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INYUVESI YAKWAZULU-NATALI

FIELD EVALUATION AND CHARACTERISATION OF THE MODE OF IMAZAPYR TOLERANCE IN THREE MUTANT SUGARCANE GENOTYPES

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By

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Abstract

Cynodon dactylon, an invasive creeping grass/weed, competes with sugarcane for sunlight, water and nutrients, thereby leading to a reduced yield of this crop. This weed can be controlled in South Africa by the herbicide Arsenal® GEN 2 (imazapyr) in conjunction with sugarcane genotypes (Mut 1, Mut 6 and Mut 7) chemically mutated using ethyl methanesulfonate (EMS) to confer tolerance to imazapyr. In this study, the agronomic characteristics of the Mut 1, Mut 6 and Mut 7 genotypes were compared with their unmutated N12 counterpart, the response of the mutants to Arsenal® GEN 2 was compared with hand weeding and a commonly used commercial herbicide cocktail treatment, and the mode of tolerance to imazapyr of the mutants was investigated.

Agronomic characterisation indicated that although the chemical mutagenesis had no effect on the number of stalks.plot^{-1}, stalk height, fibre and sucrose content of the mutant genotypes, the stalk diameter of the unmutated N12 genotype was significantly higher than Mut 1, Mut 6 and Mut 7 (23.31 vs. 19.38, 20.42 and 20.20 cm, respectively, $P \leq 0.001$). A significantly higher biomass yield in the unmutated N12 genotype was observed, compared with the Mut 1 genotype (189.73 vs. 120.52 kg.plot^{-1}, $P = 0.002$). The recoverable value sucrose content (RV per 100 g fresh mass) indicated that Mut 1 was significantly higher ($P \leq 0.05$) than Mut 7 (6.23 % vs. 5.07 %, $P = 0.014$). An assessment of the damage caused by *Eldana saccharina* indicated that the Mut 1 genotype was significantly more susceptible to infestation by the pest compared with the unmutated N12 genotype (11.14 and 3.89 % internodes bored, respectively).

A field trial designed to confirm the level of tolerance to Arsenal® GEN 2 (single application of 1254 g a.i.ha^{-1} imazapyr) compared with hand weeding and an alternative herbicide cocktail treatment (two repeated applications of 200 g a.i.ha^{-1} paraquat and 800 g a.i.ha^{-1} diuron), revealed a significant decrease in the number of stalks.plot^{-1}, stalk height and leaf chlorophyll content (SPAD unit), 12 weeks after exposure to Arsenal® GEN 2 in the unmutated N12 genotypes compared with the mutants. No significant differences in the number of stalks.plot^{-1}, stalk height and leaf chlorophyll content were observed in the Mut 1, Mut 6 and Mut 7 genotypes after exposure to Arsenal® GEN 2.

Digital image analysis to assess the effectiveness of Arsenal® GEN 2 in controlling *C. dactylon* compared with an alternative commercially used herbicide cocktail treatment showed that although the percentage of green *C. dactylon* leaves decreased significantly ($P > 0.05$) after

exposure to the herbicides, the percentage of green *C. dactylon* leaves 20 weeks after exposure to a single application of Arsenal® GEN 2 was significantly higher than that of two applications of the herbicide cocktail treatment. This indicated that the management of *C. dactylon* by a single application of Arsenal® GEN 2 was not better than that of the herbicide cocktail in this study.

A comparison of a partial acetohydroxyacid synthase (AHAS) protein sequence of Mut 1 and Mut 6 revealed an amino acid mutation at position 195 which converts an arginine to a cysteine residue, which likely confers tolerance to imazapyr. A preliminary investigation for an alternative herbicide tolerance mechanism, that of an enhanced metabolic detoxification, using the cytochrome P450 (CYP) inhibitor piperonyl butoxide (PBO) showed no effect on plant height, leaf chlorophyll content, fresh and dry root mass (g) and fresh shoot mass (g) of Mut 1, Mut 6 and Mut 7 following treatment with 100 µM imazapyr. The results obtained indicated that the mutants did not possess an enhanced metabolic detoxification mechanism because the damage caused by the imazapyr and PBO treatment to the plants was not greater than that caused by the imazapyr treatment only.

Tolerance of Mut 1 and Mut 6 to imazapyr is likely due to the mutation within the AHAS gene. Future work is required in order to determine the mode of tolerance of the Mut 7 genotype to imazapyr. This involves identifying the possibility of an enhanced detoxification mechanism with the use of a higher PBO concentration or an alternative CYP inhibitor and completing the AHAS gene sequencing.

FACULTY OF SCIENCE AND AGRICULTURE
DECLARATION - PLAGIARISM

I, **Varnika Singh**, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed

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PREFACE

The experimental work described in this thesis was carried out in the Biotechnology Department of the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, Durban, South Africa from February 2013 to December 2015, under the supervision of Prof Paula Watt (UKZN), Dr Sandra Jane Snyman (SASRI and UKZN) and Dr Richard Stuart Rutherford (SASRI and UKZN).

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

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1. Introduction

Sugarcane (*Saccharum* L.) is a member of the grass family and is cultivated mainly for the juices that are obtained from its stalks that accumulate sucrose (Grivet and Arruda, 2001; Salassi *et al.*, 2002). It is an important cash crop that forms the majority of the raw sugar produced world-wide and is cultivated in many countries (Grivet and Arruda, 2001). In South Africa, half of the raw sugar produced is exported to other continents and the remainder is marketed locally (Anon, 2013).

Weed control is an important factor when considering the cultivation of sugarcane as poor management results in substantial yield losses (Odero and Dusky, 2010; Délye *et al*., 2013; Gaur and Sharma, 2013). The management of weeds is an ongoing effort aimed at reducing the damage caused to the crop and ensuring that an optimum yield is obtained (Odero and Dusky, 2010). *Cynodon dactylon*, also referred to as creeping or bermuda grass, is a persistent problem in the South African Sugar Industry and is of increasing concern in the Kwa-Zulu Natal region (Landrey *et al*., 1993; Leibbrandt, 1995; Campbell and Bruggemann, 2008; Campbell *et al*., 2008). Infestation of plantations by *C. dactylon* is managed with repeated applications of glyphosate, paraquat and diuron at high application rates (Landrey *et al*., 1993; Leibbrandt, 1995; Campbell, 2008; Campbell *et al*., 2008). The repetitive use of the above mentioned herbicides at high application rates poses a future threat by contributing to the evolution of this weed into a herbicide tolerant plant (Campbell, 2008; Conlong and Campbell, 2010)

Imazapyr, commercially sold as Arsenal® GEN 2 (240 $g.L^{-1}$ imazapyr, BASF®), belongs to the imidazolinone class of herbicides that targets the acetohydroxyacid synthase (AHAS) enzyme (Kishchenko *et al*., 2011; Lichtfouse, 2014; Yu and Powles, 2014a). The recommended rate for the use of Arsenal® GEN 2 within sugarcane fields is 5.2 $L.ha^{-1}$ (Anon, 2015). The disadvantage associated with this herbicide class is that it requires specific protocols to be implemented before sugarcane planting can resume on treated fields such as the fields need to remain bare for long periods of time (4 months) and 600 mm rain is necessary during the warmer months after the herbicide has been applied (Anon, 2013; Anon, 2015). These protocols need to be adhered to in order to prevent crop damage and yield loss which is common in plants that are not tolerant to imazapyr. Although control of *C. dactylon* in sugarcane fields with imazapyr is more efficient and cost effective than the commonly used herbicides such as glyphosate, paraquat or diuron, the disadvantages associated with its use are currently not practical for farmers. As a result the production and use of imazapyr tolerant

sugarcane genotypes will provide a new solution to this problem by eliminating the disadvantages associated with Arsenal® GEN 2 (Campbell, 2008).

Herbicide tolerance in plants can be conferred via various mechanisms which are classed into two groups: target site and non-target site tolerance (Sala *et al*., 2012). Target site tolerance is a result of mutations that occur at the herbicide target site within the plant. These mutations lead to enzymes or proteins that have a reduced sensitivity or increased activity which limits herbicide binding (Devine and Eberlein, 1997; Tranel and Wright, 2002; Gaur and Sharma, 2013; Yu and Powles, 2014b). Plants that are tolerant to AHAS inhibting herbicides have been identified based on mutations within their AHAS enzyme, *viz.* tobacco (Chaleff and Ray, 1984), *Arabidopsis thaliana* L. (Haughn and Somerville, 1986), canola (Swanson *et al*., 1989), soybean (Sabastian *et al*., 1989), maize (Newhouse *et al*., 1991; Wright and Penner, 1998b), cotton (Rajasekaran *et al*., 1996), rice (Croughan, 1998), wheat (Ponziak and Huci, 2004), sugar beet (Hart *et al*., 1992; Wright and Penner, 1998a; Kishchenko *et al*., 2011), sunflower (Sala and Bulos, 2012) and chickpea (Thompson and Tar'an, 2014).

Non-target site tolerance involves various mechanisms with the most common being translocation and/or sequestration of the herbicide and changes in the metabolic detoxification of the herbicide (Devine and Eberlein, 1997; Yuan *et al*., 2007; Shaner *et al*., 2012; Gaur and Sharma, 2013; Yu and Powles, 2014b). An enhanced herbicide detoxifcation in plants can occur naturally or it can be modified by mutagenesis and is usally a result of an altered cytochrome P450 (CYP) enzyme (Fonné-Pfister *et al*., 1990; Gressel, 1990; Kreuz and Fonné-Pfister, 1992; Christopher *et al*., 1994; Baerg *et al*., 1996; Letrouzé and Gasquez, 2001; Yu *et al*., 2004; Yun *et al*., 2005; Yasour *et al*., 2009; Yu *et al*., 2009; Breccia *et al*., 2012; Elmore *et al*., 2015). Pot trials have been carried out in order to identify plant species that have an enhanced herbicide metabolic detoxification and this was achieved by combining an AHAS inhibiting herbicide with a CYP inhibitor and include maize (Kwon *et al*., 1995; Kotoula-Syka and Hatzios, 1996), cotton (Minton *et al*., 2008), cereal weed (Yu *et al*., 2009), sunflower (Breccia *et al*., 2012) and various grass species (Kwon and Penner, 1995; Fisher *et al*., 2000; Elmore *et al*., 2015).

Herbicide tolerance in sugarcane has been produced using genetic engineering (Gallo-Meagher and Irvine, 1996; Falco *et al*., 2000; Leibbrandt and Snyman, 2003) and *in vitro* induced mutagenesis (chemical and physical) (Irvine *et al*., 1991; Ali *et al*., 2007; Kenganal *et al*., 2008; Koch *et al*., 2012). The use of genetically engineered sugarcane, as with any plant species, has

various disadvantages compared with those obtained via mutagenesis. Mutations within important genes or traits, which are produced via mutagenesis, can be exploited by plant breeders without the restrictions, licensing costs and societal opposition linked to that of genetically engineered plants (Parry *et al*., 2009).

In a previous study at the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, KwaZulu-Natal, South Africa, Koch *et al*. (2012) produced seven mutant genotypes of the N12 sugarcane genotype by chemical mutagenesis using ethyl methanesulfonate (EMS). The mutant genotypes were screened for herbicide tolerance by an *in vitro* enzyme assay that characterised the activity of the AHAS enzyme in the presence of imazapyr and the outcome of that study indicated that three mutant genotypes (Mut 1, Mut 6 and Mut 7) had a higher AHAS enzyme activity compared with the other mutants and the unmutated N12 genotype (Rutherford *et al*., *in press*). The tolerance of the mutants to imazapyr was tested in the field using three different concentrations of Arsenal® GEN 2 (imazapyr) (Rutherford *et al*., *in press*). Based on the findings of that study, Mut 1, Mut 6 and Mut 7 were selected for the present study which involved the objectives listed below.

1. **To assess and compare the agronomic characteristics of the previously produced Mut 1, Mut 6 and Mut 7 genotypes (Koch *et al*., 2012; Rutherford *et al*., *in press*) with the unmutated N12 control.**

An agronomic field assessment was conducted on the mutants in order to assess if the EMS chemical mutagenesis resulted in any changes to the phenotypic characteristics of the unmutated N12 control. Comparisons of number of stalks.plot^{-1}, stalk height (cm), stalk diameter (cm), estimated yield (kg.plot^{-1}), fibre content (g.100g^{-1} fresh weight), sucrose content (g.100g^{-1} fresh weight) and recoverable value sucrose content (RV per 100g fresh mass). An assessment was also conducted in order to evaluate if the chemical mutagenesis altered the tolerance of N12 genotype to the sugarcane borer, *Eldana saccharina*.

2. **To compare the efficacy of Arsenal® GEN 2 (imazapyr) with that of a commercial herbicide cocktail treatment used to manage *Cynodon dactylon* and to assess the response of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes to the management of this weed by Arsenal® GEN 2 compared with the herbicide cocktail and a hand weeded treatment.**

The plant damage caused by application of Arsenal® GEN 2 (1254 g a.i.ha^{-1} imazapyr) on the unmutated N12 and mutant genotypes was compared with a hand weeded treatment and a conventionally used commercial herbicide cocktail consisting of Gramoxone® (200 g a.i.ha^{-1} paraquat) and Diuron® (800 g a.i.ha^{-1} diuron). The efficacy of this herbicide for the eradication/management of *C. dactylon* was also assessed in order to evaluate if it provided an equivalent or better herbicide regime compared with the herbicide cocktail. The herbicides were applied post-emergence of the sugarcane and *C. dactylon* which were planted within the furrows. The experimental fields were treated with a single application of Arsenal® GEN 2 at 0 weeks and two applications of the herbicide cocktail at 0 and 4 weeks. The percentage of green *C. dactylon* leaves was measured at different times. The effect of exposing the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes to Arsenal® GEN 2 was evaluated by measuring the plant damage caused by the herbicide to the number of stalks.plot^{-1}, stalk height and leaf chlorophyll content of each genotype. The effect of Arsenal® GEN 2 on the genotypes was compared with two other weed management strategies, hand weeding and the conventionally used commercial herbicide cocktail.

3. **To characterise the mode of tolerance to imazapyr in Mut 1, Mut 6 and Mut 7.**

The Mut 1, Mut 6 and Mut 7 genotypes were characterised at a molecular level by sequencing the AHAS gene, translating the gene into a protein sequence and comparing the AHAS protein sequences of the mutants with that of the unmutated N12 genotype in order to identify possible amino acid mutations. In addition, a pot trial involving an analytical standard of imazapyr and a CYP inhibitor, piperonyl butoxide (PBO), was conducted in order to assess if the mutants possessed an enhanced metabolic herbicide detoxification mechanism.

2. Literature review

2.1 Sugarcane cultivation and its challenges

Sugarcane is an important cash crop that is cultivated in tropical and subtropical regions of the world mainly for its sugar which is used in the production of food, beverages and biofuels (Grivet and Arruda, 2001; Menossi *et al*., 2008; Kinkema *et al*., 2014; Barnabas *et al*., 2015). It forms the majority (78%) of the sugar produced worldwide with Brazil and India ranking first and second, respectively, for the highest production of sugarcane (Grivet and Aruda, 2001; Barnabas *et al*., 2015; Kaur *et al*., 2015). In recent years, many countries have focused on the cultivation of sugarcane for the production of ethanol with the intention to replace fossil fuels with a renewable biofuel (Menossi *et al*., 2008; de Siqueira Ferreira *et al*., 2013). In addition to sugar and biofuel production, sugarcane can be used as a component in various other valuable products such as animal feed, dietary fibre in breads, substitute for wood, paper production and the synthesis of carbon fibres (Han and Wu, 2004; Paiva *et al*., 2004; Sangnark and Noomhorm, 2004; Sun *et al*., 2004; Menossi *et al*., 2008; Kinkema *et al*., 2014; Barnabas *et al*., 2015).

Sugarcane belongs to the genus *Saccharum* L. of the Poaceae family as do *Sorghum* and *Zea* (Kellogg, 2001). The *Saccharum* genus consists of two wild type species, *S. spontaneum* L. and *S. robustum* Brandes and Jesw. Ex Grassl and four cultivated species, *S. officinarum* L., *S. barberi* Jesw., *S sinense* Roxb. and *S. edule* Hassk (Daniels and Roach, 1987). Commercially produced sugarcane cultivars are interspecific hybrids of *S. officinarum* and *S. spontaneum* (Grivet and Arruda, 2001) and the hybrid nature of sugarcane genome contributes to its polyploidy and aneuploidy which in turn make molecular characterisation of the *Saccharum* genome extremely difficult (Hotta *et al*., 2010; Pan, 2012). Advances in the understanding and sequencing the sugarcane genome have been made (Hotta *et al*., 2010; Pan, 2012) and although the sugarcane genome has only been partially sequenced, the most recent attempt made by de Setta *et al*. (2014) resulted in the generation of a comprehensive dataset that provides information on the sugarcane genome, thereby contributing to the development of various molecular tools which can be used in gene discovery and breeding programs.

Plant stress poses a serious threat to the sustainability of crop yields by contributing to high productivity losses (Gaur and Sharma, 2013). The cultivation of sugarcane is affected by various abiotic (drought cold stress and low phosphorous) and biotic (nematodes, insects, viral,

bacterial and fungal infections) stresses (Menossi *et al*., 2008). One of the most important challenges associated with the cultivation of this crop is the control of weeds. These are a serious biotic concern as they compete for water, sunlight and nutrients and as a result lead to major yield losses (Délye *et al*., 2013). Weeds respond differently to various habitats and also provide environments for insects and disease-causing pests (Bajwa, 2014) and, as a result, their management in the sugarcane field is vital in reducing the risk of damage to crop quality and ensuring production of optimum yield (Odero and Dusky, 2010).

2.2 Management of weeds such as *Cynodon dactylon* in sugarcane fields

Creeping grasses are regarded by the South African Sugar Industry as one of the most costly weeds to control as they drastically reduce sugarcane yields and, in more severe cases, can kill the crop (Conlong and Campbell, 2010). *Cynodon dactylon* (L.) Pers, *Cynodon nlemfuensis* and *Digitaria abyssinica* (A. Rich.) Stapf are creeping grass species that are indigenous to North and East Africa and are causing an increasing concern amongst farmers in the KwaZulu-Natal region (Landrey *et al*., 1993; Leibbrandt, 1995; Campbell and Bruggemann, 2008; Campbell *et al*., 2008). Their invasive nature may lead to them out-competing other weed species and they are therefore regarded as the most detrimental weeds on sugarcane farms (Leibbrandt, 1995; Halvorson and Guertin, 2003).

Cynodon dactylon (commonly known as kweek and bermuda grass) (Campbell, 2008; Conlong and Campbell, 2010), ranked second as the world's worst weed (Holm *et al*., 1977; Heap, 2016), is a monocotyledonous, perennial rhizomatous and stoloniferous grass. As the stolons of this grass elongate, the nodes make contact with the soil producing roots which then establish into fully developed plants (Leibbrandt, 1995, Halvorson and Guertin, 2003). These vegetative propagation characteristics contribute to its weedy nature and lead to a high field persistence making it difficult to control (Burton and Hanna, 1995; Ferrell *et al*., 2005).

As previously mentioned, *C. dactylon* is known as one of the main weeds that competes with sugarcane (both plant cane and ratoon crops) and is found in regions where slow canopy development occurs and burning of old leaf trash during harvesting is practiced (Campbell, 2008). *Cynodon dactylon* grows in most soil types including sandy loam soils and in both dry and irrigated farming lands (Abdullahi, 2002). Although creeping grasses usually thrive in humid and sunny conditions thereby allowing them to inhabit large regions of

cultivated land, the deep-set root system of *C. dactylon* also facilitates its growth and survival during periods of drought (Halvorson and Guertin, 2003).

Infestation by *C. dactylon* is increasing in areas containing sandy coastal soil (Campbell, 2008) and, although it is considered the most difficult grass weed to control, it is also a valuable pasture and turf grass (Abdullahi, 2002). It invades the field from surrounding areas such as roadsides and cane breaks and requires frequent and costly control measures (Conlong and Campbell, 2010). The spread of *C. dactylon* in cane growing regions is also increased by a loss of cane canopy in dry conditions, which allows for the survival of the shade susceptible species (Leibbrandt, 1995). An increase in *C. dactylon* biomass under cane canopy can reduce yields in both plant cane and ratoon crops by 5-21 tons ha^{-1} and 5-14 tons ha^{-1}, respectively (Richard and Dalley, 2005) and if not controlled effectively the yield loss can amount to ~66% in both plant cane and ratoon crops (Landrey *et al*., 1993). This weed has been so problematic for both large and small scale farmers that severe infestations have forced farmers to abandon their lands during the cropping season (Abdullahi *et al*., 2001). Due to the high losses incurred by the influx of *C. dactylon* within cultivated sugarcane fields, the establishment of effective control regimes need to be implemented to ensure complete eradication of the weed species.

Hand weeding was once a dominant method employed to manage and control *C. dactylon*, but technology advances have led to its substitution with a mechanical method of weed control (Smith *et al*., 2001). Although the mechanical method of weed control has been proven more efficient, the high cost incurred to maintain the equipment and the harmful effects caused by the equipment on cultivated lands such as soil erosion, high energy consumption and increased soil compaction makes it undesirable (Smith *et al*., 2001). Management practices currently implemented for eradicating *C. dactylon* in sugarcane fields include mechanical mowing or repeated herbicide use (Conlong and Campbell, 2010). Small-scale sugarcane farmers generally rely on hand weeding, inter-row tillage and the use of herbicides as a weed management strategy however, the latter is expensive and small scale farmers lack the skills and equipment required to ensure the proper application of herbicides (Anon, 1994; Kaur *et al*., 2015).

The term herbicide refers to a heterogeneous group of chemicals with properties that aid in the eradication of vegetation or selectively kill weeds without seriously injuring cultivated crops (Gaur and Sharma, 2013). Herbicides can be classified into two groups, pre- or post-emergence

herbicides. The former are used to manage weeds before they begin to shoot and can be applied before the crop is planted or soon after harvest (Stewart, 1955; Iggo, 1975; Odero and Dusky, 2010). This group consists of herbicides that are absorbed rapidly by the roots and work by killing the roots before the leaves and stems begin to grow (Iggo, 1975; Odero and Dusky, 2010). In contrast, post-emergence herbicides, whose residual effect allow them to persist in the soil for short periods of time, only act once the shoots penetrate the soil surface and are consequently less effective on below ground plant material (Stewart, 1955; Iggo, 1975; Odero and Dusky, 2010).

There are various herbicides used in the South African sugarcane industry to control creeping grasses each with a different mode of action depending on their mode of uptake by the plant (Iggo, 1975; Streibig, 2003). The use of herbicides provides a better method of controlling *C. dactylon* than hand hoeing or mechanical eradication and an effective chemical regime is vital because infestation by *C. dactylon* is aggravated by residual herbicide programs which only eliminate other weeds thus leading to a more vigorous spread of *C. dactylon* (Landrey *et al*., 1993).

Numerous herbicides which are phytotoxic to *C. dactylon* have been identified in the past, each with a different mode of action (Whitewell and Santelmann, 1978; Johnson, 1988; Grichar and Boswell, 1989; Waltz *et al*., 2001; Webster *et al*., 2003). Primary herbicides used to control *C. dactylon* include non-selective ones such as glyphosate, paraquat, diuron and imazapyr that kill all plants with which they come into contact (Table 1) (Landrey *et al*., 1993; Leibbrandt, 1995; Campbell *et al*., 2008).

Table 1: Herbicides used to control *Cynodon dactylon* in sugarcane fields, their active ingredients and corresponding recommended application rates (adapted from Campbell, 2008; Herbicide Guide, 2014; Anon, 2015)

Herbicide trade name	Active ingredient	Chemical family	Recommended application rate ($kg\ ha^{-1}$)
Roundup®	Glyphosate	Glycine	2.16
Gramoxone®	Paraquat	Bipyridyliums	0.60
Diuron®	Diuron	Ureas	1.60
Arsenal® GEN 2	Imazapyr	Imidazolinone	1.25

The repeated use of the above mentioned herbicides as with any other herbicide, could lead to the development of resistance/tolerance of the weed species to the chemicals being used (Eksteen, 2007). The potential risk of *C. dactylon* becoming a herbicide resistant weed is primarily due to the current repeated field applications of glyphosate at higher than the recommended application rates (Table 1) (Conlong and Campbell, 2010). Herbicide resistant weeds are an increasing threat worldwide regardless of the herbicide effectiveness. Consequently, farmers cannot rely on a single herbicide control regime because weeds will ultimately adapt and survive (Green and Owen, 2011). To date, 249 herbicide resistant weed species have been identified worldwide of which 12 are present in South Africa, with different modes of herbicide action (Délye *et al*., 2013; Heap, 2016). The most commonly recommended strategy for farmers to combat herbicide resistance is the rotation of herbicides or the use of a mixture of herbicides. This should involve different types of herbicides with different modes of action and metabolic pathways. The rotation or mixing of herbicides allows for a more stringent control of problematic weeds than a single herbicide, without causing much injury to the crop (Duke, 2005; Délye *et al*., 2013).

The effect of the current control regimes involving glyphosate, paraquat and diuron is short lived and the current high dosages of glyphosate which are repeatedly used to control *C. dactylon* pose as a problem for the future (Campbell, 2008). Imazapyr has been identified as a promising herbicide that provides an equal and in some cases better control of *C. dactylon* than glyphosate (Richard, 1997; Broome *et al*., 2000; Ferrell *et al*., 2005). In South Africa, a study by Campbell (2008) identified imazapyr as a promising non-glyphosate herbicide candidate to control *C. dactylon*. In that study, *C. dactylon* was controlled by the application of imazapyr at different rates (0.10, 0.20, 0.30, and 0.60 kg ha^{-1}) and time intervals before the sugarcane was planted. This was done in order to identify if imazapyr effectively controls *C. dactylon* and to compare the phytotoxic effects caused to the sugarcane with that of glyphosate (2.16 kg ha^{-1}). No significant differences were found when comparing the phytotoxic effects of imazapyr on the agronomic traits (number of stalks, stalk height, yield and sucrose content) of sugarcane with that of glyphosate.

The disadvantage of applying imazapyr before planting is that the field needs to remain bare for a long period of time (4 months) before planting can resume and approximately 600 mm of rainfall is required (Anon, 2015). This is due to the persistent soil residual activity of imazapyr which leads to a high risk of herbicide carryover resulting in crop damage and in more severe

cases plant death (Goetz *et al*., 1990; Alister and Kogan, 2005). The recommended application rate of imazapyr varies from 0.75 to 1.25 kg ha^{-1} (Anon, 2015). Imazapyr differs from the more commonly used glyphosate in its mode of action and absorption by the plant making it an ideal alternative non-glyphosate herbicide for the control of *C. dactylon*. Imazapyr is absorbed by both foliage and root tissues (Tu *et al*., 2004; Manabe *et al*., 2007) and acts by inhibiting the AHAS enzyme whereas glyphosate is mainly absorbed by foliar uptake and acts by inhibition of the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Schuette, 1998; Senseman, 2007; Schirmer *et al*., 2012).

2.3 Imazapyr and inhibition of the acetohydroxyacid synthase enzyme

Imazapyr belongs to the imidazolinone class of herbicides which are used to control a wide spectrum of grass and broadleaf weeds (Kishchenko *et al*., 2011; Yu and Powles, 2014a). Imidazolinones make up one of the five structurally distinct chemical families of herbicides that inhibit the AHAS enzyme, the others include sulfonylureas, triazolopyrimidine sulphonamides, sulfonylaminocarbonyl-triazolinones and pyrimidinylsalicylates (pyrimidinylcarboxylates) (Shimizu *et al*., 2002; Tan *et al*., 2005; Sala *et al*., 2008; Lichtfouse, 2014; Yu and Powles, 2014a). These herbicide families are commonly used in agriculture because they offer a high crop weed selectivity, low application rates, high weed control efficacy and low mammalian toxicity (Shimizu *et al*., 2002; Tan *et al*., 2005; Manabe *et al*., 2007; Sala *et al*., 2008). Imidazolinones consist of six structurally similar herbicides, each containing an imidazole moiety, *viz*. imazaquin, imazamethabenz, imazamox, imazethapyr, imazapic and imazapyr (Tan *et al*., 2005). These herbicides are further classified based on their second cyclic structure to produce three structurally different groups, benzene, quinolone and pyridine imidazolinone (Figure 1) (Tan *et al*., 2005). The imidazolinones that consist of a pyridine ring differ by the functional group at position R: (1) imazapyr (R = H); (2) imazapic (R = CH_3); (3) imazethapyr (R = CH_3-CH_2); (4) imazamox (R = CH_3-O-CH_2) (Figure 1) (Tan *et al*., 2005; Sala *et al*., 2008). Four imidazolinones (imazamox, imazethapyr, imazapic and imazapyr) are active ingredients of herbicides that have been registered for use on imidazolinone tolerant plants/crops. To ensure a season-long weed control these herbicides can be applied singly, in combination with other imidazolinone herbicides or in combination with herbicides of a different mode of action (Lichtfouse, 2014).

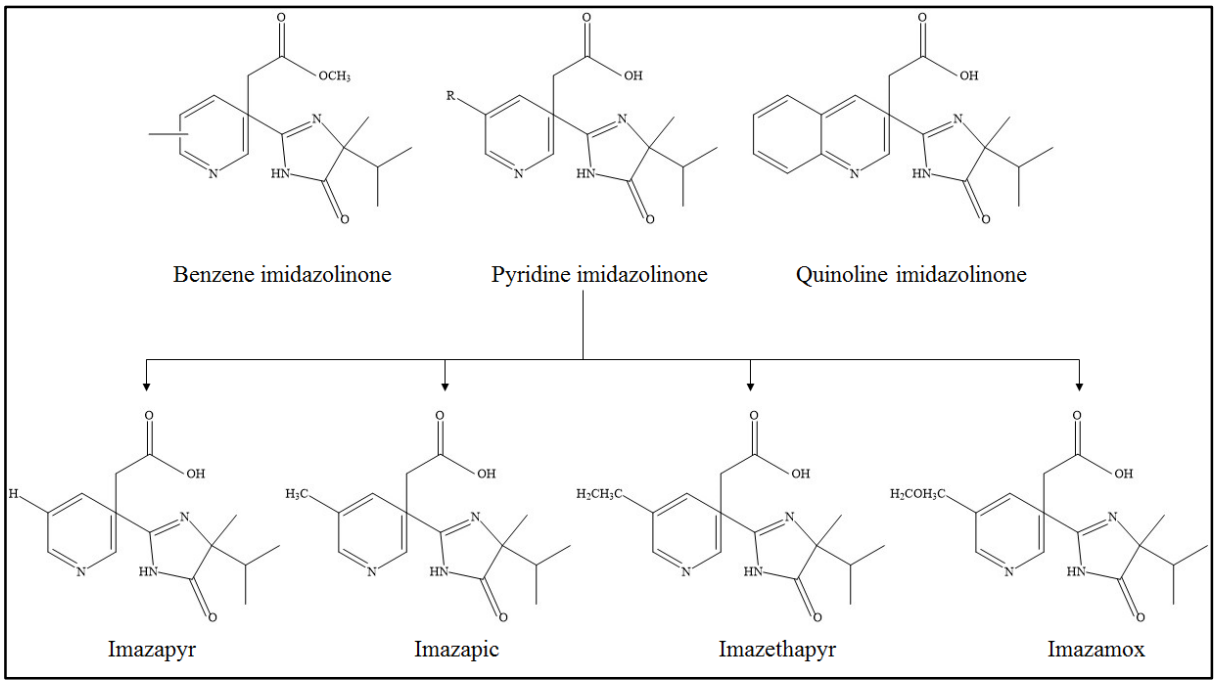

Figure 1: Chemical structure of the imidazolinone class of herbicides.

The AHAS enzyme is vital for the catalytic biosynthesis of three essential branched chain amino acids: valine, leucine and isoleucine (Shimizu *et al*., 2002; Lichtfouse, 2014; Yu and Powles, 2014a). This process is nuclear-encoded and carried out in the chloroplast (Lichtfouse, 2014). The AHAS enzyme is made up of a large catalytic subunit, consisting of approximately 670 amino acids, and a small regulatory subunit and catalyses two vital steps: the condensation of two pyruvate molecules to produce acetolactate and the combination of 2-ketobutyrate and pyruvate to produce 2-acetobutyrate (Tan *et al*., 2005; Lichtfouse, 2014). The acetolactate molecule leads to the formation of valine and leucine whereas the 2-acetobutyrate molecule is a precursor of isoleucine (Lichtfouse, 2014). Inhibition of AHAS leads to a deficiency of these amino acids resulting in plant death (Shimizu *et al*., 2002; Tranel and Wright, 2002; Tan *et al*., 2005; Lichtfouse, 2014). Apart from being deficient in these essential amino acids, secondary effects of inhibiting AHAS in plants include disruption of protein synthesis and photosynthate transport and the build-up of 2-ketobutyrate (Tranel and Wright, 2002). The herbicides such as imidazolinones which inhibit the AHAS enzyme are considered to be growth inhibitors, thereby allowing them to act faster than the more commonly used glyphosate herbicide (Lichtfouse, 2014).

The imidazolinone class of herbicides inhibit the AHAS enzyme by binding to its active site and is therefore uncompetitive with the AHAS substrate, pyruvate (Tan *et al*., 2005; Lichtfouse,

2014). Tan *et al*. (2005) argued that based on the structural composition of the imidazolinones, there is a good correlation between their chemical structure and their ability to inhibit AHAS. It has also been reported that there is a difference in the inhibition of AHAS activity by the three groups of imidazolinones, thus indicating that the secondary structure of these herbicides plays a vital role in the inhibition of AHAS (Shaner *et al*., 2012). The functional groups of the imidazolinone herbicides are also associated with their metabolic detoxification in plants (Tecle *et al*., 1997).

Despite their advantages, there are also disadvantages associated with the use of imidazolinone herbicides. They display an extended soil persistence which is usually absent in other post-emergence herbicides (Sprague *et al*., 1997; Rangel *et al*., 2010; Lee *et al*., 2011). The persistence of imidazolinone herbicides in the soil varies from 90 to 730 days and is influenced by the pH, moisture and organic matter (Alister and Kogan, 2005). This is a disadvantage because there is a high risk of residual carryover that reduces crop growth and kills rotational crops (Goetz *et al*., 1990; Alister and Kogan, 2005). Although the non-selective nature of imidazolinone herbicides makes them excellent candidates for the control of a vast range of weed species, it is disadvantageous towards sensitive crops that cannot overcome the inhibition caused by these herbicides resulting in extensive damage and yield losses in the field (Fletcher *et al*., 1993). Plants can overcome the inhibition caused by herbicides, such as imidazolinones, by various mechanisms such as an altered herbicide target site, enhanced metabolic detoxification of the herbicide and translocation of the herbicide to other parts of the plant (Délye *et al*., 2013). Although amino acid substitutions at the active site of the AHAS enzyme can lead to the identification of imazapyr tolerant crops, it can also lead to crops which are more sensitive to the harmful effects of imazapyr (Ott *et al*., 1996; Duggleby and Pang, 2000). Such approaches can be used to identify sugarcane genotypes that are tolerant to imazapyr thereby allowing for proper eradication of problematic weeds such as *C. dactylon* with the use of Arsenal® GEN 2 (Campbell, 2008).

2.4 Herbicide site of action in plants

There are various mechanisms by which herbicides can act on plants, via the inhibition of photosynthesis, metabolic synthesis, normal chloroplast development, cell division and growth regulation, to name a few (Gaur and Sharma, 2013). As previously mentioned commercially used herbicides have various mechanisms/modes of action according to the manner in which

they affect a plant, either at the tissue or cellular level (Délye *et al.*, 2013*)*. Herbicides that have similar modes of action produce similar plant injury symptoms (Duke, 1990). Based on their mode of action, they have been ranked by a Herbicide-Resistance Action Committee (HRAC) and the three most widely used herbicide groups inhibit AHAS, EPSP synthase and acetyl-CoA carboxylase (ACCase), (Table 2 and Figure 2), with those that inhibit AHAS being most popular (Devine and Shukla, 2000; Tranel and Wright, 2002). Herbicides may have single or multiple sites of action and can be differentiated according to the metabolic processes they inhibit such as inhibition of enzymes at different stages of metabolic processes or energy transfer reactions that are vital for plant survival (Duke, 1990; Délye *et al*., 2013).

The mode of herbicide action within a plant involves five consecutive steps: (1) penetration; (2) translocation to the location of the target protein; (3) accumulation at the target protein location; (4) binding to the target protein; (5) ensuing damage, cell and plant death (Figure 2) (Délye *et al*., 2013). Herbicide resistance/tolerance mechanisms in plants are identified by overcoming the above mentioned steps (Figure 2).

Table 2: A summary of the most common herbicide sites of action in plants and the metabolic processes they inhibit (Duke, 1990; Délye *et al*., 2013).

Metabolic process inhibited	Herbicide site of action	Mode of action in plants	Herbicide/chemical group
Amino acid biosynthesis	AHAS enzyme	The AHAS enzyme catalyses the first step in the biosynthesis of branched chain amino acids valine, leucine and isoleucine. Inhibition of this enzyme results in plant death due to insufficient branched chain amino acid levels.	Sulfonylureas, imidazolinones, triazolopyrimidines and pyrimidinylthiobenzoates
	EPSP synthase	The EPSP synthase is responsible for the biosynthesis of aromatic amino acids. Inhibition of this enzyme results in plant death due to insufficient aromatic amino acids.	Glycine
	Glutamine synthase	Glutamine synthase is responsible for the conversion of glutamate and ammonia to glutamine. Inhibition of this enzyme results in the accumulation of ammonia within the plant which is lethal to plant cells and tissue.	Phosphinic acid
Fatty acid biosynthesis	ACCase	Inhibition of ACCase, a vital enzyme in fatty acid/lipid biosynthesis in grasses results in plant death.	Arloxyphenoxypropionates, cyclohexanediones and phenylpyrazoles

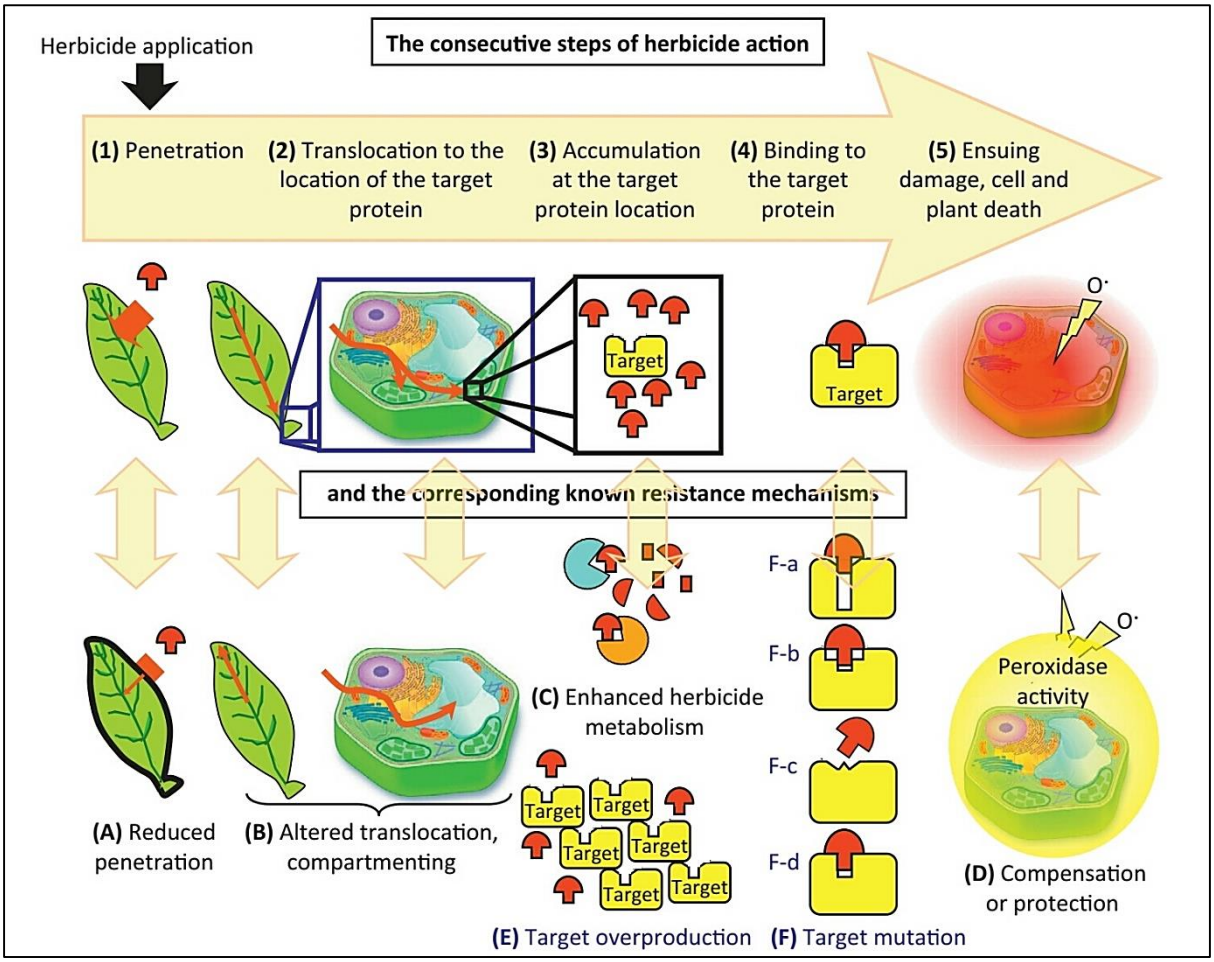

Figure 2: Herbicide mode of action (top) and the plant resistance/tolerance mechanisms corresponding to mode of action (bottom). **(1)** Herbicide molecules penetrate the plant; **(2)** herbicide molecules are translocated to the target protein; **(3)** accumulation of the herbicide molecule occurs at the site of the target protein; **(4)** herbicide molecules then bind to the target protein; **(5)** the binding causes the disruption of cellular processes or pathways which results in plant death. **(A)** reduced penetration of the herbicide; **(B)** altered translocation or compartmenting; **(C)** enhanced herbicide metabolism; **(D)** compensation or protection from herbicide action; **(E)** overproduction of target protein or enzyme; **(F)** altered enzyme or protein target (replicated from Délye *et al*., 2013).

2.5 Characterisation of herbicide tolerance in plants

Herbicide tolerance can be acquired in one of three ways, resistance at the site of action, metabolic detoxification of the herbicide and/or prevention of the herbicide from reaching the target site (Gaur and Sharma, 2013). Herbicide tolerance in plants can be further divided into two major groups, target site tolerance and non-target site tolerance (Sala *et al*., 2012). The

former is caused by mutations in the target site, which leads to enzymes or proteins that have a reduced sensitivity or increased activity thereby limiting herbicide binding (Figure 2). This type of tolerance in plants is mostly monogenic (Devine and Eberlein, 1997; Tranel and Wright, 2002; Gaur and Sharma, 2013; Yu and Powles, 2014b). In contrast, non-target site tolerance involves various mechanisms of which the most common include changes in translocation and/or sequestration of the herbicide and changes in the metabolic detoxification of the herbicide (Figure 2) (Devine and Eberlein, 1997; Yuan *et al*., 2007; Shaner *et al*., 2012; Gaur and Sharma, 2013; Yu and Powles, 2014b). An increase in gene expression could also be the basis for both target and non-target site tolerance (Yuan *et al*., 2007).

2.5.1 Target site tolerance

Target site tolerance is conferred by an altered herbicide target site (enzyme or protein) and has been identified in various plant species (Chaleff and Ray, 1984; Haughn and Somerville, 1986; Sabastian *et al*., 1989; Swanson *et al*., 1989; Newhouse *et al*., 1991; Hart *et al*., 1992; Rajasekaran *et al*., 1996; Croughan, 1998; Wright and Penner, 1998a; Wright and Penner, 1998b; Ponziak and Huci, 2004; Kishchenko *et al*., 2011; Sala and Bulos, 2012; Thompson and Tar'an, 2014). Herbicide tolerance in various plant species has revealed the most common herbicide sites of action as AHAS, EPSP synthase and ACCase (Devine and Shukla, 2000). Plants synthesise their own amino acids and because humans and animals differ from plants in this regard, the herbicides that inhibit amino acid biosynthesis are selectively toxic to plants having less or no impact on humans and animals. As a result, herbicides that inhibit amino acid biosynthesis pathways are ideal targets when synthesising new herbicides (Yu *et al*., 2004).

Herbicides which inhibit the ACCase enzyme lead to the inhibition of acyl lipid biosynthesis, eventually resulting in the death of the plant (Burton *et al*., 1987). Mutations within the ACCase gene may therefore confer different tolerance patterns (Devine and Shukla, 2000). Dicotyledonous species are naturally tolerant to ACCase inhibiting herbicides due to the presence of a prokaryotic form of ACCase. In contrast, monocotyledonous species are resistant to the ACCase herbicides due to the presence of a eukaryotic form of ACCase. This difference is used as the basis for selectivity of the herbicides between the plant species (Devine and Shukla, 2000). The tolerance of grasses and cereal crops to ACCase herbicides are based on an insensitive form of ACCase or an increased ability to metabolize the herbicides into inactive compounds (Stoltenberg *et al*., 1989; Catanzaro *et al*., 1993).

The most common herbicides are those that target enzymes and proteins responsible for the biosynthesis of amino acids (Tan *et al*., 2005). The key enzyme in the biosynthesis of the aromatic amino acids phenylalanine and tyrosine is EPSP synthase, which is inhibited by glyphosate, a non-selective herbicide that has been used for decades (Devine and Shukla, 2000; Dill, 2005; Sprenger, 2006). The non-selective nature of glyphosate has led to the genetic engineering of glyphosate-tolerant crops, which have been commercially available to farmers and include soybean, canola, cotton, sugar beet and maize (Dill, 2005; Duke, 2005; Gianessi, 2005; Green and Owen, 2011). Such crops contain an *Agrobacterium* CP4 EPSP synthase gene which confers tolerance without affecting the catalytic properties of the enzyme (Padgette *et al*., 1996; Devine and Shukla, 2000). Target site tolerance in glyphosate resistant plants can also be characterised by a double mutation in the same region of the EPSP synthase gene at positions 101, 102 and/or 106 (Degryse *et al*., 1997). Glyphosate resistance has been characterised in various ways one of which includes a single mutation within the EPSP synthase that converts $Glycine_{96}$ (Gly) to Alanine (Ala) or $Proline_{101}$ (Pro) to Serine (Ser) (Devine and Shukla, 2000; Dill, 2005). Although the presence of either one of these mutations confers tolerance to glyphosate, the catalytic properties of the enzyme is impaired, reducing the strength of the enzyme in the absence of the herbicide (Devine and Shukla, 2000).

As mentioned previously, the AHAS enzyme catalyses one of the most common biochemical reactions within plants which involves the synthesis of branched chain amino acids valine, leucine and isoleucine (Duggleby and Pang, 2000; Tan *et al*., 2005; Délye, 2013). The first AHAS inhibiting herbicide was commercialised in 1982 and has been widely used ever since (Devine and Shukla, 2000). The AHAS enzyme is also the target site for more than 50 commercial herbicides that belong to five structurally distinct herbicide families and inhibition of the enzyme leads to decreased pools of the essential branched chain amino acids thereby causing inhibition of protein synthesis (Senseman, 2007). The result of inhibiting AHAS is the slow death of the plant with first symptoms appearing in the meristematic tissues (Duggleby and Pang, 2000).

2.5.1.1 Acetohydroxyacid synthase mutations that confer tolerance to imidazolinone tolerant crops

Various plants, mainly weeds, have developed resistance to AHAS inhibiting herbicides due to their extensive use. Plants resistant to AHAS inhibitors have been identified following

mutagenesis using various chemical mutagens, the most common being EMS. These plants include tobacco (Chaleff and Ray, 1984), *A. thaliana* L. (Haughn and Somerville, 1986), canola (Swanson *et al*., 1989), soybean (Sabastian *et al*., 1989), maize (Newhouse *et al*., 1991; Wright and Penner, 1998b), cotton (Rajasekaran *et al*., 1996), rice (Croughan, 1998), wheat (Ponziak and Huci, 2004), sugar beet (Hart *et al*., 1992; Wright and Penner, 1998a; Kishchenko *et al*., 2011), sunflower (Sala and Bulos, 2012) and chickpea (Thompson and Tar'an, 2014).

Resistance to AHAS inhibiting herbicides has evolved over time and is commonly associated with a target site mutation within a discrete conserved region of the AHAS gene (Singh and Shaner, 1995; Devine and Eberline, 1997; Walsh *et al*., 2012). A review of AHAS mutations indicated that there are 22 substitutions at seven distinct sites across the AHAS gene which leads to herbicide tolerance (Powles and Yu, 2010). With reference to a mutant genotype of *A. thaliana*, there are five commonly found mutations within the AHAS gene which have been identified to confer tolerance to AHAS inhibiting herbicides: Ala_{122} (Ala represents the amino acid alanine and 122 represents the position of the amino acid within the protein sequence), Pro_{197}, Ala_{205}, $Tryptophan_{574}$ (Trp) and Ser_{653} (Bernasconi *et al*., 1995; Tranel and Wright, 2002; Whaley *et al*., 2007). The mutation at codon 574 encodes resistance to all AHAS inhibitors, whereas the mutation at codon 197 results in higher tolerance to sulfonylureas than imidazolinones and the mutations at codons 122, 205 and 653 confers tolerance to imidazolinones only (Table 3) (Tan *et al*., 2005; Lichtfouse, 2014). Other less common mutations found within the AHAS gene include $Arginine_{377}$ (Arg), $Phenylalanine_{578}$ (Phe) Trp_{591}, Gly_{654} and Ser_{670} (Jung *et al*., 2004; Laplante *et al*., 2009; Tranel *et al*., 2016). The amino acids which make-up these codons are distributed throughout the primary structure (linear amino acid sequence) of AHAS but once the enzyme is folded into its protein (quaternary structure) they are present adjacent to each other (Ott *et al*., 1996). This area represents the binding site of AHAS inhibitors (Lichtfouse, 2014). The different AHAS mutations can result in specific tolerance to one chemical family or cross tolerance to multiple chemical families such as sulfonylureas and imidazolinones (Table 3) (Devine and Shukla, 2000; Walsh *et al*., 2012). The cross tolerance occurs because imidazolinones and sulfonylureas both inhibit AHAS (Shimizu *et al*., 2002; Tan *et al*., 2005; Sala *et al*., 2008). For example, the Trp_{591} to Leu (Leucine) mutation is the only one that confers a high level of tolerance to both sulfonylureas and imidazolinone herbicides (Boutsalis *et al*., 1999), whereas the Ser_{653} to Asparagine (Asp) mutation results in tolerance to imidazolinones with much less or no tolerance to sulfonylureas (Lee *et al*., 1999) and the Pro_{197} to Ser mutation confers

tolerance to sulfonylureas with little or no tolerance to imidazolinones (Table 3) (Thill, 1997; Tranel and Wright, 2002; Yu *et al*., 2003).

The main benefit of implementing herbicide tolerant crops in the field is to eliminate the injury caused by non-selective herbicides. Other benefits include a more efficient weed control strategy which leads to higher yields and lower input costs (Green, 2012). The use of imidazolinone tolerant crops in combination with imidazolinone herbicides, referred to as the Clearfield® production system (Tan *et al*., 2005; Rosas *et al*., 2014; Sudianto *et al*., 2013), allows for the control of difficult weeds that cannot be achieved by other herbicides and is being used successfully in countries that have commercially released imidazolinone tolerant crops such as maize (Anderson and Georgeson, 1989; Newhouse *et al*., 1991; Bernasconi *et al*., 1995; Shaner *et al*., 1996; Bright *et al*., 1997; Dietrich, 1998), oilseed rape (Swanson *et al*., 1989; Hatorri *et al*., 1995; Shaner *et al*., 1996), rice (Croughan, 1998; Croughan, 2002; Croughan, 2003) and sunflower (Al-Khatib *et al*., 1998; Bruniard, 2001; White *et al*., 2003) all of which have been produced using various methods followed by selection with an imidazolinone herbicide (Table 4). The Clearfield® crops mentioned above have been characterised for tolerance to imidazolinone herbicides based on a target-site mutation which is the most common mode of tolerance (Yu and Powles, 2014a).

Table 3: Amino acid mutations within the acetohydroxyacid synthase gene (with reference to *Arabidopsis thaliana*) that confer tolerance and/or cross tolerance to sulfonylureas and imidazolinones. Ala – Alanine; Thr – Threonine; Pro – Proline; His – Histidine; Leu – Leucine; Arg – Arginine; Ile – Isolecuine; Gln – Glutamine; Ser – Serine; Asp – Asparagine; Asp – Aspartic acid; Trp – Tryptophan (adapted from Hartnett *et al*., 1990; Bernasconi *et al*., 1995; Guttieri *et al*., 1995; Devine and Eberlein, 1997; Boutsalis *et al*., 1999; Lee *et al*., 1999; Tan *et al*., 2005; Sala and Bulos, 2012; Lichtfouse, 2014; Yu and Powles, 2014a).

Amino acid mutation	**Tolerance level**	
	Sulfonylureas	**Imidazolinones**
Ala_{122} to Thr	Low/zero	High
Pro_{197} to Ala	High	Zero
Pro_{197} to Thr	High	Low/zero
Pro_{197} to His	High	Moderate
Pro_{197} to Leu	High	Moderate
Pro_{197} to Arg	High	*None
Pro_{197} to Ile	High	Moderate
Pro_{197} to Gln	High	*None
Pro_{197} to Ser	High	Zero
Ala_{205} to Asp	*None	High
Trp_{574} to Leu	High	High
Trp_{591} to Leu	High	High
Ser_{653} to Asn	Low/Zero	High
Ser_{670} to Asp	Low	High

* None – No tolerance to sulfonylureas and/or imidazolinones.

Table 4: A summary of imidazolinone tolerant crops (Clearfield® crops) that consist of mutations within the acetohydroxyacid synthase enzyme.

Crop	Mutagenic method	Codon position	Amino acid substitution	Cross tolerance to other herbicides	References
Maize	Chemical mutagenesis and selection in *in vitro* culture	653	Ser to Asp	-	Newhouse *et al*., 1991; Bernasconi *et al*., 1995;
	Selection in *in vitro* culture	574	Trp to Leu	Sulfonylureas Triazolopyrimidines Pyrimidinylthiobenzoates	Bright *et al*., 1997; Dietrich, 1998
	Chemical mutagenesis	155	Ala to Thr	Pyrimidinylthiobenzoates	
	Chemical mutagenesis	122	Ala to Thr	-	
Oilseed rape	Chemical mutagenesis	653	Ser to Asp	-	Hattori *et al*., 1995
	Chemical mutagenesis	574	Trp to Leu	Sulfonylureas	
Rice	Chemical mutagenesis	654	Gly to Glu	-	Croughan, 2003
	Chemical mutagenesis	653	Ser to Asp	-	
Sunflower	Natural selection	205	Ala to Val	-	Bruniard, 2001
	Natural selection	197	Ala to Val	Sulfonylureas	Kolkman *et al*., 2004
	Chemical mutagenesis	122	Ala to Thr	-	Sala *et al*., 2008
Barley	Chemical mutagenesis	653	Ser to Asp	-	Lee *et al*., 2011

2.5.2 Non-target site tolerance

Non-target site herbicide tolerance refers to mechanisms which prevent a lethal herbicide dose from reaching the herbicide binding site (Han *et al*., 2014). Characterisation of non-target site herbicide tolerance has been achieved mainly for glyphosate tolerant plants because it is the most widely used herbicide worldwide (Shaner *et al*., 2012). As previously mentioned, the repetitive use of glyphosate at high application rates has contributed to the evolution of many glyphosate tolerant weed species (Shaner *et al*., 2012). Non-target site herbicide tolerance has been identified in various grass species and has now been implicated as a common herbicide mode of tolerance (Powles and Yu, 2010; Délye *et al*., 2011; Délye, 2013).

As plant species differ in their response to different herbicide classes, some are more susceptible whereas others are more tolerant to herbicides, they also differ in their metabolic activity (Gaur and Sharma, 2013). According to Yuan *et al*. (2007), the metabolic detoxification of herbicides occurs within the plant in a four phase mechanism. Phase I involves oxidation of the herbicide which exposes certain enzyme functional groups involved in phase II. Phase II is the conjugation of the activated herbicide to a hydrophilic molecule which allows for the end products of this phase to be recognised by phase III transporters. Phase III involves the transportation of the conjugated molecules into the vacuole where phase IV, the degradation of the conjugated molecule occurs. Each step in the detoxification process is catalysed by a different enzyme and to date the four gene families that have been identified for their involvement in the detoxification of herbicides include: CYP enzyme family, glutathione S-transferases (GSTs), glucosyltransferases and ATP-binding cassette transporters (ABC) transporters.

The CYP gene family encodes various enzymes which play a pivotal role in phase I of the detoxification of herbicides (Kreuz *et al*., 1996; Siminszky, 2006; Kumar *et al*., 2012). These enzymes are a superfamily of ubiquitous heme-containing proteins which catalyse a series of reactions: deaminations, decarboxylations, isomerizations, epoxidations, dealkylations and hydroxylations, which produce oxygenated products for subsequent stages within the detoxification process (Kreuz *et al*., 1996; Kaspar *et al*., 2011; Kumar *et al*., 2012; Délye *et al*., 2013). The CYP enzyme family has been used as biocatalysts to create herbicide tolerant plants and although many studies have successfully described the role of plant CYP in metabolising herbicides of different families their molecular characterisation is limited (Cocker *et al*., 2001; Yun *et al*., 2005; Siminszky, 2006; Kumar *et al*., 2012). Plant CYP which

have been molecularly characterised include CYP76B1 from *Helianthus tuberosus* and CYP71A10 from soybean which metabolises phenylureas, and CYP81B2 and CYP71A11 from tobacco which metabolises chlortoluron (a urea herbicide) (Kumar *et al*., 2012). Although the CYP enzymes which have been identified do play a role in the metabolic detoxification of herbicides they do not contain an efficient and complete catabolic pathway to detoxify the herbicides and other harmful compounds (Kumar *et al*., 2012).

Some plants have an existing capacity for P450-mediated metabolism whereas others have been modified by mutagenesis (Werck-Reichhart *et al*., 2000; Siminszky, 2006). The involvement of CYP in herbicide metabolism has been used to characterise herbicide detoxification in various plant species achieved by application of a herbicide in combination with a CYP inhibitor (Fonné-Pfister *et al*., 1990; Gressel, 1990; Kreuz and Fonné-Pfister, 1992; Christopher *et al*., 1994; Baerg *et al*., 1996; Letrouzé and Gasquez, 2001; Yu *et al*., 2004; Yun *et al*., 2005; Yasour *et al*., 2009; Yu *et al*., 2009; Breccia *et al*., 2012; Elmore *et al*., 2015).

2.5.2.1 Imidazolinone tolerant plants with an enhanced herbicide detoxification mechanism

Herbicide selectivity is commonly based on the ability of a plant species to detoxify the herbicide metabolically. In weed species, the use of herbicides at a low dose results in a lower plant mortality rate and based on this some weeds overcome herbicide treatment because they possess the genes required to metabolise (or detoxify) the herbicide to an extent that ensures survival (Yu and Powles, 2014b). In crops, the trait conferring an enhanced metabolic detoxification of herbicides has been widely used in agriculture to genetically modify crops for herbicide tolerance, which allows for the implementation of stringent weed control practices (Cole, 1994; Kreuz *et al*., 1996). Enhancement of herbicide metabolism is regarded as the most useful mechanism of crop tolerance (Dekker and Duke, 1995).

The role of CYP in enhanced/altered herbicide metabolism in herbicide tolerant plants was first evident in cotton seedlings (Frear *et al*., 1969) and has been assessed in various plant species to date such as maize (Kwon *et al*., 1995; Kotoula-Syka and Hatzios, 1996), cereal weed (Yu *et al*., 2009), sunflower (Breccia *et al*., 2012), cotton (Minton *et al*., 2008) and various grass species (Kwon and Penner, 1995; Fisher *et al*., 2000; Elmore *et al*., 2015) (Table 5). There are nine classes of herbicides, including imidazolinones, known to undergo CYP-mediated

metabolism *in vivo* and there are known CYP inhibitors which suppress herbicide metabolism *in vivo* and can also reverse herbicide tolerance (Siminszky, 2006). The use of CYP inhibitors in combination with herbicides allows for the identification of an enhanced/altered herbicide metabolism in herbicide tolerant plants (Christopher *et al*., 1991; Hall *et al*., 1995; Preston *et al*., 1996; Hall *et al*., 1997; Fisher *et al*., 2000; Yun *et al*., 2005; Yasour *et al*., 2009). In contrast an enhanced herbicide metabolism in herbicide tolerant plants can also be identified by pre-treatment of plants with CYP inhibitors followed by treatment with the herbicide (Preston *et al*., 1996).

Compounds that are synergistic to herbicides, i.e. enhance their effect, are implemented to supress plants' tolerance mechanisms (Gressel and Shaaltiel, 1988). Piperonyl butoxide is a mixed function oxidase inhibitor and is widely used as a herbicide/pesticide synergist to increase the activity of the chemicals against herbicide tolerant plants and insecticide tolerant insects (O'Brien, 1967; Attia *et al*., 1980; Varsano *et al*., 1992). This pesticide synergist has a dual function because it is also used to enhance the effect of herbicides on plants (Kwon and Penner, 1995). Studies have been carried out to assess the metabolic role of CYP in herbicide tolerant plants (maize, cereal weed, sunflower and various grass species) with the use of CYP inhibitors (Table 5) (Kwon and Penner, 1995; Kwon *et al*., 1995; Kotoula-Syka and Hatzios, 1996; Fisher *et al*., 2000; Yu *et al*., 2009; Breccia *et al*., 2012; Elmore *et al*., 2015). The objective of those studies was to assess the metabolic detoxification of the herbicide by the plant with the aim of determining if herbicide metabolism catalysed by CYP contributes to herbicide tolerance. Plants with an enhanced metabolism were identified when PBO inhibited metabolism in the presence of the herbicide thus causing plant injury or death. A comparison was made amongst treatments involving: (1) no herbicide and no PBO (control); (2) PBO only; (3) herbicide only, in order to confirm an enhanced herbicide metabolism within the herbicide tolerant plant (Kwon and Penner, 1995; Kwon *et al*., 1995; Kotoula-Syka and Hatzios, 1996; Fisher *et al*., 2000; Breccia *et al*., 2012).

Table 5: A summary of studies involving the detection of an enhanced herbicide metabolism by inhibition of cytochrome P450 in plants.

Plant	Class of herbicide tested	Outcome	Reference
Maize (*Zea mays*)	Sulfonylurea	Piperonyl butoxide mixed with the herbicides nicosulfuron and primisulfuron decreased the maize height and fresh weight thus indicating that CYP plays a role in the metabolic detoxification of both sulfonylurea herbicides.	Kwon *et al*., 1995
		The CYP inhibitor PBO mixed with the tribenuron herbicide did not influence the activity of the herbicide on a sulfonylurea tolerant hybrid thus indicating that enhanced detoxification was not a mode of herbicide tolerance.	Kotoula-Syka and Hatzios, 1996
Grass (Kochia scoparia)	Sulfonylurea	A mixture of PBO with the herbicides thifiensulfuron and primisulfuron increased the injury to the grass species, which indicated an enhanced mode of herbicide detoxification.	Kwon and Penner, 1995
Watergrass (*Enchinochloa phyllopogon*)	Pyrimidinyl(thio)benzoate	Results showed that combination of the herbicide with PBO decreased the fresh weight of the plant, indicating an enhanced metabolic detoxification.	Fisher *et al*., 2000
Cereal weed (*Lolium rigidum*)	Glyphosate, sulfonylurea, aryloxyphenoxy-propionate (FOPs)	The use of CYP inhibitors malathion and amitrole with AHAS and ACCase inhibitors, respectively, reversed resistance to both the sulfonylurea and FOPs herbicides. This is an indication that herbicide tolerance is also due to a non-target site mechanism such as an enhanced detoxification.	Yu *et al*., 2009
Sunflower (*Helianthus*)	Imidazolinone	An increase in phytotoxic effects was observed for the resistant genotypes only thus indicating that CYP play a role in the metabolic detoxification of imazapyr.	Breccia *et al*., 2012
Cotton (*Gossypium hirsutum*)	Sulfonylurea	The CYP inhibitor malathion could potentially reduce metabolism of the sulfonylurea herbicide trifloxysulfuron in cotton.	Minton *et al*., 2008
Creeping grass (*Agrostis stolonifera*)	Topramezone	The response of the creeping bent grass to two CYP inhibitors, malathion and 1-aminobenzotriazole (ABT) indicated that its herbicide tolerance is influenced by a CYP catalysed metabolism.	Elmore *et al*., 2015

2.6 Herbicide tolerant sugarcane

The most widely used chemical mutagens include EMS, sodium azide, hydrogen fluoride (HF), methyl methanesulfonate (MMS), *N*-methyl-*N*-nitrosourea (MNU) and hydroxylamine (Parry *et al*., 2009). Of the above mentioned chemical mutagens, EMS is most commonly used in plants (Schy and Plewa, 1989).

Ethyl methanesulfonate is a mutagen commonly used to induce variation within plant genomes. This chemical is an alkylating agent that adds an ethyl group to thymine (T) and guanine (G) residues thereby causing them to be identified as adenine (A) and cytosine (C) residues, respectively. The mutations produced are in the form of base pair substitutions that convert GC to AT (Schy and Plewa, 1989; van Harten, 1998; Jander *et al*., 2003; Rutherford *et al*., 2014). The base pair substitutions produced by the EMS mutagenesis result in altered forms of the triplet codon sequence within a protein sequence. This merely changes the physiological characteristics of the protein without stopping its function (Rutherford *et al*., 2014). With regards to sugarcane, induced mutagenesis using EMS is achieved by addition of the chemical to embryogenic calli because it is at this stage that DNA replication is most rapid thereby ensuring the highest probability of incorrect DNA repair (Kilbey and Hunter, 1983; Rutherford *et al*., 2014).

In a study by Koch *et al*. (2012), imazapyr tolerant sugarcane genotypes were produced from the unmutated N12 genotype by an *in vitro* chemical mutagenesis protocol using EMS. The mutant genotypes were screened for tolerance to imazapyr based on increased AHAS enzymatic activity compared with that of the unmutated N12 control and seven mutants were selected. Rutherford *et al*. (*in press*) characterised these mutants in the field to determine the effect of Arsenal® GEN 2 (imazapyr) on the genotypes. Different concentrations of Arsenal® GEN 2, applied at two different time intervals were tested: (1) 0, 312 and 625 g a.i.ha^{-1} imazapyr were applied to the field 2 months after the sugarcane was planted; (2) 1248 g a.i.ha^{-1} imazapyr was applied to the field prior to planting. The results of that study showed no differences in sucrose, fibre and estimated yield amongst genotypes in unsprayed fields. Normal green leaves were observed for all genotypes in the unsprayed field and for five mutants in the fields sprayed with 312 and 615 g a.i.ha^{-1} imazapyr. All mutants germinated in the field sprayed with 1248 g a.i.ha^{-1} imazapyr prior to planting. The AHAS activity of the mutants was characterised in the presence of imazapyr (analytical grade) using an *in vitro* enzyme assay (Rutherford *et al*., *in press*). The results indicated that three mutant genotypes:

Mut 1, Mut 6 and Mut 7, had a higher AHAS enzyme activity compared with that of the unmutated N12 genotype and the other mutants. The mode of tolerance to imazapyr in the mutant genotypes was not identified.

Herbicide tolerant sugarcane has been previously produced using various methods such as genetic engineering (Gallo-Meagher and Irvine, 1996; Falco *et al*., 2000; Leibbrandt and Snyman, 2003) and induced mutagenesis (Irvine *et al*., 1991; Ali *et al*., 2007; Kenganal *et al*., 2008). There are various disadvantages associated with the former approach compared with the latter. These include a limitation regarding the commercial release of transgenic sugarcane varieties due to intellectual property restrictions and acceptance by international markets (Snyman *et al*., 2008; Koch *et al*., 2012). Mutations within important genes or traits can be exploited by plant breeders without the restrictions, licensing costs and societal opposition linked to that of genetically engineered plants (Parry *et al*., 2009). The use of chemical mutagens results in less chromosomal damage and the presence of point mutations occurs at high frequencies than in mutants produced by physical mutagenesis (van Harten, 1998; Parry *et al*., 2009). Physical mutagenesis involving either radiation or gamma rays has been used to induce mutations within the sugarcane genome that resulted in genotypes that were tolerant to salinity, drought (Saif-Ur-Rasheed *et al*., 2001; Patade *et al*., 2006; Ali *et al*., 2007; Patade and Suprasanna, 2008; Patade *et al*., 2008; Khan and Khan, 2010) and the herbicide glyphosate (Zambrano *et al*., 2003).

Previous studies involving the production of genetically modified herbicide tolerant sugarcane have been carried out using *Agrobacterium*-mediated genetic transfer (Enríquez-Obregón *et al*., 1998; Manickavasagam *et al*., 2004) and microprojectile bombardment (Leibbrandt and Snyman, 2001; Snyman *et al*., 2001) and sugarcane genotypes that were tolerant to herbicides containing the active ingredient glufosinate ammonium were produced. A transgenic sugar beet genotype tolerant to the imidazolinone herbicide imazethapyr was also produced using *Agrobacterium*-mediated transformation involving a mutated AHAS gene of *A. thaliana* with a Ser_{653} amino acid mutation (Kishchenko *et al*., 2011). The herbicide tolerant sugar beet genotypes produced using this method were tested *in vitro* and under glasshouse conditions after spraying with the herbicide (Kishchenko *et al*., 2011).

A study by van der Vyver (2013) reported on a selection system for identifying transformed sugarcane using a selectable marker targeted by an AHAS inhibiting herbicide. In that study biolistic transformation was used to transfer a mutant tobacco AHAS gene into sugarcane

plants. The plants were then proliferated, regenerated and rooted using *in vitro* culture based techniques. Thereafter, the putative transgenic plants were acclimatized and established under glasshouse conditions. After being sprayed with a sulfonylurea herbicide, five out of six plants expressed a mutant form of the AHAS gene.

2.7 Assessment of mutant sugarcane genotypes

Field evaluations of herbicide tolerant crops have been carried out on genetically engineered sugar beet (Buckmann *et al*., 2000), rice (Jiang *et al*., 2000), tobacco and potato (de Greef *et al*., 1989) but there is very little or no information available on herbicide tolerant sugarcane other than the preliminary work by Rutherford *et al*. (*in press*). A field evaluation of mutant sugarcane genotypes involves assessing various agronomic traits such as stalk height, stalk mass, stalk diameter, cane yield, sucrose yield and fibre content (Bailey and Bechet, 1989; Gilbert *et al*., 2005).

2.7.1 Agronomic assessment of sugarcane genotypes

Herbicide tolerant sugarcane genotypes produced by genetic engineering or mutagenesis (chemical or physical) need to be assessed before they can be transferred to the field. These assessments and comparisons can only be done on mature sugarcane plants (fully grown) and are carried out to ensure that the desired phenotypic and genotypic traits of interest are being expressed positively without hindering other important traits of the crop (Bailey and Bechet, 1989; Gravois *et al*., 2008; Gilbert *et al*., 2009). The assessment is done to ensure that the agronomic traits of the mutants have not been altered by previously undetected mutations and *in vitro* regeneration methods used to produce them (Bailey and Bechet, 1989; Gravois *et al*., 2008; Gilbert *et al*., 2009). In the South African Sugar Industry the quality of the cane is also assessed. Originally, this was done by considering only the sucrose content but in more recent years it is based on the recoverable value (RV) sucrose content (per 100g fresh mass or RV% cane). The RV content, which is a measure of both sugar and molasses that will be recovered from the cane, is calculated by taking into account the sucrose (S), non-sucrose (N) and fibre (F) content of the cane (Cane Testing Service, South African Sugar Association, 2015). The RV content is calculated as follows:

$$RV\ \%\ cane = S - dN - cF$$

Where:

d = 0.04 (the relative value of sucrose with each unit of non-sucrose diverts from sugar production to molasses)

c = 0.02 (the loss of sucrose from sugar production per unit of fibre)

There are studies which have been carried out on the agronomic performance of mutant sugarcane genotypes, however, these studies are limited and also contradictory. The first field analysis of transgenic sugarcane was performed by Arencibia *et al*. (1999) who tested resistance of the genotypes to stalk borers and concluded that the agronomic traits of the transgenic genotypes were similar to that of the parent. Gilbert *et al*. (2005) assessed the expression of a transgene in the transgenic sugarcane genotypes and established that these genotypes had an equivalent agronomic performance to that of the parent genotype. Although studies by Vickers *et al*. (2005) and Gilbert *et al*. (2009) concluded that a significant yield reduction was present in the transgenic sugarcane genotypes compared with that of the parent, there were no differences between the genotypes in all other agronomic traits analysed. Leibbrandt and Snyman (2001) conducted a field trial to evaluate herbicide tolerant sugarcane genotypes produced by microprojectile bombardment and the results indicated that although the stalk diameter of the control plants were significantly wider compared with the transgenic plants, no significant differences in stalk height were found. A higher fibre content, which has been associated with decreased damage by the sugarcane borer *E. saccharina* (Lepidoptera: Pyralidae) (Keeping and Rutherford, 2004), was also noted in the herbicide tolerant genotype. *Eldana saccharina* is considered as the most destructive pest in South African sugarcane which is managed through cropping practises (Carnegie and Smaill, 1982), planting of resistant cultivars (Keeping, 2006) and with the use of insecticides (Leslie, 2003). Assessment of the damage caused by this borer is based on a visual analysis of the number of damaged internodes and the larval age/instar stage of the pest. This is achieved by splitting each stalk longitudinally in order to assess borer damage (Goebel and Way, 2003). Infestation by the pest is analysed by calculating the number of internodes bored and is calculated as follows (Anon, 2005):

$$\%\ \text{Internodes bored} = \frac{\text{The number of internodes bored in a stalk}}{\text{The total number of internodes}} \times 100$$

The above calculation gives the percentage of internodes bored within one stalk. This value is calculated for each sample stalk and an average of these values is then calculated to represent the percentage of internodes bored by *E. saccharina* in the field.

2.7.2 Assessment of leaf chlorophyll content as a measure of plant stress

Herbicide tolerant sugarcane plants can be further assessed by measuring the leaf chlorophyll content which is an indicator of plant stress due to low temperatures, dehydration, freezing, flooding, disease, insects and when under the influence of herbicides (Eagles *et al*., 1983; Carter and Knapp, 2001; Adriano *et al*., 2013). Visual observations of plant stress under various circumstances can be subjective because it relies on human knowledge and interpretation which differs from person to person (Percival, 2004). The chlorophyll content meter or soil plant analysis development (SPAD) meter (Wood *et al*., 1992) is a commercially available and portable tool that is used to measure the 'greenness' of a plant. This measurement is achieved based the optical response of a leaf that is being exposed to light which in turn generates an output that is used to estimate foliar chlorophyll concentrations (Kariya *et al*., 1982).

Previously, the effect of a herbicide/s on *C. dactylon* has been assessed visually and expressed as percentages of the coverage of the weed. These assessments comprised two ratings: (1) 100% coverage which indicates one of the following (a) no weed control, (b) total/complete coverage of the weed or (c) no effect of the herbicide; (2) 0% coverage which indicates one of the following (a) no ground cover by the weed or (b) complete eradication of the weed by herbicide application (Leibbrandt, 1995; Richard Jr., 1997; Miller *et al*., 1999; Broome *et al*., 2000; Abdullahi, 2002; Ferrell *et al*., 2005; Donald, 2006). Although visual ratings are a more common practice, they are biased by various factors such as fatigue, lack of training, knowledge of treatments and complexity of observations (Nesser *et al*., 2000). On the other hand, digital image analysis measures the percentage of green leaves by quantifying the percentage of green pixels in a digital image that is bordered by a neon quadrat. At SASRI this quantification is carried out by an AutoWeedCover software designed by the institute. The output generated by the software is expressed as a percentage of green leaves with the associated image identification (Campbell *et al*., 2008). Digital image analysis overcomes the problems associated with visual ratings and is therefore the type of analysis implemented in this study to quantify the percentage of greenness of *C. dactylon*.

2.7.3 Molecular assessment of sugarcane genotypes

Isolation of mutants with altered phenotypes, generated by chemical or biological mutagenic agents, allows for identifying and learning about gene functions in a physiological and developmental context (Papdi *et al*., 2010). Depending on the intended outcome of the study, herbicide tolerance in sugarcane can be identified by molecular analysis which involves the use of various methods, *viz*. *in vitro* enzyme assays that characterise the activity of the enzyme in question (Enríquez-Obregón *et al*., 1998; Koch *et al*., 2012; Adriano *et al*., 2013; Rutherford *et al*., *in press*), Western blot analyses to characterise proteins (Manickavasagam *et al*., 2004), genotyping to identify differences within the genetic makeup of an organism (Gilbert *et al*., 2009) and the more commonly used polymerase chain reaction (PCR) to amplify a gene of interest prior to further assessment (Manickavasagam *et al*., 2004; van de Vyver *et al*., 2013). Enzyme assays are used to identify changes in the catalytic activity of a specific enzyme when put under stress for example under the influence of a gene inhibiting herbicide (Adriano *et al*., 2013; Rutherford *et al*., *in press*). Van de Vyver *et al*. (2013) used PCR to identify the AHAS gene as a selectable marker in a herbicide tolerant genotype. That study utilized a plant gene that contained a mutant form of the tobacco AHAS gene which conferred resistance to herbicides. Previous studies have used PCR to identified mutations within the AHAS gene prior to sequencing (Newhouse *et al*., 1991; Bernasconi *et al*., 1995; Hattori *et al*., 1995; Bright *et al*., 1997; Devine and Eberlein, 1997; Dietrich, 1998; Bruniard, 2001; Croughan, 2003; Sala and Bulos, 2012; Lichtfouse, 2014; Yu and Powles, 2014a). As aforementioned, there are various mutations within the AHAS gene that confer tolerance to different herbicides including imazapyr. To date, only one mutation (Ala_{559}) within the sugarcane AHAS gene has been identified to confer tolerance to imazapyr in field-grown sugarcane (Punyadee *et al*., 2007; Khruangchan *et al*., 2011).

3. Materials and Methods

3.1 Plant material

Seven mutants of the N12 sugarcane genotype were produced by chemical mutagenesis using EMS at SASRI, Mount Edgecombe, KwaZulu-Natal, South Africa (Koch *et al*., 2012). Rutherford *et al*. (i*n press*) analysed these mutants for tolerance to imazapyr in the field and although no significant differences were observed amongst the mutants and the unmutated N12 in the unsprayed plots, when the plots were sprayed with different concentrations of Arsenal® GEN 2 (imazapyr), five mutants showed a higher tolerance to imazapyr. The mutant genotypes were then screened for tolerance to imazapyr by an *in vitro* enzyme assay that characterised the activity of the AHAS enzyme in the presence of the herbicide (Rutherford *et al*., *in press*). The outcome of that study indicated that three mutant genotypes (Mut 1, Mut 6 and Mut 7) had a higher AHAS enzyme activity than the other mutants and the unmutated N12 control. As this was an indication that these three mutant genotypes were more tolerant to imazapyr, they were selected for this study.

3.2 Field assessment of Mut 1, Mut 6 and Mut 7 compared with the unmutated N12 control

3.2.1. Field trial design

The agronomic field trial was planted in October 2013 at SASRI. It consisted of 20 plots with 2 rows of 10 m in length and the plots were separated by a 2 m gap (Figure 3). The unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes were planted as five replicates using a randomised block design.

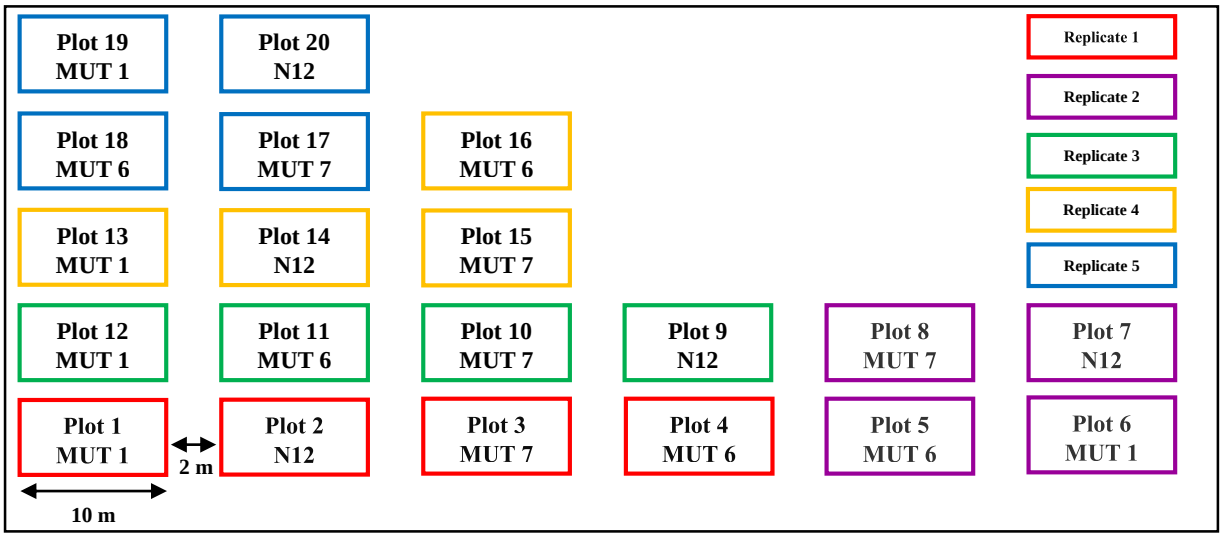

Figure 3: Representation of the way in which the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes were planted within the agronomic field trial. The planting of the mutants and the unmutated N12 was replicated in five plots using a randomised block design. Each plot consisted of 2 rows of 10 m in length and plots were separated by a 2 m gap.

3.2.2 Agronomic assessment of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes

An agronomic assessment of the plants was done after 18 months (when the cane was mature) in the field (April 2015) and included the number of stalks.plot^{-1}, stalk height (cm) and stalk diameter (cm) measurements which were taken from 20 randomly selected stalks. A millroom analysis of sucrose (g.100g^{-1} fresh weight) and fibre content (g.100g^{-1} fresh weight) (Schoonees-Muir *et al*., 2009), and an *E. saccharina* assessment (% internodes bored) were performed on 12 and 15 randomly selected stalks.plot^{-1}, respectively. The percentage of internodes bored by *E. saccharina* was calculated based on the total number of nodes and the number of damaged nodes within each stalk. The recoverable value sucrose content (RV per 100g fresh mass) was calculated in order to measure the sugar and molasses that will be recovered from each genotype (Cane Testing Service, South African Sugar Association, 2015).

3.3 Response of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes to the control of *Cynodon dactylon* by Arsenal® GEN 2 (imazapyr)

3.3.1 Field trial design

A field trial was conducted to assess the response of Mut 1, Mut 6 and Mut 7 compared with the unmutated N12 plants, after the field was sprayed with Arsenal® GEN 2 (1254 g a.i.ha^{-1} imazapyr, BASF®) to control *C. dactylon* (refer to section 3.3.2). The field trial was planted in November 2014 and was divided into three *C. dactylon* management regimes (T1, T2, T3) (Table 6). Each treatment consisted of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes which were planted as three replicates using a randomised block design. Each plot consisted of three rows of 4 m in length and plots were separated by a 0.5 m gap (Figure 4). The sugarcane was planted as vegetative stem sections/setts within the furrows and tufts of *C. dactylon* (2 x 2 cm) were planted every 0.5 m within the inter-rows of T2 and T3 with eight tufts per row. A net row of 3 m was marked within each plot to exclude the edge effect when the herbicide was applied and to act as a buffer between plots to prevent contact by drift or movement of the herbicide in the soil. All sugarcane and *C. dactylon* analyses were performed within the net row of each plot. All fields were hand weeded prior to planting the sugarcane and *C. dactylon*.

Table 6: *Cynodon dactylon* management regimes used to evaluate and compare the effect of Arsenal® GEN 2 (imazapyr, 1254 g a.i.ha^{-1}) on the unmutated N12, Mut 1, Mut 6 and Mut 7 plants.

Treatment	*Cynodon dactylon* planted	Application of imazapyr	Weed control method	Frequency of weed control	Reason
T1 Hand weeded	No	No	1. Hand weeding of all weeds. 2. No herbicide applied.	Hand weeding was performed at 0 and 4 weeks after herbicide application.	To compare the phenotypic characteristics of the sugarcane when no herbicides were applied.
T2 Commercial herbicide cocktail	Yes	No	1. Hand weeding of broad leaf weeds. 2. Cocktail of Gramoxone® (200 g a.i.ha^{-1} paraquat) and Diuron® (800 g a.i.ha^{-1} diuron) to control *C. dactylon*.	Hand weeding was performed at 0 weeks only and the herbicide cocktail was applied at 0 and 4 weeks.	The commercially used chemical method for controlling *C. dactylon*.
T3 Arsenal® GEN 2	Yes	Yes	1. Hand weeding of broadleaf weeds. 2. Application of Arsenal® GEN 2 (1254 g a.i.ha^{-1} imazapyr) to control *C. dactylon*.	Hand weeding was performed at 0 weeks only and Arsenal® GEN 2 was applied at 0 weeks.	The proposed chemical method of controlling *C. dactylon*. This method is slow acting but more effective due to its persistent residual soil activity (Anon, 2014).

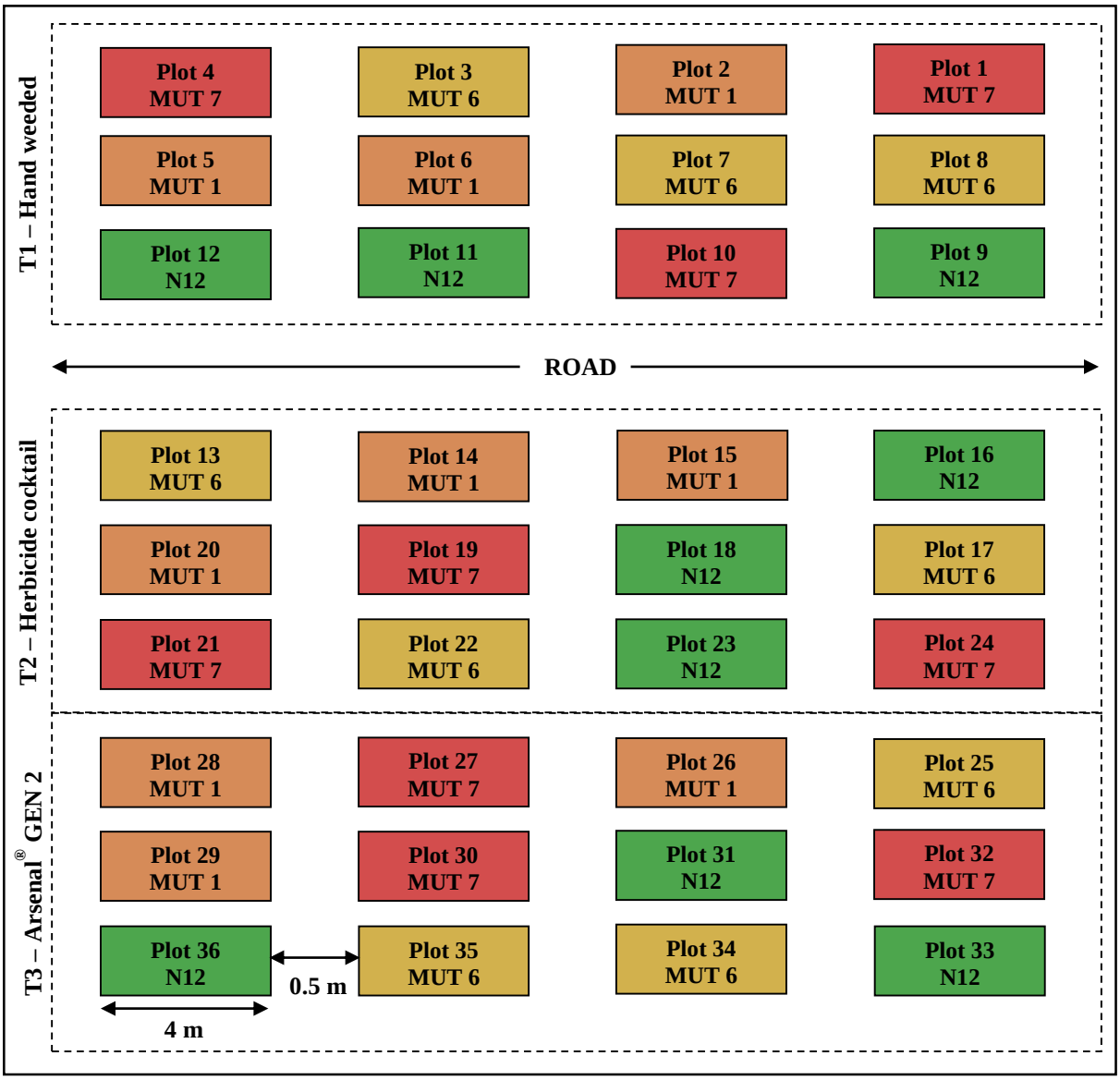

Figure 4: Representation of the way in which the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes were planted within the different treatments: **(T1)** hand weeded; **(T2)** herbicide cocktail; **(T3)** Arsenal® GEN 2, of the *Cynodon dactylon* field trial. The planting of the unmutated N12 and mutant genotypes was replicated in three plots within each treatment using a randomised block design. Each plot consisted of three rows of 4 m in length and plots were separated by a 0.5 m gap.

3.3.1 Herbicide treatment

All herbicides were applied at 2.5 months, post-emergence of the sugarcane and *C. dactylon*, to the soil using a commercial knapsack sprayer (OSATU®). Care was taken to prevent the herbicides from reaching the sugarcane in order to reduce damage. This was achieved by

erecting a cardboard shield on either side of each inter-row to block the cane from herbicide contact due to drift. The *C. dactylon* in T2 was treated with two applications of a conventionally used commercial herbicide cocktail containing Gramoxone® (200 g a.i.ha^{-1} paraquat, Syngenta®) and Diuron® (800 g a.i. ha^{-1} diuron, Arysta Lifescience®) (Table 6). In T3 a single dose of Arsenal® GEN 2 (1254 g a.i.h^{-1} imazapyr) was applied to the *C. dactylon* at 0 weeks (Table 6).

3.3.2 Measurement of plant injury caused by herbicide application on the sugarcane

3.3.3.1 Phenotypic assessment

A phenotypic assessment of the number of stalks.$plot^{-1}$ and stalk height (cm) was performed to assess the effect of the herbicides on the growth of the sugarcane after herbicide application. A total of five stalks were marked for measurement of stalk height within each plot prior to herbicide application. The measurements of the number of stalks.$plot^{-1}$ and stalk height were taken at 0, 2, 4, 6, 8, 12, 16 and 20 weeks after herbicide application. Only the viable (living) plants were measured and counted.

3.3.3.2 Leaf chlorophyll content

The leaf chlorophyll content (measured in SPAD units) of the unmutated N12, Mut 1, Mut 6, and Mut 7 plants at 0, 2, 4, 8, 12, 16 and 20 weeks after herbicide application, was measured using a soil plant analysis development device (SPAD-502 Plus Minolta). A total of five plants were marked for leaf chlorophyll measurements within each plot prior to herbicide application. Measurements were taken from the third leaf of each plant with three replicate measurements per plant and five plants per plot for each treatment. Leaf chlorophyll measurements were taken from the viable plants only.

3.4 Measurement of the effect of herbicide application on the percentage green of *Cynodon dactylon*

Tufts of *C. dactylon* (2 x 2 cm) were planted within the inter-rows of the herbicide cocktail (T2) and Arsenal® GEN 2 (T3) treatments (Table 6 and Figure 4). Two regions of *C. dactylon* (0.55 x 0.55 m) were marked per plot within these two treatments. The effect of Arsenal® GEN 2 in controlling *C. dactylon* was compared with that of the herbicide cocktail using a digital image analysis which measured the percentage of green *C. dactylon* leaves at 0, 2, 4, 8, 10, 12, 16 and 20 weeks after herbicide application (Campbell *et al*., 2008). The percentage of green *C. dactylon* leaves was measured by quantifying the percentage of green pixels in a digital image bordered by a neon orange quadrat (0.55 x 0.55 m), using an AutoWeedCover software designed at SASRI (Figure 5) (Campbell *et al*., 2008). The output generated included the percentage green and its associated image identification (Figure 6). Images of each region were captured using a Canon 650D camera and processed using the AutoWeedCover software.

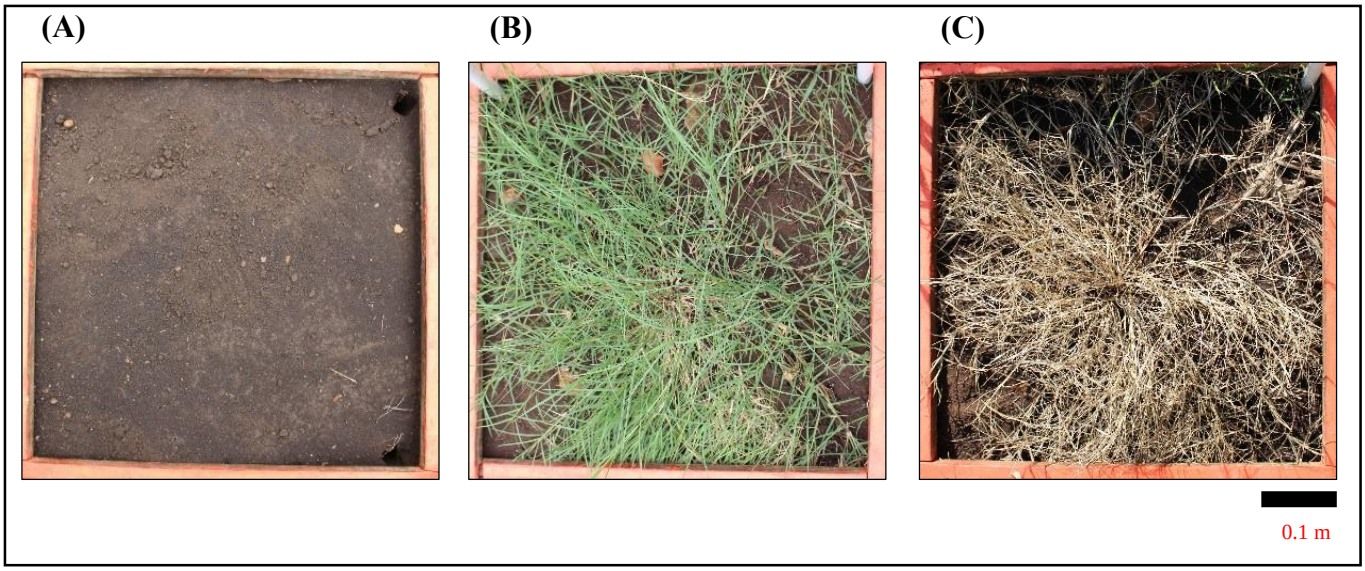

Figure 5: Regions of *Cynodon dactylon* marked using a neon orange quadrat for digital image analysis. **(A)** A control quadrat used in T1 which excludes the target *Cynodon dactylon*. **(B)** An experimental quadrat with *Cynodon dactylon* used in T2 (herbicide cocktail treatment involving Gramoxone® and Diuron®) and T3 (Arsenal® GEN 2 treatment) before herbicide application (t = 0). **(C)** An experimental quadrat with *Cynodon dactylon* from T2, 2 weeks after the herbicide cocktail application.

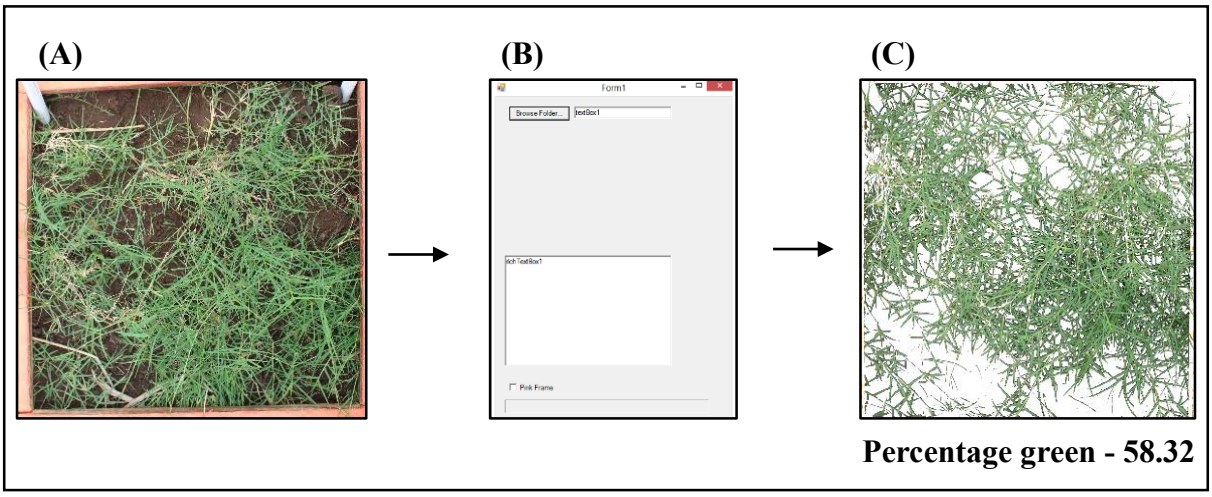

Figure 6: Representation of the digital image analysis methodology. All photographs were cropped **(A)** and uploaded into the AutoWeedCover software **(B)** which calculated the percentage of green pixels within the neon orange quadrat as shown around **(A)**. The output generated produced an image showing the percentage cover of green pixels (percentage green) within the boundaries of the neon orange quadrat **(C)**.

3.5 Characterisation of the mode of imazapyr tolerance in the Mut 1, Mut 6 and Mut 7 genotypes

3.5.1 Sugarcane acetohydroxyacid synthase gene sequencing

3.5.1.1 Experimental design used to amplify, sequence and identify mutations within the acetohydroxyacid synthase gene

The sugarcane AHAS gene was amplified and sequenced using optimized reactions (see sections 3.5.4, 3.5.5 and 3.5.6). The AHAS gene sequences of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes were analysed and AHAS mutations were identified using the approach illustrated in Figure 7.

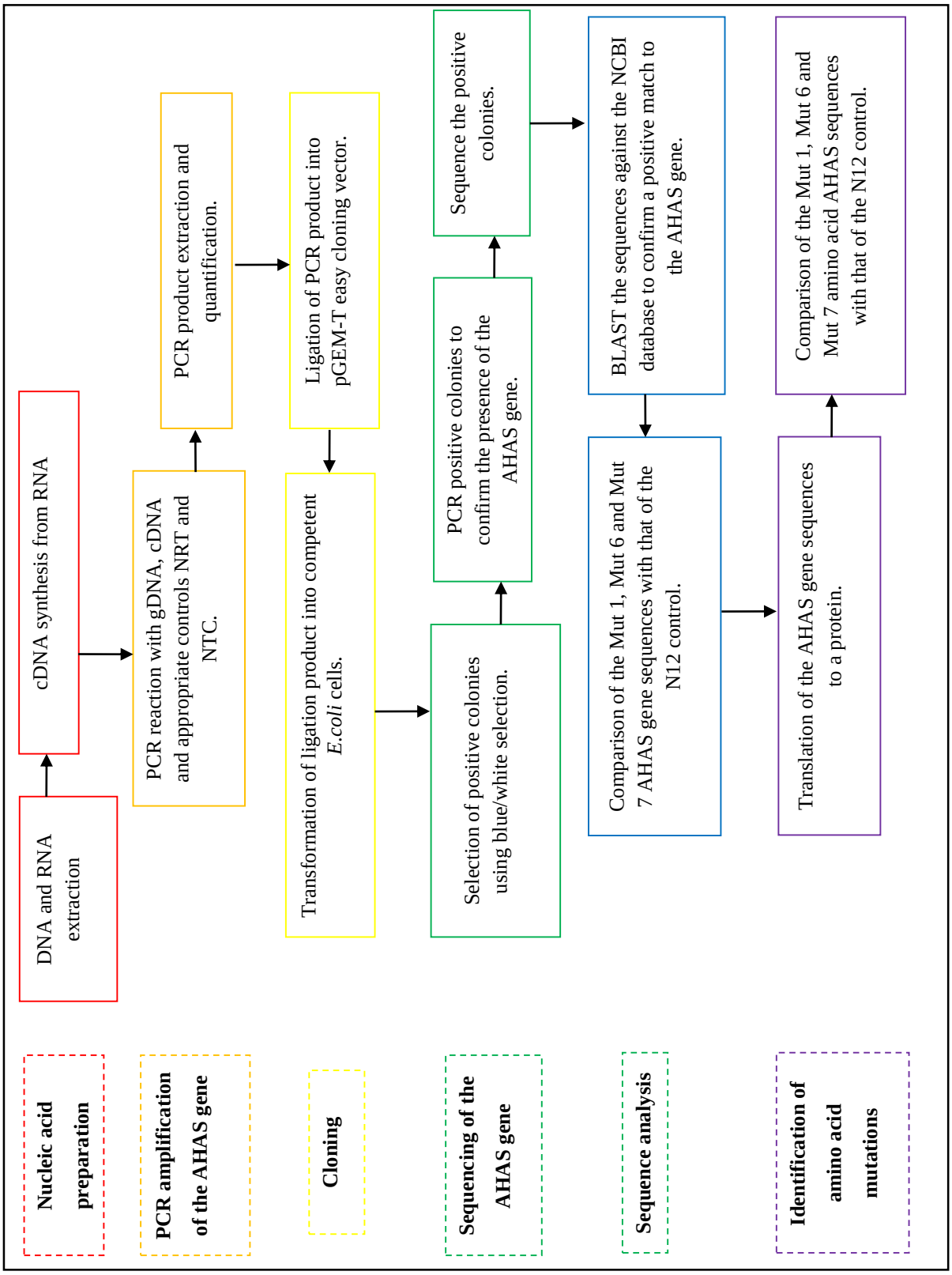

Figure 7: Experimental approach used for amplification, cloning, sequencing and identification of amino acid mutations within the acetohydroxyacid synthase gene of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes. cDNA – complementary DNA; gDNA – genomic DNA; NRT – no reverse transcriptase; NTC – no template control.

3.5.1.2 Plant material and nucleic acid preparation

The third, fully expanded leaf of each sugarcane genotype was selected for both genomic DNA (gDNA) and RNA extraction. Genomic DNA extraction was conducted using a DNeasy plant mini kit (Qiagen®), extracted samples were quantified with the Nanodrop 2000c UV-Vis Spectrophotometer (Thermo Scientific®) and stored at -20 °C. Prior to storage, all DNA samples were visualised on a 1% agarose gel run at 60 V for 1 hr. Similarly, RNA extraction was conducted using a RNeasy plant mini kit (Qiagen®), the extracted samples were analysed by gel electrophoresis, quantified with the Nanodrop 2000c UV-Vis Spectrophotometer, aliquoted as 2 µg samples and stored at -80 °C. Prior to storage, a total of 2 µg RNA in combination with a sample buffer and loading dye (ratio 1:6:1) were combined and heated for 5 min at 60 °C. The RNA samples were then loaded onto a 1.2 % agarose gel containing ethidium bromide (8 µL ethidium.120 ml agarose gel) and run at 60 V for 1 hr. The gels were then visualised under UV light.

Complementary first strand DNA (cDNA) was synthesised from a 2 µg RNA sample treated with DNase I (Thermo Scientific®) to remove any gDNA contaminants. This was carried out by combining the 2 µg RNA sample with a 2 µL DNase buffer (Thermo Scientific®), 3 µL DNase enzyme (Thermo Scientific®), 0.5 µL RiboLock RNase inhibitor (Thermo Scientific®) and nuclease free water to bring the total volume to 20 µL. The reagents were mixed by inverting the tube, spun down using a benchtop centrifuge and heated at 37 °C for 30 min after which 2 µL of ethylenediaminetetraacetic acid (EDTA) was added. The reagents were heated at 65 °C for 10 min and aliquoted into two tubes each with 11 µL: (1) cDNA synthesis; (2) No reverse transcriptase (NRT). The first strand cDNA synthesis protocol was carried out in two steps. Step 1: Each DNase treated RNA sample was combined with 1 µL random hexamer primer (10 mM) (Sigma Aldrich®), 1 µL oligo dT primer (10 mM) (Sigma Aldrich®), 1 µL dNTP mix (10 mM) (Kapa Biosystems®) and 0.5 µL nuclease free water, mixed by inversion, heated at 65 °C for 5min and cooled on ice. Step 2: To tube 1 and 2, 4 µL 5 X RT buffer and 0.5 µL RiboLock RNase inhibitor was added. To tube 1, 1 µL Maxima Reverse Transcriptase enzyme was added to initiate cDNA synthase and to tube 2, 1 µL nuclease free water was added. The reagents in each tube were mixed, spun down using a benchtop centrifuge, kept at room temperature for 10 min, heated at 50 °C for 30 min and lastly heated at 85 °C for 5 min.

3.5.1.3 Primer design used to amplify the sugarcane acetohydroxyacid synthase gene

Acetohydroxyacid synthase gene sequences from *Sorghum bicolor* (Accession number: KJ538787.1), *Zea mays* (Accession numbers: EE181466.2, EE181466.2) and *Saccharum officinarum* (Accession numbers: CA077116.1, CA095528.1, CA114581.1 and CA212516.1) were used to generate a consensus sequence (Appendix IA). Although three sets of primers (Table 7: Primer set A, B and C) were designed based on the consensus sequence there were difficulties associated with reproducing the results of the PCR using Primer set A and C (Table 7). As a result, successful PCR products of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes were sequenced based on the experimental approach illustrated in 3.5.1 to generate a new consensus sequence (Appendix IB). Subsequently, multiple primer sets were designed based on the new consensus sequence to amplify the AHAS gene (Table 7: Primer set 1-5, AHAS 1, AHAS 2, AHAS 3). Primers were designed using Primer3web (version 4.0.0, http://primer3.ut.ee/) and analysed for possible hairpins, self-dimers and hetero-dimers using OligoAnalyzer (version 3.0) (Integrated DNA Technologies®).

Table 7: A summary of primer sequences designed based on an acetohydroxyacid synthase consensus sequence of *Sorghum bicolor*, *Zea mays* and *Saccharum officinarum* (Appendix IA and Appendix IB), their corresponding PCR amplicon sizes and recommended annealing temperatures.

Primer set	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size/bp	Annealing temperature (°C)
A	GAGATCCACCAGGCACTCAC	CACATGTGGCTGCTTGTTCT	855	61
B	CGGTTTGATGATCGTGTGAC	GCGGACTTCGTTCTTCTTTG	757	61
C	AGACAGCCGCCGCAAC	ATCTGCTGCTGGATGTCCTT	715	60
1	CGCAAGAGTTCGTGGGAAT	AACCTAGTCTCCGCGCTC	1068	60 - 63
2	TCGTCCACAGTCAAGTCCAA	GAAGAGGTGGTTGGCGATGA	691	60 - 63
3	TCACCATCTGAGCCACACAT	GAGGACCAGGTAGTTGTGCT	728	60 – 63
4	TCAAAACCTCCTTGACGCAC	GGAAGAGGTGGTTGGCGA	646	60 - 63
5	AAAAGCAGGGAGGCCTCTAC	GAGGACCAGGTAGTTGTGCT	702	60 - 63
AHAS 1	TCATCGCCAACCACCTCTTC	TCACACGATCATCAAACCGC	732	60
AHAS 2	AGCACAACTACCTGGTCCTC	CCAACTCATCGTGCCATGAG	675	60
AHAS 3	CGGTTTGATGATCGTGTGAC	CAAAGAAGAACGAAGTCCGC	750	61

3.5.1.4 PCR amplification of the acetohydroxyacid synthase gene

Polymerase chain reactions were performed using the KAPA2G Robust Hotstart PCR kit (Kapa Biosystems®) in a total volume of 25 µl (Table 8). The optimized PCR reaction involved a 1 min initial denaturation step at 95 °C followed by 35 cycles of 94 °C for 30 sec, 58-62 °C for 30 sec and 72°C for 1 min with a final elongation step of 72 °C for 4 min. Each PCR reaction consisted of three controls: (1) gDNA to confirm the correct product size; (2) no reverse transcriptase (NRT) to confirm that the cDNA synthesised was not contaminated with gDNA;

(3) a no template control (NTC) to confirm that the reagents were not contaminated. The PCR products were visualised by gel electrophoresis on a 1.5% agarose gel run at 80 V for 1 hr. Positive cDNA products were extracted from the agarose gel using a Gel Extraction Kit (Zymogen®), quantified with the Nanodrop Spectrophotometer and stored at -20 °C.

Table 8: Reagents used in a single PCR reaction and their corresponding initial and final concentrations.

Reagent	Initial concentration	Final concentration	Volume required for 1 reaction (µL)
Nuclease free water	-	-	[1] X
5X KAPA 2G Buffer A	5 X	1 X	5
dNTP mix	10 mM	0.2 mM	0.5
Forward primer	10 µM	0.1 µM	0.25
Reverse primer	10 µM	0.1 µM	0.25
Kapa2GTaq DNA polymerase	5 U/µL	0.5 U	0.1
DNA template	-	-	[1] Y
Total volume	-	-	25

[1] X and Y were adjusted in accordance with the concentration of the DNA template.

Three primer sets (Table 7: AHAS 1, AHAS 2, AHAS 3) designed based on the AHAS consensus sequence (Appendix IB) were successful in amplifying 1463 base pairs of the sugarcane AHAS gene. The annealing temperature (Tm°) of each primer (P) set was calculated as:

$$Tm^{\circ} = \frac{(Tm^{\circ} \text{ forward P} + Tm^{\circ} \text{ reverse P})}{2}$$

Each primer set was optimized using ~100 ng of gDNA of the unmutated N12 genotype as a DNA source (Figure 8). Thereafter the optimized reactions were tested on ~1500 ng cDNA of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes (Figure 9). High concentrations of cDNA (~1500 ng) were used to ensure that sufficient PCR product was produced for subsequent ligation into cloning vectors. All PCR products were visualised on a 1.5 % agarose gel run at 80 V for 1 hr. It was observed that the gDNA PCR products migrated faster than that of the cDNA PCR products. These gDNA and cDNA PCR products were sequenced in order to confirm that they were AHAS genes.

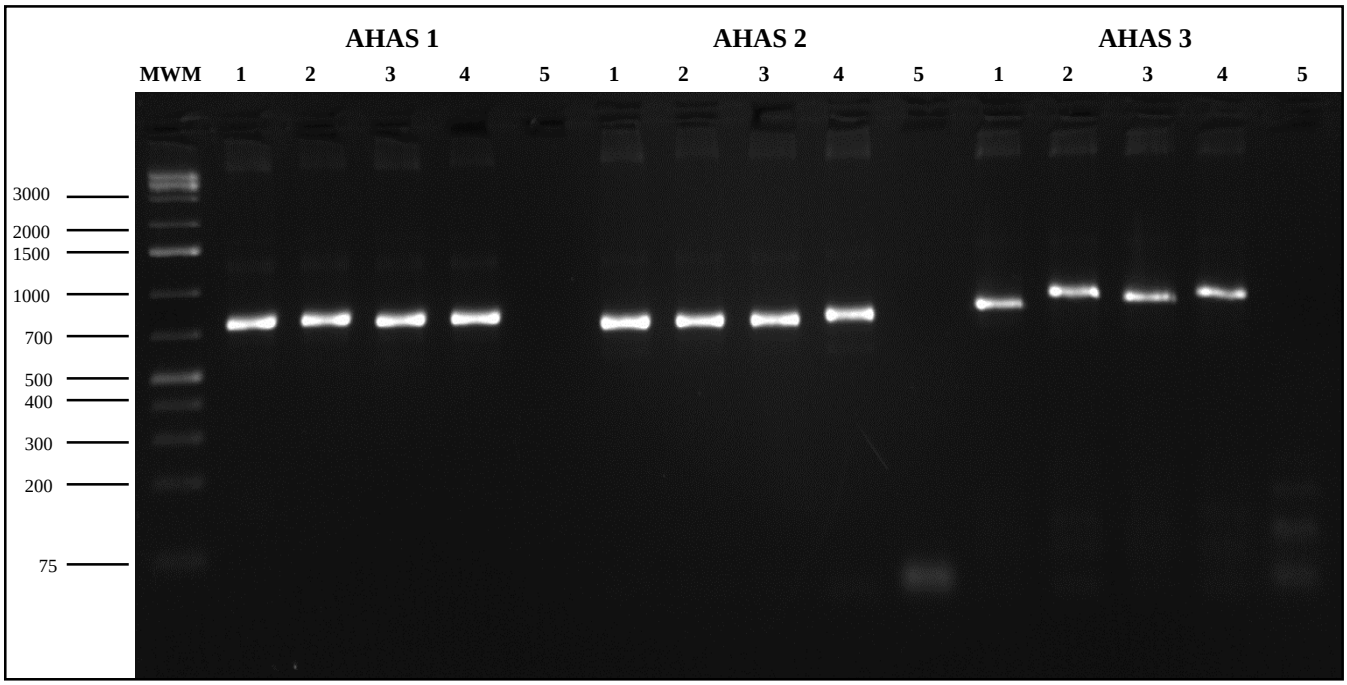

Figure 8: Optimized acetohydroxyacid synthase PCR reactions using primer sets AHAS 1, AHAS 2 and AHAS 3 on genomic DNA of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes. MWM – Molecular weight marker; Lane 1 – unmutated N12; Lane 2 – Mut 1; Lane 3 – Mut 6; Lane 4 – Mut 7; Lane 5 – no template control.

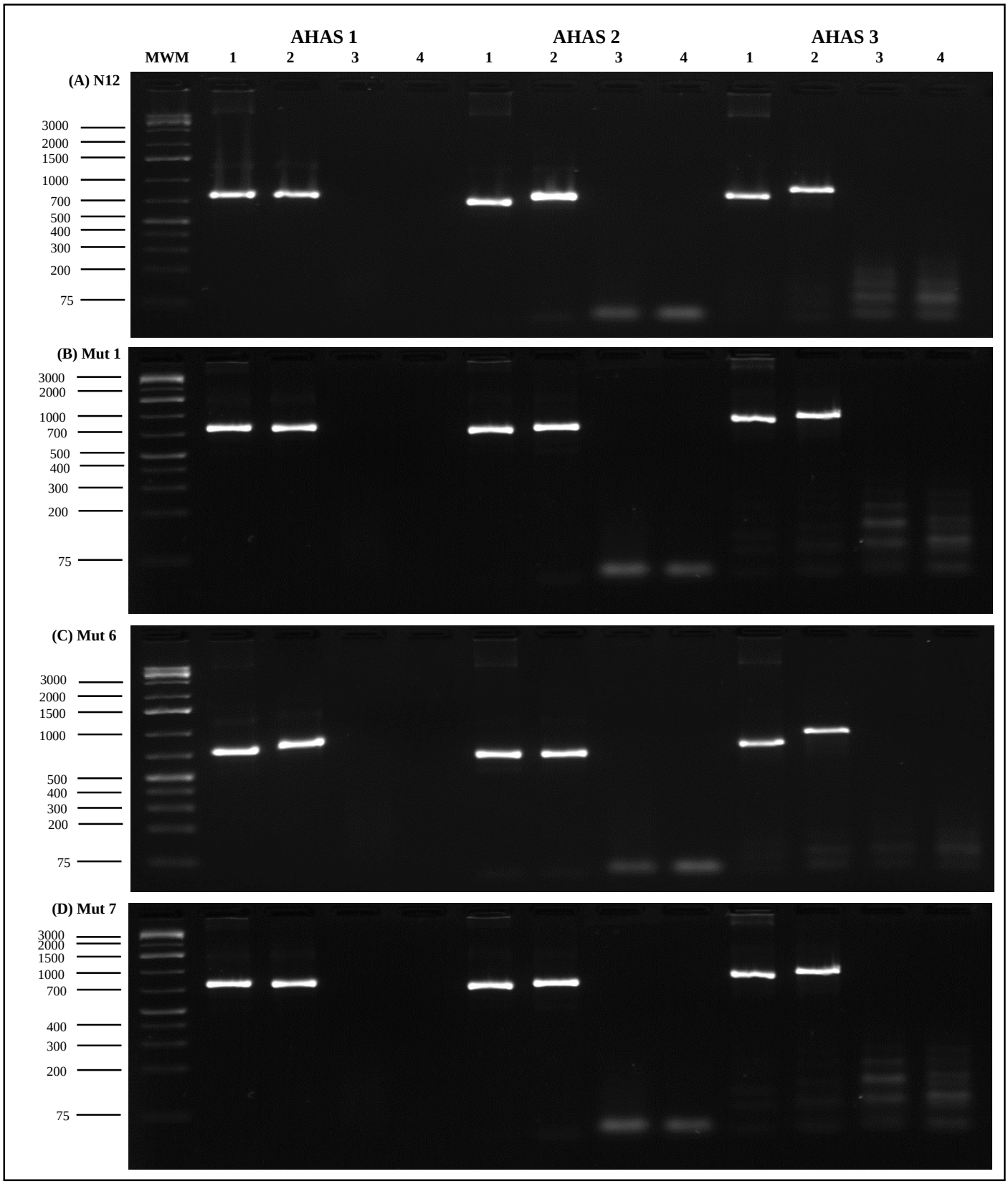

Figure 9: Optimized acetohydroxyacid synthase PCR reactions using primer sets AHAS 1, AHAS 2 and AHAS 3 on genomic DNA and complementary DNA of **(A)** unmutated N12, **(B)** Mut 1, **(C)** Mut 6 and **(D)** Mut 7 genotypes. MWM – Molecular weight marker; Lane 1 – gDNA; Lane 2 – cDNA; Lane 3 – no reverse transcriptase; Lane 4 – no template control.

3.5.1.5 Cloning and sequencing

The PCR products were ligated into pGEM-T Easy cloning vector (Promega®) and transformed using competent *Escherichia coli* cells (Untergasser, 2008). The PCR product was combined with the ligation reagents and incubated for 1 hr at room temperature (Table 9). The volume of PCR template required for the ligation reaction was calculated as follows:

$$\text{Volume of PCR template } (\mu L) = \frac{50\ ng \text{ vector x size of insert (kb)}}{3.0 \text{ kb (size of vector)}} \text{ X } \frac{3}{1}$$

Table 9: Ligation reaction components and their corresponding volumes required for a single ligation reaction.

Reaction component	Volume required for a single reaction (µL)
Nuclease free water	[1]X
2X Rapid Ligation Buffer	5
pGEM-T Easy Vector (50 *ng*)	1
T4 DNA Ligase	1
PCR temple	[1]Y

[1] X and Y were adjusted in accordance with the volume of the PCR template.

The transformation protocol was carried out by adding 1 µL of the ligation product to 50 µL of competent *E. coli* cells. The components were mixed by pipetting, transferred to an ice cold electroporation cuvette and kept on ice. The *E. coli* cells were then exposed to a single electric pulse (2.5 kV) delivered by a MicroPulser electroporator (Bio-Rad Laboratories®) and immediately thereafter, the suspension was mixed with 450 µL of Luria-Bertani (LB) medium (Maniatis *et al*., 1982) containing 10 $g.L^{-1}$ tryptone powder, 5 $g.L^{-1}$ yeast extract and 5 $g.L^{-1}$ sodium chloride (All Merck®). The solution was transferred into a microcentrifuge tube and incubated at 37 °C for 1 hr. A 100 µL volume of the transformation products were plated onto LB agar plates containing 15 $g.L^{-1}$ agar, ampicillin (0.1 $mg.L^{-1}$), Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 M) and 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-Gal) (0.012 M) (All Sigma Aldrich®). Transformed/positive colonies were identified by blue/white selection and the presence of the AHAS gene was confirmed by PCR using the reaction conditions outlined in section 3.5.1.4. The PCR products were purified using

a DNA Clean and Concentrator Kit (Zymo Research®), quantified with a Nanodrop spectrophotometer and used as the template for DNA sequencing analysis.

Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®) and 150-300 ng DNA template in a total volume of 20 µl (Table 10). The thermal cycling profile involved a 1 min initial denaturation step at 96 °C followed by 25 cycles of 96 °C for 10 sec, 50 °C for 0.05 sec and 60 °C for 4 min. A total of 8 colonies were sequenced per primer set for the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes using an Applied Biosystems® 3500 Genetic Analyser.

Table 10: A summary of the reagents required for a single sequencing reaction.

Reagent	Volume required for 1 reaction (µL)
BigDye™ Terminator 3.1 Ready reaction mix	4
BigDye™ Terminator v3.1 5X sequencing buffer	2
[1]Primer (10 µM)	0.5
DNA template	[2] X
Nuclease free water	[2] Y
Total volume	20

[1] Specific forward and reverse primers were used (Table 7: AHAS 1, AHAS 2 and AHAS 3) and sequencing reactions were performed separately.

[2] X and Y were adjusted in accordance with the concentration of the DNA template.

3.5.1.6 Sequence analysis

The sequences generated by the Applied Biosystems® Genetic Analyser 3500 were compared with the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm to confirm a positive match to the AHAS gene (97 – 100 % identity match). Geneious® was then used to generate a consensus sequence for each primer set and each genotype individually and thereafter a consensus sequence of the AHAS gene was generated for the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes. The AHAS gene sequences of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes were aligned and compared in order to identify point mutations within the expressed gene. Each consensus sequence was further translated into an amino acid sequence. The AHAS

amino acid sequences of the mutants were compared with that of the unmutated N12 control to determine the position of amino acid mutations within the protein.

3.5.2 Detoxification of imazapyr by cytochrome P450

3.5.2.1 Experimental design used to determine the concentrations of piperonyl butoxide (PBO) and imazapyr required for the detoxification trial

Single budded setts of the unmutated N12 genotype were germinated in peat moss and vermiculite (ratio 1:1) and watered twice a day for 4 weeks. The plants were transferred to pots (10 cm diameter x 10 cm height) containing Umgeni sand, placed into plastic troughs (20 L) with 2 L of a nutrient solution (5 ml.5 L^{-1} Eezi-Fert®) and plant height (cm) and leaf chlorophyll measurements (SPAD unit) were taken (t = 0). After 1 week (t = 1) the plants were exposed to different concentrations of PBO (0, 25 and 50 µM) in order to determine a concentration of PBO that did not cause injury to the plants. Similarly, plants were treated with different concentrations of an analytical standard of imazapyr (Pestanal®, Sigma Aldrich®) (0, 1, 10, 25, 50, 75 and 100 µM) in order to determine which concentration caused visible injury to the unmutated N12 plants within the shortest period of time. This was achieved by adding the imazapyr to the nutrient solution which prevented the foliar uptake of the herbicide by the plant. Plant height and leaf chlorophyll measurements were taken before treatment with PBO and imazapyr (t = 0) and every week thereafter for 4 weeks (total duration of 5 weeks) after which time the fresh and dry mass of the root and shoot samples were determined.

3.5.2.2 Determination of piperonyl butoxide concentration required for the detoxification trial

The concentration of PBO required for the detoxification trial was determined as per section 3.6.1. Data sets were analysed using the Genstat statistical package 17th edition and a one-way ANOVA and Sidak post hoc test were used to test for statistically significant differences. Comparisons were made over time for each treatment and across treatments for each time interval. This analysis was done in order identify the highest concentration of PBO that did not cause a significant degree of plant injury compared with that of the control treatment.

Based on the results obtained from the plant height and leaf chlorophyll measurements as presented in the results section 4.3.2.1, 50 µM PBO was selected for the subsequent pot trial and plants were treated with the chemical for a total of 4 weeks.

3.5.2.3 Determination of imazapyr concentration required for the detoxification trial

The concentration of imazapyr required for the detoxification trial was determined as per 3.6.1. Data sets were analysed using the Genstat statistical package 17th edition and a one-way ANOVA and Sidak post hoc test were used to test for statistically significant differences. Comparisons were made over time for each treatment in order to identify the highest concentration of imazapyr that caused visible injury to the plants within the shortest time.

Based on the results obtained from the plant height and leaf chlorophyll measurements as presented in the results section 4.3.2.1, plants were treated with 100 µM imazapyr, with and without 50 µM PBO in the subsequent pot trial for a total of 3 weeks.

3.5.2.4 Assessing the potential of an enhanced detoxification of imazapyr by cytochrome P450

Single budded setts of the unmutated N12, Mut 1, Mut 6 and Mut 7 were germinated and transferred into pots as described in section 3.5.2.1. Plants were exposed to a control treatment (0 µM PBO + 0 µM imazapyr), 50 µM PBO, 100 µM imazapyr alone and in combination with 50 µM PBO and each treatment consisted of five plant replicates. The plants were exposed to the nutrient solution for 1 week, followed by PBO for 1 week, after which time imazapyr was added to the nutrient solution. Plant height and leaf chlorophyll readings were taken before exposure to imazapyr (t = 0) and every week thereafter for 3 weeks after which time the fresh and dry root and shoot mass was obtained.

3.6 Data analyses

All data sets were analysed using the Genstat statistical package 17th edition (VSN International UK). A one-way Analysis of Variance (ANOVA) and Sidak post hoc test were used to test for statistical differences: (a) amongst the unmutated N12, Mut 1, Mut 6 and Mut 7 in section 3.2.2,

(b) amongst treatments for each genotype and amongst genotypes for each treatment in section 3.3.3 and (c) across time for each genotype in each treatment and across treatments for each genotype sections 3.5.2.2 and 3.5.2.3. A two-way T-test was carried out to test for statistically significant differences between the commercial herbicide cocktail and Arsenal® GEN 2 in section 3.4.

4. Results

As previously mentioned, Koch *et al*. (2012), produced seven N12 mutant genotypes using EMS at SASRI. Rutherford *et al*. (*in press*) continued that study by conducting a field analysis to compare the agronomic characteristics of the mutants to that of the unmutated N12, in order to assess if the chemical mutagenesis resulted in changes to the phenotypic characteristics of these mutants. The results of that study indicated that no significant differences were found amongst genotypes in the unsprayed field. When the field was sprayed with Arsenal® GEN 2 (imazapyr), five mutants showed a higher tolerance to the herbicide compared with the unmutated N12. The mutants were then screened for tolerance to imazapyr using an *in vitro* enzyme assay that characterised the activity of the AHAS gene in the presence of the herbicide (Rutherford *et al*., *in press*). The results of that study indicated that Mut 1, Mut 6 and Mut 7 were more tolerant to imazapyr because they displayed a higher enzyme activity than the other mutants and the unmutated N12.

4.1 Agronomic evaluation of Mut 1, Mut 6 and Mut 7 compared with the unmutated N12 genotype

Due to the random effect of the EMS chemical mutagenesis on the sugarcane genome the agronomic traits of the mutants were assessed to identify if any significant changes occurred as a result of the mutagenesis. Although this assessment was conducted previously by Rutherford *et al*. (*in press*), in the current study more plant replicates were used in the agronomic assessment. The agronomic traits of the mutants and unmutated N12 genotype were assessed after 18 months in the field. The assessed traits included number of stalks.plot^{-1}, stalk height (cm), stalk diameter (cm), estimated yield (kg.plot^{-1}), fibre content (g.100g^{-1} fresh weight), sucrose content (g.100g^{-1} fresh weight) and recoverable value sucrose content (RV per 100g fresh mass/RV % cane). The RV % cane is a measurement of the sugar and molasses recovered from each genotype (Cane Testing Service, South African Sugar Association, 2015). The mutant genotypes were also assessed for resistance to *E. saccharina* determined by the percentage of internodes bored by the pest.

No significant differences ($P \geq 0.05$) were found in the number of stalks.plot^{-1}, stalk height or stalk diameter amongst all genotypes (Table 11). The stalk diameter of the unmutated N12 plants was significantly wider ($P \leq 0.05$) than that of the mutant genotypes ($P \leq 0.001$). The estimated yield (kg fresh mass above ground) of the unmutated N12 genotype was significantly

higher ($P \leq 0.05$) than that of the Mut 1 genotype ($P = 0.004$) (Table 11). No significant differences ($P \geq 0.05$) in fibre and sucrose content were found amongst genotypes ($P = 0.487$ and 0.929 for each trait, respectively) (Table 11). The RV % cane of Mut 1 was significantly higher ($P \leq 0.05$) than that of Mut 7 and no significant differences ($P \geq 0.05$) in RV % cane were found amongst the Mut 1, Mut 6 and N12 plants ($P = 0.014$) (Table 11).

From previous work conducted at SASRI, the unmutated N12 genotype is known to have an intermediate resistance to the sugarcane borer *E. saccharina*, therefore an assessment was done to see if the chemical mutagenesis resulted in a change in resistance of the mutant genotypes to the pest compared with the unmutated N12 genotype (Anon, 2005). This was achieved by calculating the percentage of internodes bored by the pest (Anon, 2005). A significantly higher percentage ($P \leq 0.05$) of internodes were bored by *E. saccharina* in Mut 1 than in the unmutated N12 plants ($P = 0.013$) (Table 12).

Table 11: An assessment of agronomic traits of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes after 18 months in the field. Dissimilar alphabet characters denote statistically significant differences amongst genotypes in each row. Data sets were analysed using a one-way ANOVA and a Sidak post hoc test. $P \geq 0.05$, n = 20, mean ± SE.

Agronomic trait	**Genotype**				***P value***
	N12	**Mut 1**	**Mut 6**	**Mut 7**	
Number of stalks (per plot)	367.40 ± 14.49 [a]	359.40 ± 23.83 [a]	404.20 ± 20.79 [a]	409.80 ± 4.28 [a]	0.120
Stalk height (cm)	169.30 ± 4.88 [a]	155.70 ± 4.12 [a]	161.30 ± 6.35 [a]	170.60 ± 5.14 [a]	0.124
Stalk diameter (cm)	23.31 ± 0.75 [b]	19.38 ± 0.15 [a]	20.42 ± 0.40 [a]	20.20 ± 0.25 [a]	≤0.001
Estimated yield ($kg.plot^{-1}$)	189.73 ± 9.79 [b]	120.52 ± 10.59 [a]	155.22 ± 20.10 [ab]	161.43 ± 11.72 [ab]	0.004
Fibre ($g.100g^{-1}$ fresh weight)	14.87 ± 0.49 [a]	14.44 ± 0.36 [a]	14.50 ± 0.25 [a]	15.14 ± 0.42 [a]	0.487
Sucrose ($g.100g^{-1}$ fresh weight)	11.11 ± 0.44 [a]	11.01 ± 0.45 [a]	10.91 ± 0.50 [a]	11.34 ± 0.43 [a]	0.929
Recoverable value sucrose content (RV per 100g fresh mass)	5.63 ± 0.31 [ab]	6.23 ± 0.10 [b]	5.46 ± 0.24 [ab]	5.07 ± 0.22 [a]	0.014

Table 12: An assessment of the damage caused by *Eldana saccharina* based on the percentage of internodes bored within the stalks of the unmutated N12, Mut 1, Mut 6 and Mut 7, 18 months after being in the field. Dissimilar alphabet characters denote statistically significant differences amongst genotypes. Data sets were analysed using a one-way ANOVA and a Sidak post hoc test. $P \geq 0.05$, n = 20, mean ± SE.

Genotype	**Internodes Bored by *Eldana saccharina* (%)**
Unmutated N12	3.89 ± 0.52 [a]
Mut 1	11.14 ± 1.37 [b]
Mut 6	9.15 ± 1.52 [ab]
Mut 7	8.64 ± 1.98 [ab]
P value	0.013

4.2 Response of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes to the management of *Cynodon dactylon* with Arsenal® GEN 2 (imazapyr)

Infestation by *C. dactylon* within the sugarcane fields is a persistent problem in the sugar industry and is managed with the use of various herbicides such as Roundup® (glyphosate), Gramoxone® (paraquat) and Diuron® (diuron) (Campbell *et al*., 2008; Anon, 2014; Seeruttun *et al*., 2014). The high dosages of these herbicides repeatedly used to manage *C. dactylon* pose a future threat because they can lead to herbicide tolerant *C. dactylon* (Eksteen, 2007; Conlong and Campbell, 2010). Arsenal® GEN 2 has been identified as an excellent candidate herbicide to overcome this threat because a single application is required to supress the growth of *C. dactylon* compared with the above mentioned herbicides which involve multiple and high application rates. Although there are advantages associated with the use of Arsenal® GEN 2, its non-selective nature makes its field use restricted and limited (Campbell, 2008; Campbell, pers. comm.).

A comparison was made amongst three weed control treatments, *viz*. hand weeding (T1), a conventionally used commercial herbicide cocktail involving Gramoxone® (200 g a.i.ha^{-1} paraquat) and Diuron® (800 g a.i.ha^{-1} diuron) (T2) and Arsenal® GEN 2 (1254 g a.i.ha^{-1} imazapyr) (T3), in order to compare the effect of Arsenal® GEN 2 on the unmutated N12 and the mutant genotypes with that of the other two weed control treatments (T1 and T2). The herbicide cocktail and a recommended dose of Arsenal® GEN 2 was applied to the *C. dactylon* within the plot inter-rows of T2 and T3 respectively at 0 weeks and a second application (spot spray) of the herbicide cocktail was applied at 4 weeks. The sugarcane and *C. dactylon* was planted prior to herbicide application and care was taken to prevent contact between the herbicide and the sugarcane during spraying to prevent damage.

4.2.1 Agronomic evaluation of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes after the application of Arsenal® GEN 2 (imazapyr)

The plant injury caused by the Arsenal® GEN 2 application was compared with that caused by the hand weeded and herbicide cocktail regimes at t = 0 and at 2, 4, 8, 12, 16 and 20 weeks after application. This assessment was done by measuring the number of stalks.plot^{-1}, stalk height (cm) and leaf chlorophyll content (SPAD unit).

4.2.1.1 Number of stalks

Only the viable (living) number of stalks.plot^{-1} of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes were counted within each treatment (T1, T2 and T3 as described in section 4.2) at 0, 2, 4, 8, 12, 16 and 20 weeks after herbicide application.

The number of N12 stalks.plot^{-1} decreased significantly ($P \leq 0.05$) 4 weeks after application of Arsenal® GEN 2 and continued to decrease 8, 12 and 16 weeks thereafter in contrast to the hand weeded and herbicide cocktail treatments (P = 0.024, 0.008, 0.002 and 0.008 for each time, respectively) (Table 13). No significant differences ($P \geq 0.05$) in the number of N12 stalks.plot^{-1} were found amongst treatments 20 weeks after the application of Arsenal® GEN 2 (Table 13).

Twelve weeks after the application of Arsenal® GEN 2, the number of Mut 1 stalks.plot^{-1} were significantly lower ($P \leq 0.05$) than that of the hand weeded treatment (P = 0.008) (Table 13). No significant differences in the number of Mut 1 stalks.plot^{-1} were found amongst all three treatments thereafter (Table 13). The application of Arsenal® GEN 2 did not affect the number of Mut 6 and Mut 7 stalks.plot^{-1} ($P \geq 0.05$) (Table 13).

A comparison amongst genotypes for each weed management treatment indicated that no significant differences ($P \geq 0.05$) in the number of stalks.plot^{-1} were found in the hand weeded treatment site at each time tested (Table 13). A significantly lower ($P \leq 0.05$) number of stalks.plot^{-1} was recorded for Mut 6 than the unmutated N12 and Mut 1 genotypes at zero and 2 weeks after the application of the herbicide cocktail (P = 0.041 and 0.036 for each time, respectively); no significant differences were found subsequently ($P \geq 0.05$) (Table 13). Twelve weeks after the application of Arsenal® GEN 2 the number of N12 stalks.plot^{-1} were significantly lower ($P \leq 0.05$) than that of Mut 1 and Mut 6 (P = 0.044) (Table 13). This was expected as the N12 genotype is not tolerant to imazapyr. No significant differences ($P \geq 0.05$) were observed amongst genotypes, 16 and 20 weeks after the application of Arsenal® GEN 2 (P = 0.144 and 0.384 for each time, respectively) (Table 13). The application of Arsenal® GEN 2 had no significant effect ($P \geq 0.05$) on the number of stalks of the mutant genotypes.plot^{-1} at each time tested (Table 13).

Table 13: The effect of spraying *Cynodon dactylon* with Arsenal® GEN 2 (T3) on the number of unmutated N12, Mut 1, Mut 6 and Mut 7 stalks.plot^{-1} compared with the hand weeded (T1) and herbicide cocktail (T2) weed control regimes, 0, 2, 4, 8, 12, 16 and 20 weeks after treatment. Dissimilar alphabet characters denote statistically significant differences: (a) amongst genotypes within each treatment (A-B underlined in green in rows); (b) amongst treatments for each genotype (a-b highlighted in green in columns). Data sets were analysed using a one-way ANOVA and a Sidak post hoc test. $P \geq 0.05$, n =3, mean ± SE.

Time (w)	Treatment	Number of stalks.plot^{-1}				*P* value
		Genotype				
		Unmutated N12	M1	M6	M7	
0	T1	121.00 ± 16.44 $^{a, A}$	137.70 ± 8.41 $^{a, A}$	93.00 ± 19.29 $^{a, A}$	80.30 ± 25.99 $^{a, A}$	0.310
	T2	111.70 ± 7.54 $^{a, B}$	108.00 ± 13.87 $^{a, B}$	64.00 ± 7.09 $^{a, A}$	86.00 ± 7.00 $^{a, AB}$	0.041
	T3	127.00 ± 9.29 $^{a, A}$	105.70 ± 16.33 $^{a, A}$	93.30 ± 11.61 $^{a, A}$	81.30 ± 20.41 $^{a, A}$	0.279
	P value	0.540	0.330	0.411	0.972	
2	T1	193.30 ± 22.75 $^{a, A}$	183.70 ± 28.00 $^{a, A}$	143.00 ± 19.86 $^{a, A}$	128.00 ± 44.68 $^{a, A}$	0.482
	T2	177.00 ± 5.86 $^{a, BC}$	184.30 ± 24.73 $^{a, C}$	109.30 ± 13.86 $^{a, A}$	124.30 ± 8.45 $^{a, AB}$	0.036
	T3	140.30 ± 12.24 $^{a, A}$	136.00 ± 23.12 $^{a, A}$	111.00 ± 9.61 $^{a, A}$	99.70 ± 18.35 $^{a, A}$	0.410
	P value	0.185	0.454	0.398	0.757	
4	T1	218.70 ± 13.91 $^{b, A}$	225.30 ± 29.76 $^{a, A}$	170.00 ± 37.23 $^{a, A}$	151.30 ± 56.17 $^{a, A}$	0.538
	T2	196.30 ± 16.91 $^{ab, A}$	177.30 ± 20.22 $^{a, A}$	113.30 ± 16.83 $^{a, A}$	137.70 ± 10.35 $^{a, A}$	0.071
	T3	121.00 ± 5.86 $^{a, A}$	154.70 ± 16.90 $^{a, A}$	118.70 ± 13.53 $^{a, A}$	110.00 ± 26.21 $^{a, A}$	0.227
	P value	0.024	0.167	0.412	0.718	
8	T1	236.70 ± 21.07 $^{b, A}$	251.70 ± 15.56 $^{a, A}$	197.70 ± 28.49 $^{a, A}$	173.00 ± 56.61 $^{a, A}$	0.483
	T2	174.30 ± 9.82 $^{ab, A}$	176.70 ± 15.96 $^{a, A}$	157.70 ± 22.38 $^{a, A}$	149.30 ± 3.28 $^{a, A}$	0.618
	T3	108.30 ± 3.38 $^{a, A}$	201.00 ± 19.00 $^{a, B}$	162.00 ± 17.24 $^{a, B}$	137.30 ± 31.02 $^{a, AB}$	0.077
	P value	0.008	0.072	0.587	0.796	
12	T1	228.30 ± 27.87 $^{b, A}$	202.30 ± 9.77 $^{b, A}$	165.00 ± 18.61 $^{a, A}$	163.30 ± 42.86 $^{a, A}$	0.374
	T2	175.00 ± 8.74 $^{b, A}$	177.70 ± 1.45 $^{ab, A}$	144.00 ± 13.58 $^{a, A}$	158.70 ± 18.56 $^{a, A}$	0.381
	T3	65.30 ± 9.82 $^{a, A}$	151.00 ± 5.51 $^{a, B}$	157.70 ± 31.55 $^{a, B}$	119.70 ± 17.68 $^{a, AB}$	0.044
	P value	0.002	0.008	0.853	0.587	
16	T1	193.70 ± 23.21 $^{b, A}$	186.00 ± 15.70 $^{a, A}$	146.30 ± 19.65 $^{a, A}$	134.30 ± 32.18 $^{a, A}$	0.385
	T2	165.70 ± 2.91 $^{b, A}$	162.30 ± 13.93 $^{a, A}$	145.70 ± 23.71 $^{a, A}$	158.70 ± 3.53 $^{a, A}$	0.769
	T3	81.70 ± 17.46 $^{a, A}$	190.70 ± 12.47 $^{a, A}$	177.00 ± 31.00 $^{a, A}$	167.00 ± 37.11 $^{a, A}$	0.144
	P value	0.008	0.361	0.716	0.756	
20	T1	185.00 ± 13.65 $^{a, A}$	183.00 ± 6.43 $^{a, A}$	139.30 ± 21.50 $^{a, A}$	153.30 ± 48.91 $^{a, A}$	0.675
	T2	182.30 ± 16.33 $^{a, A}$	188.70 ± 12.13 $^{a, A}$	176.30 ± 36.06 $^{a, A}$	181.30 ± 29.18 $^{a, A}$	0.992
	T3	101.70 ± 26.46 $^{a, A}$	200.70 ± 9.35 $^{a, A}$	203.70 ± 42.65 $^{a, A}$	159.70 ± 56.10 $^{a, A}$	0.384
	P value	0.053	0.363	0.568	0.916	

4.2.1.2 Stalk height

Stalk height (cm) measurements were taken from the top visible dewlap leaf of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes at 0, 2, 4, 8, 12, 16 and 20 weeks after herbicide application.

The unmutated N12 stalks were significantly shorter ($P \leq 0.05$) 4 and 8 weeks after the application of Arsenal® GEN 2 than those of the hand weeded treatment ($P = 0.013$ and 0.001 for each time, respectively) and similarly again at 12, 16 and 20 weeks compared with that of the hand weeded and herbicide cocktail treatments ($P = \leq 0.001$, ≤ 0.001 and ≤ 0.001 for each time, respectively) (Table 14). The herbicide cocktail also negatively affected the height of the unmutated N12 stalks resulting in significantly shorter ($P \leq 0.05$) stalks 12, 16 and 20 weeks after herbicide application compared with the stalks of the hand weeded treatment ($P \leq 0.001$, ≤ 0.001 and ≤ 0.001 for each time, respectively) (Table 14).

The application of Arsenal® GEN 2 had no significant ($P \geq 0.05$) effect on the stalk height of the mutant genotypes (Table 14) which is an indication that the growth of mutant genotypes, unlike that of the unmutated N12 control, was not inhibited by the herbicide.

A comparison amongst the unmutated N12 and mutant genotypes for each treatment indicated that the hand weeded and herbicide cocktail treatments had no significant ($P \geq 0.05$) effect on the stalk height of all genotypes at each tested time (Table 14). The inhibition caused by Arsenal® GEN 2 to the unmutated N12 genotype compared with the mutants was visible at 12, 16 and 20 weeks after application of the herbicide, recorded as significantly shorter ($P \leq 0.05$) stalks at each time compared with that of the mutants genotypes ($P = 0.014$, 0.006 and 0.034 for each time, respectively) (Table 14). There were no differences in stalk height found amongst the mutant genotypes after the application of Arsenal® GEN 2 (Table 14).

Table 14: The effect of spraying *Cynodon dactylon* with Arsenal® GEN 2 (T3) on the stalk height (cm) of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes compared with that of the hand weeded (T1) and herbicide cocktail (T2) weed control regimes 0, 2, 4, 8, 12, 16 and 20 weeks after treatment. [1]Dissimilar alphabet characters denote statistically significant differences: (a) amongst genotypes within each treatment (A-B underlined in green in rows); (b) amongst treatments for each genotype (a-b highlighted in green in columns). Data sets were analysed using a one-way ANOVA and a Sidak post hoc test. $P \geq 0.05$, n =3, mean ± SE.

Time (w)	Treatment	Stalk height (cm)				*P* value
		Genotype				
		Unmutated N12	Mut 1	Mut 6	Mut 7	
0	T1	15.36 ± 0.29 a, A	14.47 ± 0.81 a, A	12.40 ± 1.27 a, A	10.89 ± 1.76 a, A	0.156
	T2	12.73 ± 1.57 a, A	14.67 ± 1.69 a, A	10.58 ± 1.09 a, A	11.56 ± 0.78 a, A	0.265
	T3	14.18 ± 0.25 a, A	15.36 ± 1.24 a, A	14.47 ± 2.40 a, A	12.36 ± 2.14 a, A	0.698
	P value	0.275	0.905	0.447	0.818	
2	T1	20.36 ± 0.33 a, A	23.93 ± 2.14 a, A	18.13 ± 1.72 a, A	16.69 ± 3.08 a, A	0.198
	T2	17.11 ± 2.78 a, A	19.24 ± 1.88 a, A	14.04 ± 1.99 a, A	15.73 ± 1.56 a, A	0.464
	T3	13.02 ± 0.30 a, A	17.33 ± 1.74 a, A	14.91 ± 3.04 a, A	13.07 ± 2.99 a, A	0.553
	P value	0.101	0.119	0.584	0.571	
4	T1	28.60 ± 0.44 b, A	32.09 ± 3.25 a, A	25.31 ± 1.84 a, A	22.64 ± 4.39 a, A	0.216
	T2	23.02 ± 2.85 ab, A	27.71 ± 2.72 a, A	19.44 ± 2.19 a, A	21.82 ± 1.87 a, A	0.236
	T3	14.18 ± 0.46 a, A	23.47 ± 3.00 a, A	17.96 ± 3.48 a, A	17.98 ± 3.69 a, A	0.247
	P value	0.013	0.125	0.306	0.577	
8	T1	48.93 ± 0.77 b, A	54.84 ± 6.46 a, A	43.62 ± 3.82 a, A	37.98 ± 9.00 a, A	0.284
	T2	39.64 ± 4.60 b, A	47.33 ± 3.59 a, A	33.71 ± 4.01 a, A	38.29 ± 4.11 a, A	0.279
	T3	15.38 ± 0.29 a, A	39.78 ± 5.83 a, A	32.04 ± 7.74 a, A	28.02 ± 7.90 a, A	0.119
	P value	0.001	0.184	0.472	0.477	
12	T1	81.58 ± 2.79 c, A	83.24 ± 7.65 a, A	70.69 ± 3.83 a, A	60.07 ± 13.11 a, A	0.172
	T2	65.07 ± 4.95 b, A	73.29 ± 4.74 a, A	55.96 ± 5.16 a, A	60.44 ± 7.96 a, A	0.334
	T3	3.49 ± 2.56 a, A	70.04 ± 6.58 a, B	48.16 ± 11.78 a, B	42.38 ± 11.51 a, B	0.014
	P value	< 0.001	0.51	0.281	0.350	
16	T1	90.04 ± 3.35 c, A	87.76 ± 10.68 a, A	76.24 ± 4.60 a, A	65.69 ± 15.10 a, A	0.245
	T2	71.76 ± 5.88 b, A	82.91 ± 4.09 a, A	64.71 ± 6.01 a, A	65.80 ± 9.17 a, A	0.337
	T3	3.62 ± 2.34 a, A	63.91 ± 6.81 a, B	53.60 ± 8.10 a, B	42.73 ± 14.03 a, B	0.006
	P value	< 0.001	0.067	0.230	0.303	
20	T1	90.44 ± 4.997 c, A	86.42 ± 9.670 a, A	76.82 ± 6.232 a, A	67.93 ± 16.318 a, A	0.388
	T2	69.47 ± 8.130 b, A	82.36 ± 5.696 a, A	64.36 ± 8.371 a, A	67.29 ± 10.980 a, A	0.569
	T3	3.89 ± 2.145 a, A	61.87 ± 5.921 a, B	49.89 ± 13.991 a, B	43.38 ± 15.529 a, B	0.034
	P value	< 0.001	0.098	0.372	0.375	

4.2.2 Comparison of leaf chlorophyll content of the N12 control, Mut 1, Mut 6 and Mut 7 genotypes after treatment with Arsenal® GEN 2

In addition to the agronomic characteristics, the chlorosis caused by the application of Arsenal® GEN 2 was identified using a soil plant analysis development (SPAD) method which assessed the leaf chlorophyll content (SPAD unit) of the unmutated N12 and mutant genotypes 0, 2, 4, 8, 12, 16 and 20 weeks after herbicide application. A comparison was made amongst treatments for each genotype and across genotypes for each treatment, at each tested time.

The application of Arsenal® GEN 2 caused chlorosis of the leaves of the unmutated N12, 2 weeks after application and, as a result, the leaf chlorophyll content was significantly lower ($P \leq 0.05$) than in the leaves of the plants subjected to the hand weeded and herbicide cocktail treatments ($P = 0.004$) (Table 15). Due to the harmful effects caused by Arsenal® GEN 2, the leaf chlorophyll content of the unmutated N12 continued to decrease thereafter and was significantly lower than that of the plants of the hand weeded and herbicide cocktail treatments at 4, 8, 12, 16 and 20 weeks after application ($P = 0.042$, 0.002, ≤ 0.001, ≤ 0.001 and ≤ 0.001 for each time, respectively) (Table 15).

The leaf chlorophyll content of the plants of the Mut 1 genotype was significantly lower ($P \leq 0.05$) 4 weeks after the application of Arsenal® GEN 2 than when treated with hand weeding ($P = 0.022$) (Table 15) and the chlorosis caused by the herbicide application was also evident at 8 weeks resulting in a significantly lower ($P \leq 0.05$) leaf chlorophyll content than that observed with the hand weeding and herbicide cocktail treatments ($P = 0.003$) (Table 15). No significant differences ($P \geq 0.05$) in the leaf chlorophyll content amongst treatments for Mut 1 were observed at each subsequent time thereafter (Table 15).

A degree of chlorosis was noted in the Mut 6 genotype 4 weeks after the application of the herbicide cocktail where the leaf chlorophyll content was significantly lower ($P \leq 0.05$) than in the hand weeded plants ($P = 0.022$) (Table 15). A significantly lower ($P \leq 0.05$) leaf chlorophyll content was evident at 8 and 12 weeks after application of Arsenal® GEN 2 compared with that of the other weed control treatments ($P = 0.002$ and 0.002 for each time, respectively) (Table 15). No significant differences ($P \geq 0.05$) in the leaf chlorophyll content amongst treatments for Mut 6 were observed at each subsequent time thereafter (Table 15).

Chlorosis of Mut 7 plants was noted 4 weeks after application of Arsenal® GEN 2 compared with that of the hand weeded treatment ($P = 0.019$) and again at 12 weeks with a significantly

lower ($P \leq 0.05$) leaf chlorophyll content than that of the plants treated with both the hand weeded and herbicide cocktail treatments ($P \leq 0.001$) (Table 15). Thereafter, no significant differences ($P \geq 0.05$) in the leaf chlorophyll content amongst treatments for Mut 7 were observed at each tested time (Table 15).

There were no significant differences ($P \geq 0.05$) amongst genotypes with respect to the leaf chlorophyll content between plants subjected to the hand weeded and the herbicide cocktail treatments (Table 15). The leaf chlorophyll content of the unmutated N12 plants were significantly lower ($P \leq 0.05$) 12 weeks after application of Arsenal® GEN 2 compared with that of the mutant genotypes ($P \leq 0.001$) and similarly again at 16 and 20 weeks ($P = 0.002$ and 0.027 for each time, respectively) (Table 15).

A decrease in leaf chlorophyll content of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes was observed in all three weed control treatments after 12 weeks. It is suggested that this might have been caused by a lack of irrigation/rainfall after 12 weeks (Figure 10).

Table 15: The effect of spraying *Cynodon dactylon* with Arsenal® GEN 2 (T3) on the leaf chlorophyll content (SPAD unit) of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes compared with the hand weeded (T1) and herbicide cocktail (T2) weed control regimes 0, 2, 4, 8, 12, 16 and 20 weeks after treatment. [1]Dissimilar alphabet characters denote statistically significant differences: (a) amongst genotypes within each treatment (A-B underlined in green in rows); (b) amongst treatments for each genotype (a-b highlighted in green in columns). Data sets were analysed using a one-way ANOVA and a Sidak post hoc test. $P \geq 0.05$, n =3, mean ± SE.

Time (w)	Treatment	Leaf chlorophyll content				*P value*
		Genotype				
		Unmutated N12	Mut 1	Mut 6	Mut 7	
0	T1	48.24 ± 1.75 a, A	46.77 ± 0.41 a, A	47.27 ± 0.48 a, A	46.54 ± 1.69 a, A	0.818
	T2	46.36 ± 1.77 a, A	46.76 ± 1.35 a, A	44.75 ± 1.30 a, A	47.01 ± 1.16 a, A	0.538
	T3	47.14 ± 0.96 a, A	48.29 ± 0.33 a, A	50.11 ± 1.63 a, A	45.29 ± 2.72 a, A	0.201
	P value	0.770	0.391	0.074	0.853	
2	T1	46.49 ± 0.24 b, A	46.53 ± 0.19 a, A	48.06 ± 0.96 a, A	47.24 ± 1.24 a, A	0.471
	T2	43.41 ± 1.03 b, A	43.63 ± 1.39 a, A	41.35 ± 2.88 a, A	45.96 ± 1.57 a, A	0.436
	T3	39.18 ± 1.02 a, A	43.76 ± 1.98 a, A	45.04 ± 0.31 a, A	43.39 ± 1.33 a, A	0.065
	P value	0.004	0.285	0.167	0.123	
4	T1	48.47 ± 1.01 b, A	48.96 ± 0.80 b, A	49.45 ± 1.34 b, A	47.24 ± 2.43 b, A	0.789
	T2	39.42 ± 3.77 ab, A	44.58 ± 0.24 ab, A	37.15 ± 2.15 a, A	43.79 ± 0.97 ab, A	0.142
	T3	35.30 ± 0.86 a, A	36.52 ± 3.31 a, A	40.91 ± 3.03 ab, A	36.69 ± 1.13 a, A	0.285
	P value	0.042	0.022	0.022	0.019	
8	T1	50.34 ± 1.04 b, A	47.19 ± 1.15 b, A	49.58 ± 0.44 b, A	48.92 ± 4.01 a, A	0.587
	T2	50.40 ± 0.46 b, A	51.22 ± 1.34 b, A	48.31 ± 1.10 b, A	48.87 ± 2.33 a, A	0.472
	T3	32.10 ± 3.47 a, A	34.05 ± 2.59 a, A	36.69 ± 1.78 a, A	38.27 ± 1.58 a, A	0.082
	P value	0.002	0.003	0.002	0.054	
12	T1	51.28 ± 0.82 b, A	52.03 ± 1.10 a, A	52.46 ± 1.14 b, A	51.79 ± 0.42 b, A	0.846
	T2	48.74 ± 0.86 b, A	50.64 ± 0.75 a, A	51.13 ± 0.11 b, A	51.52 ± 0.38 b, A	0.061
	T3	30.43 ± 1.42 a, A	43.15 ± 3.08 a, B	42.73 ± 1.67 a, B	45.54 ± 0.05 a, B	≤0.001
	P value	< 0.001	0.059	0.002	< 0.001	
16	T1	40.53 ± 2.75 b, A	44.04 ± 1.21 a, A	40.96 ± 1.88 a, A	41.25 ± 2.05 a, A	0.605
	T2	37.72 ± 1.06 b, A	43.77 ± 1.18 a, A	42.02 ± 0.92 a, A	42.79 ± 3.52 a, A	0.246
	T3	4.68 ± 2.39 a, A	40.41 ± 0.75 a, B	39.37 ± 3.56 a, B	36.97 ± 6.89 a, B	0.002
	P value	< 0.001	0.053	0.806	0.56	
20	T1	32.32 ± 0.74 b, A	32.95 ± 2.13 a, A	33.06 ± 0.27 a, A	33.29 ± 3.81 a, A	0.938
	T2	26.45 ± 3.51 b, A	35.04 ± 0.59 a, A	27.93 ± 4.78 a, A	28.82 ± 5.07 a, A	0.538
	T3	1.39 ± 1.39 a, A	30.36 ± 1.77 a, B	31.01 ± 7.29 a, B	27.35 ± 6.76 a, B	0.027
	P value	< 0.001	0.340	0.813	0.729	

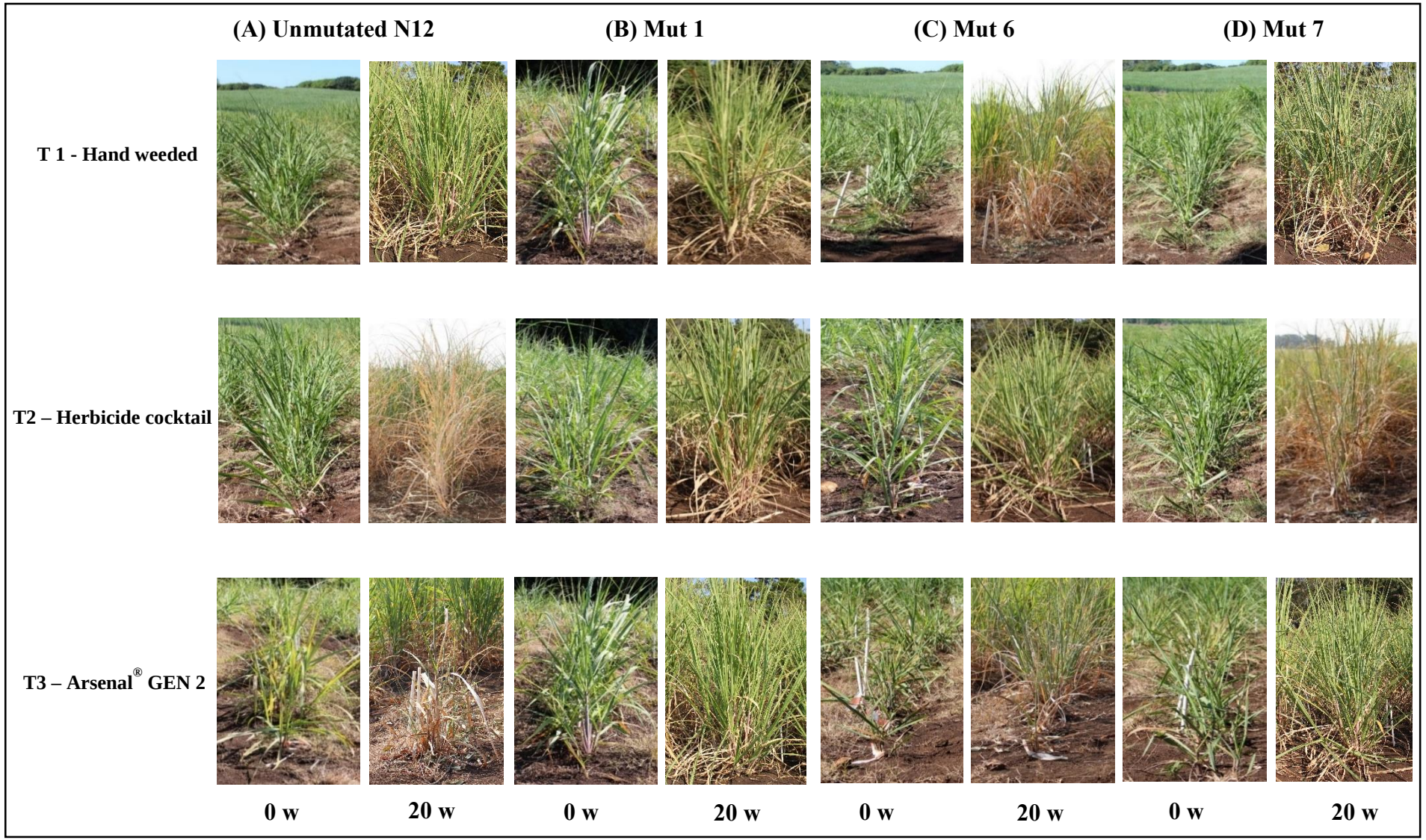

Figure 10: A visual comparison of sugarcane plants of **(A)** unmutated N12, **(B)** Mut 1, **(C)** Mut 6 and **(D)** Mut 7 plants within the different weed control treatments: **(T1)** hand weeded; **(T2)** herbicide cocktail involving Gramoxone® (paraquat) and Diuron® (diuron); **(T3)** Arsenal® GEN 2 (imazapyr), at 0 and 20 weeks.

4.2.2 Comparison of the effect of Arsenal® GEN 2 (imazapyr) and a commercial herbicide cocktail on *Cynodon dactylon*

The effectiveness of the recommended dose of Arsenal® GEN 2 (1254 g a.i.ha^{-1} imazapyr) in managing infestation by *C. dactylon* was compared with that of a conventionally used commercial herbicide cocktail, involving Gramoxone® (200 g a.i.ha^{-1} paraquat) and Diuron® (800 g a.i.ha^{-1} diuron). In this study, Arsenal® GEN 2 was applied post-emergence of both the *C. dactylon* and sugarcane in contrast with the recommended method which stipulates that cultivated fields remain fallow for a specific period of time (4 months) and only after 600 mm of precipitation (rainfall) can planting of the sugarcane resume (Anon, 2014; Anon, 2015). The sugarcane was planted in rows with tufts of *C. dactylon* within the plot inter-rows of the two treatments and the herbicides were applied after planting. Arsenal® GEN 2 and the herbicide cocktail were applied directly to *C. dactylon* within the plot inter-rows at week 0 and a second application of the herbicide cocktail was applied 4 weeks later. Spot sprays of the herbicide cocktail were done as required, every 3 to 5 weeks in order to eradicate the *C. dactylon* effectively, as per commercial practise (Anon, 2014). A digital image analysis was used to assess the percentage of green leaves of *C. dactylon* before herbicide application (t = 0) and at 2, 4, 8, 12, 16 and 20 weeks thereafter.

No significant differences ($P \geq 0.05$) in the percentage of green *C. dactylon* leaves were found between the two herbicide treatments at 0, 2, 4, 8, 12 and 16 weeks after their application (Figure 11). Although the percentage of green leaves of *C. dactylon* decreased significantly ($P \leq 0.05$) after the application of the herbicide cocktail and Arsenal® GEN 2 ($P \leq 0.001$ and ≤ 0.001 for each herbicide treatment respectively) (Figure 11), a significant difference ($P \leq 0.05$) was observed at week 20 - the percentage of green *C. dactylon* leaves after treatment with a single application of Arsenal® GEN 2 was higher than that of the herbicide cocktail treatment (which required two applications) ($P = 0.025$) (Figure 11).

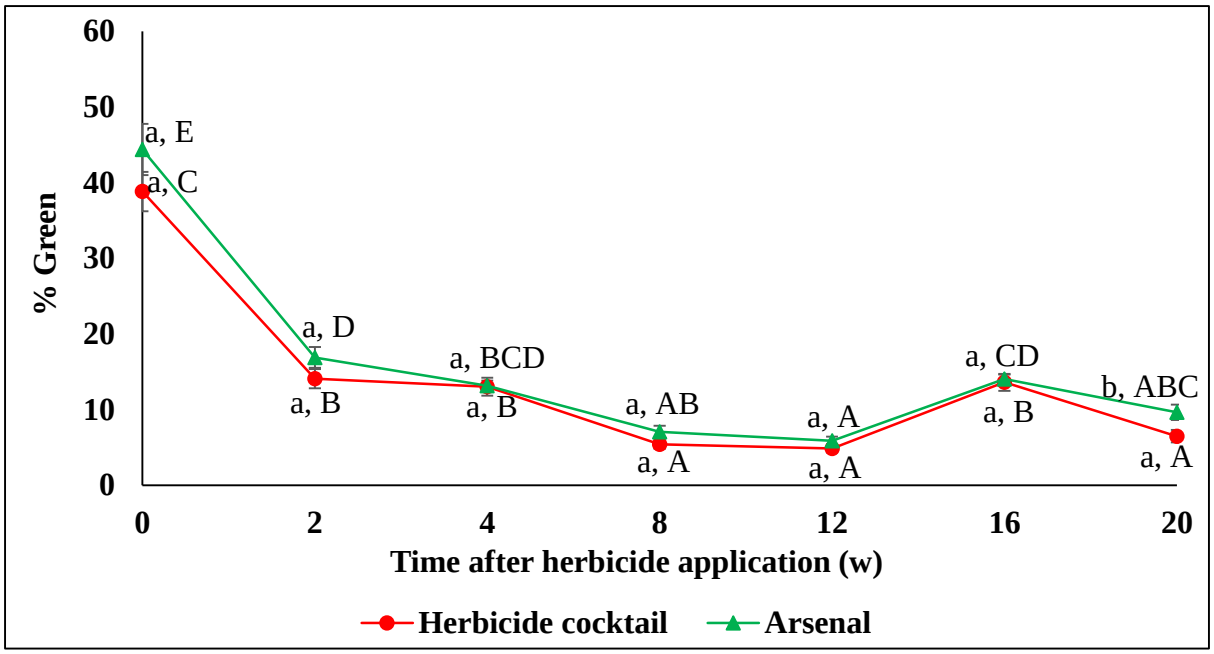

Figure 11: A comparison of the percentage green of *Cynodon dactylon* 0, 2, 4, 8, 10, 12, 16 and 20 weeks after treatment with Arsenal® GEN 2 compared with the commercial herbicide cocktail. Dissimilar alphabet characters denote statistically significant differences: (a) between herbicide treatments at each time interval (a - b); (b) over time for each herbicide treatment (A-B). Data sets were analysed using a two-sample T-test, one-way ANOVA and Sidak post hoc test. $P \geq 0.05$, n = 24, mean ± SE.

4.3 Characterisation of the mode of imazapyr tolerance in the Mut 1, Mut 6 and Mut 7 genotypes

As previously discussed, herbicide tolerance in plants can be conferred by one of two mechanisms, target site and non-target site tolerance (Sala *et al*., 2012). The former occurs due to mutations within the herbicide target site of the plant, in this case the AHAS enzyme targeted by Arsenal® GEN 2 (Devine and Eberlein, 1997; Tranel and Wright, 2002). The latter can be conferred by various mechanisms but the two most common in plants are the metabolic detoxification and a reduced translocation of the herbicide (Devine and Eberlein, 1997; Yuan *et al*., 2007; Shaner *et al*., 2012). Based on the results obtained from the field trials involving Arsenal® GEN 2, it can be confirmed that Mut 1, Mut 6 and Mut 7 have a mechanism that confers tolerance to imazapyr.

4.3.1 Identification of changes within the acetohydroxyacid synthase gene of Mut 1, Mut 6 and Mut 7 compared with the unmutated N12 control

The sugarcane AHAS gene was partially sequenced from the unmutated N12 control genotype by cloning the complementary DNA (cDNA) products from the PCR reactions into pGEM-T Easy cloning vectors. The cDNA was then used as the DNA source for sequencing because it represented the expressed AHAS gene in the mutated genotypes. The clones derived from the AHAS PCR reactions were sequenced and aligned and the AHAS gene sequence for the unmutated N12 genotype was generated. The consensus AHAS gene sequence of the unmutated N12 genotype was analysed by a National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm to identify a positive match to a known AHAS gene of a closely related species. The results of the BLAST analysis produced a positive match to the AHAS gene sequence of *S. bicolor* (Accession number: XM_002452104.1) and *Z. mays* (Accession number: X63553.1) with a 98 and 97 % identity match, respectively (Figure 12). The sugarcane AHAS gene sequence was not available at the time of this study, therefore the gene sequences of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes generated were compared with those of *Z. mays* and *S. bicolor* in order to confirm a positive match to the AHAS gene.

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                          1       10        20        30        40        50        60
                          |        |         |         |         |         |         |
  Unmutated N12 AHAS gene ----------------------CACCTCTTCCGCCACGAGCAAGGGGAGGCCTTCGCCG
Sorghum bicolor AHAS gene ------------------------------------------------------------
       Zea mays AHAS gene CCCGCTCCCCCGTCATCGCCAACCACCTCTTCCGCCACGAGCAAGGGGAGGCCTTTGCGG

  Unmutated N12 AHAS gene CCTCCGGCTTCGCGCGCTCCTCGGGCCGCGTCGGCGTCTGCGTCGCCACCTCCGGCCCCG
Sorghum bicolor AHAS gene ------------------------------------------------------------
       Zea mays AHAS gene CCTCCGGCTACGCGCGCTCCTCGGGCCGCGTCGGCGTCTGCATCGCCACCTCCGGCCCCG

  Unmutated N12 AHAS gene GCGCCACCAACCTAGTCTCCGCGCTCGCCGACGCGCTGCTCGACTCCGTCCCCATGGTCG
Sorghum bicolor AHAS gene ------------------------------------------------------------
       Zea mays AHAS gene GCGCCACCAACCTTGTCTCCGCGCTCGCCGACGCGCTGCTCGATTCCGTCCCCATGGTCG

  Unmutated N12 AHAS gene CCATC-ACGGGA-CAGGTGCCGCGGCG-CATGATTGGCACCGATGCCTTCC-AGGAGACG
Sorghum bicolor AHAS gene --ATGGACTTGAGGAAGTCCGATGGCGTCTTCACCGTC-CCGCCGGCGCCCGAGGCG-GG
       Zea mays AHAS gene CCATC-ACGGGA-CAGGTGCCGCGACG-CATGATTGGCACCGACGCCTTCC-AGGAGACG

  Unmutated N12 AHAS gene CCCATCGTCGAGGTCAC--CCGCTCCATCACCAAGCACAACTACCTG-GTCCTCGAC---
Sorghum bicolor AHAS gene CCTGTCGCTGCCGCCGCCGCCGCTGC-TCATCTTGCGC-----GCTGTGCCCCCGGCGGT
       Zea mays AHAS gene CCCATCGTCGAGGTCAC--CCGCTCCATCACCAAGCACAACTACCTG-GTCCTCGACG--

  Unmutated N12 AHAS gene GTCGACGACATCC----CCCGCGTCGTGCAGGAGGCCTTCTTCCTCGCCTCCTCTGGTCG
Sorghum bicolor AHAS gene GTCCCTGTGTTGCGGATCGCGGGTGGGCCAGGAGGCTTTCTTCCTCGCCTCCTCCGGTCG
       Zea mays AHAS gene -TCGACGACATCC----CCCGCGTCGTGCAGGAGGCTTTCTTCCTCGCCTCCTCTGGTCG
                                                                              Cont…
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Cont…

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   Unmutated N12 AHAS gene CCCGGGACCGGTGCTTGTCGACATCCCCAAGGACATCCAGCAGCAGATGGCGGTGCCGGT
 Sorghum bicolor AHAS gene CCCGGGACCGGTGCTTGTCGACATCCCCAAGGACATCCAGCAGCAGATGGCCGTGCCGGT
        Zea mays AHAS gene ACCGGGGCCGGTGCTTGTCGACATCCCCAAGGACATCCAGCAGCAGATGGCGGTGCCTGT

   Unmutated N12 AHAS gene CTGGGACACGCCCATGAGTCTGCCTGGGTACATTGCGCGCCTTCCCAAGCCTCCTGCGAC
 Sorghum bicolor AHAS gene CTGGGACACGCCCATGAGTCTGCCTGGGTACATTGCGCGCCTTCCCAAGCCTCCTGCGAC
        Zea mays AHAS gene CTGGGACAAGCCCATGAGTCTGCCTGGGTACATTGCGCGCCTTCCCAAGCCCCCTGCGAC

   Unmutated N12 AHAS gene TGAATTGCTTGAGCAGGTGCTGCGTCTTGTTGGTGAATCGCGGCGCCCTGTTCTTTATGT
 Sorghum bicolor AHAS gene TGAATTGCTTGAGCAGGTGCTGCGTCTTGTTGGTGAATCAAGGCGCCCTGTTCTTTATGT
        Zea mays AHAS gene TGAGTTGCTTGAGCAGGTGCTGCGTCTTGTTGGTGAATCCCGGCGCCCTGTTCTTTATGT

   Unmutated N12 AHAS gene TGGCGGTGGCTGCGCAGCATCTGGTGAGGAGTTGCGCCGCTTTGTGGAGATGACTGGAAT
 Sorghum bicolor AHAS gene TGGTGGTGGCTGCGCAGCATCTGGCGAGGAGTTGCGCCGCTTTGTGGAGATGACTGGAAT
        Zea mays AHAS gene TGGCGGTGGCTGCGCAGCATCTGGTGAGGAGTTGCGACGCTTTGTGGAGCTGACTGGAAT

   Unmutated N12 AHAS gene CCCAGTCACAACTACTCTTATGGGCCTTGGCAACTTCCCCGGCGACGACCCACTGTCTCT
 Sorghum bicolor AHAS gene CCCAGTCACAACTACTCTTATGGGCCTTGGCAATTTCCCTGGCGACGACCCACTGTCTCT
        Zea mays AHAS gene CCCGGTCACAACTACTCTTATGGGCCTCGGCAACTTCCCCAGCGACGACCCACTGTCTCT

   Unmutated N12 AHAS gene GCGCATGCTTGGTATGCATGGCACAGTGTATGCAAATTATGCAGTGGATAAGGCTGATCT
 Sorghum bicolor AHAS gene GCGCATGCTTGGTATGCATGGCACGGTGTATGCAAATTATGCAGTGGATAAGGCGGATCT
        Zea mays AHAS gene GCGCATGCTAGGTATGCATGGCACGGTGTATGCAAATTATGCAGTGGATAAGGCCGATCT

   Unmutated N12 AHAS gene GTTGCTTGCATTTGGTGTGCGGTTTGATGATCGTGTGACAGGGAAGATTGAGGCTTTTGC
 Sorghum bicolor AHAS gene GTTGCTTGCATTTGGTGTGCGGTTTGATGATCGTGTGACAGGGAAGATTGAGGCTTTTGC
        Zea mays AHAS gene GTTGCTTGCACTTGGTGTGCGGTTTGATGATCGTGTGACAGGGAAGATTGAGGCTTTTGC

   Unmutated N12 AHAS gene AAGCAGGGCTAAGATTGTGCACATTGATATTGATCCGGCTGAGATTGGCAAGAACAAGCA
 Sorghum bicolor AHAS gene AAGCAGGGCTAAGATTGTGCACATTGATATTGATCCCGCTGAGATTGGCAAGAACAAGCA
        Zea mays AHAS gene AAGCAGGGCTAAGATTGTGCACGTTGATATTGATCCGGCTGAGATTGGCAAGAACAAGCA

   Unmutated N12 AHAS gene GCCACATGTGTCCATCTGTGCAGATGTTAAGCTTGCTTTGCAGGGCATGAATGCTCTTCT
 Sorghum bicolor AHAS gene GCCACATGTGTCCATCTGTGCAGACGTTAAGCTTGCTTTGCAGGGCATGAATGCTCTTCT
        Zea mays AHAS gene GCCACATGTGTCCATCTGTGCAGATGTTAAGCTTGCTTTGCAGGGCATGAATGCTCTTCT
   Unmutated N12 AHAS gene GGAAGGAAGCACATCAAAGAAGAGCTTTGACTTTGGCTCATGGCACGATGAGTTGGATCA
 Sorghum bicolor AHAS gene GGAAGGAAGCACATCAAAGAAGAGCTTTGACTTTGGCTCATGGCAAGCTGAGTTGGATCA
        Zea mays AHAS gene TGAAGGAAGCACATCAAAGAAGAGCTTTGACTTTGGCTCATGGAACGATGAGTTGGATCA

   Unmutated N12 AHAS gene GCAGAAGAGAGAATTCCCCCTTGGGTATAAAACTTTTGATGAGGAGATCCAGCCACAGTA
 Sorghum bicolor AHAS gene GCAGAAGAGAGAGTTCCCCCTTGGGTATAAAACTTTTGATGACGAGATCCAGCCACAATA
        Zea mays AHAS gene GCAGAAGAGGGAATTCCCCCTTGGGTATAAAACATCTAATGAGGAGATCCAGCCACAATA

   Unmutated N12 AHAS gene TGCTATCCAGGTTCTTGATGAGCTGACAAAAGGGGAGGCCATCATTGCCACAGGTGTTGG
 Sorghum bicolor AHAS gene TGCTATTCAGGTTCTTGATGAGCTGACAAAAGGGGAGGCCATCATTGCCACAGGTGTTGG
        Zea mays AHAS gene TGCTATTCAGGTTCTTGATGAGCTGACGAAAGGCGAGGCCATCATCGGCACAGGTGTTGG

   Unmutated N12 AHAS gene GCAGCACCAGATGTGGGCGGCACAGTACTACACTTACAAGCGGCCAAGGCAGTGGTTGTC
 Sorghum bicolor AHAS gene GCAGCACCAGATGTGGGCGGCACAGTACTACACTTACAAGCGGCCAAGGCAGTGGTTGTC
        Zea mays AHAS gene GCAGCACCAGATGTGGGCGGCACAGTACTACACTTACAAGCGGCCAAGGCAGTGGTTGTC

   Unmutated N12 AHAS gene TTCGGCTGGTCTTGGGGCTATGGGATTTGGTTTGCCGGCTGCTGCTGGCGCTGCTGTGGC
 Sorghum bicolor AHAS gene TTCAGCTGGTCTTGGGGCTATGGGATTTGGTTTGCCGGCTGCTGCTGGCGCTGCTGTGGC
        Zea mays AHAS gene TTCAGCTGGTCTTGGGGCTATGGGATTTGGTTTGCCGGCTGCTGCTGGTGCTTCTGTGGC

   Unmutated N12 AHAS gene CAACCCAGGTGTCACTGTTGTTGACATCGACGGAGATGGTAGCTTCCTCATGAACATTCA
 Sorghum bicolor AHAS gene CAACCCAGGTATCACTGTTGTTGACATCGACGGAGATGGTAGCTTCCTCATGAACATTCA
        Zea mays AHAS gene CAACCCAGGTGTTACTGTTGTTGACATCGATGGAGATGGTAGCTTTCTCATGAACGTTCA

   Unmutated N12 AHAS gene GGAGCTAGCTATGATCCGAATTGAGAACCTCCCAGTGAAGGTCTTTGTGCTAAACAACCA
 Sorghum bicolor AHAS gene GGAGCTAGCTATGATCCGAATTGAGAACCTCCCAGTGAAGGTCTTTGTGCTAAACAACCA
        Zea mays AHAS gene GGAGCTAGCTATGATCCGAATTGAGAACCTCCCGGTGAAGGTCTTTGTGCTAAACAACCA

   Unmutated N12 AHAS gene GCACCTGGGGATGGTGGTGCAGTGGGAGGACAGGTTCTATAAGGCCAACAGAGCACACAC
 Sorghum bicolor AHAS gene GCACCTGGGGATGGTGGTGCAGTGGGAGGACAGGTTCTATAAGGCCAATAGAGCACACAC
        Zea mays AHAS gene GCACCTGGGGATGGTGGTGCAGTGGGAGGACAGGTTCTATAAGGCCAACAGAGCGCACAC

   Unmutated N12 AHAS gene ATACTTGGGAAACCCAGAGAATGAAAGTGAGATATATCCAGATTTCGTGACAATTGCCAA
 Sorghum bicolor AHAS gene ATACTTGGGAAACCCAGAGAATGAAAGTGAGATATATCCAGATTTCGTGACAATTGCCAA
        Zea mays AHAS gene ATACTTGGGAAACCCAGAGAATGAAAGTGAGATATATCCAGATTTCGTGACGATCGCCAA

   Unmutated N12 AHAS gene AGGGTTCAACATTCCAGCAGTCCGTGTGAC------------------------------
 Sorghum bicolor AHAS gene AGGGTTCAACATTCCAGCAGTCCGTGTGACAAAGAAGAGCGAAGTCCATGCAGCAATCAA
        Zea mays AHAS gene AGGGTTCAACATTCCAGCGGTCCGTGTGACAAAGAAGAACGAAGTCCGCGCAGCGATAAA
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Figure 12: Acetohydroxyacid synthase nucleotide sequence alignment of the unmutated sugarcane N12 control genotype, *Sorghum bicolor* and *Zea mays*.

The AHAS gene sequences of Mut 1, Mut 6 and Mut 7 were amplified, cloned and sequenced based on the method illustrated in section 3.5.1. The sequences of the mutant genotypes were then aligned to that of the unmutated N12 genotype in order to confirm a positive match and to identify possible mutations within the gene (Figure 12, Appendix II). As the AHAS copy number in sugarcane is unknown, but likely to be 8-10 copies based on the polyploidy of sugarcane (Butterfield, 2007), colony sequencing was performed to account for the different AHAS gene sequences that may be present. As a result different base pairs were identified at the same position within the gene sequence, e.g. adenine (A) and guanine (G) or cytosine (C) and thymine (Table 16).

Table 16: Point mutations within the acetohydroxyacid synthase nucleotide sequences of Mut 1, Mut 6 and Mut 7 compared with the unmutated N12 genotypes. A – Adenine; T – Thymine; G – Guanine; C – Cytosine.

Nucleotide Position	**Genotype and position of point mutation**			
	N12	**Mut 1**	**Mut 6**	**Mut 7**
319	C	C or T	C or T	C
550	C	T	T	C
620	C	C or T	C or T	C
1350	A	A or G	A or G	A or G

As aforementioned, the sugarcane AHAS gene sequence was not available at the time of this study therefore *S. bicolor* was used as a reference to identify an open reading frame (ORF). This allowed for the sequenced region to be translated into an amino acid sequence. A 477 amino acid sequence was generated and sequences of Mut 1, Mut 6 and Mut 7 were aligned to that of the unmutated N12 genotype (Figure 13). A single point mutation at position 195 was identified within Mut 1 and Mut 6, which converted an arginine (Arg) residue to a cysteine (Cys) residue (Figure 14). No amino acid mutations were found within the sequenced region of the Mut 7 genotype suggesting that a different mode of tolerance exists as reported by Kwon and Penner (1995), Kwon *et al*. (1995), Kotoula-Syka and Hatzios (1996), Fisher *et al*. (2000) and Breccia *et al*. (2012).

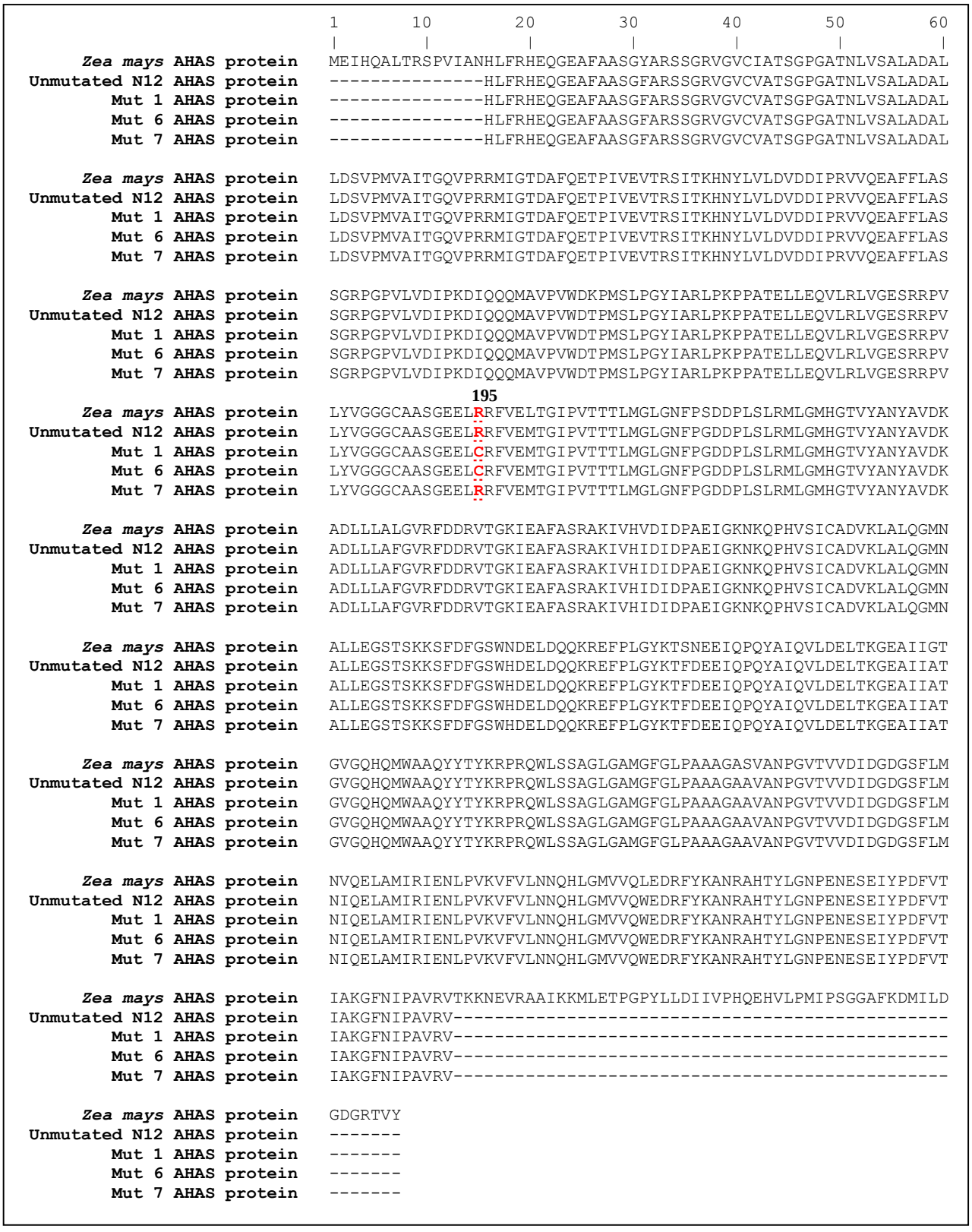

Figure 13: Acetohydroxyacid synthase protein alignment of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes with an amino acid point mutation at position 195 that converts arginine to cysteine.

4.3.2 Identification of mutant genotypes with an altered/enhanced metabolic detoxification of imazapyr

4.3.2.1 Determination of piperonyl butoxide concentration required for the detoxification trial

The concentration of PBO required for the detoxification of imazapyr was determined by measuring the height (cm) and leaf chlorophyll content (SPAD unit) of the unmutated N12 plants upon exposure to 0 (control), 25 and 50 µM PBO. In order to identify the highest concentration of PBO that did not cause plant injury compared with that of the control treatment, data sets were analysed by a one-way ANOVA and Sidak post hoc test and comparisons were made for each treatment over time and across treatments for each time.

The height of the unmutated N12 plants in the 0 µM treatment increased significantly ($P \leq 0.05$) over 3 weeks of exposure and when exposed to 25 and 50 µM PBO ($P = 0.013$, ≤ 0.001, ≤ 0.001 and respectively) (Table 17). No significant differences ($P \geq 0.05$) in leaf chlorophyll content was observed for the unmutated N12 plants within the 0, 25 and 50 µM PBO treatments ($P = 0.302$, 0.094 and 0.882, respectively) over 3 weeks of exposure (Table 18).

A comparison of plant height and leaf chlorophyll content showed no significant differences ($P \geq 0.05$) amongst treatments at each time tested, which indicated that neither 25 nor 50 µM PBO caused plant injury compared with the 0 µM treatment (Tables 17 and 18). Based on the results obtained and on the concentration of PBO used by Breccia *et al*. (2012), 50 µM PBO was selected for the subsequent trial involving the detoxification of imazapyr by CYP.

Table 17: The effect of 0, 25 and 50 µM piperonyl butoxide on the plant height (cm) of unmutated N12 plants 0, 1, 2 and 3 weeks after exposure. Dissimilar alphabet characters denote statistically significant differences: (a) across time for each treatment (A-B underlined in green in the rows), (b) amongst treatments for each time tested (a-b in columns). Data was analysed using a one-way ANOVA and a Sidak post hoc test. $P \geq 0.05$, n = 5, mean ± SE.

Piperonyl butoxide (µM)	**Plant height (cm)**				***P* value**
	Time (w)				
	***0**	**1**	**2**	**3**	
0	8.20 ± 1.44 $^{a, A}$	8.90 ± 1.13 $^{a, AB}$	9.60 ± 1.04 $^{a, AB}$	10.30 ± 1.04 $^{a, B}$	0.013
25	8.20 ± 0.72 $^{a, A}$	9.20 ± 0.68 $^{a, A}$	10.70 ± 0.77 $^{a, B}$	11.50 ± 1.04 $^{a, B}$	≤ 0.001
50	8.60 ± 1.35 $^{a, A}$	9.90 ± 1.50 $^{a, AB}$	11.10 ± 1.67 $^{a, BC}$	12.70 ± 1.73 $^{a, C}$	≤ 0.001
P value	0.909	0.677	0.520	0.292	

* Time at which PBO was introduced to the nutrient solution.

Table 18: The effect of 0, 25 and 50 µM piperonyl butoxide on the leaf chlorophyll content (SPAD unit) of unmutated N12 plants 0, 1, 2 and 3 weeks after exposure. Dissimilar alphabet characters denote statistically significant differences: (a) across time for each treatment (A-B in rows), (b) amongst treatments for each time tested (a-b in columns). Data was analysed using a one-way ANOVA and a Sidak post hoc test. $P \geq 0.05$, n = 5, mean ± SE.

Piperonyl butoxide (µM)	**Leaf chlorophyll content (SPAD unit)**				***P* value**
	Time (w)				
	***0**	**1**	**2**	**3**	
0	38.99 ± 2.32 $^{a, A}$	44.24 ± 2.43 $^{a, A}$	45.96 ± 1.66 $^{a, A}$	43.62 ± 3.04 $^{a, A}$	0.302
25	42.47 ± 2.80 $^{a, A}$	47.27 ± 1.55 $^{a, A}$	42.62 ± 1.58 $^{a, A}$	38.72 ± 3.70 $^{a, A}$	0.094
50	43.37 ± 3.97 $^{a, A}$	44.34 ± 2.33 $^{a, A}$	44.90 ± 2.51 $^{a, A}$	41.72 ± 2.64 $^{a, A}$	0.882
P value	0.623	0.504	0.438	0.607	

* Time at which PBO was introduced to the nutrient solution.

4.3.2.2 Determination of imazapyr concentration required for the detoxification trial

The concentration of imazapyr required for the detoxification trial was determined by comparing the height (cm) and leaf chlorophyll content (SPAD unit) of the unmutated N12 plants after exposure to 0 (control), 1, 10, 25, 50, 75 and 100 µM imazapyr. For this trial an analytical standard of imazapyr was added directly to the troughs containing the nutrient solution to prevent foliar uptake by the unmutated N12 plants. In order to determine the highest concentration of imazapyr that caused visible injury to the plants within the shortest time, data sets were analysed by a one-way ANOVA and Sidak post hoc test and comparisons were made for each treatment over time and amongst treatments for each time tested.

The height of the unmutated N12 plants exposed to 0 and 1 µM imazapyr increased significantly ($P \leq 0.05$) over 3 weeks ($P = 0.013$ and 0.003, respectively), whereas those within the 10 and 25 µM imazapyr treatments did not (Table 19). The growth of the unmutated N12 plants was inhibited after exposure to the 50, 75 and 100 µM imazapyr treatments resulting in a significant decrease ($P \leq 0.05$) in plant height over time ($P = 0.074$, 0.029, ≤ 0.001 and 0.001, respectively) (Table 19). The height of the unmutated N12 plants at each time showed significant differences 2 and 3 weeks after exposure to the chemical: after 2 weeks of exposure to imazapyr, the height of the unmutated N12 plants within the 0 µM treatment was significantly higher than those in the other imazapyr treatments ($P \leq 0.001$) (Table 19); after 3 weeks of exposure to imazapyr, the height of the unmutated N12 plants within the 0, 1 and 10 µM imazapyr treatments was significantly higher than those within the 75 and 100 µM imazapyr treatments ($P \leq 0.001$) (Table 19). All plants within the 75 and 100 µM imazapyr treatments were dead 3 weeks after exposure to the chemical (Table 19 and Figure 14).

After exposure to the 0 and 1 µM imazapyr treatments, the leaf chlorophyll content of the unmutated N12 plants was not significantly different ($P \geq 0.05$) to the plants exposed to the 10, 25, 50, 75 and 100 µM imazapyr treatments; the latter decreased significantly ($P \leq 0.05$) after exposure ($P = 0.025$, ≤ 0.001, 0.004, ≤ 0.001 and ≤ 0.001, respectively) (Table 20). The leaf chlorophyll content of the unmutated N12 plants exposed to 10 µM imazapyr was significantly higher ($P \leq 0.005$) at 0 weeks than that of the plants exposed to 75 and 100 µM imazapyr ($P = 0.006$) (Table 20). This difference is probably not an indication of chlorosis or plant stress but merely a lack of randomisation in the experiment. One week after exposure to imazapyr, the unmutated N12 plants exposed to the 50, 75 and 100 µM imazapyr treatments displayed a significantly lower ($P \leq 0.05$) leaf chlorophyll content than the plants exposed to the 0 and

10 µM imazapyr treatments ($P \leq 0.001$) (Table 20). The 100 µM imazapyr treatment caused chlorosis to the unmutated N12 plants 2 weeks after exposure resulting in a significantly lower ($P \leq 0.005$) leaf chlorophyll content than that of the plants exposed to 0 and 10 µM imazapyr treatments ($P \leq 0.001$) (Table 20). The 25 and 50 µM imazapyr treatments caused chlorosis to the unmutated N12 plants 3 weeks after exposure to the chemical which resulted in a significantly lower ($P \leq 0.005$) leaf chlorophyll content than that of the plants exposed to 0, 1 and 10 µM imazapyr ($P \leq 0.001$) (Table 20). All plants within the 75 and 100 µM imazapyr treatments were dead 3 weeks after exposure to imazapyr.

The aim of this trial was to determine a concentration of imazapyr that caused visible injury to the plants within the shortest time and based on the results obtained, 100 µM imazapyr was selected for use in the subsequent pot trial (section 4.3.1.3). This involved assessing if the Mut 1, Mut 6 and Mut 7 genotypes displayed an enhanced detoxification mechanism after being treated with imazapyr in combination with 50 µM PBO.

Table 19: The effect of 0, 1, 10, 25, 50, 75 and 100 µM imazapyr (analytical grade) on the plant height (cm) of unmutated N12 plants 0, 1, 2 and 3 weeks after exposure. Dissimilar alphabet characters denote statistically significant differences: (a) across time for each treatment (A-B underlined in green in the rows), (b) amongst treatments for each time tested (a-b highlighted in green in the columns). Data was analysed using a One-way ANOVA and a Sidak post hoc test. $P \geq 0.05$, n = 5, mean ± SE.

Imazapyr (µM)	**Plant height (cm)**				***P* value**
	Time (w)				
	***0**	**1**	**2**	**3**	
0	8.20 ± 1.44 $^{a, A}$	8.90 ± 1.13 $^{a, AB}$	9.60 ± 1.04 $^{c, AB}$	10.30 ± 1.04 $^{c, B}$	0.013
1	7.80 ± 0.51 $^{a, A}$	8.90 ± 0.37 $^{a, B}$	9.00 ± 0.32 $^{bc, B}$	9.40 ± 0.37 $^{c, B}$	0.003
10	7.80 ± 0.60 $^{a, A}$	8.10 ± 0.64 $^{a, A}$	7.90 ± 0.73 $^{ab, A}$	8.20 ± 0.64 $^{bc, A}$	0.341
25	8.00 ± 0.84 $^{a, A}$	8.20 ± 0.87 $^{a, A}$	7.60 ± 1.02 $^{ab, A}$	5.20 ± 2.22 $^{abc, A}$	0.068
50	7.60 ± 0.66 $^{a, C}$	7.16 ± 0.28 $^{a, BC}$	3.90 ± 1.73 $^{a, A}$	4.00 ± 1.74 $^{ab, AB}$	0.047
75	7.50 ± 0.79 $^{a, B}$	6.50 ± 0.84 $^{a, B}$	3.30 ± 1.49 $^{a, AB}$	0.00 ± 0.00 $^{a, A}$	0.001
100	7.20 ± 0.68 $^{a, B}$	6.90 ± 0.64 $^{a, B}$	3.20 ± 1.98 $^{a, AB}$	0.00 ± 0.00 $^{a, A}$	< 0.001
P value	0.808	0.088	< 0.001	< 0.001	

* Time at which imazapyr was introduced to the nutrient solution.

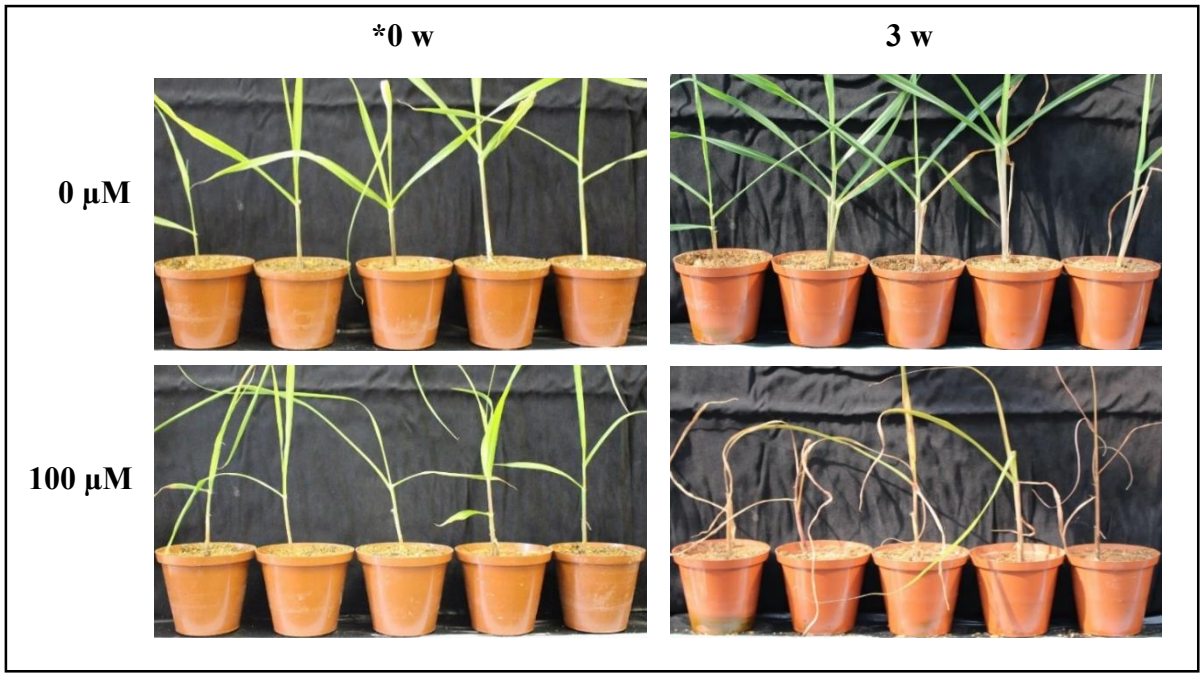

Figure 14: Visual comparison of unmutated N12 plants 0 and 3 weeks after exposure to 0 and 100 µM imazapyr (analytical grade). * indicates the time at which the plants were exposed to imazapyr.

Table 20: The effect of 0, 1, 10, 25, 50, 75 and 100 µM imazapyr (analytical grade) on the leaf chlorophyll content (SPAD unit) of unmutated N12 plants 0, 1, 2 and 3 weeks after exposure. Dissimilar alphabet characters denote statistically significant differences: (a) across time for each treatment (A-B underlined in green in the rows), (b) amongst treatments for each time tested (a-b highlighted in green in the columns). Data was analysed using a One-way ANOVA and a Sidak post hoc test. $P \geq 0.05$, n = 5, mean ± SE.

Imazapyr (µM)	**Leaf chlorophyll content (SPAD unit)**				***P* value**
	Time (w)				
	***0**	**1**	**2**	**3**	
0	38.99 ± 2.32 $^{ab, A}$	44.24 ± 2.43 $^{b, A}$	45.96 ± 1.66 $^{c, A}$	43.62 ± 3.04 $^{c, A}$	0.302
1	37.48 ± 0.95 $^{ab, A}$	35.80 ± 2.52 $^{ab, A}$	36.14 ± 6.97 $^{abc, A}$	41.32 ± 2.59 $^{c, A}$	0.705
10	44.16 ± 2.08 $^{b, AB}$	44.54 ± 1.81 $^{b, B}$	42.38 ± 3.13 $^{bc, AB}$	34.84 ± 1.61 $^{c, A}$	0.025
25	36.76 ± 2.49 $^{ab, B}$	37.68 ± 2.01 $^{ab, B}$	37.28 ± 2.04 $^{abc, B}$	14.99 ± 6.21 $^{b, A}$	< 0.001
50	39.52 ± 1.61 $^{ab, C}$	28.38 ± 5.97 $^{a, BC}$	16.20 ± 8.16 $^{ab, AB}$	14.20 ± 6.61 $^{b, A}$	0.004
75	31.08 ± 1.90 $^{a, B}$	26.36 ± 3.57 $^{a, AB}$	16.78 ± 7.10 $^{ab, A}$	0.00 ± 0.00 $^{a, A}$	< 0.001
100	32.57 ± 2.58 $^{a, C}$	26.78 ± 4.40 $^{a, BC}$	11.42 ± 7.12 $^{a, AB}$	0.00 ± 0.00 $^{a, A}$	< 0.001
P value	0.006	< 0.001	< 0.001	< 0.001	

* Time at which imazapyr was introduced to the nutrient solution.

4.3.2.3 Assessing the potential for an enhanced detoxification of imazapyr by piperonyl butoxide within the mutant genotypes

Based on reports by Kwon and Penner (1995), Kwon *et al*. (1995), Kotoula-Syka and Hatzios (1996), Fisher *et al*. (2000) and Breccia *et al*. (2012), it is expected that a plant with an enhanced detoxification mechanism would exhibit more damage when treated with a combination of a herbicide and PBO than a plant treated with only the herbicide. This is because the CYP enzyme is inhibited by the PBO which in turn inhibits the detoxification mechanism resulting in plant necrosis. In contrast, plants that do not display signs of chlorosis or necrosis after treatment with a combination of the herbicide and PBO are less likely to exhibit an enhanced metabolic detoxification mechanism. As the tolerance mechanism (target or non- target site) of mutant genotypes used in this study has not been previously characterised, an investigation into the enhanced detoxification of imazapyr by CYP was carried out. This was done by exposing the Mut 1, Mut 6 and Mut 7 plants to imazapyr with and without the CYP inhibitor PBO. The unmutated N12 plants used in this trial served as a control by mimicking the expected outcome of plants sensitive to inhibition by imazapyr.

The unmutated N12, Mut 1, Mut 6 and Mut 7 plants were exposed to a nutrient solution only for 1 week which provided the plants with all the necessary nutrients required for growth. Following this, the plants were exposed to 50 µM PBO for another week, after which time 100 µM imazapyr was added to the nutrient solution. Plant height (cm) and leaf chlorophyll measurements (SPAD unit) were taken before exposure to imazapyr (t = 0) and every week thereafter for 3 weeks at which time the fresh and dry mass (g) of the root and shoot samples were determined.

The unmutated N12 plants exposed to 100 µM imazapyr and 100 µM imazapyr + PBO exhibited severe damage defined by a significant decrease in plant height and leaf chlorophyll content. The plant height of the unmutated N12 plants exposed to the control (0 µM PBO + 0 µM imazapyr) and 50 µM PBO treatments increased significantly ($P \leq 0.05$) over 3 weeks ($P = 0.030$ and 0.008 for each treatment, respectively) (Table 21). Two weeks after exposure to 100 µM imazapyr and 100 µM imazapyr + PBO the height of the unmutated N12 plants decreased significantly ($P \leq 0.05$) compared with those exposed to the control and 50 µM PBO treatments ($P = 0.038$), this was still evident after 3 weeks of exposure to imazapyr ($P = 0.001$) (Table 21). The leaf chlorophyll content of the unmutated N12 plants exposed to 100 µM imazapyr and 100 µM imazapyr + PBO were significantly lower than that of the plants exposed

to the control and 50 µM PBO treatments after 3 weeks of exposure to the chemicals (P = 0.021) (Table 22). The trend observed with plant height and leaf chlorophyll content of the unmutated N12 plants was observed with the fresh and dry root and dry shoot mass, with plants exposed to the 100 µM imazapyr and 100 µM imazapyr + PBO treatments being significantly lower than those exposed to the control and 50 µM PBO treatments (P = 0.006, 0.004 and 0.010 for each measurement, respectively) (Figures 15, 16 and17). No significant differences in dry shoot mass of the unmutated N12 plants were observed amongst treatments (data not shown).

The Mut 1 plants exposed to imazapyr showed no significant differences ($P \geq 0.05$) in plant height amongst treatments at 0, 2 and 3 weeks after exposure (Table 21). Similarly, no significant differences ($P \geq 0.05$) in leaf chlorophyll content were found over time for the Mut 1 plants exposed to the control, 50 µM PBO and 100 µM imazapyr + PBO treatments (Table 22). Although the leaf chlorophyll content of the Mut 1 plants decreased significantly ($P \leq 0.05$) over time after exposure to 100 µM imazapyr (P = 0.039) there were no significant differences ($P \geq 0.05$) in the leaf chlorophyll content of Mut 1 found amongst treatments, 3 weeks after exposure to imazapyr (Table 22).

The height of the Mut 6 plants exposed to the 100 µM imazapyr + PBO treatment decreased significantly ($P \leq 0.05$) over time ($P \leq 0.001$) whereas those not exposed to this treatment showed no significant differences in plant height over 3 weeks ($P \geq 0.05$) (Table 21). No significant differences ($P \geq 0.05$) in the leaf chlorophyll content of the Mut 6 plants were observed for each treatment over time and amongst treatments at each time tested (Table 22).

The height of the Mut 7 plants decreased significantly ($P \leq 0.05$) after exposure to the 50 µM PBO treatment (P = 0.039) whereas those exposed to the control, 100 µM imazapyr and 100 µM imazapyr + PBO treatments showed no significant differences ($P \geq 0.05$) over 3 weeks (Table 21). Although the leaf chlorophyll content of the Mut 7 plants decreased significantly ($P \leq 0.05$) after exposure to 100 µM imazapyr (P = 0.049) there were no significant differences ($P \geq 0.05$) observed amongst treatments at each time tested (Table 22).

It is unknown if the concentration of PBO selected for this study was high enough to inhibit detoxification of the herbicide in the mutant plants as 50 µM PBO was the highest concentration tested on the unmutated 12 plants in the pre-trial. There is also no control plant species, i.e. a sugarcane genotype known to have an enhanced detoxification mechanism, to

which a comparison could be made. Regardless of this limitation, the roots were the first line of contact between the herbicide and the plants and no significant differences ($P \geq 0.05$) in fresh and dry root mass and fresh shoot mass were observed amongst treatments for the Mut 1, Mut 6 and Mut 7 plants, in contrast to the unmutated N12 plants (Figures 15, 16 and17).

As previously mentioned, the results obtained from the sequence analysis indicated that tolerance to imazapyr of Mut 1 and Mut 6 could possibly be a result of the amino acid mutation within the AHAS gene. Based on the results obtained in this trial it can be concluded that tolerance to imazapyr of Mut 1, Mut 6 or Mut 7 is not based on a non-target site tolerance such as an enhanced metabolic detoxification of the herbicide.

Table 21: The effect of 100 µM imazapyr with and without piperonyl butoxide, on the plant height (cm) of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes after 0, 2 and 3 weeks of exposure. Dissimilar alphabet characters denote statistically significant differences: (a) across time for each treatment (A-B underlined in green in the rows); (b) amongst treatments for each time tested (a-b highlighted in green in the columns).Data sets were analysed by a one-way ANOVA and Sidak post hoc test. $P \geq 0.05$, n = 5, mean ± SE.

Genotype	Treatment	Plant height (cm)			*P* value
		Time (w)			
		0	2	3	
Unmutated N12	*Control	8.28 ± 0.59 a, A	9.90 ± 0.84 bc, AB	10.50 ± 0.47 b, B	0.030
	50 µM PBO	8.80 ± 0.51 a, A	10.30 ± 0.37 c, B	10.90 ± 0.33 b, B	0.008
	100 µM imazapyr	9.00 ± 0.61 a, A	7.10 ± 1.07 a, A	6.40 ± 1.02 a, A	0.100
	100 µM imazapyr + PBO	9.40 ± 0.91 a, A	7.50 ± 0.89 ab, A	7.60 ± 0.68 a, A	0.269
	P value	0.200	0.038	0.001	
Mut 1	*Control	9.00 ± 0.47 a, A	8.30 ± 1.09 a, A	8.30 ± 1.18 a, A	0.771
	50 µM PBO	9.10 ± 0.40 a, A	7.50 ± 0.88 a, A	8.00 ± 1.04 a, A	0.347
	100 µM imazapyr	9.30 ± 0.64 a, A	7.40 ± 0.73 a, A	6.90 ± 0.58 a, A	0.099
	100 µM imazapyr + PBO	9.30 ± 0.68 a, A	8.70 ± 0.30 a, A	8.60 ± 0.51 a, A	0.311
	P value	0.915	0.709	0.568	
Mut 6	*Control	10.00 ± 1.19 a, A	9.50 ± 0.57 a, A	8.70 ± 2.21 a, A	0.743
	50 µM PBO	9.30 ± 0.46 a, A	9.20 ± 0.58 a, A	9.80 ± 0.30 a, A	0.519
	100 µM imazapyr	9.20 ± 0.44 a, A	8.70 ± 1.01 a, A	8.80 ± 0.72 a, A	0.786
	100 µM imazapyr + PBO	9.30 ± 0.41 a, B	7.90 ± 0.68 a, A	7.60 ± 0.48 a, A	< 0.001
	P value	0.510	0.366	0.622	
Mut 7	*Control	9.10 ± 0.62 a, A	8.50 ± 0.76 a, A	7.70 ± 1.06 a, A	0.148
	50 µM PBO	9.50 ± 0.55 a, B	7.00 ± 0.97 a, A	7.40 ± 1.24 a, A	0.039
	100 µM imazapyr	9.30 ± 0.46 a, A	7.20 ± 2.18 a, A	7.60 ± 2.19 a, A	0.486
	100 µM imazapyr + PBO	9.50 ± 0.91 a, A	8.60 ± 0.37 a, A	7.90 ± 1.07 a, A	0.260
	P value	0.923	0.776	0.997	

*Control = 0 µM PBO + 0 µM imazapyr

Table 22: The effect of 100 µM imazapyr with and without piperonyl butoxide, on the leaf chlorophyll content (SPAD unit) of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes after 0, 2 and 3 weeks of exposure. Dissimilar alphabet characters denote statistically significant differences: (a) across time for each treatment (A-B underlined in green in the rows); (b) amongst treatments for each time tested (a-b highlighted in green in the columns). Data sets were analysed by a one-way ANOVA and Sidak post hoc test. $P \geq 0.05$, n = 5, mean ± SE.

Genotype	Treatment	Leaf chlorophyll content (SPAD unit)			*P* value
		Time (w)			
		0	2	3	
Unmutated	*Control	36.80 ± 3.14 [a, A]	40.84 ± 1.29 [a, A]	35.99 ± 2.96 [b, A]	0.247
N12	50 µM PBO	38.58 ± 1.62 [a, A]	38.54 ± 3.53 [a, A]	40.66 ± 2.88 [b A]	0.833
	100 µM imazapyr	36.80 ± 1.37 [a, A]	29.02 ± 6.15 [a, A]	22.44 ± 4.21 [a, A]	0.100
	100 µM imazapyr + PBO	36.58 ± 0.47 [a, A]	31.82 ± 0.71 [a, A]	30.46 ± 3.28 [a, A]	0.151
	P value	0.874	0.127	0.021	
Mut 1	*Control	36.54 ± 2.80 [a, A]	40.60 ± 3.84 [a, A]	40.66 ± 2.75 [a, A]	0.434
	50 µM PBO	38.32 ± 2.70 [a, A]	47.92 ± 11.11 [a, A]	42.76 ± 1.92 [a, A]	0.567
	100 µM imazapyr	43.34 ± 2.64 [a, B]	31.06 ± 1.91 [a, A]	35.1 ± 3.89 [a, AB]	0.039
	100 µM imazapyr + PBO	40.46 ± 0.99 [a, A]	32.54 ± 4.15 [a, A]	38.38 ± 1.44 [a, A]	0.173
	P value	0.241	0.301	0.351	
Mut 6	*Control	32.86 ± 3.18 [a, A]	32.90 ± 2.72 [a, A]	25.72 ± 6.83 [a, A]	0.409
	50 µM PBO	36.58 ± 4.46 [a, A]	35.98 ± 3.53 [a, A]	38.54 ± 2.39 [a, A]	0.770
	100 µM imazapyr	34.78 ± 2.13 [a, A]	38.14 ± 1.95 [a, A]	38.46 ± 1.65 [a, A]	0.322
	100 µM imazapyr + PBO	36.02 ± 2.28 [a, A]	34.12 ± 1.35 [a, A]	34.16 ± 2.23 [a, A]	0.742
	P value	0.751	0.561	0.086	
Mut 7	*Control	36.40 ± 2.00 [a, A]	32.48 ± 4.38 [a, A]	41.56 ± 5.10 [a, A]	0.275
	50 µM PBO	41.70 ± 1.48 [a, A]	34.54 ± 2.45 [a, A]	37.64 ± 2.18 [a, A]	0.079
	100 µM imazapyr	39.32 ± 1.83 [a, B]	23.06 ± 5.84 [a, A]	23.48 ± 8.37 [a, A]	0.049
	100 µM imazapyr + PBO	32.10 ± 3.36 [a, A]	34.54 ± 3.47 [a, A]	39.28 ± 4.10 [a, A]	0.201
	P value	0.095	0.314	0.166	

*Control = 0 µM PBO + 0 µM imazapyr

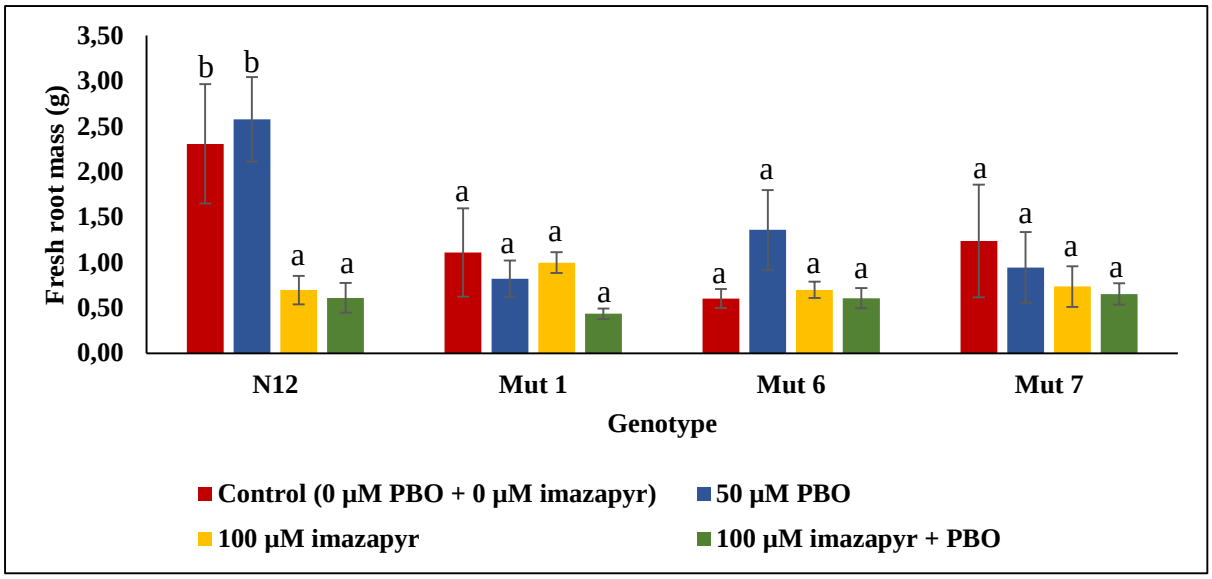

Figure 15: The effect of 100 µM imazapyr with and without piperonyl butoxide, on the fresh root mass (g) of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes after 0, 2 and 3 weeks of exposure. Dissimilar alphabet characters denotes a statistically significant difference amongst treatments for each genotype (a-b in columns). Data sets were analysed by a one-way ANOVA and Sidak post hoc test. $P \geq 0.05$, n = 5, mean ± SE.

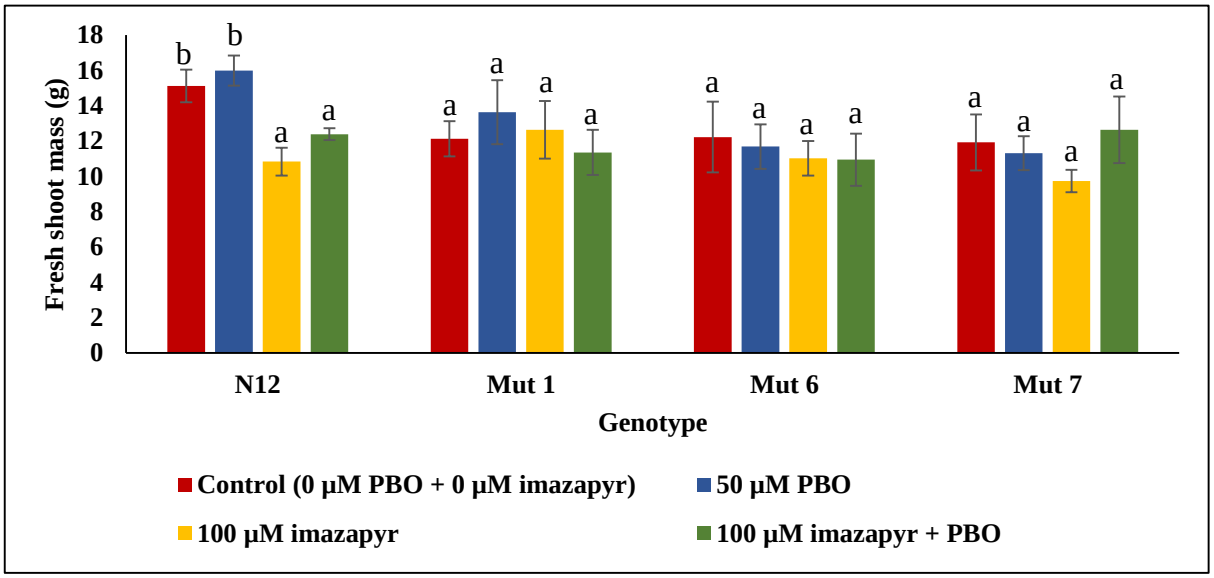

Figure 16: The effect of 100 µM imazapyr with and without 50 µM piperonyl butoxide, on the fresh shoot mass (g) of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes after 5 weeks of exposure. Dissimilar alphabet characters denotes a statistically significant difference amongst treatments for each genotype (a-b in columns). Data sets were analysed by a one-way ANOVA and Sidak post hoc test. $P \geq 0.05$, n = 5, mean ± SE.

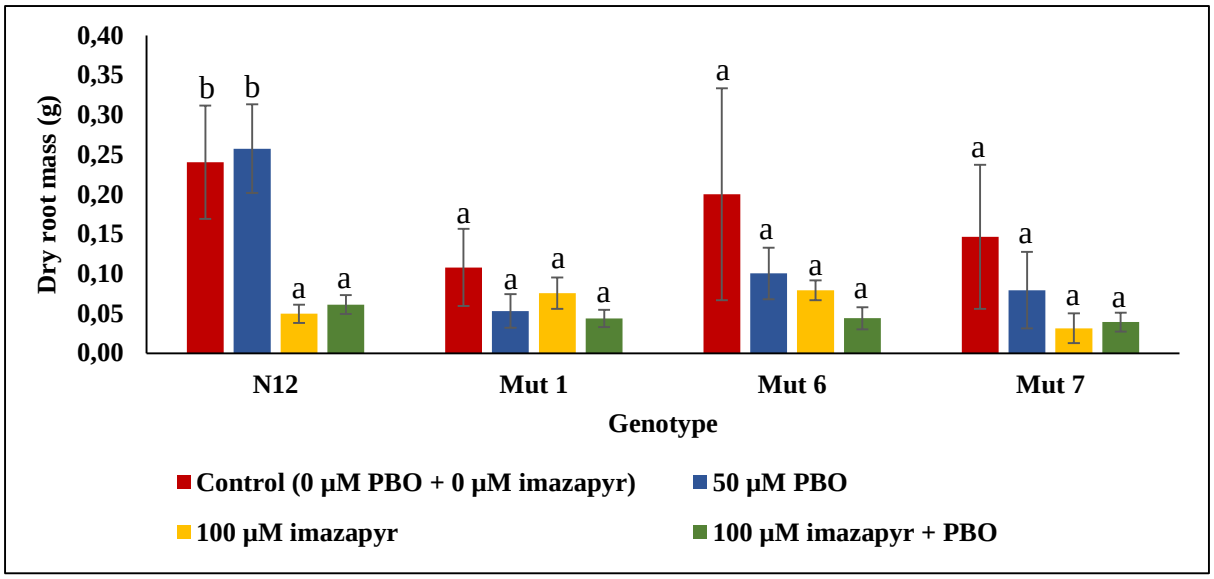

Figure 17: The effect of 100 µM imazapyr with and without 50 µM piperonyl butoxide, on the dry root mass (g) of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes after 5 weeks of exposure. Dissimilar alphabet characters denotes a statistically significant difference amongst treatments for each genotype (a-b in columns). Data sets were analysed by a one-way ANOVA and Sidak post hoc test. $P \geq 0.05$, $n = 5$, mean ± SE.

5. Discussion

Cynodon dactylon is a persistent weed in the South African Sugar Industry which can be controlled chemically using a wide range of herbicides which inhibit different plant metabolic processes (Campbell, 2008). These herbicides include glyphosate (Roundup®), paraquat (Gramoxone®) and diuron (Diuron®) which are either used alone or as a mixture with other herbicides (Campbell *et al*., 2008; Seeruttun *et al*., 2014; Anon, 2014). The high dosages of these herbicides currently being used to control *C. dactylon* pose a future threat due to the possibility of this weed becoming herbicide tolerant (Eksteen, 2007). Imazapyr has been identified as a non-glyphosate candidate herbicide that provides and equal and in some cases better control of *C. dactylon* than glyphosate (Richard, 1997; Broome *et al*., 2000; Ferrell *et al*., 2005; Campbell, 2008; Conlong and Campbell, 2010; Seeruttun *et al*., 2014).

The Mut 1, Mut 6 and Mut 7 genotypes used in this study were previously produced by mutating the N12 genotype using EMS (Koch *et al.*, 2012). Rutherford *et al*. (*in press*) subsequently compared the agronomic characteristics of the mutants to the unmutated N12 when the genotypes were treated with different concentrations of Arsenal® GEN 2 in a field trial. The agronomic characteristics of the mutants were assessed in comparison with the unmutated N12 genotype and no significant differences were observed in the unsprayed field. When the field was treated with Arsenal® GEN 2 prior to planting of the sugarcane, the Mut 1, Mut 6 and Mut 7 genotypes showed a higher tolerance to imazapyr compared with the other mutants and the unmutated N12. The activity of the AHAS enzyme (the enzyme that confers tolerance to imazapyr) of the Mut 1, Mut 6 and Mut 7 genotypes was higher after exposure to imazapyr compared with the other mutants and the unmutated N12 genotype. This was based on an *in vitro* enzyme assay that characterised the activity of the enzyme in the presence of the herbicide. Based on the results obtained by Koch *et al*. (2012) and Rutherford *et al*. (*in press*), Mut 1, Mut 6 and Mut 7 were selected for this study.

5.1 Efficacy of Arsenal® GEN 2 (imazapyr) in controlling *Cynodon dactylon*

In the present study, the effect of a lethal dose of Arsenal® GEN 2 (1254 g a.i.ha^{-1} imazapyr) in controlling *C. dactylon* was compared with two applications of a commonly used herbicide cocktail containing a mixture of Gramoxone® (200 g a.i.ha^{-1} paraquat) and Diuron® (800 g a.i.ha^{-1} diuron). These herbicides were applied after tufts of *C. dactylon* were planted within

the inter-rows of each treatment (Table 6 and Figure 4). The percentage of green *C. dactylon* leaves decreased significantly 20 weeks after treatment with Arsenal® GEN 2 (9.64 %). These results differ from those reported by Campbell (2008) because although the percentage of green *C. dactylon* leaves decreased significantly after treatment with Arsenal® GEN 2, at 20 weeks after treatment the percentage of green *C. dactylon* leaves was significantly higher ($P \leq 0.05$) than that observed in the herbicide cocktail treatment (9.64% vs. 6.5%) (Figure 11). Although the trial in this study was successful in controlling *C. dactylon* making it unable to compete with the sugarcane, it was unsuccessful in confirming imazapyr as the better herbicide of choice for the control of *C. dactylon* compared with that of the herbicide cocktail because the duration of the trial was short-lived due to time constraints (Campbell, pers. comm.). However, if the trial were to be continued for longer than 20 weeks additional applications of the herbicide cocktail would be required (every 3 to 5 weeks) to ensure complete control of *C. dactylon* compared with the single application of Arsenal® GEN 2 applied at week 0 (Anon, 2014). In contrast, previous studies have indicated that in addition to being a more effective herbicide for the control of *C. dactylon*, only a single application of imazapyr is required to control the weed compared with the multiple applications of the herbicide cocktail (paraquat + diuron) which is required every 3 to 5 weeks to ensure complete eradication of *C. dactylon*, thereby making imazapyr a more cost effect method of chemical control (Richard, 1997; Broome *et al*., 2000; Ferrell *et al*., 2005; Campbell, 2008; Conlong and Campbell, 2010; Seeruttun *et al*., 2014; Anon, 2014).

Campbell *et al*. (2008) stated in their study that although there were no phytotoxic effects, nor differences in stalk height, number of stalks.plot^{-1}, sugarcane yield and sucrose yield caused by Arsenal® GEN 2 on the sugarcane compared with that of glyphosate, a 6 week waiting period is required after treating the field with imazapyr before planting can take place which would be a disadvantage to farmers. As a result, the use of Arsenal® GEN 2 in conjunction with imazapyr tolerant sugarcane would be beneficial.

5.2 Agronomic evaluation of Mut 1, Mut 6 and Mut 7 with the unmutated N12 genotype

Chemically induced mutagenesis using EMS is achieved by addition of the mutagenic agent to embryogenic calli because it is at this stage that rapid cell division occurs thus ensuring the highest probability of incorrect DNA repair (Kilbey and Hunter, 1983; Rutherford *et al*., 2014).

As a result of the random mutations produced by EMS within the genome their effect on the agronomic performance of sugarcane genotype is unknown. Field evaluations allow for the identification of mutants that have all the desired characteristics of the parent plant (Rutherford *et al*., 2014). Although there have been many studies directed at obtaining traits of interest via mutagenic agents (chemical and physical) to generate plants with resistance to different fungal diseases (red rot and stalk rot) (Mahlanza *et al*., 2013), tolerance to salinity and drought (Saif-Ur-Rasheed *et al*., 2001; Patade *et al*., 2006; Ali *et al*., 2007; Patade and Suprasanna, 2008; Patade *et al*., 2008; Khan and Khan, 2010) and tolerance to herbicides glyphosate (Zambrano *et al*., 2003) and imazapyr (Punyadee *et al*, 2007; Koch *et al*., 2012; Rutherford *et al*., *in press*), not many of those studies have characterised these mutants in the field (Rutherford *et al*., 2014).

The field evaluation of sugarcane derived from tissue culture involves the assessment of a number of standard agronomic characteristics including stalk height, stalk diameter, number of stalks, stalk mass, biomass yield and sucrose yield (Bailey and Bechet, 1989; Gilbert *et al*., 2005). Other evaluations include quantification of resistance to the sugarcane borer *E. saccharina* (Goebel and Way, 2003; Anon, 2005) and calculation of the recoverable value sucrose content (RV per 100g fresh mass/RV % cane) which methodically assesses the commercial quality of the sugarcane (Cane Testing Service, South African Sugar Association, 2015).

Studies by Arencibia *et al*. (1999) and Gilbert *et al*. (2005) who evaluated the resistance of transgenic sugarcane genotypes to stalk borers and the expression of a transgene, respectively, found that the agronomic traits of the transgenic genotypes were similar to that of the wild type parent. Although the yield (biomass and sucrose) of the transgenic sugarcane genotypes evaluated by Vickers *et al*. (2005) and Gilbert *et al*. (2009) were significantly lower than that of the parent, no significant differences were observed in all the other evaluated agronomic characteristics such as number of stalks and stalk mass. Field trials involving herbicide tolerant transgenic sugarcane conducted by Leibbrandt and Snyman (2003) found that the stalk diameter of the transgenic plants was significantly less and a lower infestation by *E. saccharina* was noted compared with the parent. There were no significant differences observed in stalk height.

In the current study, a field assessment of sugarcane planted from setts was conducted after 18 months in the field in order to compare the effect of the chemical mutagenesis (and possibly tissue culture) on the agronomic characteristics of the mutants with the unmutated N12.

Comparisons were made on the number of stalks.plot^{-1}, stalk height (cm), stalk diameter (cm), estimated yield (kg.plot^{-1}), fibre content (g.100g^{-1} fresh mass), sucrose content (g.100g^{-1} fresh mass) and recoverable value sucrose content (RV % cane) of the mutants to those of the unmutated N12 control (Table 11). An *E. saccharina* assessment was also carried out to determine the percentage of internodes bored by the pest (Table 12).

Although the EMS mutagenesis and tissue culture had no effect on the number of stalks.plot^{-1}, stalk height, fibre content and sucrose content, the combined (i.e. EMS + tissue culture) treatment negatively affected the stalk diameter of the mutants resulting in significantly lower values compared with those of the unmutated N12 control (Table 11). In a study by Leibbrandt and Snyman (2003), where differences in *E. saccharina* infestation were observed in the transgenic line, it was postulated that a higher fibre content in that line could result in a lower number of internodes bored by the insect. Although, in the present study, there were no significant differences in fibre content and the percentage of internodes bored by *E. saccharina* amongst the mutants, the trend stated by Leibbrandt and Snyman (2003) was observed: Mut 1 had the lowest fibre content and highest percentage of damage internodes, Mut 7 had the highest fibre content and lowest percentage of damage internodes (Tables 11 and 12). Based on the data presented in Tables 11 and 12 the results of this study indicated that unmutated N12 control was least susceptible to infestation by *E. saccharina*, suggesting that some other component associated with insect-plant defences might have been altered as a result of the EMS + tissue culture treatment (Tables 11 and 12). The estimated yield (kg fresh mass above ground) of the Mut 1 genotype was affected by the EMS mutagenesis resulting in a lower value than that of the unmutated N12 (Table 11). Although the cane quality (RV % cane) of Mut 1 was better than that of the Mut 7 genotype, no variation in the cane quality of Mut 7 was observed when compared with the unmutated N12 control (Table 11). The genotypes used in the agronomic field trial are derived from vegetative setts of plant cane and, therefore, the significant differences in stalk diameter, estimated yield, cane quality and level of infestation by *E. saccharina* observed could not be attributed to any direct tissue culture effects (Lourens and Martin, 1987; reviewed in Snyman *et al*., 2011).

In this study, the tolerance of the mutants and the unmutated N12 genotype to Arsenal® GEN 2 was identified by comparing the number of stalks.plot^{-1}, stalk height and leaf chlorophyll content after exposure to the herbicide with those of two other weed control treatments, *viz*. hand weeding and a herbicide cocktail involving a mixture of Gramoxone® and Diuron®. Both

the herbicide cocktail and Arsenal® GEN 2 were applied after the sugarcane was planted, as oppose to 6 weeks before planting as stated by Campbell *et al*. (2008) or 4 months prior to planting as stated by Anon (2015). Measurements were taken at 0, 2, 4, 8, 10, 12, 16 and 20 weeks after herbicide application in order to monitor the effect of the herbicides on the sugarcane.

The Mut 1, Mut 6 and Mut 7 genotypes showed a level of tolerance after exposure to Arsenal® GEN 2 in terms of number of stalks.plot^{-1} and stalk height compared with those of the unmutated N12 control which began to die 2 weeks after herbicide application (Tables 13 and 14). Although the height of the Mut 1 stalks were significantly shorter 12 weeks after the application of Arsenal® GEN 2 compared with that of the hand weeded treatment, no significant differences in stalk height were observed at each tested time thereafter (Table 14). These results support the findings of Campbell *et al*. (2008). The number of N12 stalks.plot^{-1} (Table 13) and the stalk height (Table 14) were significantly lower over time after exposure to Arsenal® GEN 2 compared with the other two weed management regimes. This trend was expected because the unmutated N12 genotype is not tolerant to imazapyr and it also proves that the chemical mutagenesis using EMS was successful in producing imazapyr tolerant mutant genotypes. These results were similar to those obtained in the study by Rutherford *et al*. (*in press*) who reported a reduced number of stalks.plot^{-1}, stalk height and stalk diameter in the imazapyr sensitive sugarcane genotypes. Similarly, Punyadee *et al*. (2007) reported that sugarcane genotypes sensitive to imazapyr displayed shorter stalks compared with their herbicide tolerant counterparts. Other imidazolinone tolerant crops which support these findings include peanuts (Matocha *et al*., 2003), cotton (Grey *et al*., 2005; Wiatrak *et al*., 2009) and wheat (Newhouse *et al*., 1992). Although these studies involve a different imidazolinone herbicide they can still be used as a comparison because they belong to the same herbicide family and therefore have the same mode of action (AHAS inhibitors).

Leaf chlorophyll content is regarded as a good measure of plant stress (Hendry and Price, 1993). In this study, the leaf chlorophyll content of the unmutated N12 control decreased significantly over time after exposure to Arsenal® GEN 2 compared with the plants exposed to the hand weeded and herbicide cocktail management regimes (Table 15). This is a further indication that the unmutated N12 plants, unlike the mutant genotypes, are not tolerant to imazapyr. Although the Mut 1, Mut 6 and Mut 7 genotypes showed significant decreases in leaf chlorophyll content 4, 8 and 12 weeks after exposure to Arsenal® GEN 2 compared with

that of the hand weeded and herbicide cocktail treatments, there were no differences found thereafter (Table 15). A decrease in leaf chlorophyll content was observed for Mut 1, Mut 6, Mut 7 and the unmutated N12 control in all three weed treatments 12 weeks after treatment (Table 15 and Figure 10). This could possibly be a result of water stress which occurred during that time period. The results observed in this study coincide with the findings of Rutherford *et al*. (*in press*) who observed chlorosis in the leaves of the unmutated N12 plants which eventually died. The symptoms observed in that study occurred 6 weeks after the plants were treated with Arsenal® GEN 2 whereas in this study, chlorosis of the unmutated N12 leaves were observed 2 weeks after the application of imazapyr. This observation is probably due to exposure of the unmutated N12 plants to a higher dosage of Arsenal® GEN 2 and as a result the observed effect on the leaf chlorophyll content of the unmutated N12 control was expected. When plants are treated with imazapyr they stop growing immediately and symptoms of this can be visualised by chlorosis, necrosis and loss of leaf lustre (Ray, 1984; Scheel and Casida, 1985). These symptoms vary depending on the environmental conditions but usually they are expected to occur two to 4 weeks after the herbicide has been applied (Williamson, 1987; Tu *et al*., 2004).

In this study it has been observed that with the use of imazapyr tolerant sugarcane varieties the field can be treated with the recommended dose of the herbicide post-emergence of the sugarcane. The trends observed with Mut 1, Mut 6 and Mut 7 regarding the number of stalks.plot^{-1}, stalk height and leaf chlorophyll content is an indication that these genotypes are more tolerant to imazapyr than the unmutated N12 control. This is an advantage to farmers because it allows for the use of Arsenal® GEN 2 on cultivated fields after imazapyr tolerant sugarcane has been planted.

5.3 Characterisation of the mode of imazapyr tolerance in Mut 1, Mut 6 and Mut 7

Herbicide tolerance can be divided into two major groups, target site tolerance which is caused by mutations within the enzymes to which herbicides bind and non-target site tolerance which refers various mechanisms including an enhanced detoxification mechanism resulting in a decrease in the effective concentration of a herbicide, reduced herbicide translocation and an efflux of ABC transporters (Devine and Eberlein, 1997; Tan *et al*., 2005; Yuan *et al*., 2007; Kang *et al*., 2011; Shaner *et al*., 2012; Gaur and Sharma, 2013; Yu and Powles, 2014b). An increase in gene expression is also a mechanism of herbicide tolerance which contributes to

both target and non-target site tolerance (Yuan *et al*., 2007). Imazapyr belongs to the imidazolinone class of herbicides which are used to control both grass and broadleaf weed species (Kishchenko *et al*., 2011; Yu and Powles, 2014a). This class of herbicides is one of five structurally distinct chemical families that inhibit the AHAS enzyme in plants (Shimizu *et al*., 2002; Tan *et al*., 2005; Sala *et al*., 2008; Lichtfouse, 2014; Yu and Powles, 2014a). The AHAS enzyme is responsible for the biosynthesis of three essential amino acids valine, leucine and isoleucine (Shimizu *et al*., 2002; Yu and Powles, 2014a; Lichtfouse, 2014) and inhibition of the enzyme results in plant death caused by starvation of these essential amino acids (Shimizu *et al*., 2002; Tranel and Wright, 2002; Tan *et al*., 2005; Lichtfouse, 2014).

5.3.1 Identification of mutations within the acetohydroxyacid synthase protein of Mut 1, Mut 6 and Mut 7

In weeds, tolerance to imidazolinones has evolved over time and, in most instances, is due to mutations within a conserved region of the AHAS enzyme (Singh and Shaner, 1995; Devine and Eberlein, 1997). Mutations which confer tolerance to imidazolinones have been identified with reference to a mutant genotype of *A. thaliana* and include Ala_{122}, Pro_{197}, Ala_{205}, Trp_{574}, Trp_{591}, Ser_{653}, Ser_{670} (Hartnett *et al*., 1990; Bernasconi *et al*., 1995; Guttieri *et al*., 1995; Devine and Eberlein, 1997; Boutsalis *et al*., 1999; Lee *et al*., 1999; Tranel and Wright, 2002; Tan *et al*., 2005; Sala and Bulos, 2012; Lichtfouse, 2014; Yu and Powles, 2014a). Clearfield® crops, referring to imidazolinone tolerant crops used in combination with imidazolinone herbicides, which are commercially used to date each consist of one or more of the above mentioned mutations (Anderson and Georgeson, 1989; Swanson *et al*., 1989; Newhouse *et al*., 1991; Bernasconi *et al*., 1995; Hatorri *et al*., 1995; Shaner *et al*., 1996; Bright *et al*., 1997; Al-Khatib *et al*., 1998; Croughan, 1998; Dietrich, 1998; Bruniard, 2001; Croughan, 2002; Croughan, 2003; White *et al*., 2003).

The AHAS enzyme activity of the Mut 1, Mut 6 and Mut 7 genotypes was assessed by Rutherford *et al*. (*in press*) using an *in vitro* enzyme assay that characterised the activity of the AHAS enzyme in the presence of imazapyr. The basal AHAS enzyme activity of Mut 1 and Mut 6 were significantly higher than that of the Mut 7 and unmutated N12 genotypes. When the AHAS enzyme activity was assessed in the presence of imazapyr, the IC_{50} value of imazapyr (concentration of imazapyr that inhibits 50% of the AHAS enzyme activity) of Mut 1 was significantly higher than that of the unmutated N12 control and no differences in

IC_{50} values were observed amongst the Mut 6, Mut 7 and unmutated N12 genotypes. The hypothesis in that study was that the AHAS enzyme activity of the mutant genotypes was altered by the EMS chemical mutagenesis.

In the present study, the AHAS gene of the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes was sequenced in order to identify if there were mutations present in the expressed AHAS gene of the mutants. As aforementioned, because the sugarcane AHAS gene sequence was not available at the time of this study that of *S. bicolor* and *Z. mays* were used as reference sequences. Although there were various point mutations found in the expressed AHAS gene sequences of the mutants compared with the unmutated N12 genotype (Table 16, Appendix II), when the translated to a protein sequence, a single amino acid mutation was identified in Mut 1 and Mut 6 at position 195 (Figure 13). The results of this comparison indicated that the chemical mutagenesis using EMS produced a single point mutation within Mut 1 and Mut 6 that converted an arginine to a cysteine residue. This point mutation differed from those found in other imidazolinone tolerant crops such as maize (amino acid position 122, 574 and 653) (Newhouse *et al*., 1991; Bright *et al*., 1997; Dietrich, 1998), oilseed rape (amino acid position 574 and 653) (Swanson *et al*., 1989; Hatorri *et al*., 1995; Shaner *et al*., 1996) and rice (amino acid position 653) (Croughan, 2003), where each amino acid mutation correlated with the reference amino acid mutations of *A. thaliana*. There are some crops which differ in this regard such as tobacco (amino acid positions 196 and 573) (Lee *et al*., 1988), maize (amino acid position 155) (Bernasconi *et al*., 1995) and rice (amino acid positions 95, 654, 627 and 548) (Croughan, 2003; Kawai *et al*., 2007; Okuzaki *et al*., 2007; Ogawa *et al*., 2008; Endo *et al*., 2012), each containing amino acid mutations which confer tolerance to other AHAS inhibiting herbicides.

The results obtained in this study contribute to the findings of Rutherford *et al*. (*in press*) who observed an increase in basal AHAS enzyme activity in the Mut 1, Mut 6 and Mut 7 genotypes compared with that of the unmutated N12 control. The results of the present study indicated that the EMS chemical mutagenesis which resulted in an amino acid mutation contributes to the significantly higher basal AHAS activity of Mut 1 and Mut 6 and the significantly higher IC_{50} imazapyr value of Mut 1 observed by Rutherford *et al*. (*in press*). In that study, the Mut 7 genotype did not show an increased AHAS IC_{50} concentration for imazapyr which is possibly the reason why no amino acid mutations were identified within the AHAS protein sequence of

this genotype. Based on this, the possibility of a non-target site mode of tolerance to imazapyr was investigated.

5.3.2 Assessing the potential of an enhanced detoxification of imazapyr by piperonyl butoxide within the mutant genotypes

Several mechanisms are involved in non-target site herbicide tolerance but the most common found in plants is the enhanced metabolic detoxification catalysed by the CYP family of enzymes (Kreuz *et al*., 1996; Siminszky, 2006; Kumar *et al*., 2012). Although there are various studies that characterised herbicide tolerance in plants based on an altered CYP enzyme, the majority of them deal with weeds. Cytochrome P450s are the largest superfamily of enzymes in plants (Renault *et al*., 2014) and play a vital role in Phase I of herbicide (and other foreign substances) detoxification which involves conversion of these harmful chemicals to more soluble derivatives which then go through the rest of the detoxification process (Siminszky, 2006; Kang *et al*., 2011).

The protocol for assessing the enhanced detoxification of imazapyr by CYP used in this study was adapted from Breccia *et al*. (2012) who identified an enhanced metabolism in herbicide tolerant sunflower plants. The result of that study indicated that when the plants were treated with a combination of the herbicide and a CYP inhibitor, PBO, the plant growth ceased because the inhibitor prevented detoxification of the herbicide thus leading to plant death. Other studies that successfully confirmed an enhanced herbicide metabolism include those on *Z. mays* (Kwon *et al*., 1995; Kotoula-Syka and Hatzios, 1996), *K. scoparia* (Kwon and Penner, 1995), *E. phyllopogon* (Fisher *et al*., 2000), *G. hirsutum* (Minton *et al*., 2008), *L. rigidum* (Yu *et al*., 2009) and *A. stolonifera* (Elmore *et al*., 2015).

In this study, the unmutated N12, Mut 1, Mut 6 and Mut 7 genotypes were exposed to imazapyr with and without the CYP inhibitor PBO in order to identify if these genotypes possessed an enhanced detoxification mechanism. Based on the pre-trial carried out using the unmutated N12 plants to determine the concentrations of PBO and imazapyr that would be required, 50 µM and 100 µM of each chemical was selected, respectively (Tables 17, 18, 19, 20 and Figure 14).

The leaf chlorophyll content (SPAD unit) and plant height (cm) of the unmutated N12 was significantly lower over time after treatment with 100 µM imazapyr alone and in combination with PBO compared with the control treatment (Tables 21 and 22). It was observed that 3

weeks after exposure to the imazapyr treatments, the leaf chlorophyll content and height of the unmutated N12 plants were significantly lower compared with that of the control (0 µM PBO + 0 µM imazapyr) and 50 µM PBO treatments (Tables 21 and 22). This trend was observed with fresh root and shoot mass and the dry root mass of the unmutated N12 plants where plants within the imazapyr treatments were significantly lower compared with that of the control and 50 µM PBO treatments (Figures 15, 16 and 17). Unlike the unmutated N12 control, the plant height and leaf chlorophyll content of the Mut 1, Mut 6 and Mut 7 genotypes showed no significant differences over time and amongst treatments after exposure to the imazapyr (Tables 21 and 22). Similarly, there were no significant differences in fresh root and shoot and dry root mass observed amongst treatments for each of the mutant genotypes (Figures 15, 16 and 17).

Studies by Kwon and Penner (1995), Kwon *et al*. (1995), Fisher *et al*. (2000), Minton *et al*. 2008, Yu *et al*. (2009) and Breccia *et al*. (2012) observed that treating a herbicide tolerant plant with a combination of a CYP inhibitor and an AHAS inhibiting herbicide caused a greater degree of damage to the plant compared with treating the plants with the herbicide only. This was an indication that the mode of herbicide tolerance in these plants was due to an enhanced metabolic detoxification mechanism. In contrast, the study by Kotoula-Syka and Hatzios (1996) observed that the mode of herbicide tolerance in the maize genotype tested was not based on an enhanced metabolic detoxification mechanism because the CYP inhibitor mixed with the herbicide did not influence the activity of the herbicide on the plant.

It is unknown if the concentration of PBO selected in this study was adequate to inhibit the catalytic effect of CYP and there are no studies confirming if this chemical actually works on sugarcane. The use of a sugarcane genotype known to have an enhanced metabolic detoxification mechanism would allow for a comparison to a reference genotype to be made. The results of this study, given the limitations of the PBO method used above, indicated that tolerance of the Mut 7 genotype to imazapyr may not be conferred by an enhanced detoxification mechanism. The use of different parameters (concentration of PBO and imazapyr) could allow for a better analysis of the mode of tolerance of Mut 7 to imazapyr.

6. Conclusion

The results of this study indicated that the although the chemical mutagenesis using EMS (and possibly a somaclonal variation effect due to the *in vitro* process) negatively affected some of the agronomic characteristics of the mutant genotypes such as stalk diameter, estimated yield and susceptibility to *E. saccharina*, there were also some beneficial characteristics such as in increase in estimated yield and cane quality which were calculated based on the recoverable value sucrose content of each genotype. The mutant genotypes proved to be more tolerant to Arsenal® GEN 2 when applied post-emergence of the sugarcane to control *C. dactylon* and this was confirmed by comparing the number of stalks.plot^{-1}, stalk height and leaf chlorophyll content of the mutants with that of the unmutated N12 at different times after exposure to the herbicide. Based on the results of the field analysis it was confirmed that these three mutants are tolerant to imazapyr and it coincides with the findings of Koch *et al*. (2012) and Rutherford *et al*. (*in press*).

The application of Arsenal® GEN 2 (1254 g a.i.ha^{-1} imazapyr), post-emergence of the sugarcane, had no effect on the growth of the mutant plants which was an indication that these genotypes have a tolerance to the herbicide. Tolerance of the mutant genotypes to Arsenal® GEN 2 allows for these genotypes to be planted prior to herbicide application as opposed to waiting for long periods (4 months) of time before planting can resume (Anon, 2015). Tolerance of the mutant plants to imazapyr is not based on an enhanced metabolic detoxification mechanism unlike studies involving maize (Kwon *et al*., 1995; Kotoula-Syka and Hatzios, 1996), cereal weed (Yu *et al*., 2009), sunflower (Breccia *et al*., 2012), cotton (Minton *et al*., 2008) and various grass species (Kwon and Penner, 1995; Fisher *et al*., 2000; Elmore *et al*., 2015) which concluded that tolerance to the AHAS inhibiting herbicides was conferred by an enhanced detoxification mechanism. The lack of a non-target mode of herbicide tolerance indicated that tolerance to imazapyr is possibly due to a target site mutation.

A target site mode of tolerance was confirmed when the AHAS gene of the mutant genotypes (Mut 1 and Mut 6) was sequenced and compared with that of unmutated N12 genotype. The AHAS gene comparison indicated that the EMS chemical mutagenesis produced a single amino acid mutation at position 195 which converted an arginine to a cysteine residue. Tolerance to other AHAS inhibiting herbicides based on a mutation at the target site was also confirmed in tobacco (Chaleff and Ray, 1984), *A. thaliana* L. (Haughn and Somerville, 1986), canola (Swanson *et al*., 1989), soybean (Sabastian *et al*., 1989), maize (Newhouse *et al*., 1991;

Wright and Penner, 1998b), sugar beet (Hart *et al*., 1992; Wright and Penner, 1998a; Kishchenko *et al*., 2011), cotton (Rajasekaran *et al*., 1996), rice (Croughan, 1998), wheat (Ponziak and Huci, 2004), sunflower (Sala and Bulos, 2012) and chickpea (Thompson and Tar'an, 2014).

Future work should involve: (1) analysing the potential of an enhanced metabolic detoxification mechanism within the mutant genotypes with the use of a higher PBO concentration, an alternative chemical that is known to inhibit the CYP enzyme family within sugarcane or a sugarcane genotype known to have an enhanced herbicide detoxification mechanism thereby allowing for a comparison to be made; (2) identifying if herbicide tolerance is due to a different mechanism of non-target site tolerance; and (3) completion of the AHAS gene sequence which will allow for identification of amino acid mutations within the Mut 7 genotype that may or may not confer tolerance to imazapyr. In addition, the imazapyr tolerant mutant genotypes, Mut 1, Mut 6 and Mut 7, have the potential to be used as parents in a breeding program at SASRI or for commercial purposes within the field.

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Appendices

Appendix IA: Consensus sequence generated from acetohydroxyacid synthase gene sequences of *Saccharum officinarum*, *Sorghum bicolor* and *Zea mays*.

```
GATCCCCGATGGGATTTATCTCCGTGGGGAACGGGGATGGAGAAGAAATGTCTCTCGCAAGAGTTCGTGGGAATCCCCGC
GGGAAATTTTTTTGTCGCGGGGACAGATACGGATACGGCGAGCCAAAGGGTGCCATCCCTAACACCGACACCGGGCCTGA
CGCTGTCGCCGCAGCTGCTCATCTTGTGGTTGTGGGCTGTGCCCGGTTTCCCTGTTGCGGATTGCGGGTGGCAGCCTGGC
AGGTGGGTGCGACCCGTTTGGATTCCCTTGTCTGGGCCCCTTGTGTCAGTACCGTCTGTACTCCGATGACATGCACCGTC
GTCCACAGTCAAGTCCAAAATCTCCCCTCTTTTTTAACGGAACAGTTCAAAACCTCCTTGACGCACGCTGTCGTGTACCA
GCACTCGGTGGACACCACGTTTGTAATGCAGGCCGACACGTCGGTCCCACGTCGACAGGCCCCACCGTCCGGTCTGTAGC
GTGTACGTATTCGGGCGACGGACGTGTCGTCGTCGTCTTGCGAGTCCCATTCCCATCACCATCTGAGCCACACATCCTCT
GAACAAAAGCAGGGAGGCCTCTACGCACATCCCCCTTGCTCCCACTCCGYGKMCGBKKSVSCSRMCSCNNRYSSKCGCGC
CGCCTCCGAGACAGCCGCCGCAACCATGGCCANNNCCGCCGCCGCCGCBKCYRCCGCGCTMRCYGGCGCCACTACCGCTG
CGCCCAAGGCGAGGCGCCGGGCGCACCTCCTGGCCRCMCGSCGCGYCCTCGCCGCGCCCATCAGGTGCTCMGCGGCGYCA
CCCGCCAYGCNNNNNNYGAYGGCTCCCCCGGCCACCCCGCTCCGGCCGTGGGGCCCCAMCGAKCCCCGCAGGGCGCCGAC
ATCCTCGTCGAGKCYCTYGAGCGCTGCGGCGTYCGCGACGTCTTCGCCTACCCCGGCGGCGCGTCCATGGAGATCCACCA
GGCACTCACCCGYTCCCCCGTCATCGCCAACCACCTCTTCCGCCACGAGCAAGGGGAGGCCTTYGCSGCCTCYGGCTWCG
CGCGCTCCTCGGGCCGCGTCGGCGTCTGCRTCGCCACCTCCGGCCCCGGCGCCACCAACCTWGTCTCCGCGCTCGCCGAC
GCGCTGCTCGAYTCCGTCCCCATGGTCGCCATCACGGGACAGGTKCCGCGRCGCATGATTGGCACCGAYGCCTTCCAGGA
GACGCCCATCGTCGAGGTCACCCGCTCCATCACCAARCAYAACTACCTGGTCCTCGACGTCGACGACATCCCCCGCGTCG
TGCWGGARGCYTTCTTCCTCGCCTCCTCTGGTCGACCGGGGCCGGTGCTTGTCGACATCCCCAAGGACATCCAGCAGCAG
ATGGCGGTGCCTGTCTGGGACAAGCCCATGAGTCTGCCTGGGTACATTGCGCGCCTTCCCAAGCCCCCTGCGACTGAGTT
GCTTGAGCAGGTGCTGCGTCTTGTTGGTGAATCCCGGCGCCCTGTTCTTTATGTTGGCGGTGGCTGCGCAGCATCTGGTG
AGGAGTTGCGACGCTTTGTGGAGCTGACTGGAATCCCGGTCACAACTACTCTTATGGGCCTCGGCAACTTCCCCRGCGAY
GACCCACTGTCTCTGCGCATGCTWGGTATGCATGGCACRGTGTATGCAAATTATGCAGTGGATAAGGCYGATCTGTTGCT
TGCAYTTGGTGTGCGGTTTGATGATCGTGTGACAGGGAAGATTGAGGCTTTTGCAAGCAGGGCTAAGATTGTGCACRTTG
ATATTGATCCRGCTGAGATTGGCAAGAACAAGCAGCCACATGTGTCCATCTGTGCAGATGTTAAGCTTGCTTTGCARGGC
ATGAATGCTCTTCTKGAAGGAAGCACATCAAAGAAGAGCTTTGACTTTRGCTCATGGMACRATGAGTTGGATCAGCAGAA
GAGRGAATTCCCCCTTGGGTATAAAACWTYTRATGAGGAGATCCAGCCACARTATGCTATYCAGGTTCTTGATGAGCTGA
CRAAAGGSGAGGCCATCATYGSCACAGGTGTTGGGCAGCACCAGATGTGGGCGGCACAGTACTACACTTACAAGCGGCCA
AGGCAGTGGTTGTCTTCVGSTGGTCTTGGGGCTATGGGATTTGGTTTGCCGGCTGCTGCTGGYGCTKCTGTGGCCAACCC
ARGTGTYACTGTTGTTGACATCGAYSGAGATKGTAGCTTYCTCMTGAACRTTCARGARCTAGCTATKATCCGAATTGARA
ACCTCCNRGTGAAGGTCTTTGTGCTAAACAACCARCACCKNGGGATGGTGGTGCMRTGGGNNGGACAGGTTCTANTAAGG
SCMACAGARCSCACMCMTNCNTTGGGAAAMCCANRARAATGAAAGTNGARATATATNCCARATTTCSTGANCRATSGCCA
AAGGGTTCAACMTTCCAGCGGTCCGTGTGACAAAGAAGAACGAAGTCCGCGCAGCGATAAAGAAGATGCTCGAGACTCCA
GGGCCGTACCTCTTGGATATAATCGTCCCACACCAGGAGCATGTGTTGCCTATGATCCCTAGTGGTGGGGCTTTCAAGGA
TATGATCCTGGATGGTGATGGCAGGACTGTGTACTGATCTAAAATCCAGCAAGCAACTGATCTAAAATCCAGCAAGCACC
GCCTCCCTGCTAGTACAAGGTGATATGTTTATCTGTGTG
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Appendix IB: Consensus sequence generated from acetohydroxyacid synthase gene sequences of *Saccharum officinarum*, *Sorghum bicolor* and *Zea mays* and polymerase chain reaction products of the sugarcane acetohydroxyacid synthase gene.

GATCCCCGATGGGATTTATCTCCGTGGGGAACGGGGATGGAGAAGAAATGTCTCTCGCAAGAGTTCGTGGGAATCCCCGC
GGGAAATTTTTTTGTCGCGGGGACAGATACGGATACGGCGAGCCAAAGGGTGCCATCCCTAACACCGACACCGGGCCTGA
CGCTGTCGCCGCAGCTGCTCATCTTGTGGTTGTGGGCTGTGCCCGGTTTCCCTGTTGCGGATTGCGGGTGGCAGCCTGGC
AGGTGGGTGCGACCCGTTTGGATTCCCTTGTCTGGGCCCCTTGTGTCAGTACCGTCTGTACTCCGATGACATGCACCGTC
GTCCACAGTCAAGTCCAAAATCTCCCCTCTTTTTTAACGGAACAGTTCAAAACCTCCTTGACGCACGCTGTCGTGTACCA
GCACTCGGTGGACACCACGTTTGTAATGCAGGCCGACACGTCGGTCCCACGTCGACAGGCCCCACCGTCCGGTCTGTAGC
GTGTACGTATTCGGGCGACGGACGTGTCGTCGTCGTCTTGCGAGTCCCATTCCCATCACCATCTGAGCCACACATCCTCT
GAACAAAAGCAGGGAGGCCTCTACGCACATCCCCCTTGCTCCCACTCCGYGKMCGBKKSVSCSRMCSCNNRYSSKCGCGC
CGCCTCCGAGACAGCCGCCGCAACCATGGCCANNNCCGCCGCCGCCGCBKCYRCCGCGCTMRCYGGCGCCACTACCGCTG
CGCCCAAGGCGAGGCGCCGGGCGCACCTCCTGGCCRCMCGSCGCGYCCTCGCCGCGCCCATCAGGTGCTCMGCGGCGYCA
CCCGCCAYGCNNNNNNYGAYGGCTCCCCCGGCCACCCCGCTCCGGCCGTGGGGCCCCAMCGAKCCCCGCAGGGCGCCGAC
ATCCTCGTCGAGKCYCTYGAGCGCTGCGGCGTYCGCGACGTCTTCGACCTRCACCCGGCSGCGMRTYCACTAGTGATKGA
GATCCACCAGGCACTCACCCGCTCCCCCGTCATCGCCAACCACCTCTTCCGCCACGAGCAAGGGGAGGCCTTCGCCGCCT
CCGGCTTCGCGCGCTCCTCGGGCCGCGTCGGCGTCTGCGTCGCCACCTCCGGCCCCGGCGCCACNAACCTAGTCTCCGCG
CTCGCCGACGCGCTGCTCGACTCCGTCCCCATGGTCGCCATCACGGGACAGGTGCCGCGGCGCATGATTGGCACCGATGC
CTTCCAGGAGACGCCCATCGTCGAGGTCACCCGCTCCATCACCAAGCACAACTACCTGGTCCTCGACGTCGACGACATCC
CCCGCGTCGTGCAGGAGGCCTTCTTCCTCGCCTCCTCTGGTCGCCCGGGACCGGTGCTTGTCGACATCCCCAAGGACATC
CAGCAGCAGATGGCGGTGCCGGTCTGGGACACGCCCATGAGTCTGCCTGGGTACATTGCGCGCCTTCCCAAGCCTCCTGC
GACTGAATTGCTTGAGCAGGTGCTGCGTCTTGTTGGTGAATCGCGGCGCCCTGTTCTTTATGTTGGCGGTGGCTGCGCAG
CATCTGGTGAGGAGTTGCGCCGCTTTGTGGAGATGACTGGAATCCCAGTCACAACTACTCTTATGGGCCTTGGCAACTTC
CCCGGCGACGACCCACTGTCTCTGCGCATGCTTGGTATGCATGGCACAGTGTATGCAAATTATGCAGTGGATAAGGCTGA
TCTGTTGCTTGCATTTGGTGTGCGGTTTGATGATCGTGTGACAGGGAAGATTGAGGCTTTTGCAAGCAGGGCTAAGATTG
TGCACATTGATATTGATCCGGCTGAGATTGGCAAGAACAAGCAGCCACATGTGTCCATCTGTGCAGATGTTAAGCTTGCT
TTGCAGGGCATGAATGCTCTTCTGGAAGGAAGCACATCAAAGAAGAGCTTTGACTTTGGCTCATGGCACGATGAGTTGGA
TCAGCAGAAGAGAGAATTCCCCCTTGGGTATAAAACTTTTGATGAGGAGATCCAGCCACAGTATGCTATCCAGGTTCTTG
ATGAGCTGACAAAAGGGGAGGCCATCATTGCCACAGGTGTTGGGCAGCACCAGATGTGGGCGGCACAGTACTACACTTAC
AAGCGGCCAAGGCAGTGGTTGTCTTCGGCTGGTCTTGGGGCTATGGGATTTGGTTTGCCGGCTGCTGCTGGCGCTGCTGT
GGCCAACCCAGGTGTCACTGTTGTTGACATCGACGGAGATGGTAGCTTCCTCATGAACATTCAGGAGCTAGCTATGATCC
GAATTGAGAACCTCCCAGTGAAGGTCTTTGTGCTAAACAACCAGCACCTGGGGATGGTGGTGCAGTGGGAGGACAGGTTC
TATAAGGCCAACAGAGCACACACATACTTGGGAAACCCAGAGAATGAAAGTGAGATATATCCAGATTTCGTGACAATTGC
CAAAGGGTTCAACATTCCAGCAGTCCGTGTGACAAAGAAGAACGAAGTCCGCGCAGCGATAAAGAAGATSCTCGAKCTCM
TGCCCCACTSCAGGGCGATCNCGTACCTCTKGGATATAWTSGTCCCACACCAGGAGCATGTGTTSCCTATGMTCCTGCAN
CCCCTAGTGGTGGGGCTTTCAAGGATATGATCCTGGAKKSTRMTGGCAGGACCTSTGTACTGATCTMRAATYCAGCAAGC
AMCTGATCTAAMATCYAGCAAGCACCRCCTCCCTGCTAGNGTTAVARGGTGATRWSTTYATCTGKGTGGKCYRMAKCWRM
RCCWKMWSCRSYSRKCWAMYMMWWCGACGATGRGCACCCKCGTGTGTTGKACGGCCTGCGCGCGCGCCCTACMGTS

Appendix II: Comparison of the acetohydroxyacid synthase gene sequence in the unmutated N12 control, Mut 1, Mut 6 and Mut 7 genotypes.

```
Unmutated N12 AHAS gene ------------CACCTCTTCCGCCACGAGCAAGGGGAGGCCTTCGCCGCCTCCGGCTTC
        Mut 1 AHAS gene TTCATCGCCCACCACCTCTTCCGCCACGAGCAAGGGGAGGCCTTCGCCGCCTCCGGCTTC
        Mut 6 AHAS gene TTCATCGCCCACCACCTCTTCCGCCACGAGCAAGGGGAGGCCTTCGCCGCCTCCGGCTTC
        Mut 7 AHAS gene ----------ACCACCTCTTCCGCCACGAGCAAGGGGAGGCCTTCGCCGCCTCCGGCTTC

Unmutated N12 AHAS gene GCGCGCTCCTCGGGCCGCGTCGGCGTCTGCGTCGCCACCTCCGGCCCCGGCGCCACCAAC
        Mut 1 AHAS gene GCGCGCTCCTCGGGCCGCGTCGGCGTCTGCGTCGCCACCTCCGGCCCCGGCGCCACCAAC
        Mut 6 AHAS gene GCGCGCTCCTCGGGCCGCGTCGGCGTCTGCGTCGCCACCTCCGGCCCCGGCGCCACCAAC
        Mut 7 AHAS gene GCGCGCTCCTCGGGCCGCGTCGGCGTCTGCGTCGCCACCTCCGGCCCCGGCGCCACCAAC

Unmutated N12 AHAS gene CTAGTCTCCGCGCTCGCCGACGCGCTGCTCGACTCCGTCCCCATGGTCGCCATCACGGGA
        Mut 1 AHAS gene CTAGTCTCCGCGCTCGCCGACGCGCTGCTCGACTCCGTCCCCATGGTCGCCATCACGGGA
        Mut 6 AHAS gene CTAGTCTCCGCGCTCGCCGACGCGCTGCTCGACTCCGTCCCCATGGTCGCCATCACGGGA
        Mut 7 AHAS gene CTAGTCTCCGCGCTCGCCGACGCGCTGCTCGACTCCGTCCCCATGGTCGCCATCACGGGA

Unmutated N12 AHAS gene CAGGTGCCGCGGCGCATGATTGGCACCGATGCCTTCCAGGAGACGCCCATCGTCGAGGTC
        Mut 1 AHAS gene CAGGTGCCGCGGCGCATGATTGGCACCGATGCCTTCCAGGAGACGCCCATCGTCGAGGTC
        Mut 6 AHAS gene CAGGTGCCGCGGCGCATGATTGGCACCGATGCCTTCCAGGAGACGCCCATCGTCGAGGTC
        Mut 7 AHAS gene CAGGTGCCGCGGCGCATGATTGGCACCGATGCCTTCCAGGAGACGCCCATCGTCGAGGTC

Unmutated N12 AHAS gene ACCCGCTCCATCACCAAGCACAACTACCTGGTCCTCGACGTCGACGACATCCCCCGCGTC
        Mut 1 AHAS gene ACCCGCTCCATCACCAAGCACAACTACCTGGTCCTCGACGTCGACGACATCCCCCGCGTC
        Mut 6 AHAS gene ACCCGCTCCATCACCAAGCACAACTACCTGGTCCTCGACGTCGACGACATCCCCCGCGTC
        Mut 7 AHAS gene ACCCGCTCCATCACCAAGCACAACTACCTGGTCCTCGACGTCGACGACATCCCCCGCGTC

Unmutated N12 AHAS gene GTGCAGGAGGCCTTCTTCCTCGCCTCCTCTGGTCGCCCGGGACCGGTGCTTGTCGACATC
        Mut 1 AHAS gene GTGCAGGAGGCCTTCTTYCTCGCCTCCTCTGGTCGCCCGGGACCGGTGCTTGTCGACATC
        Mut 6 AHAS gene GTGCAGGAGGCCTTCTTYCTCGCCTCCTCTGGTCGCCCGGGACCGGTGCTTGTCGACATC
        Mut 7 AHAS gene GTGCAGGAGGCCTTCTTCCTCGCCTCCTCTGGTCGCCCGGGACCGGTGCTTGTCGACATC

Unmutated N12 AHAS gene CCCAAGGACATCCAGCAGCAGATGGCGGTGCCGGTCTGGGACACGCCCATGAGTCTGCCT
        Mut 1 AHAS gene CCCAAGGACATCCAGCAGCAGATGGCGGTGCCGGTCTGGGACACGCCCATGAGTCTGCCT
        Mut 6 AHAS gene CCCAAGGACATCCAGCAGCAGATGGCGGTGCCGGTCTGGGACACGCCCATGAGTCTGCCT
        Mut 7 AHAS gene CCCAAGGACATCCAGCAGCAGATGGCGGTGCCGGTCTGGGACACGCCCATGAGTCTGCCT

Unmutated N12 AHAS gene GGGTACATTGCGCGCCTTCCCAAGCCTCCTGCGACTGAATTGCTTGAGCAGGTGCTGCGT
        Mut 1 AHAS gene GGGTACATTGCGCGCCTTCCCAAGCCTCCTGCGACTGAATTGCTTGAGCAGGTGCTGCGT
        Mut 6 AHAS gene GGGTACATTGCGCGCCTTCCCAAGCCTCCTGCGACTGAATTGCTTGAGCAGGTGCTGCGT
        Mut 7 AHAS gene GGGTACATTGCGCGCCTTCCCAAGCCTCCTGCGACTGAATTGCTTGAGCAGGTGCTGCGT

Unmutated N12 AHAS gene CTTGTTGGTGAATCGCGGCGCCCTGTTCTTTATGTTGGCGGTGGCTGCGCAGCATCTGGT
        Mut 1 AHAS gene CTTGTTGGTGAATCGCGGCGCCCTGTTCTTTATGTTGGCGGTGGCTGCGCAGCATCTGGT
        Mut 6 AHAS gene CTTGTTGGTGAATCGCGGCGCCCTGTTCTTTATGTTGGCGGTGGCTGCGCAGCATCTGGT
        Mut 7 AHAS gene CTTGTTGGTGAATCGCGGCGCCCTGTTCTTTATGTTGGCGGTGGCTGCGCAGCATCTGGT

Unmutated N12 AHAS gene GAGGAGTTGCGCCGCTTTGTGGAGATGACTGGAATCCCAGTCACAACTACTCTTATGGGC
        Mut 1 AHAS gene GAGGAGTTGTGCCGCTTTGTGGAGATGACTGGAATCCCAGTCACAACTACTCTTATGGGC
        Mut 6 AHAS gene GAGGAGTTGTGCCGCTTTGTGGAGATGACTGGAATCCCAGTCACAACTACTCTTATGGGC
        Mut 7 AHAS gene GAGGAGTTGCGCCGCTTTGTGGAGATGACTGGAATCCCAGTCACAACTACTCTTATGGGC

Unmutated N12 AHAS gene CTTGGCAACTTCCCCGGCGACGACCCACTGTCTCTGCGCATGCTTGGTATGCATGGCACA
        Mut 1 AHAS gene CTTGGCAACTTCCCCGGCGACGACCCACTGTCTCTGCGCATGCTTGGTATGCATGGCACA
        Mut 6 AHAS gene CTTGGCAACTTCCCCGGCGAYGACCCACTGTCTCTGCGCATGCTTGGTATGCATGGCACA
        Mut 7 AHAS gene CTTGGCAACTTCCCCGGCGACGACCCACTGTCTCTGCGCATGCTTGGTATGCATGGCACA

Unmutated N12 AHAS gene GTGTATGCAAATTATGCAGTGGATAAGGCTGATCTGTTGCTTGCATTTGGTGTGCGGTTT
        Mut 1 AHAS gene GTGTATGCAAATTATGCAGTGGATAAGGCTGATCTGTTGCTTGCATTTGGTGTGCGGTTT
        Mut 6 AHAS gene GTGTATGCAAATTATGCAGTGGATAAGGCTGATCTGTTGCTTGCATTTGGTGTGCGGTTT
        Mut 7 AHAS gene GTGTATGCAAATTATGCAGTGGATAAGGCTGATCTGTTGCTTGCATTTGGTGTGCGGTTT
                                                                              Cont…
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Cont…

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Unmutated N12 AHAS gene GATGATCGTGTGACAGGGAAGATTGAGGCTTTTGCAAGCAGGGCTAAGATTGTGCACATT
        Mut 1 AHAS gene GATGATCGTGTGACAGGGAAGATTGAGGCTTTTGCAAGCAGGGCTAAGATTGTGCACATT
        Mut 6 AHAS gene GATGATCGTGTGACAGGGAAGATTGAGGCTTTTGCAAGCAGGGCTAAGATTGTGCACATT
        Mut 7 AHAS gene GATGATCGTGTGACAGGGAAGATTGAGGCTTTTGCAAGCAGGGCTAAGATTGTGCACATT

Unmutated N12 AHAS gene GATATTGATCCGGCTGAGATTGGCAAGAACAAGCAGCCACATGTGTCCATCTGTGCAGAT
        Mut 1 AHAS gene GATATTGATCCGGCTGAGATTGGCAAGAACAAGCAGCCACATGTGTCCATCTGTGCAGAT
        Mut 6 AHAS gene GATATTGATCCGGCTGAGATTGGCAAGAACAAGCAGCCACATGTGTCCATCTGTGCAGAT
        Mut 7 AHAS gene GATATTGATCCGGCTGAGATTGGCAAGAACAAGCAGCCACATGTGTCCATCTGTGCAGAT

Unmutated N12 AHAS gene GTTAAGCTTGCTTTGCAGGGCATGAATGCTCTTCTGGAAGGAAGCACATCAAAGAAGAGC
        Mut 1 AHAS gene GTTAAGCTTGCTTTGCAGGGCATGAATGCTCTTCTGGAAGGAAGCACATCAAAGAAGAGC
        Mut 6 AHAS gene GTTAAGCTTGCTTTGCAGGGCATGAATGCTCTTCTGGAAGGAAGCACATCAAAGAAGAGC
        Mut 7 AHAS gene GTTAAGCTTGCTTTGCAGGGCATGAATGCTCTTCTGGAAGGAAGCACATCAAAGAAGAGC

Unmutated N12 AHAS gene TTTGACTTTGGCTCATGGCACGATGAGTTGGATCAGCAGAAGAGAGAATTCCCCCTTGGG
        Mut 1 AHAS gene TTTGACTTTGGCTCATGGCACGATGAGTTGGATCAGCAGAAGAGAGAATTCCCCCTTGGG
        Mut 6 AHAS gene TTTGACTTTGGCTCATGGCACGATGAGTTGGATCAGCAGAAGAGAGAATTCCCCCTTGGG
        Mut 7 AHAS gene TTTGACTTTGGCTCATGGCACGATGAGTTGGATCAGCAGAAGAGAGAATTCCCCCTTGGG

Unmutated N12 AHAS gene TATAAAACTTTTGATGAGGAGATCCAGCCACAGTATGCTATCCAGGTTCTTGATGAGCTG
        Mut 1 AHAS gene TATAAAACTTTTGATGAGGAGATCCAGCCACAGTATGCTATCCAGGTTCTTGATGAGCTG
        Mut 6 AHAS gene TATAAAACTTTTGATGAGGAGATCCAGCCACAGTATGCTATCCAGGTTCTTGATGAGCTG
        Mut 7 AHAS gene TATAAAACTTTTGATGAGGAGATCCAGCCACAGTATGCTATCCAGGTTCTTGATGAGCTG

Unmutated N12 AHAS gene ACAAAAGGGGAGGCCATCATTGCCACAGGTGTTGGGCAGCACCAGATGTGGGCGGCACAG
        Mut 1 AHAS gene ACAAAAGGGGAGGCCATCATTGCCACAGGTGTTGGGCAGCACCAGATGTGGGCGGCACAG
        Mut 6 AHAS gene ACAAAAGGGGAGGCCATCATTGCCACAGGTGTTGGGCAGCACCAGATGTGGGCGGCACAG
        Mut 7 AHAS gene ACAAAAGGGGAGGCCATCATTGCCACAGGTGTTGGGCAGCACCAGATGTGGGCGGCACAG

Unmutated N12 AHAS gene TACTACACTTACAAGCGGCCAAGGCAGTGGTTGTCTTCGGCTGGTCTTGGGGCTATGGGA
        Mut 1 AHAS gene TACTACACTTACAAGCGGCCAAGGCAGTGGTTGTCTTCGGCTGGTCTTGGGGCTATGGGA
        Mut 6 AHAS gene TACTACACTTACAAGCGGCCAAGGCAGTGGTTGTCTTCGGCTGGTCTTGGGGCTATGGGA
        Mut 7 AHAS gene TACTACACTTACAAGCGGCCAAGGCAGTGGTTGTCTTCGGCTGGTCTTGGGGCTATGGGA

Unmutated N12 AHAS gene TTTGGTTTGCCGGCTGCTGCTGGCGCTGCTGTGGCCAACCCAGGTGTCACTGTTGTTGAC
        Mut 1 AHAS gene TTTGGTTTGCCGGCTGCTGCTGGCGCTGCTGTGGCCAACCCAGGTGTCACTGTTGTTGAC
        Mut 6 AHAS gene TTTGGTTTGCCGGCTGCTGCTGGCGCTGCTGTGGCCAACCCAGGTGTCACTGTTGTTGAC
        Mut 7 AHAS gene TTTGGTTTGCCGGCTGCTGCTGGCGCTGCTGTGGCCAACCCAGGTGTCACTGTTGTTGAC

Unmutated N12 AHAS gene ATCGACGGAGATGGTAGCTTCCTCATGAACATTCAGGAGCTAGCTATGATCCGAATTGAG
        Mut 1 AHAS gene ATCGACGGAGATGGTAGCTTCCTCATGAACATTCAGGAGCTAGCTATGATCCGAATTGAG
        Mut 6 AHAS gene ATCGACGGAGATGGTAGCTTCCTCATGAACATTCAGGAGCTAGCTATGATCCGAATTGAG
        Mut 7 AHAS gene ATCGACGGAGATGGTAGCTTCCTCATGAACATTCAGGAGCTAGCTATGATCCGAATTGAG

Unmutated N12 AHAS gene AACCTCCCAGTGAAGGTCTTTGTGCTAAACAACCAGCACCTGGGGATGGTGGTGCAGTGG
        Mut 1 AHAS gene AACCTCCCAGTGAAGGTCTTTGTGCTAAACAACCAGCACCTGGGGATGGTGGTGCAGTGG
        Mut 6 AHAS gene AACCTCCCAGTGAAGGTCTTTGTGCTAAACAACCAGCACCTGGGGATGGTGGTGCAGTGG
        Mut 7 AHAS gene AACCTCCCAGTGAAGGTCTTTGTGCTAAACAACCAGCACCTGGGGATGGTGGTGCAGTGG

Unmutated N12 AHAS gene GAGGACAGGTTCTATAAGGCCAACAGAGCACACACATACTTGGGAAACCCAGAGAATGAA
        Mut 1 AHAS gene GAGGACAGGTTCTATAAGGCCAACAGAGCRCACACATACTTGGGAAACCCAGAGAATGAA
        Mut 6 AHAS gene GAGGACAGGTTCTATAAGGCCAACAGAGCRCACACATACTTGGGAAACCCAGAGAATGAA
        Mut 7 AHAS gene GAGGACAGGTTCTATAAGGCCAACAGAGCRCACACATACTTGGGAAACCCAGAGAATGAA

Unmutated N12 AHAS gene AGTGAGATATATCCAGATTTCGTGACAATTGCCAAAGGGTTCAACATTCCAGCAGTCCGT
        Mut 1 AHAS gene AGTGAGATATATCCAGATTTCGTGACAATTGCCAAAGGGTTCAACATTCCAGCAGTCCGT
        Mut 6 AHAS gene AGTGAGATATATCCAGATTTCGTGACAATTGCCAAAGGGTTCAACATTCCAGCAGTCCGT
        Mut 7 AHAS gene AGTGAGATATATCCAGATTTCGTGACAATTGCCAAAGGGTTCAACATTCCAGCAGTCCGT

Unmutated N12 AHAS gene GTGAC
        Mut 1 AHAS gene GTGAC
        Mut 6 AHAS gene GTGAC
        Mut 7 AHAS gene GTGAC
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