Field assessment of agronomic traits and *in vitro* acetolactate synthase characterisation of imazapyr herbicide tolerant sugarcane

by

Kwanele Zakhele Maphalala

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Abstract

Weed control is a major cost for growers in the sugarcane industry, especially for monocotyledonous species such as *Cynodon* and *Rottboellia* spp. The introduction of imazapyr-tolerant sugarcane would be advantageous as this herbicide has shown to be effective against the above-mentioned weeds but it also kills sugarcane. In a previous study in our laboratory, several sugarcane putative-mutant lines of variety N12 were generated by *in vitro* exposure of embryogenic callus to 16 mM ethyl methanesulfonate (EMS), followed by selection on imazapyr-containing medium. Tolerance to a low dose of imazapyr was confirmed in seven of those lines when the herbicide was applied (182 g a.i. ha^{-1}) to 3 month-old plants in pots. The aim of the present study was to identify which of the seven herbicide mutant lines had agronomic characteristics at least equivalent to un-mutated N12. The objectives were to: 1) confirm tolerance to increased rate (312 and 625 g a.i. ha^{-1}) of imazapyr in field plants; 2) measure the agronomic characteristics of these lines; 3) determine the effect of residual soil herbicide activity on germination of sugarcane setts.

The seven mutant lines (Mut1-Mut7) and un-mutated N12 were clonally propagated *in vitro* by shoot multiplication followed by rooting and planted in three plots (untreated, sprayed with 312 or 625 g a.i. ha^{-1} imazapyr), in the field, in a randomized complete block design. In the untreated control plot there were no significant differences between the control and the mutant plants for agronomic traits (tiller number/plot, stalk height and stalk diameter) or estimated yield (kg/plot) after 10 months, indicating that the mutation process had no effect on general plant phenotype. In the sprayed (312 and 625 g a.i. ha^{-1}) plots, Mut1, Mut4, Mut5, Mut6 and Mut7 plants showed tolerance to imazapyr as the leaves remained green compared with Mut2, Mut3 and N12 control plants, which displayed chlorotic leaves and eventually died in the plot sprayed with 625 g a.i. ha^{-1}. Post-herbicide application, the yields of Mut5, Mut6 and Mut7 (52.33, 43.43 and 41.43 kg/plot, respectively) from the 312 g a.i. ha^{-1} plot were not significantly different from that of N12 control (53. 61 kg/plot) in the untreated plot. However, in the 312 g a.i. ha^{-1} plot, the yield and agronomic trait measurements of the untreated N12 control were significantly higher than those of the herbicide-susceptible plants Mut2 and Mut3. Similarly, in the 625 g a.i. ha^{-1} plot, the recorded yields for Mut4, Mut6 and Mut7 were 41.60, 43.44 and 36.30 kg/plot, respectively, indicating that their imazapyr tolerance and yield characteristics were comparable to the untreated N12 control.

Imazapyr is conventionally applied to a fallow field 3-4 months prior to planting sugarcane as there is residual herbicide activity in the soil that suppresses sugarcane germination and growth. Therefore, in order to establish if the herbicide-tolerant mutants could germinate in

an imazapyr-treated field, 3-budded setts of the mutant lines (Mut1-Mut7) and N12 control were planted in two plots, one unsprayed and one sprayed with 1254 g a.i. ha^{-1} imazapyr, 2 weeks previously. Germination was calculated after 3 weeks as the number of germinated setts in each plot/no. germinated setts in unsprayed plot x100. In the sprayed plot, the setts from Mut1, Mut4 and Mut6 displayed the highest germination percentages (60, 71 and 74%, respectively) compared with Mut2 (24%), Mut3 (46%), Mut5 (34%), Mut7 (40%) and the N12 control (12%).

The *in vitro* acetolactate synthase (ALS) enzyme activity of 10 month-old plants from the untreated plot was assessed in the presence of 0-30 µM imazapyr to determine the herbicide concentration that inhibited ALS activity by 50% (IC_{50}). The IC_{50} values for the mutated lines were between 3 and 30 µM, i.e. 1.5-8.8 times more tolerant to imazapyr than the N12 control plants, with Mut6 displaying the highest IC_{50} value (30 µM).

On the basis of the results, it was concluded that Mut1, Mut6 and Mut7 lines were more tolerant to imazapyr than N12 and the other tested lines. Future work includes phenotypically assessing these lines for traits including sucrose content, fibre content, actual yield (tons cane ha^{-1}) and altered pest and disease resistance. Once isolated and sequenced, the ALS gene conferring imazapyr tolerance can be used in genetic bombardment in the genetic modification approach as the gene of interest or as a selectable marker. In addition, the imazapyr-tolerant line can be used for commercial purposes in the field and as the parent plant in the breeding programme.

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DECLARATION 1-PLAGIRISM

I, Kwanele Zakhele Maphalala, 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.

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Preface

The experimental work described in this dissertation was carried out in the Biotechnology Department of the South African Sugarcane Research Institute (SASRI), Mount Edgecombe, from January 2012 to October 2013, under the supervision of Prof. Paula Watt (UKZN), Dr Sandra Jane Snyman (SASRI) and Dr Stuart Rutherford (SASRI).

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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List of abbreviations

2, 4-D	2,4-dichloro-phenoxyacetic acid
DNOC	4, 6- dinitro-o-cresol
A	adenine
a.i.	active ingredient
AHAS	acetohydroxyacid synthase
Ala	alanine
ALS	acetolactate synthase
Asn	asparagine
AU	absorbance unit
bp	base pairs
C	cytosine
cDNA	complementary DNA
DNA	deoxyribonucleic acid
EMS	ethylmethane-sulphonate
G	guanine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GM	genetically modified
h	hour/s
Ha	hectare
His	histidine
Ile	isoleucine
Leu	leucine
LSU	large subunit
min	min/s

MSMA	monosodium methylarsonate
Mut	mutant
NCBI	national centre for biotechnology information
NIR	near infrared spectroscopy
PCP	pentachlorophenol
Pro	proline
SASRI	South African Sugarcane Research Institute
Ser	serine
SPAD	soil plant analysis development meter
SSU	small subunit
T	thymine
Thr	threonine
Trp	tryptophan

1. Introduction

Sugarcane is an important commercial crop cultivated both in tropical and subtropical regions of the world. It belongs to the genus *Saccharum* of the Poaceae family (Menossi *et al*., 2008). The crop is a major source of raw material for the sugar industry producing 80% of the world's sugar (Zucchi *et al*., 2002; FAO, 2007; 2012). It occupies about 20.4 million hectares of land providing 1392.4 million tons of cane (FAO, 2007).

Worldwide, the sugar industry is driven by the pressure of producing sugarcane that will sustain increased demand for human consumption. However, productivity is declining in many production areas due to abiotic and biotic factors such as salinity (Rozeff, 1998; Nelson and Ham, 2000), fungal (Mahlanza *et al*., 2013) and phytoplasma diseases (Gonçalves, 2012), insects (Rutherford and Conlong, 2010) and weeds (Richard, 1990). The latter are a major problem as they affect the growth of sugarcane by competing for water, nutrients, light and space, sheltering diseases and pests, and excreting toxic chemicals in the soil that result in crop damage (Khan *et al*., 2004a; Cheema *et al*., 2010). They are capable of reducing cane and sugar yield by more than 40% and cause unnecessary harvesting expenses (Richard, 1990; Millhollon, 1995). The monocotyledonous plant, *Cynodon dactylon,* also referred to as creeping grass, is the main weed species in sugarcane growing areas of South Africa. It is a serious problem as sugarcane is also a monocotyledonous species and, consequently, broad spectrum herbicides cannot be utilised to control it. The development of a sugarcane genotype tolerant to herbicides of interest would greatly increase the options of weed control (Newhouse *et al*., 1990).The herbicides currently used to control weeds in sugarcane include trazines, glyphosate and acetolactate synthase (ALS)-inhibiting herbicides such as sulfonylureas and imidazolinones.

Imazapyr belongs to the imadazalinone family of herbicides, and is a non-selective, broad-spectrum herbicide that is used to control grass and broad leaf weeds in non-crop areas, and in crop plantations such as rubber, oil palm and sugarcane (Cox, 1996; Osuna *et al*., 2003). It is effective because it inhibits the activity of the ALS enzyme that is involved in the biosynthesis of branched chain amino acids valine, leucine and isoleucine. Imidazolinone-tolerant plants with altered ALS genes and enzymes have been reported in many crop species such as corn (Anderson and Georgeson, 1989), canola (Swanson *et al*., 1989), soybean (Sebastian *et al*., 1989), tobacco (Chaleff and Mauvais, 1984) and sugarcane (Punyadee *et al*., 2007; Koch *et al*., 2012; Rutherford *et al*., 2014).

Herbicide tolerance in sugarcane, as in many other crops, is achieved by conventional plant breeding, transgenic approaches (Sebastian *et al*., 1989; Swanson *et al*., 1989; Newhouse *et al*., 1990, 1992; Gallo-Meagher and Irivine, 1996; Rajasekaran *et al*., 1996; Wright and Penner, 1998a, 1998b; Falco *et al*., 2000; Bae *et al*., 2002; Bailey and Wilcut, 2003; Leibbrandt and Snyman, 2003) and induced mutagenesis (Irvine *et al*., 1991; Ali *et al*., 2007; Kenganal *et al*., 2008). However, the use of either or both conventional breeding and genetic modification is difficult. Sugarcane breeding is limited by factors such as high polyploidy, the long periods (8-10 years) to develop and release new improved varieties and the transfer of unwanted traits along with the desired traits into newly developed varieties (Butterfield *et al*., 2001; Sengar *et al*., 2011). Genetic modification is presently not an option as commercialization of transgenic sugarcane is restricted because of lack of acceptance by international markets and intellectual property-protected technologies (Snyman *et al*., 2008).

Currently, *in vitro*-induced mutagenesis is considered a suitable approach for obtaining imazapyr-tolerant sugarcane (Rutherford *et al*., 2014). At the South African Sugar Research Institute (SASRI), Koch *et al.* (2012) produced herbicide-tolerant somaclonal variants using a chemical mutagenic agent, followed by *in vitro* micropropagation. With this approach, they generated seven putative imazapyr-tolerant sugarcane mutant plants from variety N12 by *in vitro*-induced mutagenesis. These putative-mutants displayed higher imazapyr tolerance than that of the N12 variety when they were screened for herbicide tolerance in pots. However, that study did not include assessment of the ALS enzyme activity of the mutants to confirm tolerance at the genetic level, nor did it include field evaluation of the imazapyr putative-mutants to determine their yield and other agronomic characteristics.

Field evaluation of *in vitro* micropropagated sugarcane plants has been conducted to check that they are ‘true-to-type’ and that no unintended phenotypic changes occurred due to culture-induced somaclonal variation (Lourens and Martin, 1986; Bailey and Bechet, 1989; Irvine *et al*., 1991; Burner and Grisham, 1995). In some instances phenotypic variability was recorded in micropropagated lines, e.g. reduced stalk diameter and decreased sucrose yield (Bailey and Bechet, 1989; Burner and Grisham, 1995; Gravois *et al*., 2008). However, some of the changes were found to be epigenetic in that they reverted to the normal phenotype in subsequent ratoons (Lourens and Martin, 1986; Irvine *et al*., 1991; Burner and Grisham, 1995; Taylor *et al*., 1995; Snyman *et al*., 2011).

In studies where the aim was to create mutant plants with improved traits, field evaluation was undertaken to ensure that both the trait of interest was expressed and that no unintended phenotypic changes occurred due to the mutagenic treatment. In this regard, in

sugarcane, the standard phenotypic characters that have been evaluated include yield, sugar content, stalk height, stalk diameter and tiller number (Ali *et al*., 2007; Suprasanna, 2010; Oloriz *et al*., 2012). Screening for new traits, introduced via mutagenesis, has been reported for salt tolerance (Kenganal *et al*., 2008), smut resistance (Munsamy *et al*., 2013), imazapyr tolerance (Koch *et al*., 2012; Munsamy *et al*., 2013) and *Fusarium sacchari* tolerance (Mahlanza *et al*., 2013). All of these studies have emphasized the need for the phenotypic evaluations of the mutated plants under field conditions. Consequently, the main aim of this study was to continue the work of Koch *et al.* (2012) and further investigate the tolerance and field characteristics of the seven putative imazapyr-mutant lines. In this context, the objectives were as follows:

1. **Conduct field trials to identify which of the seven N12 herbicide putative-mutant lines (Mut1-Mut7) had agronomic characteristics equivalent to un-mutated N12**
   Standard agronomic characterization using tiller number, stalk height and stalk diameter was used to assess any unintended effects of the mutagenic treatment on the sugarcane mutant lines compared with N12 control sugarcane plants in plant cane.

2. **Determine the level of herbicide tolerance in 10 month-old putative-mutant plants under field conditions**
   Investigations were carried out to confirm tolerance of the mutant plants to two rates (312 and 625 g a.i. ha^{-1}) of imazapyr in the field, to measure the agronomic characteristics of these lines after herbicide application and to determine the effect of residual soil imazapyr activity (1254 g a.i. ha^{-1}, the commercial rate of imazapyr) on sett germination of sugarcane.

3. **Characterize acetolactate synthase activity by *in vitro* enzyme assays in the presence of imazapyr**
   The ALS enzyme activity of the Mut1-Mut7 and N12 control plants was tested at different imazapyr concentrations (0-30 µM). This information was used to calculate the IC_{50} values to compare herbicide tolerance levels amongst the mutants.

2. Literature review

2.1 Sugarcane cultivation and challenges

Sugarcane is a large grass cultivated in tropical and subtropical regions (Jannoo *et al*., 1999). It belongs to the genus *Saccharum* of the Poaceae family composed of hybrids derived from *Saccharum officinarum, S. sinense* (Chinese clones)*, S. baberi* (North Indian clones) and *S. spontaneum.* The hybrids are characterized by both a high ploidy level and frequent aneuploidy. On average they contain about 100-120 chromosomes estimated to have a 10,000 Mbp somatic cell size (Menossi *et al.,* 2008).

Sugarcane is cultivated for its potential to produce high amounts of sucrose and because of its vegetative propagation simplicity, through stem sections called setts. It is ranked as one of the 10 top food crops worldwide and is the major source of raw material in sugar industries (Filho *et al*., 2011). The major countries cultivating sugarcane currently are Brazil, India, China, Thailand, Pakistan, Mexico, Colombia, Australia, USA, Indonesia, Philippines, South Africa, Argentina and Cuba (Scortecci *et al*., 2012).

Brazil is the largest sugarcane producing country, accounting for one third of the world's production and India, China and Thailand account for the other third (FAO, 2012). Across the world, 80% of sugar is produced from sugarcane and 20% from sugarbeet. Sugarcane is cultivated on more than 20 million hectares (2% of total cropped area of the world) of land, producing 1392.4 million tons of the crop (FAO, 2007; 2012). In addition to sugar production, the crop is also used for cogeneration of electricity, paper making, livestock feed, fertilizer, syrup, mulch, chipboard, cane wax and bioethanol (Chaudhry and Naseer, 2008).

Sugarcane cultivation in South Africa began in 1848 (O'Reilly, 1998), at the southernmost region of the world where it is grown commercially and for subsistence farming. The country is a major producer of the crop on the African continent (Lebaron *et al*., 2008). Production in KwaZulu-Natal is on the eastern coast which is fed by moist trade winds from the Indian Ocean (Lebaron *et al*., 2008). However, some production is at higher elevations in the interior. Historically, the sugar industry in South Africa has successfully met domestic needs and supported regional exports (Lebaron *et al*., 2008).

Sugarcane cultivation is limited by climatic conditions at Northern Eastern Cape to Mpumalanga (htt://www.sasa.org.za). Despite this challenge, the industry produces approximately 22 million tons of sugarcane annually (http://www.sasa.org.za). Sucrose obtained from sugarcane ranks among South Africa's top three most important agricultural

exports, producing about 2.5 million tons of sugar per annum with a contribution of ZAR6 billion to the country's foreign exchange on an annual basis (http://www.sasa.org.za).

The maturation or ripening of sugarcane is through the accumulation of sucrose in the internodes (Dalley and Richard, 2010). This is due to several environmental factors that include cooling temperatures, high daily sunlight, low soil moisture, and non-limiting nutrient content (Dalley and Richard, 2010). When approaching harvesting, sugarcane requires dry, sunny and cool conditions for ripening. Optimum temperature for rooting and sprouting of the planted stem pieces occur at 25^{o}C. Sugarcane is capable of growing in any soil type with a pH range of 5-8.5 (Tammisola, 2010). The crop requires high levels of nitrogen and potassium and minimal amounts of phosphorus for optimal growth (Tammisola, 2010).

Sugarcane is a long duration crop, it is important to develop early maturing, high yielding and abiotic and biotic tolerant varieties that will meet this demand (Dalvi *et al*., 2012). Using approaches such as conventional plant breeding, genetic modification and somaclonal variation, sugarcane research institutes have successfully developed improved cultivars with high yielding potential (Khan *et al*., 2009), high sugar content (Hoy *et al*., 2003; Suprasanna *et al*., 2006), increased resistance to diseases (Zambrano *et al*., 2003a; Ali *et al*., 2007; Oloriz *et al*., 2012; Mahlanza *et al*., 2013) and high salt conditions (Patade and Suprasanna, 2008).

Sugarcane productivity is declining in many production areas worldwide due to abiotic and biotic stresses (Sengar *et al.,* 2011). To counter-act this, sugar growing industries need to consider factors such as productivity and tolerance, nutrient management and improved sugar recovery (Kenganal *et al*., 2008). Salinity is one of the major abiotic factors that results in declined sugarcane yield worldwide (Rozeff, 1998; Nelson and Ham, 2000). Sugarcane is a typical glycophyte and its growth is inhibited or stunted in increased saline conditions (Kenganal *et al*., 2008), up to 50% or less than the normal yield. Injudicious use of water for irrigation has worsened the situation in salinity stressed sugarcane cultivated areas (Kenganal *et al*., 2008).

Sugarcane is susceptible to viral, bacterial, fungal and phytoplasma diseases (Gonçalves, 2012). Most sugarcane industries control diseases by an integrated approach that uses resistant cultivars, clean planting material and correct farming practices (Wada *et al*., 1999; Malathi *et al*., 2002; Zeng, 2004; Lakshmanan *et al*., 2005; Malathi and Viswanathan, 2013). Sugarcane insect pests are another major cause for declining sugarcane productivity and economic loss. For example, eldana (*Eldana saccharina*), can totally damage the crop if

uncontrolled. Similarly, fungal diseases rust (*Puccinia melanocephala* H&P Sydow) and smut (*Ustilago scitaminea* H&P Sydow) can reduce sugarcane yield by 30% (Rutherford *et al*., 2003; Campbell *et al*., 2009). In addition, high populations of nematode can cause 60-80% yield losses (Campbell *et al*., 2009).

Currently integrated pest management approaches comprising biological, cultural, and chemical approaches are used to control sugarcane pests (Malathi and Viswanathan, 2013). Another approach to help maximize and sustain productivity is using sugarcane that already comprise tolerance and increasing pest tolerance by introducing insecticidal genes (e.g. Bt gene) using transgenic strategies (Arencibia *et al*., 1997; Bohorova *et al*., 2001; Falco and Silva-Filho, 2003; Lakshmanan *et al*., 2005; Christy *et al*., 2009).

Weeds are also a major biotic problem in plantations. They are described as plants that are 'out of place', and inhibit efficient sugarcane growing (Richard, 1990). When they are not controlled, they compete with the sugarcane plants, reduce yields by more than 40% and may cause unnecessary harvesting expenses (Richard, 1990; Lencse and Griffin, 1991). Consequently, currently hand-hoeing and chemical control approaches are used to control weeds on sugarcane plantations (Preston and Powles, 2002; Punyadee *et al*., 2007).

2.2 Weed control

2.2.1 Hand-hoeing

To obtain high yields and good quality plants in any crop production system, the effective control of weeds is a necessity (Tranel, 2003; Cheema *et al*., 2010). This was initially achieved by hand weeding but later replaced by cost-effective mechanical methods (Mulwa and Mwanza, 2006). From 1910, the South African sugar Industry established progressive research programs for weed control which includes development of tolerant varieties (LeBaron *et al*., 2008).

Sugarcane is a perennial grass and it is generally replanted every 3-8 years but some growers in some countries can keep the crop for up to 15 ratoons (Cheema *et al*., 2010). There are a number of ways through which weeds affect the growth of sugarcane including competition for water, nutrients, light and space, harboring diseases and pests, and excretion of crop damaging chemicals into the soil (Khan *et al*., 2004a; Cheema *et al*., 2010). Even a single weed plant when left to grow to maturity can produce seeds that can be problematic in the future (Cheema *et al*., 2010).

Sugarcane weeds are either dicotyledonous or monocotyledonous species. As sugarcane is monocotyledonous species, the monocotyledonous weeds (e.g. *Cynodon dactylon*, *C.*

plectostachyus, C. nlemfuensis, *Digitaria longifolia D. abyssinica* and *Cyperus rotundus* L.) are the most problematic to control using herbicides. In South Africa, the negative effect of these weeds was shown by Turner (1984), who reported that *C. rotundus* could decrease sugarcane yield by 83-85%. Hence, early and effective weed control is essential to prevent competition of weeds and sugarcane at critical stages of sugarcane growth. In addition, this reduces further possible weed control problems, i.e. the production of weed seeds (Rainbolt and Dusky, 2007).

Hand-hoeing is sometimes the best approach to control weeds found in sugarcane plantations (Campbell, 2008). According to Dreistadt and Clark (2004), in this technique, weeds are eliminated using tools that chop, cut or scrape weeds. It has little or no damage to other crop plants and causes minimal environmental impact. It is performed when crop plants are at their early stage of growth (with one or two 'true' leaves), in soil that is relatively dry with no expected rain and planned irrigation several days after weeding. The weed roots and shoots are left to dry in an open area. However, hand-hoeing is labour intensive for the control of perennial weeds (Gill, 1982). Further, it has been reported that when this mechanical method is applied on sugarcane plantations it may result in the damage of the root system leaving the plant susceptible to diseases (Leibbrandt and Snyman, 2003). Because of these limitations sugarcane growers have found controlling weeds by chemicals more effective (Leibbrandt and Snyman, 2003).

2.2.2 Chemical weed control

Herbicides have been used to facilitate crop productivity by killing the weeds competing with cultivated plants. Chemical weed control for all crops began in 1932 with 4, 6-dinitro-o-cresol (DNOC) (van Rensen, 1989). However, many of the chemicals used also damaged crops and were corrosive to machinery, poisonous to humans and expensive (Stewart, 1955). In 1940, a synthetic plant growth hormone (2 methyl, 4- chlorophenoxyacetic acid) was developed, which had the potential of selectively killing some plants and allowing others to survive (Stewart, 1955). Later another such compound, 2, 4-dichlorophenoxy acetic acid (2, 4-D), which had similar herbicidal properties, was reported (Zimmerman and Hitchcock, 1948). This synthetic phytohormone was widely used in the past in sugarcane due to its low cost and effectiveness (Bovey and Young, 1980). However, it was found to be toxic to other plants and animals and causing phenotypic abnormalities in other crop plants (Zimmerman and Hitchcock, 1948). In the 1950's, ureas, trazines and bipyridiniums were discovered and released commercially (reviewed by Dodge, 1989). Their potential for weed control in sugarcane plantations was first discussed in 1949 (McMartin, 1950). LeBaron *et al*. (2008) reported that chemical weed control in sugarcane may have begun in Hawaii. According to

that author, the first herbicide, sodium arsenite, was initially used in rubber plantations but it ended up being employed in sugar industries for weed control in sugarcane plantations. Later, other new herbicides discovered were used including pentachlorophenol (PCP), 2, 4-D, dalapon, trazine, monosodium methylarsonate (MSMA), paraquat, glyphosate as well as other herbicides such as imazapyr that inhibit the acetolactate synthase (ALS) enzyme [also referred to as acetohydroxyacid synthase (AHAS)] (Table 1).

Table 1: Summary of some herbicides previously and currently used in the Sugar Industry.

Herbicides	**References**
2,4-dichlorophenoxy acetic acid (2, 4-D)	Zimmerman and Hitchcock, 1948
2 methyl, 4-chlorophenoxyacetic acid	Stewart, 1955
Pentachlorophenol (PCP)	Steward, 1956
PCP and 2,4-D, sodium chlorate trichloro-acetic acid	Thompson and Trichardt, 1957
Glyphosate, N-(phosphonomethyl) glycine	Turner, 1980
Hexazinone, monosodium methylarsonate (MSMA, paraquat, dalapon and paraquat)	Turner, 1984
Glyphosate and imazapyr	Campbell, 2008

Herbicides generally function by disrupting primary metabolic processes shared by crop and weed plants which includes amino acid biosynthesis, photosynthesis, pigment biosynthesis and mitosis (Mulwa and Mwanza, 2006). Their selectivity is mainly based on herbicide uptake between weeds and crops, managed timing and application or crop potential to detoxify the herbicide (Mulwa and Mwanza, 2006). Herbicides are generally used for weed control in different agro-ecosystems (Blanco *et al.*, 2012). This has been successfully established in sugarcane plantations and is important during establishment of seedlings and ratoon crops (Campbell, 2008).

Herbicide activity can either be non-selective (broad-spectrum herbicides) or selective. Glyphosate and paraquat are examples of non-selective herbicides used in sugarcane plantations. They kill most types of weed plants if applied at an adequate rate (Mohr and Schopfer, 1995). Effective broad spectrum herbicides are unable to kill some weeds, whilst some eliminate crops plants due to affecting process (e.g. photosynthesis and amino acid biosynthesis) shared with weeds (Sandhu *et al*., 2002; Mulwa and Mwanza, 2006). This has limited their use in some cropping operations (Mulwa and Mwanza, 2006). The development of new herbicides is expensive and they are not easily introduced because of the increased concern for the environment (Burnside, 1992; Goldberg, 1992). Selective herbicides (kill only a specific targeted set of weed plant species without harming the crop (Mohr and Schopfer, 1995). As continuous use of a few selective herbicides has resulted in the development of resistant weeds, this has created difficulties in effectively controlling weeds in some crop plants (Stewart, 1955). A major advantage would be to have herbicide tolerant plants as discussed in the next section.

Herbicides are either applied directly over sugarcane plants for foliar absorption (post-emergence treatment) by weeds, or on soil to be roots. This is before the plant is exposed to the soil surface (pre-emergence) treatment (Blanco *et al.*, 2012). Most pre-emergence herbicides used require moist soil conditions for its molecules to distribute through the soil solution and for absorption by weeds (Martini and Durigan, 2004). Thus, effectiveness of chemical weed control is best during the rainy season, as water availability in the soil and weed development favours herbicide absorption (Azania *et al*., 2010).

Herbicides have a great impact on modern agricultural practices due to shortage of farm labour and energy resources (Pimentel *et al*., 2005; Kughur, 2012). They have the potential of eliminating weeds from fields with reduced soil disturbance and allow increased productivity and viable economic weed control (Kughur, 2012). Herbicides with the ability to kill weeds, while having low or no environmental persistence (Mulwa and Mwanza, 2006), are not toxic to crop plants, mammals and invertebrates, have low production costs and display relatively short residual properties are the most preferred by growers (de Greef *et al.*, 1989).

2.3 Strategies for inducing herbicide tolerance to sugarcane

2.3.1 Conventional plant breeding

Conventional plant breeding is the recombination of desired genes from crop varieties and related species by sexual hybridization to develop new cultivars with required traits of

interest such as high yield, tolerance to herbicides, diseases, salinity, insects, pests and drought.

Although breeding in sugarcane has created new cultivars with desired traits such as high yields, improved ratooning ability and disease tolerance (Lakshmanan *et al*., 2005; Sengar *et al*., 2011; Snyman *et al*., 2011), this remains a challenge due to limited flower production, large complex genome, slow breeding advances, difficulties in back crossing and susceptibility to diseases, insects and pests (Gururaj, 2001; Sengar *et al*., 2011; Snyman *et al*., 2011). In addition, getting desired traits using sexual hybridization is limited by high polyploidy of the sugarcane genome (Rutherford *et al*., 2014). Further, it takes a long period (8-10 years) to develop and release a new improved sugarcane cultivar using this approach (Gururaj, 2001; Sengar *et al*., 2011; Snyman *et al*., 2011). Other factors limiting the use of this method include low fertility, a large genotype by environment interactions, perpetuation of diseases from one generation to the next, and the transfer of unwanted traits along with the desired traits into the newly developed variety (Butterfield *et al*., 2001; Sengar *et al*., 2011; Dalvi *et al*., 2012).

For these reasons new approaches in plant biotechnology have been introduced to complement conventional breeding in the areas of: (i) cell and tissue culture for rapid propagation and molecular breeding (Patade and Suprasanna, 2008; Snyman *et al*., 2011); (ii) commercial cultivars engineered with novel genes (Borrás-Hidalgo *et al*., 2005); (iii) sugarcane molecular pathogen diagnostics for exchange improvement between *Saccharum* germplasm and closely related genera (Patade and Suprasanna, 2008); (iv) identification of newly created varieties (Khan *et al*., 2009); (v) and evaluation of various traits within the varieties (Gururaj, 2001; Sengar *et al*., 2011).

2.3.2 Genetic modification

Genetic modification is the method of direct transferring a gene or genes from one plant (of the same or different species) to another as well as from another organism in order to obtain plants expressing the desired traits. The plants acquire the genes artificially instead of obtaining them under natural conditions of crossing or natural recombination. The newly created plants are termed transgenic or genetically modified (GM). Genetic modification has become an important tool in developing plants with improved traits to survive abiotic and biotic stresses (Lakshmanan *et al*., 2005). There are several steps involved in genetic modification, including identification of the gene of interest, cloning of the gene into an appropriate plasmid vector, insertion of the vector into the plant and expression of the gene

encoding a polypeptide (Wang *et al*., 1988; Christou *et al.*, 1989; Gordon-Kamm *et al.,* 1990; Bower and Birch, 1992; Vasil *et al*., 1992; Becker *et al*., 2000).

Traits previously developed by genetic modification in soybean, corn, cotton, canola and potato include herbicide tolerance, insect resistance, and high quality trait (Tripathi, 2005). Herbicide tolerance is the most common trait in commercial crops since 1996; in the year 2003, the trait comprised 82% of all GM plants and in 2011, 59% or 93.9 million hectares were planted with herbicide tolerance plants globally (James, 2011).

Genetic modification has been implemented to induce herbicide tolerance in sugarcane (Bower *et al*., 1996; Gallo-Meagher and Irvine, 1996; Enríquez-Obregón *et al*., 1998; Snyman and Meyer, 2012). According to Mulwa and Mwanza, (2006), there are several mechanisms that can be employed to confer herbicide tolerance. These include: (i) introduction of a gene encoding the enzyme inducing herbicide tolerance; (ii) modification of the enzyme to hinder binding of the herbicide molecule; (iii) expressing more of the gene (s) coding for the enzyme that induces herbicide tolerance, without any changes in the normal function of the plant, although this may result in the inhibition of some enzymes. The process of genetic modification for selecting the herbicide tolerant trait involves the identification of the herbicide tolerant gene from the plant or bacterium, isolation and expression of the gene encoding an enzyme conferring herbicide tolerance.

Transgenic sugarcane expressing various herbicide tolerance traits have been developed via microprojectile bombardment and *Agrobacterium tumefaciens* (Table 2). Transgenic sugarcane plants resistant to other herbicides like imidazolinone and chlorimuron, which inhibit the ALS enzyme, have not yet been developed.

Genetic modification is a good strategy for introducing herbicide tolerance in sugarcane. However, it is limited by number of factors including reduction of gene expression and limited transformation frequency (Rai *et al*., 2011). This is caused by a number of internal cellular processes, e.g. post-translational gene silencing linked to promoter methylation (Snyman and Meyer, 2012).

Table 2: Herbicide tolerance traits in sugarcane created by genetic modification.

Herbicide tolerance	Method	References
Glufosinate ammonium	micro-projectile	Bower *et al*., 1996 Gallo-Meagher and Irvine, 1996 Falco *et al*., 2000 Leibbrandt and Snyman, 2003
	Agrobacterium tumefaciens	Enríquez-Obregón *et al*., 1998
Glyphosate	micro-projectile	Snyman and Meyer, 2012

In South Africa, other barriers to the commercialization of GM sugarcane plants with desired traits include limitations in access to intellectual property-protected technologies due to high costs and the small sugarcane industry. However, current collaboration between national sugar industries and private companies are aiming at the commercial release of GM sugarcane within the next five-ten years (Snyman and Meyer, 2012).

2.3.3 *In vitro*-induced somaclonal variation

Tissue culture refers to the culture of plant cells, tissues and organs under defined laboratory conditions (Jain, 2006; Thorpe, 2007) to regenerate whole plants (Poehlman and Sleper, 1995). The initiated explant may be any plant organ such as embryos, microspores, roots, leaves and protoplasts (Chawla, 2002). The plants can be regenerated *in vitro* via organogenesis or somatic embryogenesis and each of the two morphogenesis routes has wide applications (Table 3). Organogenesis involves the regeneration of plants either directly from tissues or indirectly from callus, an undifferentiated mass of cells (George, 1993). Somatic embryogenesis involves the regeneration of plants either directly, when embryos are obtained directly from explant tissue creating identical clones or indirectly from callus (George, 1993) which result in somaclonal variation (Lakshmanan *et al*., 2006).

Table 3: Examples of application of *in vitro* culture via different morphogenic pathways.

Application	Morphogenic route	Reference
Micropropagation	Direct organogenesis	Baksha *et al*., 2002
	Indirect organogenesis	Behera and Sahoo, 2009
	Direct somatic embryogenesis	Meyer *et al*., 2007
	Indirect somatic embryogenesis	Mittal *et al*., 2009
Pathogen elimination		
Sugarcane mosaic virus	Direct organogenesis	Irvine and Benda 1985
		Uzma *et al*., 2012
Yellow leaf syndrome	Indirect somatic embryogenesis	Parmessur *et al*., 2002
Ratooning Stunting disease	Direct somatic embryogenesis	Snyman *et al*., 2005
Sugarcane mosaic virus	Indirect somatic embryogenesis	Ramgareeb *et al*., 2010
	Direct and indirect organogenesis	
Genetic transformation	Direct and indirect somatic embryogenesis	Snyman *et al*., 2000
	Direct somatic embryogenesis	Snyman *et al*., 2006
	Indirect organogenesis	Anjum *et al*., 2012

It has been reported that all plants regenerated from tissue culture are not always exactly the same as the parent plants and some may show high variability in agronomic traits (Larkin and Scowcroft, 1981). This genetic alteration is termed somaclonal variation (Larkin and Scowcroft, 1981). In sugarcane, somaclonal variation has been exploited to reduce time needed to develop varieties with desirable traits, e.g. herbicide (Koch *et al*., 2012), disease (Larkin and Scowcroft, 1983; Mahlanza *et al*., 2013) and salt tolerance (Patade and Suprasanna, 2008).

There are several mechanisms which have been reported to lead to somaclonal variation. They include: (i) change of chromosome number and structure: (ii) point mutations induced by exposing cells to chemicals in the medium; (iii) deoxyribonucleic acid (DNA) methylation; and activation of transposons; (iv) alteration in the mitochondrial DNA; (v) changes in plastid

DNA; and (vi) epigenetic variation due to micro environmental conditions in tissue culture (Jain, 1998; Kaeppler *et al*., 2000; Miguel and Marum, 2011).

Somaclonal variation is both an advantage and a disadvantage of *in vitro* culture systems. The advantages are: (i) it is cheaper than other methods such as hybridization and transformation (Doule *et al.,* 2008); (ii) the culture process may lead to desired variability in plants (Doule *et al.,* 2008); (iii) culture systems are available for more plant species than somatic hybridisation and transformation methods which are limited only to a few (Doule *et al.,* 2008); and (iv) knowledge of the genetic basis of the trait is not necessary compared to transformation which requires isolation and cloning of the gene (Karp, 1995). The disadvantages of somaclonal variation include: (i) unexpected resulting in development of inferior lines (Larkin and Scowcroft, 1981); and (ii) unstable changes in the genome of the developed varieties (Larkin and Scowcroft, 1981).

The potential of somaclonal variation for the generation of improved somaclones by callus culture was first realized in sugarcane by Nickel (1964). Since then, somaclones have been found that displayed variation for different traits including plant morphology (Heinz and Mee, 1969; Nagai *et al*., 1991), disease resistance (Krishnamurthi and Tlaskal, 1974), early maturity (Heinz *et al*., 1977; Niaz and Quraishi, 2002; Khan *et al*., 2004b), chromosome number (Sreenivasan and Jalaja, 1982; Sreenivasan and Sreenivasan, 1984), salt tolerance (Patade and Suprasanna, 2008) and high yield (Khan *et al*., 2009). Somaclonal variation in sugarcane is increased and quickly facilitated by the combination of *in vitro* culture and the use of mutagenic agents and various traits including disease resistance, yield and salt tolerance have been produced using these methods (Table 4).

Induced mutations are defined as changes in the organism genetic material which are not originally from genetic segregation or recombination (Coimbra *et al*., 2004). Since spontaneous mutations rates are extremely low, techniques that induce mutations has been successfully used for the rapid creation and increase of genetic variants in sugarcane (Coimbra *et al*., 2004) but caused by environmental causes. The main advantage of inducing mutations in sugarcane is the ability of improving one or more characters of the crop without changing the entire genotype (Sengar *et al*., 2011).

Table 4: Example of important traits in sugarcane developed by the combination of *in vitro* culture and induced mutation.

Trait	Mutagenic agent	References
Sugarcane Mosaic Virus resistance	Gamma rays	Zambrano *et al*., 2003b
Brown rust resistance	Gamma rays	Oloriz *et al*., 2012
Red rot resistance	Nitroso methyl urethane	Srivastava *et al*., 1986
	Di ethylsulphate	Srivastava *et al*., 1986
	Sodium azide	Ali *et al*., 2007
Salt tolerance	Gamma rays	Patade and Suprasanna, 2008
Yield gain	Gamma rays	Khan *et al*., 2009
Imazapyr (Herbicide) tolerance	Ethylmethane-sulphonate (EMS)	Koch *et al.,* 2010
Fusarium sacchari tolerance	EMS	Mahlanza *et al*., 2013

In vitro-induced somaclonal variation (with or without the use of mutagens) has become a useful tool in sugarcane breeding programmes because it is obtained readily, and allows new genotypes with improved economically vital agronomic characteristics to be selected (Khan *et al*., 2000; Wagih *et al*., 2004; Rutherford *et al*., 2014) (Table 4) to overcome various biotic and abiotic stresses limiting sugarcane production (Kumar and Shekhawat, 2009; Rutherford *et al*., 2014).

Mutations can be induced by either using physical agents (e.g. gamma rays) or chemical alkylating agents [e.g. (EMS) (Coimbra *et al*., 2004)]. Physical mutagenesis has been used more frequently in sugarcane than chemical mutagenesis. However, chemical agents have more potential of leading to specific and predictable mutations (Luan *et al*., 2007). This is

because base pair substitutions from GC to AT are normally produced by chemical mutagen-induced mutations leading to amino acid sequence changes that alter the function of the protein rather than inhibit it (Khan *et al*., 2009).

Ethylmethanesulphonate has been commonly used in plant breeding because of its high frequency of gene mutation (primarily point mutations) (Schy and Plewa, 1989) and low frequency of chromosomal aberrations (van Harten, 1998). It has an alkylating ability that causes complementary bases to mispair by formation of adducts with nucleotides, resulting in the changing of bases after replication (Van *et al*., 2008). Srivastava *et al*. (1986) reported that 0.8% nitroso methyl urethane, di ethylsulphate and EMS are effective mutagenic agents in sugarcane. Koch *et al*. (2010) reported that EMS can be used as a chemical mutagen to induce imazapyr (herbicide) tolerance in sugarcane (Table 4). The plants were screened *in vitro* against the selective herbicide to target the specific character. Plants that survived in the presence of the herbicide were regarded as herbicide tolerant. They were then selected and grown to maturity for further screening with the herbicide.

2.4 Acetolactate synthase inhibiting herbicides

Acetolactate synthase Inhibiting herbicides were discovered in 1975 (Brown and Cotterman, 1994). They are classified into five different chemical families: sulfonylureas, triazolopyrimidine, pyrimidyl-oxy-benzoic acid, sulfonlycarboxamide and imidazolinones (Fig. 1) (Lee *et al*., 2011). They act upon a specific plant enzyme ALS that is absent in mammals or other animals (Brown, 1990). These herbicides were first commercialized in 1982 for the control of broadleaf weeds (Burgos *et al*., 2001).

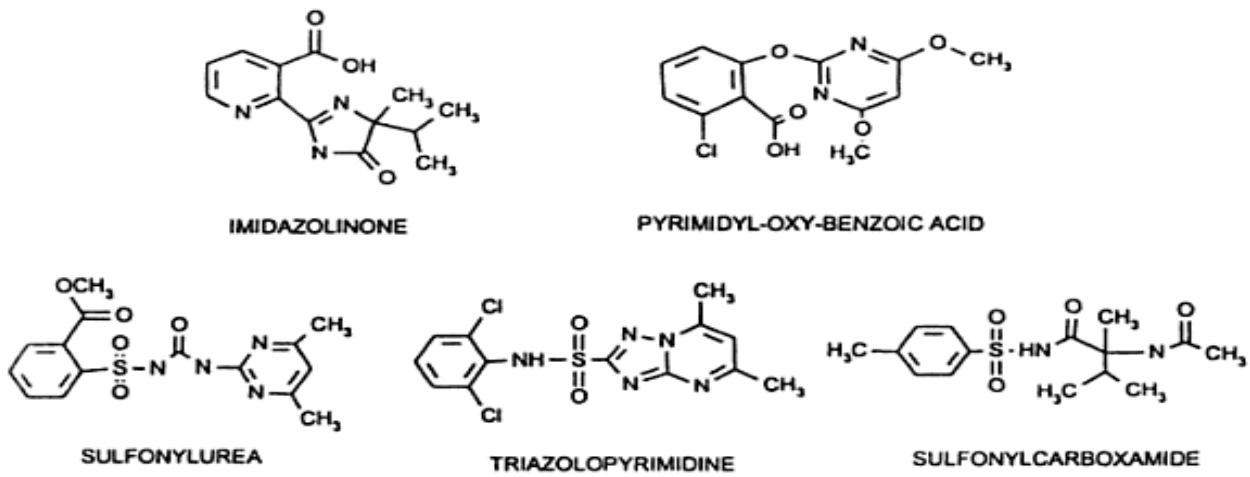
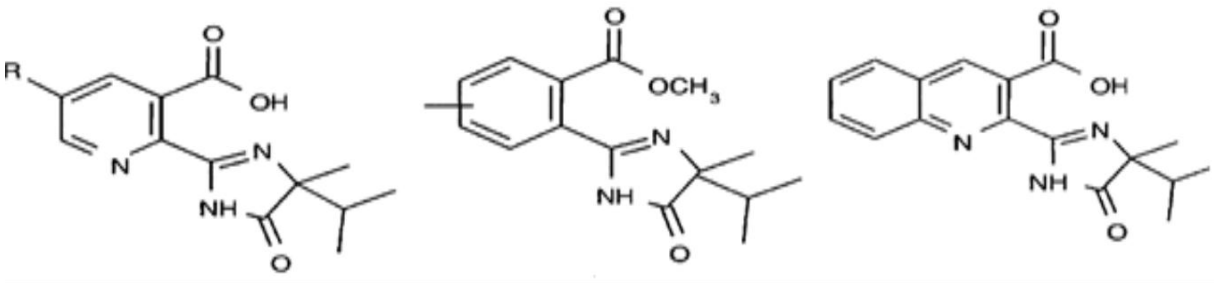


Fig.1. Molecular structures of ALS inhibiting herbicides (Roe *et al*., 1997).

2.4.1 The imidazolinone family of herbicides

Imidazolinones include imazapyr, imazapic, imazethapyr, imazamox, imazamethabenz and imazaquin (Fig. 2). This family of herbicides have a toxic effect on both monocotyledonous and dicotyledonous species (Rangel *et al*., 2010).

As reflected in the names, imidazolinones consist of an imidazole moiety in their molecular structure (Tan *et al.,* 2005). The herbicides are further divided into three groups based on the presence of the cyclic structure composed in their molecular structure (Tan *et al.*, 2005). This separation excludes the imidazole ring. Imazaquin has a quinoline, imazamethabenz has a benzene ring and the rest of the herbicides have a pyridine ring (Tan *et al*., 2005). The pyridine ring-containing imidazolinones are differentiated by four chemical functional groups that differ only at position five of the pyridine ring. These chemical groups include hydrogen (H) (in imazapyr), methyl (CH3) (in imazapic), ethyl (CH3-CH2) (in imazethapyr) and methoxymethyl (CH3-O-CH2) (in imazamox) (Fig. 2).



Pyridine imidazolinone | **Benzene imidazolinone (imazamethabenz methyl ester)** | **Quinoline imidazolinone (imazaquin)**

Fig. 2. Molecular structure of imidazolinone herbicides. Imazapyr: R=H, imazapic: R=CH3, imazethapyr: R=CH3-CH2, and imazamox: R=CH3-O-CH2. (Tan *et al*., 2005).

Since all six imidazolinone compounds have an imidazole ring in their molecular structure it was previously reported that there must be a strong link between this and ALS inhibition caused by them (Tan *et al*., 2005). Because of the inhibition difference observed in the ALS activity among the three groups of imidazolinone having quinoline, benzene and pyridine, it is thought that the second cyclic structure also contributes to inhibition (Tan *et al*., 2005). The different functional groups at the pyridine ring participates the least in inducing inhibition, but it is related to some characteristics of the imidazolinone herbicides such as plant metabolism (Tecle *et al*., 1997).

2.4.2 Application of imidazolinone herbicides

Imidazolinones herbicides are widely used for their potential in effectively controlling weeds at low application rates, high crop safety, low mammalian toxicity and high selectivity against weed populations (Tan *et al*., 2005; Rangel *et al*., 2010; Lee *et al*., 2011). In addition, most of these herbicides display extended soil persistence, which is absent in the most post-emergence herbicides (Sprague *et al*., 1997; Rangel *et al*., 2010; Lee *et al*., 2011).

As they are absorbed by plant roots and leaves imidazolinones herbicides can control weeds by both foliar and soil application (Schirmer *et al*., 2012). They are normally applied when the crops have appeared on the soil surface. The soil half-life of sulfonylureas, triazolopyrimidines and imidazolinones is within the range of 1 to 25 weeks depending on the soil pH and temperature (Goetz *et al.,* 1990). Soil persistence of imidazolinones can either be increased by low soil pH, soil moisture or high organic matter and it has been determined that for imazapyr it varies from 90 to 730 days (Alister and Kogan, 2005), and from 60 to 360 days for imazethapyr (Goetz *et al.,* 1990; Alister and Kogan, 2005). The high soil persistence of these herbicides indicates that there is a high risk of carryover which reduces growth and kills rotational crops (Goetz *et al.,* 1990; Alister and Kogan, 2005). Imidazolinones are degraded by microorganisms and photolysis accelerated by warm, moist and low organic soil (Goetz *et al*., 1990; Alister and Kogan, 2005).

Crops that have been treated with sulfonylureas, triazolopyrimidines and imidazolinones include barley, corn, spring wheat, winter wheat, durum wheat, peanuts, rice, soybeans and sugarcane (Punyadee *et al*., 2007). There is a wide range of sensitivity revealed by crop and noncrop plants to these herbicides with greater than 10,000 fold difference in observed toxicity levels for some compounds (Peterson *et al*., 1994). Field studies in most sensitive crops showed there was some yield loss after application of these herbicides (Fletcher *et al*., 1993).

2.4.3 Imidazolinone herbicides mode of action

Previous studies on the structural modeling of plant ALS and its crystal structure revealed that the binding site of ALS-inhibiting herbicides is located near the active site at the interface of the two catalytic subunits on the enzyme (Pang *et al*., 2002; Pang *et al*., 2003; Tan *et al*., 2005; McCourt *et al*., 2006). Once bound, they replace the enzyme substrate by blocking its binding to the active site stopping the synthesis of plant essential amino acids (Pang *et al*., 2002). These herbicides do not act as analogs of the substrates and cofactors suggesting that the inhibition mechanism is complex (Sikdar and Kim, 2010). The deficiency of the amino acids results in a deficit in important proteins required for plant survival, and as

a result the cell division rate is slowed down (Lee *et al*., 2011). Herbicide-treated plants show symptoms in the meristematic tissues where the primary synthesis of amino acids occurs followed by death after days or weeks after application (Schirmer *et al*., 2012).

2.4.4 Tolerance to imidazolinone herbicides

The basis for crop selectivity of the imidazolinones are the differences in the nature or rate of metabolism displayed by the herbicide (Brown *et al*., 1987; Newhouse *et al*., 1992). The *in vitro* ALS activity of species with natural tolerance to this class of herbicides displays sensitivity to inhibition by them (Singh *et al*., 1990). Development of crop varieties with an ALS enzyme activity that is insensitive to inhibition by imidazalinones would greatly increase the option of weed control in those plants. (Newhouse *et al*., 1990). Imidazalinone-tolerant plants could enhance the use of more effective, safer and more cost-effective weed control options than the ones which are currently available (Newhouse *et al*., 1992). For such plants, any of the imidazalinone herbicides could be used in controlling weeds without concern about phytotoxicity, and the choice of herbicide could be made independently without worrying about crop selectivity (Newhouse *et al*., 1992).

Commercial crops resistant to ALS-inhibiting herbicides (sulfonylureas and imidazolinones) have been developed (Table 5). Tolerance may result from one of the three mechanisms: (i) resistance at the herbicide target site caused by mutation of the ALS gene; (ii) metabolic detoxification; and (iii) inhibition of herbicides from binding to the active site (Tan *et al*., 2005; Pozniak *et al*., 2004). Example of studies which reports resistance caused by mutation of the ALS gene, include studies by Chaleff and Ray (1984), who reported ALS resistance by selecting sulfometuron methyl (sulfonylurea)-resistant tobacco cell lines with an altered ALS enzyme which had developed resistance against sulfonylureas. This herbicide tolerance was inherited as a single semi-dominant trait. A subsequent report by Anderson and Georgeson (1989) on imidazolinone resistant maize (Table 5) regenerated from cell culture, showed that resistant resulted from an altered ALS enzyme resistant against imidazolinone herbicides.

Multiple herbicide applications on sugarcane plantations have increased weed herbicide tolerance (Punyadee *et al*., 2007). A study by Campebell *et al*. (2008) showed that when sugarcane fields are invaded by running grasses, e.g. *Cynodon dactolyn*, repeated applications of the herbicide glyphosate are required and this can increase herbicide resistance in weeds. To avoid this it is, therefore, important to: (i) practice efficient management of herbicide application (Odero *et al*., 2011); (ii) have a basic understanding on the herbicide mode of action and herbicides sharing the same site of action as the weed plants (Odero *et al*., 2011); (iii) apply a herbicide like imazapyr, with a mode action different

to that of glyphosate, might be valuable for minimum tillage in sugarcane infested fields (Campbell, 2008). The use of herbicide-tolerant sugarcane varieties can increase or provide additional economical weed control options for sugarcane growers. Such imazapyr-tolerant varieties generated by somaclonal variant cells *in vitro* in the presence of imazapyr have been used successfully for controlling a wide spectrum of grass and broadleaf weeds (Punyadee *et al*., 2007). Those authors showed that ALS activity from cells with resistance at the imazapyr target site of action was greater than that from normal cells (Fig. 3). Therefore, growth of normal cells was strongly inhibited in the presence of the herbicide compared with resistant cells.

Table 5: Example of crops tolerant to ALS inhibiting herbicides.

Crop	Herbicide	References
Tobacco	Sulfonylurea	Chaleff and Ray, 1984
		Gabard *et al*., 1989
		Harms and DiMaio, 1991
	Imidazolinone	Shimizu *et al*., 2008
Camelina	Imidazolinone	Walsh *et al*., 2012
	Sulfonylurea	Walsh *et al*., 2012
Corn	Imidazolinone	Newhouse *et al*., 1990
Soybean	Sulfonylurea	Sebastian and Chaleff, 1987
Canola	Imidazolinone	Swanson *et al*., 1989
Maize	Imidazolinone	Anderson and Georgeson, 1989
		Shaner *et al*., 1990
Sugarbeet	Sulfonylurea	Hart *et al*., 1992
Oilseed rape	Imidazolinone	Anderson and Georgeson, 1989
		Shaner *et al*., 1990
Rice	Sulfonylurea	Li *et al*., 1992
	Imidazolinone	Gealy *et al*., 2003
Wheat	Imidazolinone	Newhouse *et al*., 1992
	Sulfonylurea	Pozniak *et al*., 2004
Barley	Sulfonylurea	Baillie *et al*., 1993
Sunflower	Imidazolinone	Brighenti *et al*., 2011
	Sulfonylurea	Sala and Bulos, 2012

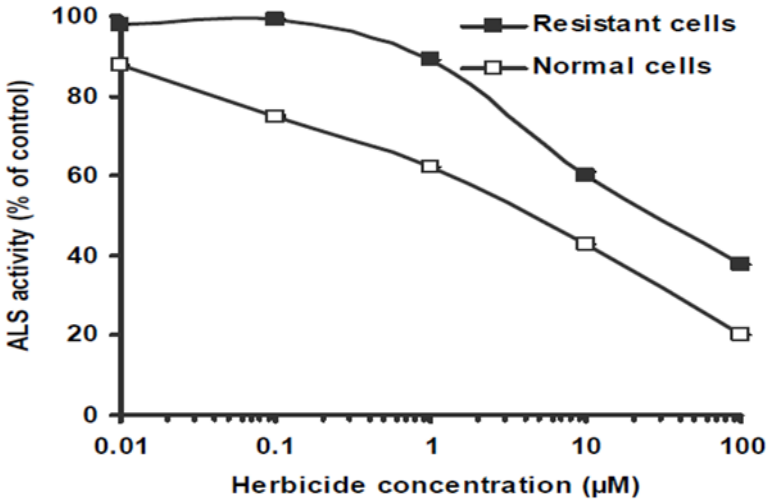


Fig. 3. Evaluation of imazapyr-tolerance by ALS activity in normal (—■—) and resistant (—□—) sugarcane cells (Punyadee *et al*., 2007).

2.5 Acetolactate synthase gene mutation and imidazolinone-tolerance trait in plants

Reports on ALS sequencing show that the amino acid sequence of the enzyme is highly conserved among plants species (Mazur *et al*., 1987). Acetolactate synthase genes among plant species are either a single copy as in *Arabidopsis thaliana* and sugarbeet, or multicopy as in corn, soybean, and tobacco (Mazur *et al.,* 1987; Keeler *et al*., 1993). Hence, multiple isozymes of ALS appear not to contribute to plant growth and development, although some plant species maintain them for the purpose of developmental regulation (Keeler *et al*., 1993).

Mutations in the ALS gene that confer herbicide tolerance in various agricultural crops have been characterized (Table 6). They result mainly from one single-base-pair changes in the genes encoding ALS (Tan *et al*., 2005). These changes occur in a number of highly conserved regions in ALS; this does not affect the functionality or inhibit the enzyme but alters its function by inducing herbicide tolerance in plants where they occur (Tranel and Wright, 2002; Mulwa and Mwanza, 2006). The most commonly occurring mutations conferring resistance to ALS inhibiting herbicides occur at ALa122, Pro197, Ala205, Trp574 and Ser653 (*A. thaliana* is used as a reference for the position of codons) (Tan *et al*., 2005). However, the development of commercialized imidazolinone-tolerant crops is currently from either one or a combination of Ala205, Trp574, and Ser653 mutations (Bernasconi *et al*., 1995; Dietrich, 1998).

Table 6: Amino acid changes in ALS as the result of single point mutations in the ALS gene of some agricultural crops.

Crop	Codon position	Amino acid change	References
Tobacco	196	Pro-Gln	Lee *et al*., 1988
	196	Pro-Ala	Lee *et al*., 1988
	573	Trp-leu	Lee *et al*., 1988
			Van der Vyver *et al*., 2013
Arabidopsis	197	Pro-Ser	Haughan *et al*., 1988
thaliana	653	Ser-Asn	Schnell *et al*., 2012
Maize	653	Trp-Leu	Dietrich, 1998
	122	Ala-Thr	Bright *et al*., 1992
	155	Ala-Thr	Bernasconi *et al*., 1995
	574	Trp-Leu	Bernasconi *et al*., 1995
Rice	653	Ser-Asn	Croughan, 2003
	654	Gly-Glu	Croughan, 2003
	95	Gln-Ala	Okuzaki *et al*., 2007
	627	Asn-Ser	Ogawa *et al*., 2008
	548	Trp-leu	Kawai *et al*., 2007
			Endo *et al*., 2012
	627	Ser-Ile	Endo *et al*., 2012
Oilseed rape	574	Ser-Asn	Hottori *et al*., 1995
			Tan *et al*., 2005
Wheat	653	Ser-Asn	Ponziak *et al*., 2004
Sugarbeet	122	Ala-Thr	Wright and Penner, 1998b
	197	Pro-Ser	Wright and Penner, 1998b
Lettuce	197	Pro-His	Eberlein *et al*., 1999

2.6 Evaluation of herbicide tolerance by acetolactate synthase *in vitro* enzyme assay

Organisms that contain anabolic ALS include bacteria, fungi, algae and plants (Duggleby *et al*., 2008). In higher plants, ALS is nuclear-encoded and found in the chloroplast (Chaleff and Ray, 1984; Smith *et al*., 1989). The amino sequence of the enzyme has an N-terminal extension that is absent in prokaryotic ALS proteins. This N-terminal region functions as a transit peptide to direct the enzyme into the correct subcellular organelles during protein biosynthesis (Smith *et al*., 1989). Studies have reported that this enzyme is composed of two large subunits (LSU) (catalytic subunits) arranged as a homotetramer and two small subunits (SSU) (regulatory subunits) (Lee and Duggleby, 2001; Pang *et al*., 2002; Pang *et al*., 2004). The primary structure of the LSU is comprised of about 670 amino acids, varying among different species (Tan *et al*., 2005).

No SSU has been reported to be required for catalytic activity of plant ALS enzymes (Smith *et al*., 1989). However, they stimulate the activity of the catalytic subunits. Plants ALSs consists of catalytic subunits similar to yeast (*Saccharomyces cerevisiae*) and bacteria (Smith *et al*., 1989). Genes that express the plant regulatory subunit have been previously cloned and characterized (Shimizu *et al*., 2008). The length of the deduced amino acid sequences in plants is twice that of bacterial sequences and comprises two domains thought to have a role in feedback inhibitor mechanisms (Shimizu *et al*., 2008).

Herbicide tolerance in plants is evaluated by characterizing the activity of the enzyme conferring herbicide tolerance using enzyme assays in the presence of the herbicide (Monquero *et al*., 2003). Whether or not an enzyme is obtained commercially or isolated using a multistep procedure, it is important that an experimental method used to detect and quantify the specific enzyme activity is developed. During enzyme isolation and purification, the assay is vital in determining the amount and purity of the enzyme and for the study of enzyme kinetics and enzyme inhibition. An assay is also important if a further study of the mechanism of the catalysed reaction is to be performed. The design of an assay requires the following knowledge: the complete stoichiometry, substances required (substrate, metal ions, cofactors etc.), and effect of pH, temperature and ionic strength (Duggleby *et al*., 2008).

The use of an *in vitro* ALS enzyme assay in the presence of ALS inhibiting herbicides (e.g. imazapyr) for evaluating herbicide tolerance in sugarcane has been reported (Punyadee *et al*., 2007; Koch *et al*., 2012). It determines the activity of ALS, the enzyme involved in the biosynthesis of branched-chain essential amino acids isoleucine, valine and leucine in plants (Newhouse *et al*., 1992; Duggleby *et al*., 2008). The formation of isoleucine involves four enzyme-catalysed steps, beginning with 2-ketobutyrate and pyruvate (Fig. 4a). Valine is

formed by a parallel pathway starting with two pyruvates substrates, in which the product of the fourth step of the valine pathway is combined with acetyl-CoA in the first reaction (Figure 4b). The formation of leucine involves four catalysed-enzyme steps beginning with acetyl-CoA (Fig. 4c) (Duggleby *et al.,* 2008).

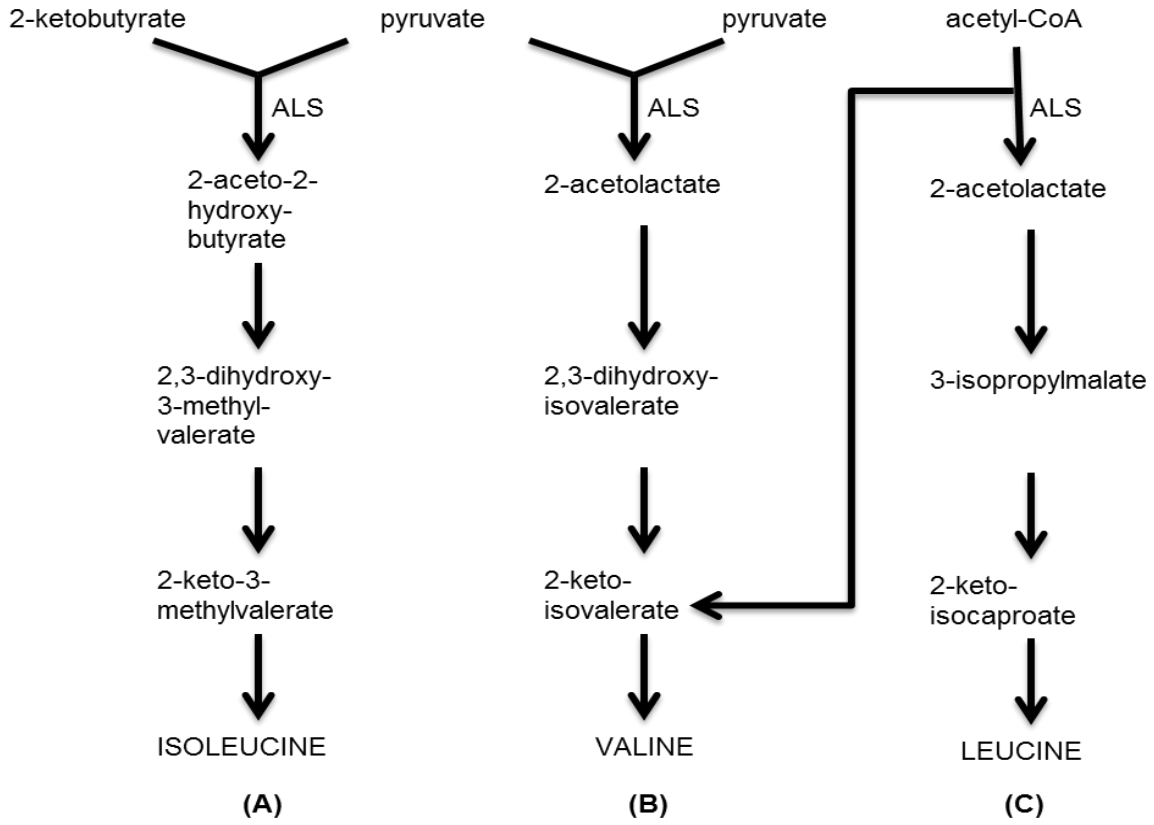


Fig. 4. Reactions of isoleucine, valine and leucine biosynthesis pathways. (a) Isoleucine biosynthesis starting with the combination of 2-ketobutyrate and pyruvate in a pathway that parallels that of valine biosynthesis. (b) Valine biosynthesis beginning with the condensation of two pyruvate molecules. (c) The formation of leucine starting with the condensation 2-keto-isovalerate, a product from the valine pathway with acetyl-CoA. Adapted from Duggleby *et al*., (2008).

The most commonly-used method for detection of ALS is colorimetric (Singh *et al*., 1988) and involves the indirect detection of the product acetolactate. This is formed from the conversion of two pyruvate molecules in the presence of ALS and cofactors (Singh *et al*., 1988; Duggleby and Pang, 2000). The method involves the incubation of samples containing the enzyme and pyruvate and other additives at 37^{o}C for a fixed time that is between 30 minutes and 2 hours (Duggleby and Pang, 2000). This reaction is stopped by the addition of sulphuric acid (H_2SO_4) and incubated at 60^{o}C to convert acetolactate to acetoin which reacts with creatine and naphthol, forming a red complex (Simpson *et al*., 1995). If herbicide

resistant plants are sprayed with ALS inhibiting herbicides, creatine is formed as the results of the accumulation of acetolactate. However, formation of creatine is hindered in susceptible plants with sensitive ALS enzymes due to inhibition by the herbicides (Monquero *et al*., 2003). ALS enzyme assay is a very sensitive assay and allows the measurement of small enzyme activities (10^{-4} units). However, this is limited when working with tissue extracts because ALS occurs in low amounts (ALS activities lower than 10^{-4} units) in its natural sources (Duggleby and Pang, 2000).

2.7 Phenotypic assessment of sugarcane mutant plants

Sugarcane mutant plants produced and multiplied using *in vitro* technologies need to be acclimatised before being transferred to the field for assessment and comparisons of specific agronomically-beneficial traits (e.g. stalk height, mid-stalk diameter, tiller number, fibre content, sucrose content and juice purity) (Bailey and Bechet, 1989; Gravois *et al*., 2008; Gilbert *et al*., 2009). However, assessment and comparisons can only be performed in mature fully grown plants. This is to check that phenotypic traits of interest are improved and expressed positively and other traits are not altered in a negative way. The phenotypic changes are further evaluated to study whether using plants obtained *in vitro* for clonal propagation is advisable (Lourens and Martin, 1986; Rutherford *et al*., 2014). Studies on sugarcane checking true-to-type have reported phenotypic changes generated from indirect somatic embryogenesis including reduced stalk diameter, decreased sucrose yield and increased susceptibility to smut disease (Bailey and Bechet, 1989; Gravois *et al*., 2008; Gilbert *et al*., 2009). However, such changes are not always stable because plants can possibly return to their originally characteristics after some time (Lourens and Martin, 1986; Watt *et al*., 2009; Snyman *et al*., 2011).

Plants can further be assessed by measuring and using leaf chlorophyll content as an indicator of many plant stresses (Palta, 1990) including low temperatures (Eagles *et al*., 1983) and herbicide stress (Adriano *et al*., 2013). Such measurements were traditionally performed by extraction of leaf materials and spectrophotometric determination (Arnon 1949; Porra *et al*., 1989) using wavelengths in the red region of visible spectrum where the chlorophyll pigment is the primary absorbing molecule (Markwell *et al*., 1995). However, the spectrophotometric determination of chlorophyll content was not clearly straightforward thus, modifications of this technique have been developed (Holden 1976). A new commercial chlorophyll meter or Soil Plant analysis Development (SPAD) meter is now available and it is a simple and portable tool that measures the ‘greenness’ or relative chlorophyll content of leaves (Inada, 1963; Kariya *et al*., 1982; Inada, 1985). Meter readings are given in Minolta Company-defined SPAD values that specify relative chlorophyll contents.

3. Materials and Methods

3.1 Plant material

Mutant sugarcane plantlets (Mut1-Mut7 of cultivar N12) with higher tolerance to the herbicide imazapyr than N12 were produced at South African Sugar Research Institute (SASRI) at Mount Edgecombe, KwaZulu Natal, South Africa (Koch *et al.,* 2012). They were multiplied *in vitro* via meristems (Ramgareeb *et al.,* 2010). Together with N12 plantlets (umutated control), Mut1-Mut7 plants were acclimated by planting in polystyrene speedling trays (670 x 330 mm) containing a substrate composed of peat moss (Nirom, Alberta, Canada) and vermiculite (Hygrotech, Pretoria, SA) (1:1) (v v^{-1}). The mixture was supplemented with 0.5 g kg^{-1} of dolomitic lime (Calmsil®, Middleburg, SA). The plants were grown in a polytunnel, watered using automatic sprayers for 5 min (600 ml min^{-1}) twice a day and fertilized every 2 weeks (NPK 5:1:5, Profert, Noordsberg, SA). They were maintained under these conditions for 3 months until they were approximately 200 mm in length before being transferred to the field.

3.2 Field trial design

The field experiments were performed for 10 months at SASRI (29° 42' 24.5585" S, 31° 02' 45.1735 E") under rainfall conditions (1023 mm) (Fig. 5) (September 2012). The field had 4 plots (Fig. 6) and in each, 10 plants from each clone were planted in 3.5 m rows with1.5 m row spacing. There were 3 rows per sugarcane line (i.e. a total of 30 plants per plot), planted in a randomized complete block design. Plot D (Fig. 6) was initially left unplanted to mimic conventional herbicide application to soil prior to planting. This plot was planted 2 weeks after herbicide was applied to a fallow field (see 3.4.2).

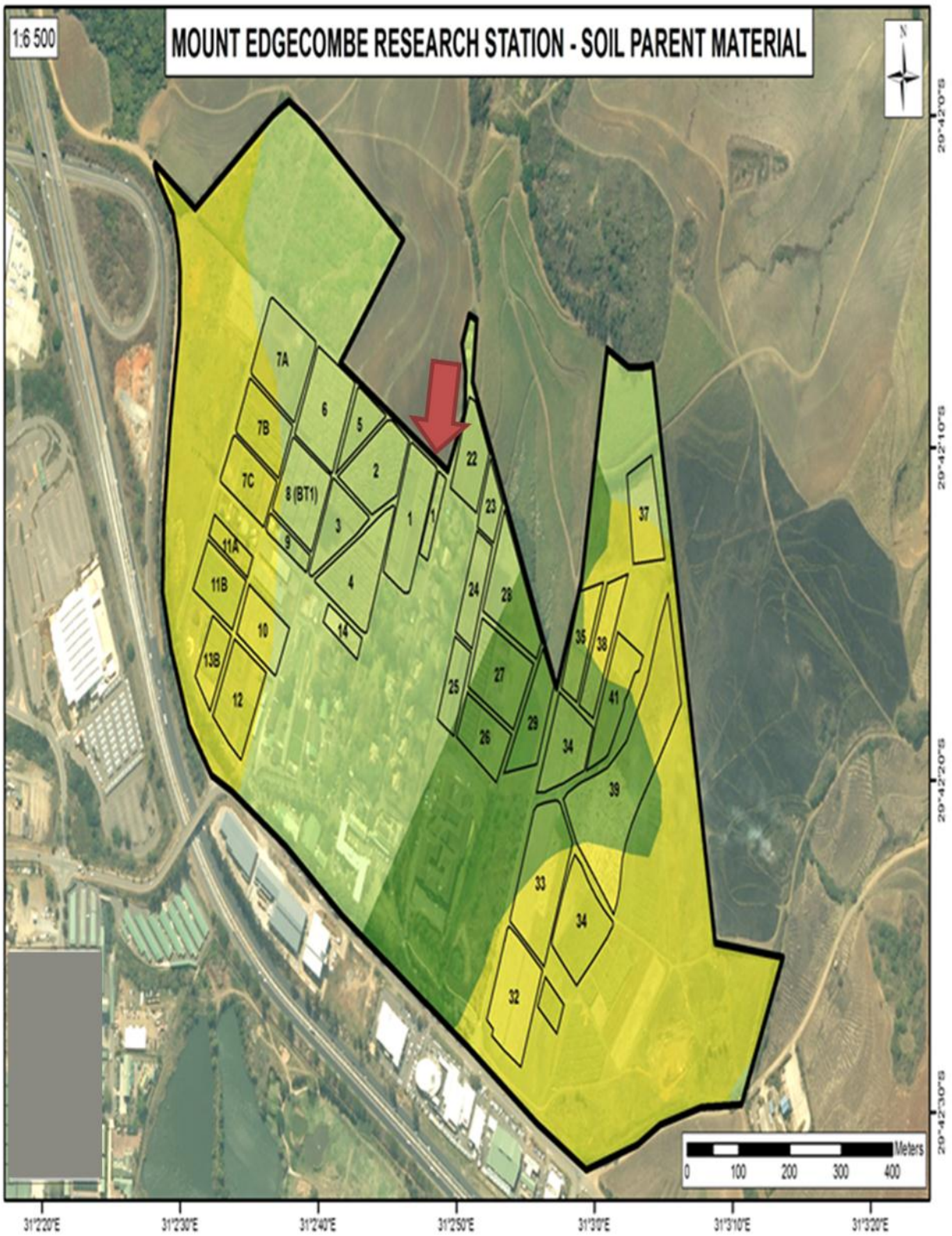


Fig. 5. Map of the South African Sugarcane Research Institute. The red arrow shows the location of field 1 where field experiments were performed in the current study.

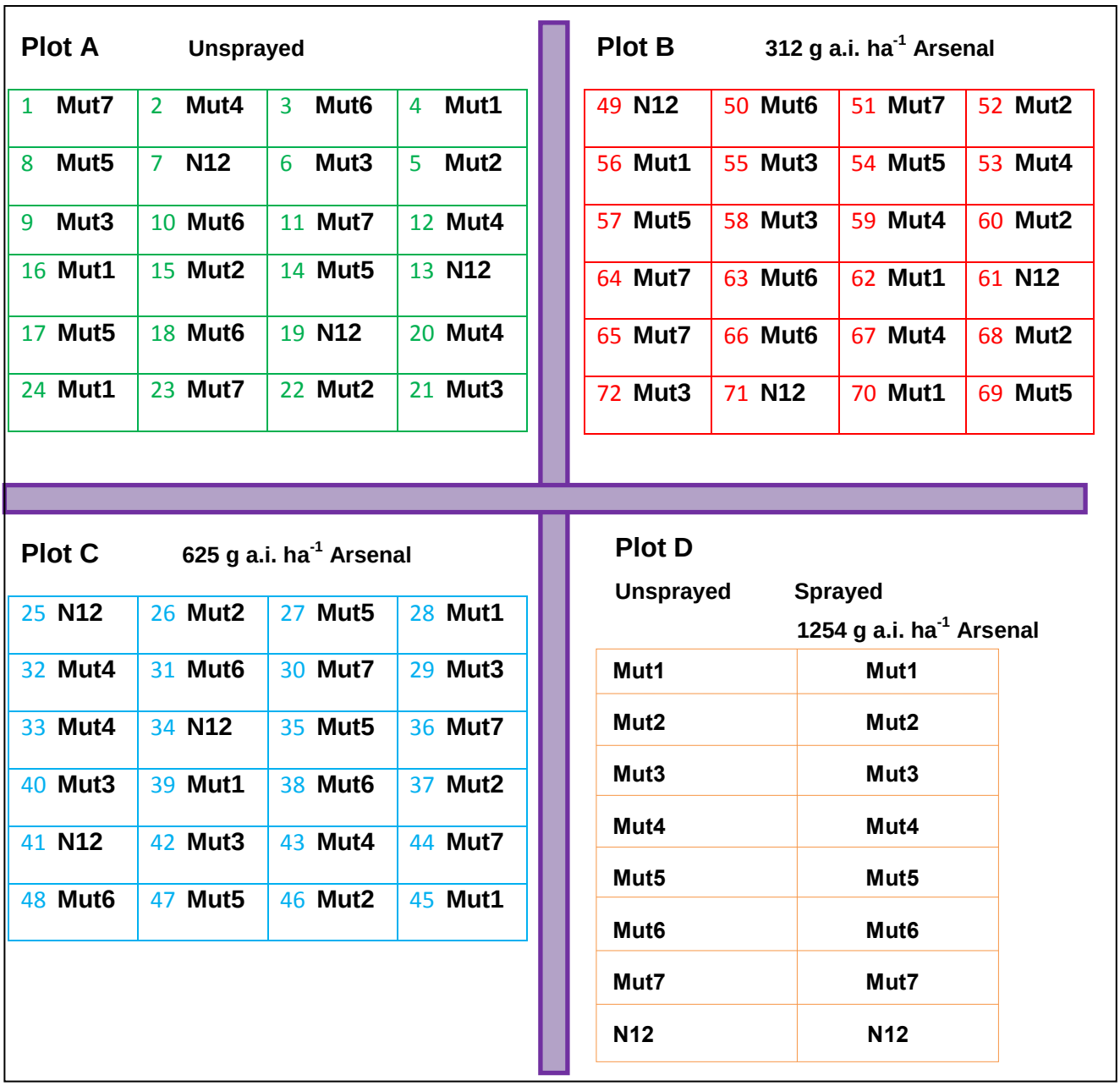


Fig. 6. Field planting showing the randomized complete block design for herbicide tolerant lines Mut1-Mut7 and N12 control. Treatment with two imazapyr concentrations and an unsprayed control was performed at 2 months after planting in plots A, B and C. Plot D was initially left unplanted and untreated, but sprayed with arsenal 2 weeks prior to planting, and planted using setts from plot A.

3.3 Experimental design

The approach followed in this study is shown in Fig. 7. Four field plots were used for generation of material for acetolactate synthase enzyme assays. In addition, field material was used for measuring agronomic and yield characteristics and for identification of herbicide-tolerant lines, after herbicide application.

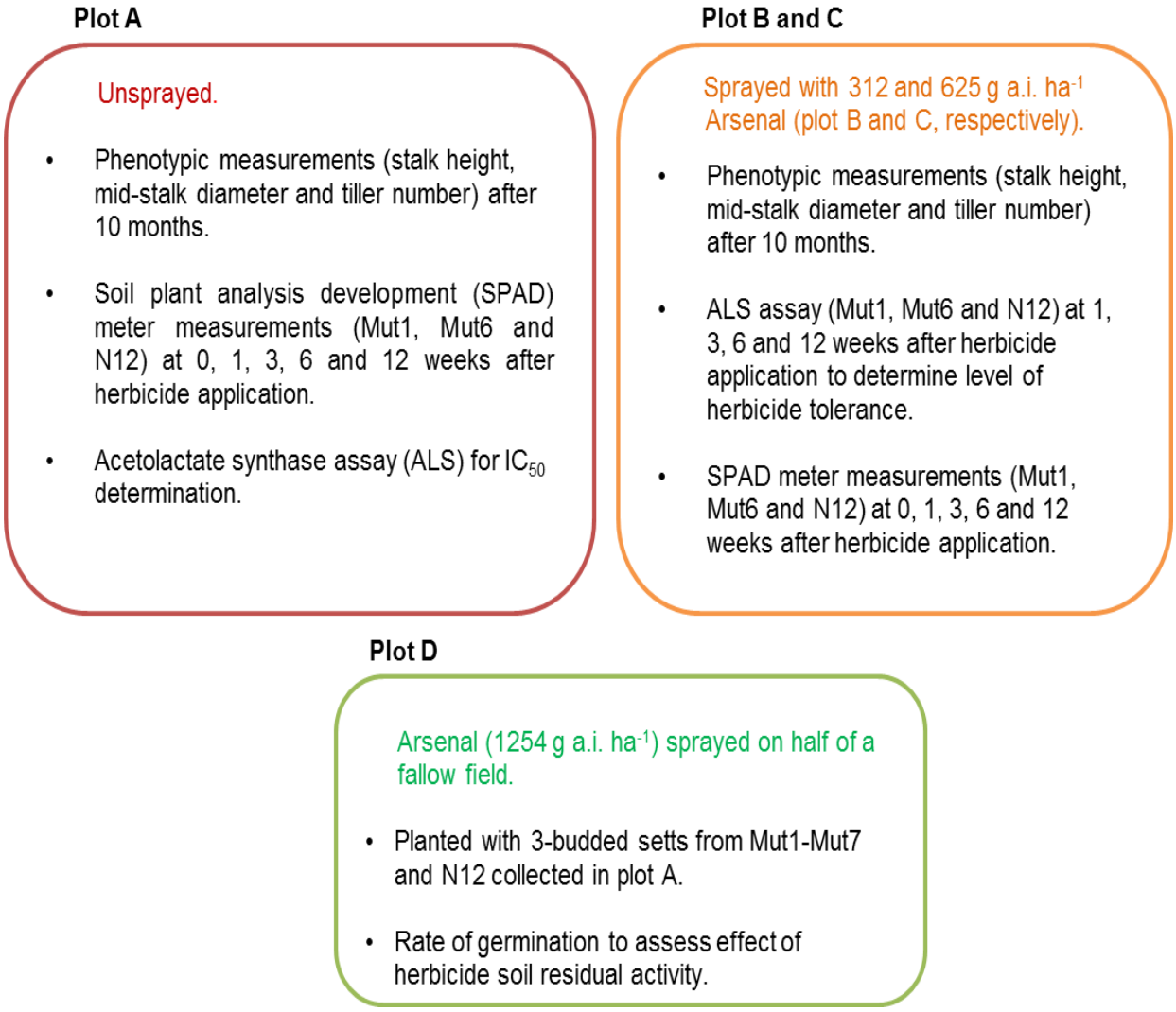


Fig. 7. Experimental design used to identify the mutant line with highest imazapyr tolerance. The laboratory experiments were performed in field plants from plots, A, B and C.

3.4 Imazapyr application

3.4.1 Foliar application

Two months after planting, when plants were at 4-6 leaf stage, Arsenal® [240 g active ingredient (a.i) ha^{-1}, imazapyr; BASF, Ago BV Arnhem, Switzerland] was applied directly over the top of the plants at 312 and 625 g a.i. ha^{-1} (Fig. 5; plots B and C) using a gas-regulated sprayer fitted with a flat-fan nozzle (Albuz APE 110°) at 194.2 l ha^{-1} application volume (1.515 l min^{-1}). Plot A was unsprayed. The level of tolerance was assessed at 6, 12, and 16 weeks after herbicide application by visually evaluating plants for chlorotic and necrotic symptoms.

3.4.2 Application to a fallow field

Arsenal® (1254 g a.i. ha^{-1}) was applied to half of a fallow field (Fig. 6, plot D) 2 weeks prior to planting. The field was divided into 2 halves of 8 x 9.5 m plots. Each half was planted with 3-budded setts from mutant plants (Mut1-Mut7) and the N12 control. Rainfall (of 77.9 mm from 8 February to 26 February 2013) was monitored between the time of herbicide application and planting. Sugarcane stalks of Mut1-Mut7 and the N12 control, collected from the control plot A and cut into 3-budded setts, were planted in plot D and the rate of shoot growth of the germination (length of shoot) and final germination percentage were monitored and recorded after 1 month.

3.5 Agronomic assessment of field plants

All phenotypic measurements were taken from plants in plots A, B and C (Fig. 6) 10 months after planting to determine if there were any observed differences amongst the seven lines and the N12 control. These were tiller number, stalk height, and mid-stalk diameter. The entire plot A was harvested and some of the stalks were cut into 3-budded setts and used for planting in plot D (Fig. 6).

3.6 SPAD meter measurements

Leaf greenness and relative chlorophyll content was determined using a SPAD-502 Plus Minolta. Measurements were taken from the middle third of leaf 3 of Mut1, Mut6 and N12 control (5 month-old) from plot A, B and C on 3 replicates per plot (Fig. 6). This was performed at 0, 1, 3, 6 and 12 weeks after imazapyr application.

3.7 Acetolactate synthase enzyme assay

3.7.1 Establishment of method

The required fresh sample mass to be used in the ALS assay to obtain an initial absorbance of approximately 1.5 at 0 uM imazapyr in the standard assay was established and was used in all subsequent assays (Fig. 8). The activity of ALS was measured by recording absorbance readings of acetoin. Leaves (3 per plot) were collected from 2 month-old mutant and N12 control plants from 3 plots in the field (Fig. 6, plot A) and weighed into 4 different masses (0.5, 1, 1.5 and 2 g). The assay was repeated three times for each mass. However, there was a lot of variation in mass replicates of Mut2 and Mut6 which was possibly due to experimental errors in the assay.

From these investigations, it was determined that the leaf mass from each mutant and control N12 plants required to achieve an absorbance of 1.5 AU was 0.43-1.20 g (Fig. 8).

Mut6 plants displayed the highest ALS activity which was significantly higher (p <0.001) (appendix 1) than that of other mutant plants and the N12 control. The line Mut6 required the least amount of plant material (0.43 g) to give a standard absorbance reading of 1.5 (Fig. 8). The leaf masses from Mut3, Mut4, Mut5 and Mut7 plants required for a standard absorbance reading of 1.5 were 0.92, 0.91, 0.94 and 0.97 g, respectively (Fig. 8). Mut1 and Mut2 required significantly more leaf material (1.20 and 1.05 g) to achieve an absorbance of 1.5 AU. These masses were used in the subsequent assay studies.

In order to calculate the rate of ALS enzyme activity (AU h^{-1} mg protein), the total protein concentration at each of the above leaf masses used was determined (Bradford, 1976). The rate of ALS activity was determined by dividing the absorbance unit from the ALS enzyme assay of the corresponding mass by one hour and then by the determined protein concentration.

The levels of imazapyr tolerance over time were determined after field application of imazapyr (refer to 3.4.1). The ALS assay was initially performed on field leaf material to test if the ALS enzyme degraded when material was stored at -80°C. Findings were that material could be sampled, immediately flash-frozen and stored at -80°C. Consequently, the leaf material of Mut1, Mut6 and N12 control was collected (from plot A, B and C, Fig. 6) at 1 and 3, 6, and12 weeks after imazapyr application and stored in this manner.

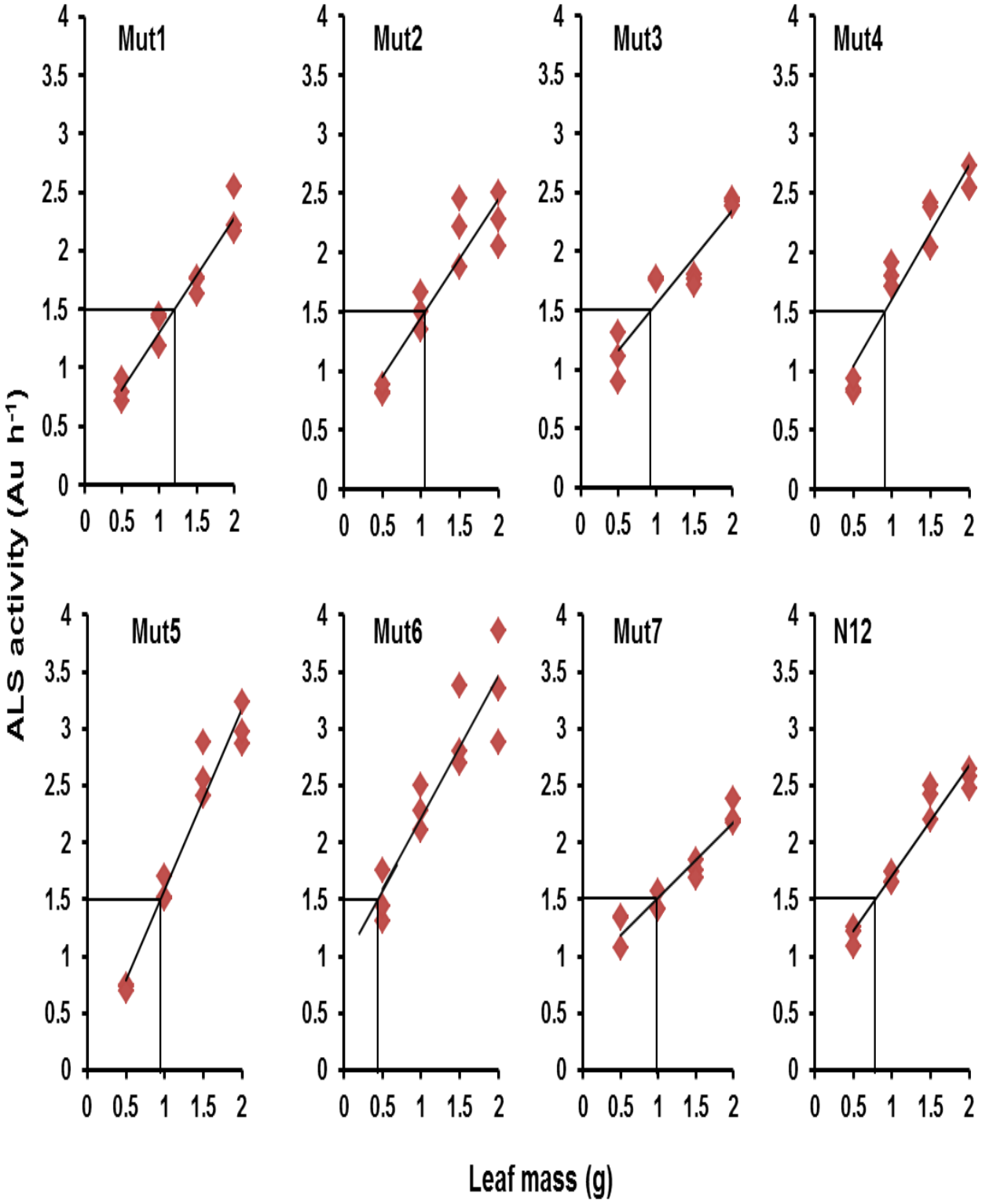


Fig. 8. The effect of leaf mass on the ALS activity assay. The ALS assay was performed on leaf material of plants (Mut1-Mut7 and N12 control) collected from the field 2 months after planting. The leaves (3 per plot) were weighed into 4 different masses (0.5, 1, 1.5 and 2 g) and each mass was assayed 3 times. Acetolactate synthase activity is expressed as absorbance units (AU h^{-1}).

3.7.2 Assay procedures

The ALS enzyme assay was conducted according to the method of Yu *et al*. (2010) with modifications. Fresh leaf material (0.43-1.20 g, as per Fig. 8 depending on Mutant) was collected and ground in liquid nitrogen using an electric grinder (IKA®A11 basic, SA) and then mixed with extraction buffer (7.5 ml g^{-1} fresh weight) and polyvinylpolyrrolidone (PVP; 0.004 g ml^{-1}) in 50 ml tubes (Corning, Massachusetts, USA). The extraction buffer contained 0.1 M potassium phosphate (K_2HPO_4) at pH 7.5, 0.1 M magnesium chloride ($MgCl_2$), 0.01 M thiamine pyrophosphate (TPP), 0.002 M flavin adenine dinucleotide (FAD), 0.5 M sodium pyruvate, glycerol (1:9 v v^{-1}), 0.01 M dithiothreitol (DTT) and protease inhibitor cocktail (for plant cell and tissue extracts; Sigma-Aldrich, USA used according to the manufactures recommendations). The homogenate was lightly vortexed, filtered through one layer of cheesecloth and centrifuged (23 200 x g for 15 min, Avanti™ J-25 I, Beckman). The protein fraction was precipitated from the crude extract at 3.78 M saturation of ammonium sulphate [$(NH_4)_2SO_4$] by addition of an equal volume of saturated $(NH_4)_2SO_4$ and allowed to stand on ice for 10 min, with slow stirring, before being centrifuged (23 200 x g for 25 min).

To assess the IC_{50}, the assay mixture (1.2 ml assay buffer) contained 0.5 M HEPES, pH 7.5, 0.5 M sodium pyruvate, 0.1 M $MgCl_2$, 0.01 M TPP and 0.002 M FAD. ALS activity was assayed in a 96 well plate (Costar®, LASEC SA) containing 55 µl of various imazapyr concentrations (0, 2, 5, 10, 15, 20 and 30 µM) PESTANAL® (Sigma-Aldrich), the pure imazapyr analytical standard and 55 µl of plant protein extract. The assay mixture was incubated at 37°C for 1 h and the reaction was stopped by the addition of 22 µl 3 M sulphuric acid (H_2SO_4).

The decarboxylation of acetolactate to acetoin was enhanced by heating the reaction at 60°C for 15 min. A coloured complex of acetoin (A_{530} nm) was detected after the addition of 0.042 M freshly prepared creatine (Sigma-Aldrich), 0.38 M α-naphthol (Sigma-Aldrich) freshly prepared in 2.5 M sodium hydroxide (NaOH) and incubation at 60°C for 15 min (Westerfeld, 1945). Absorbance readings were taken at 530 nm using a microplate reader (Synergy HT, Bio Tek® instruments, Vermont USA). Background readings were determined by initially stopping the reaction prior to incubation and subtraction of the background value from the corresponding assay value. Enzyme activity (expressed as mmol acetoin mg^{-1} protein h^{-1}) was determined colourimetrically (530 nm) by measuring the amount of acetoin formed using commercial acetoin (Sigma-Aldrich) using a standard curve (appendix 2).

3.8 Data collection and statistical analyses

The statistical program Genstat, version 14, was used for all analysis and data were initially tested for normality using the Shapiro-Wilk test ($P < 0.05$).

3.8.1 IC_{50} determination

To obtain the IC_{50} the concentration of imazapyr required to inhibit ALS activity by 50%, the ALS enzyme assay was performed on three plants replicates for each mutant (Mut1-Mut7 and N12 control, from plot A, Fig. 6) 5 months after planting. The IC_{50} values were calculated from the nonlinear regression analysis of log (inhibitor) vs. response (Graph Pad Prism 5.0., Graph pad software Inc., San Diego, CA, USA). Comparisons of plant IC_{50} values was performed using a One-way analysis of variance (ANOVA) supported by a Holm-Sidak test ($P<0.05$).

3.8.2 Effect of imazapyr on ALS activity

The imazapyr tolerance levels in Mut1, Mut6 and N12 control plants over time was evaluated at 1 and 3, 6, and 12 weeks after imazapyr application by performing ALS assays on leaf material of the plants. ALS assay data was collected and analysed using a One-way ANOVA.

3.8.3 Acetolactate synthase enzyme assay optimization

A general linear regression model was used to compare the gradients of the Mut1-Mut7 and N12 control plants in order to determine the significant differences between ALS activities expressed on a mass basis. The regression analysis was run eight times. All the lines were used as references for comparison (of regression parameter estimates) purposes between genotypes using the Student's t-test.

3.8.4 Field measurements

Application to a fallow field

The effect of imazapyr on plant shoot length over time on a fallow field previously treated with the herbicide was evaluated. Data were analysed using a One-way ANOVA supported by a Holm-Sidak test ($P<0.05$).

Agronomic assessment of field plants

The effect of imazapyr on mutant (Mut1-Mut7) and N12 control plants visual appearance and agronomic characteristics (tiller number, stalk height and diameter) and estimated yield was evaluated 5 and 10 months after planting. Data were analysed using a Two-way ANOVA supported by a Holm-Sidak test ($P<0.05$).

SPAD meter measurements

Soil Plant analysis Development (SPAD) readings were taken from leaf material of mutant and N12 control plants12 weeks after imazapyr application. Data analysis was performed using a Two-way ANOVA supported by a Holm-Sidak test ($P<0.05$).

3.9 Photography

Photographs were taken with a Nik DS-Fil camera (2/3 inch, colour 5.24 megapixels).

4. Results

4.1 Field assessment of immature mutant plants

4.1.1 Visual assessment of plant response to imazapyr after application

Imazapyr was applied at two rates, 312 and 625 g a.i. ha^{-1}, to 2 month-old plants of Mut1-Mut7 lines and to the N12 control to assess tolerance in the field. The level of tolerance to imazapyr was assessed 6 weeks after foliar spray application by visually evaluating the plants for chlorotic and necrotic symptoms in their leaves. As expected, all plants in the untreated plot showed no symptoms and the leaves were green (Fig. 9a). Within the treated plots (312 and 625 g a.i. ha^{-1}), Mut1, Mut4, Mut5, Mut6 and Mut7 displayed no symptoms as their leaves remained as green as those in the untreated plot, indicating tolerance to imazapyr (Fig. 9b and c). However, the leaves of Mut2, Mut3 and N12 control plants turned red-brown, as expected from herbicide-sensitive genotypes (Fig. 9b and c).

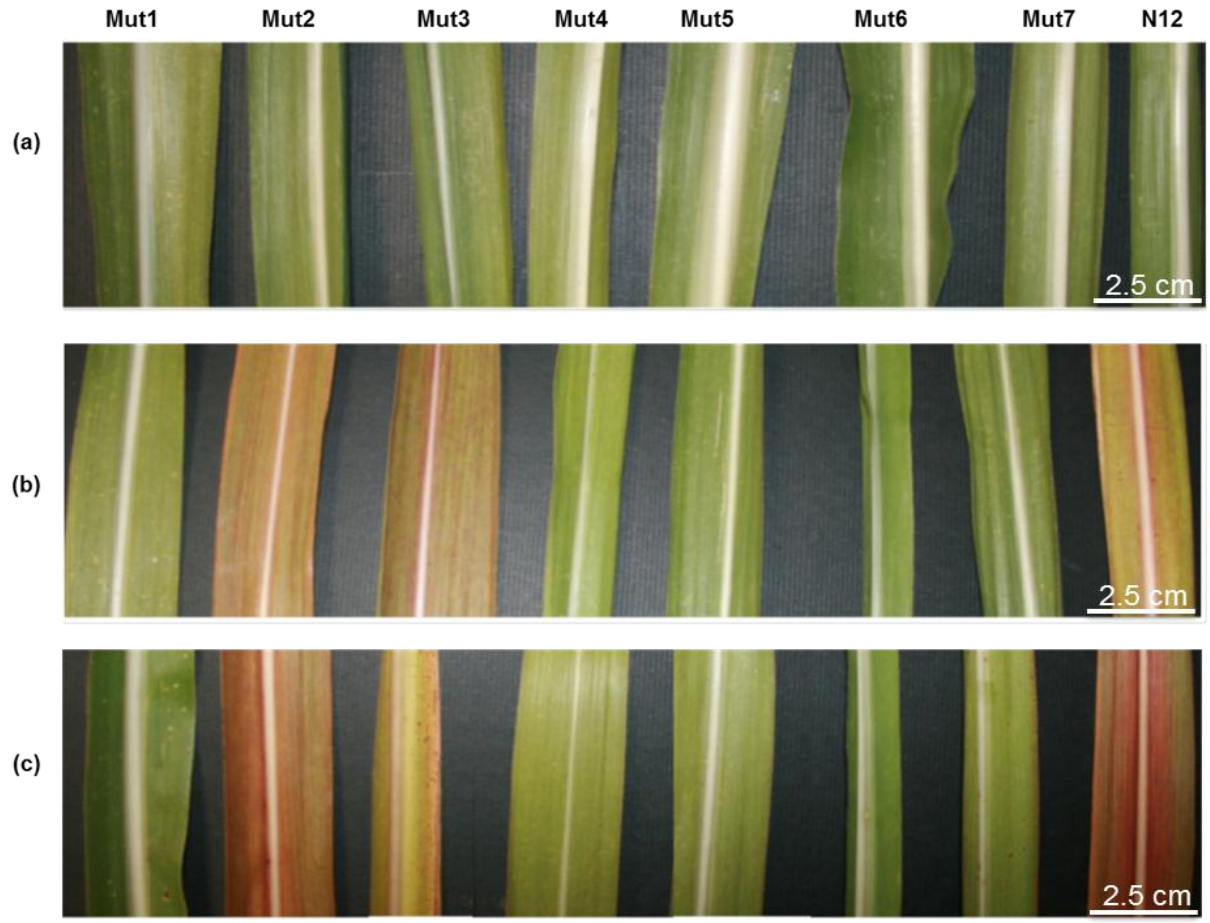


Fig. 9. A visual comparison of the effect of imazapyr on leaf appearance of plants Mut1-Mut7 and N12 control 6 weeks after foliar application. Leaves were collected from (a) untreated; (b) 312 g a.i. ha^{-1}; and (c) 625 g a.i. ha^{-1} sprayed plots.

At week 12, the plants from the untreated plot were again compared to those from the treated plot (625 g a.i. ha^{-1}) to observe differences in visual appearance of the plants (Fig. 10). All mutants and the N12 control plants in the untreated plot remained green (Fig. 10, red arrow). In the plot sprayed with 625 g a.i. ha^{-1}, Mut1, Mut4, Mut5, Mut6 and Mut7 exhibited neither chlorosis nor necrosis suggesting tolerance to imazapyr. However, 12 weeks after treatment with imazapyr they displayed stunted growth compared with the same genotypes in the untreated plot (based on a subjective visual comparison). By week 16, they appeared to have recovered as there was no visual difference in height between unsprayed and sprayed plots for those genotypes.

At week 16, the herbicide-sensitive genotypes Mut2, Mut3 and the N12 control in the sprayed plot showed stunted of growth compared with the tolerant mutants, and did not recover (Fig. 10, white arrow). The plants showed chlorotic and necrotic symptoms in the leaves and all plants died, including the N12 control.

Fig. 10. Visual appearance of imazapyr untreated and treated plants (Mut1-Mut7 and N12 control). The plants indicated by a red arrow were untreated while the white arrow donates those sprayed with 625 g a.i. ha^{-1} imazapyr. Plots were assessed for symptoms 12 weeks after application.

4.1.2 Comparison of SPAD meter readings

In this study, the leaf 'greenness' or relative chlorophyll content of Mut1 and Mut6 plants (5 month-old) was determined by taking Soil Plant analysis Development (SPAD) measurements (Inada, 1963; Kariya, 1982; Inada, 1985) and compared with those of the N12 control plants at 0, 1, 3, 6 and 12 weeks after imazapyr application. Comparisons of SPAD readings were performed across treatments and between genotypes within each plot (sprayed 312 and 625 g a.i. ha^{-1} and unsprayed plots). The SPAD readings were also compared over time for each genotype within each plot.

The SPAD readings for the Mut1, Mut6 and N12 control plants in the unsprayed plot at week 6 were significantly higher ($p < 0.001$) (appendix 3) than those of the sprayed plots (312 and 625 g a.i. ha^{-1}) (Fig. 11). In addition, at week 12 the N12 control plants also displayed SPAD readings that were significantly lower ($p < 0.001$) (appendix 3) than those at week 12 in the untreated plot (Fig. 11).

In the untreated plot, the SPAD readings for the Mut1, Mut6 were significantly higher N12 ($p < 0.001$) (appendix 6) compared with those of N12 control plants at week 3 (Fig. 11a). The SPAD readings for the Mut1 plants at weeks 1 and 3 decreased significantly ($p < 0.002$) (appendix 4) when compared with those of week 0. This decrease was possibly due to environmental stresses such as dry spell, cloudy days or imazapyr drift. However, there was an increase in SPAD readings at 6 and 12 weeks (Fig. 11a), suggesting that the plants had recovered from the negative effects of imazapyr application. There were no significant differences observed in the SPAD readings for Mut6 and N12 control plants over time (week 0-12). There was a significant decrease ($p < 0.001$) (appendix 4) in SPAD readings for N12 control plants at 1 and 3 weeks compared with week 0, followed by recovery at week 12.

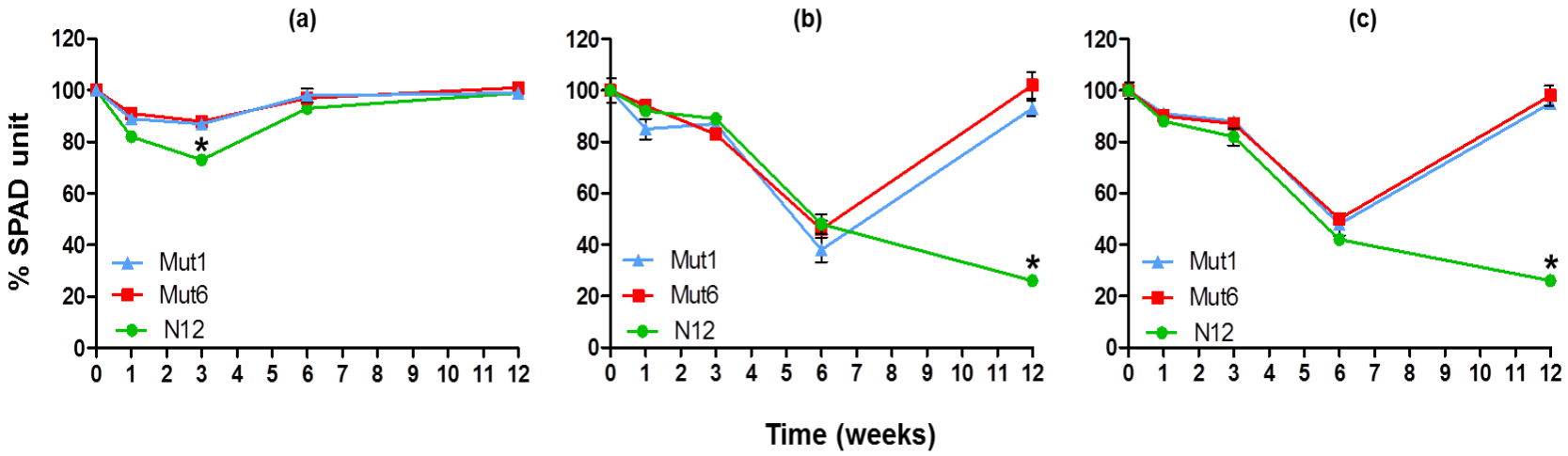


Fig. 11. A comparison of SPAD meter measurements on leaves of Mut1, Mut6 and N12 control under different imazapyr dosages at 0, 1, 3, 6 and 12 weeks after herbicide application. SPAD eadings were taken on the third leaf of Mut1, Mut6 and N12 from (a) untreated; (b) 312 g a.i. ha^{-1}; (c) 625 g a.i. ha^{-1} treated plots. *Indicates significant difference between the mutant plants and the N12 control at each week within a treatment (P <0.05; n=3, mean ± SE).

In the plot sprayed with 312 g a.i. ha^{-1} imazapyr, The SPAD readings in Mut1 and Mut6 plants were significantly higher ($p < 0.001$) (appendix 5) than those of the N12 control plants at week 12, indicating that the mutant plants recovered after herbicide application while the N12 control became chlorotic and eventually died (Fig. 11b), supporting the observations discussed previously (4.1.1; Fig. 10). There was a significant decrease ($p < 0.001$) (appendix 6) in SPAD readings of Mut1 plants at week 6 when compared with those of weeks 0, 1 and 3, followed by an increase in SPAD readings at week 12 (Fig. 11b). A similar trend was observed for Mut6, except that values at week 3 were not significantly different to that at week 6. Although visually the leaves of Mut1 and Mut6, 6 weeks after imazapyr application, remained green (Fig. 11b), the SPAD measurements taken at this time were significantly lower ($p < 0.001$) (appendix 6), indicating that the mutants were also affected by the herbicide (Fig. 11b). The SPAD readings of the N12 control plants decreased significantly ($p < 0.001$) between 6 and 12 weeks and 0, 1 and 3 weeks (Fig. 11b) (appendix 6).

In the plot sprayed with 625 g a.i. ha^{-1} imazapyr, the SPAD readings for Mut1 and Mut6 plants were significantly higher ($p < 0.001$) (appendix 5) compared with those of the N12 control plants at week 12 (Fig. 11c). There was a significant decrease ($p < 0.001$) (appendix 7) in SPAD readings at 6 weeks for both the Mut1 and Mut6 plants when compared with those of 0, 1 and 3 weeks, followed by an increase in SPAD readings at week 12 (Fig. 11c). The SPAD readings of N12 control plants in 6 and 12 weeks decreased significantly ($p = 0.001$) (appendix 7) when compared with those of 0, 1 and 3 weeks (Fig. 11c).

4.2 Acetolactate synthase activity in plants

4.2.1 Rate of ALS activity

The rate of ALS activity from 2 month-old field plants (Mut1-N12) was calculated by using absorbance values from the ALS assay performed in the absence of imazapyr and determining the total protein content using a Bradford assay (refer to 3.7). The Mut6 and Mut7 plants had ALS rates (11.41 and 11.86 AU h^{-1} mg^{-1} protein) that were significantly higher compared with the rest of the plants (Table 7).

Table 7: The rate of ALS activity of mutant (Mut1-Mut7) and N12 control plants, expressed on a protein basis. Different alphabet characters indicate a statistical significance between each genotype. Data was analysed using a One-way ANOVA and Holm-Sidak test, P <0.05; n=12, mean ± SE (appendix 8).

Genotype	Rate of ALS activity (AU h^{-1} mg^{-1} protein)
Mut1	8.12±0.04[b]
Mut2	8.10±0.02[b]
Mut3	8.09±0.04[b]
Mut4	8.09±0.02[b]
Mut5	8.09±0.04[b]
Mut6	11.41±0.02[a]
Mut7	11.86±0.02[a]
N12	8.03±0.01[b]

4.2.2 Effect of imazapyr foliar application on ALS activity of field (5 month-old) plants

The imazapyr dose response effect on Mut1, Mut6 and N12 control plants grown in the field for 5 months, was investigated to assess their levels of tolerance. The herbicide was applied to plants in two plots, one with 312 g a.i. ha^{-1} and the other with 625 g a.i. ha^{-1}. In addition to those plots, another was left untreated and used as a control. The ALS activity was assayed in leaf material from untreated, 312 and 625 g a.i. ha^{-1} plots at 1 and 3, 6 and 12 weeks after imazapyr application. Comparisons of ALS activity was performed across treatments and between genotypes within each plot (sprayed 312 and 625 g a.i. ha^{-1} and unsprayed plots). The ALS activity was also compared over time for each genotype within each plot.

The ALS activity for the Mut1 and Mut6 plants in the sprayed plots (312 and 625 g a.i. ha^{-1}) at week 6 was significantly lower ($p < 0.001$) (appendix 9) than that of the plants in the unsprayed plot (Fig. 12). The N12 control plants displayed decreased ALS activities at weeks 3, 6 and 12 in the 625 g a.i. ha^{-1} plot that were significantly lower ($p < 0.001$) (appendix 9) than those in corresponding weeks in the untreated plot.

In the untreated plot, the ALS activity of Mut1 and Mut6 was significantly higher ($p < 0.001$) (appendix 10) compared with that of the N12 control plants at week 12 (Fig. 12a). There was a slight significant decrease ($p < 0.001$) (appendix 11) in ALS activity for the Mut1 plants from week 1 to 3 and 6 weeks (Fig. 12a), then ALS activity increased at 12 weeks. A similar trend was observed in Mut6 plants, except that there was no significant difference in ALS activity between 3 and 6 weeks (Fig. 12a). However, there were no significant differences in ALS activity observed between Mut1 and Mut6 plants over time (1, 3, 6 and 12 weeks). There was a significant decrease ($p < 0.001$) (appendix 11) in ALS activity over time for the N12 control plants (Fig. 12a).

In the plot sprayed with 312 g a.i. ha^{-1} imazapyr, at week 12, the Mut1 and Mut6 plants displayed ALS activities that were significantly higher ($p < 0.001$) (appendix 10) than that of the N12 control plants, indicating tolerance to imazapyr (Fig. 12b). There was a significant decrease in ALS activity at 6 weeks for Mut1 and Mut6 plants, but then it increased at week 12 (Fig. 12b). Again, there was a significant decrease ($p < 0.001$) (appendix 12) in ALS activity over time for the N12 control plants (Fig. 12b). The high ALS activity observed in the Mut1 and Mut6 plants compared with the N12 control plants suggested that tolerance to imazapyr was possibly due to the overproduction of ALS at the target site or a mutation in the ALS gene.

In the plot sprayed with 625 g a.i. ha^{-1} imazapyr, at week 12, Mut1 and Mut6 plants displayed ALS activities that were significantly higher ($p < 0.001$) (appendix 10) than that of the N12 control plants (Fig. 12c). The ALS activities of Mut1 and Mut6 decreased significantly ($p < 0.001$) (appendix 13) at week 6 compared with that of week 1 (Fig. 12c). The ALS activity in N12 decreased over the 12 weeks and was always significantly lower ($p < 0.001$) (appendix 13) than those of Mut1 and Mut6 plants (Fig. 12c).

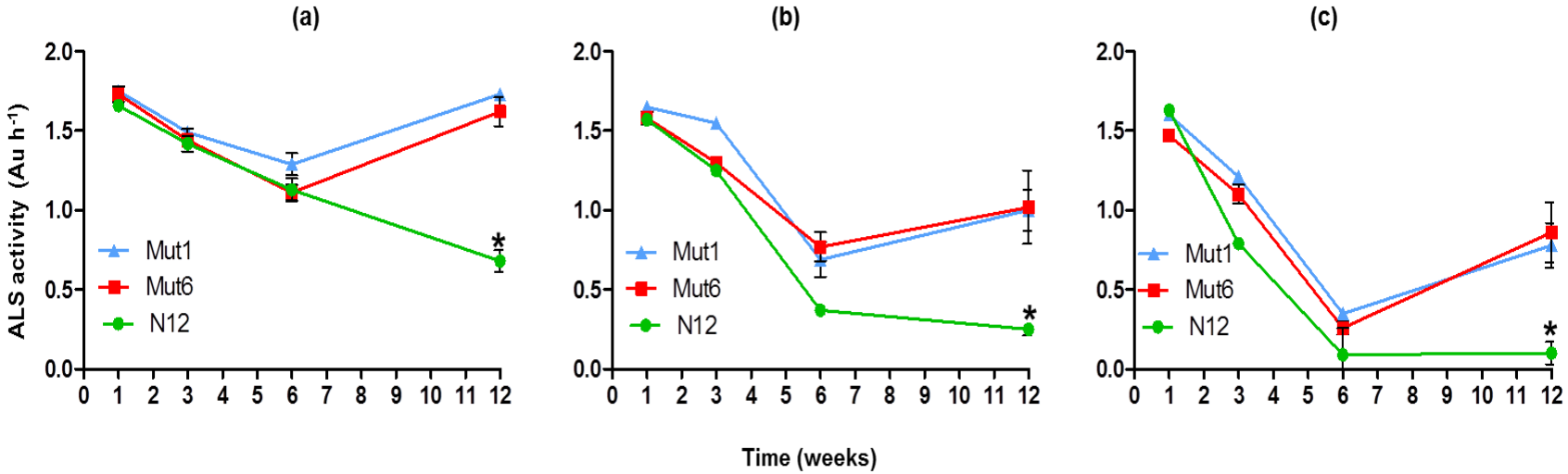


Fig. 12. The effect of imazapyr on ALS activity for Mut1, Mut6 and N12 control in field material as determined by a spectrophotometric assay for acetoin at 530 nm. Leaf material was collected from (a) untreated; (b) 312 g a.i. ha^{-1}; (c) 625 g a.i. ha^{-1} treated plots. The ALS enzyme assay was performed on the third leaf of Mut1, Mut6 and N12 control 1, 3, 6 and 12 weeks after herbicide application.* Indicates significant difference (P <0.05; n=3, mean ± SE) between the mutant plants and the N12 control at 12 weeks.

4.2.3 The effect of imazapyr on *in vitro* ALS activity incorporated in the enzyme assay and calculation of IC_{50}

The ALS enzyme activity of the leaves of Mut1-Mut7 and N12 control plants was tested at different imazapyr concentrations (0-30 µM). The ALS activity was assayed on field leaf material for all plants, 5 months after planting. There was a decrease in the total ALS activity of the mutants and N12 control when the concentration of imazapyr was increased from 0-30 µM (Fig. 13).

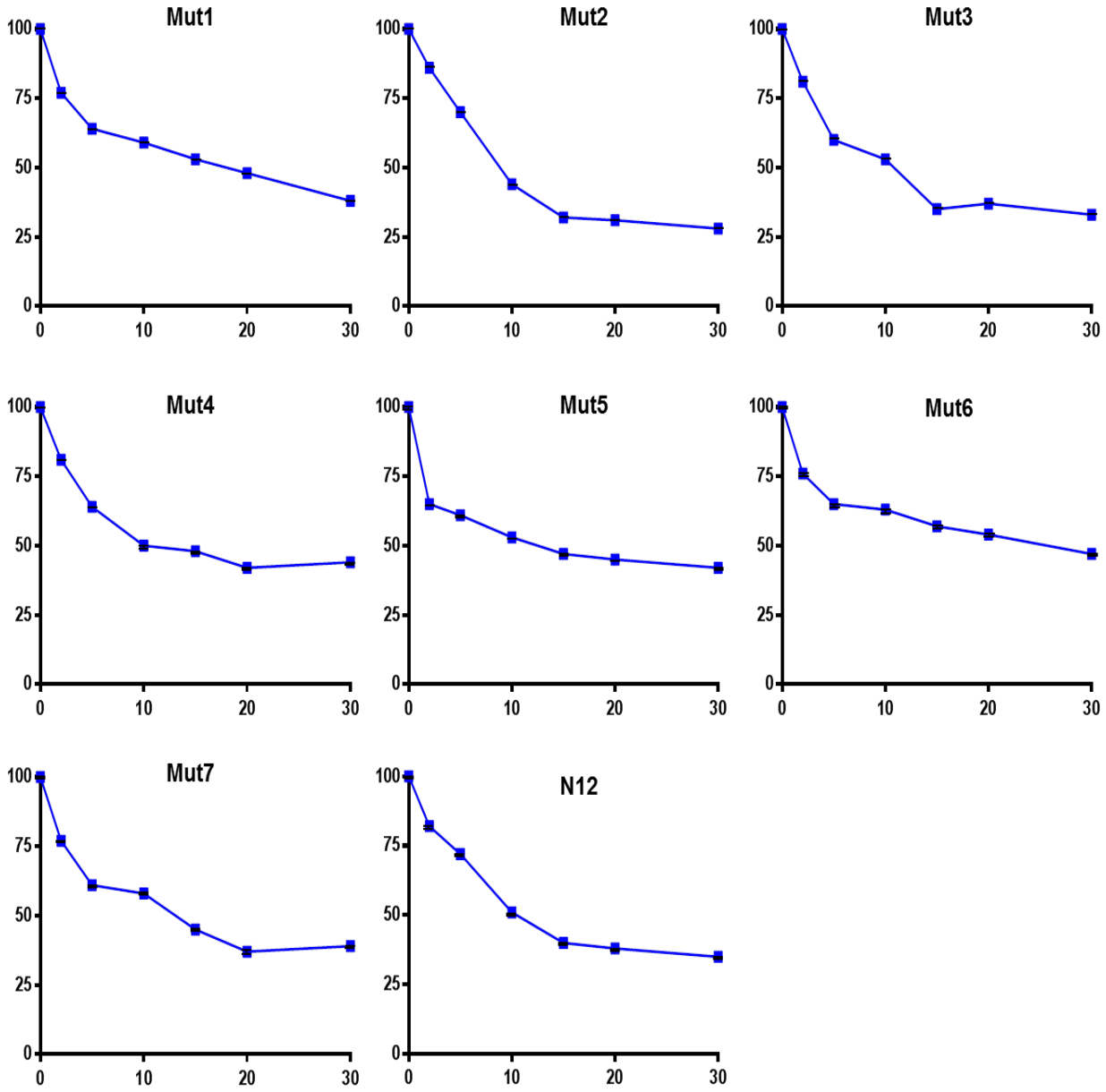


Fig. 13. Effect of imazapyr concentration on ALS activity for Mut1-Mut7 and the N12 control plants as determined by a spectrophotometric assay for acetoin at 530 nm. Mean ± SE.

In this investigation, IC_{50} values of the mutant (Mut1-Mut7) and N12 control plants were calculated from data generated in Fig. 13 to evaluate imazapyr tolerance levels. An IC_{50} is the concentration of imazapyr required to reduce ALS enzyme activity by 50%. The ALS enzyme assay was performed on leaf material from field plants at imazapyr concentrations 0, 1, 2, 5, 10, 20 and 30 µM, and 6 months after planting. The mutants (Mut1, Mut4, Mut5, Mut6 and Mut7) displayed IC_{50} values 1.5-8.9 times greater than that of the Mut2, Mut3 and N12 control plants (Fig. 14).

The IC_{50} value from Mut6 was significantly higher (p = 0.005) (appendix 14) than that from Mut3, but there were no significant differences between Mut6 and the other mutant and the N12 control plants. There were also no observed significant differences in IC_{50} amongst Mut1, Mut2, Mut3, Mut4, Mut5, Mut7 and N12 control (Fig. 14). This study suggested that the higher ALS activity observed in Mut4, Mut5, Mut6 and Mut7 was probably due to a mutation in the ALS gene resulting to imazapyr tolerance.

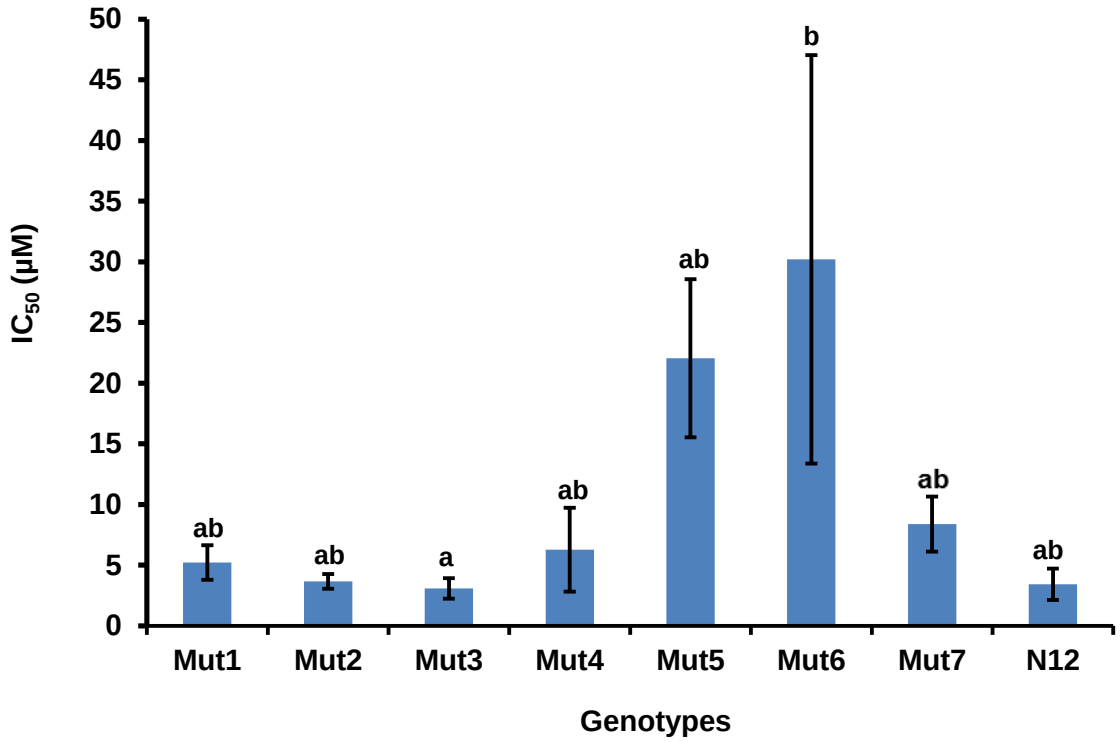


Fig. 14. Comparison of IC_{50} values as a measure of imazapyr tolerance amongst sugarcane genotypes. Plants were tested 5 months after planting. Different alphabet characters indicate a statistical significance between each line, (One-way Anova and Holm-Sidak test, P <0.05; n=3, mean ± SE. For analysis purposes data were log_{10} transformed, but untransformed data is presented).

4.3 Agronomic assessment of mature plants

The effect of imazapyr on the agronomic traits of the mutant (Mut1-Mut7) and N12 control plants in the treated plots (312 and 625 g a.i. ha^{-1}) was determined by comparing each of the investigated characteristics of those plants with those of the untreated control plot plants. The comparisons of agronomic traits were performed across the three treatments and between the mutant lines and untreated N12 control (Table 8). The comparisons of agronomic characteristics were made amongst Mut1, Mut4, Mut5, Mut6, Mut7 plants in the 625 g a.i. ha^{-1} plot that survived the treatment. Those that did not survive were Mut2, Mut3 and N12 control (Table 8).

The number of tillers/plot for the plants in the sprayed (312 and 635 g a.i. ha^{-1}) plots decreased significantly ($p < 0.001$) (appendix 15) when compared with those of the untreated plot (Table 8). Excluding dead plants, the mean number of tillers/plot was 109.3-160.0 in the untreated plot, 11.0-120.7 in the 312 g a.i.ha^{-1} plot, and 112.3-150.0 in the 625 g a.i. ha^{-1}plot (Table 8). In the untreated plots, the number of tillers/plot in the mutant and N12 control plants were not significantly different from each other (Table 8). In the treatment sprayed with 312 g a.i. ha^{-1} imazapyr, the N12 control died and the tiller number of Mut5, Mut6 and Mut7 plants were significantly higher ($p < 0.001$) (appendix 15) than that of Mut2 and Mut3 plants, indicating tolerance (Table 8). The Mut2 and Mut3 plants had tiller numbers that were significantly lower ($p < 0.001$) (appendix 15) than those of the N12 control plants in the untreated plot (Table 8), indicating sensitivity to the herbicide. In the plots sprayed with 625 g a.i. ha^{-1} imazapyr, the Mut1, Mut4, Mut5, Mut6 and Mut7 plants displayed tolerance to imazapyr compared with herbicide-sensitive Mut2, Mut3 and N12 control plants which died (Table 8). There were no significant differences amongst tiller number in the surviving mutants Mut1, Mut4, Mut5, Mut6 and Mut7 plants and the N12 control from the untreated plot (Table 8), indicating the potential commercial significance of those mutant lines.

The stalk height for the plants in the sprayed plots decreased significantly ($p < 0.001$) (appendix 15) with increased imazapyr concentration when compared with the untreated plot: excluding dead plants, the stalk heights were 121.2-139.0 in the untreated plot, 46.5-115.6 in the 312 g a.i.ha^{-1} plot, and 72.9-81.3 cm in the 625 g a.i. ha^{-1} plot (Table 8). Within the untreated plot, there were no significant differences in stalk heights amongst the mutants and N12 control. In the plot sprayed with 312 g a.i. ha^{-1} imazapyr, Mut6 and Mut7 plants had stalk heights that were significantly taller ($p < 0.001$) (appendix 15) than those of Mut2 and Mut3 plants. Mut2 plants were significantly shorter ($p < 0.001$) (appendix 15) than Mut1 plants (Table 8). A similar trend was observed when Mut2 and Mut3 plants were compared

with mutant and N12 control plants in the untreated plot. However, the Mut2 and Mut3 plants were 89.1 and 80.3 cm significantly shorter ($p < 0.001$) (appendix 15), respectively, than the untreated N12 control plants (Table 8). In the plot sprayed with 625 g a.i. ha^{-1} imazapyr, the Mut1, Mut4, Mut5, Mut6 and Mut7 plants survived but Mut2, Mut3 and N12 control plants died (Table 8). The Mut1, Mut4, Mut5 and Mut6 plants were significantly shorter ($p < 0.001$) (appendix 15) than the untreated N12 control (Table 8).

There was a significant decrease ($p < 0.001$) (appendix 15) in stalk diameter of the plants in the sprayed plots as the concentration of imazapyr increased (Table 8).The stalk diameter was 1.7-2.3 cm in the untreated plot, 1.3-2.1 cm in the 312 g a.i.ha^{-1} plot, and 1.8-2.1 cm in the 625 g a.i. ha^{-1} plot (dead plants excluded) (Table 8). Within the untreated plot, the differences in diameter amongst the mutants and the N12 control were not statistically significant. In the plot sprayed with 312 g a.i. ha^{-1} imazapyr, the Mut5, Mut6 and Mut7 plants had stalks that were significantly thicker ($p < 0.001$) (appendix 15) than those of Mut2 plants (Table 8), whilst the N12 control died. A similar trend was observed when Mut2 plants in the 312 g a.i. ha^{-1} treatment were compared with the plants (Mut1, Mut3, Mut4, Mut5, Mut6, Mut7 and N12 control) in the untreated plot, except for Mut2 (Table 8). In the treatment sprayed with 625 g a.i. ha^{-1} imazapyr, there were no significant differences in stalk diameter amongst the surviving Mut1, Mut4, Mut5, Mut6 and Mut7 plants. However, these plants, with the exception of Mut7, were significantly thinner ($p < 0.001$) (appendix 15) than the N12 control plants from the untreated plot. The Mut2, Mut3 and N12 control plants died in the 625 g a.i. ha^{-1} plot (Table 8).

The estimated yield in the sprayed plots decreased significantly ($p < 0.001$) (appendix 15) with an increase in imazapyr concentration (Table 8). The estimated yields were 42.5-74.3 kg/plot in the untreated plot, 2.7-52.3 kg/plot in the 312 g a.i.ha^{-1} plot, and 26.0-43.4 kg/plot in the 625 g a.i. ha^{-1} plot (dead plants excluded) (Table 8). Within the untreated plots, the differences in yield amongst the mutants (Mut1-Mut7) and N12 control plants were not statistically significant (Table 8). In the treatment sprayed with 312 g a.i. ha^{-1} imazapyr, the estimated yield of Mut5 plants was significantly higher ($p < 0.006$) (appendix 15) than that of Mut2 and Mut3 plants (Table 8). However, the estimated yields of Mut1, Mut3, Mut5 and N12 control plants from the untreated plot were significantly higher ($p < 0.006$) (appendix 15) than that of Mut2 and Mut3 plants in the 312 g a.i. ha^{-1} treatment (Table 8).

Table 8: An assessment of the agronomic traits and estimated yield from field-grown plants after 10 months. Two months after planting, imazapyr was applied at 312 and 625 g a.i. ha^{-1} leaving one plot untreated. Different alphabet characters indicate a statistical significance between each genotype and plot. Data was analysed using a Two-way ANOVA and Holm-Sidak test, P <0.05; n=3, mean ± SE (appendix 15).

Treatment	Genotype	Parameter measured			
		Tiller number/plot	Stalk height (cm)	Stalk diameter (cm)	Estimated yield (kg/plot)
Untreated	Mut1	133.67 ± 6.94^{d}	124.65 ± 11.38^{def}	2.21 ± 0.08^{c}	55.04 ± 11.83^{cd}
	Mut2	143.67 ± 21.94^{d}	123.39± 9.39^{def}	1.70 ± 0.11^{bc}	43.18 ± 12.63^{abcd}
	Mut3	133.67 ± 3.84^{d}	138.97 ± 5.23^{f}	2.26 ± 0.05^{c}	59.67 ± 2.60^{cd}
	Mut4	126.67 ± 12.99^{d}	126.93 ± 2.41^{def}	1.96 ± 0.15^{c}	50.16 ± 11.60^{bcd}
	Mut5	160.00 ± 20.00^{d}	136.45 ± 7.88^{ef}	2.10 ± 0.10^{c}	74.32 ± 6.58^{d}
	Mut6	110.33 ± 10.27^{cd}	121.15 ± 11.49^{def}	1.89 ± 0.12^{c}	51.16 ± 6.36^{cd}
	Mut7	109.33 ± 11.20^{cd}	123.08 ± 10.69^{def}	1.90 ± 0.11^{c}	42.48 ± 1.12^{abcd}
	N12	128.67 ± 5.61^{d}	135.88 ± 3.06^{ef}	1.97 ± 0.04^{c}	53.61 ± 4.51^{cd}
Treatment mean		130.75^{A}	128.81^{A}	1.95^{A}	53.70^{A}
312 g a.i. ha^{-1}	Mut1	105.67 ± 12.68^{cd}	110.88 ± 11.49^{cdef}	1.71 ± 0.06^{bc}	27.53 ± 6.90^{abc}
	Mut2	28.00 ± 16.17^{bc}	46.47 ± 13.69^{b}	1.30 ± 0.10^{b}	4.20 ± 1.72^{ab}
	Mut3	11.00 ± 11.00^{b}	55.58 ± 14.04^{bc}	1.57 ± 0.13^{bc}	2.65 ± 0.00^{a}
	Mut4	97.33 ± 17.02^{bcd}	92.28 ± 9.05^{bcdef}	1.67 ± 0.03^{bc}	20.42 ± 5.61^{abc}
	Mut5	142.67 ± 27.63^{d}	110.90 ± 15.78^{cdef}	2.07 ± 0.07^{c}	52.33 ± 12.24^{cd}
	Mut6	119.33 ± 8.11^{d}	112.45 ± 1.07^{def}	2.05 ± 0.10^{c}	43.97 ± 1.07^{abcd}
	Mut7	120.67 ± 9.13^{d}	115.62 ± 10.41^{def}	1.94 ± 0.03^{c}	41.43 ± 5.60^{abcd}
	N12	-	-	-	-
Treatment mean		78.08^{B}	86.71^{B}	1.75^{B}	21.82^{B}
625 g a.i. ha^{-1}	Mut1	112.33 ± 19.43^{cd}	79.02 ± 15.25^{cd}	1.78 ± 0.14^{bc}	26.01 ± 11.13^{abc}
	Mut2	-	-	-	-
	Mut3	-	-	-	-
	Mut4	150.00 ± 10.26^{d}	77.58 ± 14.59^{bcd}	1.86 ± 0.15^{c}	41.60 ± 13.85^{abcd}
	Mut5	117.00 ± 34.60^{d}	72.87 ± 13.73^{bcd}	1.78 ± 0.16^{bc}	27.21 ± 12.83^{abc}
	Mut6	129.00 ± 11.24^{d}	77.58 ± 14.59^{bcd}	2.06 ± 0.10^{c}	43.44 ± 8.08^{abcd}
	Mut7	139.00 ± 22.81^{d}	81.30 ± 13.03^{bcde}	1.93 ± 0.11^{c}	36.30 ± 15.26^{abcd}
	N12	-	-	-	-
Treatment mean		80.92^{B}	48.54^{C}	1.18^{C}	24.07^{C}

- Plants died after herbicide application
Treatment mean with capital letters is used to compare differences between treatments
Underlined are N12 control values used for comparative purposes.

The Mut4 plants in the untreated plot displayed yields that were significantly higher (p < 0.006) (appendix 15) than those of Mut2 plants from the 312 g a.i. ha^{-1} plot (Table 8). The N12 control plants died (Table 8). When the concentration of imazapyr was increased to 625 g a.i. ha^{-1}, there were no significant differences in yield amongst the surviving mutant plants (Mut1, Mut4, Mut5, Mut6 and Mut7) and the untreated N12 control, indicating the potential commercial significance of those mutant lines. The Mut2, Mut3 and N12 control plants died (Table 8).

Based on the agronomic traits, the Mut1, Mut4, Mut5, Mut6 and Mut7 plants were not severely affected by imazapyr and they survived in all the treated plots. This indicated that these plants were more tolerant to the herbicide than the N12 control plants which died. Amongst the herbicide-tolerant plants, Mut5, Mut6 and Mut7 displayed the best traits (number of tillers, height and diameter) and yield than the plants of the other mutants in the untreated, 312 and 625 g a.i. ha^{-1} treatments. Further, those plants were significantly better (p < 0.001) (appendix 15) than the Mut2 and Mut3 plants in the 312 g a.i. ha^{-1} plot. However, Mut5 did not perform very well in the higher dose plot (625 g a.i. ha^{-1} imazapyr) when compared with Mut4, Mut6 and Mut7. The Mut2, Mut3 and N12 control plants were severely affected by the herbicide in the 312 g a.i. ha^{-1} plot, as they displayed a reduction in agronomic traits and yield. The herbicide-sensitive Mut2, Mut3 and N12 control plants died when imazapyr concentration was increased to 625 g a.i. ha^{-1}.

4.4 Assessment of the stability of imazapyr tolerance in plants arising from setts planted in soil treated with imazapyr

Imazapyr is conventionally applied to a fallow field 3-4 months prior to planting sugarcane as there is residual herbicide activity in the soil that suppresses sugarcane sett 'germination' and growth. Hence, in order to establish if setts of the herbicide-tolerant mutants were able to germinate in a field recently sprayed with imazapyr, 3-budded setts of the mutant lines (Mut1-Mut7) and N12 control were planted in each of two plots: unsprayed and sprayed with 1254 g a.i. ha^{-1} imazapyr, 2 weeks prior to planting. Germination in the sprayed plot (11.3-73.6%) was calculated as a percentage of control (untreated plot). However, no stats were performed because there were not enough setts and space for planting more than one line. The setts from Mut1, Mut4 and Mut6 showed higher germination percentages (60.3, 71.0 and 73.7%, respectively) than those from Mut2, Mut3, Mut5, Mut7 and N12 control (23.0, 46.3, 33.3 and 11.3%, respectively) (Fig. 15). The plants Mut2 and N12 had the lowest germination percentages indicating susceptibility to the herbicide.

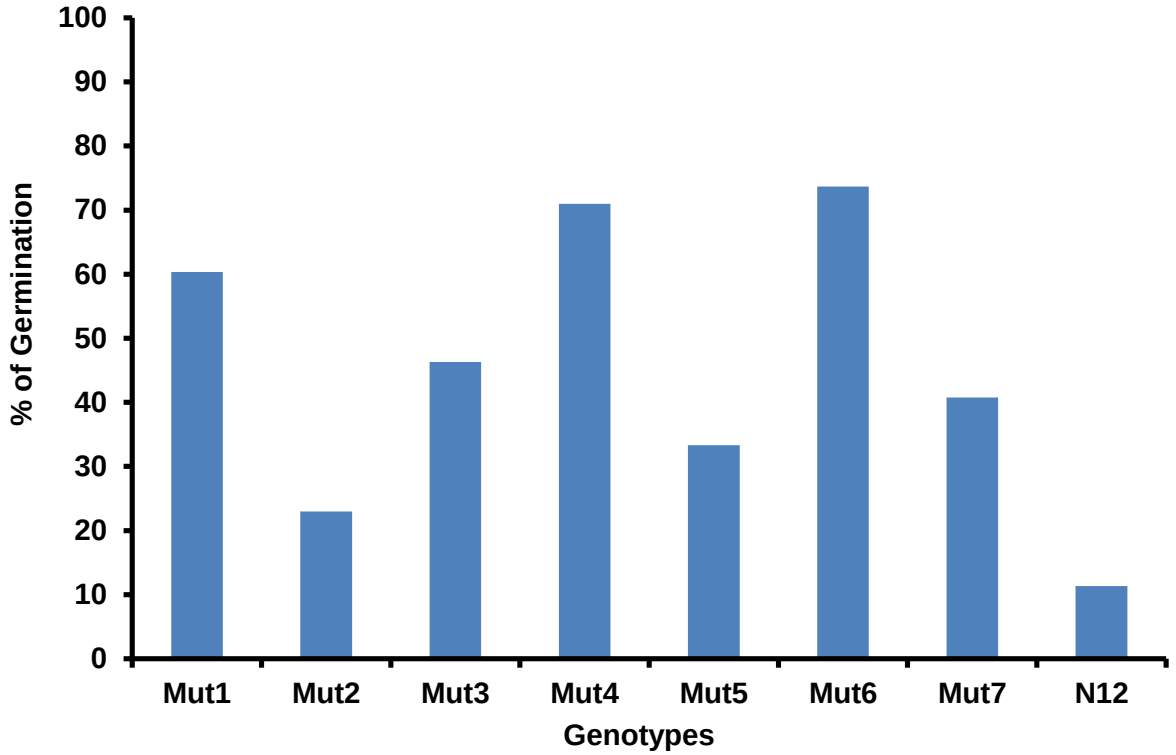


Fig. 15. Comparison of sett germination between the mutants (Mut1-Mut7) and N12 control. The field was sprayed with imazapyr (1254 g a.i. ha^{-1}) 3 weeks prior to planting. Plants were assessed for germination 3 weeks after planting of 3-budded setts. Germination of plants in the sprayed plot was expressed as percentage of germination observed in the unsprayed plot.

The effect of imazapyr on plant shoot length over time was also evaluated (Fig. 16). The percentage shoot length in the plot sprayed with 1254 g a.i. ha^{-1} imazapyr was calculated as the shoot length in each sprayed plot/shoot length in unsprayed plot x 100. The calculated percentage shoot length for each line was then averaged. In the first 4 weeks after germination, the mean percentage shoot length of all the mutants (Mut1-Mut7) and of the N12 control were significantly higher ($p< 0.001$) (appendix 16) than at 8 and 12 weeks after herbicide application (Fig. 16). However, Mut1 and Mut6 showed no significant differences between weeks 4 and 12. At week 4, the mean shoot lengths of Mut4 and Mut6 were significantly higher ($p< 0.001$) (appendix 17) than those of Mut2, Mut3, with the N12 control plants having the lowest mean shoot length (Fig. 16). However, the shoot length of Mut4 and Mut6 were not statistically significant to those of Mut5 and Mut7 (Fig. 16).

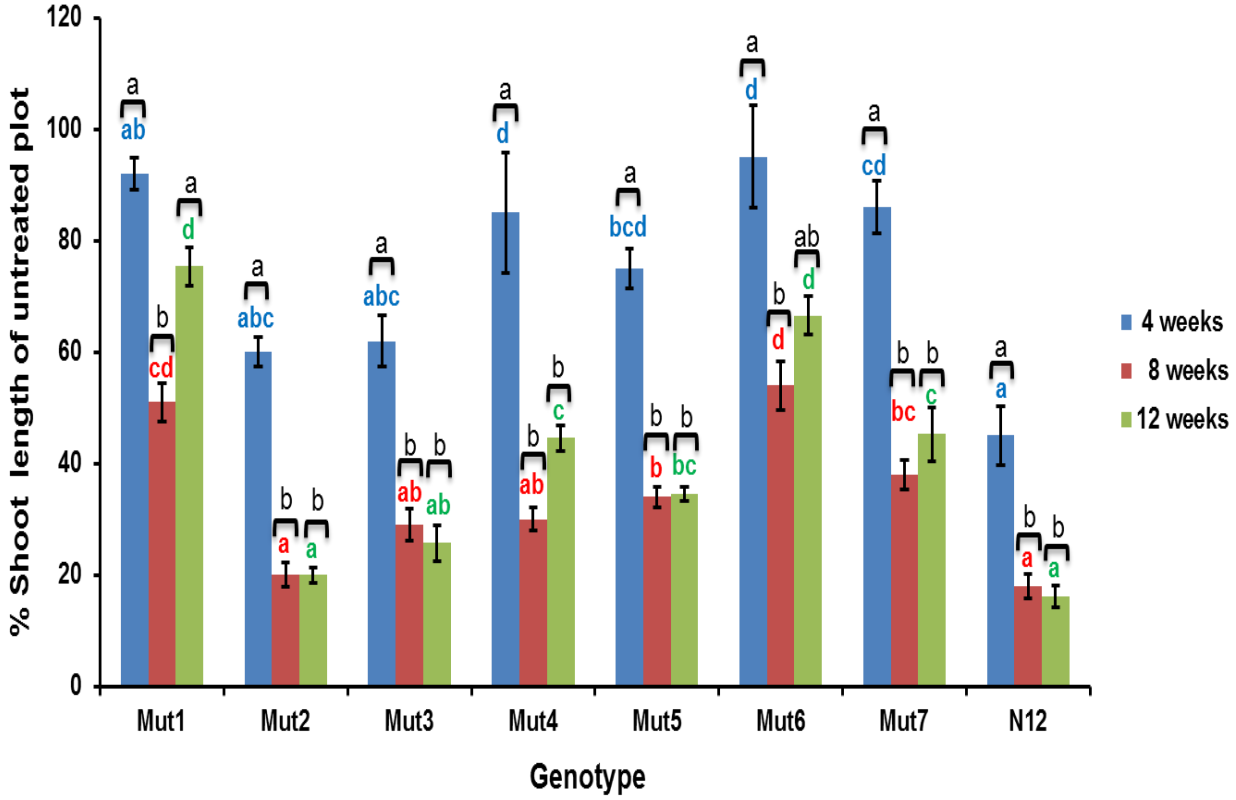


Fig. 16. The effect of imazapyr on shoot length in the germinating setts of plants Mut1-Mut7 and N12 control. The field was sprayed with 1254 g a.i. ha^{-1}, 3 weeks before planting. Shoot length was recorded 4, 8 and 12 weeks after planting**.** Shoot length percentages in the sprayed plot were calculated as percentages of shoot lengths observed in the untreated plot. Different black alphabet characters indicate a statistical significance between each week and are limited to each genotype. Different colour coded alphabet characters indicate a statistical significance between each genotype and are limited to each week. (One-way Anova and Holm-Sidak test, *P* <0.001; n=10, mean ± SE).

At 8 weeks, the percentage shoot length of plants in the treated plot was lower than the shoot length of the corresponding plants in the untreated plot. The mean percentage shoot length of Mut6 plants was significantly higher ($p< 0.001$) (appendix 17) than those of the Mut2, Mut3, Mut4, Mut5 and the N12 control, but was similar to that of Mut1. The mutant Mut3 displayed the highest mean shoot length percentage when compared to Mut2 and N12 control. In week 12, Mut1 and Mut6 had the highest average shoot length and were significantly taller ($p< 0.001$) (appendix 17) than the rest of the mutants and N12 control (Fig. 16 and Fig. 17). The mutants Mut4 and Mut5 were also significantly taller ($p< 0.001$) (appendix 17) than Mut2 and N12 control (Fig. 16 and Fig. 17). However, there were no statistically significant differences with respect to shoot length between Mut4 and Mut5. All herbicide-sensitive plants Mut2, Mut3 and N12 control showed stunting of growth compared with the mutants Mut1, Mut4, Mut5, Mut6 and Mut7 (Fig. 17). However, these differences were not statistically significant.

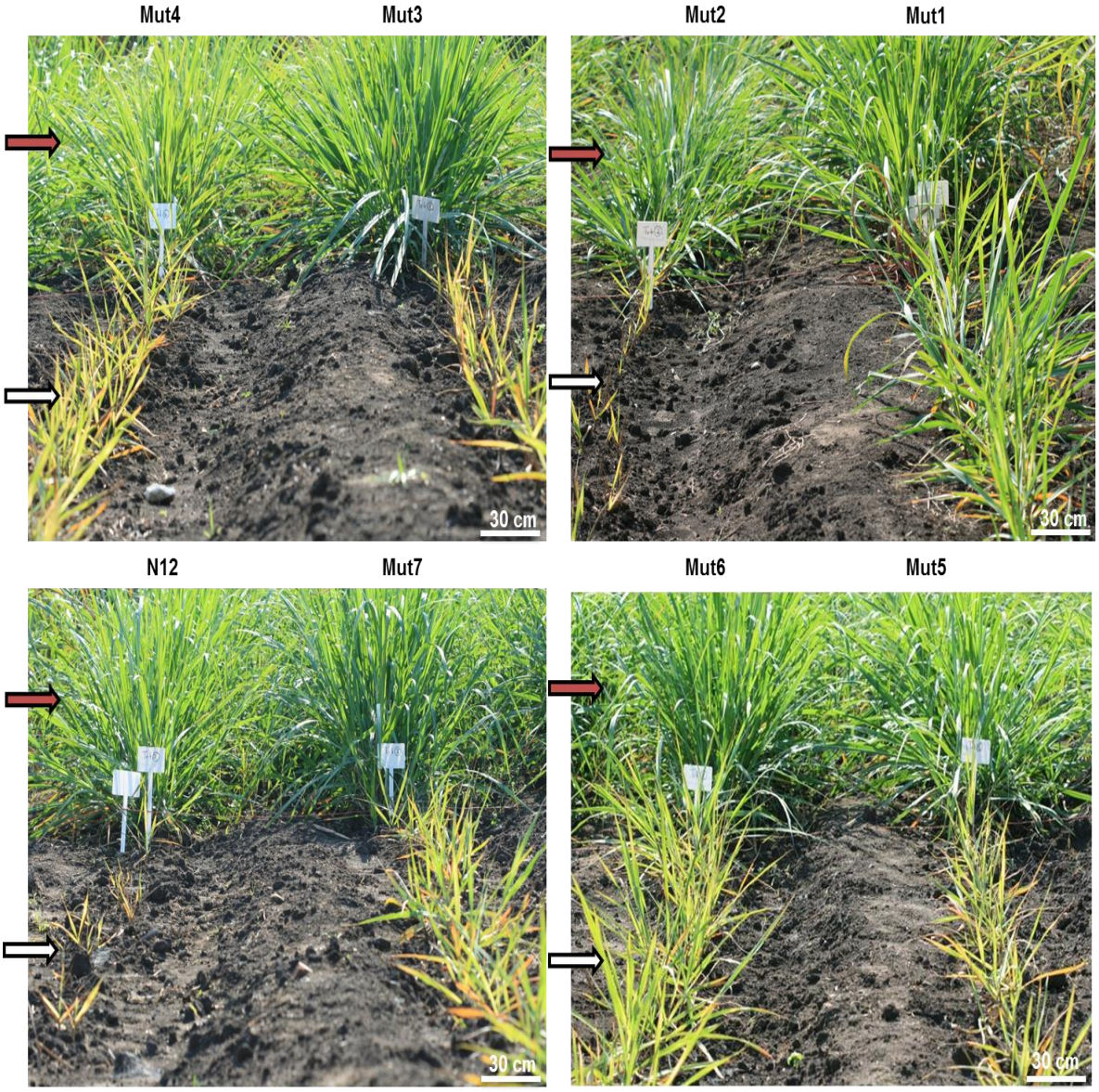


Fig. 17. Field assessment of growth response in Mut1-Mut7 and N12 control in a field sprayed with imazapyr 3 weeks prior to planting. The red arrows indicate plants appearance in the untreated control plot. White arrows show plants in the plot treated with 1254 g a.i. ha^{-1} imazapyr. Plants were assessed for visual injury 12 weeks after planting.

5. Discussion

5.1 Identification of imazapyr-tolerant mutant lines using field evaluation

The use of plants generated by *in vitro* culture techniques and induced mutagenesis have been an important resource in plant breeding (van Harten, 1998) and subsequently in plant genomic research studies (Henikoff *et al*., 2004). Various studies have reported deriving tolerant crops from tissue culture selection, such as imidazolinone-tolerant sugarbeet cells generated using somatic cell selections (Wright and Penner 1998a, c), soybean cells tolerant to protox-inhibiting herbicides (Pornprom *et al*., 1994; Warabi *et al*., 2001) and to glufosinate ammonium (Pornprom *et al*., 2000), rice cells tolerant to cyhalofopbutyl (Bae *et al*., 2002) and a glyphosate-tolerant sugarcane cellular line (Zambrano *et al*., 2003). Similarly, in a previous study at SASRI, seven putatively imazapyr-tolerant sugarcane mutant plants (Mut1-Mut7) were generated from the variety N12 by *in vitro* exposure of embryogenic callus to 16 mM EMS, followed by selection on imazapyr-containing medium (Koch *et al*., 2010). Those plants were then clonally propagated *in vitro* according to Meyer *et al*. (2007) and planted in the field for the current study.

Field trials play a vital role in the identification of important specific agronomic traits of interest expressed by any new pre-released varieties, as well as for mutated plants. This evaluation also ensures that the plants retain the original characters of the parent plant or indicate if they have been altered in a positive way (Rutherford *et al*., 2014). Previous work to evaluate *in vitro*-derived mutagenic plants in the sugar industry has focused mainly on the use of field trials for the identification of plants resistant to the fungal diseases including red rot, smut, brown rust, stalk rot, and sugar mosaic virus in the presence or absence of the mutagenic treatments (Rutherford *et al*., 2014). On the other hand, although traits obtained *in vitro* through mutations conferring tolerance to salt (Gandonou *et al*., 2005; Gandonou *et al*., 2006) and herbicides glyphosate (Zambrano *et al*., 2003) and imazapyr (Punyadee *et al*., 2007; Koch *et al*., 2012; Adriano *et al*., 2013; Munsamy *et al*., 2013) have been reported, there is little published work on field trials of such mutants. This could be due to the multiple years required for sugarcane field evaluation and unstable epigenetic nature of such mutants (Rutherford *et al*., 2014).

Tolerance to imidazolinone herbicides with altered ALS genes and enzymes for weed control has been developed in many crops including maize (Anderson and Georgeson, 1989), wheat (Newhouse *et al*., 1992), oil seed rape (Anderson and Georgeson, 1989), sunflower (Brighenti *et al*., 2011; Sal *et al*., 2012) and sugarcane (Koch *et al*., 2012). Such tolerant crops have the potential to rapidly metabolize the herbicide to nontoxic compounds. However, weeds and herbicide-sensitive crops are unable to do the same, or do so more

slowly (Brown, 1990; Wright and Panner, 1998b). The generation of imidazolinone-tolerant rice, best known as Clearfield® varieties, have had success in selectively controlling red rice (Masson and Webster, 2001), and indicates that it is possible to develop crops that are tolerant to imidazolinones based on the resistance at the site of action caused by the mutation of the ALS gene for these crops. Consequently, this mutagenic approach was taken by Koch *et al*. (2012) to generate the seven putative imizapyr-tolerant sugarcane plants that were evaluated for imazapyr tolerance in the field in the current study.

Sugarcane plots sprayed with 312 and 625 g a.i. ha^{-1} imazapyr were assessed by visually monitoring the change in leaf colour at 0, 1, 6 and 12 weeks. Owen (2013) stated that symptoms (e.g. chlorosis and nicrosis) in plants normally show at 1-4 weeks after herbicide application, depending upon the herbicide applied, dose, type of plant species and environmental conditions. Similar symptoms have been observed in crops such as barley, corn, spring wheat, winter wheat, durum wheat, peanuts, rice, soybeans and sugarcane where imidazolinone herbicides have been used (Punyadee *et al*., 2007). A study by Adriano *et al.* (2013) reported initial yellowing of the sugarcane leaves that later evolved into necrosis and total death of plants, especially in plants treated with higher dosages (2880, 3600 and 4320 g a.i. ha^{-1}) of glyphosate, indicating susceptibility to herbicide.

Visual assessment of the leaves of Mut1, Mut4, Mut5, Mut6 and Mut7 plants (Fig. 9 and Fig. 10) indicated herbicide tolerance as they remained green after the herbicide application. In contrast, the Mut2, Mut3 and the N12 control plants were regarded as herbicide-sensitive as they showed little growth, chlorotic and necrotic symptoms of their leaves, and eventually whole plant necrosis (Fig. 9 and Fig. 10). These symptoms were evident 6 weeks after foliar spray with imazapyr and at week 16 the plants died (data not shown). These data supports studies in the early 1980s that reported that ALS-sensitive plants treated with imidazolinone herbicides displayed stunted growth, chlorosis and eventually necrosis (Ray, 1984; Scheel and Casida, 1985). This is because imidazolinone herbicides can inhibit the ALS activity in herbicide-sensitive plants resulting in plant death (Ray, 1984; Scheel and Casida, 1985).

In the current study, the loss of leaf ‘greenness’ or chlorophyll content over time was further investigated in Mut1, Mut6 and the N12 control plants by taking SPAD measurements (Fig. 11). Chlorophyll loss is associated with adverse environmental conditions and is a good indicator of stress in plants (Hendry and Price, 1993). The SPAD results indicated a slight decrease in chlorophyll content at weeks 1 and 3 for Mut1, Mut6 and N12 control plants in the unsprayed plot (Fig. 11a). This could be due to environment factors at the time of measurements or due to imazapyr drift, as it has been reported that this herbicide can affect

crops even at small amounts (Cox, 1996). However, all plants in the treated plots displayed a reduction in chlorophyll content over time, especially 6 weeks after herbicide application (Fig. 11b and c). These results were similar to those reported by Adriano *et al*. (2013) on sugarcane cultivars treated with glyphosate. The loss of chlorophyll content without other apparent damages indicated that the sensitive mutant and N12 control plants experienced herbicide-induced stress. However, the N12 control plants that were not mutated were affected negatively by imazapyr and were regarded as herbicide-sensitive because low dosages of imazapyr were sufficient to reduce chlorophyll content in their leaves, chlorotic symptoms were observed and no recovery was apparent (Fig. 11b).

The effect of imazapyr on chlorophyll content was also tested by Spencer *et al*. (2009) on giant reed (*Arundo donax*), also known as giant cane. Their results showed reduced leaf chlorophyll content in less than 30 days after herbicide application, but the plants recovered in the following spring. In soybean plants, SPAD measurements were performed by Zobiole *et al*. (2010) to estimate chlorophyll content levels. They reported a reduction in chlorophyll content after herbicide application even in cultivars that were known to be glyphosate-tolerant. SPAD measurements are, therefore, useful when attempting to 'quantify' levels of stress and/or recovery between mutant lines, as was the case in the present study.

As previously mentioned, to date, studies examining the response of sugarcane cultivars to imazapyr relied mainly on phenotypic observations such as tiller number, stalk height, stalk diameter and mass, etc. (Punyadee *et al*., 2007; Koch *et al*., 2012; Adriano *et al*., 2013; Munsamy *et al*., 2013). However, these agronomic traits are influenced by environmental factors and the evaluation period is long (Souza *et al*., 2009; Zera *et al*., 2011), during which time the environmental factors vary substantially. Biochemical alterations can also occur concurrently with the phenotypic alterations that occur in response to herbicide application (Adriano *et al*., 2013).

In this study, herbicide-tolerant mutants and N12 un-mutated control plants were compared with respect to phenotypic characterisitics such as tiller number, stalk height and diameter (Table 8). Comparisons were made amongst plants in the untreated and imazapyr treated plots (312 and 625 g a.i. ha^{-1}) and amongst control and Mut plants in all treatments (Table 8). In the untreated plot, there was a lot of variation observed in the Mut2 and Mut5 plants for the tiller number parameter compared with the other genotypes (Mut1, Mut3, Mut4, Mut6, Mut7 and N12 control) (Table 8). In the plot treated with 312 g a.i. ha^{-1} imazapyr, Mut2, Mut4 and Mut5 displayed much variation in tiller number compared with the other mutant lines (Mut1, Mut3, Mut6 and Mut7) (Table 8). In the 625 g a.i. ha^{-1} plot, Mut1, Mut5 and Mut7 also

showed more variation for the number of tillers than that observed in other mutants (Mut2, Mut3, Mut4 and Mut6) (Table 8). Variations in height measurements were also observed across all plants in the untreated and treated plots, but to a lesser degree than tiller number (Table 8).

In the untreated plot, tiller number and height parameters amongst the genotypes were within the 'normal' range expected for N12 and showed no statistical significant differences, indicating that there were no negative changes in these traits that occurred concurrently with the ALS gene mutation that resulted in imazapyr tolerance (Table 8). However, positive changes were observed for the Mut1, Mut2 and Mut5 plants because they produced more tillers than the N12 parent control plants (Table 8). The changes could be the result of initial *in vitro* culture of the plants. It is known that tissue culture plants produce more tillers in plant cane, but in the first ratoon crop, this positive change is no longer observed. In addition, Mut3 and Mut5 plants were taller than the N12 control plants (Table 8). A similar trend was observed for the stalk diameter parameter for Mut1, Mut3 and Mut5 plants (Table 8).

With respect to the responses that indicated sensitivity to the imazapyr treatment, as expected, the N12 control plants in both spay treatments died (Table 8) confirming that the variety N12 is sensitive to imazapyr at 312 and 625 g a.i. ha^{-1}. Of the putative-mutant lines, Mut2 and Mut3 displayed significantly reduced morphological parameters (tiller number, stalk height and stalk diameter), compared with those in the untreated plot, in response to both 312 and 625 g a.i. ha^{-1} (Table 8). This response was, therefore, regarded as being due to susceptibility of the Mut2 and Mut3 plants to imazapyr. These results were similar to those reported by Punyadee *et al*. (2007) in herbicide-sensitive sugarcane clones which showed a stunted growth with significantly shorter stalks compared with imazapyr-tolerant clones. Their study also showed that treatment of sugarcane clones with 156, 312 and 625 g a.i. ha^{-1} imazapyr, significantly affected the relative number of stalks per hectare, which ranged from 48.15% to 80.83%. Those authors suggested that increasing the concentration of imazapyr from 156 to 625 g a.i. ha^{-1} would result in an increased visual injury and a significant decrease in relative plant height and relative stalk number per hectare. A study by Newhouse *et al*. (1992) on herbicide-sensitive wheat also showed decreased plant height and grain yields after treatment with 300 g a.i. ha^{-1} imazethapyr compared with plants in the untreated control. Similar results were reported by Wiatrak *et al*. (2009) and Grey *et al*. (2005) in cotton

[1]Campbell. S.A., 2013 South African Sugarcane Research Institute (SASRI), Private Bag X02, Mount Edgecombe, Durban, 4300, South Africa

and Matocha *et al*. (2003) in peanut. Those studies on imazethapyr are appropriate for comparisons with the effect of imazapyr used in the current study because both herbicides have the same mode of action and belong to the same herbicide group (imidazolinone).

The observations that Mut1, Mut4, Mut5, Mut6 and Mut7 plants survived and exhibited significantly better agronomic traits than Mut2, Mut3 and N12 plants indicated that the former were tolerant and the latter sensitive to imazapyr (Table 8). Further, the measured agronomic traits and yields of the most imazapyr-tolerant Mut5, Mut6 and Mut7 plants in the treated and untreated plots were the same as those of N12 control plants in the untreated plot. James *et al*. (2001) reported similar results on maize tolerant to imazethapyr and imazapyr. They inspected the crops for signs of injury and found that there were no reduced stalk height and diameter after herbicide application. Newhouse *et al*. (1992) also reported similar results in imazethapyr-tolerant wheat.

As previously mentioned, one of the effects of spraying with ALS-inhibiting herbicides such as imazapyr, is the soil residual activity that can result in weed control throughout the growing season (Corbucci *et al*., 1998). However, this can also result in crop damage and economic loss due to its phytotoxic effect on herbicide-sensitive crops (Corbucci *et al*., 1998). Conventionally, soil treatment with imazapyr is performed 3-4 months prior to planting sugarcane because it has been reported that the residual activity of the herbicide suppresses sugarcane sett germination and growth ([1]Campbell, 2013 pers. comm.). The degree to which the herbicide can persist in the soil and cause crop damage depends on factors such as soil properties (pH), environmental conditions between time of application and time of planting of crops and sensitivity of the crops planted (Ayeni *et al*., 1998; Krieger *et al*., 2000; Moyer and Hamman, 2001; Schoenau *et al*., 2005). In this regard, sugarbeet (*Beta vulgaris* L.) has shown that sensitivity to imadazolinone herbicides and injury (e.g. chlorosis, necrosis and stunted growth) can occur at low soil pH (Renner *et al*., 1991). Corbucci *et al*. (1998) also reported that soil pH can affect the response of crops such as sugarbeet and canola (*Brassica napus* L.) to imazamox and imazethapyr. Studies by Bresnahan *et al*. (2000) and Bresnahan *et al*. (2002) showed that the response of sugarbeet and canola to the same residue levels of these herbicides was greater when the soil pH was below 6. They concluded that such imadazolinone herbicide's bioavailability increases with the decrease of pH. Low temperatures can also delay the degradation process and increase the potential of injury to herbicide-sensitive crops. In addition, it is now known that, if not sufficiently degraded in the soil between time of application and planting, imadazolinone herbicides can cause damage to sensitive crops due to reduced microbial degradation of the herbicide (Shaner and Hornford, 2005).

In this study, the levels of imazapyr tolerance in the seven mutant lines were further evaluated by investigating sett germination and shoot length when planting 'sugarcane setts' in a fallow field previously treated with 1254 g a.i. ha^{-1} imazapyr to prevent weed growth. Germination and subsequent growth of the setts of Mut1, Mut4, and Mut6 and Mut7 tolerant lines was similar in the treated and untreated plots. Further, the tolerant mutants displayed better germination and subsequent growth than the herbicide-sensitive Mut2, Mut3 and N12 control plants (Fig. 15 and Fig. 16). In comparison, the herbicide-sensitive Mut2, Mut3 and N12 control plants displayed yellow-red leaves with stunted growth in the imazapyr-treated plots (Fig. 16 and Fig. 17). Similar symptoms in sunflower, including reddening of the stem and leaves, stunted growth, dark green colouration of leaves and a less dense secondary root system have been reported by Alonso-Prados *et al*. (2002) for the effect of 20 and 40 g a.i. ha^{-1} sulfosulfuron imidazolinone herbicide on susceptible genotypes.

Even though, in the present study, Mut1, Mut4, Mut6 and Mut7 displayed tolerance to the residual effect of imazapyr at weeks 8 and 12, their growth was significantly reduced by the herbicide as observed for the herbicide-sensitive Mut2 and Mut3 plants (Fig. 16 and Fig. 17). Further, they did not recover in the subsequent weeks (data not shown), indicating that they were also affected by the residual effect of the imazapyr herbicide. The height of Mut2, Mut3 and N12 control plants in weeks 8 and 12 was approximately half of that recorded in week 4 (Fig. 16), and in the subsequent weeks they remained stunted and did not recover. This prolonged persistence of the herbicide and consequent sugarcane damage may have been influenced by the winter season (when data were collected) as imadazolinone activity depends on environmental conditions (Ayeni *et al*., 1998; Krieger *et al*., 2000; Moyer and Hamman, 2001). These results supported the findings of previous studies which stated that imazapyr has a long residual activity in soil. Alister and Kogan (2005) reported that the phytotoxic effect caused by soil residual-herbicides such as imazapyr combined with either imazapic or imazethapyr can last more than a year after application resulting in reduced yields of oats, barley, pea, alfalfa, sugarbeet, chili, tomato and cantaloupe. Shinn *et al.* (1998) reported injury to barley, peas and canola a year after sulfosulfuron was applied.

In conclusion, of all of the tested mutants Mut1, Mut4, Mut5, Mut6 and Mut7 were regarded as tolerant to imazapyr, due to the following results: 1) significantly better agronomic traits (tiller number, stalk height and diameter) and yield in the treated plots (Fig 9, Fig. 10 and Table 8) compared with the N12 control; 2) better germination and growth (Fig 15, Fig 16 and Fig 17) than N12 control on a fallow field previously treated with imazapyr. The Mut2, Mut3 and N12 plants were sensitive in all experiments that involved treatment with imazapyr (Fig. 9, Fig.10, Fig. 15, Fig 16, Fig. 17 and Table 8).

5.2 Confirmation of tolerance by *in vitro* ALS activity levels

There are several reports on increased ALS activity in various weed biotypes (Boutsalis *et al*. , 1999), mutant cell lines (Chang and Duggleby, 1998; Purrington and Bergelson, 1999), transgenic plants (Purrington and Bergelson, 1999) and in yeasts (Duggleby *et al*., 2003). In the current study, the *in vitro* ALS activity was assayed to assess the response of the mutant plants (Mut1-Mut7) to different imazapyr concentrations (0-30 µM) (Fig. 13), using leaf material of plants collected from the untreated plot. The ALS activity in the herbicide-treated mutant and N12 control plants decreased with the increase in imazapyr concentration (Fig. 13). Studies by Ray (1984), Sebastian *et al*. (1989), Stidham and Singh (1991) and Simpson *et al*. (1995) reported similar results. Simpson *et al*. (1995) stated that a reduction in metabolism or an increase in herbicide absorption may be due to the higher concentration of the herbicide that accumulates at the target site of the ALS enzyme and thus increases inhibition.

The *in vitro* ALS activity in the plants with supplied imazapyr was also assessed. There was a decrease in the total ALS activity of the mutants and N12 control when the concentration of imazapyr was increased from 0-30 µM (Fig. 13), indicating susceptibility of ALS to higher concentrations of the herbicide. Newhouse *et al*. (1992) reported similar results on wheat. Consequently, the ALS activity of the mutants and N12 control plants based on the IC_{50} values were also evaluated (Fig. 14). The ALS activities in the tolerant mutant plants Mut1, Mut4, Mut5, Mut6 and Mut7 were 5.2, 6.3, 22.0, 30.0 and 8.4 µM respectively, which was approximately 1.5-8.9-fold greater than that of the herbicide-sensitive Mut2, Mut3 and N12 control plants (Fig. 14). These results indicated that the target site of ALS in these plants was less sensitive to imazapyr than that of the Mut2, Mut3 and N12 control plants. This tolerance was probably due to the mutation of the ALS gene. Punyadee *et al*. (2007) reported ALS IC_{50} values in tolerant sugarcane cells that were 6.5 times that of the herbicide-sensitive cells. Koch *et al.* (2012) also found that tolerant sugarcane plants generated from the previous study had IC_{50} values that were between 2.8-4.8 times that of sensitive plants. The levels found in this study are similar. In addition, the rate of ALS activity of 2 month-old Mut1-Mut7 and N12 control plants from the untreated control plot was determined and compared amongst the plants. The Mut6 and Mut7 plants displayed the highest rate of ALS activities, which were approximately 1.4-1.5 times greater than those recorded for the N12 control and the other mutant lines, indicating rapid production of the final product acetoin (Table 7).

In addition to the IC_{50} results, a more detailed investigation was done by determining the *in vitro* ALS activity of 5 month-old Mut1, Mut6 plants and N12 control plants at 1, 3, 6 and 12 weeks after imazapyr application. Comparisons were performed across treatments and between genotypes within each plot (sprayed 312 and 625 g a.i. ha^{-1} and unsprayed plots). The ALS activities were also compared over time for each genotype within each plot. There was a decrease in ALS activity at weeks 1, 3 and 6 for Mut1, Mut6 and N12 control plants in the unsprayed plot (Fig. 12a). Muhitch (1988) and Duggleby and Pang (2000) reported that the ALS enzyme assay is a very sensitive assay and allows the measurement of small enzyme activities. The possibility of rapid loss of ALS activity from plant tissue extracts during *in vitro* assays is because ALS occurs in low amounts in its natural sources (Muhitch, 1988; Duggleby and Pang, 2000). However, in this case there was recovery at week 12 for Mut1 and Mut6 (Fig. 12a), indicating that the decrease in ALS activity in the assay was not caused by the low amounts of ALS. This trend was similar to that of measured chlorophyll content indicating that ALS activity is reduced with chlorophyll content. Consequently, possible reasons for the observations include the effect of environmental factors at the time of measurement or imazapyr drift. Recovery of all the mutants indicated herbicide tolerance, likley due to overexpression of ALS or metabolic detoxification of the 'drifted' herbicide by the enzyme.

The Mut1, Mut6 and N12 control plants in the treated plots displayed a significantly reduced ALS activity over time, especially at 6 weeks after herbicide application, compared with those in the untreated plot (Fig. 12a, b and c). The ALS activity of the plants in the higher dose treated plot (625 g a.i. ha^{-1}), was significantly reduced over time compared with that of plants in the 312 g a.i. ha^{-1} plot (Fig. 12b and c), indicating more sensitivity to the high levels of the herbicide. These results support previous studies that reported that when plants were treated with an imidazolinone herbicide and ALS was then extracted from them and measured *in vitro*, there was a reduction in the extractable ALS activity when compared to that of the untreated plants (Muhitch *et al*., 1987). According to that author, the reduction is specific for ALS and is not a result of general loss in enzymatic activity caused by the herbicide. Hawkes (1989) speculated that the loss of ALS activity in plants treated with imidazolinones is caused by the formation of an enzyme/inhibitor complex that destabilizes the enzyme leading to its degradation.

At week 12 in both dosage treatments, the ALS activities of mutant plants increased indicating recovery and tolerance of these plants, which may have been the result of overproduction of ALS at the target site, or a mutation in the ALS gene or a metabolic detoxification of the herbicide (Fig. 12b and c) (Tan *et al*., 2005). Sweetser *et al*. (1982)

reported that plants that are tolerant due to metabolic detoxification of the herbicide recovered over time, and this could be an explanation for observations with Mut1 and Mut6 in the present study (Fig12b and c). The N12 control plants were regarded as herbicide-sensitive as they did not survive the negative effects of imazapyr even at a low concentration and did not recover over time (Fig. 12b). The ALS activity for both treatments in the N12 control plants was significantly reduced compared with that of the Mut1 and Mut6 plants (Fig. 12b and c). These results suggest that the higher ALS activity in imazapyr-tolerant mutant plants (Mut1 and Mut6) was due to either metabolic detoxification of the herbicide or less sensitivity to imazapyr conferring the resistance mechanism (Punyadee *et al*., 2007). Similar results were reported earlier in canola (Swanson *et al*., 1989), soybean (Sebastian *et al*., 1989), maize (Newhouse *et al*., 1991; Bailey and Wilcut, 2003), wheat (Newhouse *et al*., 1992), cotton (Rajasekaran *et al*., 1996), sugarbeet (Wright and Penner, 1998c), rice (Bae *et al*., 2002), and sugarcane (Punyadee *et al*., 2007; Koch *et al*., 2012).

In summary, based on the significantly higher ALS activity of plants in the imazapyr-treated plots, Mut1, Mut4, Mut5, Mut6 and Mut7 are considered to be imazapyr-tolerant and Mut2, Mut3 and N12 control are imazapyr-sensitive (Fig. 12, Fig. 13 and Fig. 14). Based on the results, the herbicide tolerance observed in Mut4, Mut5, Mut6 and Mut7 was possibly due to a mutation of the ALS gene conferring tolerance as they displayed higher IC_{50} values than Mut1, Mut2, Mut3 and N12 control plants.

5.3 Concluding remarks and future work

Of the seven tested mutant plants, the field trial results gave no evidence of negative effects on general plant phenotype due to the EMS mutagenesis treatment as, in general, there were no significant differences in agronomic parameters when compared with the un-mutated N12. The phenotypic characteristics such as tiller number, stalk height, stalk diameter and estimated yield in the untreated and sprayed (312 and 625 g a.i. ha^{-1}) plots were comparable to those of the untreated N12 control. Based on the imazapyr residual activity and ALS activity results, imazapyr tolerance was confirmed in three (Mut1, Mut6 and Mut7) of the seven tested mutant lines. However, further testing is necessary to establish the response of these Mut1, Mut6 and Mut7 plants under standard weed control practices because herbicide application to control weeds needs to be established without compromising yield parameters. Future work will also need to focus on phenotypically assessing these mutant lines for traits including sucrose content, fibre content because there was not enough plant material to perform this in the current study. In addition, their actual

yield (tons cane ha^{-1}) and response to standard pests and diseases also need to be determined.

The ALS gene has been successfully identified and isolated from many other crop plants species including tobacco (Van der Vyver *et al*., 2013), oil seed rape (Tan *et al*., 2005), *Arabidopsis thaliana* (Schnell *et al*., 2012), rice (Ogawa *et al*., 2008; Endo *et al*., 2012), sugarbeet (Wright and Penner, 1998a) and wheat (Ponziak *et al*., 2004) but not in sugarcane. To-date, only a single base pair mutation on the ALS gene, which confers tolerance to the imadazolinone class of herbicides (e.g. imazapyr), has been identified in field-grown sugarcane plants (Punyadee *et al*., 2007; Khruangchan *et al*., 2011). Boutsalis *et al*. (1999), White *et al*. (2003); Yu *et al*. (2003) and Sala *et al*. (2012) reported that the amplification of the ALS gene requires oligonucleotide primer design based on conserved region of the gene. Future work will involve designing primers to amplify the ALS gene from Mut1, Mut6, Mut7 and N12 control plants using known sequences in several plants (*Saccharum* hybrid cultivars, sorghum and maize) and analysed through the National Center for Biotechnology Information (NCBI) site (http:// www.ncbi.nlm.nih.gov). The ALS gene from the mutants and N12 will be isolated and sequenced, and compared for base pair differences. Once isolated, and if found to be different to that of the N12, the mutated ALS gene can either be used as a gene of interest and/or as a selectable marker, for example in genetic bombardment in the genetic modification approach. In addition, the identified imazapyr-tolerant lines have the potential to be used for commercial purposes in the field and as a parent plant in the breeding programme of SASRI.

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Appendices

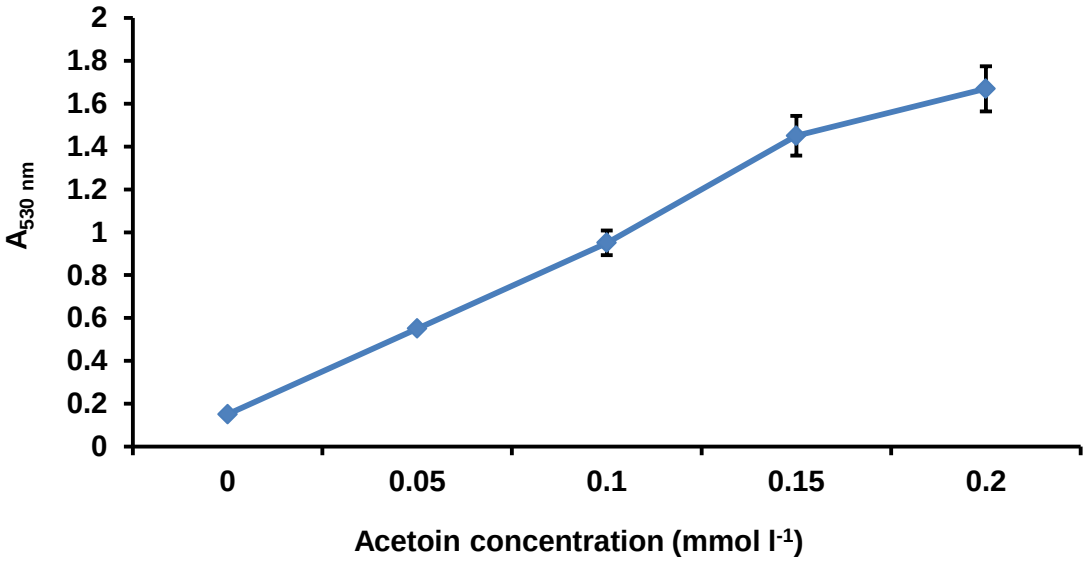
Appendix 1

Summary of analysis of genotype masses determined using a Student's t-test. The genotypes in the table below were used as reference varieties allowing comparisons (of regression parameter estimates)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Mut1, Mut3, Mut4, Mut5, Mut6, Mut7, N12 reference level					
Regression	15	42.6	2.8	65.3	<0.001
Residual	80	3.5	0.04		
Total	95	46.1	0.5		
Mut2 reference level					
Regression	8	40.5	5.1	78.3	<0.001
Residual	87	5.6	0.06		
Total	95	46.1	0.5		

Appendix 2

The Acetoin standard curve was used to express ALS activity of mutant plants (Mut1-Mut7) and N12 control in mmol l^{-1}



Acetoin standard curve. The r^2 =0.9.275 between 0.15-1.45 absorbance, representing 0-0.15 mmol l^{-1} acetoin linear range.

Appendix 3

Statistical significance of SPAD readings of 0, 1, 3, 6 and 12 weeks of Mut1, Mut6 and N12 compared across sprayed (312 and 625 g a.i. ha^{-1}) and unsprayed plots using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Mut1					
Plot.Week	8	983.5	122.9	6.8	<0.001
Residual	28	505.4	18.1		
Total	44	3173.4			
Mut6					
Plot.Week	8	559.2	69.90	6.04	<0.001
Residual	28	324.1	11.6		
Total	44	2720.7			
N12					
Plot.Week	8	1700.3	212.54	19.2	<0.001
Residual	28	310.0	11.1		
Total	44	5968.4			

Appendix 4

Statistical significance of SPAD readings (in the untreated) of Mut1, Mut6 and N12 control over time were determined using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
untreated plot					
Mut1					
Week	4	376.8	94.2	22.8	<0.001
Residual	8	33.0	4.1		
Total	14	412.1			
Mut6					
Week	4	65.0	16.3	2.6	0.115
Residual	8	49.7	6.2		
Total	14	122.7			
N12					
Week	4	215.0	54.0	10.5	0.003
Residual	8	55.0	6.9		
Total	14	282.1			

Appendix 5

Statistical significance of SPAD readings (in the 312 and 625 g a.i. ha^{-1} plots) between Mut1, Mut6 and N12 control plants for week 12 were determined using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
312 g a.i. ha^{-1} plot					
Genotype.Week	8	1608.1	201.0	8.8	<0.001
Residual	28	642.3	22.9		
Total	44	5533.8			
625 g a.i. ha^{-1} plot					
Genotype.Week	8	1135.8	142.0	9.0	<0.001
Residual	28	443.0	15.8		
Total	44	5825.2			

Appendix 6

Statistical significance of SPAD readings (in the 312 g a.i. ha^{-1} plot) of Mut1, Mut6 and N12 control over time were determined using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
312 g a.i. ha^{-1} plot					
Mut1					
Week	4	1132.6	283.2	31.0	<0.001
Residual	8	73.2	9.1		
Total	14	1583.2			
Mut6					
Week	4	796.6	199.1	13.6	<0.001
Residual	8	116.9	14.6		
Total	14	1053.7			
N12					
Week	4	2214.2	553.6	63.2	<0.001
Residual	8	70.0	8.8		
Total	14	2327.7			

Appendix 7

Statistical significance of SPAD readings (in the 625 g a.i. ha^{-1} plot) of Mut1, Mut6 and N12 control over time were determined using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
625 g a.i. ha^{-1} plot					
Mut1					
Week	4	967.8	241.9	13.8	<0.001
Residual	8	139.9	17.5		
Total	14	1113.8			
Mut6					
Week	4	877.8	219.4	20.4	<0.001
Residual	8	86.2	10.8		
Total	14	1027.7			
N12					
Week	4	877.8	219.4	20.4	<0.001
Residual	8	161.5	20.2		
Total	14	1981.8			

Appendix 8

Statistical significance of ALS rate of activity (in the 625 g a.i. ha^{-1} plot) for mutant (Mut1-Mut7) and N12 control plants were determined using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Mut	11	10.8	0.1	2.3	<0.001
Residual	77	33.2	0.4		
Total	95	265.0			

Appendix 9

Statistical significance of ALS activity of 0, 1, 3, 6 and 12 weeks for Mut1, Mut6 and N12 plants compared across sprayed (312 and 625 g a.i. ha^{-1}) and unsprayed plots using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Mut1					
Plot.Week	6	1.0	0.2	10.6	<0.001
Residual	22	0.4	0.02		
Total	35	7.3			
Mut6					
Plot.Week	6	0.5	0.09	3.3	0.02
Residual	22	0.6	0.03		
Total	35	6.7			
N12					
Plot.Week	6	1.0	0.16	39.8	<0.001
Residual	22	0.09	0.004		
Total	35	11.0			

Appendix 10

Statistical significance of ALS activities (the 312, 625 g a.i. ha^{-1} and untreated plots) between Mut1, Mut6 and N12 control plants for week 12 were determined using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Untreated plot					
Genotype.Week	6	1.3	0.2	24.0	<0.001
Residual	22	0.2	0.01		
Total	35	3.7			
312 g a.i. ha^{-1} plot					
Genotype.Week	6	0.5	0.1	3.1	0.02
Residual	22	0.6	0.03		
Total	35	8.1			
625 g a.i. ha^{-1} plot					
Genotype.Week	6	0.7	0.1	8.4	<0.001
Residual	22	0.3	0.01		
Total	35	9.9			

Appendix 11

Statistical significance of ALS activity (Untreated plot) of Mut1, Mut6 and N12 control over time were determined using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Untreated					
Mut1					
Week	3	0.4	0.1	30.9	<0.001
Residual	6	0.02	0.004		
Total	11	0.5			
Mut6					
Week	3	0.7	0.2	16.32	0.003
Residual	6	0.1	0.01		
Total	11	0.8			
N12					
Week	3	1.6	0.5	144.3	<0.001
Residual	6	0.02	0.003		
Total	11	1.6			

Appendix 12

Statistical significance of ALS activity (in the 312 g a.i. ha^{-1} plot) of Mut1, Mut6 and N12 control over time were determined using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
312 g a.i. ha^{-1} plot					
Mut1					
Week	3	1.90	0.6	20.1	0.002
Residual	6	0.2	0.03		
Total	11	2.1			
Mut6					
Week	3	1.1	0.42	7.3	0.020
Residual	6	0.3	0.05		
Total	11	1.5			
N12					
week	3	3.7	1.2	492.7	<0.001
Residual	6	0.01	0.002		
Total	11	3.7			

Appendix 13

Statistical significance of ALS activity (in the 625 g a.i. ha^{-1} plot) of Mut1, Mut6 and N12 control plants over time were determined using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
625 g a.i. ha^{-1} plot					
Mut1					
Week	3	2.6	0.9	53.66	<0.001
Residual	6	0.1	0.02		
Total	11	2.7			
Mut6					
Week	3	2.3	0.8	45.92	<0.001
Residual	6	0.1	0.02		
Total	11	2.6			
N12					
Week	3	4.1	1.4	1828.3	<0.001
Residual	6	0.004	0.001		
Total	11	4.1			

Appendix 14

Statistical significance of IC_{50} of mutant plants (Mut1-Mut7) and N12 control was determined using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	7	2.7	0.4	5.0	0.005
Residual	14	1.1	0.1		
Total	23	4.6			

Appendix 15

Statistical significance of plant agronomic traits was determined using a Two-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Tiller number					
Plot	2	42121.3	21060.7	30.0	<0.001
Treatment	7	99780.8	14254.4	20.3	<0.001
Plot.Treatment	14	68964.7	4926.0	7.01	<0.001
Residual	46	32324.1	702.7		
Stalk height					
Plot	2	77380.9	38690.4	133.9	<0.001
Treatment	7	32022.9	4574.7	15.8	<0.001
Plot.Treatment	14	23103.3	1650.2	5.71	<0.001
Residual	46	13292.6	289.0		
Stalk diameter					
Plot	2	773.1	386.6	150.0	<0.001
Treatment	7	1056.6	151.0	58.6	<0.001
Plot.Treatment	14	1120.7	80.1	31.1	<0.001
Residual	46	118.5	2.6		
Estimated yield					
Plot	2	15198.0	7599.0	38.7	<0.001
Treatment	7	11357.9	1622.6	8.3	<0.001
Plot.Treatment	14	7290.8	520.8	2.7	0.006
Residual	46	9022.1	196.1		

Appendix 16

Statistical significance of shoot length over time (at 4, 8 and 12 weeks) of mutant (Mut1-Mut7) and N12 control plants was determined using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Mut1					
Week	2	8820.6	4410.3	60.7	<0.001
Residual	18	1308.1	72.7		
Total	29	10948.3			
Mut2					
Week	2	10829.1	5414.5	112.7	<0.001
Residual	18	864.9	48.1		
Total	29	12043.4			
Mut3					
Week	2	8026.5	4013.2	30.6	<0.001
Residual	18	2360.9	131.2		
Total	29	11578.2			
Mut4					
Week	2	15947.5	7973.7	48.6	<0.001
Residual	18	2951.9	164.0		
Total	29	20121.5			
Mut5					
Week	2	11346.2	5673.1	91.4	<0.001
Residual	18	1117.8	62.1		
Total	29	12916.0			
Mut6					
Week	2	7841.9	3920.9	11.6	<0.001
Residual	18	6095.5	338.6		
Total	29	17485.9			
Mut7					
Week	2	11425.9	5712.9	27.2	<0.001
Residual	18	3786.1	210.3		
Total	29	16659.9			
N12					
Week	2	5280.3	2640.1	26.7	<0.001
Residual	18	1778.4	98.8		
Total	29	8505.4			

Appendix 17

Statistical significance of shoot length percentage of mutant (Mut1-Mut7) and N12 control plants was determined using a One-way ANOVA

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	7	24934.0	3562.0	8.75	<0.001
Residual	63	25652.0	407.2		
Total	79	51859.0			