tolerance of inbreeding, existence of multiple breeding products (inbreds, hybrids, synthetic cultivars, open-pollinated varieties, improved landraces) and wide adaptability including good sources of resistance to environmental stresses (Xu et al., 2009).

In SSA, maize is predominantly grown in smallholder farming systems under rain-fed conditions with limited inputs (Cairns et al., 2013). Low yields in this region are largely associated with drought stress, low soil fertility, weeds, pests, diseases, low farm input and inappropriate seeds (Cairns et al., 2013). Over reliance on rainfall increases the vulnerability of maize systems to climate variability and change (Cairns

et al., 2013).

In Kenya, current maize production capacity is below consumption demand (Omondi et al., 2014). High population pressure and continued subdivision of arable land, coupled with limited resources severely constrains maize production (Omondi et al., 2014). Although most farmers in Kenya recognize that modern maize hybrids generally yield more than local open pollinated varieties, they believe that hybrid maize can perform well only under high input management practices (Omondi et al., 2014). The high cost of certified seed and fertilizer forces majority of farmers (most of whom are small scale) in Kenya to either use low-yielding farm-saved seeds derived from local varieties and/or apply fertilizers below the recommended rates (Omondi et al., 2014).

2.2 Diversity of maize germplasm in Africa

A range of maize landraces, improved local Open Pollinated Varieties (OPVs) and hybrids, as well as transgenic varieties exist and form major maize germplasm reservoirs in Africa (Westengen et al., 2014). Despite decades of attempts to formalize the African maize seed sector, the seed management fora and exchange rates taking place outside the formal institutions of variety development and distribution, that is "the informal seed system", supplies a far larger share of the seeds than the formal system in Africa especially in SSA (Westengen et al., 2014). Informal seed systems include saving from own harvest, farmer-to-farmer seed exchange, and purchase from local markets (Westengen et al., 2014). Maize is a diverse crop whose adaptations are highly location specific. The formal seed system has not been able to deliver adequate locally adapted varieties to the heterogeneous agroecologies in the region (Westengen et al., 2014). Some of the challenges facing maize seed system in SSA include predominantly poor seed quality (health and germination viability) as well as compromised genetic quality (purity) of the seed (Wambugu et al., 2012; Westengen et al., 2014).

Maize has enormous genetic diversity that offers incredible opportunities for genetic enhancement despite the challenges mentioned above (Prasanna, 2012). Knowledge on the genetic diversity and relationships among maize inbred lines is indispensable in identification of promising combinations for exploitation of heterosis and

establishment of heterotic groups for use as source materials in a breeding program (Prasanna, 2012).

The ability to broaden the genetic base of maize, breed climate-resilient and high-yielding maize cultivars adaptable to diverse agro-ecologies will undoubtedly depend on the efficient and rapid discovery and introgression of novel/ favourable alleles and haplotypes (Prasanna, 2012).

2.3 Biotic and abiotic constraints to maize production in SSA

Maize, like any other plant, faces a couple of growth-limiting factors (stresses) in the environment (Robert-Seilaniantz et al., 2010). Two types of these stresses exists namely; abiotic and biotic stresses (Pathi et al., 2013; Robert-Seilaniantz et al., 2010). Abiotic stresses are caused by drought, salinity, heat, cold, chilling, freezing, nutrient, high light intensity, ozone (O_3) and anaerobic stresses (Amara et al., 2013; Robert-Seilaniantz et al., 2010; Suzuki et al., 2014; Wambugu et al., 2012). The main abiotic stresses that affect plants and crops such as maize in the field have extensively been studied (Cairns et al., 2013; Mittler and Blumwald, 2010; Shiferaw et al., 2011; Suzuki et al., 2014). Of great concern is the current climate prediction models that indicate likelihood of gradual increase in ambient temperature that may result in high frequency and amplitude of heat stress in the near future (Mittler and Blumwald 2010; Mittler et al., 2012; Li et al., 2013; Suzuki et al., 2014). Biotic stresses on the other hand are caused by living organisms (Robert-Seilaniantz et al., 2010; Shiferaw et al., 2011). These living organisms, also known as pathogens include bacteria, viruses, fungi, oomycetes, plants and animals (Robert-Seilaniantz et al., 2010; Shiferaw et al., 2011). They are broadly categorized in two classes depending on their life style: biotrophs and necrotrophs. Biotrophic pathogens keep the plant cell alive and absorb nutrients from living tissues such as *S. hermonthica*, which parasitizes maize (Adetimirin et al., 2000) while Necrotrophic pathogens kill the living cells or tissues of the host and then feed on the dead matter (Robert-Seilaniantz et al., 2010; Shiferaw et al., 2011).

The most noxious biotic stress affecting maize production in SSA is *S. hermonthica* (Atera et al., 2013; Oswald, 2005). *S. hermonthica* has a devastating impact on maize and other cereal crops because of its effective competitive ability in depriving

the host plant of carbon, nitrogen and inorganic salts while at the same time inhibiting the growth and impairing the photosynthesis of its host (Atera et al., 2012). Control of this parasite is a challenge because it attacks the host plant under the ground and, by the time the flowering stem of the parasite appears above the ground, the damage has already been caused (Atera et al., 2012). Another biotic stress is maize diseases such as southern corn leaf blight (*Bipolaris maydis*), southern rust (*Puccinia polysora*), northern corn leaf blight (*Exserohilum turcicum*), common rust (*Puccinia sorghi*), gray leaf spot (*Cercospora spe*cies), stalk and ear rots caused by *Diplodia* and *Fusarium*, and kernel and ear rots caused by several *Fusarium* and *Aspergillus* species, which also contaminate grain with mycotoxins thereby reducing grain quality and safety (Shiferaw et al., 2011).

Therefore, to meet future food demands for the increasing population of SSA, development of maize varieties with enhanced tolerance to abiotic and biotic stress or combined effect of both is needed (Suzuki et al., 2014).

2.4 Rhizosphere microorganisms and their significance

Associations between plants and microorganisms are varied, complex and have been the subject of considerable research and diverse applications (Mastretta et al., 2006; Weyens et al., 2009). The microbe-plant interaction in the rhizosphere can be beneficial, neutral or deleterious for plant growth (Mastretta et al., 2006). The potential of exploiting natural antagonists (biocontrol) to control weeds such as *S. hermonthica* has received increasing attention (Charudattan, 2001; Gafar et al., 2015; Watson, 2013; Weyens et al., 2009). A considerable number of plant pathogens have been studied for their possible use as bioherbicides (Pilgeram et al., 2010). Only a few have proven to be adequately virulent to control weed species and to compete with commercial chemical herbicides (Pilgeram et al., 2010). This is because majority of plant-pathogenic bacteria and fungi evaluated for the control of weeds cause inconsequential disease symptoms or reductions in a weed population (Pilgeram et al., 2010).

There are two strategies by which microbial agents are often used for biological control of weeds: Classical and inundative approaches (Boyetchko et al., 2002).

Classical biological control is characterized by the importation, introduction and release of a natural enemy from the same geographic origin of the weed into an area where the weed is a problem. Following release, the natural enemy (bacterial or fungal pathogen) is allowed to self-perpetuate, survive and establish, thus providing long-term weed control over a period of several years (Boyetchko et al., 2002). Classical biological control is often more appropriate in rangeland where site disturbance is minimal. The inundative approach is also known as the bioherbicide approach (Boyetchko et al., 2002). The bioherbicide is defined as a plant pathogen used as a weed control agent through inundative and repeated applications of its inoculum (Charudattan et al., 2000). The pathogens are often indigenous, artificially mass-produced and applied during the growing season. Weed control is short term, compared to classical biocontrol agents, and the microbes are not expected to persist in the environment. The majority of microbes used as bioherbicides are predominantly fungal pathogens, while there are a growing number of examples of foliar and soil-applied bacterial agents being explored and developed as bioherbicides as well (Bailey et al., 2011).

Although limited work on bacteria as *S. hermonthica* suppressants has been reported, the potential of such an approach and the anticipated ease of application in comparison to other biological agents is likely to be embraced by anti transgenic farmers and consumers (Hassan et al., 2009). Among the microorganisms colonizing the root surface are bacteria of the genus *Pseudomonas* (Babalola, 2002). *Pseudomonas* sp., *Klebsiella oxytoca* and *Enterobacter sakazakii* have been evaluated for their potential to inhibit *S. hermonthica* seed germination (Babalola and Odhiambo, 2008). Other studies have also shown that *Pseudomonas fluorescens* and *Pseudomonas putida* isolates significantly inhibit germination of *S. hermonthica* seeds (Babalola et al., 2007). About 52 fungal species belonging to 16 genera have been isolated from diseased *S. hermonthica* plants with majority of them being pathogenic to the parasitic weed (Berner et al., 2003).

Of particular interest is the use of plant pathogenic fungi that infect *S. hermonthica* and other parasitic weeds including Orobanche spp. (Müller-Stöver et al., 2004; Nemat Alla et al., 2008; Watson, 2013). In West Africa, fungi of the genus *Fusarium*

have been isolated from diseased *S. hermonthica* plants and their potential of being biocontrol agent against *S. hermonthica* evaluated (Berner et al., 2003; Ciotola et al., 2000; Marley et al., 1999). Critical in the evaluation process is the specificity of the potent weed biocontrol agent to the target (weed) plant and non-pathogenicity to the *S. hermonthica* host crop (Beed et al., 2007;Elzein and Kroschel, 2006). For instance, *F. oxysporum f.sp.* (Foxy2) has specificity towards *S. hermonthica* species, and is non-pathogenic to cereal crops. It exerts pathogenicity on *S. hermonthica* seedlings while still underground by destroying the appressorium, the hyaline tissue, xylem vessels and cortical parenchyma (Elzein et al., 2010; Ndambi et al., 2011). In emerged *S. hermonthica*, the fungal hypha adheres to the root surface, penetrates and enters the apical region of the root, grows through the endodermis and reaches the xylem (Elzein et al., 2010; Ndambi et al., 2011).

However, host range studies show that *Solanaceous* plants (Irish potato, tomato and eggplant) are susceptible to Foxy2, hence discouraged in intercropping farming system (Musyoki et al., 2015; Zarafi et al., 2014). Other techniques such as formulation or encapsulation of fungal propagules in a solid matrix to prevent rapid desiccation or microbial competition have been developed (Aly, 2007). A successful example of granular formulation called "Pesta" showed high efficacy in controlling *S. hermonthica* and *O. Cumana* in the greenhouse (Elzein et al., 2008; Kroschel & Muller-stover, 2004; Zahran et al., 2008).

2.5 Plant Parasitism, its origin, Taxa and Diversity

Parasitism is a successful life strategy and means of survival that originated independently several times during angiosperm evolution, and today parasitic angiosperms are found in a variety of ecological habitats around the world (Timko et al., 2012; Runyon et al., 2010). Parasitic plants derive all or part of their nutrition from the host by attaching to the roots or aerial parts of a host species with an unique organ known as a haustorium that forms a physical and physiological conduit between host and parasite (Timko et al., 2012).

Parasitic plants vary widely in their degree of host dependence, some of them are facultative parasites (able to survive in the absence of hosts) while others are obligate

parasites and cannot develop independently (Runyon et al., 2010). Hemiparasitic plants possess chlorophyll and are able to produce some of their required nutrients through photosynthesis while holoparasitic plants lack chlorophyll and are completely dependent on host resources (Runyon et al., 2010). Another set of parasitic plants exist comprising of parasitic plants that make below-ground attachments (*Orobanche* and *S. hermonthica*) to host-plant roots and parasitic plants that attach above ground (*Dodders* and *Mistletoes*) to host-plant shoots (Aly, 2007; Runyon et al., 2010).

While many parasitic species remain innocuous members of larger plant communities, some (*Orobanche* and *S. hermonthica*) have established themselves as noxious and persistent pests in farmers' fields and pose a serious constraint to crop productivity (Timko et al., 2012).

Parasitic weeds such as *Orobanche* and *S. hermonthica* are difficult to control because the parasite has ability to produce numerous seeds with long fecundity period of more than 15 years and the intimate physiological interaction of the parasite with host plants (Aly, 2007; Mbuvi et al., 2016).

The high selection pressure associated with host co-evolution can drive a parasite's morphology and physiology in new directions, making parasites valuable subjects for studying diversification and the evolution of phenotypic changes associated with heterotrophy (Westwood et al., 2010).

2.5.1 Parasitism of *Striga hermonthica* and its significance

The genus *S. hermonthica* belong to Orobanchaceae family and includes 28 species and six subspecies, over 80% of which are found in Africa, while the rest occur in Asia and the United States (Runo et al., 2012). The name *S. hermonthica* (Latin for "witch"), and its common names, both in English (witchweed) and various African local names, refer to the host symptoms which appear before the parasite emerges normally as if a hex had befallen the crop (Rich and Ejeta, 2008; Spallek et al., 2013). Among the five major *S. hermonthica* species, *S. hermonthica* (Del.) Benth and S. *asiatica* Kuntze are the most important cereal weeds. *S. gesnerioides* (Willd.) Vatke is the only *Striga* species known to parasitize cowpea and other legumes and is

a serious constraint to legume production (Bozkurt et al., 2014; Runo et al., 2012; Spallek et al., 2013).

S. hermonthica is an obligate out-crossing species and hence has greater diversity within populations than seen in related autogamous species (Estep et al., 2011). *S. hermonthica* is an endemic parasitic weed of maize and other gramineous plants including sorghum, millet, rice, sugarcane, pasture and wild grasses in SSA (Avedi et al., 2014; Rich and Ejeta, 2008). Previously, the most widely affected crops were sorghum and pearl millet, but the problem has increased in maize, which is partly due to the increased acreage of maize (Parker, 2009). The successful lifecycle of *S. hermonthica* is attributed to its elevated fecundity and remarkable intimate synchronized host-parasite life cycle (Mbuvi et al., 2017). *S. hermonthica* population densities can reach thousands, sometimes millions, of individuals per hectare and more than one hundred thousand seeds per m^2 in the soil (Van Mourik, 2007). An individual *S. hermonthica* plant can produce more than a hundred thousand tiny seeds in a single season (Van Mourik, 2007). These seeds survive for only a few days after germination unless they attach to a host and establish xylem connection (Bouwmeester et al., 2003; Shen et al., 2006).

These tiny seeds are mainly dispersed by anthropogenic activity, but wind, water and forage animals have also been shown to play a dispersal role (Berner et al., 1994; Van Mourik, 2007). A seed bank study in the USA showed germination viability of upto 12 years after burial (Bebawi et al., 1984; Van Mourik, 2007). Other studies in Africa have shown considerable portions of the total seeds (>40%) germinated in any given growing season (Oswald et al., 2001; Van Mourik, 2007).

The life cycle of this noxious parasite is highly synchronized with that of the host and generally involves the stages of germination, attachment to host, haustorial formation, penetration, establishment of vascular connections, accumulation of nutrients, flowering and seed production (Runo et al., 2012). Seed of this parasite only germinate after sensing presence of specific chemical cues (strigolactones, sequiterpenes, and lactones) in root exudates of susceptible host plants (Bouwmeester et al., 2003; Yoder and Yoder, 1999; Yoneyama et al., 2010). Each

germinating *S. hermonthica* seed forms a radicle-like structure called haustorium (Cissoko et al., 2011). Upon forming haustoria, *S. hermonthica* elicits the host to produce a signal necessary for parasite development in a process termed semagenesis (Keyes et al., 2007). On contact with a host root, the haustorium develops a wedge-shaped group of cells that penetrates the host root cortex and endodermis to establish parasite-host xylem-xylem connections (Keyes et al., 2007). These root parasites depend on host plants for nutrients and water and cannot survive without parasitizing the host (Yoneyama et al., 2009). Once attached to the host root, the parasite grows towards the soil surface, emerges above ground and flowers to produce many tiny seeds (Cissoko et al., 2011). Beyond the burden of losing food and water to this parasite, host plants suffer from a characteristic malady resembling the symptoms of severe drought, including leaf scorching and increased root: shoot ratios (Rich et al., 2008; Satish et al., 2012).

In Kenya, *S. hermonthica* is the dominant parasitic weed in western Kenya, especially in areas such as Busia, Homabay, Siaya and Vihiga counties (Ajanga and Avedi, 2013; Jamil et al., 2012; Khan et al., 2008). This parasitic weed can cause crop losses as high as 70% (Khan et al., 2008), especially under low soil fertility and drought conditions (Avedi et al., 2014; Kamara et al., 2012; Stringer et al., 2009). In western Kenya, a survey revealed that 73% of the farms are infected with *S. hermonthica* (Atera et al., 2013). The average yield loss due to *S. hermonthica* menace is 1.15, 1.10 and 0.99 tons per hectare for maize, sorghum and millet, respectively (Atera et al., 2013). However, the damage can reach as high as 2.8 tons per ha in maize and sorghum in some locations with high *S. hermonthica* densities (Atera et al., 2013). The loss represents 12.3% of the 2.4 million metric tonnes of maize that Kenya produces annually (Atera et al., 2013). This translates to about 39.6 kg of maize loss per capita, amounting to about 20% of a typical person's annual food requirement (Atera et al., 2013). Clearly, this shows that the consequences of *S. hermonthica* infestation are severe and often render small-scale farmers helpless and often bewildered.

2.5.2 Current *Striga hermonthica* management strategies

Two strategies for *S. hermonthica* management have extensively been explored. One

aims at soil fertility improvement (improved crop rotation, use of mineral and organic fertilization) and the other aims at preventing *S. hermonthica* development and seed production through weeding, sowing of early maturing host-plant, integrating trap crops into the crop rotation and breeding of resistant host varieties (Ayongwa et al., 2011). *S. hermonthica* infestation is generally much less severe where water and soil fertility are optimal for crop growth (Rich & Ejeta, 2008). Growing conditions are, however, rarely optimal in Africa (Rich & Ejeta, 2008). *S. hermonthica* can be controlled through various cultural practices such as application of fertilizers and herbicides and also through genetic manipulation of crop plants, however these solutions are beyond the financial capacity of resource-poor farmers (Ayongwa et al., 2011; Rich et al., 2008).

Crop rotation with non-host crops reduces *S. hermonthica* seed bank but is becoming less feasible with the dwindling farm sizes in the populated rural areas (Sunda et al., 2013; Van Mourik et al., 2011). Intercropping with allelopathic legumes have also been proposed, though not widely deployed (Sunda et al., 2013; Van Mourik et al., 2011). Use of imazapyr resistant maize is toxic to other crops that are planted in the same hole, therefore, not suitable in mixed cropping system. Use of tolerant maize varieties such as GAF4 and KSTP'94, which does not kill the *S. hermonthica*, however does not reduce the weed seed bank. Use of *Fusarium oxysporum* that manages the *S. hermonthica* to a small extent, and the push and pull technology using desmodium, which is not feasible for non-dairy farmers, do not significantly control *S. hermonthica* (Müller-Stöver et al., 2004; Nemat Alla et al., 2008; Rich and Ejeta, 2008; Sunda et al., 2013; Watson, 2013).

Currently, Integrated *S. hermonthica* Management (ISM) strategies are being developed for the control of *S. hermonthica* in Africa, however affordability of such strategies by small scale holder farmers remains a challenge (Avedi et al., 2014; Beed et al., 2007; Berner et al., 2003; Vanlauwe et al., 2008; Venne et al., 2009).

Most of the above-mentioned recommended weed control measures have not been successful in addressing the *S. hermonthica* problem, while others are not sustainable (Bozkurt et al., 2014; Kanampiu et al., 2002; Sunda et al., 2013). Persistent effort in

the identification of sources of resistance genes and development of improved selection schemes holds potential of controlling *S. hermonthica* menace (Rich & Ejeta, 2008). The ability of *Striga*, particularly *S. hermonthica*, to overcome resistance is known (Bozkurt et al., 2014; Rich & Ejeta, 2008). Deliberate stacking of resistance traits (genes) into deployed cultivars decreases likelihood of resistance breakdown (Rich and Ejeta, 2008). Virulent *S. hermonthica* races would be less likely to emerge if multiple mutations were required to overcome host resistance genes (Rich & Ejeta, 2008).

In the short-run, improved crop varieties with resistance to *S. hermonthica* remain the more feasible technology for the resource poor small-scale farmers in SSA (Rich & Ejeta, 2008). Integration of various cost-effective *S. hermonthica* control options with improved agronomic practices will be the ultimate solution to *S. hermonthica* menace (Rich & Ejeta, 2008).

2.6 Phosphorus Conundrum in Ecosystems and Agriculture

Phosphorus (P) is an integral element of all living cells and organisms (Zhang et al., 2012). In plants, phosphorus (P), is the second most essential nutrient after nitrogen, that is required for different biochemical processes, plant development, reproduction and growth (López-Arredondo et al., 2014; Mehra *et al*, 2015). Phosphorus also contributes to cellular signaling cascades by functioning as the mediators of signal transduction and its ester bond universally serves as a vital energy source for a wide range of biological functions (Ha and Tran, 2014). Inorganic phosphate (Pi) is the only form of P that can be assimilated by plant roots from soil. Availability of Pi in the soil is a great challenge in agriculture and agroecosystems (Miller et al., 2001).

It is estimated that approximately 70% of the world's arable lands suffer from Pi deficiency, and its concentration is far below the optimum level for best plant growth (López-Arredondo et al., 2014; Wang et al., 2013) which in turn limits crop growth and yield (Younessi-hamzekhanlu et al., 2016). This is because, although the total rhizosphere phosphate content is quite high, the concentration of utilizable Pi by plants in soil solution is usually about 1mM (Baker et al., 2015; Li et al., 2007; Schachtman et al., 1998; Taurian et al., 2010). Studies have shown that more than

80% of P becomes immobile and unavailable for plant uptake because of adsorption, precipitation or conversion to organic form (Kapri & Tewari, 2010; Lambers and Plaxton, 2015; Taurian et al., 2010). This paradox is because phosphorus forms sparingly soluble phosphate compounds with either aluminium or iron ($FePO_4$ and $AlPO_4$) in acidic soils and with calcium ($Ca_3(PO_4)_2$) in alkaline soils yet plants preferentially take up phosphorus in the inorganic form of hydrogen phosphate (H_2PO_4) or orthophosphate(Pi) (Kapri & Tewari, 2010). Insufficiency of bioavailable Pi in soil and lack of rock Pi reserves are potential Pi crisis for agriculture in the future (Baker et al., 2015; Heckenmüller et al., 2014; Li et al., 2007).

To cope with inadequate Pi supply, plants activate a set of adaptive responses. Such counter-measures include the redesign of root morphology, adjustment of metabolism pathways, synthesis increment of APase and Pi transporter proteins (Heckenmüller et al., 2014; Jones et al., 2004; Li et al., 2007; Raghothama, 1999; Zhu & Lynch, 2004). However, these measures are not sufficient enough to stop continuous phosphorus application in agricultural farms.

The need for continuous phosphorus input in agriculture can be attributed to two different rationales. First, phosphorus is removed with each farm harvest at a higher rate than the soil can naturally provide (Baker et al., 2015; Van Vuuren et al., 2010). This is because the natural rate of phosphorus replenishment from the environment to the soil cannot adequately sustain perennial farming. Therefore, phosphorus needs to be supplied artificially in order to keep crop yields constant and the soil's phosphorus stock from getting depleted (Baker et al., 2015; Van Vuuren et al., 2010). The second reason is, there is a strong economic incentive for farmers to apply large quantities of phosphate fertilizer during farming in order to realize high crop yields (Baker et al., 2015; Heckenmüller et al., 2014). Whereas some of these phosphorus inputs are returned to arable land in the form of manure and crop residues, the majority is not (Heckenmüller et al., 2014). Instead, various inefficiencies along the value-chain in fertilizer and food production as well as natural soil erosion entail considerable phosphorus losses to the environment. Eventually, a significant amount of phosphorus ends up in the aquatic environment (Baker et al., 2015; Cordell et al., 2009). The two rationales therefore clearly indicate that volatile phosphate rock and

unpredictable fertilizer prices pose a risk on crop farming, especially to farmers in developing and emerging nations (Baker et al., 2015; Heckenmüller et al., 2014).

2.7 Molecular Plant Responses to low Phosphorus

Phosphorus (P) is an essential nutrient for plant growth: it is required for the majority of metabolic processes, including photosynthesis and respiration, and is a key structural component of macromolecules such as phospholipids and nucleic acids (González-Muñoz et al., 2015). Phosphate esters in general act as energy carriers in various metabolic pathways while phospholipids play an important role in membrane integrity and function (Calderon-Vazquez et al., 2011). In addition, phosphorylation and dephosphorylation of proteins are crucial for signal-transduction pathways in plants (Raghothama and Karthikeyan, 2005). Furthermore, phosphate homeostasis in the chloroplast regulates the transport of phosphorylated sugars across the membrane and synthesis of starch (Raghothama & Karthikeyan, 2005).

In agricultural systems P is typically limiting and, as a consequence, large quantities of P additions are required to maintain productivity (Schröder et al., 2011; Temegne et al., 2015). It is now widely accepted that global P reserves are limited and need to be better managed to achieve maximum benefit (Schröder et al., 2011). Although rhizosphere P content is high, plants acquire phosphorus as phosphate anions from the soil solution (González-Muñoz et al., 2015). This form of P is one of the least available plant nutrients found in the rhizosphere (González-Muñoz et al., 2015). This phosphorus deficiency is considered to be one of the major limitations for crop production, particularly in the tropics (Li et al., 2012).

Interestingly the ability of plants to acquire Pi increases significantly under Pi deficiency as shown by the great disparity in distribution of Pi between plant cells which is mM quantities and soil solution which is in μM quantities (Raghothama et al., 2005; Temegne et al., 2015). The performance of a genotype grown under low-P conditions relative to its performance under high-P conditions can be considered as its P tolerance (Leiser et al., 2015).

A combination of efficient uptake and translocation mechanisms of Pi is essential for plants to maintain adequate levels of cellular Pi necessary for normal functioning

(Rose et al., 2013). Plants have to acquire Pi against a steep concentration gradient (three orders of magnitude or greater) across the plasma membrane (González-Muñoz et al., 2015). In addition electrical gradients also play a major role in nutrient uptake. Many uptake models have been proposed to explain the plant's ability to acquire Pi both under deficiency and sufficiency conditions (González-Muñoz et al., 2015). A dual uptake model for ions involving both the high- and low-affinity uptake mechanisms is widely used to explain the concentration-dependent acquisition of Pi (Raghothama & Karthikeyan, 2005). Both soil and plant factors such as Pi supply to roots by mass flow and diffusion, changes in root geometry and size, Pi uptake in relation to Pi concentration at the root surface, and root competition influence Pi uptake by plants (Raghothama & Karthikeyan, 2005).

There is genetic and molecular evidence for the presence of transporters operating efficiently at both high and low concentration of Pi in the medium (Baker et al., 2015; Chen et al., 2008). Interestingly, the low-affinity transport system appears to be expressed constitutively in plants; in contrast, the high-affinity uptake system is strongly enhanced during Pi deficiency (Chen et al., 2008). In general the orthophosphate ion (H_2PO_4) is the preferred form of phosphate for translocation by transporters (Tang et al., 2013).

Plants have evolved two broad strategies for P acquisition and use in nutrient-limiting environments. The first strategy aims at conservation of use of P and the second strategy is directed toward enhanced acquisition or uptake of P (Vance et al., 2003). Processes that conserve the use of P involve decreased growth rate, increased growth per unit of P uptake, remobilization of internal Pi, modifications in carbon metabolism that bypass P-requiring steps, and alternative respiratory pathways (Vance et al., 2003). By comparison, processes that lead to enhanced uptake include increased production and secretion of phosphatases, exudation of organic acids, greater root growth along with modified root architecture, expansion of root surface area by prolific development of root hairs, and enhanced expression of Pi transporters (Li et al., 2007). To cope with inadequate Pi supply, plants activate a set of adaptive responses (Li et al., 2007). Such countermeasures include the remodeling of root architecture (Tian & Liao, 2015), Arbuscular mycorrhizal (AM) fungi and

rhizobia symbioses with roots, adjustment of metabolism pathways, elevated synthesis of APase and Pi transporter proteins (Baker et al., 2015; Guo et al., 2011; López-Arredondo et al., 2014), to increases the surface area for Pi uptake. Root morphology may be especially important for P acquisition efficiency since the relative immobility of P makes its acquisition dependent on soil exploration in time and space (Li et al., 2007).

2.7.1 *Lupinus albus*, *Medicago truncatula* and their adaptations to low P

White lupin (*Lupinus albus L.*) is a species of the genus *Lupinus*, family Leguminosae (Christian, 1997). *Lupinus albus* is a nitrogen-fixing legume plant that can grow in soils with poorly available nutrients and low pH, despite its lack of mycorrhizal symbioses (Esteban et al., 2003; Gilbert et al., 1999; Kerley, 2000; Sbabou et al., 2010; Skene, 2000). Four species of this genus (*L. albus, L. angustifolius, L. luteus* and *L. mutabilis*) are cultivated in the world with three main uses namely; for human nutrition because of their high protein and oil contents, as a green manure contributing to improved soil structure with an increase of the organic matter content and through nitrogen and phosphorus accumulation in poor sandy soils and as such, is it used as a pioneer crop in land reclamation (Esteban et al., 2003; Skene, 2000). It is also used as ruminant feed either as green forage in the areas of traditional cultivation or as grains introduced as protein supplements in the diets of ruminants (Christian, 1997).

Medicago truncatula is an annual plant from the *Fabaceae* family and a close relative of alfalfa (Ané et al., 2008). Key attributes of *M. truncatula* include its small, diploid genome (~5 $X10^8$ bp); its self-fertile nature; its prolific seed production; and its rapid regeneration time. Numerous ecotypes of *M. truncatula* have been collected throughout its Centre of origin in the Mediterranean Basin, and because these populations exhibit considerable phenotypic variation for features such as growth habit, flowering time, symbiotic specificity, and disease resistance they represent an important resource to examine the genetic basis of legume functions (Cook, 1999).

Growth of white lupin is more vigorous than any other plant under condition of low phosphorus (Tang et al., 2013; Wasaki et al., 2003). It has a suite of adaptations to P

deficiency that facilitates efficient P acquisition, including a highly synchronous, coordinated expression of genes which results in proliferation of densely clustered roots, root exudation of organic acids and acid phosphatase, as well as the induction of numerous transporters (Neumann et al., 2002; Schulze et al., 2006; Tang et al., 2013; Uhde-Stone et al., 2003; Vance et al., 2003). These roots excrete organic acids exudates (mainly citrate and malate) and acidify the rhizosphere, which allows the mobilization of phosphate bound to Ca, Al and Fe and also from P-adsorbing Fe/Al humic acid complexes (Neumann et al., 2000; Sbabou et al., 2010). It also exudes acid phosphatases that hydrolyse organic forms of phosphate (Tang et al., 2013). A novel isoform of acid phosphatase (orthophosphoric-monoester phosphohydrolyases) is induced in proteoid roots under P-deficient conditions, hydrolysing mono-ester soil organic P at low pH, and thereby increasing orthophosphate availability (Gilbert et al., 1999; Sbabou et al., 2010). Moreover, the formation of cluster roots results in an increase in root surface area, thereby providing enhanced zones for P uptake (Lamont, 2003; Schulze et al., 2006; Tang et al., 2013).

2.8 Purple Acid Phosphatase (PAPs)

Adaptive mechanisms that help plants survive and grow under Pi deprivation include, the ability of the plant to access the 80% of Pi that is immobile and not readily available to plants via production of PAPs (González-Muñoz et al., 2015; Wang et al., 2013). These enzymes catalyze Pi hydrolysis from a broad range of phosphomonoesters that cleaves Pi from ester linkage sites. During Pi stress, intracellular APases remobilize and recycle Pi from expendable P-monoesters and anhydrides of older tissues. This is accompanied by a marked reduction in cytoplasmic P-metabolites during extended Pi stress (Tawaraya et al., 2014; Tran et al., 2010). Extracellular APases belong to a group of Pi starvation inducible (PSI) phosphohydrolases secreted by roots and cell cultures of P-deficient plants to scavenge and hydrolyse Pi from external organophosphates, the predominant form of P in soil solutions (Richardson et al., 2009; Tawaraya et al., 2014; Tran., et al., 2010). *PAPs* represent a specific class of PSI characterized by their pink or purple colour in solution (because of a bimetallic active centre), and insensitivity to l-tartrate inhibition (Tawaraya et al., 2014; Tran et al., 2010).

Among these APases, purple acid phosphatases (PAPs) are primarily non-specific, the largest group and special class of APases. These enzymes exhibit distinct characteristics including a purple color in aqueous solution and they contain a bimetallic site (González-Mendoza et al., 2013; Tawaraya et al., 2014). Purple Acid phosphatases are known plant enzymes with phytase activity i.e. they initiate sequential liberation, transport and recycling of orthophosphate groups from phytate compounds, which is crucial for cellular metabolism and bioenergetics (Dionisio et al., 2011; Qian, et al., 2010). PAPs release Pi from a broad spectrum of P-monoesters over a wide pH range (4-7) and they are commonly found in a wide range of plant species (Younessi-hamzekhanlu et al., 2016).

Lupinus albus and *Medicago truncatula* are highly efficient with respect to P uptake and the utilization of sparingly available sources of soil Phosphorous (Christian, 1997; Cook, 1999; Tang et al., 2013). Under P deficient conditions, the secretion of PAPs from *L. albus* and *M. medicago* is high and usually catalyze the hydrolysis of organic P thereby increasing P available to these plants (Richardson et al., 2004; Tang et al., 2013). The expression of PAP gene in other plant is relatively low while in others its absolutely absent (Richardson et al., 2004).

It has been documented that transgenic plants overexpressing *PAP* genes show altered root architecture under various P levels (Kong et al., 2014). In addition, some of these genes participate in root growth, including mutation in AtPAP10, and pap12 /pap26 double mutant (mutation in both PAP12 and PAP26) lead to attenuated root growth (Younessi-hamzekhanlu et al., 2016)

2.9 Phosphorus, *Striga hermonthica* and *Arbuscular Mycorrhizal* Fungi nexus

The *Arbuscular mycorrhiza* is the name given to a symbiotic association of plants and *Arbuscular mycorrhizal* (AM) fungi (Gomez et al., 2009). The AM symbiosis occurs widely throughout the plant kingdom (especial terrestrial plants) and involves angiosperms, gymnosperms, pteridophytes and some bryophytes (Gomez et al., 2009; Smith & Read, 2008;Yoneyama et al., 2015). The fungi that participate in this symbiosis are all members of the Glomeromycota (Gomez et al., 2009). Because of the low mobility of phosphorus in the soil solution, plants rapidly create a depletion zone for phosphorus around the root. The symbiosis develops in the plant roots,

where AM fungi form extensively branched hyphae, called arbuscules, in the cortical cells (Gomez et al., 2009). The external hyphae of AM fungi facilitate phosphorus uptake by extending beyond the depletion zone (Gomez et al., 2009). A root associated with AM fungi may transport phosphate at a rate more than four times higher than that a root without AM fungi. Inorganic phosphate (Pi) and nitrogen (N), acquired by the extra-radical hyphae, particularly under P-deficient conditions, are translocated to the arbuscules and released to the plant (Gomez et al., 2009; Smith & Read, 2008;Yoneyama et al., 2015). In return, the plant provides the fungus with carbon. Phosphorus and nitrogen are the two mineral nutrients that plants require in the greatest quantities and therefore the symbiosis has an important influence on plant health and consequently on ecosystem function (Gomez et al., 2009).

There has been significant progress in understanding the signaling process associated with the early stages of development of AM symbiosis (Gomez et al., 2009). Some of the signal molecules produced by host plant have been identified, including strigolactone, which act as pre-contact signals to stimulate AM fungal growth and metabolism, which increases probability of contact between the fungi and the roots of the host (Gomez et al., 2009). Strigolactone stimulates not only hyphal branching of AM fungi but also *S. hermonthica* seed germination (Akiyama et al., 2005;Yoneyama et al., 2015).

The relationship of roots of some AM-host plant with the parasitic weeds *S. hermonthica* and *Orobanche* is a known case in which major parallelism with the AM signaling pathway has been observed (Garcia-Garrido et al., 2009; Mbuvi et al., 2017). This is because, signaling mechanism of AM fungi–host plant root interactions seems to have been adapted by the parasitic plants to establish a parasitic relationship with roots of the same host plant (Garcia-Garrido et al., 2009). Studies have shown that deficiency in mineral nutrients, especially phosphorus (P) and nitrogen (N), promotes strigolactone exudation into the rhizosphere by host plants (Jamil et al., 2007, 2012;Yoneyama et al., 2015). The exudates are aimed at stimulating the symbiotic relationship between host plant and *Arbuscular mycorrhizal* (AM) fungi that can help the plant overcome nutrient deficiency (Bouwmeester et al., 2007, 2003). However, parasitic plants also use these signaling

molecules to detect the presence of a suitable host (Jamil et al., 2012).

2.10 *Agrobacterium*-mediated plant transformation: Biology and Biotechnology

Agrobacterium species are known as the only organisms capable of inter-kingdom gene transfer (Pitzschke and Hirt, 2010). This soil-borne Gram-negative bacterium is a broad-host range plant pathogen, which initiates tumour formation on most dicotyledonous and some monocotyledonous species (Pitzschke and Hirt, 2010).

Such tumours do not require the continuous presence of the bacteria for proliferation, showing that the plant cells have been transformed genetically (Pitzschke and Hirt, 2010). This is because *Agrobacterium*-plant interaction is a highly complex molecular choreography process that is greatly controlled by bacterial Virulence (Vir) proteins that impinge on multiple processes including transport, transcription, and chromosome status of the host cells.

The factors required for tumour formation are encoded on a large tumour-inducing (Ti) plasmid of virulent *Agrobacterium* strains. The Ti plasmid also serves as a source for the transfer DNA (T-DNA), a DNA region that is imported into plant cells and integrated into the host chromosomal DNA resulting in genetic manipulation of the host (Pitzschke and Hirt, 2010). The expression of T-DNA-encoded bacterial genes in the host cell results in the production of enzymes that catalyze the synthesis of plant hormones, which are responsible for tumour growth and the formation of novel amino acid–sugar conjugates, termed as opines. As opines can serve as carbon and sometimes nitrogen sources for *Agrobacterium* to the exclusion of most other microorganisms, they provide a selective advantage for this species (Pitzschke and Hirt, 2010).

The capacity for gene transfer into plants has been explored to develop *Agrobacterium tumefaciens* as a vector for genetic manipulation of plants (Pitzschke and Hirt, 2010). Engineered DNA segments of interest, which are first cloned into the T-DNA region of ‘disarmed’ plasmids, are then introduced into *Agrobacterium* and subsequently transferred into plants. The plasmids are ‘disarmed’ through a process in which genes responsible for the growth of tumours are removed this ensures that the transformed cells can be regenerated into fertile plants that transmit

the engineered DNA to their progeny (Pitzschke and Hirt, 2010).

The molecular perceptive of the interaction between *Agrobacterium* and plant cells is divided into several steps comprising of: recognition of the host, expression of virulence (*vir*) genes, attachment to the host cell, targeting of Vir factors and T-DNA into the host cell, and chromosomal T-DNA integration (Pitzschke and Hirt, 2010).

Induction of *Agrobacterium vir* genes depends on production of phenolic molecules such as acetosyringone by the host plant. Acetosyringone is now routinely used artificially for enhancing the efficiency of *Agrobacterium*-mediated plant transformation (Pitzschke and Hirt, 2010). Six types of bacterial *vir* genes (coded as *vir A, B, D, E, F* and *G*) are involved in the entire process of transformation. The production of phenolic molecules by the host plant activates *virA* and *virG* of *Agrobacterium*. The capability of the *virA*/*virG* system to recognize a diversity of plant phenols and sugars is a likely explanation for the broad-host range exhibited by *Agrobacterium* (Pitzschke and Hirt, 2010). The *virA* and *virG* genes encode a two-component phospho-relay system in which VirA is a membrane-bound sensor and VirG is the intracellular response regulator. On signal sensing, the histidine kinase VirA activates VirG through transferring its phosphate to a particular aspartate of VirG, thereby activating VirG to function as a transcription factor. Phosphorylated VirG then binds at specific 12 bp DNA sequences of the *vir* gene promoters (*vir* boxes), thereby activating transcription (McCullen and Binns, 2006). Attachment of *Agrobacterium* to plant cells is mediated by chromosomally encoded *Agrobacterium* genes *chvA*, *chvB*, and *pscA* (exoC).

After *vir* gene activation and attachment of *Agrobacterium* to plant cells, a transporter complex formed by VirB proteins and VirD4 enables Vir proteins and T-DNA to cross the inner bacterial membrane, the peptidoglycan layer, and outer membrane, as well as the plant host cell wall and membrane. The VirB complex belongs to the class of type IV secretion systems (T4SS), which are found across a broad range of Gram-negative bacteria and are involved in the conjugative transfer of plasmids between bacteria as well as the translocation of Vir factors from pathogens to host cells during infection (Cascales and Christie, 2003).

The bacterial factors transported into host cells by the VirB complex include the VirD2-T-DNA, VirE2, VirE3, VirF, and VirD5 (Vergunst et al., 2005). Once inside the plant cell, the T-DNA must find its way into the nucleus. The proteins VirD2 and VirE2 contain plant-active nuclear localization signal (NLS) sequences. VirD2, which is covalently linked to the 50 bp-end of the T-DNA, contains two NLS regions, both of which can direct chimeric proteins to the nucleus. VirE2 protein contains two separate bipartite NLS regions that can target fusion reporter proteins to plant nuclei. VirF is involved in the degradation of host cell defense factors during infection. In the host nucleus, VirF, in concert with the host proteasome machinery, also mediates degradation of the T-DNA complex, thus facilitating the release of the T-DNA and its subsequent chromosomal integration (Tzfira et al., 2004).

Relatively little is known about the precise mechanism of T-DNA integration into the plant genome or the function specific proteins have in this process. The major mode foreign DNA integrates in plants is by illegitimate recombination or non-homologous end joining and T-DNA integrates into plant chromosomes by a similar mechanism. However, this transformation process has its own share of molecular obstacles. For instance, the opines produced by host plant stimulates the synthesis of a quorum-sensing (QS) signal called 3-oxo-octanoylhomoserine lactone (OC8HSL) in bacterial cells (Haudecoeur et al., 2009). This signal in turn activates the horizontal transfer and amplification of the copy number of the Ti plasmid, thereby increasing the aggressiveness of *A. tumefaciens* populations and contributing to the dissemination of the Ti plasmid (Haudecoeur et al., 2009).

In response to *A. tumefaciens* infection, plants activate a complex program of defense which includes the synthesis of γ-aminobutyric acid, abbreviated as GABA (Djamei et al., 2007; Haudecoeur et al., 2009; Yuan et al., 2007; Zipfel et al., 2006). In *A. tumefaciens*, GABA promotes degradation of the OC8HSL signal by the lactonase AttM, thereby attenuating expression of the OC8HSL dependent functions (Haudecoeur et al., 2009). Paradoxically, tumours simultaneously accumulate opines and GABA which induce two opposite functions in *A. tumefaciens* (Haudecoeur et al., 2009). Because the plant tumour is a unique ecological niche in which *A. tumefaciens* expresses OC8HSL-dependent functions including horizontal transfer of

the Ti plasmid (white and winans, 2003), this bacterial pathogen has evolved mechanisms to escape plant GABA-defense such use of proline (White and Winans, 2007).

Plant cells produces proline depending on nitrogen source assimilated and is involved in the defense response of the host plant challenged by bacterial pathogens where it contributes to the hypersensitive response and cell death of infected tissues (Haudecoeur et al., 2009). Proline antagonizes the GABA-induced degradation of OC8HSL. Therefore proline, which accumulates in the tumour, could be used by the pathogen to by-pass the plant GABA-defense (Haudecoeur et al., 2009).

2.11 Progress in Regeneration and Transformation of Maize (*Zea mays L.*)

Reliable *in vitro* regeneration techniques permitting the production of whole maize plant from single or a group of cells is of central importance to both clonal propagation and successful genetic engineering of maize (Ahmadabadi et al., 2007). Green and Philips (1975) first reported *in vitro* maize regeneration from immature embryos. Since then, many different explants, such as shoot apices, leaf and stem segments, hypocotyls, epicotyls, immature embryos and mature intact seeds, have been tested for their regeneration capacity (Oneto et al., 2010).

In maize, successful whole plant *in vitro* regeneration has been reported from immature embryos (Aguado-Santacruz et al., 2007; Bohorova et al., 1995; Duncan et al., 1985; Ishida et al., 1996), mature embryos (Al-Abed et al., 2006; Huang and Wei, 2004), nodal regions (Vladimir et al., 2006), leaf tissues (Ahmadabadi et al., 2007; Conger et al., 1987), anthers (Barloy and Beckert, 1993; Ting et al., 1981), tassel and ear meristems (Pareddy and Petolino, 1990), protoplast (Morocz et al., 1990), and shoot meristems (Sairam et al., 2003). However, immature zygotic embryos are predominantly used for establishing regeneration competent cells or callus cultures for genetic transformation (Frame et al., 2011; Gui-rong et al., 2013; Wang et al., 2012).

Transgenic maize plants were first obtained from protoplasts by an electroporation method (Rhodes et al., 1988), but fertile plants have never been produced by this method. Other direct gene transfer methods, which did not require the prior culture

of protoplasts, were then tried (D'Halluin et al., 1992; Frame et al., 1994; Fromm et al., 1990), and microprojectile bombardment of cells in suspension cultures or immature embryos became quite popular in basic and applied studies (Koziel et al., 1993). Gordon Kamm et al., (1990) was the first scientific team to established bialophos resistant transgenic maize. In 1993, Koziel et al., developed the first insect-resistant transgenic maize with *cry* 1Ab. Efficiency of transformation by microprojectile bombardment has been higher than other direct methods however, quite a few fertile plants are regenerated by this technique (Armstrong, 1993). Microprojectile bombardment is also useful for the analysis of the transient expression of foreign genes in intact and fully developed tissues. High copy numbers and extensive rearrangement of the foreign DNA have however been frequently reported in plants transformed with most of direct gene transfer methods (Register, 1994; Shou et al., 2004).

The first successful *Agrobacterium*-mediated transformation was reported using A188, an amenable maize inbred (Cho et al., 2014). Other successful maize transformation findings in Hi-II, a hybrid have also been reported (Cho et al., 2014). In addition, Huang and Wei (2005) developed an *Agrobacterium*-mediated transformation system for maize inbred lines using EHA105 with optimal culturing and infection conditions (Cho et al., 2014). A major advantage of *Agrobacterium*-mediated transformation is the ability to deliver a small number of copies (often one or two) of relatively large segments (can be larger than 10 kb) of T-DNA. The T-DNA has defined ends that often integrate stably into the plant genome with minimal rearrangement, resulting in transgenic plants of high quality (Cho et al., 2014). Initially, it was not clear if this technology could be extended to monocotyledonous plants, as they are not natural hosts of *A. tumefacien*. Nevertheless, a highly efficient method of transformation of rice by *A. tumefacien* was reported by Hiei et al., (1994), followed by successful reports of the *A. tumefacien*-mediated transformation of important cereals such as maize (Ishida et al., 2003), wheat (Cheng et al., 1997), barley (Tingay et al., 1997) and sorghum (Zhao et al., 2000). Key factors in these achievements include the optimization of types of explant material for infection with

A. tumefacien, choice of vectors, choice of *A. tumefacien* strains and optimization of tissue culture techniques (Ishida et al., 2007).

For the successful production of transgenic plants in any species, foreign genes must be delivered to undifferentiated, dedifferentiated or dedifferentiating cells that are actively dividing or about to divide and that are capable of regenerating plants. In maize, the material of choice is immature embryos, and all protocols mediated by particle bombardment or *A. tumefacien* for efficient production of transgenic maize have solely employed immature embryos (Ishida et al., 2007). Thus, the primary determinants of a successful transformation are the response of immature embryos in tissue culture, the types of cells that grow from immature embryos and subsequent characteristics in growth and regeneration. Unfortunately, many varieties of maize, especially the so-called elite varieties, are poor in these aspects, and thus only a limited number of varieties (adapted to temperate regions) have been efficiently transformed so far (Bohorova et al., 1995; Prioli et al., 1989).

Most of *Agrobacterium*-mediated maize transformation protocols in principle follow the five step procedure namely; *Agrobacterium* infection, co-cultivation, resting, selection and finally regeneration (Frame et al., 2002; Ishida et al., 2007; Zhao et al., 2001). Different studies have been done to optimize maize transformation frequency by introducing modifications both in the steps and media used for transformation, however Ishida *et al.* (2007) protocol remain the most popular and widely acceptable protocol.

Although *Agrobacterium*-mediated transformation is the most commonly used method for maize transformation, the resulting transgenic plants are of course subject to biosafety issues related to the presence of vector backbone sequences and/or selectable marker genes, irrespective of the delivery method used (Barampuram and Zhang, 2011).

There is therefore a need to develop advanced transformation methods that would not only incorporate the required characteristics (stable and desirable transgene integration and expression) into plants but also enable generation of transgenic events in a high-throughput manner (Barampuram and Zhang, 2011). These

requirements are particularly relevant now in the crop post-genome era in which ever-increasing amounts of genome sequence information, BAC clones, ESTs, and full-length cDNAs are available (Barampuram and Zhang, 2011). This situation presents both new challenges and opportunities for plant transformation research.

The use of the Phosphomannose isomerase (PMI) gene as a selectable marker in plant biotechnology has been steadily increasing during the last decade (Stoykova and Stoeva-Popova, 2011). Its appropriateness comes from its absence in plant genomes as well as the fact that Mannose is in itself not toxic to plants (Stoykova and Stoeva-Popova, 2011). The Mannose A (*manA*) gene has been incorporated into different constructs and successfully expressed under CaMV35S, CMPS, actin, or ubiquitin promoters, of which the most commonly used is the CMPS promoter. This gene has also been successfully transferred to maize (Stoykova and Stoeva-Popova, 2011).

3.2.7 Genetic transformation of maize with novel traits against biotic stresses

Formidable efforts have been made to generate biotic resistant transgenic crops. For instance, production of herbicide resistant crops has been achieved via insertion of either one or two genes that encode inactivation of the herbicide by either overproduction of a herbicide-sensitive biochemical target, alteration in biochemical target resulting in altered binding sites to the herbicide or detoxification or degradation of the herbicide, before it reaches its target site in the plant cell (Chaudhary et al., 2014).

Gordon-Kamm *et al.* (1990) reported transformation of cells from embryogenic maize suspension cultures with the bacterial gene bar encoding for the enzyme phosphinothricin acetyltransferase (PAT) that inactivates the herbicidal compound phosphinothricin (PPT) by acetylation (Chaudhary et al., 2014). Likewise, resistance to maize has been incorporated for non-selective herbicides such as glufosinate (GLU) which disrupts the enzyme pathway for glutamine synthetase and hence the formation of acid glutamine from glutamate is prevented, which leads to the accumulation of ammonia to levels that are toxic to the plant and glyphosate (GLY) which inhibits the shikimate pathway in plants, in turn inhibiting the biosynthesis of

amino acids important for protein production and plant growth (Chaudhary et al., 2014; Devos et al., 2008; Yann Devos et al., 2008; Zhu et al., 2000).

The development of insect-resistant crops was initiated by the discovery that a Gram-positive soil bacterium, *Bacillus thuringiensis* (Bt), produces insecticidal crystal proteins (δ-endotoxins) during sporulation (Chaudhary et al., 2014). The initial attempts to confer insect resistance by insertion of the crystal gene (*cry*) coding for δ-endotoxin did not provide expected levels of insect resistance due to low *cry* gene expression, partly due to the high A–T content of the gene, which was subsequently modified to fit the higher level G–C content of plant genes, and especially monocot genes (Chaudhary et al., 2014). Expression of the altered versions of cry genes, *cry*lA(b) and *cry*lA(c), resulted in a 100-fold higher level of δ–endotoxin production in corn (Chaudhary et al., 2014).

Transgenic corn expressing the *Bt*-gene *cry*1A(b) was developed in the USA for protection against the devastating European corn borer, *Ostrinia nubilalis*, and was approved for commercial cultivation in the 1990s (Chaudhary et al., 2014). In elite tropical maize lines, CML67, CML72 and CML216, direct transformation of δ-endotoxin, *cry*lA(c), expressing varying levels of resistance to South-western corn borer has been done (Chaudhary et al., 2014). Transgenic corn plant (MON 810), expressing the *Bt* protein, CrylA(b), was reported to provide effective protection against maize stem borer, *Chilo partellus*, even under high level of larval infestation in the greenhouse (Chaudhary et al., 2014; Singh et al., 2005). Various Cry proteins entailing resistance genes against insect-pests, namely, *cry*lA(b), *cry*IA(c), *cry*IF, *cry*3B(b), *cry*34Ab1, *cry*35Ab1, modified *cry*3A, cry1A.105, *cry*2Ab2 and *Vip*3Aa20 have been widely used in maize transformation (Chaudhary et al., 2014).

The *cry* genes exhibit specificity for different insect species, and each protein is active in only one or a few insect species, specificity to a large extent determined by the toxin-receptor interaction, although solubility of the crystal and protease activation also play a role (Chaudhary et al., 2014). The members of the *cry* gene family are grouped in subfamilies according to their specificity for members of the insect families *Lepidoptera* (caterpillars), *Diptera* (flies and mosquitoes) and

Coleoptera, beetles (Chaudhary et al., 2014). It is also significant that several important insect-pests appear to be insensitive to known Cry proteins e.g. the corn rootworm, aphids and white flies (Chaudhary et al., 2014).

To overcome this limitation another source of resistance against insect-pests was used, gene *gna* encoding for snowdrop lectin under control of phloem-specific promoter taken from *Galanthus nivalis* L (Chaudhary et al., 2014). This toxic agglutinin is toxic to insects such as corn leaf aphid (*Rhopalosiphum maidis*) under greenhouse conditions (Chaudhary et al., 2014; Wang et al., 2005). The toxicity of the endotoxins was significantly enhanced by use of fusion proteins like BtRB, combining the endotoxin Cry1A(c) with the galactose-binding domain of the nontoxic ricin B-chain (RB) which provides the toxin with additional binding domains, thus increasing the potential number of interactions at the molecular level in target insects (Chaudhary et al., 2014). Apart from increased toxicity from the *Bt* gene, the resistance was also transferred against a wider range of insects, including important pests that are not normally susceptible to *Bt* toxins (Chaudhary et al., 2014; Mehlo et al., 2005).

Another biotic stress is Maize dwarf mosaic virus (MDMV), a worldwide pathogen that causes chlorosis, stunting, and significant loss of yield in maize (Uzarowska et al., 2009; Zhang et al., 2010). Transgenic resistant lines have been obtained by introduction of the sense (Liu et al., 2009; Zhang et al., 2010) or the antisense (Bai et al., 2008; Zhang et al., 2010) sequence of the MDMV CP gene (Zhang et al., 2010)**.**

2.13 Genetic transformation of maize with novel traits against abiotic stresses

Tolerance to abiotic stress may be achieved through the modification of endogenous plant pathways, often by manipulating important regulatory proteins such as transcription factors (Chaudhary et al., 2014). Altering the level of expression of key transcription factors involved in abiotic stress pathways has been shown to enhance tolerance to drought stresses in maize (Chaudhary et al., 2014; Nelson et al., 2007). Introgression of transgenes expressing manganese superoxide dismutase that entails foliar tolerance to chilling and oxidative stress and for iron superoxide dismutase entailing enhanced tolerance towards methylviologen and increased growth rates,

respectively have been reported (Chaudhary et al., 2014).

2.14 Gene overexpression as a tool in genetic transformation of maize

The ability to alter the genetic composition hence manipulating biological processes of a plant is fundamental to crop improvement and development of new cultivars with desirable characters (Ganeshan and Chibbar, 2010). The availability and the versatility of different plant DNA delivery methods have become even more pertinent in recent years with the availability of gene sequence data and the need for functional analysis of cloned and sequenced genes (Ganeshan and Chibbar, 2010). Technologies such as antisense RNA, dominant negative mutants, dominant gain-of-function mutants, ectopic expression, and overexpression have provided powerful tools to investigate a broad range of plant biochemical pathways. However, some of these molecular genetic approaches are not easily applied to genes that control fundamental processes of plant growth, differentiation, and reproduction. This is primarily because manipulating such critical genes can be severely detrimental to plant growth and survival and so preclude the generation and propagation of useful transgenic plants.

One solution has been the use of ‘‘tissue-specific’’ promoters to restrict the activity of transgenes to certain tissues (Que et al., 2014). This approach is far from satisfactory as many such promoters are active during the process of regenerating transgenic plants. Tissue specific expression also restricts the scope of the analysis to a few cell types and does not always allow the appropriate tissues to be studied. Another option is the use of heat-shock promoters that can achieve relatively high level expression in many cell types (Ahanger et al., 2017; Moore et al., 1998). This has the disadvantage that the subsequent analysis is performed on plants subjected to heat stress, growth conditions must be carefully controlled, and problems have been encountered with expression of genes in plants not induced by heat shock (Que et al., 2014).

To overcome these limitations several attempts have been made to develop chemically regulated gene expression systems (Ahanger et al., 2017; Que et al., 2014). Again, these are not entirely satisfactory as they are either relatively

inefficient (high background and/or only modest induction), dependent on sustained gene repression, not applicable to commonly studied plant species, or reliant on the application of chemicals at concentrations that may be toxic to plants (Ahanger et al., 2017; Moore et al., 1998). The best characterized system is a tetracycline inducible promoter developed for tobacco (Ahanger et al., 2017; Moore et al., 1998). This chemical induction system provides useful temporal control of gene expression in tobacco cells though its usefulness and reliability for spatial control of gene expression in the various tissues of whole plants is less clear. Also it appears that this system is not effective in Arabidopsis (Ahanger et al., 2017; Moore et al., 1998). It is necessary to establish a generally applicable system for effective spatial control of transgene expression that does not require external intervention or imposition of environmental stress. A promoter and transcription factor system that allows a gene of interest to be expressed only after crossing of reporter and activator plants is the most effective way of controlling transgene expression (Ahanger et al., 2017; Moore et al., 1998).

This system may be useful under the following scenarios; if the gene of interest is expected to interfere with regeneration or propagation of transgenic material, if its activity is required only in the progeny of a cross, or if it must be expressed in several different specific tissues. It may also allow the expression of several transgenes to be regulated coordinately without multiplication of plant promoter sequences. High transformation efficiency in maize has been reported in studies where *Agrobacterium* strains carrying a “Super-binary” vector were used (Zhi et al., 2015). Recently, an *Agrobacterium* standard binary vector system was also successfully used to transform maize with novel traits that are either constitutively or inducibly expressed in a stable manner (Zhi et al., 2015).

CHAPTER THREE

BIOCONTROL OF *S. HERMONTHICA* BY BACTERIA AND FUNGI FROM SUPPRESSIVE SOILS IN WESTERN KENYA

3.1 Introduction

Striga hermonthica infestation in cereal fields results in about 30 to 90% yield losses hence emerging as a major constraint to cereal production in Sub-Saharan Africa (Musyoki et al., 2015). The yield loss can be attributed to two aspects of *S. hermonthica* lifecycle namely elevated seed fecundity and its remarkable synchronized lifecycle to that of its host (Mbuvi et al., 2017). Studies show that *S. hermonthica* flower spike can produce over 50,000 seeds with 14 years of germination viability (Yoder and Scholes, 2010). Interestingly, most damage by this weed occurs before it emerges above the ground (Ahonsi et al., 2002; Musyoki et al., 2015). The prevailing weed management strategy against *S. hermonthica* relies on cultural, chemical and manual control techniques (Nzioki et al., 2016). These options are rarely adopted by farmers because they are expensive, take long to make an impact and are often ineffective since they are applied when the damage on the host has already occurred at the subterranean stage of growth (Nzioki et al., 2016).

In the last decade, there has been extensive exploration studies on novel biological control agents for management of *S. hermonthica* weed prior to occurrence of its subterranean host damage (Avedi et al., 2014; Nzioki et al., 2016). While biological weed control strategy can be effective, sometimes it is faced with uneconomical production options, inconsistent results at different locations due to lack of biocontrol agent adaptability (Evans, 2013; Pereg and Mcmillan, 2015; Teka, 2014). To date, there exists no single effective and sustainable *S. hermonthica* control/management option that has widely been adopted by small-scale farmers in western Kenya (Avedi et al., 2014). This scenario has resulted in continued spread of the weed and increased grain yield loss over the years (Nzioki et al., 2016).

Given the potential of the biological control option in management of *S. hermonthica,* it is important to explore biocontrol agent(s) that are effective and adapted to the region of application (Atera et al., 2012; Musyoki et al., 2015). The

mechanism by which these biological agents exhibit biocontrol action is believed to be through direct antibiosis, competitive exclusion, interference with pathogen signaling and/or induction of plant resistance mechanisms (Compant et al., 2012). Most biocontrol agents are specific in action, they do not contaminate the environment through residues and are more acceptable and affordable than inorganic fertilizers or genetically engineered crops (Heydari et al., 2010; Whipps, 2001). For example, *Pseudomonas fluorescens* and *P. putida* have been reported to significantly inhibit *S. hermonthica* seed germination with no reported negative effect on cereal host plant (Babalola et al., 2007). In the case of fungal biocontrol option, *Fusarium oxysporium* f. sp. *Strigae* (Foxy2) has been shown to reduce emergence of *S. hermonthica* and *S. asiatica* plants through destruction of appressorium, hyaline tissue, xylem vessels or cortical parenchyma (Avedi et al., 2014; González-Fernández et al., 2010). However, there exists no known report of a biocontrol agent with potential to cause *S. hermonthica* seed decay. The use of a combination of compatible biocontrol agents with different modes of action is likely to yield better results than the use of any single biocontrol agent (Midthassel et al., 2016).

This study analyzed the bioactivity of culturable bacterial and fungal isolates from *S. hermonthica* suppressive soils of western Kenya with the intention of designing maize probiotics capable of suppressing, triggering suicidal seed germination and/or perturbing early developmental stages of *S. hermonthica*. The findings of this study form the basis of robust bioprospection efforts to identify microbial isolates that could be exploited as *S. hermonthica* biocontrol agents in the region.

3.2 Materials and Methods

3.2.1 Description of study sites, sample collection criteria and processing

Purposive sampling technique (Bernard, 2002) was used to collect soil and *S. hermonthica* plant samples from six counties in Western region of Kenya in the months of April and May in 2012 and 2013. Twenty samples of soil and *S.hermonthica* plants were collected within a diameter of 10km of each of the marked sites shown in the map (**Fig. 3.1,** page 41). This region had previously been mapped out as *S. hermonthica*-prone region (Omondo, 2013). In each county, twenty fields were sampled and samples collected from ten random spots in each field

infested with *S. hermonthica*. Global positioning system (GPS) coordinates and height above sea level for each site where samples were collected were recorded and used to generate spatial analytical map of the sampled region using ArcGIS Desktop version 10.5 software (**Fig. 3.1**).

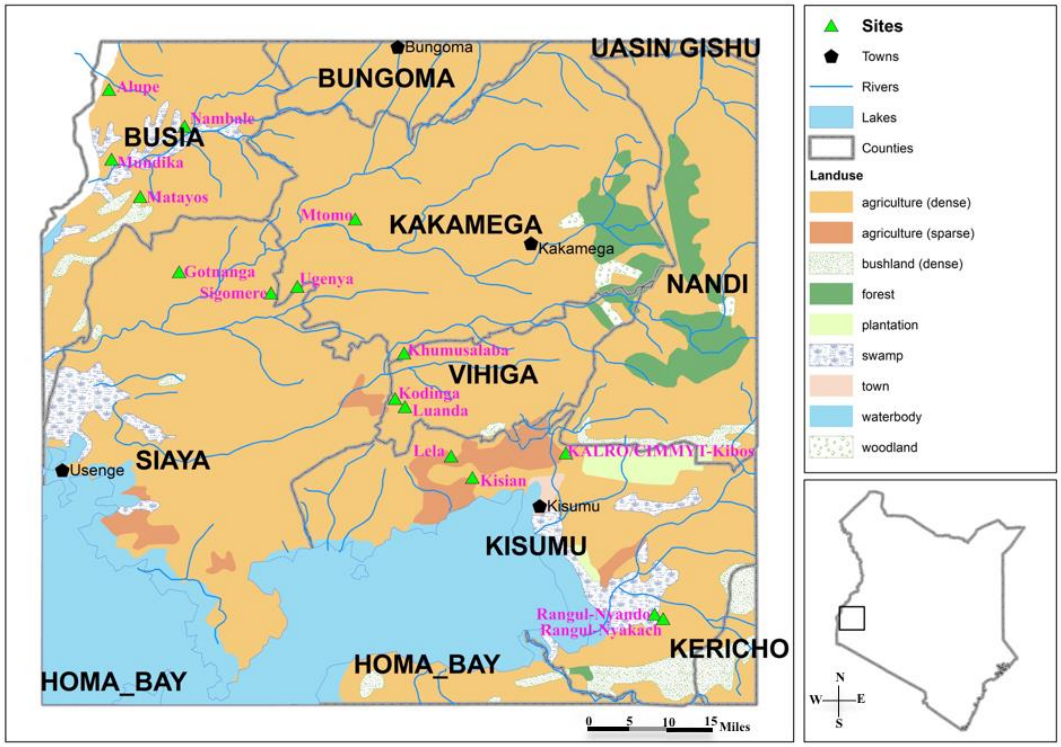

Figure 3.1: Spatial analytical map of sites where *S. hermonthica* was collected

During the sampling exercise, unhealthy/dead and healthy *S. hermonthica* plants together with their respective rhizosphere soil were collected from sites marked green on the map (**Fig. 3.1**). *Striga hermonthica* plants that appeared to be wilting, drying or had soft rot, complete blight of the stem and/or black floral parts (unhealthy/dead) and growing in close proximity with healthy host plants were collected (**Plate 3.1,** page 42). About 500g of rhizosphere soil of each sampled plant was also taken from a depth of up to 20cm. All the samples were transported to the molecular biology laboratory at Institute for Biotechnology Research (IBR) at JKUAT, Kenya.

Plate 3.1: *Striga hermonthica* infestation and existence of suppressive soils
Legend:
A) Farmer's maize field infested by *S. hermonthica* in western Kenya. **B)** Healthy and diseased *S. hermonthica* plant growing in close proximity to each other. **C)** An example of healthy and diseased *S. hermonthica* plants collected near a host plant.

The collected plant samples were first washed under running tap water for 20min. The plant samples were cut into 2cm-length pieces and then surface sterilized by soaking in 1% sodium hypochlorite solution in beakers that were swirled gently for 2 min and then rinsed twice using double distilled water. The explants were then immersed in 70% ethanol for 5min and then rinsed twice using double distilled water. The explants were further soaked in 2.5% redomil solution fortified with two drops of tween twenty for 10 min and finally rinsed thrice with double distilled water to ensure that the isolates to be cultured were from the endorhizosphere (Kwaśna and Bateman, 2007).

For soil samples, 10g of each of the bulked soil samples was shaken in 1000 ml of 0.01 M phosphate buffer (pH stabilizer). The solution contain (0.88% (w/v) NaCl, 2.9 mM KH_2PO_4, 7.1 mM K_2HPO_4, pH 7.2) for 2 h on a rotary shaker (orbit shaker, Labtech, Daihan Co.Ltd) at 200rpm (Janssen et al., 2002). Subsequently, serial dilutions (up to 10^{-8}) were prepared, beginning with a 1 ml aliquot of the stock suspension.

3.2.2 Isolation of Bacteria from samples

The pre-sterilized explants (collected samples) were air-dried on sterile Whatman No.1 filter paper and further chopped into small pieces (approximately 0.5 cm long) and used as source of inoculum on five different types of media namely; Peptone pentachloronitrobenzene agar (PPA), Potato dextrose agar (PDA) Spezieller nähtoffarmer Agar (SNA), nutrient agar (NA) and Babalola (2007) media with

freshly cut edges being in contact with the media at 37°C. For soil samples, aliquots (0.1 ml) of the soil suspensions of pre-phosphate buffered solution from the dilution levels of 10^{-3} to 10^{-8} was dispensed in separate 9-cm diameter sterilized Petri-dishes containing five different types of media namely; Peptone pentachloronitrobenzene agar (PPA), Potato dextrose agar (PDA) Spezieller nähtoffarmer Agar (SNA), nutrient agar (NA) and Babalola (2007) media in triplicate. For each media, five treatments based on pH values (pH 3, 6, 7, 8 and 11), were prepared. Two petri plates per treatment were marked at the bottom with the dilution level prepared for all the soil samples. For each dilution (starting with 10^{-3}), 0.1 ml was spread onto each of the two-petri plates. The plates were swirled gently in a clockwise motion to mix the suspension and spread over the agar using a sterile glass rod. The plates were then sealed with parafilm, inverted and placed in an incubator at 37°C for 24 h. After incubation for 24 h at 37°C, representative types of bacterial colonies emerging in the media were further purified by sub culturing on fresh nutrient agar medium and stored in 35% glycerol at -80°C.

3.2.3 Isolation of fungi from samples

The pre-sterilized explants (collected samples) were air-dried on sterile Whatman No.1 filter paper and further chopped into small pieces (approximately 0.5 cm long) and inoculated on five different types of media namely; Peptone pentachloronitrobenzene agar (PPA), Potato dextrose agar (PDA) Spezieller nähtoffarmer Agar (SNA), nutrient agar (NA) and Carnation Leaf Agar (CLA) media with freshly cut edges being in conduct with the media at 28°C. For soil samples, aliquots (0.1 ml) of the soil suspensions of pre-phosphate buffered solution from the dilution levels of 10^{-3} to 10^{-8} was dispensed in sterile Petri-dishes containing five different types of media namely; Peptone pentachloronitrobenzene agar (PPA), Potato dextrose agar (PDA) Spezieller nähtoffarmer Agar (SNA), nutrient agar (NA) and Carnation Leaf Agar (CLA) media in triplicate. For each media, five treatments based on pH values (pH 3, 6, 7, 8 and 11), were prepared. Two petri plates per treatment were marked at the bottom with the dilution level prepared for all the soil samples. For each dilution (starting with 10^{-3}), 0.1 ml was spread onto each of the two-petri plates. The plates were swirled gently in a clockwise motion to mix the

suspension and the suspension was spread over the agar using a sterile glass rod. The plates were then sealed with parafilm, inverted and placed in an incubator at 28°C for 24 h. Emerging bacterial colonies were individually streaked on fresh nutrient agar media while emerging fungal colonies were cultured on PDA, SNA and Carnation Leaf Agar (CLA) to obtain pure fungal isolates for further characterization. Fungal isolates were further grown on PDA, SNA and CLA for 14 days to allow sporulation. Fifteen milliliters of sterile distilled water was added to the collected spores/mycelia and the mixture thoroughly ground. The mixture was filtered using an 80-µm filter sieve and the spore concentration standardized to 10^6-spore ml^{-1} using haemacytomer.

3.2.4 Coding of microbial isolates

The emerging bacterial and fungal isolates were assigned coded names for easy traceability of their origin (site of collection and type of sample). To achieve this, isolates from rhizosphere soils of diseased *S. hermonthica* plants were labeled ISS, while isolates from rhizosphere soils of healthy *S. hermonthica* plants were labeled HSS. Isolates from diseased *S. hermonthica* plants were labeled ISP while isolates from healthy *S. hermonthica* plants were labeled HSP. The numerical number of the code refers to the sampled site, assigned in a sequential order of the collection exercise. Bacterial isolates were tagged with a prefix SM to differentiate them from fungal isolates, which were tagged SH. Isolates with alphabet letter after the numeric code refers to the viable strain that occurred in all samples collected within a single site. Examples of coded names of the isolates in this study include bacterial isolates such as SM103ISS, SM12ISP and SM63C and fungal isolates such as SH_8HSP, SH_ISS01F and SH_12ISS.

3.2.5 Purification and morphological identification of bacteria and fungi

Emerging bacterial colonies were individually streaked on fresh nutrient agar media in petri plates while emerging fungal colonies were inoculated on fresh PDA media amended with 0.1g/l streptomycin (PDAS) to obtain pure colony isolates for further characterization. Pure cultures of fungi isolates were obtained by a single spore/colony isolation technique and maintained on PDA slants (Johnston and Booth,

1983). Gram staining and description of cell shape and colour as well as colony structure following Bergey's Manual of Determinative Bacteriology were used to characterize bacterial isolates morphologically. Fungal morphology descriptors such as colour, shape and texture of pure colonies as well as spore/mycelia-staining technique according to Saccardian System was described and used to presumptively classify the fungal isolates morphologically (Barnett, 1962; Barnett et al., 1998).

3.2.6 Criteria of selecting test microorganisms for antibiosis assay

Exploration for biocontrol microbes among the large number culturable bacterial and fungal isolates purified from the collected samples required appropriate screening technique. In this study, several pre-selection assays were performed to select isolates with high potential of causing *S. hermonthica* seed decay. Isolates that grew on media with different pH levels and also recorded high antibiosis and enzymatic activity values were selected and used in the *S. hermonthica* biocontrol-screening assay. Bacterial isolates 1HSP, 5b1, 3b2, 3ISS, and 11HSP grew very fast (based on periodical OD measurement) and were highly susceptible to inhibition by ampicillin, tetracycline, centramaxazole, streptomycin, kanamycin, gentamicin, sulphamohoxazole and chloramphenicol antibiotics. Based on the ability to grow very fast and high susceptibility to the antibiotics used, bacterial isolates that exhibited these characteristics were used as test microorganism in the antibiosis experiment. The bacterial isolates 1HSP, 5b1, 3b2, 3ISS, and 11HSP were decoded as BUT101, BUT501, BUS202, BUS203 and SIA102 respectively to differentiate them from the microbes selected for *Striga* seed decay experiment.

3.2.7 Bacterial and Fungal Antibiosis assays

The interaction between bacterial isolate (four points of inoculation per plate) and test microorganism formed bacterial antibiosis treatments (sterile distilled water served as a negative control) and all the treatments were replicated three times. Standardized bacterial suspensions were prepared based on 0.5McFarland Turbidity Standard (appendix 2) which is presumed to have approximately $1.5X10^8$ bacterial cells per ml (Nuneza et al., 2015). Aliquot of 100µL of each of the test microorganism was separately spread onto Mueller Hinton Agar (MHA) media in

petri plates. Sterile Whatman No.1 filter paper discs (1cm-diameter) were saturated with isolates as disc inoculants (4 discs per plate replicated six times) following Kirby-Bauer disc diffusion method. The plates were sealed with parafilm and incubated at 37°C for 24 h. Emerging zones of inhibition after 24 h were measured and recorded. Similarly, fungal spore paste, prepared by mixing 10^6-spores per ml suspension with one drop of triton X-100 was used to saturate fungal inoculant discs. Three replicates, each consisting of four saturated discs per isolate, were inoculated onto Muller Hinton agar media previously spread with test microbe, incubated at 30°C and monitored routinely after every 24 h. The different fungal-bacteria isolate combinations formed fungal treatments and the emerging zones of inhibition around the discs after 7 days (considered as indicators of efficiency) were measured and recorded.

3.2.8 Bacterial and fungal extracellular enzymatic assays

Minimum salt media (MSM) fortified with special carbon source (based on target extracellular enzymatic test) were prepared. The constituents of MSM were; 25g of $MgSO_4.7H_2O$, 2.8g of $FeSO_4.7H_2O$, 1.7g of $MnSO_4$, 0.6g of NaCl, 0.1g of $Na_2MoO_4.2H_2O$, 0.1g of $ZnSO_4.7H_2O$ and 8.54 ml of 32% HCl solution dissolved in 1 litre of deionized distilled water (Khalil 2011).

Bacterial and fungal isolates with standardized concentration levels as described in the bioassay experiment above (sub heading 3.2.7) formed treatments and were inoculated separately and individually per media type. Four inoculations per plate with each inoculum consisting of 100µL of the standardized isolate concentration were made and replicated six times.

For extracellular cellulase enzyme assay treatments, individual isolates were inoculated separately on nutrient agar media fortified with 1% (w/v) Carboxymethyl cellulose (Teather et al., 1982) in petri plates, respectively. The petri plates with bacterial and fungal isolates were incubated at 37°C and 30°C for 24 h and 48 h, respectively. Cellulase-producing isolates were identified after flooding the plates with 0.1% aqueous Congo red for 15 min followed by repeated washing with $1mol^{-1}$ NaCl (Teather et al., 1982). Diameters of visible clearance zones around the

inoculants were measured and used to compare extracellular enzyme activity profiles.

For extracellular xylanase enzyme assay treatments, individual isolates were inoculated separately on nutrient agar media fortified with 1% (w/v) Xylan (Gessesse et al., 1997) in petri plates, respectively. The petri plates with bacterial and fungal isolates were incubated at 37°C and 30°C for 24 h and 48 h, respectively. Xylanase-producing isolates were identified after flooding the plates with 0.1% aqueous Congo red for 15min followed by repeated washing with $1mol^{-1}$ NaCl (Teather et al., 1982). Diameters of visible clearance zones around the inoculants were measured and used to compare extracellular enzyme activity profiles.

The ability of the isolates to produce extracellular amylase enzyme was tested by culturing individual isolate on autoclaved medium composed of 1% (w/v) soluble starch and 1% (v/v) MSM salts and solidified using bacteriological agar. The petri plates with bacterial and fungal isolates were incubated at 37°C and 30°C for 24 h and 48 h, respectively. After incubation period, plates were flooded with 1% Lugol's iodine to detect the presence of clear halos around extracellular amylase-producing isolates.

Ability of the isolates to produce extracellular protease was assayed by inoculating individual isolates on solid media containing 0.1% NaCl, 0.1% K_2HPO_4 (RANKEM), 0.01% $MgSO_4.7H_2O$ (RANKEM), 0.05% yeast extract (FLUKA), 1% skim milk (PRADIP) and 2% bacteriological agar (FLUKA). The petri plates with bacterial and fungal isolates were incubated at 37°C and 30°C for 24 h and 48 h, respectively. The clear zone forming around the inoculant indicated the ability of the isolate to produce extracellular proteases (Zilda et al., 2012).

Screening for extracellular pectinase activity of isolates was done using Huang et al., (2012) protocol. The media used contained; 1.9 g/l K_2HPO_4, 0.94 g/l KH_2PO_4, 1.6 g/l KCl, 1.43 g/l NaCl, 0.15 g/l NH_4Cl, 0.037 g/l $MgSO_4 \cdot 7H_2O$, 0.017 g/l $CaCl_2 \cdot 2H_2O$, 0.1 g/l yeast extract and 10 g/l Carboxymethyl cellulose (CMC). The petri plates with bacterial and fungal isolates were incubated at 37°C and 30°C for 24 h and 48 h, respectively. After the incubation period, plates were flooded with 1% Lugol's

iodine to detect the presence of clear halos, as opposed to the blue-black color typical of the reaction of Lugol's solution to starch, around those bacterial colonies capable of secreting pectinase (Huang et al., 2012). Diameters of emerging zones of clearance around the discs in all the extra cellular enzymatic experiments (considered as indicators of efficiency) were measured and recorded.

3.2.9 Conditioning of *S. hermonthica* seeds and determination of their fecundity

S.hermonthica seeds were surface-sterilized in punches of 10mg of (approximately 1,500 seeds) and preconditioned according to the protocol of Lendzemo et al., (2009). The seeds were then placed in a beaker containing 2% (v/v) NaOCl and vortexed for 5 min. The seeds were later emptied on a funnel lined with folded filter paper and rinsed thoroughly with autoclaved distilled water to get rid of traces of NaOCl. Two hundred sterilized *S. hermonthica* seeds from each bunch were transferred into a petri plate lined with moist Whatman GFA filter paper, wrapped with Aluminum foil and incubated at 28°C for 11 days for pre-conditioning. Three plates of preconditioned *S. hermonthica* seeds of each bunch were germinated by adding 3ml of 0.1ppm GR24 and incubated overnight at 28°C. The number of germinating *S. hermonthica* seeds was determined using a Leica MZ7F stereomicroscope fitted with a DFC320FX camera. The remaining *S. hermonthica* seeds from each bunch that had an average germination mean rate of >69% were used in the *S. hermonthica* seed decay experiments.

3.2.10 Bioherbicidal activity of the selected isolates on *S. hermonthica* seed

A total of sixteen pre-selected isolates were screened for their ability to induce *S. hermonthica* seed decay that consequently resulted in the reduction of the number of germinating *S. hermonthica* seeds after exposure to GR24. Two controls comprising pre-conditioned *S. hermonthica* seeds to which 3ml of sterile distilled water was added (negative control) and *S. hermonthica* seeds treated with 3ml of 0.1ppm GR24 (positive control) were included in the experiment. Petri plates used in this assay were lined with two sterile filter papers (Whatman GFA). In the bacterial assay, six replicates each consisting of 100 pre-conditioned *S. hermonthica* seeds per petri plate were carefully spread on the bottom filter paper and covered with the second filter

paper. Ten millilitres of 10^{-1} serial dilution of standardized bacterial suspension was used to wet the top filter paper. The petri plates were sealed with parafilm and incubated at 28°C in the dark for 48 h. After 48 h, the top filter paper was carefully removed, 3ml of 0.1ppm GR24 added and further incubated overnight at 28°C.

In a separate experiment, standardized fungal spore concentrations (10^6 spores per ml) were evaluated for their ability to cause *S. hermonthica* seed decay (Kroschel et al., 1996). Bunches of 10mg *S. hermonthica* seeds that had been pre-conditioned for 4 days were carefully spread on Whatman GFA filter paper and covered with another filter paper in petri plates. Ten milliliters of standardized fungal spore suspensions were used to wet the top filter papers that had been sprinkled with 0.05g of PDA. The petri plates were sealed with parafilm and incubated in the dark at 28°C for 10 days. After incubation, the top filter paper was carefully removed and seeds washed with sterile distilled water through two filter sieves with mesh size of 315-µm and 80-µm. The first sieve facilitated the separation of *S. hermonthica* seeds and fungal spores from the mycelium while the second sieve enabled the separation of *S. hermonthica* seeds from fungal spores. One hundred *S. hermonthica* seeds of each treatment were transferred onto Whatman GFA filter paper moistened with 3ml of 0.1ppm GR24 in a petri plate (replicated six times) and incubated overnight in the dark at 28°C.

In both bacterial and fungal screening assays, the petri plates were observed under a Leica MZ7F stereomicroscope fitted with a DFC320FX camera, for presence of decaying *S. hermonthica* seed coat. The number of decaying *S. hermonthica* seed per replicate was recorded and subjected to ANOVA.

3.2.11 Partial characterization of bacterial 16S rRNA gene

Pre-selected bacterial isolates were grown in Luria broth (LB) medium (10g/l Tryptone, 5g/l yeast extract, 10 g/l NaCl, pH 7.0) at 37°C in a rotary shaker at a speed of 120rpm for 48 h. The cultures were then centrifuged at 13,000rpm in a centrifuge (AG 22331 Hamburg, 5415D) for 1 min and the supernatant discarded. DNA extraction from the pelleted bacterial cells was performed according to Sambrok and Russell (2001) protocol and stored at 4°C. Bacterial 16S rRNA gene

universal primers 8F (5'-AGRCTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGCTACCTTGTTACGACTT-3') were used to partially amplify the 16S rRNA gene for all selected bacterial isolates (Heuer et al., 1997). Polymerase chain reaction (PCR) was performed in a thermocycler (PeQLab, VWR, Germany). Amplification was carried out in a 30µL mixture containing 3µl of 10X PCR buffer, 4µl of 2.5mM dNTPs, 2.5µl of 8F forward primer (5pmol), 2.5µl of 1492R reverse primer (5pmol), 0.4µl of 5 U/µl Taq polymerase, 1.5µl of DNA template and 16.1µl PCR grade water (all reagents were products of BIOLINE Co.). The amplification was performed as follows; initial denaturation at 94°C for 5 min, followed by 30 cycles each of denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, chain extension at 72°C for 1.5 min, and a final extension at 72°C for 8 min. The PCR amplicons (≈1.5kb) were checked by gel electrophoresis, labeled and shipped to Macrogen, South Korea for sequencing.

Sequencing was conducted in one direction using the forward primer (27 F) according to Sanger et al., (1977). BioEdit program was used to remove ambiguity in the sequences. Edited sequences were submitted to the GenBank database and assigned the following accession numbers KY038852- KY038856 and KY041695-KY0041697. Sequence comparisons analysis was done with the NCBI GenBank databases using the Basic Local Alignment Tool (BLAST) algorithm (Altschul et al., 1990). The sequences were aligned along with selected sequences from the GenBank/EMBL/DDBJ databases by using the CLUSTAL X program (version 1.81). The distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura, (1980). The differences in the nucleotides were converted into genetic distance matrices using neighbor joining method (Saitou et al., 1987). Construction of a phylogenetic tree was done using MEGA 7 (Kumar et al., 2016). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (1985) based on 1000 samplings (Sumpavapol et al., 2010).

3.2.12 Partial characterization of fungal 18S rRNA gene

To characterize fungal 18SrRNA gene of selected isolates, pure fungal cultures were revived using PDA medium. Fungal DNA was extracted using Brandfass and

Karlovsky (2008) protocol. The primer set used to study the biodiversity of the selected isolates were; Forward primer (566F) 5'-CAG CAG CCG CGG TAA TTC C-3' and Reverse primer (1200R) 5'-CCC GTG TTG AGT CAA ATT AAG C-3'. These primers amplify on average a 650bp long fragment from the V4 and V5 regions. PCR was performed using BIOLINE Taq polymerase masterMix (ready to use, CAT. No.BIO-2541) following the manufacturer's instructions and at the following conditions; initial denaturation at 95°C for 15 min, 35 cycles each of denaturation at 95°C for 45 sec, primer annealing at 60°C for 45 sec, chain extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR amplicons were checked by gel electrophoresis, labeled and shipped to Macrogen in South Korea for sequencing.

The obtained 18S rRNA gene partial sequences were deposited in the NCBI GenBank database where they were assigned accession numbers ranging from KY04168 to KY04176. The sequences were aligned along with selected sequences from the largest database tailored for fungal ITS sequences UNITE (Abarenkov et al., 2010; Https://unite.ut.ee/, 2nd June 2016; Nilsson et al., 2014) using the CLUSTAL X program (version 1.81). UNITE was used in this study because it mirrors and curates the International Nucleotide Sequence Database Collaboration (INSDC GenBank, ENA, and DDBJ) for fungal ITS sequences and offers extensive capacities for analysis and third-party annotation of sequences to its users (Nilsson et al., 2014). The distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura (1980). The differences in the nucleotides were converted into distance matrices using neighbor joining method (Saitou et al., 1987). Construction of a phylogenetic tree was done using MEGA 7 (Kumar et al., 2016). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (1985) based on 1000 samplings (Sumpavapol et al., 2010).

3.3 Statistical analysis

Data on antibiosis and extra cellular enzymatic experiments was recorded in excel sheets. General linear model (PROC GLM) procedure of SAS software version 9.1 (SAS Institute, Cary.NC) was used to perform analysis of variance (ANOVA) for all the measured data. The ANOVA graphs, presented as mean ± standard error (SE), in the result section were generated using graphpad prism software (version 7). Tukey's honest significant difference (HSD) test was used to compare and separate the means of diameter of zones of inhibition and clearance (presented in form of alphabet letters in the graphs).

In the evaluation of bioherbicidal activity of the selected isolates on *S. hermonthica* seed decay, the number of decaying *S. hermonthica* seeds were arcsine transformed into percent values prior to statistical analysis (Gomez et al., 1984). Similarly, General linear model (PROC GLM) procedure of SAS software version 9.1 was used to perform Analysis of variance (ANOVA) of transformed data (supplementary data in appendix 3). The ANOVA graph, presented as mean ± standard error (SE), in the result section was generated using graphpad prism software (version 7). Tukey's honest significant difference (HSD) test was used to compare and separate the means of the number of decaying *S. hermonthica* seed among the different treatments (presented in form of alphabet letters in the graph).

Correlation profiles of zones of inhibition, zones of clearance and number of decaying *S. hermonthica* seed with respect to selected isolates were visualized as heatmaps generated by a hierarchical clustering R script using R version 3.3.1 software. Genetic affiliation of the screened isolates was deduced from phylogenetic tree generated using MEGA 7 as described earlier.

3.4 Results

The specific areas in Western region where the samples were collected lay between 1000m to 2000m above sea level (**Appendix 1**). During isolation, a total of 128 bacterial isolates and 46 fungal isolates were obtained. Gram staining technique was used to profile the morphology of bacterial isolates (**Plate 3.2**).

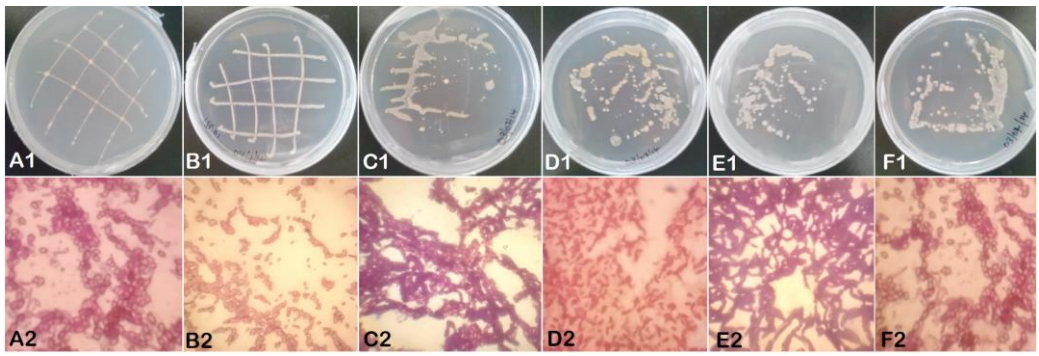

Plate 3.2: Bacterial isolates on culture media and their respective microscopic images

Legend
(**A**) SM103ISS, (**B**) SM63C, (**C**) SM5ISS, (**D**) SM8F2, (**E**) SM12ISP, (**F**) SM5ISP. The roman numbers 1 and **2** refers to macroscopic and microscopic images, respectively.

The fungal isolates exhibited distinct phenotypic characteristics especially the colony colour, margin, elevation and form as well as microscopic configuration (**Plate 3.3** and **Appendix 3**).

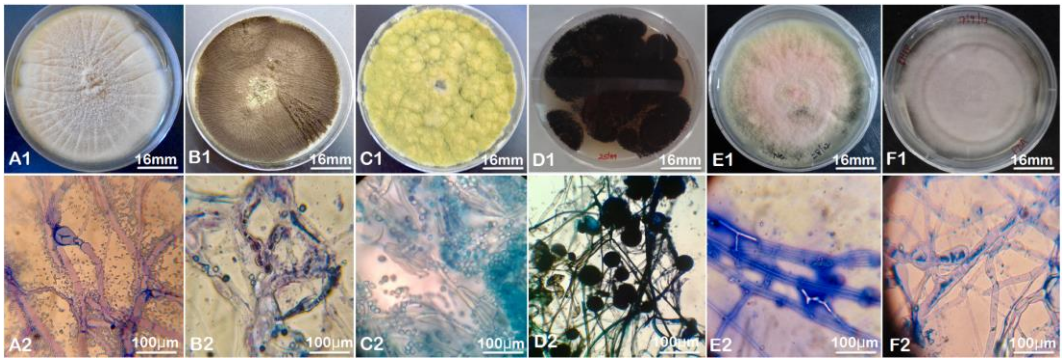

Plate 3.3: Fungal isolates on culture media and their respective microscopic images

Legend
(**A**) SH_9ISP, (**B**) SH_ISS01F, (**C**) SH_12ISS, (**D**) SH_S15, (**E**) SH_S17b, (**F**) SH_8HSP. The numerical numbers 1 and 2 refers to macroscopic and microscopic images, respectively.

Analysis of bacterial antibiosis and fungal antibiosis assays revealed that eight bacterial isolates and eight fungal isolates exhibited high and consistent antibiotic characteristic against the selected test microorganisms (**Plate 3.4**).

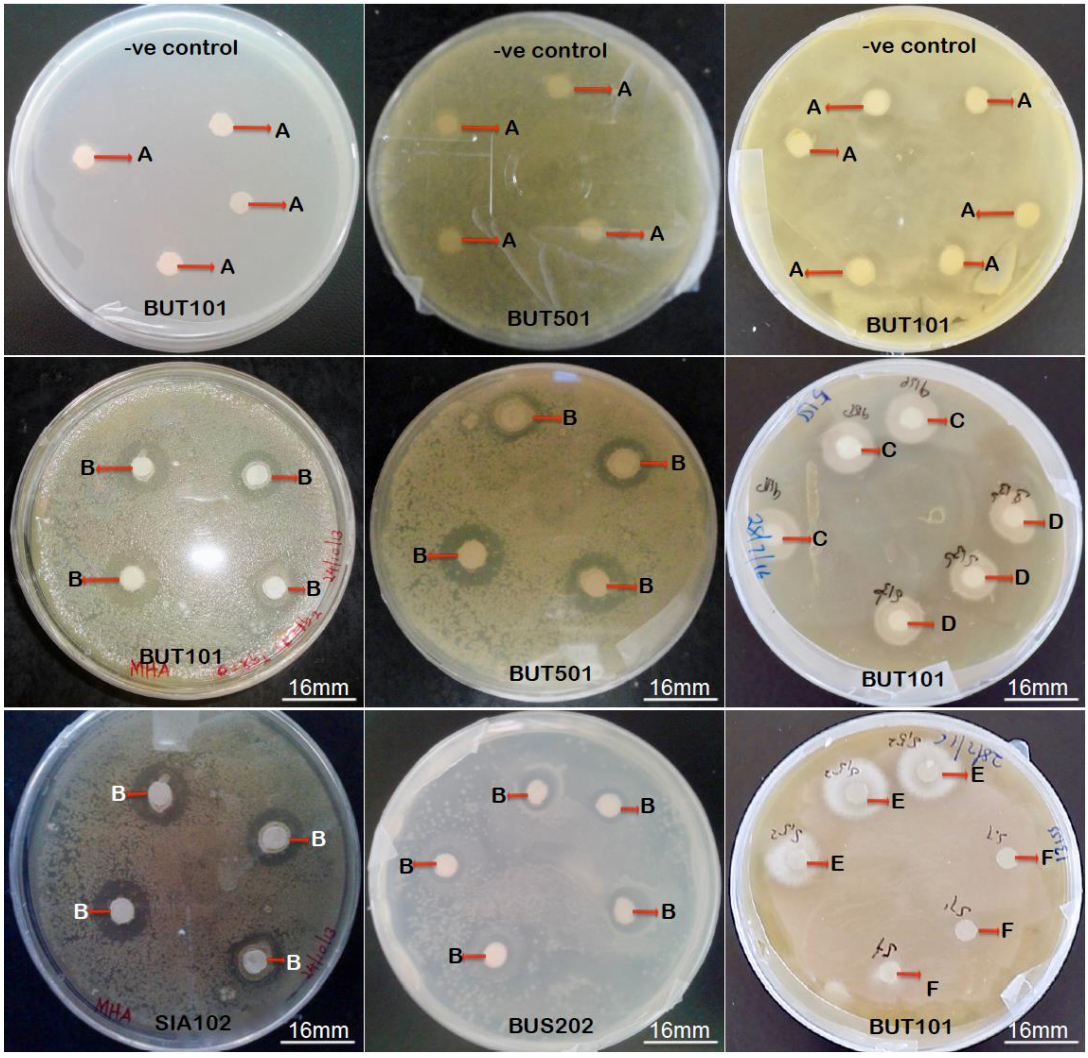

Plate 3.4: Antibiosis profile of microbial isolates against selected test microorganisms

Legend:
A) Disc inoculum of sterile distilled water (negative control), **B**) Disc inoculum of SM5ISS, **C**) Disc inoculum of SH_9ISP, **D**) Disc inoculum of SH_13B, **E**) Disc inoculum of SH_17b and **F**) Disc inoculum of SH_152.

Quantitative comparative assessment of bacterial antibiosis properties presented in Figure 3.2 (page 55) clearly shows the variation in the strength of the eight selected bacterial isolates to inhibit other bacterial strains from growing in the same nutrient niche. For instance, isolate SM5ISS exhibited significantly high antibiosis property

against test organisms namely BUT101, BUT 501, BUS201 and SIA102 recording an average inhibition diameter mean of 6.4±0.33cm, 11.0±0.58cm, 6.0±0.01cm and 8.4±0.33cm respectively ($F_{(7,16)}$ = 153, 8.4, 901.83 and 86.07 respectively and P value =0.0001). Interestingly, isolate SM12ISP scored significantly only high antibiosis property against test organisms BUS202 and BUS203 by recording 6.3±0.33cm and 7.3±0.33cm respectively ($F_{(7,16)}$=11907 and 15987 respectively and P value = 0.0001). The susceptibility of the test organisms to inhibition by the screened bacterial isolates as indicated by number of asterisk in Figure 3.2 revealed that, BUT501 was the most susceptible test organism with mean inhibition diameter of 7.6±0.12cm while BUS 202 was the most resistant test organism with mean inhibition diameter of 0.79±0.01cm ($F_{(5, 17)}$ = 2997.82 and P value = 0.0001).

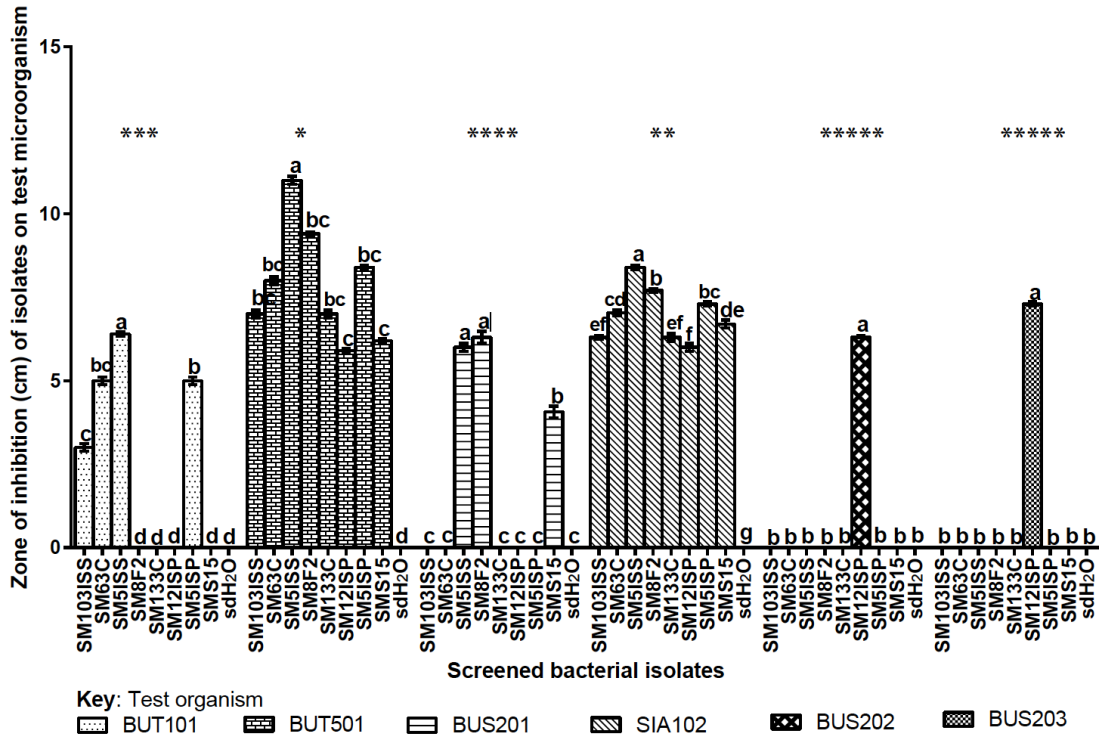

Figure 3.2: Antibiotic effect of bacterial isolates on growth of selected microbes

Legend:
Bars represent screened isolates while identical stacked bars (treatments) with the same alphabet letter represent means of bacterial isolate with no significant difference at *Tukey's* test (P≤0.05). Treatments with grand means that are not significantly different at *Tukey's* test (P≤0.05) have the same number of asterisk(s) above the stacked bars (treatment).

Quantitative comparative assessment of antibiosis properties of the selected fungal

isolates is presented in Figure 3.3. Results shows that isolate SH12ISS exhibited significantly high antibiosis property against all test organisms compared to other fungal isolates. This isolate recorded mean inhibition diameter of 7.6±0.17cm, 12.2±0.11cm, 9.2±0.11cm, 10.0±0.16cm, 7.0±0.06cm and 8.3±0.07cm against test organisms BUT1, BUT501, BUS201, SIA102, BUS202, and BUS203 respectively ($F_{(7,16)}$ =513.52, 588.86, 587.87, 540.28, 36.04 and 41.63 respectively and P value = 0.0001). Analysis of the susceptibility of the test organisms to inhibition by the screened fungal isolates as indicated by number of asterisk in Figure 3.3 revealed that BUT501 was the most susceptible test organism with mean inhibition diameter of 7.2±0.12cm while BUT101 was the most resistant test organism with mean inhibition diameter of 4.8±0.02cm ($F_{(5, 17)}$=190.46 and P value = 0.0001).

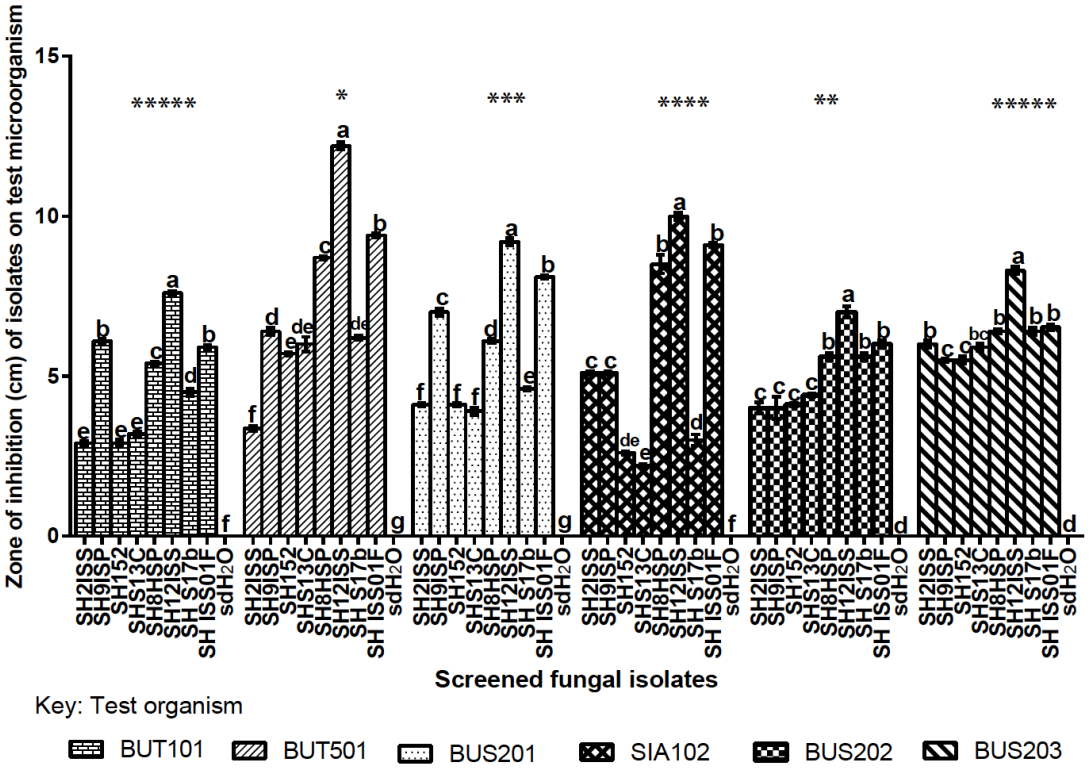

Figure 3.3: Antibiotic effects of fungal isolates on growth of selected microbes

Legend:
Bars represent screened isolates while identical stacked bars (treatments) with the same alphabet letter represent fungal isolate means with no significant difference at *Tukey's* test (P≤0.05). Treatments with grand means that are not significantly different at *Tukey's* test (P≤0.05) have the same number of asterisk(s) above the stacked bars (treatment).

Assessment of the ability of bacterial isolates to produce extra cellular lytic enzymes

revealed that all the isolates were capable of producing different extra cellular enzymes in varying magnitude (**Plate 3.5**).

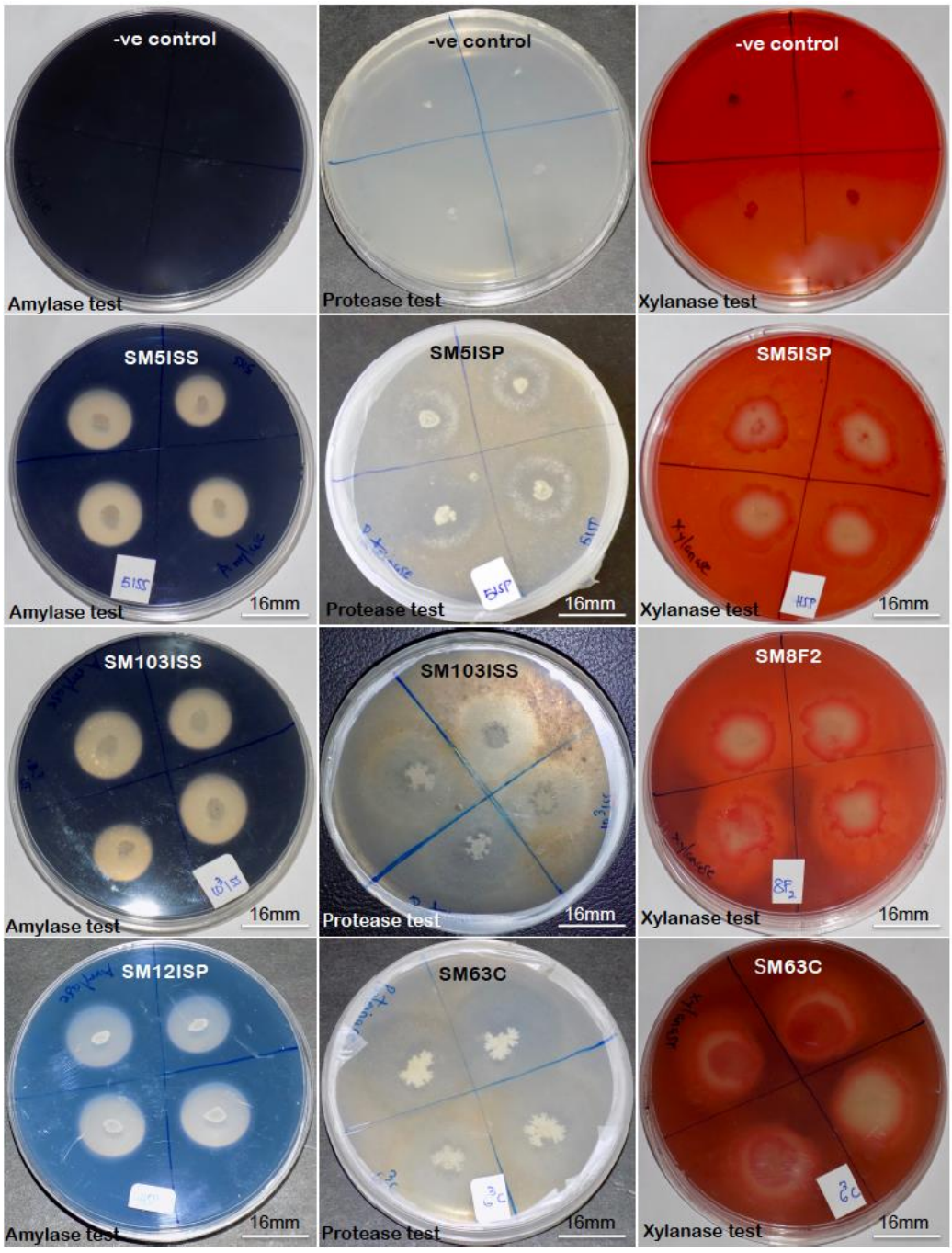

Plate 3.5: Profile of extra cellular lytic enzyme assay of selected bacterial isolates

Isolate SM7H (does not produce extra cellular enzymes tested) served as the negative control.

The expression of all measured extra cellular enzymatic activities as illustrated in plate 3.5 (page 57) differed significantly within and among the treatments as indicated by alphabet letters and the number of asterisk in Figure 3.4.

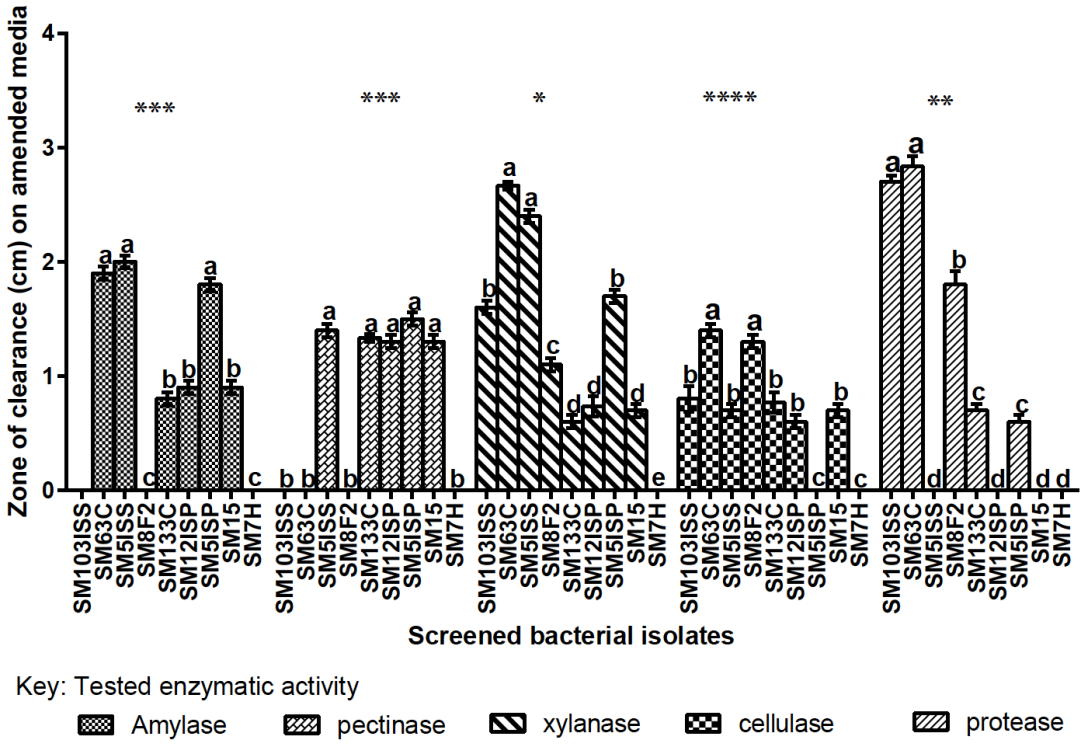

Figure 3.4: Extra cellular enzymatic activity of selected bacterial isolates

Legend:
Bars (screened isolates) in each treatment (tested enzymatic activity) with the same alphabet letter represents isolate means with no significant difference at *Tukey's test* (*P≤0.05*). Treatments with grand means that are not significantly different at *P≤0.05* according to *Tukey's test* have the same number of *asterisk(s)* above the stacked bars (treatment)

Assessment of extra cellular pectinase activity presented in Figure 3.4 show that isolate SM5ISP recorded the highest zone of clearance (average diameter of 1.5±0.01cm) while isolates SM103ISS, SM63C and SM8F2 recorded no extra cellular digestion activity ($F_{(7,16)}$ = 240 and P value = 0.0001).

For xylanase test, bacterial isolates SM63C and SM133C recorded the highest and least zones of clearance of average diameter of 2.6±0.08cm and 0±0.00cm, respectively ($F_{(7,16)}$=133.25 and P value = 0.0001).

Analysis of cellulase enzymatic activity revealed that isolates SM63C and SM5ISP recorded significantly high and low zones of clearance of average diameters of

1.4±0.09cm and 0±0.00cm, respectively ($F_{(7,16)}$ =33.97 and P value = 0.0001).

For proteolytic activity, isolates SM63C and SM15 recorded the highest (2.9±0.05cm) and least (0±0.00cm) zones of clearance respectively ($F_{(7,16)}$=221.33 and P value = 0.0001).

In the same figure, evaluation of amylase extra cellular enzymatic activity revealed that isolates SM5ISS and SM8F2 recorded the highest and least zones of clearance of average diameters of 2.0±0.1cm and 0±0.00cm respectively ($F_{(7,16)}$ = 257.07 and P value = 0.0001).

Overall comparative evaluation of tested extra cellular enzymatic activities showed that xylanase enzyme was produced in large quantities by all the screened bacterial isolates (clearance diameter of 1.4± 0.02cm) while Cellulase enzyme was the least produced enzyme, recording an average clearance diameter of 0.78±0.06cm ($F_{(4,10)}$ =19.14 and P value = 0.0001).

In contrast to bacteria, fungi exhibited higher specific enzyme activity patterns for the assayed test treatments (**Plate 3.6** on page 60). Analysis of extra cellular enzymatic activity for individual fungal isolate revealed that, isolate SHS13C recorded high extra cellular activity for amylase, pectinase and xylanase tests of average clearance diameter of 4.3±0.07cm, 2.8±0.11cm and 2.7±0.06cm respectively ($F_{(7,16)}$=68.45, 22.40 and 25.66, respectively and P value = 0.0001).

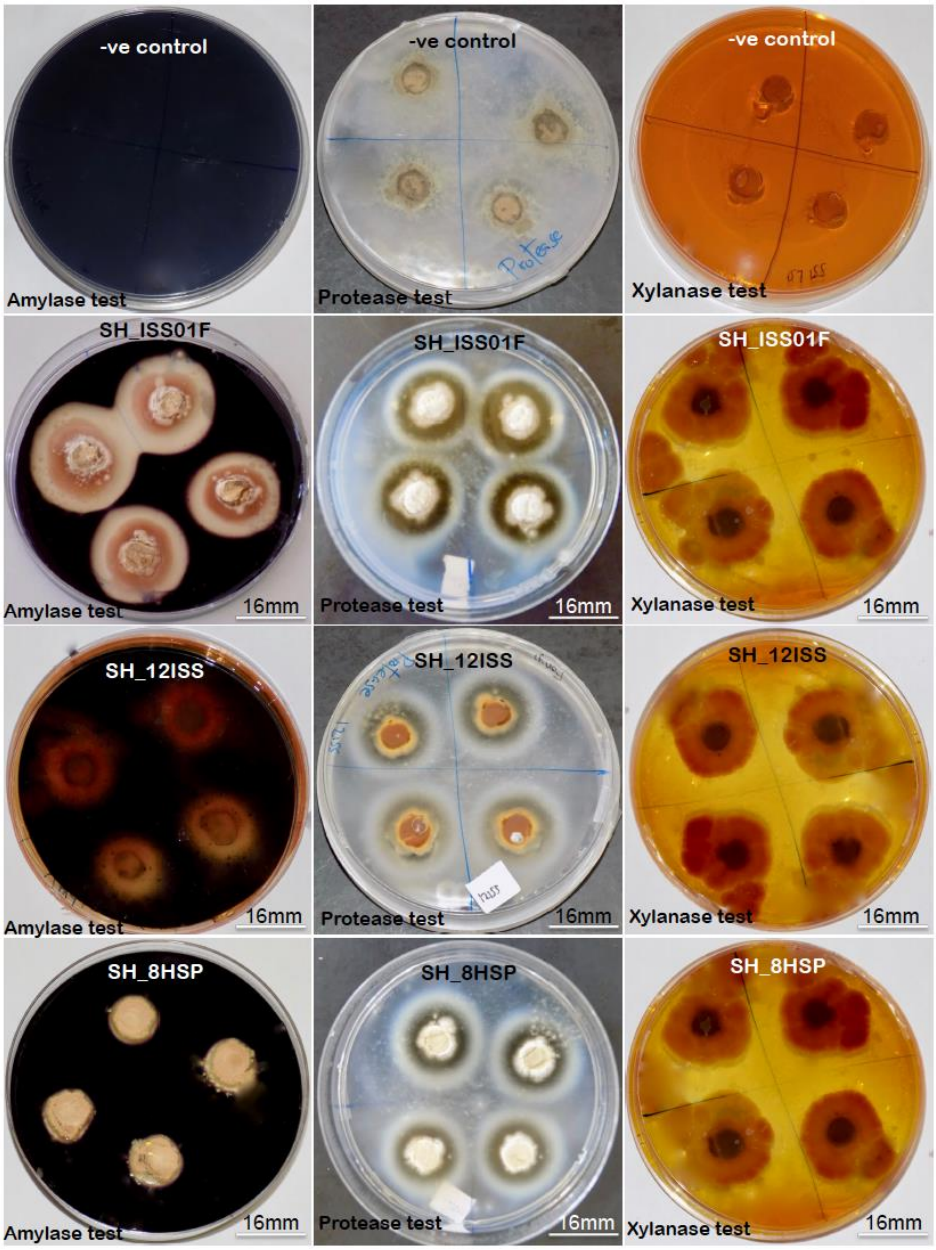

Plate 3.6: Profiles of extra cellular lytic enzyme assay of selected fungal isolates

Isolate SM7H (does not produce extra cellular enzymes tested) served as the negative control.

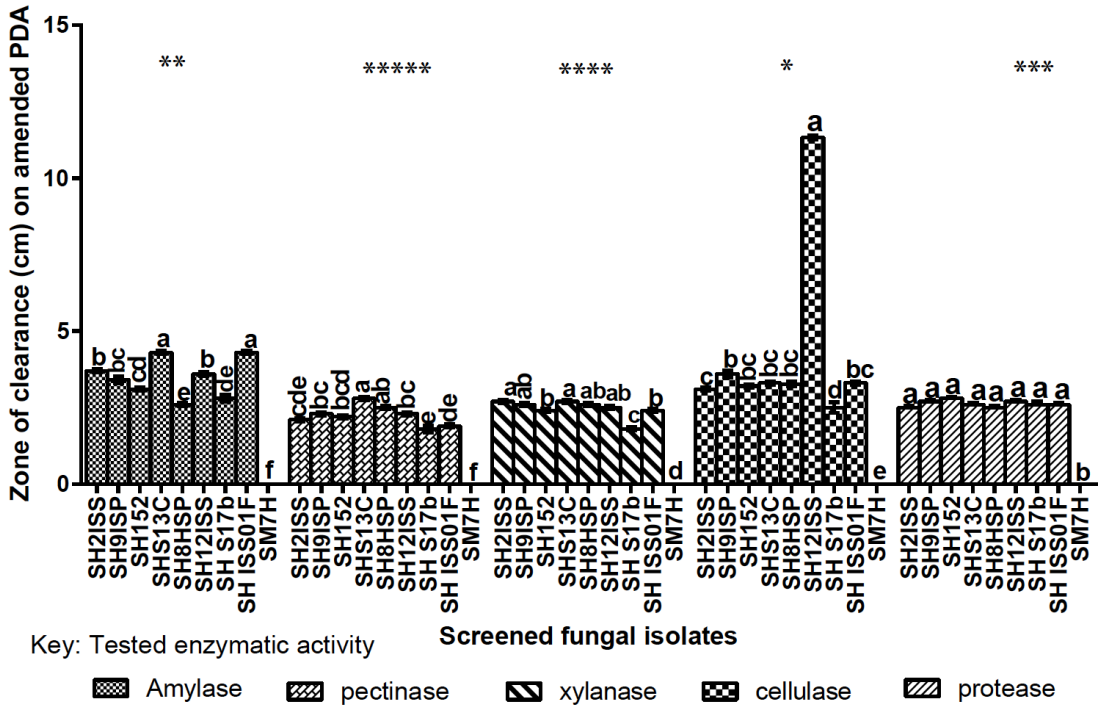

Figure 3.5: Extra cellular enzymatic activity of selected fungal isolates

Legend:
Bars (screened isolates) in each treatment (tested enzymatic activity) with the same alphabet letter represents isolate means with no significant difference at *Tukey's test* (*P≤0.05*). Treatments with grand means that are not significantly different at *P≤0.05* according to *Tukey's test* have the same number of *asterisk(s)* above the stacked bars (treatment).

Isolates SH8HSP and SHS17b on the other hand performed dismally for a number of treatments as reported in Fig. 3.5. Results of cellulase assays showed that isolate SH12ISS recorded significantly high cellulase activity of average clearance diameter of 11±0.16cm ($F_{(7,16)}$=976.07 and P value = 0.0001). Analysis of fungal extra cellular proteolytic activity show that all the isolates expressed same level of ability of digesting extra cellular protein substrate ($F_{(7,16)}$ = 2.34 and P value = 0.0756).

Evaluation of digestion of the different substrates by fungal isolate, show that cellulase enzymes was the most efficient extra cellular enzyme, recording an average clearance diameter of 4.2±0.13cm while pectinase was the least effective extra cellular enzyme recording an average clearance diameter of 2.2±0.12cm ($F_{(4,10)}$ = 93.41 and P value = 0.0001).

Figure 3.6 shows the quantified potential of bacterial isolates to induce decay of pre-conditioned *S. hermonthica* seeds (**Plate 3.7**).

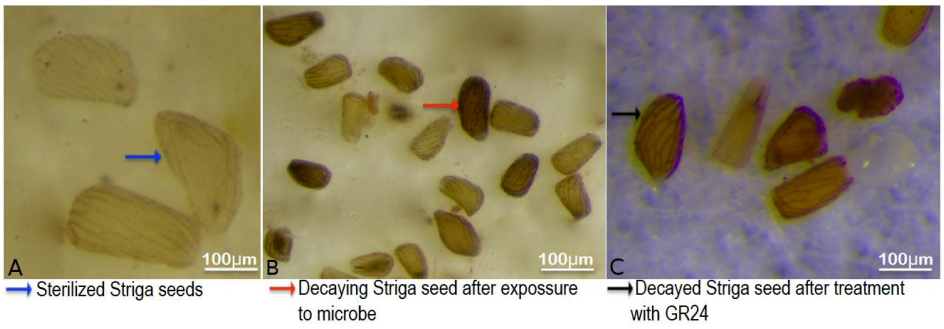

Plate 3.7: Photographs of *S. hermonthica* seed decay experiment

Legend:
A) Healthy sterilized *Striga* seeds, **B**) *Striga* seeds after exposure to microbial treatment and **C**) Preconditioned *Striga* seeds after exposure to microbes and then GR24.

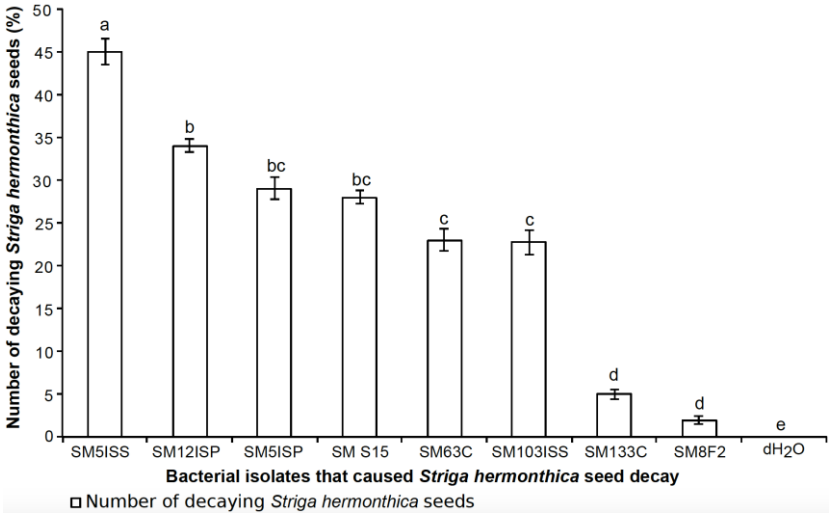

Figure 3.6: Effect of bacterial exudates on pre-conditioned *S. hermonthica* seeds.

Legend:
Bars with same alphabet letters represents means that are not significantly different at Tukey's test (0.05).

Results presented in Figure 3.6 shows empirical proportion of bacterial isolates with ability to cause *S. hermonthica* seed decay. All the decayed *Striga* seeds failed to germinate after exposure to GR24. Results of this assay (**Fig. 3.6**) revealed that isolate SM5ISS recorded significantly high *S. hermonthica* seed decay percentage (45±0.23%) while isolates SM133C and SM8F2 recorded significantly low *S.*

hermonthica seed decay (6±0.6% and 2±0.6% respectively) with L.S.D $_{(0.05)}$ =3 .951, $F_{(8,26)}$ = 143.08 and P value = 0.001). Interesting, fungal isolates did not affect the physiological appearance of the preconditioned *S. hermonthica* seeds (no symptom of *S. hermonthica* decay) and hence no significant statistical analysis was generated.

Analysis of the relationships among the bacterial isolates with respect to antibiosis, enzymatic activity and *S. hermonthica* seed decay estimates (**Fig. 3.7**) show that isolates SM5ISS and SM12ISP formed solitary clades. The remaining isolates formed two clades, each consisting of three isolates as shown in Fig 3.7. On average, isolate SM5ISS had consistently high antibiosis, extra cellular enzymatic activity and *S. hermonthica* seed decay as indicated by colours dominating its column in Figure 3.7. On the contrary, isolate SM8F2 recorded relatively low expression values for most of the assayed tests among all isolates evaluated (**Fig.3.7**).

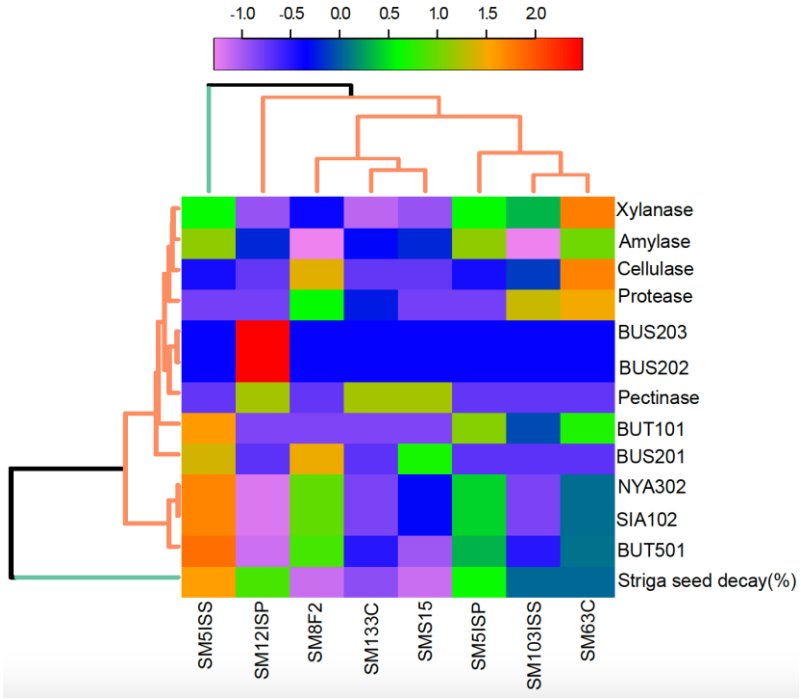

Figure 3.7: Hierarchical clustergram of assayed bacterial isolates

Legend:
Hierarchical clustergram generated using means of zones of inhibition, clearance and number of decaying *S. hermonthica* seeds caused by selected bacterial isolates. The heatmap (based on Manhattan metric) shows relationship between selected isolates and the measured morphometric descriptor. The coloured scale bar indicates the quantified significant strength of the assayed morphometric descriptor. Red and purple colours in the heatmap indicate the highest and the least recorded significant mean values respectively at P≤0.05 for the assayed treatments.

Correlation profile between morphometric descriptors (antibiosis and enzymatic activity estimates) and fungal isolates revealed that there are three functional clades (**Fig.3.8**). Isolate SH12ISS recorded a unique antibiosis and extra cellular enzymatic correlation profile (the only member in this functional clade) while the other isolates clustered in two clades as shown in Figure 3.8.

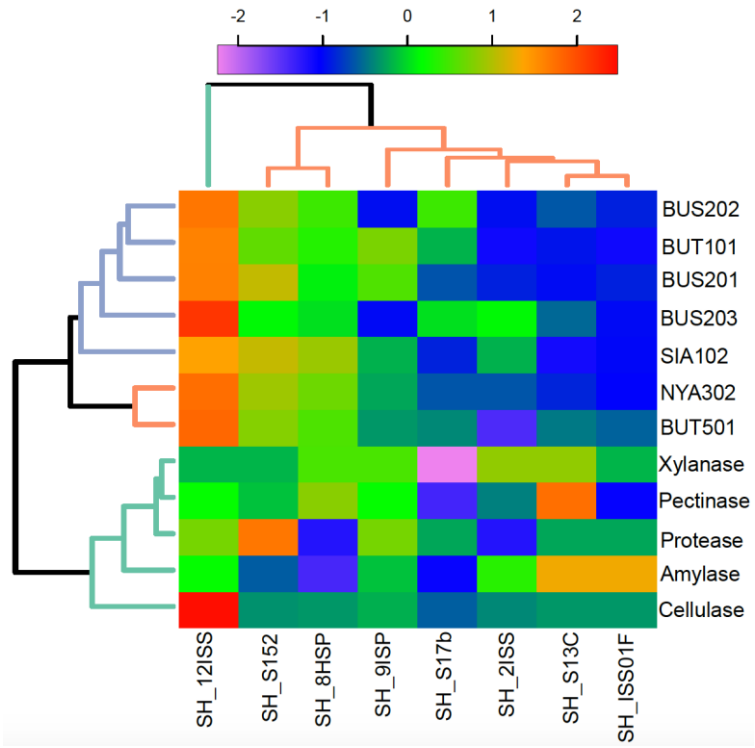

Figure 3.8: Hierarchical clustergram of assayed fungal isolates

Legend:
Hierarchical clustergram generated using means of zones of inhibition and clearance of the selected fungal isolates. The heatmap (based on Manhattan metric) shows relationship between fungal isolates and the measured morphometric descriptors. The coloured scale bar indicates the quantified strength of the assayed morphometric descriptor. Red and purple colours in the heatmap indicate the highest and the least recorded significant mean values respectively at $P \leq 0.05$ for the assayed treatments.

Molecular characterization of selected bacterial isolates was done by performing sequencing of PCR products of 16S rRNA gene (**Plate 3.8**, page 65). These products were purified, sequenced and analyzed. The results were analyzed and used to generate the output presented in Figure 3.9 (page 65).

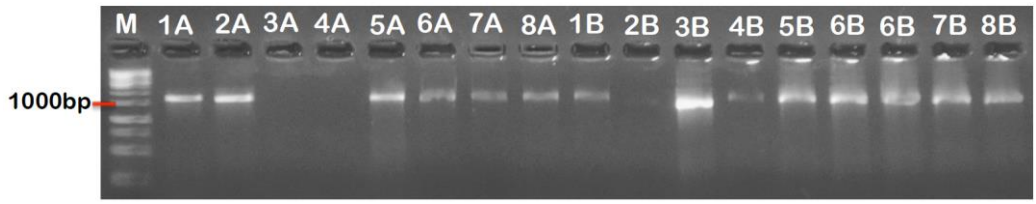

Plate 3.8: Gel photograph of bacterial 16S rRNA gene PCR products

Legend:
M) Molecular marker (1kb plus ladder), **1**) SM5ISP, **2**) SM5ISS, **3**) SM63C, **4**) SM8F2, **5**) SM103ISS, **6**) SM12ISP, **7**) SM133C, **8**) SM15. **A** and **B** are replicates of each reaction.

Phylogenetic analysis of bacterial isolates revealed that the isolates clustered in three main genus clades namely *Bacillus*, *Streptomyces* and *Rhizobium* all with bootstrap values of 99 (**Fig.3.9**). The *Bacillus* clade had the highest number of screened isolates (5) distributed in five sub clades (**Fig.3.9**).

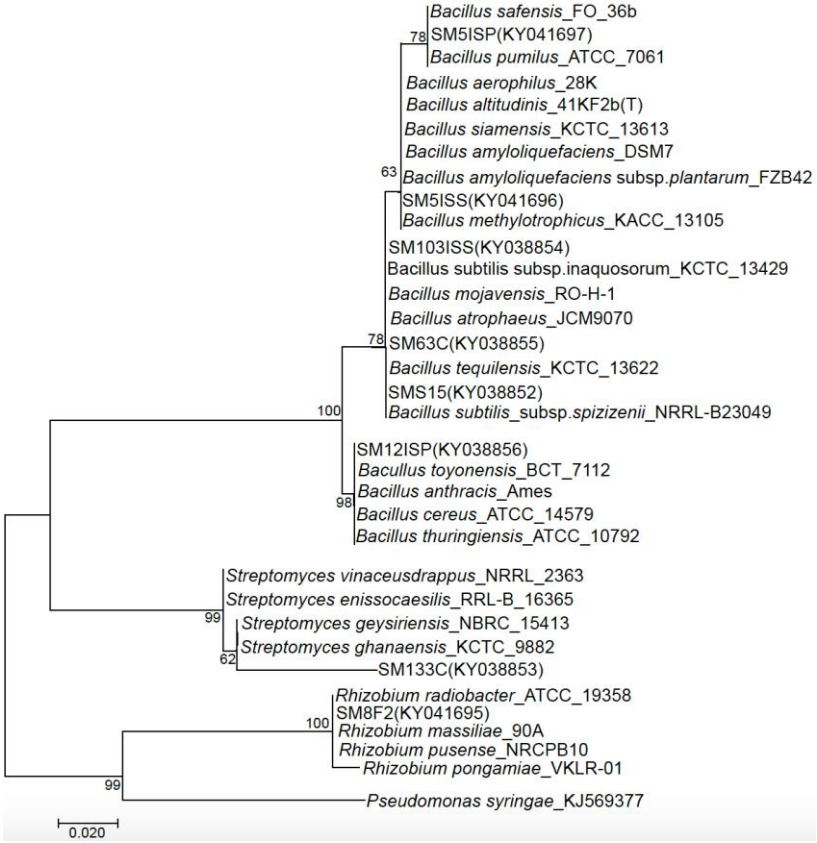

Figure 3.9: Phylogenetic tree of bacterial isolates based on 16S rRNA sequences

Legend:
Names of all screened bacterial isolates have NCBI accession codes in brackets. The scale bar refers to 0.02 substitutions per nucleotide position. Bootstrap values obtained with 1000 resampling are referred to as percentages at all branches.

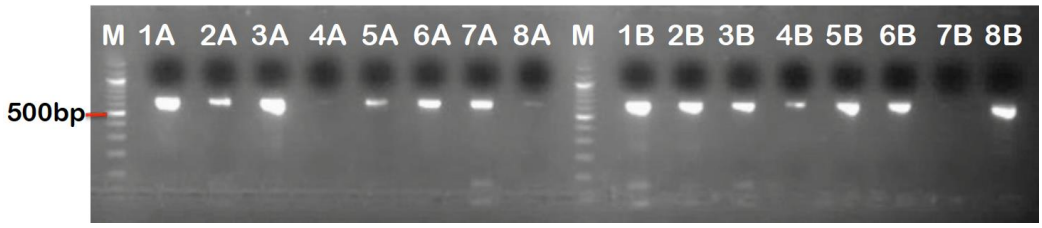

Plate 3.9: Gel photograph of fungal 18S rRNA gene PCR products

Legend:
M) Molecular marker (100bp ladder), **1**) SH_ISS01F, **2**) SH_2ISS, **3**) SH_8HSP, **4**) SH_9ISP, **5**) SH_12ISS, **6**) SH_13C, **7**) SH_152, **8**) SH_S17b. **A** and **B** are replicates of each reaction.

Plate 3.9 is a photograph of 18S rRNA PCR products of selected fungal isolates. The products were purified, sequenced and the results analyzed and used to generate the phylogenetic relationship (**Fig. 3.10**).

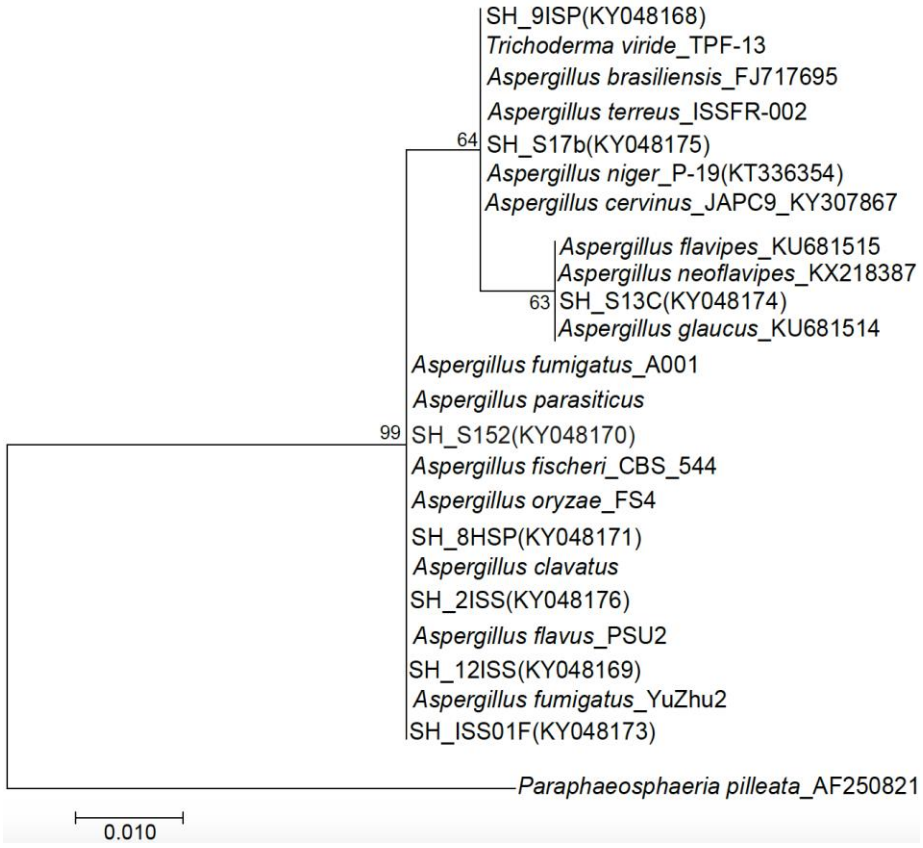

Figure 3.10: Phylogenetic tree of fungal isolates based on 18S rRNA sequences

Legend:
Names of all screened fungal isolates have NCBI accession codes in brackets. The scale bar refers to 0.007 substitutions per nucleotide position. Bootstrap values obtained with 1000 resampling are referred to as percentages at all branches.

The deduced fungal phylogenetic tree profile revealed that all the screened isolates were close closely affiliated to members of *Paraphaeosphaeria pilleata* (**Fig.3.10**).

3.5 Discussion

Most microbes used in biological control of crop pests, weeds and diseases secrete myriad metabolites that act on the pathogen by either depriving the pathogens of nutrients and space (exclusive competition), lysing cell and/or blocking specific functions related to pathogen growth (antibiosis) or inducing host plant resistance (Compant et al., 2012; Zhao et al., 2013). Understanding the mechanisms through which the biocontrol of plant weeds/pests/diseases occur is critical to the eventual improvement and wider use of biocontrol methods. These mechanisms are generally classified as competition, parasitism/predation, and antibiosis.

In this study, bacterial isolate KY041696 and fungal isolate KY048169 exhibited significantly high antibiosis and extra cellular enzymatic properties, which probably confers an exclusive competitive survival advantage to them over non antibiotic-producing microbes in the same environment. Earlier studies had made similar proposition and several other research findings reported a positive correlation profile between *in vitro* antibiosis assays and *in vivo* biocontrol experiments (Fravel, 1988; Kämpfer, 2006). For instance, production of the antibiotic chetomin by *Chaetomium globosum in vitro* was positively correlated with antagonism of *Venturia inequalis* on apple seedlings in a growth chamber (Fravel, 1988).

In this study, bacterial isolate KY041696 deduced to be a *Bacillus* species and fungal isolate KY048169 deduced to be an *Aspergillus* species recorded high extracellular enzymes and antibiosis effect. The extra cellular enzymes produced are presumably responsible for the hyperparasitism expressed against *S. hermonthica* in the form of seed decay by KY041696 (Sicuia et al., 2015).

The study also revealed that most of the bacterial isolates that caused *S. hermonthica* seed decay had close genetic relationship with members of the genus called *Bacillus* with only single bacterial isolate having close genetic affiliation to *Streptomyces* and *Rhizobium*. This study established that most bacterial isolates belonged to the *Bacillus* species at the taxonomic level and this was also noticeable for their

metabolic features, which probably explain their inherent ability to decay *S. hermonthica* seed. For instance, some *Bacillus subtilis* and *Bacillus amyloliquefaciens* strains have been investigated and found to be environmentally safe biological agents with excellent colonization capacity and remarkable versatility in protecting plants from phytopathogenic fungi (Torres et al., 2015; Zhao et al., 2014). Earlier studies have also shown that *Bacillus spp*. are spore-forming bacteria and hence can easily be stored and transported as stable biological control agent products (Choudhary et al., 2009; Hobley et al., 2013). This study established that isolates KY038852 and KY038854 are genetically related to members of *B. subtilis* (98% similarity based on 16S rRNA partial gene sequence). Previous studies reported that some *B. subtilis* strains, used in environmental bioremediation and industrial application, secrete different extra cellular enzymes which enables them to use external cellulosic and hemicellulosic substrates present in plant cell walls (Borriss et al., 2011). The extra cellular enzymes produced by isolates KY038852 and KY038854 possibly acted on cellulosic and hemicellulosic components of the cell wall as established by Borris *et al.,* (2011) hence the seed decay.

Borriss et al (2011) also demonstrated that *B. amyloliquefaciens* subsp. plantarum is able to colonize plant roots and produce plant growth hormone known as indole-3-acetic acid. Other studies also established that *B. amyloliquefaciens* has ability to produce numerous antimicrobial and bioactive metabolites such as surfactin, iturin and fengycin which have well established *in vitro* activity (Xiao-yan et al., 2014). Its antifungal activity is due to non-ribosomal synthesis of the cyclic lipopeptides bacillomycin and fengycin while its antibacterial activity is mainly due to non-ribosomally synthesized polyketides (Chowdhury et al., 2013). However these metabolites were not assayed in this study. The production of these compounds highlights *B. amyloliquefaciens* and its close relative in the study (KY041696) as good candidates for the development of biocontrol agents (Xiao-yan Liu et al., 2014). *Bacillus atrophaeus* strain on the other hand have capacity to adapt and use several external nutrients as energy sources (Sella et al., 2008). This probably explains why isolate KY038855 which is closely related to *B. atrophaeus* caused *S.hermonthica* seed decay.

Soil microorganisms have been reported to alter soil pH and also modify the equilibrium of many chemical and biochemical reactions in the rhizosphere (Li et al., 2015). The biological activities of these microorganisms in the soil largely mediate solubilization of both macro and micronutrients (biofertilizers) thereby making them available for plant uptake at the root surface (Li et al., 2015). One such important agricultural microorganism is *B. methylotrophicus.* This strain is believed to have ability to solubilize insoluble phosphorous (P) and inhibit mycelial growth of phytopathogenic fungi (Mehta et al., 2010, 2013). In this study, isolate KY041696 clustered together with *B. methylotrophicus* in the same phylogenetic clade (98% similarity based on 16S rRNA partial gene sequence). This, therefore implies that isolate KY041696 possibly have the capacity to solubilize insoluble phosphorous (P) and make it available to plants.

Equally important are members of *Rhizobium* genus which have been reported to be nitrogen-fixing rhizobacteria with some members having capacity to solubilize insoluble phosphorous (Ahemad et al., 2014; Pereg and Mcmillan, 2015). This study established that isolate KY041695 is genetically affiliated to members of *Rhizobium* genus (99% similarity based on 16S rRNA partial gene sequence). Other studies have shown that low rhizosphere phosphorus (P) content not only affects soil productivity/fertility but also impairs proper plant growth and exacerbates *S. hermonthica* infection (Jamil et al., 2012). This tripartite relationship is vicious in farms under cereal production (Ransom, 2000). Most cereals growing in phosphorous and nitrogen deficient soils produce natural compounds referred to as strigolactones (Yoneyama et al., 2013). These compounds are essential recognition signals that aid germination of root parasitic weeds such as *S.hermonthica* (Yoneyama et al., 2013). This therefore implies that any biological strategy aimed at increasing rhizosphere P content ultimately ameliorates *S.hermonthica* suppression in infested farms. This probably rationalizes the classification of soils with isolates KY041696 (*Bacillus* species) and KY041695 (*Rhizobium* species) as *S. hermonthica* suppressive soil. This is because these two isolates probably enhance P mobilization in the soil thereby scaling down the production of *S. hermonthica* seed germination-stimulant by the host plant.

The presence of *Streptomyces* strains among the screened isolates is consistent with other research findings which indicate that *Streptomyces* do exist in soils of arable land (Kämpfer, 2006; Tarkka et al., 2008). In this study isolate KY038853 is genetically related to *Streptomyces* (99% similarity based on 16S rRNA partial gene sequence). The success of these filamentous bacteria in terrestrial environments is attributed to their ability to produce an array of catabolic enzymes that degrade biopolymers (Tarkka et al., 2008). The potential of this isolate to control *S. hermonthica* can not be underestimated because members of *Streptomyces* have been shown to have versatile biodegradatve activity (Sousa et al., 2008). Evidence indicates that *Streptomyces* are quantitatively and qualitatively important in the plant rhizosphere where they influence plant growth and protect plant roots against invasion by root pathogens (Tarkka et al., 2008).

Although no single fungal isolate caused observable *S.hermonthica* seed decay, their role as biofertilizers in the rhizophere underscores their importance in *S. hermonthica* suppression mechanism (Singh et al., 2011). In this study, isolate KY048168 is genetically related to *Trichoderma viride*, a widely used biocontrol agent against phytopathogenic fungi, and as a biofertilizer because of its ability to establish mycorrhiza-like association with plants (Pereg and McMillan, 2015; Saba et al., 2012). The key factor to the ecological success of this genus is the combination of very active mycoparasitic mechanisms plus effective defense strategies induced in plants (Saba et al., 2012). Major mechanisms involved in the biocontrol activity of *Trichoderma* spp. are competition for space and nutrients, production of diffusible and/or volatile antibiotics and hydrolytic enzymes like chitinase and β-1,3-glucanase. These hydrolytic enzymes partially degrade the pathogen cell wall and lead to its parasitization (Saba et al., 2012).

Several mechanisms by which *Trichoderma* is believed to influence plant development have been proposed and these include, production of phytohormones, the solubilization of sparingly soluble minerals, induction of systemic resistance in the host plant, reduction in pollutant toxicity (organic or heavy metal), and the regulation of rhizospheric microflora (Li et al., 2015).

All other screened fungal isolates in this study have genetic affiliation to *Aspergillus*. The *Aspergillus spp.* are a fascinating group of fungi exhibiting immense ecological and metabolic diversity (Keietsu et al., 2010). Several members of *Aspergillus* spp. have been found to be involved in P solubilization and bioremediation processes in the rhizosphere (Singh et al., 2011). However, given their association with aflatoxins, which are cancer causing chemicals, utilization of *Aspergillus* as a biological control agent especially in maize/cereal/grain fields needs to be carefully scrutinized to avoid grain contamination. The close genetic affiliation to genus *Aspergillus* by most of the screened fungal isolates cultured from *S. hermonthica* suppressive soils underlines their role in *S. hermonthica* suppression. It is interesting that in the areas sampled, *F. oxysporium* fsp. *Strigae* which has been widely associated with *S.hermonthica* suppression in the regions was not isolated (Nzioki et al., 2016; Avedi et al., 2014).

3.6 Conclusion

This study has demonstrated that soil microbes have potential to be used as biocontrol agents as they produce extra cellular lytic enzymes and antibiotic compounds that can induce a biocontrol effect. However, more studies are needed to determine the functions and mode of action of the lytic enzymes of these microbes in order to develop novel biological control remedies against *S. hermonthica*.

CHAPTER FOUR

DEVELOPMENT OF REGENERATION AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION PROTOCOLS FOR FARMER PREFERRED MAIZE VARIETIES

4.1 Introduction

Majority of Kenyan farmers, especially in the western region, are small-scale farmers and prefer using their own open pollinated maize varieties (OPV) namely Kakamega *Striga* tolerant population 94 (KSTP'94) and 'Namba nane' as planting seed (Wambugu et al., 2012; Omondi et al., 2014). KSTP'94 is a certified drought tolerant and partially *S. hermonthica*-tolerant maize variety while 'Namba nane' is a traditional OPV that is distinct for its eight rows of kernels per cob (Omondi et al., 2014). Given the high price of certified hybrid maize seed, high input level and their longer maturation period, small-scale farmers in western Kenya predominantly cultivate OPVs especially during the short rains (Omondi et al., 2014; Pathi et al., 2013; Warburton et al., 2010).

These OPVs are preferred by farmers because of their early maturity, low input tolerance, culture and culinary qualities such as superior taste and better keeping quality (Anjichi et al., 2005; Denning et al., 2009; Gudu et al., 2005; Macharia et al., 2010; Omondi et al., 2014). These varieties also offer the economic advantage of seed recycling without the risk of yield loss, as is the case with hybrid varieties (Omondi et al., 2014).

Demand for maize in this region outstrips supply largely due to farmer's over reliance on poor yielding local OPVs such as KSTP'94 and Namba Nane, poor farm management practices and heavy *S. hermonthica* infestation (Wambugu et al., 2012). The risk of declining seed quality and low farm yield of these varieties calls for better breeding and biotechnological remedies. It is therefore important to set up a platform for genetic improvement of the OPVs. One way of achieving this is by developing a reliable *in vitro* regeneration and genetic transformation protocols to enable rapid improvement of the farmer preferred OPVs (Oneto et al., 2010; Sairam et al., 2008).

The development of reliable and robust *in vitro* regeneration protocols from single cells or callus cells capable of being genetically transformed at high frequency is critical for production of both clonal propagation materials and successful genetic improvement of local maize varieties (Oneto et al., 2010; Sairam et al., 2008). A number of factors have been investigated to assess their effect on *in vitro* regeneration of maize. For instance, the regenerability of different explants, such as shoot apices, leaf, stem segments, hypocotyls, epicotyls, immature embryos, and mature intact seeds, have been tested (Oneto et al., 2010). Among the fore-mentioned explants, immature zygotic embryos have been found to be the best explants for establishing regenerable maize callus cultures for genetic transformation (Aguado-Santacruz et al., 2007; Mohammad Ahmadabadi et al., 2007; Anami et al., 2010; Fernandes et al., 2008; Frame et al., 2011; Gordon-Kamm et al., 2002). Despite immature embryos being the best explants, *in vitro* establishment of maize callus cultures has also been reported to be significantly affected by type of maize variety being used largely due to differences in hormonal regulation during embryogenesis (Jimenez et al., 2001).

A number of basal media such as Murashige and Skoog (MS), Chu (N6), Linsmaier and Skoog (LS), Shenck and Hildebrandt (SH), and Gamborg's B5 have been used to initiate maize callus cultures and regenerate maize plants (De-yi et al., 2011). These media are often fortified with plant growth hormones (PGRs) to aid induction of primary and embryogenic callus as well as shoot/root formation (Songstad, 2010). Incorporation of an auxin such as 2,4-dichlorophenoxyacetic acid (2,4-D) or dicamba (3,6-dichloro-o-anisic acid) in culture media has been shown to be a key prerequisite in tissue culture of monocotyledonous plants such as maize (Songstad, 2010). The best concentration level of auxins used in the maize culture media typically ranges between 1μM to 10μM (Cheng-hao et al., 2008; Songstad, 2010).

Furthermore, studies have shown that addition of silver nitrate ($AgNO_3$), and amino acids such as L-proline and casein hydrolysate enhances induction of embryogenic callus especially type II callus in maize (Armstrong et al., 1985; Duncan et al., 1985; Songstad et al., 1988; Songstad, 2010; Vain et al., 1989).

Development of an efficient *in vitro* regeneration protocol is essential in the establishment of a good genetic transformation system for maize (Cheng et al., 2008). Assessment of transformation potential of a crop using *β*-glucuronidase (GUS) gene is important in evaluating regeneration efficiency of the transformants (Witcher et al., 1998). GUS gene is a superior sensitive and versatile gene fusion marker in higher plants and has widely been used in transformation studies of most economically important plants (Jefferson et al., 1987; Witcher et al., 1998). Plants lack detectable glucuronidase activity, providing a null background in which to assay chimeric gene expression in transformed plants (Jefferson et al., 1987). This gene fusion system therefore is easy to quantify, highly sensitive to histochemical assays of localized gene activity and completely independent of any intrinsic reporter enzyme activity in plants (Jefferson et al., 1987). The activity of this gene is assayed after transforming target plant material using a suitable gene transfer technique. *Agrobacterium*-mediated transformation is the most preferred gene transfer technique because of its remarkable advantages over direct gene transfer methodologies (Tripathi et al., 2005). For instance, it reduces the copy numbers of the transgene, potentially leading to fewer problems with transgene co-suppression and instability and also has a higher transformation frequency (Kumar and Singh, 2013; Tripathi et al., 2005).

The ability to improve important agronomic traits of local OPVs such as KSTP'94 and Namba Nane could be achieved via biotechnological techniques such as development of efficient and reliable genetic transformation system.

In this study, immature embryos of KSTP'94, Namba nane and CML144 maize varieties were evaluated for their ability to form somatic embryos and subsequently regenerate plantlets. Their transformability using *Agrobacterium*-mediated *GUS* transformation was also assayed. The findings of this study provide a solid ground for incorporation of important agronomic traits in these varieties by genetic transformation.

4.2 Materials and Methods

4.2.1 Plant material and explant preparation

Seeds of the selected local OPVs namely Namba Nane and KSTP'94 were acquired from Kenya Agricultural and Livestock Research Organization (KALRO) while Plant Transformation Laboratory (PTL) of Kenyatta University provided the tropical maize inbred line CML144. PTL acquired CML144 from International Maize and Wheat Improvement Center commonly called by its Spanish acronym CIMMYT (*Centro Internacional de Mejoramiento de Maízy Trigo*) and grown in the research field at Jomo Kenyatta University of Agriculture and Technology (JKUAT).

Maize ears and tassels were covered with paper bags before silk emerged. The ears were self-pollinated at optimum length of silk-receptivity and then harvested between the 9^{th} and 11^{th} day after pollination (DAP) to obtain immature embryos. Harvested ears were taken to PTL at Kenyatta University where they were carefully de-husked. The de-husked corns were placed in a sterile container to which 70% ethanol was added to cover all ears for 2 min and then rinsed thrice with double-distilled water in a laminar flowhood. The corn were further sterilized in 50% dilution (v/v) of 3.85% (active ingredient) solution of sodium hypochlorite in which 10µl of Tween® 20 (Polyoxyethylene sorbitan monolaurate) had been added for 30 min. The ears were then rinsed thrice with double-distilled water. The kernel crowns were aseptically excised and immature zygotic embryos (1mm - 1.5 mm in size) isolated using a sterile blunt spatula. Immature embryos of all varieties were then transferred to sterile Petri dishes containing 10 ml of embryo suspension media (ESM).

4.2.2 Media preparation

Four types of embryo suspension media (ESM), namely Murashige and Skoog (MS), Linsmaier and Skoog (LS) and N6 and N6* medium (N6 medium supplemented with 0.8mg/l$AgNO_3$ and 0.35g/l L-proline) were prepared and used to hold excised immature embryos before plating them on callus induction media (Chu et al., 1975; Linsmaier and Skoog, 1965; Murashige and Skoog, 1962). The same four types media (MS, LS, N6 and N6*) were prepared, solidified using gelrite and served as

callus induction media (CIM). The four media were fortified with 30g/l sucrose, 2,4-D at six concentration levels (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) and solidified using 3g/l gelrite (Duchefa Biochemie). For further subculture, only MS and N6 media were used for callus maturation (CMM). These two media were supplemented with 2,4-D at five concentration levels (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l, 60g/l sucrose and 3g/l gelrite. The regeneration media (RM) used in this study were similar to the callus maturation media but lacked 2,4-D (without PGR) and the sucrose content was reduced to 30g/l. 8g/l phytoagar (Duchefa Biochemie Lot No. 004016.04) was used to solidify these media. The pH of all the media was adjusted to 5.8 before autoclaving at 121°C for 15 min. Vitamins and $AgNO_3$ were filter sterilized and added to autoclaved callus induction and callus maturation media.

4.2.3 Callus induction

Callus was induced in the three maize varieties using the above-described four callus induction media (CIM). In total, 1728 embryos were used whereby 576 immature embryos of each maize variety were used to set up primary callus induction experiment. A treatment in this experimental design referred 8 immature embryos of a specific maize variety cultured on a petri plate of a particular CIM medium fortified with a specific 2,4-D concentration (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) totaling to 48 embryos per variety and replicated three times (8 immature embryos X 3 maize varieties X 4 CIM media X 6 concentration levels of 2,4-D X 3 replicates = 1728 embryos). Immature embryos with undamaged scutellar tissues were cultured on the four media with the embryo axis in contact with the medium and incubated in the dark at 25 ± 2°C. After two weeks of incubation on CIM, the number of immature embryos producing primary callus was recorded in each variety on all the CIM used.

4.2.4 Callus maturation

For maturation of somatic embryos, primary calli emerging from the callus induction experimental design were subcultured onto two callus maturation media (CMM) fortified with five concentration levels of 2,4-D (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l. At this stage, four callus from each CIM media were separately cultured on individual

CMM (MS and N6) which had been fortified with five concentration levels of 2,4-D (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l in an experimental design as defined as 4 primary calli X 4 CIM X 2 CMM X 5 concentration levels of 2,4-D X 3 maize varieties X 3 replicates totaling to 1440 calli. They were incubated in the dark at 25±2°C for four weeks, subcultured once after two weeks. The calli were regularly screened (at three days interval) under microscope (Leica MZ10F/CLS150LED installed with Leica application suite V4) for formation of embryogenic calli (globular and coleoplitar shapes). The number of primary callus obtained from different CIM in each variety that produced embryogenic callus on the different ECIM was recorded.

4.2.5 Plant regeneration

The induced embryogenic calli were further subcultured to hormone-free regeneration media (RM) namely MS and N6 supplemented with 30g/l sucrose. The experimental design deployed for plant regeneration was defined as 80 embryogenic calli X 3 maize varieties X 2 RM X 3 replicates totaling to 1440 calli. The cultures were maintained at 25 ± 2°C under a 16-h photoperiod using cool white fluorescent lights (40 mmol m^{-2} s^{-1}) for four weeks with one subculture (after two weeks of incubation). The number of embryogenic calli that produced maize plantlets was recorded. The plantlets were left to grow until they were about 10 cm tall and those with healthy roots were removed from culture, rinsed in water to remove media, and transplanted into a mixture of equal parts (v/v) of sterilized peat moss and vermiculite (ratio of 1:1), and grown under humid conditions in a growth room for 2 weeks. Surviving plantlets were then transplanted in loam soil in larger bucket in the greenhouse.

4.2.6 Transformation of the selected maize varieties using GUS gene construct

Maize transformation and detection of GUS sterilized immature embryos of CML144, KSTP'94 and Namba nane were used as explants while *Agrobacterium tumefacien* strain EHA 105 carrying plasmid pTF102 with GUS gene (binary vector for GUS) was used to drive the gene into the embryos. The binary vector contains the P35S/T35S (CaMV 35S promoter/terminator), GUS intron (an intron-containing β-glucuronidase gene cloned from *Escherichia coli*), phosphinothricin (PPT)

acetyltransferase gene (*bar*) cloned from *Streptomyces hygroscopicus* conferring PPT resistance for selection, pBR322 (origin of replication for *E. coli*), pVS1 (origin of replication for *Agrobacterium*), left border (LB) and right border (RB). This binary vector was transformed into *Agrobacterium* strain EHA105 by freeze thaw method (Jyothishwaran et al., 2007). The transformed EHA105 colonies were maintained on solid YEP medium (Chilton et al., 1974) supplemented with 100 mgl^{-1} kanamycin sulfate. A single colony was transferred to 50 ml YEP broth medium containing the same selective antibiotics and incubated overnight on a shaker (200 rpm) at 28°C. When the culture was at log phase, which corresponded to an OD_{600} of 0.5, cells were pelleted by centrifugation at 10000rpm for 10 min and then resuspended in infection medium (Inf).

The five steps of Zhao et al., (2001) transformation protocol namely; explants infection with *Agrobacterium*, co-cultivation, resting, selection and plant regeneration of transformed Namba nane, KSTP'94 and CML144 were followed. Thirty immature embryos of each maize variety infected with GUS-carrying *Agrobacterium* were cultured in separate plates of co-cultivation medium and replicated six times for each variety (30 embryos X 3 maize varieties X 2 CIM X 6 replicates). The plates were placed in the dark at 22°C for three days (co-cultivation stage). The embryos were then transferred to resting medium at 28°C in the dark for seven days. They were later transferred to selection medium I (SM I) for two weeks and then sub cultured in selection medium II (SM II) for two more weeks in the same experimental design. Both selection steps were done in darkness at 28°C. The surviving calli were subjected to histochemical staining using Jefferson et al., (1987) protocol. Total genomic DNA was extracted from calli using Savini *et al.* (2008) protocol. PCR was performed using Bioline Taq Master mix Kit (Cat No. BIO 25043). The GUS primers used to perform PCR were; Forward primer; 5'-TTTAACTATGCCGGGATCCATCGC-3' and Reverse primer; 5'-CCAGTCGAGCATCTCTTCAGCGTA-3'. The conditions for amplification were 95°C for 10 min, 40 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 1min followed by a final extension of 72°C for 7min resulting in a DNA fragment of 528bp. The transformation frequency was calculated as the proportion of the number

of biolaphos resistant callus events over the number of immature embryos of individual variety infected with *Agrobacterium*. Transformation efficiency (%) was calculated as the number of calli that tested positive for PCR screening per number of immature embryos of individual variety infected with *Agrobacterium*.

4.3 Data analysis

Data was collected and keyed in excel spreadsheets. Statistical analyses were performed using Genstat version 17 and SAS version 9.2 softwares to determine the frequency of callus induction, somatic embryogenesis, regeneration and transient Gus expression. Analysis of variance (ANOVA) was performed for individual experiment and Tukey's 95% confidence intervals statistic used to determine significant differences in callus induction, embryogenic callus formation, shoot induction efficiency (%) and GUS transformation frequency (surviving calli at selection media II) and efficiency (proportion of calli that are +ve for GUS gene PCR amplification).

4.4 Results

In this study, callus induction process was initiated by swelling of the scutellum of the immature embryos by the fourth day. The swollen scutellum formed a mass of irregular shaped cells (primary calli) within 10 days of incubation (**Plate 4.1,** page 80). Miniature globular somatic embryos were observed to emerge at the surface of the soft friable callus of all varieties by 16^{th} day of incubation on CIM (**Plate 4.1**). At this embryogenesis phase, compact, friable, fast-growing coleoptilar-like callus, typical embryogenic callus began to form (**Plate 4.1**).

Evaluation of effect of variety, media and the concentration of 2,4-D on callus induction revealed that induction of callus from immature maize embryos is influenced by genetic and environmental factors. Based on analysis of variance, variety, media and the concentration of 2,4-D influenced the frequency of callus induction (**Table 4.1,** page 81).

Frequency of callus induction of the three varieties cultured on different CIM supplemented with 2,4-D ranged from 45.6±2.05% for Namba nane cultured on either MS or LS fortified with 0.5 mg/l of 2,4-D to 85.6±2.04% for CML144

cultured on LS fortified with 2 mg/l of 2,4-D (**Table 4.1,** page 81).

Analysis of callus induction for individual variety revealed that Namba Nane recorded the highest callus induction frequency (83.9±3.75%) on N6* supplemented with 2.5 mg/l of 2,4-D while the lowest (45.6±2.05%) on MS supplemented with 0.5 mg/l of 2,4-D (**Table 4.1**, page 81).

For KSTP'94, embryos cultured on N6* fortified with 1.5mg/l of 2,4-D recorded the highest callus induction frequency (84.4±3.18%) and the lowest (46.7±1.72%) on MS at 0.5 mg/l of 2,4-D (**Table 4.1**, page 81).

On the other hand, CML144 cultured on LS media supplemented with 2 mg/l of 2,4-D recorded the highest callus induction frequency (85.6±2.04%) while the lowest frequency (48.9±2.22%) was recorded on MS fortified with 0.5 mg/l of 2,4-D (**Table 4.1**, page 81).

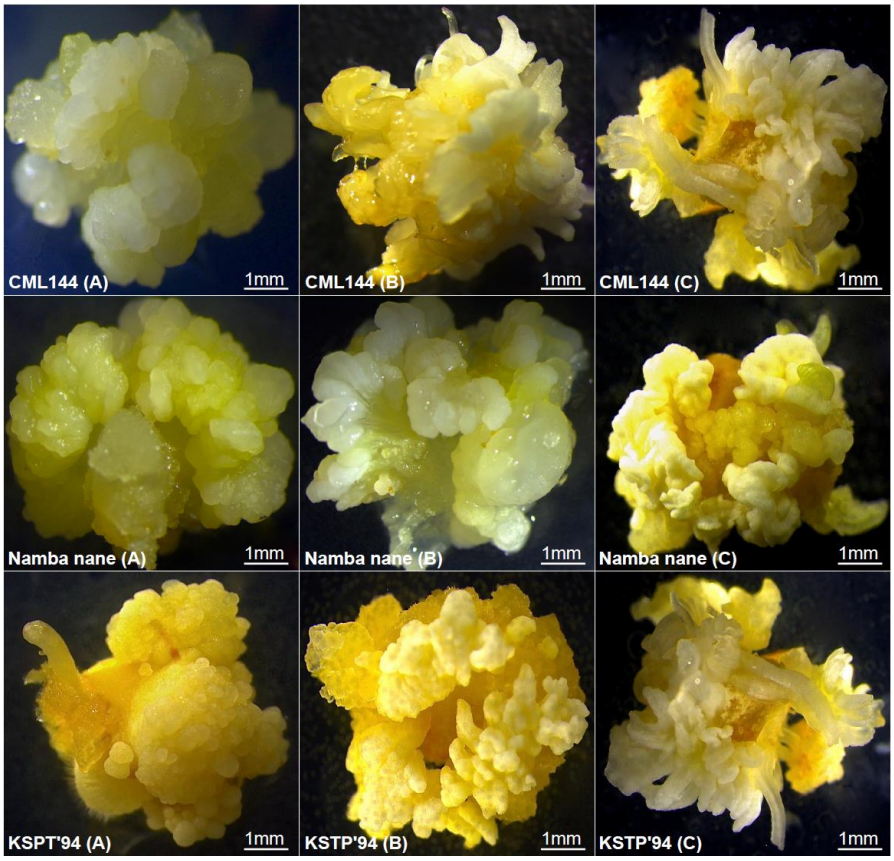

Plate 4.1: *In vitro* developmental stages of maize callus

Legend
(A) Primary callus, **(B)** Globular-shaped embryogenic callus and **(C)** Coleoptilar-shaped embryogenic callus**.**

Table 4.1: Callus induction percentage on media fortified with different 2,4-D conc. levels

Maize variety	2,4-D (mg/l)	Induction Media				Statistic			
		MS	LS	N6	N6*	$F_{(3, 20)}$	P value	$L.S.D_{(0.05)}$	CV%
CML144	0.0	$0.0\pm0.00^{a}{}_{d}$	$0.0\pm0.00^{a}{}_{d}$	$0.0\pm0.00^{a}{}_{f}$	$0.0\pm0.00^{a}{}_{c}$	0.0	1.0	0.000	0.000
	0.5	$48.9\pm2.22^{a}{}_{c}$	$51.1\pm1.41^{a}{}_{c}$	$54.4\pm1.11^{a}{}_{de}$	$53.3\pm2.43^{a}{}_{b}$	1.73	0.1940	7.425	8.845
	1	$72.2\pm3.62^{b}{}_{ab}$	$82.2\pm2.22^{a}{}_{ab}$	$70.0\pm2.85^{b}{}_{abc}$	$83.3\pm3.33^{a}{}_{a}$	4.98	0.0097	10.085	9.719
	1.5	$77.8\pm3.72^{a}{}_{a}$	$82.2\pm2.22^{a}{}_{ab}$	$78.9\pm4.10^{a}{}_{ab}$	$83.9\pm2.50^{a}{}_{a}$	0.78	0.5214	12.794	9.812
	2	$73.3\pm2.98^{b}{}_{ab}$	$85.6\pm2.04^{a}{}_{a}$	$84.4\pm2.38^{a}{}_{a}$	$81.7\pm2.81^{a}{}_{ab}$	3.36	0.0393	11.934	9.089
	2.5	$72.2\pm2.05^{b}{}_{ab}$	$83.3\pm2.28^{a}{}_{ab}$	$81.7\pm3.42^{a}{}_{a}$	$84.4\pm3.18^{a}{}_{a}$	4.42	0.0154	10.512	8.089
KSTP'94	0.0	$0.0\pm0.00^{a}{}_{d}$	$0.0\pm0.00^{a}{}_{d}$	$0.0\pm0.00^{a}{}_{f}$	$0.0\pm0.00^{a}{}_{c}$	0.0	1.0	0.000	0.000
	0.5	$46.7\pm1.72^{a}{}_{c}$	$48.9\pm1.41^{a}{}_{c}$	$52.2\pm2.05^{a}{}_{de}$	$51.1\pm2.05^{a}{}_{b}$	1.82	0.1758	7.227	8.994
	1	$68.9\pm2.81^{bc}{}_{ab}$	$78.9\pm2.05^{a}{}_{ab}$	$66.7\pm1.72^{c}{}_{bcd}$	$77.2\pm2.50^{a}{}_{a}$	6.86	0.0023	9.133	7.751
	1.5	$71.1\pm1.41^{b}{}_{ab}$	$78.9\pm2.05^{ab}{}_{ab}$	$77.8\pm3.30^{ab}{}_{abc}$	$84.4\pm3.18^{a}{}_{a}$	4.41	0.0155	10.314	8.177
	2	$71.1\pm1.41^{ab}{}_{ab}$	$82.2\pm1.41^{a}{}_{ab}$	$80.6\pm3.69^{a}{}_{ab}$	$81.1\pm4.01^{a}{}_{a}$	3.21	0.0450	11.352	8.921
	2.5	$67.8\pm2.05^{b}{}_{ab}$	$80.0\pm1.72^{a}{}_{ab}$	$80.6\pm3.69^{a}{}_{ab}$	$82.8\pm3.30^{a}{}_{a}$	6.79	0.0024	10.291	8.188
Namba 8	0.0	$0.0\pm0.00^{a}{}_{d}$	$0.0\pm0.00^{a}{}_{d}$	$0.0\pm0.00^{a}{}_{f}$	$0.0\pm0.00^{a}{}_{c}$	0.0	1.0	0.000	0.000
	0.5	$45.6\pm2.05^{a}{}_{c}$	$45.6\pm2.05^{a}{}_{c}$	$50.0\pm1.49^{a}{}_{e}$	$50.0\pm1.22^{a}{}_{b}$	2.18	0.1225	6.884	8.916
	1	$66.7\pm2.98^{b}{}_{ab}$	$74.4\pm2.05^{ab}{}_{b}$	$64.4\pm1.41^{b}{}_{cde}$	$79.4\pm3.27^{a}{}_{a}$	7.49	0.0015	10.041	8.721
	1.5	$68.9\pm1.41^{b}{}_{ab}$	$78.9\pm1.11^{a}{}_{ab}$	$76.1\pm3.49^{ab}{}_{abc}$	$81.1\pm3.06^{a}{}_{a}$	4.57	0.0136	9.847	7.991
	2	$68.9\pm1.41^{b}{}_{ab}$	$78.9\pm1.11^{a}{}_{ab}$	$79.4\pm3.27^{a}{}_{abc}$	$79.9\pm4.22^{a}{}_{a}$	3.19	0.0461	11.698	9.429
	2.5	$65.6\pm1.11^{b}{}_{b}$	$77.8\pm1.41^{a}{}_{ab}$	$78.9\pm3.62^{a}{}_{abc}$	$83.9\pm3.75^{a}{}_{a}$	8.51	0.0008	10.558	8.537
$F_{(17, 75)}$		18.69	54.96	15.51	16.92				
P value		<.0001	<.0001	<.0001	<.0001				
$L.S.D_{(0.05)}$		11.612	8.999	14.542	15.414				
CV(%)		8.728	6.012	10.011	10.037				

Mean values with the same ***superscript*** letters within the same row are not significantly different at *Tukey's* test (P<0.05). Mean values with the same ***subscript*** letters within the same column are not significantly different at *Tukey's* test (P<0.05).

The inherent varietal effect on callus induction is a critical factor in designing *in vitro* regeneration protocols for tropical maize lines as revealed by results in Table 4.2. A significant difference in callus induction frequency was observed among the selected maize varieties as shown in Table 4.2. CML144 recorded the highest primary callus induction frequency (73.8±1.37%), while Namba nane recorded the least primary callus induction frequency (69.7±1.24%), at $p \leq 0.05$.

Analysis of the effect of media (**Table 4.2**) on callus induction showed that N6* was the best callus induction medium (75.3±1.71%) while MS was the least efficient callus induction medium (65.7±1.18%). Assessment of the effect of 2,4-D concentration on callus induction revealed that optimum primary callus initiation was achieved in the presence of a limited range of 2,4-D concentration (**Table 4.2**). The results revealed that 1.5mg/l was the minimum 2,4-D concentration level that recorded the highest (78.6±0.90%) callus induction frequency.

Table 4.2: Effect of variety, media and 2,4-D conc. on callus induction frequency

Variety	Primary Callus (%)	Induction media	Primary Callus (%)	2,4-D Conc.(mg/l)	Primary Callus (%)
CML144	73.8±1.37[a]	N6*	75.3±1.71[a]	0.0	0.0±0.00[c]
KSTP'94	71.7±1.24[ab]	LS	73.9±1.44[ab]	0.5	49.8±0.59[c]
Namba 8	69.7±1.24[b]	N6	72.1±1.39[b]	1	74.0±1.05[b]
		MS	65.7±1.18[c]	1.5	78.6±0.90[a]
				2	77.8±1.40[a]
				2.5	78.5±1.06[a]
$F_{(2, 350)}$	8.28	$F_{(3, 350)}$	26.27	$F_{(5, 350)}$	180.60
P value	0.0003	P value	<.0001	P value	<.0001
$L.S.D_{(0.05)}$	2.3797	$L.S.D_{(0.05)}$	3.0137	$L.S.D_{(0.05)}$	3.579

Mean values in the same column with same letters are not significantly different at *Tukey's* test (P<0.05).

The transition from primary callus to embryogenic callus was aided by two callus maturation media (CMM) supplemented with 2,4-D at five concentration levels following the primary callus induction experimental design (outlined in materials and methods). Analysis of embryogenic callus induction frequency shows that media (both CIM and CMM), 2,4-D concentration and maize variety affected embryogenesis process. Assessment of the embryogenic callus induction response of the tested varieties cultured on different media regimes is presented in Table 4.3 (page 84).

For CML144, immature embryos that were initially cultured on N6* (CIM) and later transferred to N6 (CCM) fortified with 1.5 mg/l of 2,4-D recorded the highest (66.7±1.9%) embryogenic callus frequency (**Table 4.3**, page 84).

Interestingly, CML144 embryos cultured on MS (CIM) and transferred to MS (CCM) fortified with 0.5 mg/l of 2,4-D recorded the least (10±1.9%) embryogenic callus frequency (**Table 4.3**). Immature embryos of KSTP'94 that were initially cultured on N6*(CIM) and later transferred to either MS or N6 (CCM) fortified with 1.5 mg/l of 2,4-D recorded the highest (63.3±1.9%) embryogenic callus induction frequency (**Table 4.3**).

On the contrary, KSTP'94 embryos cultured on MS (CIM) and transferred to MS (CCM) fortified with 0.5 mg/l of 2,4-D recorded the least (12.2±1.1%) embryogenic callus frequency (**Table 4.3**). Lastly, for Namba nane, immature embryos that were initially cultured on N6* (CIM) and later transferred to N6 (CCM) fortified with 1.5 mg/l of 2,4-D recorded the highest (61.1±1.1%) embryogenic callus induction frequency (**Table 4.3**). However, KSTP'94 embryos cultured on MS (CIM) and transferred to MS (CCM) fortified with 0.5 mg/l of 2,4-D recorded the least (5.6±1.1%) embryogenic callus frequency (**Table 4.3**).

Table 4.3: Effect of variety, media and PGR on embryogenic callus frequency (%) of selected maize varieties

	1°callus Induction	Embryogenic callus induction media											
		MS Concentration of 2,4-D (mg/l)					N6 Concentration of 2,4-D (mg/l)						
Variety	Media	0.5	1	1.5	2	2.5	0.5	1	1.5	2	2.5	L.S.D (0.05)	CV%
CML144	**MS**	$10.0\pm1.9^{d}_{cd}$	$32.2\pm1.1^{c}_{d}$	$48.9\pm1.1^{ab}_{cd}$	$47.8\pm1.1^{ab}_{cd}$	$45.6\pm1.1^{b}_{cde}$	$14.4\pm1.1^{d}_{d}$	$35.6\pm1.1^{c}_{ef}$	$53.3\pm1.9^{a}_{cd}$	$52.2\pm1.1^{a}_{cde}$	$51.1\pm1.1^{ab}_{cde}$	6.584	5.822
	LS	$17.8\pm1.1^{f}_{bc}$	$33.3\pm1.9^{e}_{d}$	$54.4\pm1.1^{abc}_{bc}$	$50.0\pm1.9^{bc}_{bcd}$	$47.8\pm1.1^{cd}_{bcd}$	$22.2\pm1.1^{f}_{bc}$	$41.1\pm1.1^{d}_{de}$	$58.9\pm1.1^{a}_{abc}$	$56.7\pm1.9^{ab}_{bcd}$	$55.6\pm1.1^{ab}_{abcd}$	7.038	5.561
	N6	$25.6\pm2.9^{c}_{ab}$	$51.1\pm1.1^{b}_{bc}$	$58.9\pm1.1^{ab}_{ab}$	$56.7\pm1.9^{ab}_{abc}$	$56.7\pm1.9^{ab}_{a}$	$27.8\pm1.1^{c}_{b}$	$52.2\pm1.1^{ab}_{abc}$	$60.0\pm1.9^{a}_{abc}$	$56.7\pm1.9^{ab}_{bcd}$	$57.8\pm1.1^{ab}_{abc}$	8.620	5.923
	N6*	$31.1\pm1.1^{d}_{a}$	$58.9\pm1.1^{bc}_{a}$	$63.3\pm1.9^{abc}_{a}$	$61.1\pm1.1^{abc}_{a}$	$57.8\pm1.1^{c}_{a}$	$34.4\pm1.1^{d}_{a}$	$57.8\pm1.1^{c}_{a}$	$66.7\pm1.9^{a}_{a}$	$64.4\pm1.1^{ab}_{a}$	$61.1\pm1.1^{abc}_{a}$	6.584	4.091
KSTP'94	**MS**	$12.2\pm1.1^{e}_{cd}$	$21.1\pm1.1^{d}_{e}$	$44.4\pm1.1^{b}_{de}$	$41.1\pm1.1^{b}_{de}$	$40.0\pm1.9^{bc}_{ef}$	$12.2\pm1.1^{e}_{d}$	$34.4\pm1.1^{c}_{ef}$	$56.7\pm1.9^{a}_{bcd}$	$52.2\pm1.1^{a}_{cde}$	$51.1\pm1.1^{a}_{cde}$	6.584	6.229
	LS	$12.2\pm1.1^{d}_{cd}$	$35.6\pm1.1^{c}_{d}$	$52.2\pm1.1^{b}_{bcd}$	$50.0\pm1.9^{b}_{bcd}$	$48.9\pm1.1^{b}_{bcd}$	$14.4\pm1.1^{d}_{d}$	$38.9\pm1.1^{c}_{ef}$	$60.0\pm1.9^{a}_{abc}$	$54.4\pm1.1^{ab}_{bcde}$	$53.3\pm1.9^{ab}_{bcde}$	7.038	5.796
	N6	$25.6\pm1.1^{c}_{ab}$	$48.9\pm1.1^{b}_{bc}$	$58.9\pm1.1^{a}_{ab}$	$56.7\pm1.9^{a}_{abc}$	$54.4\pm1.1^{ab}_{ab}$	$21.1\pm1.1^{c}_{c}$	$50.0\pm1.9^{b}_{bc}$	$58.9\pm1.1^{a}_{abc}$	$54.4\pm1.1^{ab}_{bcde}$	$54.4\pm1.1^{ab}_{abcde}$	6.584	4.711
	N6*	$25.6\pm1.1^{c}_{ab}$	$55.6\pm1.1^{ab}_{ab}$	$63.3\pm1.9^{a}_{a}$	$58.9\pm2.9^{ab}_{ab}$	$56.7\pm1.9^{ab}_{a}$	$23.3\pm1.9^{c}_{bc}$	$54.4\pm1.1^{b}_{ab}$	$63.3\pm1.9^{a}_{ab}$	$61.1\pm1.1^{ab}_{ab}$	$58.9\pm1.1^{ab}_{ab}$	8.620	5.721
Namba 8	**MS**	$5.6\pm1.1^{d}_{d}$	$17.8\pm1.11^{c}_{e}$	$36.7\pm1.9^{b}_{e}$	$34.4\pm1.1^{b}_{e}$	$33.3\pm1.9^{b}_{f}$	$10.0\pm1.9^{d}_{d}$	$32.2\pm1.1^{b}_{e}$	$53.3\pm1.9^{a}_{cd}$	$51.1\pm1.1^{a}_{de}$	$47.8\pm1.1^{a}_{e}$	7.465	8.013
	LS	$8.9\pm2.2^{e}_{d}$	$31.1\pm2.2^{d}_{d}$	$44.4\pm2.2^{bc}_{de}$	$42.2\pm2.2^{bc}_{de}$	$43.3\pm1.9^{bc}_{de}$	$12.2\pm1.1^{e}_{d}$	$38.9\pm1.1^{cd}_{ef}$	$54.4\pm1.1^{a}_{cd}$	$50.0\pm1.9^{ab}_{de}$	$50.0\pm1.9^{ab}_{de}$	9.311	8.575
	N6	$21.1\pm1.1^{d}_{b}$	$47.8\pm1.1^{bc}_{c}$	$56.7\pm1.9^{a}_{abc}$	$54.4\pm1.1^{ab}_{abc}$	$52.2\pm1.1^{abc}_{abc}$	$21.1\pm1.1^{d}_{c}$	$46.7\pm1.9^{c}_{cd}$	$50.0\pm1.9^{abc}_{d}$	$47.8\pm1.1^{bc}_{e}$	$51.1\pm2.2^{abc}_{cde}$	7.670	5.909
	N6*	$25.6\pm1.1^{c}_{ab}$	$52.2\pm1.1^{b}_{abc}$	$58.9\pm1.1^{ab}_{ab}$	$52.2\pm2.9^{b}_{abc}$	$52.2\pm1.1^{b}a_{bc}$	$27.8\pm1.1^{c}_{b}$	$53.3\pm1.9^{b}_{abc}$	$61.1\pm1.1^{a}_{abc}$	$58.9\pm1.1^{ab}_{abc}$	$57.8\pm1.1^{ab}_{abc}$	7.465	5.164
$F_{(11, 24)}$		29.83	107.22	29.26	17.67	24.78	34.84	41.92	7.72	12.55	8.63		
P value		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
L.S.D (0.05)		7.844	6.744	7.844	9.676	7.671	6.542	6.939	8.654	6.939	7.129		
CV%		14.460	5.661	4.987	6.513	5.310	11.060	5.281295	5.064	4.285	4.471		

Means values with the same **superscript** letters within the same row are not significantly different at turkey's test (P<0.05). Mean values with the same **subscript** letters within the column are not significantly different at *Tukey's* test (P<0.05).

The medium that recorded the highest embryogenic callus induction irrespective of the maize variety, 2,4-D concentration level and initial callus induction medium used was N6 (**Table 4.4**). Analysis of the effect of 2,4-D concentration level on maize embryogenesis shows that maize primary callus require a precise 2,4-D concentration threshold to develop embryogenic callus. Results show that 1.5 mg/l of 2,4-D was the optimal concentration regime for embryogenesis in all varieties and further increase in 2,4-D concentration beyond this level had negative effect (Table 4.4). Evaluation of the varietal effect on induction of embryogenic callus show that indirect somatic embryogenesis in maize is genotype dependent (**Table 4.4**). Results in table 4.4 show that CML144 and Namba nane recorded significantly high and low embryogenic callus induction frequency, respectively.

The emerging embryogenic calli were then transferred to PGR-free media in a similar experimental design as that of callus maturation stage (without PGR factor) where they were observed for formation of juvenile shoots (**Plate 4.2,** page 86). In plate 4.2 juvenile shoots and roots began to emerge simultaneously from embryogenic calli after two weeks of culture in RM (photos in column 1 of plate 4.2). Well-rooted plantlets were formed within two weeks after subculture to fresh RM (photos in column 2 of plate 4.2) and later hardened for one week (photos in column 3 of plate 4.2) before transplanting them in buckets in the Glasshouse where they were maintained to maturity (photos in column 4 of plate 4.2). Upon maturity, corn was harvested from all the plants that set seed (photos in column 5 of plate 4.2).

Table 4.4: Embryogenic callus frequency of maize varieties cultured on N6 and MS media at different 2,4-D concentration

Variety	Callus (%)	Media	Callus (%)	2,4-D Conc. (mg/l)	Callus (%)
CML144	47.22±1.361[a]	N6	46.39±1.142[a]	0.5	19.3±0.94[d]
KSTP'94	44.75±1.460[ab]	MS	42.37±1.179[b]	1	42.5±1.35[c]
Namba 8	41.17±1.428[b]			1.5	55.7±0.86[a]
				2	52.7±0.84[b]
				2.5	51.6±0.79[b]
$F_{(2, 344)}$	15.63	$F_{(1, 344)}$	80.49	$F_{(4, 344)}$	713.28
P value	<0.0001		<.0001		<.0001
L.S.D $_{(0.05)}$	4.716	L.S.D $_{(0.05)}$	0.884	L.S.D $_{(0.05)}$	2.1607

Means in the same column with same letters are not significantly different at *Tukey's* test (P<0.05).

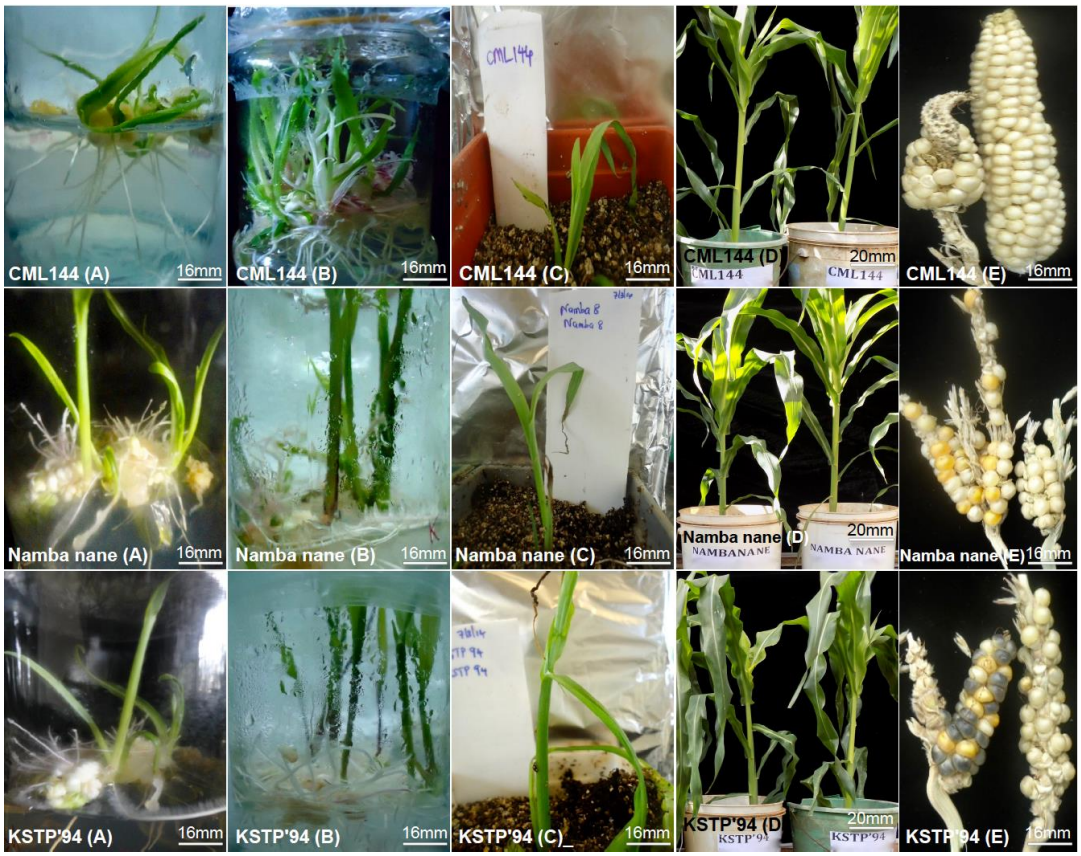

Plate 4.2: *In vitro* regeneration and maturation phases of selected maize varieties

Legend:

(A) Juvenile rooted shoots, (B) Mature plantlets, (C) Acclimatized plantlets, (D) Glasshouse-grown plants and (E) Harvested maize.

Analysis of shoot induction frequencies of the three varieties cultured in MS and N6 media is presented in table 4.5 (page 87).

CML144, initially cultured on N6* (CIM) and later subcultured on N6 (CCM) recorded the highest shoot induction frequency in both MS and N6 regeneration media (**Table 4.5**, page 87).

Namba nane initially cultured on MS (CIM) and later subcultured on MS (ECM) recorded the least shoot induction frequency in both MS and N6 regeneration media.

Table 4.5: Effect of media on maize shoot induction frequency (%)

Variety	Induction media	Shoot induction media				Statistic	
		MS	N6	$F_{(1, 24)}$	P value	$L.S.D_{(0.05)}$	CV%
CML144	MS	$30.0\pm3.55^{a}_{cde}$	$30.9\pm3.37^{a}_{de}$	1.64	0.2124	1.432	6.242
	LS	$30.2\pm326^{a}_{dc}$	$30.9\pm3.84^{a}_{de}$	0.30	0.5879	2.505	10.879
	N6	$34.0\pm2.78^{a}_{c}$	$36.2\pm2.85^{a}_{c}$	2.96	0.0981	2.665	10.070
	N6*	$42.2\pm3.39^{b}_{a}$	$48.9\pm3.34^{a}_{a}$	23.74	<.0001	2.824	8.226
KSTP'94	MS	$22.0\pm2.62^{b}_{fg}$	$31.3\pm3.90^{a}_{de}$	51.25	<.0001	2.691	13.389
	LS	$20.2\pm2.04^{b}_{g}$	$30.4\pm3.03^{a}_{de}$	63.96	<.0001	2.638	13.818
	N6	$35.1\pm3.10^{a}_{bc}$	$34.4\pm3.08^{a}_{cd}$	0.68	0.4165	1.664	6.350
	N6*	$39.8\pm3.34^{b}_{ab}$	$43.8\pm3.58^{a}_{b}$	13.74	0.0011	2.227	7.074
Namba 8	MS	$12.9\pm1.69^{b}_{h}$	$21.3\pm2.78^{a}_{g}$	41.96	<. 0001	2.691	10.865
	LS	$24.4\pm2.77^{a}_{efg}$	$25.9\pm3.20^{a}_{f}$	1.53	0.2275	2.444	12.880
	N6	$26.4\pm2.09^{a}_{def}$	$29.1\pm2.53^{a}_{ef}$	3.37	0.0789	2.999	14.325
	N6*	$32.4\pm2.92^{b}_{c}$	$36.2\pm3.16^{a}_{c}$	18.25	0.0003	1.932	7.444
$F_{(11, 24)}$		15.07	7.44				
P value		0.0350	0.0003				
$L.S.D_{(0.05)}$		5.557	4.449				
CV%		15.747	11.033				

Means with same superscript letters within the same row are not significantly different while means with same subscript letters within the same column are not significantly different at *Tukey's* test (P<0.05).

It is evident in table 4.6 that the best regeneration media (RM) for the tested maize varieties is N6. The results revealed that CML144 and KSTP'94 recorded the highest shoot induction frequency whereas 'Namba nane' recorded statistically low shoot induction frequency (**Table 4.6**).

Table 4.6: Shoot induction frequency of selected varieties on N6 and MS media

Variety	Shoot induction (%)	Shooting media	Shoot induction (%)
CML144	35.4 ± 1.24^{a}	N6	29.1 ± 1.00^{b}
KSTP'94	32.1 ± 1.28^{a}	MS	33.3 ± 1.03^{a}
Namba 8	26.1 ± 1.11^{b}		
$F_{(2, 59)}$	17.31	$F_{(1, 59)}$	24.10
P value	<.0001	P value	<.0001
$L.S.D_{(0.05)}$	4.029	**$L.S.D_{(0.05)}$**	0.884

Means in the same column with same letters are not significantly different at *Tukey's* test (P<0.05).

In a separate experiment, immature embryos of CML144, KSTP'94 and 'Namba Nane' maize varieties were co-cultivated with *Agrobacterium tumefaciens* harboring binary vector pTF102 with a GUS reporter gene according to Frame et al., (2002).

Selection of putative transformed callus tissues was achieved using Bialaphos with the compact calli surviving herbicide action used for histochemical GUS assay to assess the expression of the *uid*A gene according to Jefferson (1987) protocol.

Untransformed callus were used as a negative control and showed no blue coloration characteristic of GUS expression while GUS transformed callus had spots of blue colouration illuminating on parts of the callus as shown in plate 4.3.

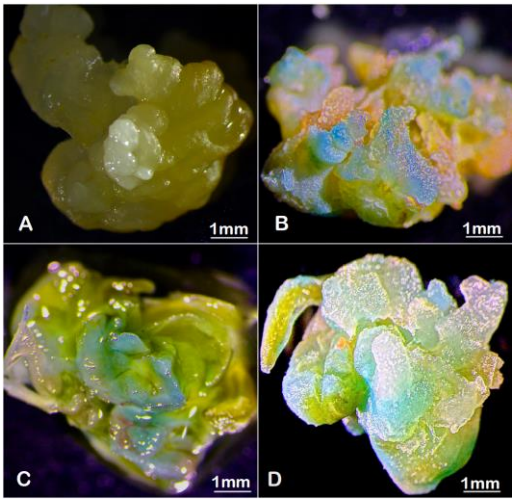

Plate 4.3: GUS assay of maize callus

Legend:
A) Untransformed CML144, B) transformed CML144, C) Transformed KSTP'94 and D) Transformed Namba nane**.**

The transformation frequency of the three maize varieties (expressed as a percentage of the proportion of total number of callus that transformed i.e. callus surviving selection) is presented in table 4.7 (page 89).

Analysis of frequency of the callus GUS expression of the three varieties indicated that the recceptability of the three maize varieties to GUS gene varied significantly in the two media used (**Table 4.7**, page 89).

Embryos of CML144 transformed using agrobacterium technique with N6* as the medium recorded the highest GUS transformation frequency. Embryos of Namba

nane transformed using the same technique with LS as the medium recorded the least GUS transformation frequency (**Table 4.7**).

Analysis of varietal receptivity to transformation (Table 4.8) revealed that CML144 was the most receptive (71.2±0.90%).

Table 4.7: GUS transformation frequency of maize callus on LS and N6* media

Variety	Gus +ve callus (%) Transformation media		Statistic			
	LS	N6*	$F_{(1, 10)}$	P value	$L.S.D_{(0.05)}$	CV%
CML144	$69.5\pm0.87^{b}{}_{a}$	$72.9\pm1.27^{a}{}_{a}$	5.13	0.0470	3.421	3.735
KSTP'94	$61.5\pm0.44^{b}{}_{b}$	$65.0\pm0.18^{a}{}_{b}$	53.21	<.0001	1.059	1.303
Namba nane	$51.7\pm0.62^{b}{}_{c}$	$54.1\pm0.39^{a}{}_{c}$	11.21	0.0074	1.639	2.410
$F_{(2, 15)}$	178.48	149.50				
P value	<.0001	<.0001				
$L.S.D_{(0.05)}$	2.451	2.837				
CV%	2.686	2.956				

Means with same superscript letters within the same row are not significantly different while means with same subscript letters within the same column are not significantly different at *Tukey's* test (P<0.05).

Analysis in table 4.8 also revealed that the best medium for *Agrobacterium*-mediated transformation protocol for maize is N6* medium (64%).

Table 4.8: Effect of variety and media on GUS transformation frequency

Variety	Gus +ve callus (%)	Media	Gus +ve callus (%)
CML144	71.2 ± 0.90^{a}	N6*	64.0 ± 1.92^{a}
KSTP'94	63.2 ± 0.57^{b}	LS	60.9 ± 1.80^{b}
Namba 8	52.9 ± 0.51^{c}		
$F_{(2, 32)}$	337.68	$F_{(1, 32)}$	29.57
P value	<.0001	P value	<.0001
$L.S.D_{(0.05)}$	1.736	**$L.S.D_{(0.05)}$**	1.175

Means in the same column with same letters are not significantly different at *Tukey's* test (P<0.05).

Evaluation of transformation efficiency expressed as a percentage of the proportion of the total number of transformed callus that were positive in the PCR amplification of GUS gene of transgenic callus (**Plate 4.4**) is presented in table 4.9 (page 90).

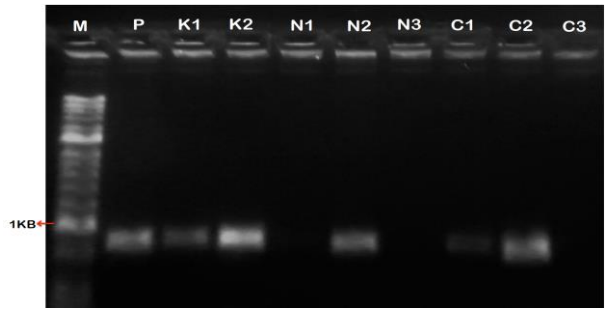

Plate 4.4: Gel photograph of PCR products of GUS transgenic plantlets

Legend:
M) Molecular marker (10kb ladder), **P**) Plasmid with GUS gene, **K**) KSTP'94, **N**) Namba nane, **C**) CML144. Numerical numbers (**1,2,3**) refers to transformation events of each variety.

Analysis presented in table 4.9 below clearly indicate that maize variety have a significant influence on transformation process.

Although transformation efficiency of individual maize variety in the two media was not significantly different (P<0.05), CML144 recorded a higher frequency than KSTP'94 and Namba nane in both transformation media (**Table 4.9**).

Both transformation media (N6* and LS) did not significantly affect transformation efficiency of maize callus, however the observed difference in the transformation efficiency was a varietal response (**Table 4.10,** page 91).

This study has established that the transformation efficiency of the selected maize varieties ranges from 0.8% to 2.1% (**Table 4.10**).

Table 4.9: GUS transformation efficiency of maize callus on LS and N6* media

Variety	**Gus transformation efficiency (%)**				
	LS	**N6***	$F_{(1, 10)}$	**P value**	**L.S.D $_{(0.05)}$**
CML144	$2.1\pm0.29^{a}{}_{a}$	$2.2\pm0.292^{a}{}_{a}$	0.01	0.9266	0.917
KSTP'94	$1.4\pm0.27^{a}{}_{ab}$	$1.4\pm0.28^{a}{}_{ab}$	0.01	0.9262	0.866
Namba nane	$0.8\pm0.36^{a}{}_{b}$	$0.8\pm0.37^{a}{}_{b}$	0.00	0.9799	1.156
$F_{(2, 15)}$	4.47	4.47			
P value	0.0301	0.0300			
L.S.D $_{(0.05)}$	1.141	1.161			

Means with same letters within the same column are not significantly different at *Tukey's* test (P<0.05).

Table 4.10: Effect of media and variety on GUS transformation efficiency

Variety	T. Efficiency (%)	Media	T. Efficiency (%)
CML144	2.1±0.20[a]	N6*	1.5±0.22[a]
KSTP'94	1.4±0.19[b]	LS	1.4±0.21[a]
Namba 8	0.8±0.25[b]		
$F_{(2, 32)}$	337.68	$F_{(1, 56)}$	29.57
P value	<.0001	P value	<.0001
L.S.D $_{(0.05)}$	0.746	**L.S.D $_{(0.05)}$**	0.505

Means with same letters in the same column are not significantly different at *Tukey's* test (P<0.05).

4.5 Discussion

In this study, immature embryos of the three maize varieties were used as explants. This part of the embryo has numerous competent cells for somatic embryogenesis with high potential of producing fertile plantlets and hence the reason for its preference as explant material (Binnot et al., 2008; Oduor et al., 2006; Ombori et al., 2008). The immature embryos were cultured with their axis being in contact with the medium and the scutellum exposed. This placement technique together with the high sucrose levels aided high callus induction frequency with limited direct embryo germination, as was the case in the hormone-free media used. Previous studies where this technique was applied also show that the precocious germination of embryos was significantly reduced (Vasil et al., 1985). Sustained cell divisions in localized areas of scutellum is believed to give rise to embryogenic callus where numerous somatic embryos have formed (Vasil, 2005).

Assessment of amenability of KSTP'94, Namba nane and CML144 (a reference variety) to *in vitro* regeneration and *Agrobacterium*-mediated transformation process revealed that these two open pollinated maize varieties can be genetically modified and regenerated for commercial purposes. Several similar studies have reported *in vitro* regeneration of economically important maize varieties with sole aim of creating platforms for genetic improvement of such germplasm (Abebe et al., 2008; Zhong et al., 2011).

This study revealed that CML144 significantly differed with KSTP'94 and Namba nane in terms of callus induction and maturation frequencies. This is probably due to

their inherent genetic make up, which makes them respond differently to regeneration and transformation processes. Similar findings by other studies have also shown that *in vitro* regeneration of maize is variety-dependent (Oduor et al., 2006; Binnot et al., 2008; Ombori et al., 2008 and Omer et al., 2012).

This study also evaluated the effect of a number of *in vitro* culture conditions such as type of media used, Plant growth regulators (PGRs) regimes and addition of L-proline and silver nitrate in culture media on *in vitro* regeneration of maize. Results show that these factors significantly influenced callus induction, embryogenesis and ultimate *in vitro* regeneration of maize and similar phenomenon was observed by Zhao et al., (2008). For instance, among the four types of media used, N6* not only recorded the highest frequency of primary callus induction but also recorded a high number of embryogenic calli formed.

It is documented that PGRs play a critical role in maize callus culture, and their effect on *in vitro* regeneration of maize has been sufficiently validated (Omer et al., 2012; Xu et al., 2014). In this study, five levels of 2,4-D concentration (0.5, 1.0, 1.5, 2.0 and 2.5) mg/l were used for induction of primary callus and embryogenic callus. Results of this study revealed that 1.5mg/l (6.787 µM) was the least 2,4-D concentration level that induced optimum primary and embryogenic callus. Higher concentrations of 2,4-D beyond this level appeared to have negative effect on induction of embryogenic callus. Earlier studies have shown that use of 2,4-D to induce callus from immature maize embryos is a critical factor (Armstrong et al., 1985; Bohorova et al., 1999). Bronsema et al., (2001) reported that atleast 0.2mg/l is needed to cross the threshold level for the transition from germination to callus induction. Concentration range of 1 mg/l to 3 mg/l (4.5-13.6 µM) have been shown to be essential for the formation of embryogenic callus in cereal tissue culture (Cheng-hao et al., 2008).

Addition of L-proline and silver nitrate ($AgNO_3$) to N6 nutrient medium (N6*) probably accounts for the better performance of N6* compared to its precursor medium (N6) with regard to induction and maturation of callus. Other studies have shown that inclusion of these two compounds in callus induction and maturation

media have the tendency of increasing the formation of Type II callus, ideal for transformation (Sairam et al., 2008; Songstad, 2010). Other studies have demonstrated that incorporation of silver nitrate ($AgNO_3$) into the culture medium enhances the frequency of initiation of type II callus from immature embryos (Anami et al., 2010; Songstad et al., 1988, 1991; Vain et al., 1989). Earlier studies argued that ethylene produced by cells in culture has an inhibitory effect on formation and growth of type II callus and therefore the beneficial effect of $AgNO_3$ may be due to its antagonistic effect on functioning of ethylene in the cell culture (Songstad et al., 1991).

In this study, the induced calli from maize cultures were classified into three types namely; NE (non-embryogenic), type I (embryogenic), and type II (embryogenic). Non-embryogenic calli appeared watery (translucent), often turned brown and lost their regeneration ability (Sairam et al., 2008). Type I calli were white, compact and juvenile maize shoots appeared to emerge from them spontaneously (Binnot et al., 2008; Oduor et al., 2006; Sairam et al., 2008). The usefulness of this type of callus however gradually diminishes as it cannot be maintained for long periods of time (Sairam et al., 2008). Type II embryogenic calli were friable, soft and yellowish in colour (Sairam et al., 2008). This type of callus is the best for transformation assays (Sairam et al., 2008). Fransz and Schel (1991) showed that type II calli of A188 inbred line originated from the abaxial scutellar cells, including the epidermis section. Similar findings were reported by Songstad et al., (1996), who provided histological evidence of cell division at the scutellar surface of pre-cultured immature embryos leading to an embryogenic response. These friable calli are desirable as starting material for a variety of maize transformation experiments including protoplasts, biolistics and selection for somaclonal variants (Songstad, 2010). Unfortunately, type II callus forms at a lower rate than type I. Armstrong and Green (1985) made a significant contribution regarding the formation of type II callus in maize. They discovered that addition of L-proline to N6 medium enabled routine initiation of type II callus from immature A188 embryos.

In the present study, N6*(N6 medium fortified with 0.35g/l L-proline and 0.8mg/l$AgNO_3$) not only recorded the highest primary callus induction efficiency but

also had high frequency of embryogenic callus formation probably due to the presence of L-proline and silver nitrate in the medium. Similar findings were also reported by Anami *et al.* (2010). Some studies argue that L-proline plays a role in the protection of plant tissues from desiccation (Haudecoeur et al., 2009).

To evaluate the transformation potential of the three maize varieties, immature maize embryos were transformed with β-glucuronidase gene (GUS gene). β-glucuronidase gene has widely been used as a reporter gene system for evaluating transformability potential of plants *(*Jefferson et al., 1987). *Agrobacterium*-mediated transformation of immature embryos of these three maize varieties used Zhao et al., (2001) media in which L-proline, silver nitrate and acetosyringone were added. The function of silver nitrate in transformation of immature embryos of maize is the same as outlined in the *in vitro* regeneration section. For L-proline, Haudecoeur et al., (2009) demonstrated that proline antagonizes synthesis of γ-aminobutyric acid (GABA) by plant cells which is a defense response against infection by *A. tumefaciens* thereby increasing the aggressiveness of *A. tumefaciens* populations and contributing to the dissemination of the vector carrying GUS gene. Acetosyringone on the other hand is a natural inducer of virulence (*vir*) gene expression of *A. tumefaciens* hence its inclusion in the media (Pitzschke et al., 2010).

Many plants assayed to date lack detectable glucuronidase activity, providing a null background in which to assay chimaeric gene expression (Jefferson et al., 1987). The β-glucuronidase gene (GUS gene) is easily and highly expressed in plant cells where it encodes a stable enzyme that catalyzes the cleavage of a wide variety of β-glucuronides, many of which are available commercially as spectrophotometric, fluorometric and histochemical substrates (Karcher, 2002). In this study, β-glucuronidase enzyme cleaved the chromogenic (colour generating) substrate X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid), resulting in the production of an insoluble blue colour in transformed maize callus displaying GUS activity. Untransformed maize callus do not contain any GUS activity and did not change colour to blue but the production of a blue colour when stained with X-gluc in particular cells of transformed calli indicated the activity of the promoter that drives the transcription of the *gus* A-chimeric gene in the particular cells.

The expression of the GUS-intron gene as detected by GUS colour staining and PCR assay are reliable indicators of transformability of a plant since the GUS-intron gene can only be expressed efficiently in plant cells but not in *Agrobacterium* (Jefferson et al., 1987). Findings of transformation efficiency of the tested maize varieties as reported in this study provide a statistical assessment of the potential of introducing useful genes into these maize varieties.

4.6 Conclusion

Derivative medium of N6 (N6* medium) was the suitable medium for *in vitro* regeneration of the three maize genotypes. The optimum 2,4-D concentration level for inducing indirect organogenesis in the selected open pollinated tropical maize genotypes was 1.5 mgl^{-1}. Further, assessment of transformation of these genotypes revealed that they are amenable to transformation therefore setting up a platform for their potential improvement with novel genes. This developed regeneration and transformation protocol when optimized will provide a critical foundation for future genetic improvement of these genotypes.

CHAPTER FIVE

TRANSFORMATION OF ADAPTED MAIZE VARIETIES WITH *PURPLE ACID PHOSPHATASE* (*PAP*) GENES FROM *LUPINUS ALBUS* AND *MEDICAGO TRUNCATULA* TO IMPROVE P MOBILIZATION

5.1 Introduction

Maize (*Zea mays*) is a widely cultivated food crop worldwide (Calderon-Vazquez et al., 2011). However, it faces an array of biotic and abiotic stresses that include drought, low soil fertility, weeds, pests and diseases (Campos et al., 2004; Kumar, 2002; Mwangi et al., 2001; Ombori et al., 2013). While conventional breeding of maize has led to improvement against these challenges, this approach has been unable to sustainably counter these effects to meet the increasing maize demand. More importantly, improvement of maize via conventional breeding is laborious, require a lot of space, are time consuming and faces genetic barriers due to incompatibility of heterotic groups (Diallo et al., 2001). The risk of declining seed quality and maize yield due to on-farm seed production, calls for the use biotechnological approaches to improve these varieties for the benefit of resource poor rural famers hence the need for a platform for genetic improvement of the OPVs (Ombori et al., 2013). Transgenic plants are rapidly becoming a common feature of modern agriculture in many parts of the world (Ombori et al., 2013). Genetic transformation holds a great potential for obtaining improved varieties of cereals in a shortened period of time (5–6 years) compared to long (7–10 years) the conventional breeding technique (Sharma et al., 2002; Shrawat and Lo¨rz, 2006).

Even when adapted maize is developed, the high cost of fertilizers and certified maize seed coupled with long maturity period of such hybrid maize varieties have forced small-scale farmers to adopt the cultivation of Open Pollinated Varieties (OPVs) especially during the short rains (Omondi et al., 2014). Some of these varieties have *S. hermonthica* tolerant traits that are beneficial in *S. hermonthica*-infested regions (Omondi et al., 2014; Wambugu et al., 2012). Furthermore, these OPVs have been reported to have superior taste and better keeping quality than hybrids (Macharia et al., 2010; Omondi et al., 2014). Over-reliance on the OPVs by

these farmers and the continued lack of fertilizer application especially in *S. hermonthica*-infested maize fields have elicited fruitless weed control efforts in the past (Omondi et al., 2014). Even where efforts to reduce *S. hermonthica* infestation has been emphasized, the focus has been short-lived, totally ignoring exploration of sustainable *S. hermonthica* control options such as bioremediation of inorganic rhizosphere phosphorus (Omondi et al., 2014).

Phosphorus, a critical factor in plant growth and agricultural sustainability, is by far the least mobile and available nutrient element to maize in most soil conditions compared with other major nutrient elements (Li et al., 2012). In acidic and alkaline soils under maize cultivation, farmers apply large quantities of P fertilizer in order to maximize yields (Calderon-Vazquez et al., 2011; Rose et al., 2013). Phosphorus fertilizers, however, are becoming increasingly costly owing to dwindling non-renewable natural reserves of rock Pi and an increase in the costs of extracting P from the remaining reservoirs (Calderon-Vazquez et al., 2011; Rose et al., 2013).

Due to its low bioavailability, the high rate of fixation by metal oxides and the low rate of diffusion in soils, plants have developed different adaptive mechanisms to deal with low-P conditions and maintain Pi homeostasis (Li et al., 2012; Younessi-hamzekhanlu et al., 2016). These mechanisms include remodeling of root architecture and *Arbuscular mycorrhizal* (AM) fungi and rhizobia symbioses with roots to increase the surface area for Pi uptake (Guo et al., 2011; López-Arredondo et al., 2014; Tian and Liao, 2015; Younessi-hamzekhanlu et al., 2016). Some of the adaptive root characteristics for phosphorus uptake in P deficiency circumstances include root/shoot ratio, root secretory proteins, root surface binding proteins, root hair formation and formation of cluster roots (Younessi-hamzekhanlu et al., 2016).

Studies show that multi-dimensional gene regulation networks are involved in response to low-P conditions (Younessi-hamzekhanlu et al., 2016). Among these genes and networks, root exudates including acid phosphatases and high affinity transporter are important (Younessi-hamzekhanlu et al., 2016). Phosphate starvation-inducing (PSI) genes in plants such as Purple acid phosphatases (*PAPs*) are key during plant response to Pi starvation (Robinson et al., 2012). Some of the *PAPs* play

important roles in internal P recycling by remobilization of Pi from intracellular P monoesters and anhydrides of older tissues (intracellular *PAPs*) while extracellular *PAPs* scavenge for Pi through liberation of orthophosphate groups from organic phosphate compounds (phytate such as myoinositol 1,2,3,4,5, 6-hexakisphosphate) in the rhizosphere hence contributing to P acquisition in deficient environments (Tawaraya et al., 2014). *PAPs* with phytase activity operate over a wide pH range (4.0–7.6) and appear to be restricted to plants (Younessi-hamzekhanlu et al., 2016).

For instance, *Lupinus albus* and *Medicago tranculata* (secrete *PAPs* with phytase activity) form cluster roots and grow vigorously than any other plant under low phosphorus condition (Tang et al., 2013). This special root structure increases the availability of soil P by expanding the root surface area, acidifying the rhizosphere and increasing the exudation of citrate (Tang et al., 2013). The acquisition of soil P by *L. albus* and *M. tranculata* is also associated with the secretion of *PAPs* which hydrolyzes soil organic P thereby increasing the rhizosphere Pi (Tang et al., 2013). The ability of maize root to secrete *PAPs* has been well characterized at a physiological level with some variations observed in maize *PAPs* successfully mapped genetically (González-Muñoz et al., 2015). The maize intracellular *PAPs* activities have been less characterized, although isozyme variation in seedlings has been used successfully for analyses of genetic diversity. Although the number of genes encoding PAP activities in maize has been established to be 33, they remain largely uncharacterized (González-Muñoz et al., 2015). The study by Zhang *et al.* (2015) suggests that only a handful of maize genotypes perform well under low P conditions. Comparatively, *M. truncatula* and *Lupinus albus* display an array of adaptations against low P (González-Muñoz et al., 2015; Zhang et al., 2014; Zhang et al., 2015).

Previous *in silico* analysis and comparison of some of the cloned maize *PAPs* with *M. truncatula* and *Lupinus albus PAPs* (designated as *Zea mays PAP*-1 and 2, *Mt*PAP and *La*PAP, respectively) revealed 6.3% identity positions and 92.2% consensus positioning between the *PAPS* (Alakonya, 2011). Interestingly this study determined that all conserved amino acid residue domains in Plant *PAP* genes which are GDLG, G**D**LS**Y**, VLM**H**, G**H**V**H** and G**N**LE are present in *Mt*PAP and *La*PAP.

The maize *PAPs* however lack VLM**H** and G**N**LE domains hence a possible explanation for poor P mobilization from Pi deficient soils (Alakonya, 2011; Li et al., 2002; Olczak et al., 2003; Schenk et al., 2000). This poor response becomes of more concern because maize responds to P deficiency by also producing root exudates with high concentration of Strigolactones (SLs). This response is meant to initiate a symbiotic association with arbuscular mycorrhizal fungi so as to enhance P access. Unfortunately, the SLs are intercepted by a group of destructive, parasitic, flowering plants, such as *Striga* species to signal presence of a host hence resulting in higher parasitic plant colonization (Calderon-Va´zquez et al., 2012). This therefore implies that soil P deficiency not only affects growth of maize but also intensifies *S. hermonthica* infestation in maize fields.

The development of P efficient maize varieties that can grow and produce more yield in P deficient soils is likely to be a cost-effective means of improving maize yields in low-input farming systems (Rose et al., 2011). Such P-efficient maize would ideally combine enhanced P uptake attributes with efficient use of P in biomass accumulation (Rose et al., 2011). Given the genetic barrier between maize and P efficient species like *M. truncatula* and *L. albus,* genetic transformation of maize with target *PAP* genes could be a viable approach. It was envisaged that transformation of adapted maize with *PAP* genes from *M. truncatula* and *L. albus* could enhance phosphorus acquisition/utilization, improve plant growth, reduce *S. hermonthica* infestation and optimally improve maize grain yield. The aim of this study therefore was to determine the effect of transforming three selected maize genotypes with two *PAP* genes from low P adapted species namely *M. truncatula* and *L. albus*.

5.2 Materials and Methods

5.2.1 Plant materials and explant preparation

Seeds of the selected local OPVs namely Namba Nane and KSTP'94 were acquired from Kenya Agricultural and Livestock Organization (KALRO) while tropical maize inbred line CML144 was provided by the Plant Transformation Laboratory (PTL) of Kenyatta University who acquired it from International Maize and Wheat

Improvement Center commonly called by its Spanish acronym CIMMYT (*Centro Internacional de Mejoramiento de Maízy Trigo*). CML 144 was selected for this study because it is a regenerable temperate maize variety that is easily transformed and served as a control. Namba nane was chosen because it is a preferred farmer-selected OPV maize and has no Plant Breeders Rights. Lastly, KSTP'94 was selected for this study because it is a *S. hermonthica*-tolerant maize variety and helped evaluate if overexpression of PAP genes would have a significant contribution to its resistance to *Striga*.

All the three maize varieties were grown in the research field at Jomo Kenyatta University of Agriculture and Technology (JKUAT). Maize ears and tassels were covered with paper bags before silk emerged. The ears were self-pollinated at optimum length of silk-receptivity and then harvested between 9^{th} and 11^{th} day after pollination (DAP) to obtain immature embryos. Harvested ears were taken to PTL at Kenyatta University where they were carefully de-husked. The de-husked corn were placed in a sterilized container with 70% ethanol to cover all ears for 2 min and then rinsed thrice with double-distilled water in a laminar flowhood. The corn were further sterilized in 50% dilution (v/v) of 3.85% (active ingredient) solution of sodium hypochlorite in which 10µl of Tween® 20 (Polyoxyethylene sorbitan monolaurate) had been added for 30 min. During the 30-minute disinfection, occasional tapping of the Mason jar was done to dislodge air bubbles for thorough surface sterilization of ears. The bleach solution was poured off and the ears rinsed three times in sterile distilled water. In a laminar flow hood bench and on sterile petri-plates the kernel crowns of the ear (the top 1-2 mm) were aseptically cut off using a new, sharp sterile scalpel blade. Immature zygotic embryos (1mm - 1.5 mm in size) were then excised from the kernels by inserting the narrow end of a blunt spatula between the endosperm and pericarp at the basipetal side of the kernel (towards the bottom of the cob) popping the endosperm out of the seed coat. This exposed the untouched embryo, which sits at the topside of the kernel, close to the kernel base. The embryo was gently coaxed onto the spatula tip and submerged in 10 ml of embryo suspension media (ESM).

5.2.2 Processing of *PAP* gene expression cassettes and transformation of *Agrobacterium tumefaciens* (EHA105)

Maize and other selected plant *PAP* genes with phytate activity were searched and retrieved from GenBank. The retrieved sequences were aligned using MUSCLE of MEGA 7 software (Tamura et al., 2013). The resulting alignment was used to construct a maximum likelihood phylogenetic tree with 1000 bootstrap replicates.

Assembled *Mt*PAP and *La*PAP gene expression cassettes were cordially provided by Dr. Amos Alakonya. The gene expression cassettes were developed from modified binary vector pTF 101.1. The modification involved introduction of ubiquitin promoter, a rice intron and a multi-cloning site harbouring a NOS terminator fragment from the binary vector NC 1100. The new plasmid was renamed PAJ101.1. The *Mt*PAP and *La*PAP genes were then ligated into pAJ101.1 (**Fig. 5.1**) using restriction digestion and ligation methods.

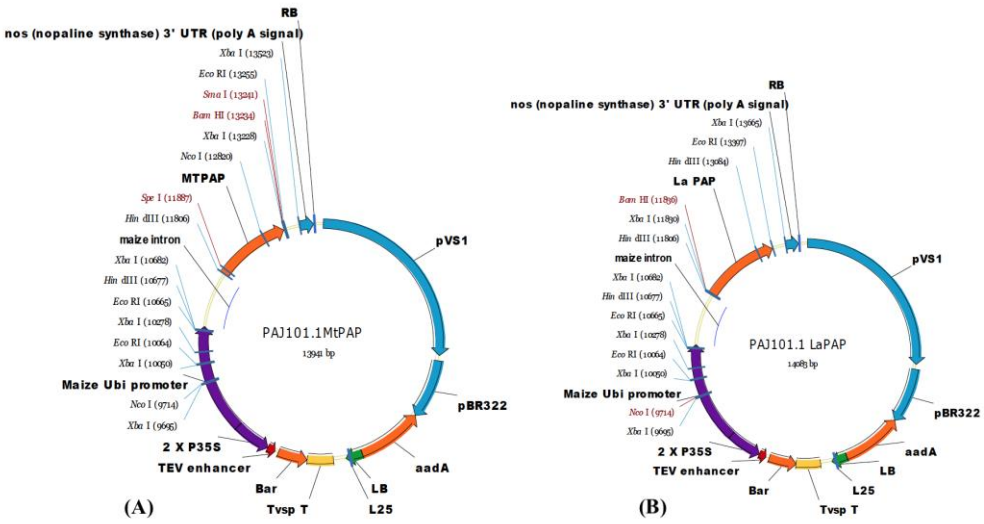

Figure 5.1: Plasmid map of MtPAP (A) and LaPAP (B) genes in pAJ101.1 binary vector

Competent cells of *Agrobacterium tumefaciens*, strain EHA 105, were prepared using chemical ($CaCl_2$) technique (Froger and Hall, 2007) and transformed with *La*PAP and *Mt*PAP gene cassettes using heat and shock technique (Li et al., 2010). The transformed EHA105 bacteria cells were maintained on LB medium fortified with 100mg/l spectinomycin (antibiotic for pTF101.1 selection), 1mg/l Rifampicin and 30mg/l Chloramphenicol (antibiotic for EHA105 selection). Colony PCR using

PAP-specific primers was performed on surviving EHA 105 colonies to identify putative PAP-transgenic EHA105 bacteria cells. The *La*PAP primer set used was as follows; Primer-F (5'-GATCCCCGGGTACCGAGCTCATGGG TTATAGTAGTTTTGTTGCAAT-3') and Primer-R(5'-CCGCCACCGCGGTGGAGCTCAGAACATTATAAATCTTGGTATCAAGG-3') while the *Mt*PAP primer set used was as follows; Primer-F (5'-CTTACTAGTTGAGTTTTGAAGAAAAATGGG-3') and Primer-R (5'-GGATCCATGAGTTGTGGAATCATCAACT-3'). Polymerase chain reaction (PCR) was performed in a thermocycler (PeQLab, VWR, Germany). Amplification was carried out in a 30µl reaction mixture containing 3µl of 10X PCR buffer, 4µl of 2.5mM dNTPs, 2.5µl of 8F forward primer (5pmol), 2.5µl of 1492R reverse primer (5pmol), 0.4µl of 5 U/µl Taq polymerase, 1.5µl of DNA template and 16.1µl PCR grade water (all reagents were products of BIOLINE Co.). The amplification of *La*PAP and *Mt*PAP were performed using the following conditions; initial denaturation at 94°C for 3 min, 30 cycles each of denaturation at 94°C for 30 sec, primer annealing at 59°C for 30 sec, chain extension at 72°C for 1 min, and a final extension at 72°C for 8 min. The *La*PAP and *Mt*PAP primer sets amplified a 1200bp DNA fragment of PAP gene. Transformed Agrobacterium *tumefaciens* bacterial cells for weekly experiments were revived from stock plates that were stored at 4°C for up to one month before being refreshed from long-term, –80°C glycerol stocks.

5.2.3 Transformation of maize with *PAP*-transformed *A. tumefaciens*

Agrobacterium tumefacien (EHA105) cultures were grown for three days at 19°C on LB medium amended with 1mg/l Rifampicin and 30mg/l Chloramphenicol (for selection of EHA105) and 100mg/l spectinomycin (for selection of EHA105 bearing pTF101.1). One full loop of EHA105 culture was scraped from the three-day old plate and suspended in 5 ml of liquid infection medium (Inf) supplemented with 100µM acetosyringone (AS) in a 50 ml centrifuge tube. The constituents of infection medium were; N6 salts and vitamins (Chu et al., 1975), 1.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.7g/l L-proline, 68.4g/l sucrose, and 36g/l glucose (pH 5.2). This medium was filter-sterilized and 100 µM of filter-sterilized AS added prior to use. The centrifuge tube was then fixed horizontally to a bench-top

shaker and shaken at low speed (~75 rpm) for two to four hours at room temperature. This pre-induction step was carried out for all experiments.

During infection of embryos with *Agrobacterium*, immature zygotic embryos from one ear (1.5 mm in size) were washed twice using bacteria-free Infection medium. The final wash was decanted off and 1.5 ml of *Agrobacterium* suspension (OD550 = 0.3 to 0.4) added to the embryos. Embryo infection was accomplished by gently inverting the tube 20 times before resting it upright for five min with embryos submerged.

Infected embryos were then transferred to the surface of co-cultivation medium and excess *Agrobacterium* suspension pipetted off the medium surface. The co-cultivation medium consisted of N6 salts and vitamins supplemented with 1.5 mg/l 2,4-D, 0.7g/l L-proline, 30g/l sucrose, and 3g/l gelrite (pH 5.8). Filter sterilized 0.85 mg/l silver nitrate, 100 μM AS, and N6 Vitamins were added to the medium that had been autoclaved at 121°C for 15min. The immature embryos were oriented with the embryo-axis side in contact with the co-cultivation medium (scutellum side up). The Petri Plates were then wrapped with parafilm and incubated in the dark at 20°C for three days.

After three days of co-cultivation, all embryos were transferred to resting medium and incubated at 28°C in darkness for 7 days. This resting medium was made up of N6 salts and vitamins supplemented with 1.5 mg/l 2,4-D, 0.7g/l L-proline, 30g/l sucrose, 0.5g/l2-(4-morpholino)-ethane sulfonic acid (MES), and $8gl^{-1}$ purified agar (pH 5.8). Filter sterilized N6 vitamins, $250mgl^{-1}$carbenicillin and 0.85mg/l silver nitrates were added to the autoclaved medium prior to use.

After seven days on resting medium, all embryos were transferred to selection medium I and incubated at 28°C in the dark for 2 weeks. This first selection medium was made up of N6 salts and vitamins fortified with 1.5mg/l 2,4-D, 0.7g/l L-proline, 30g/l sucrose, 0.5g/l MES, and 8g/l purified agar (pH 5.8). Filter sterilized N6 vitamins, 250mg/l carbenicillin, 0.85 mg/l silver nitrate, and 1.5mg/l Bialaphos were added to the autoclaved medium prior to use. The embryos were then sub-cultured after two weeks on selection medium II. This second selection medium was similar

to the first selection medium I except for Bialaphos whose concentration was doubled (3mg/l). Putatively transformed events were visible as rapidly growing Type II callus as early as five weeks after *Agrobacterium* infection.

Regeneration of transgenic plants (T_0) from Type II embryogenic callus was accomplished by a three-week maturation step on Regeneration Medium I (RM I) followed by full plant development in the light on Regeneration Medium II (RM II) as described in Frame *et al*. (2000). The first regeneration medium was made up of MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 60g/l sucrose, 100mg/l myo-inositol, no hormones and 3g/l gelrite (pH 5.8). Filter sterilized carbenicillin ($250mgl^{-1}$) and Bialaphos (3mg/l) were added to this medium after autoclaving. The second regeneration medium was similar to the first regeneration medium except for sucrose whose concentration was reduced to 30g/l. Stable transformation efficiency (%) was calculated as the number of Bialaphos resistant callus events recovered per 100 embryos infected. Acclimatization of regenerated plants was done on autoclaved pit moss for two weeks and transferred to soil (in buckets) until maturity in the glasshouse.

5.2.4 Molecular characterization of transgenic maize

During the growth period of the plants (T_0) in the glasshouse, leaf samples were collected from individual plant and DNA extracted using Qiagen kits following manufacturers instructions. Molecular screening of T_0 plants to check for the presence of PAP gene was done by PCR using PAP specific primers as described in section 5.2.2. Data collected was used to assess transformation efficiency of the different varieties. PCR positive plants Putative transgenic maize plants (**T_0**) in glasshouse were grown to maturity, self-pollinated and their seed bulked to obtain **T_1** plants.

5.2.5 Conditioning of *S. hermonthica* seeds and determination of their fecundity

Striga hermonthica seeds used in this study were supplied by KARI-Kakamega and Alupe research station. These seeds were surface-sterilized in bunches of 10mg of (approximately 1,000 seeds) and preconditioned according to the protocol of

Lendzemo et al., (2009). The seeds were then placed in a beaker containing 2% (v/v) NaOCl and vortexed for 5 min. The seeds were later emptied on a funnel lined with folded filter paper and rinsed thoroughly with autoclaved distilled water to get rid of traces of NaOCl. The approximately 1000 seeds were transferred into 10ml of sterile distilled water. Two hundred sterilized *S. hermonthica* seeds (applied as 2ml of suspended *S. hermonthica* seeds) from each bunch were transferred into a petri plate lined with moist Whatman GFA filter paper, wrapped with Aluminum foil and incubated at 28°C for 11 days for pre-conditioning. Three plates of preconditioned *S. hermonthica* seeds of each bunch were germinated by adding 3ml of 0.1ppm GR24 and incubated overnight at 28°C. The number of germinating *S. hermonthica* seeds was determined by counting under the Leica model MZ7F stereomicroscope fitted with a DFC320FX camera. The remaining *S. hermonthica* seeds from each bunch that had an average germination mean rate of >69% were used later in the *S. hermonthica* germination (exudate experiment) and *Striga* parasitization (bucket and rhizotron) evaluation experiments.

5.2.6 Evaluation of PAP overexpression effect in maize on *S. hermonthica*

A total of 100 seeds of each varietal event (for instance, CML144-*La*PAP event 1, 2 and 3) for all the three varieties were pre-germinated on wet cotton wool in germination troughs (**T_1**). Two leaves of each seedling (7day old) were harvested and DNA extracted from them using Keb *et al*'s (2002) protocol and PCR was performed using PAP-specific primers. The *La*PAP primer set used was as follows; Forward primer-F (5'-GATCCCCGGGTACCGAGCTCATGGG TTATAGTAGTTTTGTTGCAAT-3') and Reverse primer-R (5'-CCGCCACCGCGGTGGAGCTCAGAACATTATAAATCTTGGTATCAAGG-3') while the MtPAP primer used was as follows; Forward primer-F (5'-CTTACTAGTTGAGTTTTGAAGAAAAATGGG-3') and Reverse primer-R(5'GGATCCATGAGTTGTGGAATCATCAACT3'). The PCR procedure was performed in a thermocycler (PeQLab, VWR, Germany). Amplification was carried out in a 30µl reaction mixture containing 3µl of 10X PCR buffer, 4µl of 2.5mM dNTPs, 2.5µl of 8F forward primer (5pmol), 2.5µL of 1492R reverse primer (5pmol), 0.4µl of 5 U/µl Taq polymerase, 1.5µl of DNA template and 16.1µl PCR

grade water (all reagents were products of BIOLINE Co.). The amplification of *La*PAP and *Mt*PAP were performed using the following conditions; initial denaturation at 94°C for 3 min, 30 cycles each of denaturation at 94°C for 30 sec, primer annealing at 59°C for 30 sec, chain extension at 72°C for 1 min, and a final extension at 72°C for 8 min. The *La*PAP and *Mt*PAP primer sets amplified a 1200bp DNA fragment of PAP gene.

A pre-trial *Striga* colonization rhizotron experiment using CML144 as a host was set up to determine the period of subterranean development stages of *Striga* for purposes of establishing the ideal time for collecting data in the rhizotron experiment. Pre-germinated CML144 seeds were infected with preconditioned *S. hermonthica* seeds (20mg) in a rhizotron filled with vermiculite. The rhizotron was constantly kept wet using Long Ashton medium solution. The rhizotron was assessed on daily basis to monitor emergence (germination) and development of *Striga* in association with roots of host plant.

Transgenic T_1 plants that were confirmed (through PCR) to be carrying PAP gene were screened in the glasshouse using growth medium with known phosphorus content (low) and viable *S. hermonthica* seeds (viability tested using GR24) to ascertain P mobilization and *S. hermonthica* suppression. For the Pi starvation-time-course experiment, half-strength Ashton and Hoagland's solutions were applied to transgenic plants in experiment 1 and 2 below. All evaluation experiments were laid out in a complete randomized block design (CRBD). Three bioassay experiments were setup as follows;

In the Rhizotron bioassay (Experiment 1), Twenty milligrams of *S.hermonthica* seeds (used per treatment) were surface sterilized in 5% (v/v) commercial bleach (NaOCl) and rinsed thrice with distilled sterile water. The seeds were then placed on Whatman filter paper moistened with double distilled water in a petri dish. The sterilized seeds were preconditioned at 28°C in the dark for 12 days. Twenty seeds, bulked from each transgenic event (3 events) per variety and their respective wild type varieties, initially pre-germinated on wet cotton wool and later transferred in rhizotron filled with vermiculite and kept in glasshouse were infected with the

preconditioned *S. hermonthica* seeds (20mg). The experimental design used in this bioassay was; 15 host plants X 3 maize varieties X 3 gene effect X 3 replicates. Observation and data collection were performed on the 'rhizotron' system as described by Gurney *et al*. (2006) and Yoshida and Shirasu (2009). In this protocol, *S. hermonthica*-infected transgenic/wild maize varieties in rhizotron were routinely monitored for emergence of *S. hermonthica* plants on the host plant after 4 weeks. The seedlings of transgenic/wild maize varieties in experimental blocks were maintained by routine application of half-strength Long Ashton's nutrient solutions (Temegne et al., 2015).

In the Bucket bioassay (Experiment 2), twenty seeds of each transgenic event of each variety together with their corresponding wild type varieties were planted in buckets (one plant per bucket) containing soil thoroughly mixed with equal amount of *S. hermonthica* seeds (2g). The experimental design used in this bioassay was; 15 host plants X 3 maize varieties X 3 gene effect X 5 replicates. The number of germinating *S. hermonthica* seeds were counted for each variety.

Finally, in the root exudate bioassay (Experiment 3), twenty pre-germinated seedlings of each transgenic event (3 events) of each variety together with their corresponding wild type varieties were transferred into Aluminium-wrapped glass test tubes containing half strength Ashton and Hoagland's nutrient solutions separately (Tariq and Mott, 2006; Temegne et al., 2015) and anchored upright using cotton wool in the glasshouse. After one week in nutrient solution, the seedlings were washed and the nutrient solution replaced with sterile distilled water. Water rich in exudates was collected after 7 days and used to evaluate their potential to induce *S. hermonthica* germination. A standardized serial dilution (taking into consideration root mass) was determined (5mg/l) for each host plant for purposes of uniformity in root exudate concentration. Synthetic germination stimulant GR24 (10^{-9}M) and demineralized water were included as positive and negative controls in the bioassay. Twenty milligrams (20mg) of pre-conditioned *S. hermonthica* seeds were subjected to 5ml of the standardized root exudate in an experimental design defined as; 5ml root exudate of each of the 15 T_1 plants per variety event X 3 maize varieties X 5 gene expression X 2 nutrient solutions X 9 replicates. The number of

germinating *S. hermonthica* seeds were counted for each variety. Seeds were considered germinated when the radicle had protruded through the seed coat.

5.3 Data analysis

Data generated from *in vitro* transformation cultures (especially selection and regeneration phases) and greenhouse screening experiments (*S. hermonthica* emergence) was subjected to analysis of variance (ANOVA) with means being tested for significance by LSD at 5% (Tukey's test) using statistical package SAS® System for Windows (9^{th} edition) and Genstat version 17. For *in vitro* regeneration experiments, ANOVA tests showing the effects of variations (maize varieties and gene construct) in induction and selection of callus and subsequent regeneration of transgenic events. Molecular data was qualitatively analyzed and the proportion of transgenic events and plants used in ANOVA analyzing of transformation efficiency. Data collected from *Striga* germination (exudate assay) and *Striga* colonization (rhizotron and bucket assays) experiments were subjected to ANOVA analysis and their corresponding means separated using Tukey's test (0.05). The analysis output was presented in form of graphs showing the relationship profiles at transgenic event level within each variety and their respective grand treatments.

5.4 Results

To compare the underlying genetic differences in *PAP* encoding genes of maize and selected plant species, *PAP* genes with phytate activity were retrieved from GenBank and aligned using MUSCLE. The PAP activity, under low P conditions, for the selected plants have previously been studied. On the basis of this alignment, the family of maize PAPs (family 2) were confirmed to have significantly diverged from PAPs family of other plants (family 1) whose PAP activity has extensively been studied and characterized (**Fig. 5.2**).

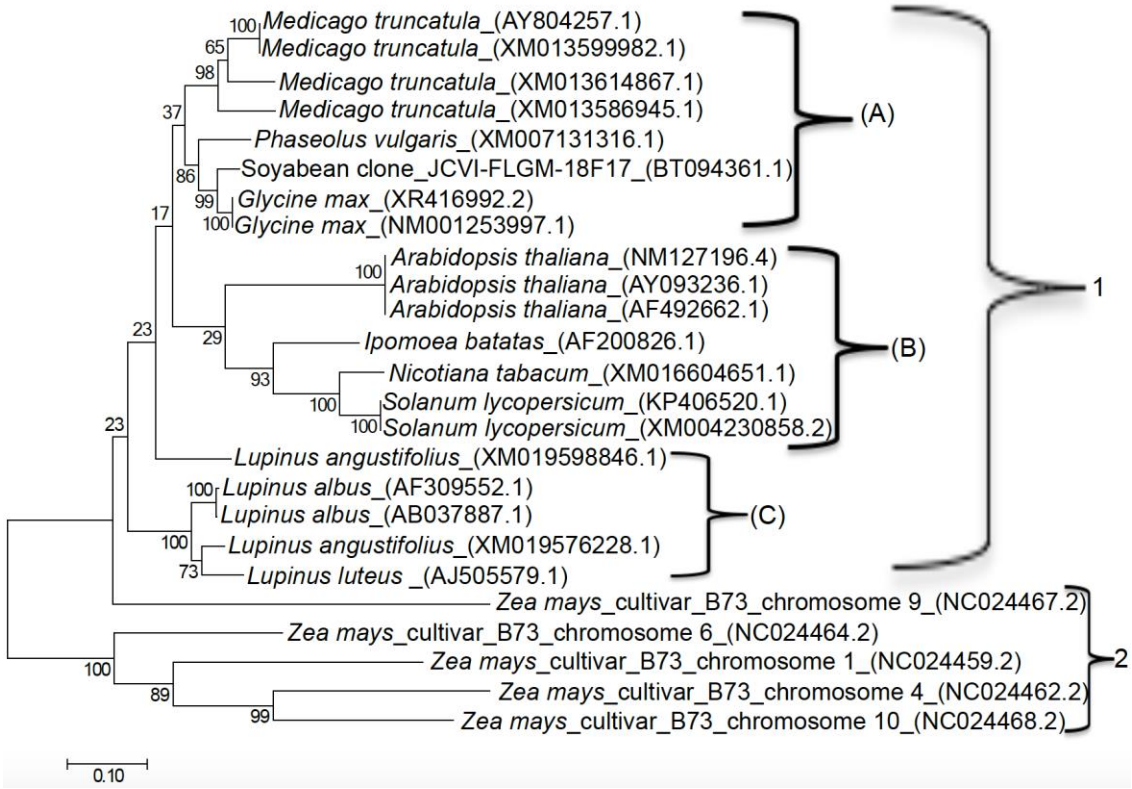

Figure 5.2: Phylogenetic analysis of PAP gene sequences of maize and selected plant species.

Legend:
The scale bar refers to 0.10 substitutions per nucleotide position. Bootstrap values obtained with 1000 resampling are presented to as percentages at all branches.

Successful transformation of *A. tumefaciens* (EHA 105) with *PAP* gene was assayed on selective bacterial medium. Transformed EHA105 bacterial cells grew on LB media amended with 100mg/l spectinomycin as shown in plate 5.1 (page 110). Colony PCR using gene-specific primers was performed to validate the presence of target gene in the resistant bacterial colonies as shown in plate 5.2 (page 110).

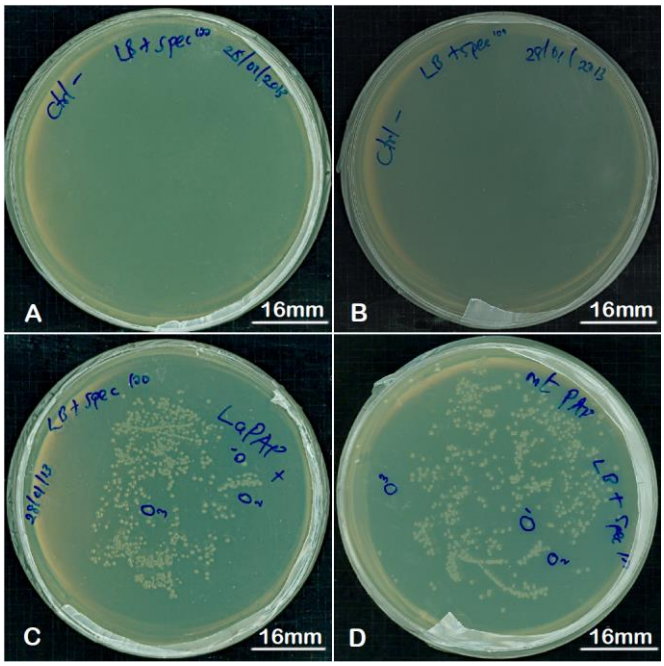

Plate 5.1: Growth of *PAP*-transformed EHA 105 on selection medium.

Legend:
(A and B) EHA105 transformed with unmodified binary vector pTF101.1 (negative control). (C) EHA105 transformed with pTF101.1 containing *bar* and *La*PAP genes, (D) EHA105 transformed with pTF101.1 containing *bar* and *Mt*PAP genes.

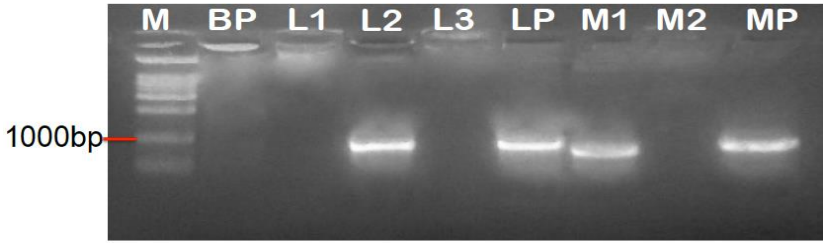

Plate 5.2: PCR products of EHA 105 transformed with PAP genes

Legend:
(M) Molecular marker, 1KB ladder, (BP) PCR masterMix without DNA, (L) EHA105 transformed with LaPAP, (LP) Plasmid containing LaPAP, (M) EHA105 transformed with MtPAP, (MP) Plasmid containing MtPAP. Numerical numbers 1,2 and 3 refers to the selected EHA105 colonies (replicates) used.

Immature maize embryos transformed with *PAP* gene displayed a distinct growth characteristic especially on selection media (**Plate 5.3,** page 111). A proportion of calli that survived selection turned embryogenic and subsequently formed juvenile plantlets upon transfer onto regeneration media (**Plate 5.3**).

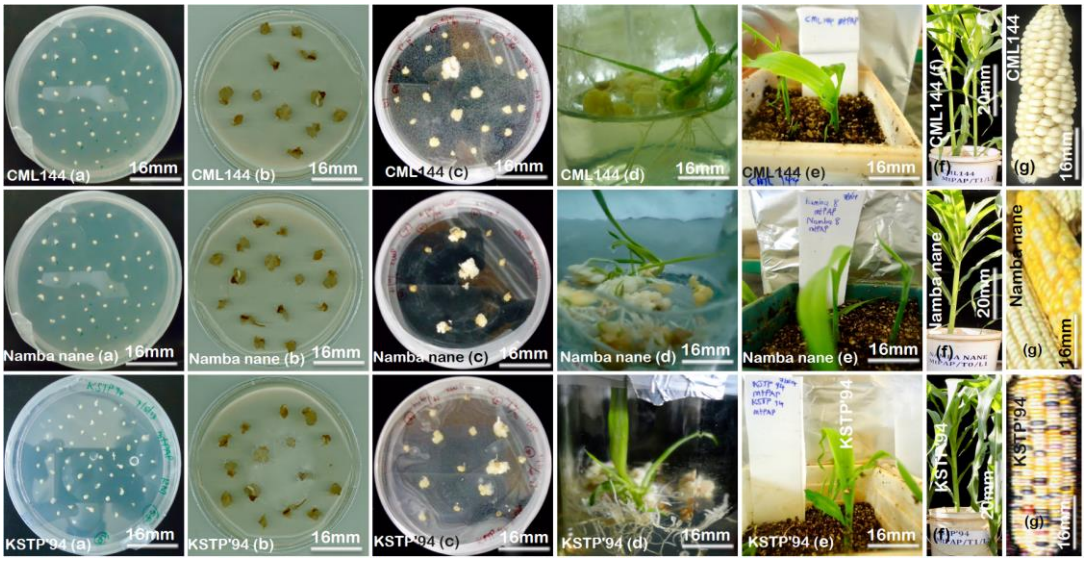

Plate 5.3: *In vitro* developmental and maturation phases of transformed maize varieties

Legend:
a) Co-cultivation stage of embryos with transgenic EHA105, **b**) Embryos on selection media I, **c**) Embryos on selection media II, **d**) Juvenile maize shoots on regeneration media, **e**) Maize plantlets in hardening troughs, **f**) Maize plants in the glasshouse, **g**) Seeds of T_0 plants.

Regenerated plantlets in the culture bottles were hardened and later acclimatized in the greenhouse (**Plate 5.3**). These plants were screened for the presence of the *PAP* gene by PCR technique using PAP-specific primers (**Plate 5.4**) and monitored until maturity stage (**Plate 5.3**). Corn of individual plant (transformation event) was harvested and labeled accordingly (**Plate 5.3**). The seeds were then replanted, monitored to maturity and harvested for seed bulking purposes.

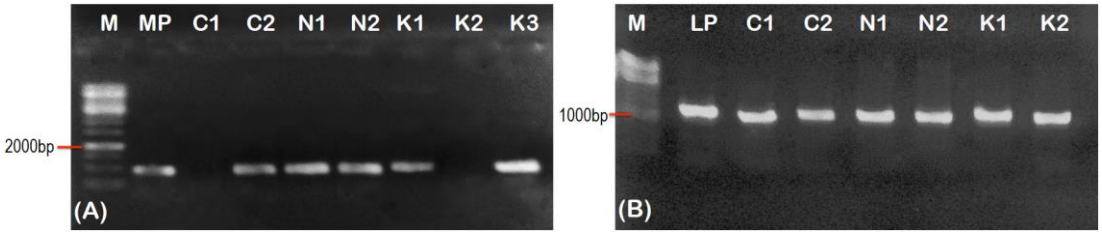

Plate 5.4: PCR products of *Mt*PAP and *La*PAP transgenic maize events

Legend:
(M) 1Kb plus (A) and 1Kb (B) Molecular marker, 1Kb ladder, (MP) Plasmid containing *Mt*PAP, (C) CML144, (N) Namba nane, (K) KSTP'94, (LP) Plasmid containing *La*PAP Numerical numbers 1, 2 and 3 refers to selected transgenic events.

Analysis of the interaction effect of gene construct type and maize variety at selection stage on callus transformation frequency was found to be significantly different among the interaction treatments (**Table 5.1**). It is important to note at this stage that, most of the surviving calli during selection process were due to the presence of the *bar* gene in the plasmid pTF101.1 in the callus. The computed analysis therefore revealed the general transformability potential (transformation frequency) of the maize varieties (**Table 5.1**). CML144-*La*PAP recorded the highest (9.9%) transformation frequency while Namba nane *Mt*PAP recorded the least (3.5%) transformation frequency (**Table 5.1**).

Table 5.1: Transformation frequency of *PAP*-specific maize varieties

Variety	**% Mean of surviving callus**
CML144 LaPAP	9.9 ± 1.58^{a}
Namba nane LaPAP	6.4 ± 1.29^{ab}
CML144 MtPAP	5.4 ± 1.47^{ab}
KSTP'94 MtPAP	5.1 ± 0.99^{ab}
KSTP'94 LaPAP	4.7 ± 1.19^{ab}
Namba nane MtPAP	3.5 ± 0.02^{c}
$F_{(5, 56)}$	32.14
P value	0.0497
L.S.D.$_{(0.05)}$	**3.71**

Means with same letters are not significantly different at *Tukey's* test (*P<0.05*).

Irrespective of the *PAP* gene construct used, analysis of the receptivity of the callus of maize varieties to plasmid pTF101.1 (varietal transformation frequency) clearly shows that CML 144 recorded the highest transformation frequency (7.6%) while KSTP'94 and Namba nane were not significantly different (**Table 5.2,** page 113). Analysis of the receptivity of maize callus to plasmid pTF101.1 having different *PAP* gene construct (gene construct transformation frequency) clearly shows that plasmid pTF101.1 with *la*PAP gene construct recorded a significantly high transformation frequency than the one with *mt*PAP gene construct (**Table 5.2**).

Table 5.2: Effect of variety and gene-construct on transformation frequency

Variety	% mean of surviving callus	Gene	% mean of surviving callus
CML 144	7.6±1.39[a]	LaPAP	6.9±1.02[a]
KSTP' 94	4.9±0.69[b]	MtPAP	4.7±0.59[b]
Namba 8	4.7±0.85[b]		
$F_{(2, 15)}$	15.06	$F_{(1, 16)}$	0.22
P value	<.0001	P value	<.0001
L.S.D $_{(0.05)}$	**2.63**	**L.S.D $_{(0.05)}$**	**2.14**

Means with same letters are not significantly different at *Turkey's* test (*P<0.05*).

The presumed transgenic T_0 plants in the greenhouse were further screened using PCR technique (using *PAP* gene-specific primers) to identify putative transgenic events. The proportion of *PAP* transgenic events with respect to the initial total number of calli of individual variety subjected to transformation was computed in table 5.3. Analysis of the interaction effect presented in table 5.3 shows that there was no significant difference in the transformation efficiency among the screened interaction treatments **(Table 5.3)**.

Table 5.3: Transformation efficiency of gene-specific maize varieties

Variety	% mean of transgenic plants
KSTP'94 *La*PAP	0.69±0.047[a]
CML144 *La*PAP	0.65±0.027[a]
KSTP'94 *Mt*PAP	0.37±0.032[a]
Namba nane *Mt*PAP	0.36±0.035[a]
CML144 *Mt*PAP	0.34±0.032[a]
Namba nane *La*PAP	0.33±0.031[a]
$F_{(5, 56)}$	3.76
P value	0.7166
L.S.D.$_{(0.05)}$	**1.070**

Means with same letters are not significantly different at *Tukey's* test (*P<0.05*).

There was no significant difference noted in transformation efficiency for both varietal and gene treatment assessment (**Table 5.4,** page 114).

Table 5.4: Effect of variety and gene-construct on transformation efficiency

Variety	Transgenic plants (%)	Gene	Transgenic plants (%)
CML 144	0.50±0.223[a]	*La*PAP	0.56±0.177[a]
KSTP'94	0.53±0.238[a]	*Mt*PAP	0.36±0.180[a]
Namba 8	0.35±0.219[a]		
$F_{(2, 56)}$	2.33	$F_{(1, 16)}$	3.84
P value	0.131	P value	0.068
L.S.D $_{(0.05)}$	**0.756**	**L.S.D $_{(0.05)}$**	**0.618**

Means with same letters in the same column are not significantly different at *Tukey's* test (*P<0.05*).

In the pre-trial *Striga* colonization experiment, it was established that *Striga* seed takes twenty-one days to establish its parasitic life rostrum in the rhizotron (**Plate 5.5**). In this study, *Striga* seed began to germinate with their radicle growing towards the root of the host on the third day of incubation. By the sixth day, *Striga* haustorium had firmly created a connection with the host root. Between the ninth and twelfth day, *Striga* haustoria enlarged significantly with emerging shoot primordium forming true leaves. From the fifteenth to eighteenth day, hair-like papillae appeared on the elongating *Striga* shoot, which developed fully by the twenty-first day.

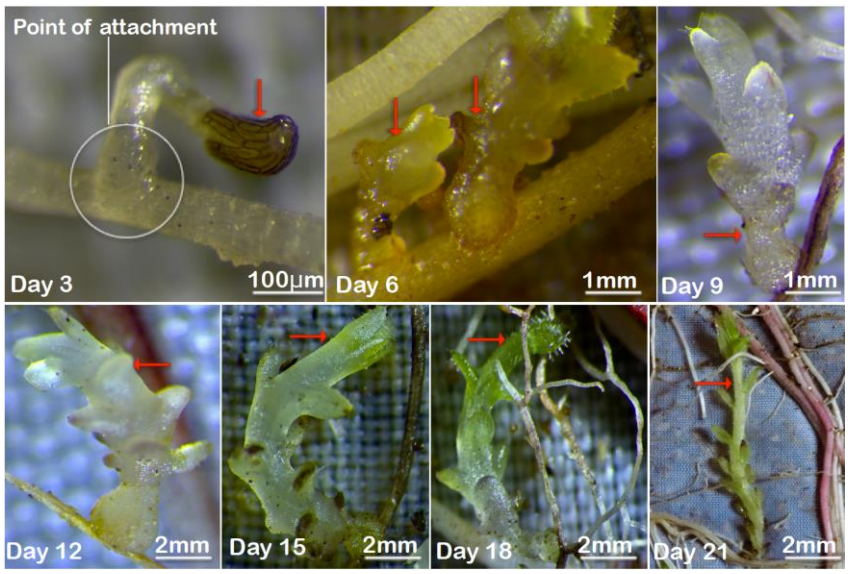

Plate 5.5: *Striga hermonthica* parasitic life infestation stages on CML144.

Legend:
Day3) emergence of radicle, **Day6**) establishment of firm haustorium-host plant connection, **Day9**) enlargement of haustorium, **Day12**) emergence of numerous primordial, **Day15**) formation of true leaves from shoot primordial, **Day18**) emergence of hair-like papillae on the elongating *Striga* shoot and **Day21**) fully developed *Striga* plants.

Seeds from T_0 plants that tested positive in PCR screening were planted, monitored to maturity and harvested for seed bulking purposes. The bulked transgenic seeds of corn were pre-germinated and screened for presence of *PAP* gene. The *PAP* positive juvenile plantlets together with their wild types were used to set-up rhizotron experiment where all *in vitro* developmental stages of *S. hermonthica* parasitization processes were monitored and observed (**Plate 5.5**).

Data collection of the rhizotron maize-*Striga*-screening assay commenced on twenty-first day (**Plate 5.6**, page 117). First, wild type of CML144, Namba nane, KSTP'94 appeared to visibly support more S. *hermonthica* plants than their corresponding transgenic lines (**Plate 5.6**). A further counting of the number of *Striga* plants parasitizing maize host plants of each variety, in both wild type and their corresponding transgenic line showed that wilt type were indeed more colonized than transgenic counterparts (**Fig.5.3,** page 118). Specifically in the case of Namba nane, an average of 9 *Striga* plants parasitizing a single wild-type maize plant was recorded ($F_{(6,14)}$ = 27.29, P value <.001). The average number of *Striga* plants parasitizing Namba nane transformed with *LaPAP* gene was 4, 4 and 5 for transgenic event 1, 2 and 3, respectively ($F_{(3,8)}$ = 54.0, P value <.001). However there was no significant difference within the three transgenic events ($L.S.D_{0.05}$=1.087, ($F_{(3,8)}$ = 54.0, P value <.001). On the other hand, the average number of *Striga* plants parasitizing Namba nane transformed with *Mt*PAP gene was 5, 5 and 6 for transgenic event 1, 2 and 3, respectively ($F_{(3,8)}$ = 36.0, P value <.001). However there was no significant difference within the three events ($L.S.D_{0.05}$=1.101, ($F_{(3,8)}$ = 36.0, P value <.001).

Results for KSTP'94 show that, an average of 5 *Striga* plants parasitized a single wild-type maize plant ($F_{(6,14)}$ = 3.42, P value 0.027). The average number of *Striga* plants parasitizing KSTP'94 transformed with LaPAP gene was 2, 3 and 3 for transgenic event 1, 2 and 3, respectively ($F_{(3,8)}$ = 14.67, P value 0.08). However there was no significant difference within the three events ($L.S.D_{0.05}$=0.769, ($F_{(3,8)}$ = 14.67, P value 0.08). Similarly, the average number of *Striga* plants parasitizing KSTP'94 transformed with *Mt*PAP gene was 3, 4 and 4 for transgenic event 1, 2 and 3,

respectively ($F_{(3,8)}$ = 1.71, P value 0.241). However there was no significant difference within the three events ($L.S.D_{0.05}$=1.138, ($F_{(3,8)}$ = 1.71, P value 0.241).

Results for CML144 show that, an average of 12 *Striga* plants parasitizing a single wild-type maize plant was recorded ($F_{(6,14)}$ = 16.29, P value <.001). The average number of *Striga* plants parasitizing CML144 transformed with *La*PAP gene was 6, 7 and 6 for transgenic event 1, 2 and 3, respectively ($F_{(3,8)}$ = 23.22, P value <.001). However there was no significant difference within the three events ($L.S.D_{0.05}$=1.883, ($F_{(3,8)}$ = 23.22, P value <.001). Relatedly, the average number of *Striga* plants parasitizing CML144 transformed with *Mt*PAP gene was 8, 7 and 8 for transgenic event 1, 2 and 3, respectively ($F_{(3,8)}$ = 28.17, P value <.001). However there was no significant difference within the three events ($L.S.D_{0.05}$=1.331, ($F_{(3,8)}$ = 28.17, P value <.001).

In general, there was a significant difference noted between wild type, *Mt*PAP and *La*PAP maize lines of Namba nane which recorded an average 9, 5 and 4 *Striga* plants per host plant, respectively ($L.S.D_{0.05}$=0.730, $F_{(6,14)}$ = 27.29, P value <.001). In KSTP'94, although there was a significant difference noted between wild type and the transgenic maize lines (4 and 3 *Striga* plants per host plant respectively), there was no significant difference noted between *La*PAP and *Mt*PAP maize lines of KSTP'94 which recorded an average 3 *Striga* plants per host plant ($L.S.D_{0.05}$=0.924, $F_{(6,14)}$ = 3.42, P value 0.027). Similarly, there was a significant difference noted between wild type and the transgenic maize lines of CML144 (12 and 7 *Striga* plants per host plant, respectively, $F_{(6,14)}$ = 16.29, P value <.001). However no statistical difference was noted between *La*PAP and *Mt*PAP maize lines of KSTP'94 which recorded an average 6 and 8 *Striga* plants per host plant ($L.S.D_{0.05}$=2.133).

Plate 5.6: Effect of rhizotron-grown transgenic maize on *Striga* infestation

Arrows point to *S. hermonthica* plant, (WT) wild plant, (*Mt*PAP) *Mt*PAP transgenic event and (*La*PAP) *La*PAP transgenic event.

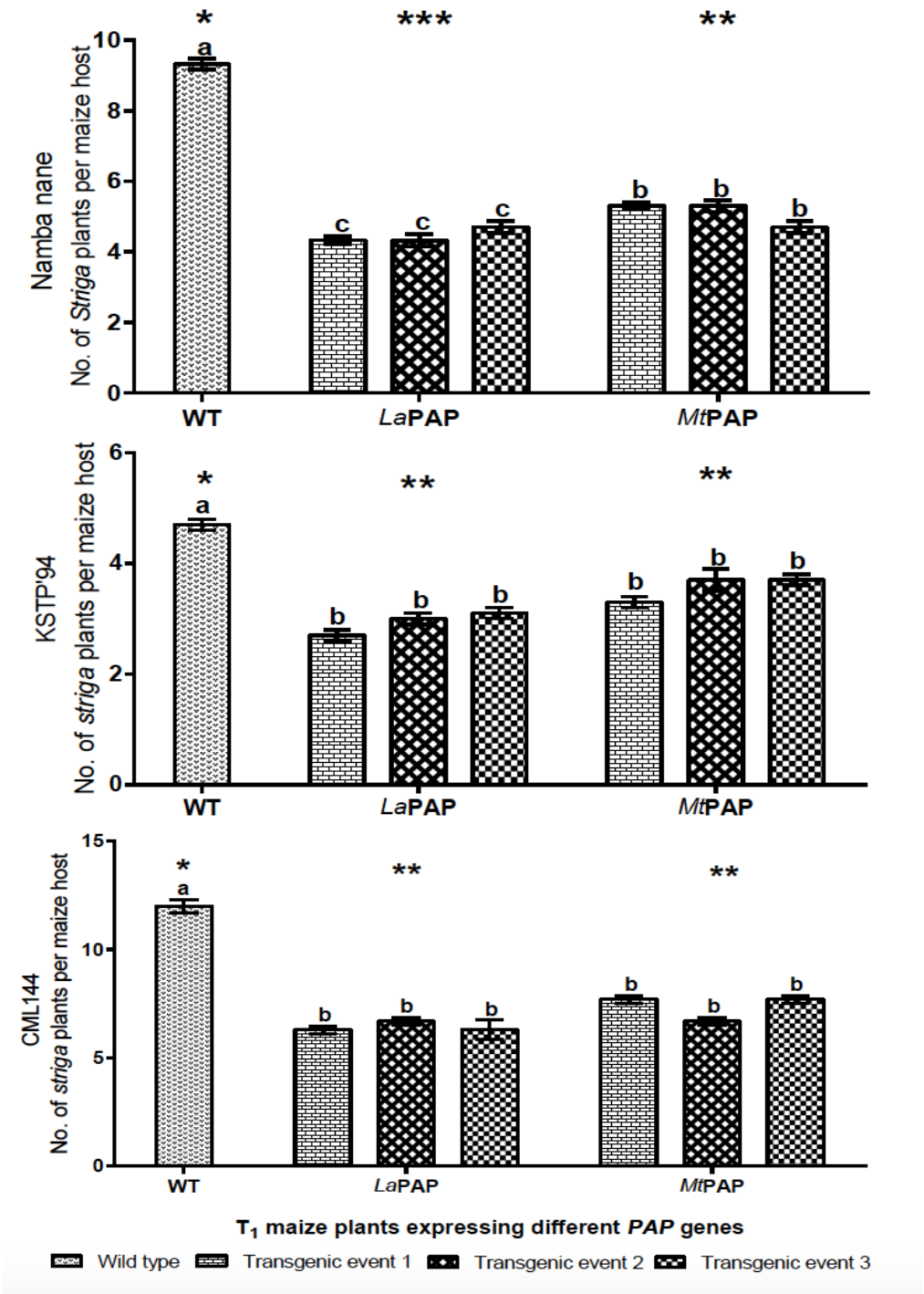

Figure 5.3: Number of *S. hermonthica* colonizing rhizotron-grown wild type and transgenic events of maize varieties used.

Legend:
Bars within a treatment bearing the same alphabet letter represent mean number of *Striga* plants parasitizing independent maize lines which are not significantly different at Tukey's test (0.05). Treatments with the same number of asterisk(s) are not significantly different at Tukey's test (0.05).

In the bucket experiment, the number of *S. hermonthica* seeds emerging from the pots was less compared to the rhizotron experiment. In the bucket experiment, the average number of *Striga* plants emerging alongside maize host plant of each variety, in both wild type and their corresponding transgenic lines, is presented in Fig.5.4 (page 121).

In the case of Namba nane ($F_{(6,14)}$ = 12.67, P value <.001), an average of 5 *Striga* plants emerged from pot with wild-type maize plant. The average number of *Striga* plants parasitizing Namba nane transformed with *La*PAP gene was 1, 1 and 2 for transgenic event 1, 2 and 3, respectively ($F_{(3,8)}$ = 19.11, P value <.001). However there was no significant difference within the three events ($L.S.D_{0.05}$=1.331). On the other hand, the average number of *Striga* plants emerging in pots with Namba nane maize plant transformed with *Mt*PAP gene was 2, 3 and 3 for transgenic event 1, 2 and 3, respectively ($F_{(3,8)}$ = 14.07, P value 0.001). However there was no significant difference within the three events ($L.S.D_{0.05}$=1.215).

Results for KSTP'94 ($F_{(6,14)}$ = 3.42, P value 0.027) show that, an average of 3 *Striga* plants parasitized a single wild-type maize plant. The average number of *Striga* plants parasitizing KSTP'94 transformed with *La*PAP gene was 0.8, 1 and 1 for transgenic event 1, 2 and 3, respectively ($F_{(3,8)}$ = 14.67, P value 0.001). However there was no significant difference within the three events ($L.S.D_{0.05}$=0.769). Similarly, the average number of *Striga* plants parasitizing KSTP'94 transformed with *Mt*PAP gene was 1.6, 1.3 and 1.6 for transgenic event 1, 2 and 3, respectively ($F_{(3,8)}$ = 1.71, P value 0.241). However there was no significant difference within the three events ($L.S.D_{0.05}$=0.838).

Results for CML144 ($F_{(6,14)}$ = 9.75, P value <.001) show that, an average of 7 *Striga* plants parasitizing a single wild-type maize plant was recorded.

The average number of *Striga* plants parasitizing CML144 transformed with *La*PAP gene was 2, 2.7 and 2 for transgenic event 1,2 and 3, respectively ($F_{(3,8)}$ = 15.64, P value 0.001). However there was no significant difference within the three events ($L.S.D_{0.05}$=1.883).

Relatedly, the average number of *Striga* plants parasitizing CML144 transformed with *Mt*PAP gene was 3.7, 3.7 and 4 for transgenic event 1,2 and 3, respectively ($F_{(3,8)}$ = 11.79, P value 0.003). However there was no significant difference within the three events ($L.S.D_{0.05}$=1.537).

In general, there was a significant difference noted between wild type, *Mt*PAP and *La*PAP maize lines of Namba nane which recorded an average 5, 2 and 1 *Striga* plants per host plant, respectively ($L.S.D_{0.05}$=0.794, $F_{(6,14)}$ = 12.67, P value <.001).

In KSTP'94, although there was a significant difference noted between wild type and the transgenic maize lines (3 and 1.5 *Striga* plants per host plant, respectively) there was no significant difference between *La*PAP and *Mt*PAP maize lines which recorded an average 0.9 and 1.6 *Striga* plants per host plant, respectively ($L.S.D_{0.05}$=0.794, $F_{(6,14)}$ = 3.42, P value 0.027).

Lastly, there was a significant difference noted between wild type, *Mt*PAP and *La*PAP maize lines of CML144 which recorded an average 7, 3.7 and 2.4 *Striga* plants per host plant, respectively ($L.S.D_{0.05}$=1.133, $F_{(6,14)}$ = 9.75, P value <.001).

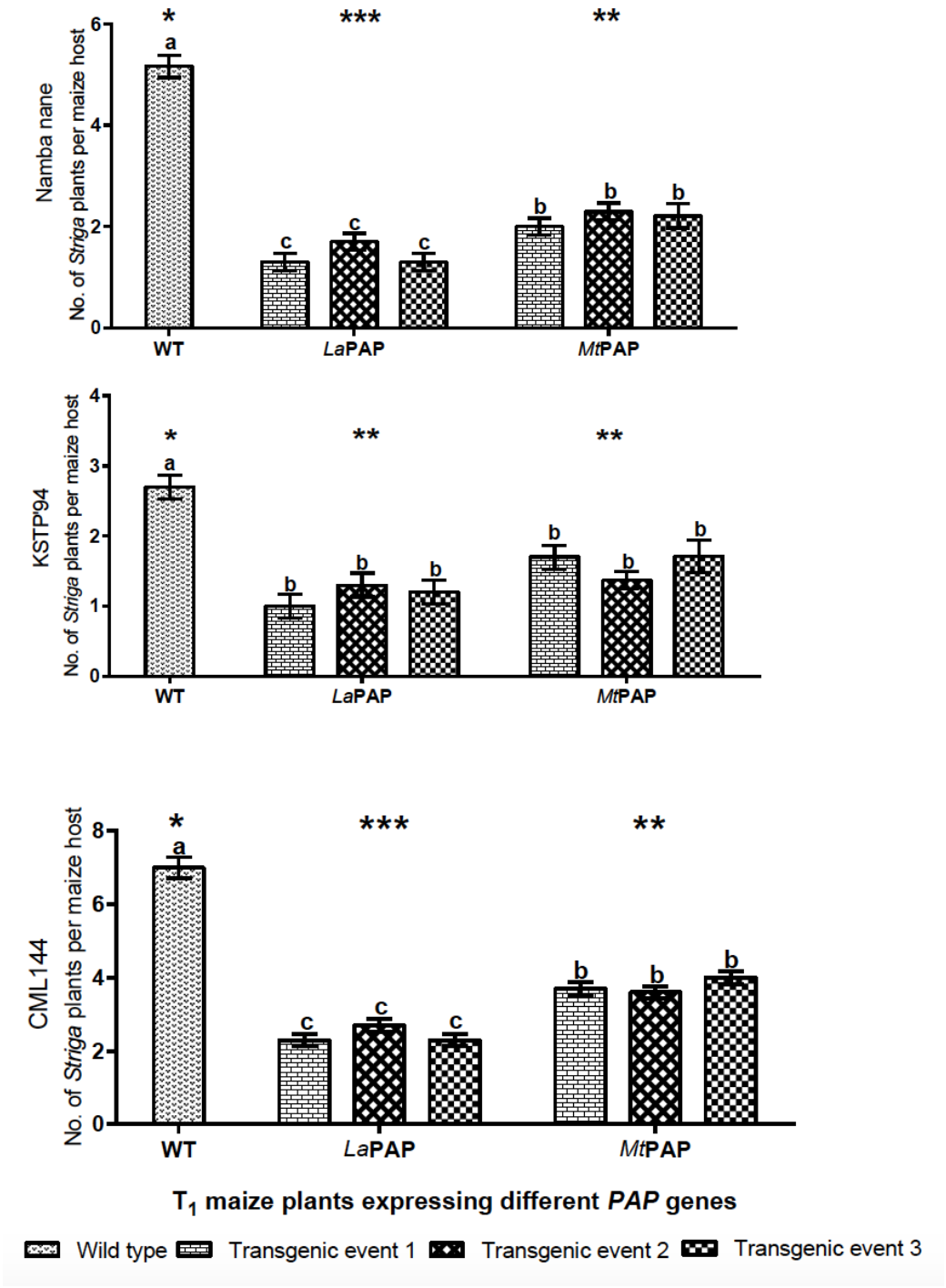

Figure 5.4: Number of *S. hermonthica* colonizing bucket-grown wild type and transgenic events of maize varieties used.

Legend:
Bars within a treatment bearing the same alphabet letter represent mean number of *Striga* plants parasitizing independent maize lines which are not significantly different at Tukey's test (0.05). Treatments with the same number of asterisk(s) are not significantly different at Tukey's test (0.05).

The ability of root exudates of wild type and transgenic maize grown on phosphorous deficient media to stimulate *Striga* germination is presented in Plate 5.7 (page 124) and Fig. 5.5 (page 125). The average number of *Striga* seeds germinating after exposure to GR24 and sterile distilled water (positive and negative generic controls respectively) were 18 and 0 respectively.

In the case of Namba nane, the results ($F_{(5,12)}$ = 4.19, P value 0.0195) of exudates collected from test tubes of Namba nane wild-type plants that had earlier been maintained in Hoagland and Long Ashton nutrient solutions induced the second and third highest number of germinating *Striga* seeds (7 and 6 *Striga* plants, respectively). Exudates collected from plants of *La*PAP transgenic events TE-1, TE-2 and TE-3 that had been maintained on Ashton nutrient medium and those of *La*PAP transgenic events TE-1, and TE-2 that had been maintained on Hoagland nutrient medium recorded an average of 5 germinating *Striga* seeds. Exudates collected from plants of *La*PAP transgenic event TE-3 that had been on Hoagland nutrient medium recorded an average of 5 germinating *Striga* seeds, which was slightly significant than the earlier mentioned exudates ($L.S.D_{0.05}$=0.983). Exudates collected from plants of *Mt*PAP transgenic events TE-1 and TE-2 (maintained on Ashton) and events TE-2 and TE-3 (maintained on Hoagland) recorded 6 germinating *Striga* seeds, which was significantly higher than 5 germinating *Striga* seeds recorded in TE-3 and TE-1 exudates of plants that had been maintained in Ashton and Hoagland media, respectively ($L.S.D_{0.05}$=0.983).

In summary, there was a significant difference noted among all the treatments. The positive generic control (GR24), wild-type exudate, *Mt*PAP exudate, *La*PAP exudate and the negative generic control recorded 18, 7, 6, 4 and 0 germinating *Striga* seeds respectively ($L.S.D_{0.05}$=0.794, $F_{(4, 24)}$ = 4.57, P value 0.0208).

Results for KSTP'94 variety ($F_{(5,12)}$ = 5.74, P value 0.0062) show that, exudates collected from wild-type plants that had earlier been maintained in Hoagland and Long Ashton nutrient solutions induced the second and third highest number of germinating *Striga* seeds (5 and 4 *Striga* plants, respectively). Exudates of *La*PAP transgenic plants of the events TE-1, TE-2 and TE-3 that had been maintained on Hoagland solution medium recorded an average of 4 germinating *Striga* seeds.

Exudates of *La*PAP transgenic plants of the events TE-1, TE-2 and TE-3 maintained on Ashton solution medium recorded an average of 1 germinating *Striga* seed ($L.S.D_{0.05}$=0.983).

Exudates of MtPAP transgenic plants of events TE-1, TE-2 and TE-3 maintained on Hoagland solution medium recorded an average of 3 germinating *Striga*. Exudates of *Mt*PAP transgenic plants of events TE-1, TE-2 and TE-3 maintained on Ashton solution medium recorded an average of 2 germinating *Striga* seeds. These two types of MtPAP exudates were significantly different ($L.S.D_{0.05}$=1.031).

In summary, there was a significant difference noted among all the treatments. The positive generic control, wild-type exudate, MtPAP exudate, *La*PAP exudate and the negative generic control recorded 18, 5, 3, 2 and 0 germinating *Striga* seeds, respectively ($L.S.D_{0.05}$=0.786, $F_{(4, 24)}$ = 11.64, P value 0.0049).

Results for CML144 variety ($F_{(5,12)}$ = 5.24, P value 0.0088) show that, exudates collected from wild-type plants that had earlier been maintained in either Hoagland or Long Ashton nutrient solutions induced the second highest number of germinating *Striga* seeds (14 *Striga* plants). Exudates of *La*PAP transgenic plants of events TE-1, TE-2 and TE-3 that had been maintained on either Hoagland or Ashton solution media recorded an average of 6 germinating *Striga* seeds, hence no significant difference ($L.S.D_{0.05}$=1.077). Similarly, exudates of *Mt*PAP transgenic plants of events TE-1, TE-2 and TE-3 that had been maintained on either Hoagland or Ashton solution media recorded an average of 7 germinating *Striga* seeds, hence no significant difference ($L.S.D_{0.05}$=1.244).

In summary, there was a significant difference noted among all the treatments. The positive generic control, wild-type exudate, *Mt*PAP exudate, *La*PAP exudate and the negative generic control recorded 18, 14, 7, 6 and 0 germinating *Striga* seeds, respectively ($L.S.D_{0.05}$=0.641, $F_{(4, 24)}$ = 5.64, P value 0.0052).

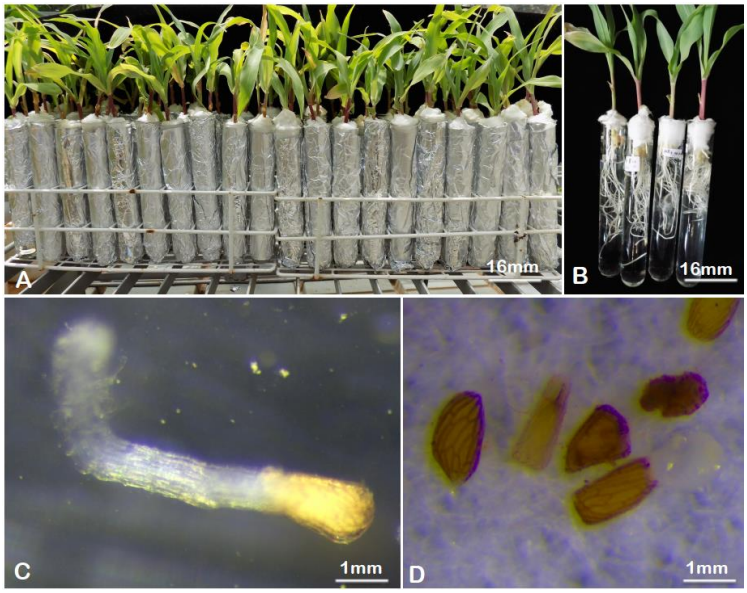

Plate 5.7: *In vitro* stimulation of *Striga* germination using root exudates

Legend:
A) Root exudate assay set up for Namba nane, KSTP'94 and CML144 varieties, B) Collection of root exudate from the three varieties, C) Germinating *Striga* seed after exposure to GR24, D) Germinating *Striga* seed after exposure to root exudate.

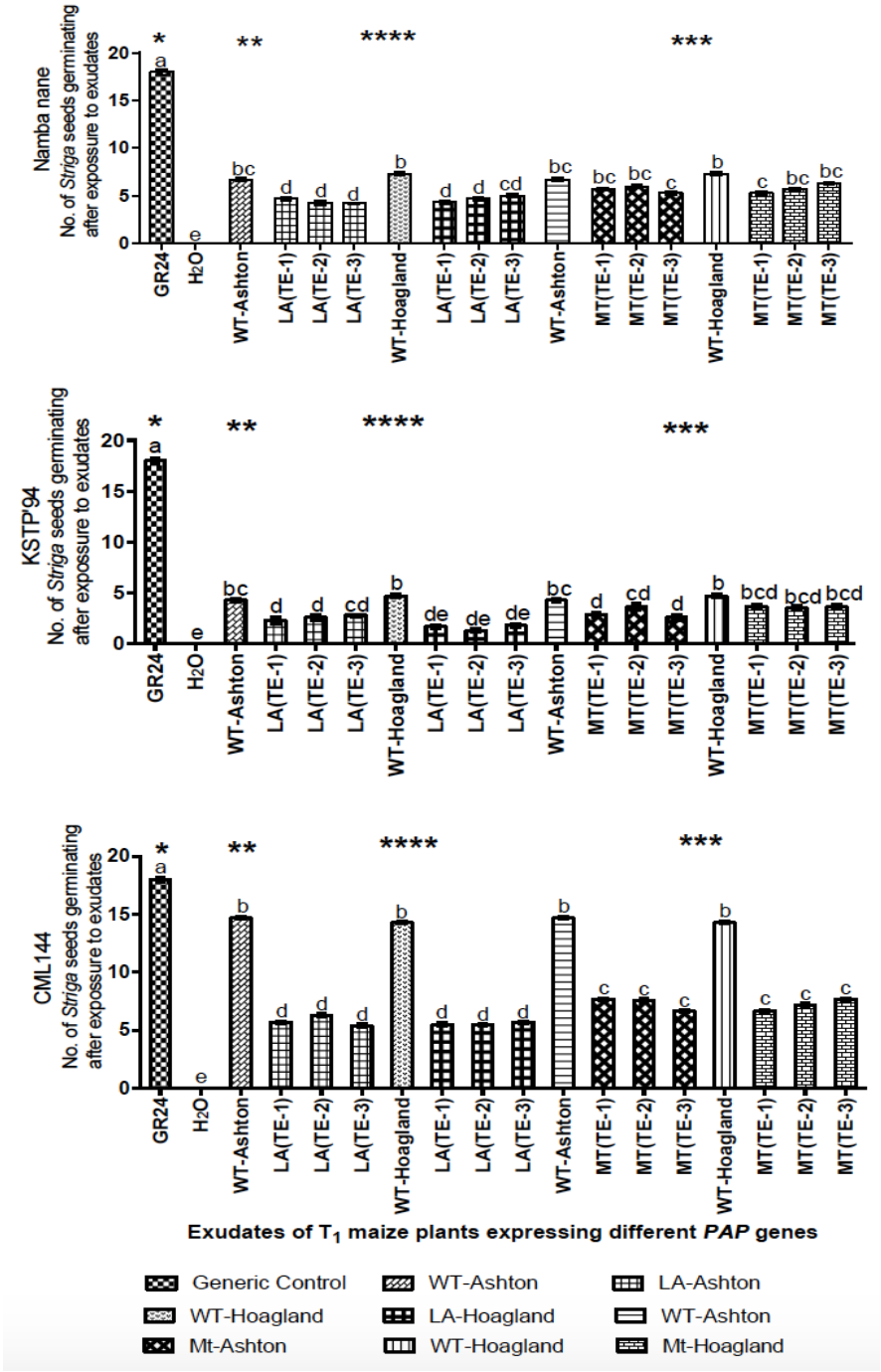

Figure 5.5: Effect of root exudates of transgenic maize varieties on *Striga* germination induction rate.

Legend:

Bars within a treatment bearing the same alphabet letter represent mean number of germinating *Striga* seeds stimulated by root exudate of independent maize lines which are not significantly different at Tukey's test (0.05). Treatments with the same number of asterisk(s) are not significantly different at Tukey's test (0.05).

5.5 Discussion

Plant genomes encode a variety of putative APases, the largest group being PAP family that plays the most significant role in production, transport and recycling of Pi, which is crucial for cellular metabolism and bioenergies (González-Muñoz et al., 2015). Plant *PAPs* are induced under various environmental and developmental conditions, including salt, water-deficit stress, flowering, fruit ripening, seed germination, senescence and pathogen infection. Different *PAPs* orthologs in plants are involved in either intracellular or extracellular APases activities (Younessi-hamzekhanlu et al., 2016). This is important because, despite the abundance of organic P reserves in the soil, the bioavailability of assimilable inorganic P is often suboptimal for plant growth and productivity (Robinson et al., 2012). In the present study, alignment of selected PAP gene sequences shows that maize *PAPs* have a marked difference in sequence code when compared to *PAPs* of other plants. This finding is in agreement with results reported by González-Muñoz et al., (2015) and Alakonya (2011) and formed the basis of selecting well characterized *PAP* gene from the *L. albus* and *M. truncatula.*

This study demonstrated that genetic transformation of maize depends largely on the ability of transformed tissues to proliferate in selection medium and subsequently regenerate plants from the transformed cells or tissues. Similar findings had earlier been reported by Sahrawat et al., (2003). This study revealed that, although callus of CML144 was highly transformable (transformation frequency of 7.6%), there was no significant difference in *PAP* transformation efficiency of the three maize varieties. Similarly, the delivery frequency of *La*PAP gene construct was higher than that of *Mt*PAP gene construct at callus stage, however the delivery efficiency of the two genes constructs did not differ significantly. Transformation efficiency of the three maize varieties used in this study ranged from 0.35 to 0.53%.

The main aim of this study was to enhance the ability of maize to mobilize phosphorus from external sources by transforming the selected maize genotypes with *L. albus* and *M. truncatula PAP* genes. Overexpression of these two types of *PAPs* in the selected maize varieties enhanced the ability of the maize varieties to utilize fixed rhizosphere phosphorus content. Earlier studies have shown that production

and secretion of *PAPs* is a universal response by higher plants to P-starvation which allows them to cope with this challenge and restore their growth in low-P conditions, however maize is deficient of this natural capability (González-Muñoz et al., 2015; Wang et al., 2013). Results of this study however indicate that after transformation of maize with the two *PAP* genes and a preliminary study on the sequence of the subterranean development stages of *S. hermonthica* revealed that, there are seven distinct subterranean development phases of *Striga* parasitic stages. These phases comprise of; radicle germination, connection of haustorium to host root, haustorium enlargement, emergence of shoot primordium, formation of true leaves, emergence of hair-like papillae on the shoot tip and finally complete development of *Striga* plant prior to emergence on the soil surface. Earlier studies had already pointed out that root parasitism behavior of *S. hermonthica* occur underground followed by aboveground flowering shoot (Catarina et al., 2014).

To establish whether the overexpression of *La*PAP and *Mt*PAP in maize down-regulated the production of SLs, the transformed maize varieties were infected with pre-conditioned *S. hermonthica*. This pre-condition step was necessary because mature *S. hermonthica* seeds are always in a state of primary dormancy and hence preconditioning (under humid and warm condition) for several days (at least 12days) is required to allow the seeds to imbibe water and be able to germinate (Karaya et al., 2012).

In this study, the effect of overexpressing *PAPs* in maize on *S. hermonthica* infestation (host root-*S. hermonthica* association) was evaluated using three different approaches namely rhizotron assay, potted plant (bucket) assessment and root exudate experiment. Emergence of the radicle on *S. hermonthica* seed in the root exudate assay and *Striga* plant in the rhizotron and bucket experiments were used as indicators of stimulation of *S. hermonthica* seed germination.

In the rhizotron experiment, assessment of the effect of *Mt*PAP and *La*PAP genes in transgenic maize lines on *Striga* parasitization under low P condition (use of Long Ashton medium) clearly shows that, the two genes conferred a significant level of *S. hermonthica* resistance in the transgenic lines compared to their corresponding wild-

type varieties. In this experimental set up, wild type variety of CML144 was the most susceptible variety to *S. hermonthica* attack (12 *Striga* plants) followed by Namba nane (9 *Striga* plants) while KSTP'94 displayed a remarkable level of resistance to *S. hermonthica* attack (5 *Striga* plants). This probably explains why KSTP'94 is a certified *S. hermonthica*-tolerant maize variety as the name 'Kakamega *S. hermonthica* Tolerant Population 94' suggests (Omondi et al., 2014). The corresponding *La*PAP transgenic lines of the three varieties recorded an average of 6, 4 and 3 *Striga* plants while the corresponding *Mt*PAP transgenic lines recorded an average of 8, 5 and 4 *Striga* plants. However it was noted that there was no significant difference in the number of *Striga* attacking host plant among the plants raised from different transformation events. Previous studies indicate that maize *PAP* gene play a major role in the breakdown of P containing compounds in senescing leaves thereby facilitating remobilization of P to more active photosynthetic tissues (González-Muñoz et al., 2015; Li et al., 2010). This therefore implies that maize has well-established intracellular *PAP* activity but lacks the ability to scavenge for P from rhizosphere sources, especially under low P rhizosphere conditions (González-Muñoz et al., 2015). However maize is known to produce strigolactones (SLs) under P deficiency that stimulates development of arbuscular mycorrhizae (AM), which is essential for establishment of a symbiotic relationship with maize (Guan et al., 2012). In this association, AM secretes PAPs through hyphae which accelerate mineralization of organic P into inorganic P in the rhizosphere thereby availing the much-needed form of phosphorus to maize (Li et al., 2012). The SLs produced by maize also induce seed germination of *Striga* species that target maize and other crops (Guan et al., 2012; Xie et al., 2010). Transformation of these three maize varieties with *La*PAP and *Mt*PAP genes therefore enhanced maize ability to mobilize extracellular P. This in turn affected the development of the signal that directs the production of strigolactone necessary for both AM symbiosis relationship and *Striga* germination and hence the low number of *Striga* plants colonizing transgenic maize.

In the bucket experiment, suppression of *Striga* colonization pattern similar to the one recorded in rhizotron was noted. This is because the mode of action of the transgenic lines on *Striga* colonization in both experiments is the same. In this case,

the wild type variety of CML144 was the most susceptible variety to *S. hermonthica* attack (7 *Striga* plants) followed by Namba nane (5 *Striga* plants) while KSTP'94 displayed a remarkable level of resistance to *S. hermonthica* attack (3 *Striga* plants). The corresponding *La*PAP transgenic lines of the three varieties recorded an average of 2, 1 and 1 *Striga* plants while the corresponding *Mt*PAP transgenic lines recorded an average of 4, 3 and 2 *Striga* plants. This huge difference in the number of *Striga* colonizing same maize lines between rhizotron and bucket experiment could partly be as a result of uniformity in nutrient supply and close proximity of *Striga* seeds to the root of host plant in the rhizotron assay.

Evaluation of the effect of root exudates of maize varieties overexpressing *La*PAP and *Mt*PAP genes on stimulation of *Striga* germination revealed interesting *Striga* resistance profiles. While the mode of action by transgenic maize lines on stimulation of *Striga* germination is similar to that of rhizotron and bucket, in this assay the effect of the two genes at two levels of P deficiency was evaluated. Given that the concentration of phosphorus ion in Hoagland and Long Ashton media is 195mg/l and 41mg/l respectively, it is evident that all the *La*PAP transgenic plants of the three maize varieties maintained on Ashton media ($F_{(5,12)} = 4.26$, P value 0.0185) consistently recorded a low number of *Striga* seeds stimulated to germinate compared to the corresponding treatments in Hoagland media ($F_{(5,12)} = 2.62$, P value 0.0798). This is possibly because of the overexpression of *La*PAP gene in the process of scavenging P from the highly P-deficient medium. The consistency in the results collected under root exudate assay could possibly be due to uniformity in the media used, standardized root exudate concentration applied and highly regulated culture conditions.

The findings of these three experiments demonstrates that the complex spatial host-parasite interaction observed *in vivo* during early growth of the parasite is mediated by the intensity of the levels of the germination stimulants produced by host plants (Yoneyama et al., 2013).

This study has therefore demonstrated that genetic modification of maize using efficient *PAP*s resulted in reduction in the amounts of SLs produced by maize roots.

It is therefore possible that transgenic maize produced low levels of *S. hermonthica* germination stimulant and that this is the probable mechanism by which the transgenic maize lines gained resistance against *S. hermonthica* attack (Karaya et al., 2012). The inherent genetic variation of host plants with regard to *S. hermonthica* resistance exists and plays a role in conferring pre-attachment resistance which is further enhanced by genetic transformation using *PAP* genes. This outcome resonates with findings of Catarina et al., (2014) which established that there is extensive structural variation in strigolactones (SLs) and plants usually produce blends of different SLs. The structural variation among natural SLs has been shown to impact their biological activity as hyphal branching and parasitic plant seed germination stimulants.

5.6 Conclusion

Transformation of KSTP'94 with *La*PAP gene recorded the highest transformation efficiency (0.69%) while Namba nane transformed with *La*PAP recorded the least transformation efficiency (0.33%). The overexpression of PAP genes in the three maize varieties reduced colonization of maize by *S. hermonthica* possibly due to enhanced P mobilization that further resulted into production of low levels of strigolactones by transgenic maize compared to their wild type counterparts.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

6.1 General discussion

This study highlights two paradigms of parasitic weed management. The first approach involved exploration of rhizosphere microbes with ability to cause *Striga hermonthica* seed decay while the second approach involved the use of genetic engineering to down-regulate production of strigolactones.

In the last decade, there has been extensive exploration studies on novel biological control agents for management of *S. hermonthica* weed (Avedi et al., 2014; Nzioki et al., 2016). The reported success of these biocontrol agents against *S. hermonthica* is largely dependent on mutual recognition, signaling, and the expression of pathogenicity and virulence factors of the microbe used (Compant et al., 2012). To date, there exists no single effective and sustainable *S. hermonthica* control option that is widely adopted by small-scale farmers in Kenya (Avedi et al., 2014).

This study clearly describes reproducible sampling design, isolation and screening procedures for novel *S. hermonthica* biocontrol agents from soil and plant tissues. This study quantified extracellular enzymes and antibiotic compounds produced by selected microbes and also correlated their production to *S. hermonthica* seed decay. This control option has potential of reducing imperishable rhizosphere *S. hermonthica* seed bank which the available commercialized biocontrol agents lack capacity to extinguish. This is because most of the available biocontrol agents exclusively cause destruction of preformed *S. hermonthica* plant tissues with no reported incidences of seed decay possibilities.

This study also underscores the indirect role played by biofertilizer microbial communities (particularly P and N rhizosphere mobilizers) in the management of *S. hermonthica* scourge. Previous studies had shown that low rhizosphere phosphorus (P) content not only affects soil productivity/fertility but also impairs proper plant growth and exacerbates *S. hermonthica* infection (Jamil et al., 2012). This therefore implies that isolates such as KY041696 and KY041695, identified in the present

study, have ability to increase rhizosphere P content that ultimately ameliorates *S. hermonthica* suppression rates in infested farms.

Development of reliable and robust *in vitro* regeneration protocols for CML144, KSTP'94 and Namba nane maize varieties from callus cells capable of being genetically transformed at high frequency was critical for successful genetic improvement of the selected maize varieties (Oneto et al., 2010; Sairam et al., 2008). This study established that N6 media fortified with 1.5mg/l of 2,4-D, 0.35g/l L-proline and 0.8mg/l $AgNO_3$ was the suitable callus induction media while unfortified N6 medium was the suitable regeneration medium. This study also established that the transformability potential (transformation efficiency) of the three varieties using modified Frame et al. (2002) protocol ranged from 0.8 to 2.1%. This transformation efficiency empirical score forms a reliable statistical estimate of transforming the three maize varieties with novel plant genes.

This study also explored genetic transformation of the same maize varieties using phosphorus efficient gene (*PAP*) with the objective of down-regulating production of host plant exudates that act as *S. hermonthica* germination stimulant, which in turn reduces their infestation rates. The overexpression of *PAP* genes in the three maize varieties reduced the production of volatiles chemical cues (strigolactone) by maize that induces *S. hermonthica* seed germination. This effect was demonstrated by the reduced number of *S. hermonthica* plants parasitizing transgenic maize plants. It is therefore evident that the low production of *S. hermonthica* germination stimulant by *PAP*-transformed maize lines is the probable mechanism by which the transgenic maize lines gained resistance against *S. hermonthica* attack (Karaya et al., 2012). It is the projection of this study that adoption of these transgenic maize varieties by small-scale farmers is expected to remedy the negative impact of *S. hermonthica.*

Integration of biological approach and genetic engineering strategy in the management of *S. hermonthica* combines the positive synergistic responses of both approaches with prospects of better results than the use of any single weed management scheme.

6.2 Conclusion

In Africa, particularly Kenya, dependency on monocotyledonous crops especially maize as the main source of carbohydrate exposes the consumers to frequent hunger outbreaks due to production challenges. In the current reality of climate change, untenable crop pest/diseases management strategies and the shrinking arable land (due to settlement and loss of soil fertility), modern farming require use of adaptable crop varieties and versatile pest/disease management options that are environmental friendly and cost effective to resource-poor farmers in Africa. This study aimed at evaluating two biotechnological frontiers (bioprospection for bioherbicide and genetic modification of preferred maize varieties) with view of addressing two maize productions constrains namely *S. hermonthica* infestation menace and low soil phosphorous content in arable farms due to residual precipitation process. With understanding that deficiency in rhizosphere phosphorous not only affects crop production but also escalates *S. hermonthica* infestation in farms under monocotyledonous crop farming, integrated *S. hermonthica* management techniques and phosphorous provision options that addressed both short term (cost effectiveness) and long term (sustainability) production targets is inevitable.

Findings of this study revealed that there exist economically important microbes in *S. hermonthica*-resistant soils that can be exploited as Biocontrol agents against *S. hermonthica.* There exist two operational modules in the biological control of *S. hermonthica*. The first module consists of microbes with ability to decay *S. hermonthica* seeds thereby gradually reducing *S. hermonthica* seed bank in the soil. The second module comprises microbes that are involved in phosphorus mobilization and/or nitrogen fixation which indirectly down-regulate production of strigolactones by host plants which subsequently scales down *S. hermonthica* germination rates. These two operational modules clearly demonstrate that *S. hermonthica* menace can be managed using biological agents and hence the stated null hypothesis for objective one of this research work is therefore rejected.

The two local maize OPVs (KSTP'94 and Namba nane) used in this study had never been evaluated for their potential to regenerate *in vitro* and also their potential to be genetically transformed. Results of this study show that these two maize varieties can

be regenerated *in vitro* with a significant level of success. It is also evident that these two maize varieties have reliable empirical chance of being transformed with novel genes using *Agrobacterium*-mediated transformation technique. Numerical estimates and validation of the *in vitro* regeneration and transformation protocols are sufficient to reject the stated null hypothesis for objective two of this study.

The ultimate transformation of these two maize varieties with PAP gene conferred a significant level of *S. hermonthica*-resistance trait to putative transgenic maize events. Results on effects of root exudates of transgenic maize grown on phosphorous deficient media clearly show that considerable expression of PAP hence a reduction in the number of *S. hermonthica* seeds stimulated to germinate by exudates of transgenic maize compared to their wild type relatives. It is therefore correct to reject the null hypothesis of objective three of this study.

6.3 Recommendation/Further studies

Findings of this study have revealed that there is a reliable chance that microbes particularly those with ability to decay *S. hermonthica* seeds can be used to control *S. hermonthica* menace. This study also established that PAP gene cloned from *Medicago truncatula* and *Lupinus albus* can be used to transform maize to enhance their *Striga* resistance trait. It is therefore recommended that:

1. The assayed microbes be evaluated for their ability to reduce *S. hermonthica* seed bank *in vivo*.
2. The regeneration and transformation protocols described in this study be adopted and used in improving the agronomic potential of these maize varieties as well as other varieties in the region
3. Finally, further studies should be undertaken to determine the effects of overexpressing this *PAPs* on non-target organisms/plants in the environment.

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APPENDICE

Appendix 1: GPS coordinates of sampled sites

Sample site no.	Coordinates of sample site	Height a.s.l (m)
1	N00.44856° : E034.25100°	1189
2	N00.50301° : E034.13894°	1141
3	N00.39950° : E034.14284°	1239
4	N00.34398° : E034.18515°	1244
5	N00.23227° : E034.24291°	1224
6	N00.20099° : E034.37874°	1309
7	N00.21051° : E034.41785°	1311
8	N00.31069° : E034.50345°	1331
9	S00.03680° : E034.81570°	1197
10	S00.27597° : E034.94675°	1145
11	S00.28261° : E034.95967°	1198
12	S00.07300° : E034.67724°	1170
13	S00.04131° : E034.64549°	1363
14	N00.03213° : E034.57728°	1497
15	N00.04408° : E034.56271°	1528
16	N00.11162° : E034.57589°	1449

Appendix 2: Morphological description of fungal isolates

Isolate code (Antibiosis level)	Location code	Top colour	Botton colour	Margin	Elevation	Form
ISSS01f	BUT101	Dark green	Green	Entire	Umbonate	Circular
ISS01d	BUT501	Green	Green	Undulate	Umbonate	Circular
11HSP	BUS102	White center, Green periphery	Redcenter, green periphery	Undulate	Umbonate	Irregular
ISS011d	BUS201	White with green ring	White	Filamentous	Umbonate	Irregular
S13b	BUS203	White center,green periphery	Red/green	Undulate	Raised	Irregular
$4F^2$	NYA301	White	White center,	Filiform	Flat	Filamentous

			black margin			
S17	NYA302	White center,dark green periphery	Green	Curled	Flat	Irregular
S17[b]	KIC101	Yellow center,white margin	Yellow	Undulate	Umbonate	Circular
8HSP	KIC301	White	White	Undulate	Umbonate	Circular
12ISS	KIC302	Yellow whitish	White	Entire	Umbonate	Circular
	SIA101	White	White	Entire	Raised	Circular
10F	SIA102	Orange	Brown	Entire	Umbonate	Circular
	SIA103	White	White	Filiform	Flat	Circular
S7[2]	SIA301	Black center,white margin	white	Entire	Convex	Circular
	SIA302	White	White	Undulate	Raised	Circular
S5[2]	SIA303	Brown center,white margin	Brown	Entire	Raised	Circular
	SIA304	Gray	Brown	Entire	Raised	Circular
12HSP	SIA305	White	Dark brown	Undulate	Convex	Irregular
9ISP	SIA306	Dark gray	Dark gray	Entire	Crateriform	Circular
S13C	NYA101	Red center,white margin	White	Entire	Umbonate	Circular
S14	NYA201	Red center,white margin	White	Entire	Umbonate	Circular
S16	NYA203	Dark red center,white margin	White	Entire	Umbonate	Circular

Appendix 3: McFarland turbidity standard

McFarland turbidity standards was prepared by mixing various volumes of 1% sulfuric acid and 1% barium chloride to obtain solutions with specific optical densities. 0.5 McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension $1.5x10^8$ colony forming units (CFU/ml). For performing antimicrobial susceptibility testing using Kirby Bauer disc diffusion

method, a cell suspension of organisms equivalent to a 0.5 McFarland standard was used.

Procedure of preparing 0.5McFarland turbidity standard

1. Prepared a 1% solution of anhydrous barium chloride ($BaCl_2$).
2. Prepared a 1% solution of sulfuric acid (H_2SO_4)
3. Combined and completely mixed the barium chloride and sulfuric acid solutions to form a turbid suspension and BaSO4 in a specific proportion for each McFarland turbidity standard as shown in **Table below.**
4. Placed the resulting mixture in a foil-covered screw –cap tube.
5. Stored the McFarland standard at room temperature (25 °C) when not in use.

Note: McFarland standard density solution will precipitate and clump over time, and it needs vigorous vortexing before each use. Mark the tube to indicate the level of liquid, and check before use to be sure that evaporation has not occurred.

6. Prepare a fresh standard solution every 6 months.

Table 5: McFarland turbidity standards

McFarland turbidity standard no.	0.5	1	2	3	4
1% barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1% sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1×10^8 CFU/ml)	1.5	3	6	9	12

Matching with turbidity standards

Density of the suspension of bacterial cells is compared to the McFarland turbidity standard *(0.5 McFarland turbidity standard for antimicrobial susceptibility testing purpose)* by holding the suspension and McFarland turbidity standard in front of a light against a white background with contrasting black lines.

If the density is too heavy, the suspension should be diluted with saline or broth (whichever was used to make the suspension). If the density is not sufficient, additional bacteria should be added to the suspension. The adjusted suspensions should be used as inocula within 15 min.

Appendix 4: ANOVA analysis summaries of bioprospection objective

Bacterial isolates

Table 4 (a): Dependent Variable: BUT101

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	145.9200000	20.8457143	153.00	<.0001
Error	16	2.1800000	0.1362500		
Corrected Total	23	148.1000000			

Table 4 (b): Dependent variable: BUT101

R-Square	Coefficient of variation	Root MSE	BUT101 Mean
0.985280	16.04872	0.369121	2.300000

Table 4 (c): Dependent variable: BUT 101

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	145.9200000	20.8457143	153.00	<.0001

Table 4 (d): Dependent Variable: BUT501

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	92.3062500	13.1866071	8.40	0.0002
Error	16	25.1200000	1.5700000		
Corrected Total	23	117.4262500			

Table 4 (e): Dependent Variable: BUT501

R-Square	Coefficient of variation	Root MSE	BUT101 Mean
0.786078	16.45972	1.252996	7.612500

Table 4 (f): Dependent Variable: BUT501

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	92.30625000	13.18660714	8.40	0.0002

Table 4 (g): Dependent Variable: BUS201

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	176.2329167	25.1761310	901.83	<.0001
Error	16	0.4466667	0.0279167		
Corrected Total	23	176.6795833			

Table 4 (h): Dependent Variable: BUS201

R-Square	Coefficient of variation	Root MSE	BUS201 Mean
0.997472	8.166981	0.167083	2.045833

Table 4 (i): Dependent Variable: BUS201

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	176.2329167	25.1761310	901.83	<.0001

Table 4 (j): Dependent Variable: SIA102

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	13.80666667	1.97238095	86.07	<.0001
Error	16	0.36666667	0.02291667		
Corrected Total	23	14.17333333			

Table 4 (k): Dependent Variable: SIA102

R-Square	Coefficient of variation	Root MSE	SIA102 Mean
0.974130	2.172955	0.151383	6.966667

Table 4 (l): Dependent Variable: SIA102

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	13.80666667	1.97238095	86.07	<.0001

Table 4 (m): Dependent Variable: BUS202

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	104.1862500	14.8837500	11907.0	<.0001
Error	16	0.0200000	0.0012500		
Corrected Total	23	104.2062500			

Table 4(n): Dependent Variable: BUS202

R-Square	Coefficient of variation	Root MSE	BUS202 Mean
0.999808	4.489567	0.035355	0.787500

Table 4 (0): Dependent Variable: BUS202

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	104.1862500	14.8837500	11907.0	<.0001

Table 4 (p): Dependent Variable: BUS203

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	139.8862500	19.9837500	15987.0	<.0001
Error	16	0.0200000	0.0012500		
Corrected Total	23	139.9062500			

Table 4 (q): Dependent Variable: BUS203

R-Square	Coefficient of variation	Root MSE	BUS203 Mean
0.999857	3.874558	0.035355	0.912500

Table 4 (r): Dependent Variable: BUS203

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	139.8862500	19.9837500	15987.0	<.0001
Error	16	0.0200000	0.0012500		
Corrected Total	23	139.9062500			

Fungal isolates

Table 4 (a): Dependent Variable: BUT101

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	62.90625000	8.98660714	513.52	<.0001
Error	16	0.28000000	0.01750000		
Corrected Total	23	63.18625000			

Table 4 (b): Dependent Variable: BUT101

R-Square	Coefficient of variation	Root MSE	BUT101 Mean
0.995569	2.748833	0.132288	4.812500

Table 4 (c): Dependent Variable: BUT101

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	62.90625000	8.98660714	513.52	<.0001

Table 4 (d): Dependent Variable: BUT501

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	156.2929167	22.3275595	588.86	<.0001
Error	16	0.6066667	0.0379167		
Corrected Total	23	156.8995833			

Table 4 (e): Dependent Variable: BUT501

R-Square	Coefficient of variation	Root MSE	BUT501 Mean
0.996133	2.687365	0.194722	7.245833

Table 4 (f): Dependent Variable: BUT501

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	156.2929167	22.3275595	588.86	<.0001

Table 4 (g): Dependent Variable: BUS201

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	87.44625000	12.49232143	587.87	
Error	16	0.34000000	0.02125000		
Corrected Total	23	87.78625000			

Table 4 (h): Dependent Variable: BUS201

R-Square	Coefficient of variation	Root MSE	BUS201 Mean
0.996127	2.475988	0.145774	5.887500

Table 4(i): Dependent Variable: BUS201

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	87.44625000	12.49232143	587.87	<.0001

Table 4 (j): Dependent Variable: SIA102

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	203.2800000	29.0400000	540.28	<.0001
Error	16	0.8600000	0.0537500		
Corrected Total	23	204.1400000			

Table 4 (k): Dependent Variable: SIA102

R-Square	Coefficient of variation	Root MSE	SIA102 Mean
0.995787	4.067377	0.231840	5.700000

Table 4 (m): Dependent Variable: SIA102

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	203.2800000	29.0400000	540.28	<.0001

Table 4 (n): Dependent Variable: BUS202

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	26.48625000	3.78375000	36.04	<.0001
Error	16	1.68000000	0.10500000		
Corrected Total	23	28.16625000			

Table 4 (o): Dependent Variable: BUS202

R-Square	Coefficient of variation	Root MSE	BUS202 Mean
0.940354	6.369278	0.324037	5.087500

Table 4 (p): Dependent Variable: BUS202

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	26.48625000	3.78375000	36.04	<.0001

Table 4 (q): Dependent Variable: BUS203

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	16.51291667	2.35898810	41.63	<.0001
Error	16	0.90666667	0.05666667		
Corrected Total	23	17.41958333			

Table 4 (r): Dependent Variable: BUS203

R-Square	Coefficient of variation	Root MSE	BUS203 Mean
0.947951	3.731641	0.238048	6.379167

Table 4 (s): Dependent Variable: BUS203

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	16.51291667	2.35898810	41.63	<.0001

Extra cellular enzyme analysis

Bacterial isolates

Table 4-i (a): Dependent Variable: Pectinase

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	10.50000000	1.50000000	240.00	<.0001
Error	16	0.10000000	0.00625000		
Corrected Total	23	10.60000000			

Table 4-i (b): Dependent Variable: Pectinase

R-Square	Coefficient of variation	Root MSE	Pectinase Mean
0.990566	9.300817	0.079057	0.850000

Table 4-i (c): Dependent Variable: Pectinase

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	10.50000000	1.50000000	240.00	<.0001

Table 4-i (d): Dependent Variable: Xylanase

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	12.82500000	1.83214286	133.25	<.0001
Error	16	0.22000000	0.01375000		
Corrected Total	23	13.04500000			

Table 4-i (e): Dependent Variable: Xylanase

R-Square	Coefficient of variation	Root MSE	Xylanase Mean
0.983135	8.228800	0.117260	1.425000

Table 4 -i(f): Dependent Variable: Xylanase

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	12.82500000	1.83214286	133.25	<.0001

Table 4-i (g): Dependent Variable: Cellulase

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	3.96291667	0.56613095	33.97	<.0001
Error	16	0.26666667	0.01666667		
Corrected Total	23	4.22958333			

Table 4-i (h): Dependent Variable: Cellulase

R-Square	Coefficient of variation	Root MSE	Cellulase Mean
0.936952	16.74804	0.129099	0.770833

Table 4-i (i): Dependent Variable: Cellulase

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	3.96291667	0.56613095	33.97	<.0001

Table 4-i (j): Dependent Variable: Protease

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	30.98625000	4.42660714	221.33	<.0001
Error	16	0.32000000	0.02000000		
Corrected Total	23	31.30625000			

Table 4-i (k): Dependent Variable: Protease

R-Square	Coefficient of variation	Root MSE	Protease Mean
0.989778	13.00426	0.141421	1.087500

Table 4-i (l): Dependent Variable: Protease

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	30.98625000	4.42660714	221.33	<.0001

Table 4-i (m): Dependent Variable: Amylase

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F

Model	7	13.49625000	1.92803571	257.07	<.0001
Error	16	0.12000000	0.00750000		
Corrected Total	23	13.61625000			

Table 4-i (n): Dependent Variable: Amylase

R-Square	Coefficient of variation	Root MSE	Amylase Mean
0.991187	8.347233	0.086603	1.037500

Table 4-i (m): Dependent Variable: Amylase

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	13.49625000	1.92803571	257.07	<.0001

Fungal isolates

Table 4-i (a): Dependent Variable: Pectinase

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	2.15625000	0.30803571	22.40	<.0001
Error	16	0.22000000	0.01375000		
Corrected Total	23	2.37625000			

Table 4-i (b): Dependent Variable: Pectinase

R-Square	Coefficient of variation	Root MSE	Pectinase Mean
0.907417	5.240688	0.117260	2.237500

Table 4-i (c): Dependent Variable: Pectinase

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	2.15625000	0.30803571	22.40	<.0001

Table 4-i (d): Dependent Variable: Xylanase

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1.79625000	0.25660714	25.66	<.0001
Error	16	0.16000000	0.01000000		
Corrected Total	23	1.95625000			

Table 4-i (e): Dependent Variable: Xylanase

R-Square	Coefficient of variation	Root MSE	Xylanase Mean
0.918211	4.060914	0.100000	2.462500

Table 4-i (f): Dependent Variable: Xylanase

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	1.79625000	0.25660714	25.66	<.0001

Table 4-i (j): Dependent Variable: Cellulase

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	176.5066667	25.2152381	976.07	<.0001
Error	16	0.4133333	0.0258333		

Corrected Total	23	176.9200000			

Table 4-i (k): Dependent Variable: Cellulase

R-Square	Coefficient of variation	Root MSE	Cellulase Mean
0.997664	3.826846	0.160728	4.200000

Table 4-i (l): Dependent Variable: Cellulase

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Isolate_	7	176.5066667	25.2152381	976.07	<.0001

Table 4-i (m): Dependent Variable: Protease

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.22500000	0.03214286	2.34	0.0756
Error	16	0.22000000	0.01375000		
Corrected Total	23	0.44500000			

Table 4-i (n): Dependent Variable: Protease

R-Square	Coefficient of variation	Root MSE	Protease Mean
0.505618	4.467063	0.117260	2.625000

Table 4-i (o): Dependent Variable: Protease

Source	DF	Anova SS	Mean Square	F Value	Pr > F
isolate_	7	0.22500000	0.03214286	2.34	0.0756

Table 4-i (p): Dependent Variable: Amylase

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	8.38500000	1.19785714	68.45	<.0001
Error	16	0.28000000	0.01750000		
Corrected Total	23	8.66500000			

Table 4-i (q): Dependent Variable: Amylase

R-Square	Coefficient of variation	Root MSE	Amylase Mean
0.967686	3.806836	0.132288	3.475000

Table 4-i (r): Dependent Variable: Amylase

Source	DF	Anova SS	Mean Square	F Value	Pr > F
isolate_	7	8.38500000	1.19785714	68.45	<.0001

Appendix 5: ANOVA analysis summaries for in vitro regeneration objective

Table 5 (a): Primary callus (column analysis-MS medium)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	17	8605.43210	614.67372	18.69	<.0001
Residual	75	2466.66667	32.88889		
Total	89	11072.09877			

Table 6.1: **Primary callus** (column analysis-LS medium)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	17	15198.02469	1085.57319	54.96	<.0001
Residual	75	1481.48148	19.75309		
Total	89	16679.50617			

Table 6.2: **Primary callus** (column analysis-N6 medium)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	17	11203.20988	800.22928	15.51	<.0001
Residual	75	3868.51852	51.58025		
Total	89	15071.72840			

Table 6.3: **Primary callus** (column analysis-LS medium)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	17	13731.08148	980.79153	16.92	<.0001
Residual	75	4346.29630	57.95062		
Total	89	18077.37778			

Table 5 (a): Primary callus (Row analysis 0.5mg/lCML144)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	3	109.2592593	36.4197531	1.73	0.1940

Residual	20	2466.66667	32.88889		
Total	23	11072.09877			

Table 6.4: **Primary callus** (Row analysis 1.0mg/lCML144)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	3	835.185185	278.395062	4.98	0.0097
Residual	20	1118.518519	55.925926		
Total	23	1953.703704			

Table 6.5: **Primary callus** (Row analysis 1.5mg/lCML144)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	3	11203.20988	800.22928	15.51	0.5214
Residual	20	3868.51852	51.58025		
Total	23	15071.72840			

Table 6.6: **Primary callus** (Row analysis 2.0mg/lCML144)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	3	549.537037	183.179012	3.36	0.0393
Residual	20	1090.740741	54.537037		
Total	23	1640.277778			

Table 6.7: **Primary callus** (Row analysis 2.5mg/lCML144)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	3	560.648148	186.882716	4.42	0.0154
Residual	20	846.296296	42.314815		
Total	23	1406.944444			

Table 5(b): Variate: efficiency of embryogenic_calli induction

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	2	1230.28	615.14	7.93	<.001
Residual	69	5351.42	77.56		
Total	71	6581.69			

Table 5(c): Variate: shoot induction efficiency

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	2	1322.12	661.06	7.74	<. 001
Residual	69	5891.65	85.39		
Total	71	7213.77			

Table 5(d): Variate: %_of_primary_callus

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Induction_media	3	3811.54	1270.51	47.11	<.001
Residual	68	1833.82	26.97		
Total	71	5645.36			

Table 5(e): Variate: efficiency_of_embryogenic calli induction

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype*induction media*embryo genic media	23	5312.81	230.99	8.74	<.001
Residual	48	1268.89	26.44		
Total	71	6581.69			

Table 5(f): Variate: shoot induction efficiency

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype*induction media*embryogenic media	23	6087.29	264.66	11.28	<. 001
Residual	48	1126.48	23.47		
Total	71	7213.77			

Table 5(g): Media used in Gus transformation of maize

Media	Composition
YEP	10gl^{-1} peptone, 5gl^{-1} NaCl, 5gl^{-1} yeast extract, 1.5% (w/v) agar pH 6.8
Inf	N6* and LS salts and vitamins, 1.5 mgl^{-1} 2,4-D, 0.7 g l^{-1}L-proline, 68.4 gl^{-1} sucrose, 36 gl^{-1}glucose, pH 5.2. Add 100µM acetosyringone[ab] before using.
CM	Inf without glucose reduced sucrose to 30gl^{-1} and supplemented with 0.5gl^{-1} MES buffer, 3gl^{-1} gelrite, pH 5.8 and autoclaved. Added filter sterilized 0.85mgl^{-1} silver nitrate, 300 mgl^{-1} Cysteine and100µM acetosyringone after autoclaving
REM	Resting medium: N6* and LS salts and vitamins, 1.5 mgl^{-1} 2,4-D, 0.7 g l^{-1}L-proline, 30 gl^{-1} sucrose with 0.5gl^{-1} MES buffer, 8gl^{-1} phytoagar, pH 5.8. Added filter sterilized N6 vitamins, 0.85mgl^{-1} silver nitrate and 100 mgl^{-1} Cefotaxime after autoclaving
SM	SM I: REM supplemented with 250mgl^{-1} carbenicillin[ac], 1.5 mgl^{-1} L-phosphinothricin[a] (Bialaphos), pH 5.8

	SM I: same as SM I except for Bialaphos which is increased to 3mgl^{-1}

[a]Filter-sterilized. [b]Sigma-Aldrich products. [c]Cabenicillin disodium salt, Sigma products [d]Cefotaxim sodium salt, Sigma products

Appendix 6: PAP gene constructs for maize transformation

Modification of pTF101.1 for maize transformation

The binary vector pTF 101.1 (Figure 27) was modified by introducing the ubiquitin promoter, a rice intron, a multicloning site and a NOS terminator fragment from the binary vector NC 1100 in to the pTF101.1 multicloning site. The end plasmid was renamed PAJ101.1 (Figure 28).

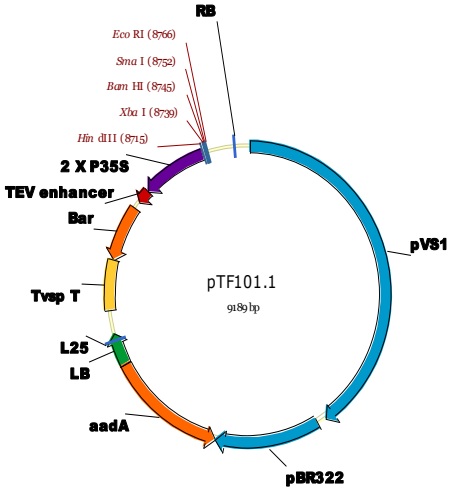

Figure 6.3(a): The binary vector pTF101.1

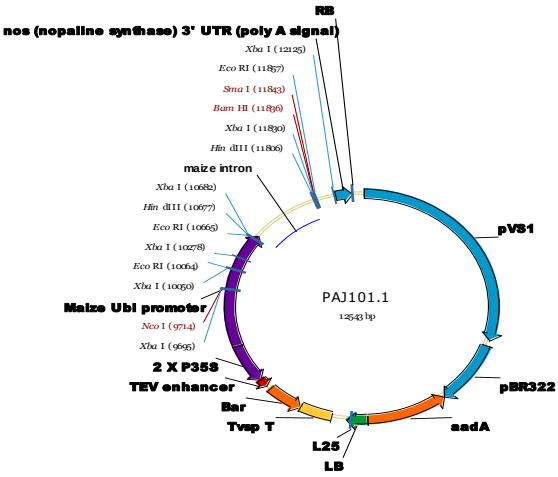

Figure 6:1(b): The binary vector pAJ101.1

MtPAP construct for maize transformation

MtPAP gene was ligated into pAJ101.1 (Fig.5-1b) using restriction digestions and ligations methods. The MtPAP gene was cloned from PCR 8 plasmid using PCR Primers MtPAP-F: 5' CTTACTAGTTGAGTTTTGAAGAAAAATGGG 3' and MtPAP-R: 5' GGATCCATGAGTTGTGGAATCATCAACT 3'. The unique restriction sites SpeI (ACTAGT) and BamHI site (GGATCC) of pAJ101.1 were used hence the forward primer had the Spe1 site while the reverse primer had the BamH1 site. Polymerase chain reaction was performed first with PWo enzyme which is a proofreading DNA polymerase that leaves the PCR product with blunt ends. Addition of 3'A overhangs was performed by incubating the blunt PCR product at 65^{o}C with Tag polymerase for 30 min. TA cloning into TOPO TA 2.1 proceeded as per the kit instructions (Invitrogen San Diego, USA). Before TA cloning into TOPO

TA 2.1, the pCR2.1 reaction was transformed into electro competent TOPO 10 high efficiency cells from Invitrogen, San Diego USA and plated on Kanamycin (50 mg/l) treated plates. Four kanamycin resistant colonies were selected and cultured in antibiotic treated LB medium before running minipreps. EcoRI digestion was performed to confirm insert presence while insert orientation was done using PvuII and XbaI restriction enzymes. A 1.3 Kb band size confirmed right orientation. Two separate restriction digestions on modified pAJ101.1 and pCR21.1 + MtPAP were done with BamHI and SpeI. The two fragments (MtPAP and linearised pAJ101.1) were ligated and later transformed into DH5α *E.coli* cells and spectinomycin treated LB plates. The same enzymes (BamHI and SpeI) were used to screen for insert presence in the vector on selected colonies. Minipreps were done on selected colonies and later electroporated into *Agrobacterium tumefasciens* EHA105 and selection done on LB spec + Kan plates. Restriction digestions (BamHI and SpeI) were done on selected *Agrobacterium tumefasciens* EHA105 colonies to check and confirm presence of the MtPAP gene.

Appendix 7: ANOVA analysis summaries for maize transformation with PAP gene

Table 7.1: Transformation efficiency of gene specific maize varieties

Source of variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
Variety	2	1140.31	570.16	11.32	0.002
Gene	1	11.30	11.30	0.22	0.644
Variety.Gene	2	75.48	37.74	0.75	0.494
Residual	12	604.67	50.39		
Corrected Total	17	1831.75			

Table 7.2: Number of germinating *S. hermonthica* seeds after root exudates exposure

Source of variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	19	2250.983333	118.472807	215.41	<.0001
Error	40	22.000000	0.550000		

Corrected Total	59	2272.983333			

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Stimulant type	19	2250.983333	118.472807	215.41	<.0001

Table 7.3: Effect of variety and Gene construct on *Striga* parasitization of bucket-grown maize

Source of variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	45.11111111	11.27777778	9.97	<.0001
Error	22	24.88888889	1.13131313		
Corrected Total	26	70.00000000			

Source of variation	DF	Sum of Squares	Mean Square	F Value	Pr > F
Variety	2	45.11111111	11.27777778	9.97	<.0001
PAP type	2	24.88888889	1.13131313		

Variety comprise of CML144, KSTP'94 and Namba Nane while PAP type comprise of wild type, LaPAP and MtPAP.