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Seasonal variation in acetohydroxy acid synthase inhibition by imazapyr in *Cynodon dactylon*

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ABSTRACT

Cynodon dactylon (cynodon) is a weed that is particularly difficult to control due to its ability to reproduce readily using stolons and rhizomes. Although glyphosate is the most commonly used herbicide to control cynodon, current recommendations involve repetitive applications, which appear likely to select for resistance. Rotating glyphosate with herbicides that have different modes of action will be a more sustainable strategy for cynodon control. Earlier work suggested that the growth of cynodon is strongly inhibited by imazapyr, an herbicide that inhibits the acetohydroxy acid synthase (AHAS) enzyme. The optimum time for application of imazapyr on cynodon, and the subsequent inhibition of AHAS activity in cynodon are unknown. The aims of this study were firstly, to determine the effectiveness of inhibiting AHAS activity when applied at different times of the year, and secondly to compare the efficacy of foliar and root applications. Results showed that application time has a significant effect on the efficacy of imazapyr to inhibit enzyme activity, with mid–late summer being optimal, and furthermore that the herbicide can be absorbed by both the shoots and the roots.

1. Introduction

Creeping grasses *Cynodon dactylon* (L.) Pers. (cynodon), *Digitaria abyssinica* (A. Rich.) Stapf (digitaria) and *Cynodon nlemfuensis* (Vanderyst) (stargrass) are common weeds in sugarcane fields in southern Africa, especially in fields where cane growth tends to be poor (Landrey et al., 1993). At present, glyphosate is the most commonly used herbicide to control these infestations, and was first introduced in 1974 (Campbell, 2008). Glyphosate acts by inhibiting the enzyme 5-enoloyruvylshikimate-3-phosphate synthase (EPSPS), blocking the formation of essential aromatic amino acids, and as a result plant death (Myers et al., 2016). While glyphosate has been effective for general weed control, higher dose rates are currently required to control creeping grasses. Continued application of glyphosate or paraquat could lead to herbicide resistance in cynodon, and therefore herbicides with new modes of action must be developed.

Imazapyr is a broad-spectrum herbicide, originally introduced for use on fallow fields in South Africa in 2013, and is found in products such as Arsenal Gen 2®, Chopper®, and Assault®. Imazapyr has been shown to provide effective control for various broadleaf and grass species, including cynodon. Unlike contact herbicides, imazapyr has been shown to be absorbed by roots, leaves and stems (Senseman, 2007). Imazapyr has a mode of action that differs from the herbicides normally used for cynodon control in the sugarcane industry. Imazapyr acts by inhibiting the enzyme acetohydroxy acid synthase (AHAS), also referred to as acetolactate synthase (ALS) (Sathasivan et al., 1991). AHAS has become the target enzyme of various herbicides since the 1980s (Sathasivan et al., 1991). There are five families of herbicides that inhibit the AHAS enzyme namely: imidazolinone (IMI), sulfonylurea (SU), triazolopyrimidine, pyrimidinyl-thiobenzoates, and sulfonylaminocarbonyltriazolinone. Imazapyr belongs to the imidazolinone family (Osuna et al., 2003, Yu et al., 2003). AHAS catalyses the first two reactions in the synthesis of the branched chain amino acids isoleucine, valine and leucine (Osuna et al., 2003; Yu et al., 2003). According to Lee and Duggleby (2001), AHAS catalysed steps produce isoleucine, beginning with 2-ketobutyrate and pyruvate, while valine is synthesised by a parallel pathway from two molecules of pyruvate. Leucine is formed by an extension of the valine pathway (Lee and Duggleby, 2001; Duggleby et al., 2008). Inhibition of AHAS by imazapyr results in death due to the failure of the plants to produce these essential amino acids.

Herbicides such as glyphosate are usually applied annually between September and March to control cynodon, often in fallow fields, verges or as spot spray treatments under the crop canopy (Campbell, 2008). The optimal timing of imazapyr application is unclear. If it is to be used successfully to control cynodon, it is important to understand how imazapyr is taken up and translocated. Furthermore, imazapyr has a residual effect, unlike glyphosate, thereby, providing better control of weeds. In general, it appears that in most plants imazapyr is rapidly translocated via the xylem and phloem to meristems where it

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accumulates (Duggleby et al., 2008). The main aims of the present study were firstly to determine the optimal season for foliar application of imazapyr to control cynodon, and secondly to test the efficacy of root uptake of this herbicide. We hypothesised that autumn would be the most appropriate time for application of herbicide. A foliar application of imazapyr in autumn, combined with natural senescence and the translocation of photosynthate toward roots, could lead to more rapid translocation of imazapyr from shoots and result in faster plant death. We therefore sprayed established cynodon with herbicide at different times of the year, and estimated the effectiveness of the herbicide by measuring AHAS activity over the following weeks. Activity was also measured in plants to which imazapyr was applied only to the roots, to determine if root applications can inhibit AHAS activity more effectively than a foliar spray.

2. Materials and methods

2.1. Establishment of plant material

The main trial tested the efficacy of seasonal foliar application of imazapyr to inhibit AHAS. Five litre pots for each season were prepared (4 seasons × 3 replicates × 4 to 8 time periods × 2 treatments). Each pot contained a sod (grass with soil) of cynodon grass taken from verges surrounding the South African Sugarcane Research Institute, which had never been treated with imazapyr. Smaller pots, containing a sandy soil, were prepared to test the efficacy of imazapyr as a root treatment.

2.2. Foliar application of imazapyr to pots of cynodon

All pots were sprayed with imazapyr at the recommended rate of 5.225 l ha^{-1} mixed in 300 l ha^{-1} (1254 g active ingredient ha^{-1}). Pots were sprayed using a CO_2 gas regulated sprayer set at 0.2 MPa pressure and fitted with a blue 110° fanjet nozzle (APE). Foliar applications were carried out in spring (10 October 2011), mid-summer (04 January 2016), late summer (03 February 2012) and autumn (03 April 2012). Enzyme assays were conducted, where possible, at 1, 2, 4, 6, 8, 13 and 16 weeks after treatment (WAT).

2.3. Root application of imazapyr to pots of cynodon

To test the effect of imazapyr on roots, cynodon plants were grown in a glasshouse, and each pot placed in a saucer. Following establishment of sufficient green leaf material, imazapyr was applied as a single dose of a 30 ml aliquot to each saucer, which corresponded to the recommended application rate of 5.225 l ha^{-1} (1254 g active ingredient ha^{-1} mixed in 300 l water). Herbicide was absorbed into the pot by capillary action. Enough water was then fed into the saucer to keep the entire pot moist. To prevent loss of the herbicide from the pot, all pots were watered into the saucer throughout the duration of the trial. Enzyme assays were conducted at 1, 2, 4, 6, 8 and 13 weeks after treatment.

2.4. Acetohydroxy acid synthase assay

Enzyme activity was measured in material from imazapyr treated and untreated pots by estimating acetohydroxy acid production, which was quantified by using acid to decarboxylate it to acetoin (Osuna et al., 2003; Koch et al., 2012).

Each sample (2 g) comprised approximately half leaf and half stem material. Samples were crushed using mechanical grinders, and mixed with 7.5 ml of extraction buffer containing 0.003 g of polyvinylpolypyrrolidone (PVPP) to bind phenolics. The extraction buffer contained 0.1 M (pH 7.5) potassium phosphate (KH_2PO_4/KH_2PO_4), 0.1 M $MgCl_2$, 0.01 M thiamine pyrophosphate (TPP), 0.01 M dithiothreitol, glycerol, 1 μM flavin adenine dinucleotide (FAD) and protease inhibitor (added in the ratio of 10 μl to 1 ml of buffer). The homogenate was filtered through one layer of cheesecloth, and centrifuged at 23,200*g* for 15 min. The protein fraction was precipitated from the supernatant by drop wise addition of an equal volume of cold 50% $(NH_4)_2SO_4$. The solution was then allowed to stand on ice for 10 min with slow stirring before being centrifuged at 23,200*g* for 20 min. The resultant pellet was then re-dissolved in 2.25 ml of assay buffer consisting of 0.5 M HEPES (pH 7.5), 0.5 M sodium pyruvate, 0.1 M MgCl2, 0.01 M TPP and 134 μM FAD. The extract was then added to wells containing 55 μl of autoclaved, distilled water before being incubated at 37 °C for 90 min, and then the reaction stopped by adding 22 μl of 3 M H_2SO_4. After 15 min, 105 μl of 0.55% solutions of alpha naphthol and creatine was added to produce a colour, which was measured spectrophotometrically 530 nm. Activity was expressed as absorption units mg^{-1} fresh mass.

2.5. Tetrazolium tests on root material for uptake trail

After all enzyme assays a sample of root material was collected and soaked in 1.5% of tetrazolium salts, pH 7, (UNIVAR, South Africa, Cape Town) at 30 °C for 4 h to indicate viability of root material. Three pots, with three roots per pot, were sampled, and the percentage of red stained roots was recorded. Red staining indicated viability.

2.6. Statistical analyses

All enzyme assay results were compared using ANOVA with GenStat 18th edition. All data was tested for normality. Foliar treatment data was analysed using an unbalanced ANOVA. Following analysis, a Sidak test was conducted to show the strength of interactions. In addition to the unbalanced ANOVA, which included all four seasons, spring data was also analysed separately to provide Sidak results for 13, and 16 WAT (not common to any other season). The root uptake trial was analysed using a two-way ANOVA, with post hoc Sidak tests conducted to show the strength of each interaction.

3. Results and discussion

3.1. Effect of imazapyr applied as a foliar spray

In the main experiment reported here, imazapyr was applied at an equivalent of standard field rates (5.225 l ha^{-1} or 1254 g active ingredient ha^{-1}) to stems and leaves of potted cynodon. During application, almost all applied herbicide was intercepted by shoots, although a little may have reached the soil, and been available for root uptake. An unbalanced ANOVA analysis showed that significant differences ($P < 0.001$) were present for all interactions (Table 1). Assays of AHAS activity clearly showed that the effect of imazapyr varied according to application time. In spring, there was strong and rapid inhibition in AHAS activity, but activity soon increased (Fig. 1A). In these plants, shoots remained alive for at least 16 WAT, suggesting that spring application of imazapyr is ineffective in cynodon control. The spring in which

Table 1
Unbalanced ANOVA results of AHAS inhibition between 0 and 8 WAT, following imazapyr application to test for fixed effects and interactions between seasons, time and treatments.

Change	d.f.	s.s.	m.s.	v.r.	F pr.
Rep	2	0.02276	0.01138	1.05	0.353
Time	5	7.96592	1.59318	147.69	<0.001
Season	3	0.97851	0.32617	30.24	<0.001
Treatment	1	10.30654	10.30654	955.42	<0.001
Time · Season	13	1.89967	0.14613	13.55	<0.001
Time · Treatment	5	2.68009	0.53602	49.69	<0.001
Season · Treatment	3	0.74555	0.24852	23.04	<0.001
Time · Season · Treatment	13	0.84	0.06462	5.99	<0.001
Residual	86	0.92772	0.01079		