MEAT & LIVESTOCK AUSTRALIA

on farm

Innovative breeding of high-digestibility kikuyu cultivators to increase milk production

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Feedbase and Pastures

Plain English Summary

Kikuyu grass is a highly productive forage species with only intermediate digestibility. It is probably the second most important grass for the Australian dairy industry (after ryegrass) but has received comparatively little breeding attention. The germplasm base of kikuyu in Australian agriculture is certainly narrow - most likely, dangerously so. There is currently no kikuyu breeding program operating in Australia and earlier work consisted primarily of selection from very limited introductions.

Milk production would be significantly increased if a kikuyu cultivar with improved digestibility were available. One method of increasing disgestibility is to lower the lignin content of the plant (lignin is an indigestible fibre component of all plants). This has been achieved in maize, sorghum and millet by the production of so-called 'brown mid-rib' types. The brown mid-rib type is caused by a single gene mutation which results in lower lignin content, and results in a characteristic brown-coloured mid-vein on the undersides of leaves.

There is no germplasm collection available for kikuyu and no brown mid-rib types are known. The aim of this project was to produce a brown mid-rib cultivar and, thereby, raise the digestibility of kikuyu pastures and their value to the dairy industry. Kikuyu digestibility needs to be raised by about 10 percentage points to be comparable with ryegrass.

Two chemicals were used (singly and in combination) to treat seeds of the kikuyu cultivar Whittet. The chemicals were chosen to cause genetic damage to the kikuyu hereditary material (DNA) and result in new genotypes (mutants). It was hoped that the new genotypes would include either a brown mid-rib type, or some other mutant that resulted in increased digestibility.

This mutagenic approach was a high-risk strategy compared to conventional plant breeding but was relatively low-cost and rapid. There was a small probability of total success using this 'hit-and-miss' method and for certain progress over the long-term a full, conventional breeding program for kikuyu would need to be funded.

Forty thousand single plants of kikuyu were grown in the field under irrigation for two years. In the second year, repeated visual selection was used to identify 212 unusual-looking individuals. In addition, a number of random selections were made as controls, bringing the total to 318. These plants were transplanted to the glasshouse, and 30-day leaf regrowth was harvested. The leaf samples were analysed for digestibility in the laboratory using the sheep rumen-fluid method.

Seventy four plants were identified which had a digestibility greater than 73%, six of which were greater than 76%. This is a marked improvement over normal kikuyu, which is usually around 65-68% digestible under field conditions, and averaged 71% in the untreated glasshouse controls in this project. These promising lines require further testing prior to their release to industry.

Residual seed from this work was sent to Wollongbar where (outside the contract for this project) one thousand individual plants were screened in the glasshouse for resistance to kikuyu yellows disease. Yellows is becoming a serious problem, particularly in older coastal pastures, and there is no cheap, effective control option. Therefore, genetic resistance to yellows would be

a valuable character in any new kikuyu cultivar. One kikuyu plant has survived three challenges from the disease and appears to be resistant - five others have survived two tests. These lines too require further testing with a view to releasing a new cultivar.

Given the promising results to date, DRDC and NSW Agriculture need to negotiate the funding of further glasshouse and field experiments to establish the value of the lines produced in this project. Potentially they could have a very significant effect on the Australian dairy industry.

DRDC and its research providors should discuss the need for a kikuyu germplasm collection and funding for a kikuyu breeding progam in Australia.

Background

Kikuyu grass is a highly productive pasture species but its digestibility is low compared to ryegrass and other temperate species. If low-lignin mutants could be produced by mutagenesis then this would be likely to increase digestibility. New kikuyu cultivars with improved digestibility would dramatically increase milk production.

Milk production in NSW from coastal kikuyu pastures between December and April has been estimated at 80 million litres. In summer, kikuyu is the principal pasture species on 80% of farms and may provide 75% of basal ration.

The release of a new kikuyu cultivar with reduced lignin content and a digestibility increase of 10 percentage points would increase the productivity of a major pasture plant supporting the NSW (and parts of WA and QLD) dairy industry and would increase milk production by 30%. With 50% adoption of the new cultivar the value of milk production (12 million litres @ 24.5 c per litre) would increase by $2.94 million per year in NSW alone. This does not take into account the potential expansion of the area of kikuyu grass into new areas, e.g. the inland irrigation areas of NSW.

Objectives

To improve the digestibility, and hence the milk production potential, of kikuyu grass by producing and isolating a low-lignin (brown mid-rib) mutant using chemical mutagenesis. The longer-term aim is to release any confirmed improved genotype as a new cultivar resulting in pastures with higher digestibility.

Achievement of Objectives

We have succeeded in isolating several genotypes (which are currently represented by single plants) which have substantially increased digestibility. Thess data need confirmation in larger-scale trials but we appear to have made good progress and have achieved an increase in the best lines to over 75% digestibility compared to an average of about 65% in normal kikuyu. Without further trials and chemical analysis we do not know whether our elite genotypes are, in fact, brown mid-rib types or whether their increased digestibility is due to some other component affecting that character (e.g. increased sugar or protein content).

Introduction

Kikuyu digestibility is too low and needs to be raised by 10 percentage points to approach the quality of ryegrass. We proposed to improve the milk production potential of kikuyu by producing a new low-lignin cultivar.

Two photographs showing the field set-up for growing the M_1 and M_2 generations. Note the multi-welled trays, weed matting, irrigation sprinklers, and frost cover.

Methodology

Kikuyu (*Pennisetum clandestinum*) is largely an unimproved species which is difficult to breed because: (a) it is tetraploid (2n = 4x = 36); (b) many genotypes are male-sterile and apomictic; (c) it is outcrossing and may have a self-incompatibility system; and (d) it usually requires close, repeated clipping to induce flowering. Consequently, kikuyu improvement has relied solely on selection from existing material. However, innovative breeding approaches to increase pasture quality were recommended by Dr Rex Oram in his review for DRDC (Oram, 1992).

The Whittet cultivar of kikuyu readily produces seed by sexual reproduction and, is agronomically acceptable to coastal milk producers. Therefore, it was decided that seed of cv. Whittet would be the starting material for mutagenesis. The production of seed by normal sexual reproduction was an important criterion since any mutants produced are likely to be recessive and in a heterozygous condition. It is only through segregation that homozygous mutant genotypes would be produced so that the mutant character could be identified and selected. Our aim is to isolate a single low-lignin mutant but to leave the rest of the genetic background of the cultivar intact in order to reduce the need for any follow-up breeding.

This mutagenic approach to producing a high-digestibility, low-lignin, *bmr*-type kikuyu was a high-risk strategy compared to conventional plant breeding. However, its advantages were it being a relatively low-cost and rapid method. There was a small probability of total success using this 'hit-and-miss' or 'shot-gun' method plus some additional chance of partial success. For certain progress in digestibility (and other desirable characteristics) over the long-term, a full, conventional breeding program for kikuyu would be needed. Given its importance to industry and its genetic vulnerability, kikuyu is under-researched.

Research Results

It was decided that the mutagenesis would use the approach advocated by Rédie (1974). The aim was to maximise the mutant recovery rate by attempting to sample each M_1 individual once in the M_2 generation. It was impractical to individually harvest the number of single plants involved, so we needed to be able to make seed production per plant as even as possible to minimise the sampling effect. In order to keep the plants small and manageable, and also to facilitate watering, it was decided to grow the plants in multi-welled plastic trays set out on a bed of sand.

Our best guess was that we would be able to handle about 40,000 individuals in both the M_1 and M_2 generations. This was the number we worked with but due to lack of experience we seriously under-estimated the amount of manual work involved. Delays in recruitment of the Technical Assistant for this project also caused early difficulties.

Two mutagens were chosen for the task of treating the Whittet seeds. The first was sodium azide which is known in other species to produce point mutations. It was decided to also use diethyl sulphate (DES) which is known to cause chromosomal aberrations. The idea here was that the DES might uncover some of the important tetrasomic loci in kikuyu and make them effectively diploid. This would then greatly increase the chance of mutants being isolated.

Since the two mutagens employed were expected to act in different ways and to have different genetic consequences , it was decided to test their joint effect in both sequential combinations, i.e. azide followed by DES, and DES followed by azide.

Two small experiments were conducted early in the project to investigate: 1) the dose response of kikuyu seeds to azide, and 2) the response of kikuyu seeds to various post-treatment recovery regimes which might influence the degree of DNA repair (we obviously wanted to minimise any post-treatment repair). However, the results from these experiments were difficult to interpret (data not presented here) and illustrated that much time and energy could be spent on examining these, and other, issues in some depth. Time did not allow this luxury. In both of these experiments mutagenic effect was measured by delay in germination and total proportion of seeds germinating. However, it was difficult to equate mutagen LD_{50} to the degree of genetic damage. It was decided to use mutagen doses and treatment times that had been reported in the literature for other species. It was also decided to use a simple (and therefore labour-saving) post-treatment regime. i.e. briefly rinse the seeds and sow immediately.

The mutagenic treatments were applied on four separate occasions (= blocks or replicates) using lots of 500 cv. Whittet seeds held loosely in muslin bags to facilitate aeration, agitation and rinsing. We had decided that each treatment x block combination was to consist of 60 x 42-well trays = 2520 individuals. Since the mutagenic treatments were aimed at killing a large proportion of the seeds, 8 bags (4000 seeds) were treated.

In addition there were five control treatments. Each control consisted of 120 treated seeds, of which 84 were sown per block (=2 x 42-well trays). The full list of treatments was:

Control 1	Pre-soak and sow
Control 2	Pre-soak, soak in azide buffer, sow
Control 3	Pre-soak, soak in DES buffer, sow
Control 4	Pre-soak, soak in azide buffer, soak in DES buffer, sow
Control 5	Pre-soak, soak in DES buffer, soak in azide buffer, sow
Treatment 1	Pre-soak, treat with azide, sow
Treatment 2	Pre-soak, treat with DES, sow
Treatment 3	Pre-soak, treat with DES, treat with azide, sow
Treatment 4	Pre-soak, treat with azide, treat with DES, sow

Pre-soaking and all treatments were carried out at room temperature, with constant aeration of the solution provided by an aquarium bubbler with diffuser tips on the ends of the micro-tubing. Pre-soaking was overnight for 12 hrs in tap water. Sodium azide was used at a concentration of 0.001 molar in a phosphate buffer solution at pH 3.0. Azide treatment was for 2 hrs at room temperature. After all treatments the seed samples were rinsed in running tap water for 10 minutes.

DES was used at a concentration of 0.01 molar in a phosphate buffer at pH 7.0. As with azide, treatment was for 2 hrs at room temperature with aeration, followed by a 10 minute rinse. In both of the double-mutagen treatments the second mutagen was applied after the 10 minute rinse.

The first block of treated seed samples were germinated on moist paper in plastic boxes and the survivors transplanted into potting mix in multi-welled trays by hand. This took far too long, so from then on we sowed two seeds directly into each well and thinned the seedlings later to one per well. Transplanted seedlings were used to fill any gaps that existed after about 2 weeks.

In the field each treatment x block combination was arranged as a 30 x 2-tray lattice. This meant that each plant could be identified by its row and well position. Each tray was approximately 25 x 35 cm with each well holding about 75 ml of sand and peat potting mix. The overall size of the experiment was, therefore: 4 blocks x 4 treatments x 60 trays x 42 wells = 40,320 treated seedlings. In addition there were 4 blocks x 5 controls x 2 trays x 42 wells = 1,680 control seedlings. The layout of the M_1 and M_2 generations was the same.

We had concerns about general aspects of growing kikuyu under our experimental conditions. These problems were all overcome as we gained more experience working with this species:
1) What was the best irrigation regime? (sprinkle system with watering frequency adjusted depending on weather - usually 5 times per day in summer for 10 minutes each time);
2) How much fertilizer was required? (used continuous flow of liquid complete fertilizer via Mazzie injector - about 60 litres of complete fertiliser per week over the whole experiment);
3) Would the plants successfully over-winter and how would we protect them from frost? (used cloth covers held in place by clips);
4) What was the flowering frequency and probable seed set? (trimmed regularly to promote flowering and control runners, raising the cutting height each time to leave previously-set seeds undamaged);
5) How long did seed take to reach maturity (about 4 weeks);
6) Was there seed dormancy? (no - we did some germination tests); and
7) Could we control weeds in the experiment? (used permanent weed-mat between trays and sprayed glyphosate herbicide on the perimeter).

M_1 generation

The seed mutagenic treatment, and particularly sowing, took a lot longer than expected. Consequently the M_1 generation could not be grown, seed set, and harvest completed before winter. The plants were maintained over winter, they grew on in spring and early summer, and were harvested just after Christmas (January 1995). The plants in each treatment x block combination were harvested as a bulk using a "whipper-snipper", by cutting level with the top of the wells. A timber screen was used to contain the debris. The cuttings were vacuumed and swept up, and then air-dried for a week in a glasshouse in plastic tubs. The cuttings were then stored in Hessian bags. Before the bulk harvest a number of plants were harvested individually using secateurs. These individuals were hand threshed to measure seed number per plant (see below). The 20 control x block combinations were each harvested by hand as a bulk.

The 16 treated bulk harvests plus the 20 controls were threshed and seed-cleaned at Grafton using various equipment, including a hammer mill. The seed was tested for viability, ready for second season, by germinating a small sample of seeds on moist filter paper in petri dishes. Viability was high and there was plenty of seed produced in each bulk.

In the M_1 generation two visual single plant inspections were made for possible mutant

phenotypes. No particularly unusual-looking plants were identified and so no selections were made. The M_1 plants were trimmed five times using a "whipper-snipper" and secateurs to promote flowering and control runners.

In the M_1 we tested various possible aids to the visual identification of the *bmr* (brown mid-rib) phenotype. This was done in conjunction with growth chamber-grown maize, sorghum and millet, of both normal and *bmr* genotype. The aids included the use of filters (coloured and polarized), and UV light. None were successful at making the *bmr*-type easier to see than the normal type.

M_2 generation

The M_2 generation was sown again as quickly as possible following threshing and seed cleaning of the M_1 (sowing was completed by March 1996). The M_2 generation grew over winter as before and the second bulk harvest was made in February 1996.

We tried filters again early in the M_2 generation to look for unusual plants but to no effect. At this stage we realised that visual inspection was the only method we had available and that even in known *bmr* types it was difficult to see the difference in phenotype (i.e. a darker mid-rib on the undersides of leaves). Given that it was becoming less likely that we would actually find a *bmr* kikuyu plant, we decided that it would be highly desirable to be able to rapidly screen some individual plants by near infra-red spectroscopy (NIR) to look for quantitative variation in digestibility.

We began working on an NIR calibration (correlated to *in vitro* digestibility and fibre content) using some material we had grown in the glasshouse plus some samples from Wollongbar with known variation in digestibility. This work progressed well until the staff member on the project resigned. Given the time remaining on the project and the fact that considerable training was required to continue the calibration, this approach had to be abandoned. We were then left with visual selection as our only option for selecting promising plants.

The individual M_2-generation plants were screened five times, on the following dates: 24/5/95, 9/10/95, 26/10/95, 8/12/95 and 10/1/96. They were also trimmed five times during the growing season (as in M_1) to promote flowering and control runners. During these inspections a total of 212 single plants were identified as putative mutants. Individuals were selected if they exhibited any unusual morphological of phenological features, such as, leaf colouration, leaf size or shape, internode length, early flowering, and particularly erect or prostrate plants. These plants were transplanted to 130 mm diameter pots in the glasshouse and grown on to produce leaf material for *in vitro* digestibility analysis. Open-pollinated seed was harvested from each plant, dried and stored just before completion of the project. The bulk M_2 harvest was carried out as for the M_1. The M_3 seed samples were stored at low temperature for possible future use.

In addition to the selected plants from the various treatments, we also made 106 random selections: approximately 20 from each of the treatment x block combinations and approximately seven from Control-1 in each block. No aberrant plants were selected from the controls so it was not considered necessary to sample individuals from each of the other controls (nos. 2-4). The origins of the individual plants are summarized in Table 1.

Table 1. Numbers of individual plants in each treatment

Treatment	Number of selected plants	Number of random plants	Total
Controls	none	27	27
Azide	64	20	84
DES	54	19	73
Azide + DES	57	21	78
DES + Azide	37	19	56
Total	212	106	318

Two leaf harvests were taken consisting of 30-day re-growth leaf material. Care was taken to avoid including runners, stems and heads. These two samples were dried, combined, and finely ground then used to conduct *in vitro* digestibility analysis.

Statistical modelling

The individual plant harvests from the M_1 generation showed that seed number per plant averaged 32 over all treatments but there were significant differences between treatments and blocks. The range per plant was from zero to over 200 seeds.

Statistical modelling of the distributions conducted by Mr Damian Collins (NSW Agriculture, Wagga) showed that the actual number of M_1 parent plants sampled to produce the M_2 was about 21,000 (21,000/40,320 = 52%). The mean per treatment was 1,312 with a 95% confidence interval of 1,100-1,430 (= 17,500-23,000 for the whole experiment).

Modelling also showed that even if 10,000 M_2 seeds had been sown per treatment block instead of 2,520, then we would still only have sampled 2,100 of the M_1 parent plants (2,100/2,520 = 83%).

Digestibility Analysis

Two consecutive 30 day leaf re-growths were harvested from the individual selected plants, dried at 80°C in a forced-air dehydrator for 24 hrs, and then ground through a 1 mm screen. *In vitro* organic matter (OM) digestibility was determined using the rumen inoculum/pepsin method. Duplicate analyses were conducted on each sample with the exception of 5 samples for which there was insufficient material.

Digestibility varied from 52 to 78%. Previous work with kikuyu grass has shown that OM digestibilities of 70-72% are rarely achieved, and then only with short re-growth (≤ 30 days) from kikuyu grown under favourable conditions. In this study, a significant number of plants had OM digestibilities above 73% (Table 2). The raw data for each sample are too voluminous to be presented here but the distribution of kikuyu plants into the various digestibility classes is presented in Table 2.

Table 2. Number of kikuyu plants (both selected and randomly-chosen) falling into various *in vitro* organic matter digestibility classes.

Digestibility class (%)	Mutagenic Treatment					Total
	Control	Azide	DES	Azide + DES	DES + Azide	
< 61	0	2	1	1	1	5
61-64	0	5	3	1	0	9
64-67	2	11	15	6	7	41
67-70	6	16	21	19	16	78
70-73	10	33	19	29	20	111
73-76	8	15	13	21	11	68
> 76	1	2	1	1	1	6
Total	27	84	73	78	56	318
%age with digestibility >73%	33	20	19	28	21	

There was a tendency for each of the mutagenic treatments to increase the range of digestibility compared to the controls. This was particularly obvious at the low end of the distribution (see Table 2). This is good evidence that the mutagenic treatments were somewhat effective and had induced additional variation. Most mutations are deleterious and, therefore, would be expected to produce increased variation at the low end of the scale. It remains to be seen whether this variation is genetically controlled and what the biochemical or morphological basis is

(particularly in the high-digestibility lines which are the ones with agricultural significance).

Future analysis of these lines would include fibre estimation to determine whether any of the lines were indeed low-lignin or *bmr* types. No genotypes with obvious brown mid-rib type leaves were detected but it is possible that in this species the *bmr* phenotype does not include the mid-vein colouration. Alternatively, we may not have left the kikuyu to grow long enough to clearly exhibit any *bmr* characteristics. In addition, in other species such as maize there is quantitaive variation in lignin content at least equal to that provided by *bmr* types. Therefore, conventional breeding can achieve the same result as *bmr*, in maize at least. Increased digestibility could also be due to other factors unrelated to lignin content, such as, increased sugar or protein content.

Kikuyu Yellows Resistance

The disease 'kikuyu yellows' has become a major agronomic limitation on kikuyu pasture production on the north coast of NSW. The disease (caused by a soil-borne fungus) is becoming more prevalent in older coastal pastures and there is no cheap and reliable control option available.

Dr Percy Wong and Dr Bill Fulkerson (NSW Agriculture) suggested that it might be useful to use our mutagenised material to screen for resistance to yellows disease. Some of the residual M_2 seed from the 16 treatment x block combinations was sent to Wollongbar. Glasshouse screening for resistance was conducted using individual plants growing in pots. This work was additional to the DRDC contract. Selection for yellows resistance is much simpler than *bmr* screening because it is a qualitative 'dead-or-alive' classification. Therefore, selection is relatively efficient and unambiguous.

One thousand plants were grown in the presence of yellows-infected leaf material. Plants that survived the first challenge were tested again in larger pots. Five plants have survived to this stage. In addition, one plant has survived a third challenge and appears to be fully resistant. During the screening process the control pots of cvv. Noonen and Whittet always died.

The preliminary results are very encouraging and these lines require multiplication and field testing to establish their value with a view to commercialisation of a new yellows resistant kikuyu cultivar. Obviously, it would also be of interest to check the digestibility of these resistant lines. The combination of resistance and high digestibility in a single new cultivar would be a major achievement indeed!

Discussion

This project has identified some 100 kikuyu lines worthy of more detailed examination and multiplication. There is a strong possibility that a new cultivar could be selected from this material bringing substantial benefits to Australian dairy farmers.

There were a number of control plants that had high digestibility (see Table 2). It remains a

possibility that high-digestibility lines could have been isolated by intra-varietal selection in cv. Whittet without mutagenesis.

Industry Implications

Kikuyu grass with the potential to produce forage of 75% digestibility would be a particularly valuable pasture species on dairy farms. It is, therefore, important that further work be conducted with the plants selected in this project to confirm the high digestibility in replicated pot (and later plot) trials, and to evaluate agronomic and quality characteristics under field conditions.

Future Research

No future research with this kikuyu material is planned. The selected plants are being maintained in the glasshouses at Wagga ARI and Wollongbar subject to a decision being made on future investment. NSW Agriculture and DRDC need to hold discussions on this issue. It is highly unlikely that NSW Agriculture will conduct any substantial, additional research without industry support.

Intellectual Property

The promising lines generated by this project are jointly owned by DRDC and NSW Agriculture. If further research confirmed that one or other of these lines had significantly increased digestibility and/or kikuyu yellows resistance, then its commercial release as a new cultivar would be warranted (subject to there being no difficulties with its other agronomic characteristics). Seed production and sales could be commercialised and result in some income from royalties. Kikuyu is also a valuable forage grass in a number of other countries (e.g. South Africa) and there may well be export possibilities for any new cultivar.

Communication

The preliminary work in this project was presented as a poster at the NSW Dairy Expo in September 1994.

Recommendations

DRDC and NSW Agriculture to hold discussions on the funding of further research to confirm (or otherwise) the value of the selected kikuyu lines with a view to releasing one or more new cultivars.

Publications

There have been no scientific publications to date but one is planned. Several articles have appeared in the local (NSW) print media reporting progress on this project.

Acknowledgements

Most of the field and glasshouse work in this project was carried out by Ms Peta Welsh (nee Hassell) and Mr Tom Clarke. Several casual staff assisted with sowing and harvest. Mr John Piltz performed the *in vitro* digestibility analyses and Mr Damian Collins conducted the statistical analysis and simulation modelling. Kikuyu yellows screening was conducted at Wollongbar by Dr Bill Fulkerson and Ms Katrina Slack.

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Two photographs showing the field set-up for growing the M_1 and M_2 generations. Note the multi-welled trays, weed matting, irrigation sprinklers, and frost cover.

Methodology

Kikuyu (*Pennisetum clandestinum*) is largely an unimproved species which is difficult to breed because: (a) it is tetraploid (2n = 4x = 36); (b) many genotypes are male-sterile and apomictic; (c) it is outcrossing and may have a self-incompatibility system; and (d) it usually requires close, repeated clipping to induce flowering. Consequently, kikuyu improvement has relied solely on selection from existing material. However, innovative breeding approaches to increase pasture quality were recommended by Dr Rex Oram in his review for DRDC (Oram, 1992).

The Whittet cultivar of kikuyu readily produces seed by sexual reproduction and, is agronomically acceptable to coastal milk producers. Therefore, it was decided that seed of cv. Whittet would be the starting material for mutagenesis. The production of seed by normal sexual reproduction was an important criterion since any mutants produced are likely to be recessive and in a heterozygous condition. It is only through segregation that homozygous mutant genotypes would be produced so that the mutant character could be identified and selected. Our aim is to isolate a single low-lignin mutant but to leave the rest of the genetic background of the cultivar intact in order to reduce the need for any follow-up breeding.

This mutagenic approach to producing a high-digestibility, low-lignin, *bmr*-type kikuyu was a high-risk strategy compared to conventional plant breeding. However, its advantages were it being a relatively low-cost and rapid method. There was a small probability of total success using this 'hit-and-miss' or 'shot-gun' method plus some additional chance of partial success. For certain progress in digestibility (and other desirable characteristics) over the long-term, a full, conventional breeding program for kikuyu would be needed. Given its importance to industry and its genetic vulnerability, kikuyu is under-researched.

Research Results

It was decided that the mutagenesis would use the approach advocated by Rédie (1974). The aim was to maximise the mutant recovery rate by attempting to sample each M_1 individual once in the M_2 generation. It was impractical to individually harvest the number of single plants involved, so we needed to be able to make seed production per plant as even as possible to minimise the sampling effect. In order to keep the plants small and manageable, and also to facilitate watering, it was decided to grow the plants in multi-welled plastic trays set out on a bed of sand.

Our best guess was that we would be able to handle about 40,000 individuals in both the M_1 and M_2 generations. This was the number we worked with but due to lack of experience we seriously under-estimated the amount of manual work involved. Delays in recruitment of the Technical Assistant for this project also caused early difficulties.

Two mutagens were chosen for the task of treating the Whittet seeds. The first was sodium azide which is known in other species to produce point mutations. It was decided to also use diethyl sulphate (DES) which is known to cause chromosomal aberrations. The idea here was that the DES might uncover some of the important tetrasomic loci in kikuyu and make them effectively diploid. This would then greatly increase the chance of mutants being isolated.

Since the two mutagens employed were expected to act in different ways and to have different genetic consequences , it was decided to test their joint effect in both sequential combinations, i.e. azide followed by DES, and DES followed by azide.

Two small experiments were conducted early in the project to investigate: 1) the dose response of kikuyu seeds to azide, and 2) the response of kikuyu seeds to various post-treatment recovery regimes which might influence the degree of DNA repair (we obviously wanted to minimise any post-treatment repair). However, the results from these experiments were difficult to interpret (data not presented here) and illustrated that much time and energy could be spent on examining these, and other, issues in some depth. Time did not allow this luxury. In both of these experiments mutagenic effect was measured by delay in germination and total proportion of seeds germinating. However, it was difficult to equate mutagen LD_{50} to the degree of genetic damage. It was decided to use mutagen doses and treatment times that had been reported in the literature for other species. It was also decided to use a simple (and therefore labour-saving) post-treatment regime. i.e. briefly rinse the seeds and sow immediately.

The mutagenic treatments were applied on four separate occasions (= blocks or replicates) using lots of 500 cv. Whittet seeds held loosely in muslin bags to facilitate aeration, agitation and rinsing. We had decided that each treatment x block combination was to consist of 60 x 42-well trays = 2520 individuals. Since the mutagenic treatments were aimed at killing a large proportion of the seeds, 8 bags (4000 seeds) were treated.

In addition there were five control treatments. Each control consisted of 120 treated seeds, of which 84 were sown per block (=2 x 42-well trays). The full list of treatments was:

Control 1	Pre-soak and sow
Control 2	Pre-soak, soak in azide buffer, sow
Control 3	Pre-soak, soak in DES buffer, sow
Control 4	Pre-soak, soak in azide buffer, soak in DES buffer, sow
Control 5	Pre-soak, soak in DES buffer, soak in azide buffer, sow
Treatment 1	Pre-soak, treat with azide, sow
Treatment 2	Pre-soak, treat with DES, sow
Treatment 3	Pre-soak, treat with DES, treat with azide, sow
Treatment 4	Pre-soak, treat with azide, treat with DES, sow

Pre-soaking and all treatments were carried out at room temperature, with constant aeration of the solution provided by an aquarium bubbler with diffuser tips on the ends of the micro-tubing. Pre-soaking was overnight for 12 hrs in tap water. Sodium azide was used at a concentration of 0.001 molar in a phosphate buffer solution at pH 3.0. Azide treatment was for 2 hrs at room temperature. After all treatments the seed samples were rinsed in running tap water for 10 minutes.

DES was used at a concentration of 0.01 molar in a phosphate buffer at pH 7.0. As with azide, treatment was for 2 hrs at room temperature with aeration, followed by a 10 minute rinse. In both of the double-mutagen treatments the second mutagen was applied after the 10 minute rinse.

The first block of treated seed samples were germinated on moist paper in plastic boxes and the survivors transplanted into potting mix in multi-welled trays by hand. This took far too long, so from then on we sowed two seeds directly into each well and thinned the seedlings later to one per well. Transplanted seedlings were used to fill any gaps that existed after about 2 weeks.

In the field each treatment x block combination was arranged as a 30 x 2-tray lattice. This meant that each plant could be identified by its row and well position. Each tray was approximately 25 x 35 cm with each well holding about 75 ml of sand and peat potting mix. The overall size of the experiment was, therefore: 4 blocks x 4 treatments x 60 trays x 42 wells = 40,320 treated seedlings. In addition there were 4 blocks x 5 controls x 2 trays x 42 wells = 1,680 control seedlings. The layout of the M_1 and M_2 generations was the same.

We had concerns about general aspects of growing kikuyu under our experimental conditions. These problems were all overcome as we gained more experience working with this species:
1) What was the best irrigation regime? (sprinkle system with watering frequency adjusted depending on weather - usually 5 times per day in summer for 10 minutes each time);
2) How much fertilizer was required? (used continuous flow of liquid complete fertilizer via Mazzie injector - about 60 litres of complete fertiliser per week over the whole experiment);
3) Would the plants successfully over-winter and how would we protect them from frost? (used cloth covers held in place by clips);
4) What was the flowering frequency and probable seed set? (trimmed regularly to promote flowering and control runners, raising the cutting height each time to leave previously-set seeds undamaged);
5) How long did seed take to reach maturity (about 4 weeks);
6) Was there seed dormancy? (no - we did some germination tests); and
7) Could we control weeds in the experiment? (used permanent weed-mat between trays and sprayed glyphosate herbicide on the perimeter).

M_1 generation

The seed mutagenic treatment, and particularly sowing, took a lot longer than expected. Consequently the M_1 generation could not be grown, seed set, and harvest completed before winter. The plants were maintained over winter, they grew on in spring and early summer, and were harvested just after Christmas (January 1995). The plants in each treatment x block combination were harvested as a bulk using a "whipper-snipper", by cutting level with the top of the wells. A timber screen was used to contain the debris. The cuttings were vacuumed and swept up, and then air-dried for a week in a glasshouse in plastic tubs. The cuttings were then stored in Hessian bags. Before the bulk harvest a number of plants were harvested individually using secateurs. These individuals were hand threshed to measure seed number per plant (see below). The 20 control x block combinations were each harvested by hand as a bulk.

The 16 treated bulk harvests plus the 20 controls were threshed and seed-cleaned at Grafton using various equipment, including a hammer mill. The seed was tested for viability, ready for second season, by germinating a small sample of seeds on moist filter paper in petri dishes. Viability was high and there was plenty of seed produced in each bulk.

In the M_1 generation two visual single plant inspections were made for possible mutant

phenotypes. No particularly unusual-looking plants were identified and so no selections were made. The M_1 plants were trimmed five times using a "whipper-snipper" and secateurs to promote flowering and control runners.

In the M_1 we tested various possible aids to the visual identification of the *bmr* (brown mid-rib) phenotype. This was done in conjunction with growth chamber-grown maize, sorghum and millet, of both normal and *bmr* genotype. The aids included the use of filters (coloured and polarized), and UV light. None were successful at making the *bmr*-type easier to see than the normal type.

M_2 generation

The M_2 generation was sown again as quickly as possible following threshing and seed cleaning of the M_1 (sowing was completed by March 1996). The M_2 generation grew over winter as before and the second bulk harvest was made in February 1996.

We tried filters again early in the M_2 generation to look for unusual plants but to no effect. At this stage we realised that visual inspection was the only method we had available and that even in known *bmr* types it was difficult to see the difference in phenotype (i.e. a darker mid-rib on the undersides of leaves). Given that it was becoming less likely that we would actually find a *bmr* kikuyu plant, we decided that it would be highly desirable to be able to rapidly screen some individual plants by near infra-red spectroscopy (NIR) to look for quantitative variation in digestibility.

We began working on an NIR calibration (correlated to *in vitro* digestibility and fibre content) using some material we had grown in the glasshouse plus some samples from Wollongbar with known variation in digestibility. This work progressed well until the staff member on the project resigned. Given the time remaining on the project and the fact that considerable training was required to continue the calibration, this approach had to be abandoned. We were then left with visual selection as our only option for selecting promising plants.

The individual M_2-generation plants were screened five times, on the following dates: 24/5/95, 9/10/95, 26/10/95, 8/12/95 and 10/1/96. They were also trimmed five times during the growing season (as in M_1) to promote flowering and control runners. During these inspections a total of 212 single plants were identified as putative mutants. Individuals were selected if they exhibited any unusual morphological of phenological features, such as, leaf colouration, leaf size or shape, internode length, early flowering, and particularly erect or prostrate plants. These plants were transplanted to 130 mm diameter pots in the glasshouse and grown on to produce leaf material for *in vitro* digestibility analysis. Open-pollinated seed was harvested from each plant, dried and stored just before completion of the project. The bulk M_2 harvest was carried out as for the M_1. The M_3 seed samples were stored at low temperature for possible future use.

In addition to the selected plants from the various treatments, we also made 106 random selections: approximately 20 from each of the treatment x block combinations and approximately seven from Control-1 in each block. No aberrant plants were selected from the controls so it was not considered necessary to sample individuals from each of the other controls (nos. 2-4). The origins of the individual plants are summarized in Table 1.

Table 1. Numbers of individual plants in each treatment

Treatment	Number of selected plants	Number of random plants	Total
Controls	none	27	27
Azide	64	20	84
DES	54	19	73
Azide + DES	57	21	78
DES + Azide	37	19	56
Total	212	106	318

Two leaf harvests were taken consisting of 30-day re-growth leaf material. Care was taken to avoid including runners, stems and heads. These two samples were dried, combined, and finely ground then used to conduct *in vitro* digestibility analysis.

Statistical modelling

The individual plant harvests from the M_1 generation showed that seed number per plant averaged 32 over all treatments but there were significant differences between treatments and blocks. The range per plant was from zero to over 200 seeds.

Statistical modelling of the distributions conducted by Mr Damian Collins (NSW Agriculture, Wagga) showed that the actual number of M_1 parent plants sampled to produce the M_2 was about 21,000 (21,000/40,320 = 52%). The mean per treatment was 1,312 with a 95% confidence interval of 1,100-1,430 (= 17,500-23,000 for the whole experiment).

Modelling also showed that even if 10,000 M_2 seeds had been sown per treatment block instead of 2,520, then we would still only have sampled 2,100 of the M_1 parent plants (2,100/2,520 = 83%).

Digestibility Analysis

Two consecutive 30 day leaf re-growths were harvested from the individual selected plants, dried at 80°C in a forced-air dehydrator for 24 hrs, and then ground through a 1 mm screen. *In vitro* organic matter (OM) digestibility was determined using the rumen inoculum/pepsin method. Duplicate analyses were conducted on each sample with the exception of 5 samples for which there was insufficient material.

Digestibility varied from 52 to 78%. Previous work with kikuyu grass has shown that OM digestibilities of 70-72% are rarely achieved, and then only with short re-growth (≤ 30 days) from kikuyu grown under favourable conditions. In this study, a significant number of plants had OM digestibilities above 73% (Table 2). The raw data for each sample are too voluminous to be presented here but the distribution of kikuyu plants into the various digestibility classes is presented in Table 2.

Table 2. Number of kikuyu plants (both selected and randomly-chosen) falling into various *in vitro* organic matter digestibility classes.

Digestibility class (%)	Mutagenic Treatment					Total
	Control	Azide	DES	Azide + DES	DES + Azide	
< 61	0	2	1	1	1	5
61-64	0	5	3	1	0	9
64-67	2	11	15	6	7	41
67-70	6	16	21	19	16	78
70-73	10	33	19	29	20	111
73-76	8	15	13	21	11	68
> 76	1	2	1	1	1	6
Total	27	84	73	78	56	318
%age with digestibility >73%	33	20	19	28	21	

There was a tendency for each of the mutagenic treatments to increase the range of digestibility compared to the controls. This was particularly obvious at the low end of the distribution (see Table 2). This is good evidence that the mutagenic treatments were somewhat effective and had induced additional variation. Most mutations are deleterious and, therefore, would be expected to produce increased variation at the low end of the scale. It remains to be seen whether this variation is genetically controlled and what the biochemical or morphological basis is

(particularly in the high-digestibility lines which are the ones with agricultural significance).

Future analysis of these lines would include fibre estimation to determine whether any of the lines were indeed low-lignin or *bmr* types. No genotypes with obvious brown mid-rib type leaves were detected but it is possible that in this species the *bmr* phenotype does not include the mid-vein colouration. Alternatively, we may not have left the kikuyu to grow long enough to clearly exhibit any *bmr* characteristics. In addition, in other species such as maize there is quantitaive variation in lignin content at least equal to that provided by *bmr* types. Therefore, conventional breeding can achieve the same result as *bmr*, in maize at least. Increased digestibility could also be due to other factors unrelated to lignin content, such as, increased sugar or protein content.

Kikuyu Yellows Resistance

The disease 'kikuyu yellows' has become a major agronomic limitation on kikuyu pasture production on the north coast of NSW. The disease (caused by a soil-borne fungus) is becoming more prevalent in older coastal pastures and there is no cheap and reliable control option available.

Dr Percy Wong and Dr Bill Fulkerson (NSW Agriculture) suggested that it might be useful to use our mutagenised material to screen for resistance to yellows disease. Some of the residual M_2 seed from the 16 treatment x block combinations was sent to Wollongbar. Glasshouse screening for resistance was conducted using individual plants growing in pots. This work was additional to the DRDC contract. Selection for yellows resistance is much simpler than *bmr* screening because it is a qualitative 'dead-or-alive' classification. Therefore, selection is relatively efficient and unambiguous.

One thousand plants were grown in the presence of yellows-infected leaf material. Plants that survived the first challenge were tested again in larger pots. Five plants have survived to this stage. In addition, one plant has survived a third challenge and appears to be fully resistant. During the screening process the control pots of cvv. Noonen and Whittet always died.

The preliminary results are very encouraging and these lines require multiplication and field testing to establish their value with a view to commercialisation of a new yellows resistant kikuyu cultivar. Obviously, it would also be of interest to check the digestibility of these resistant lines. The combination of resistance and high digestibility in a single new cultivar would be a major achievement indeed!

Discussion

This project has identified some 100 kikuyu lines worthy of more detailed examination and multiplication. There is a strong possibility that a new cultivar could be selected from this material bringing substantial benefits to Australian dairy farmers.

There were a number of control plants that had high digestibility (see Table 2). It remains a

possibility that high-digestibility lines could have been isolated by intra-varietal selection in cv. Whittet without mutagenesis.

Industry Implications

Kikuyu grass with the potential to produce forage of 75% digestibility would be a particularly valuable pasture species on dairy farms. It is, therefore, important that further work be conducted with the plants selected in this project to confirm the high digestibility in replicated pot (and later plot) trials, and to evaluate agronomic and quality characteristics under field conditions.

Future Research

No future research with this kikuyu material is planned. The selected plants are being maintained in the glasshouses at Wagga ARI and Wollongbar subject to a decision being made on future investment. NSW Agriculture and DRDC need to hold discussions on this issue. It is highly unlikely that NSW Agriculture will conduct any substantial, additional research without industry support.

Intellectual Property

The promising lines generated by this project are jointly owned by DRDC and NSW Agriculture. If further research confirmed that one or other of these lines had significantly increased digestibility and/or kikuyu yellows resistance, then its commercial release as a new cultivar would be warranted (subject to there being no difficulties with its other agronomic characteristics). Seed production and sales could be commercialised and result in some income from royalties. Kikuyu is also a valuable forage grass in a number of other countries (e.g. South Africa) and there may well be export possibilities for any new cultivar.

Communication

The preliminary work in this project was presented as a poster at the NSW Dairy Expo in September 1994.

Recommendations

DRDC and NSW Agriculture to hold discussions on the funding of further research to confirm (or otherwise) the value of the selected kikuyu lines with a view to releasing one or more new cultivars.

Publications

There have been no scientific publications to date but one is planned. Several articles have appeared in the local (NSW) print media reporting progress on this project.

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