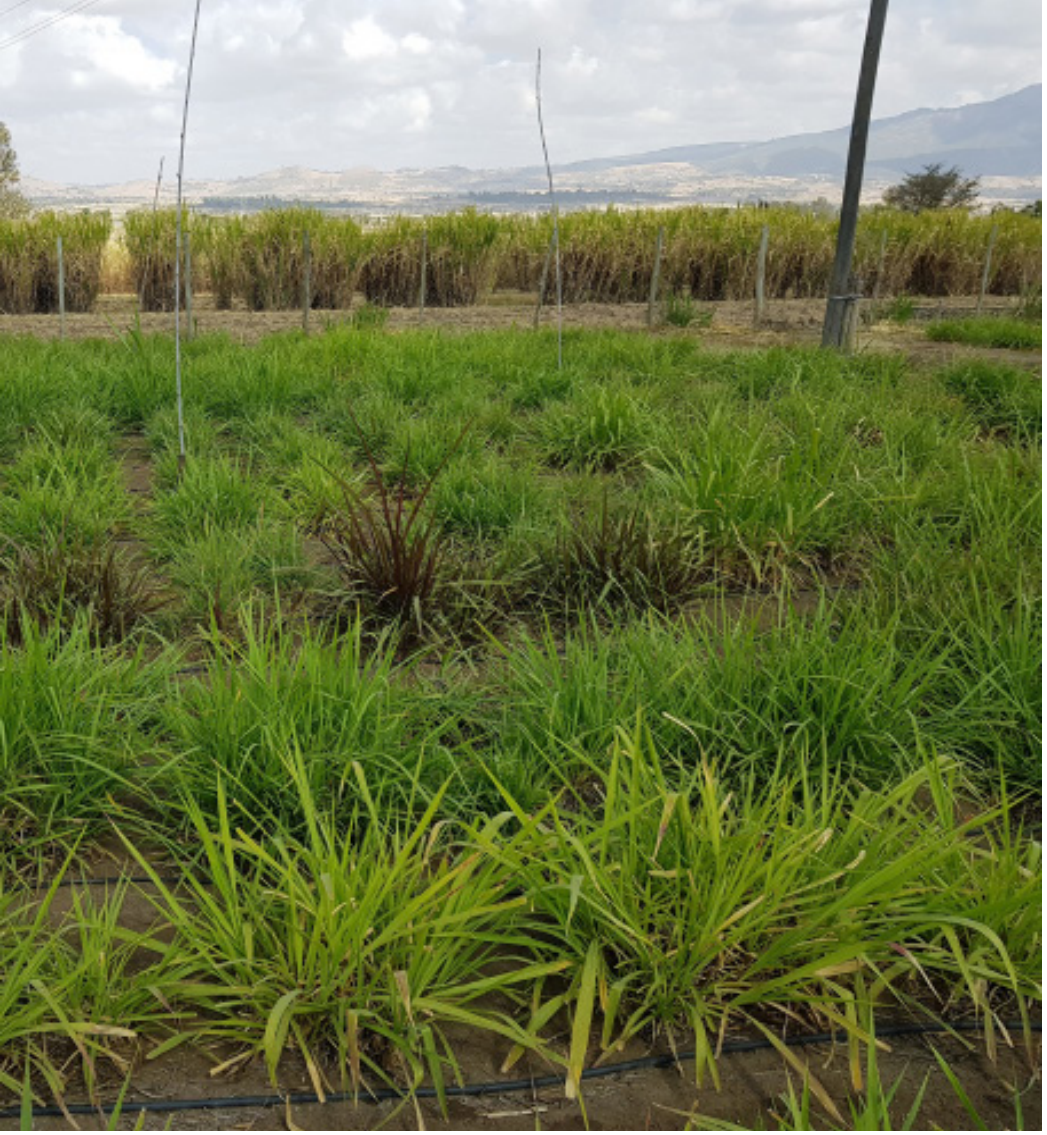


ILRI MANUAL

64

CGIAR

INITIATIVE ON
Sustainable Animal Productivity

Methods and practices for the evaluation of forage legumes, grasses and fodder trees for use as livestock feed

Second edition

Methods and practices for the evaluation of forage legumes, grasses and fodder trees for use as livestock feed

Second edition

Abel Teshome, Ermias Habte, Alemayehu Teressa Negawo, Meki Shehabu Muktar, Yilikal Assefa and Chris S. Jones.

April 2023

ILRI thanks all donors and organizations which globally support its work through their contributions to the CGIAR Trust Fund

Editing, design and layout—ILRI Editorial and Publishing Services, Addis Ababa, Ethiopia.

Cover photo—ILRI/Ermias Habte

ISBN: 92–9146–757–x

Citation: Teshome, A., Habte, E., Negawo, A.T., Shehabu, M.S., Assefa, Y. and Jones C.S. 2023. *Methods and practices for the evaluation of forage legumes, grasses and fodder trees for use as livestock feeds: Second edition*. ILRI Manual 64. Addis Ababa, Ethiopia: ILRI.

Patron: Professor Peter C Doherty AC, FAA, FRS
Animal scientist, Nobel Prize Laureate for Physiology or Medicine–1996

Box 30709, Nairobi 00100
Kenya
Phone +254 20 422 3000
Fax +254 20 422 3001
Email ilri-kenya@cgiar.org

ilri.org
better lives through livestock

ILRI is a CGIAR research centre

Box 5689, Addis Ababa,
Ethiopia
Phone +251 11 617 2000
Fax +251 11 667 6923
Email ilri-ethiopia@cgiar.org

ILRI has offices in East Africa • South Asia • Southeast and East Asia • Southern Africa • West Africa

Contents

Figures

Acknowledgements

This work was conducted as part of the CGIAR Initiative on Sustainable Animal Productivity and is supported by contributors to the CGIAR Trust Fund. CGIAR is a global research partnership for a food-secure future dedicated to transforming food, land, and water systems in a climate crisis.

We are grateful to the stakeholder experts for their contributions during the creation of this manual.

Preface

This manual describes the principles, methods and practices of experimental research used in tropical and sub-tropical forage research, that will enable the implementation of standard procedures to conduct screening of large numbers of forage genotypes or accessions (>100) and subsequent variety tests to identify best performing forage varieties and cropping systems that will support increased livestock production.

List of abbreviations and acronyms

ADF	acid detergent fibre
AMMI	Additive Main effective Multiplicative Interaction
APG	**Australian** Pastures Genebank
ASV	AMMI Stability Value
CEC	cation-exchange capacity
CIAT	International Center for Tropical Agriculture
CIFOR	Center for International Forestry Research
DAP	diammonium phosphate
DM	dry matter
FAO	Food and Agriculture Organization of the United Nations
FW	fresh weight
GBS	genotyping by sequencing
GEI	genotype by environment interaction
GHU	Germplasm Health Unit
GS	genomic selection
ICRAF	World Agroforestry Centre
IITA	International Institute of Tropical Agriculture
ILCA	International Livestock Centre for Africa
ILRI	International Livestock Research Institute
LSD	least significant difference
MAS	marker-assisted selection
NDF	neutral detergent fibre

NGS	next-generation sequencing
NIRS	near-infrared spectroscopy
PPE	personal protective equipment
QTL	quantitative trait locus
RABAOC	Reseau de Recherche en Alimentation du Bétail en Afriqua Occidentale et Centrale
RCBD	randomized complete block design
rpm	rotations per minute
SNP	singe nucleotide sequence
SSP	single superphosphate
WGS	whole genome sequencing
WUE	water use efficiency
YSI	yield stability index

1 Introduction

The evaluation of forage crops is a common practice which aims to support the development of improved forage cultivars for livestock feed. The main purpose of the field trials of forage species is to screen and evaluate large genotype collections of legumes, grasses or fodder trees, and develop compatible forage cropping systems adapted to local and wider agro-ecologies. However, to date, the field trial approach adopted by various organizations and institutes is not uniform due to differences in knowledge and experience of researchers, plus resources available and the aim of the specific forage project. Although this has generated a lot of data, variation in the methodology has precluded the comparison of results across trials and sites. This manual is thus developed to provide a standard evaluation methodology, which will enable researchers to obtain meaningful results from their forage evaluation field trials, whilst allowing comparisons to be made between trials and sites. A companion manual covering the evaluation of feed value (Osuji et al. 1993) has been produced. The present document is a second and revised edition of Tarawali et al. (1995) which was developed based on experiences from the forage and fodder-tree evaluation trials carried out at the International Livestock Research Institute (ILRI).

Generally, this publication describes the principles, methods and practices of experimental research used in tropical and subtropical forage research, that will enable the implementation of standard procedures to conduct screening of large numbers of forage genotypes or accessions (>100) and subsequent variety tests to identify best performing forage varieties and cropping systems that will support increased livestock production. The experimental procedures in forage crops described in chapters 2, 3 and 4 deal with methodologies for initial evaluation, including suitable methodologies for small-plot evaluation trials, conducted either in single or multiple locations, of herbaceous legumes, grasses and fodder trees. Subsequently, chapters 4 and 5 cover the methodologies suitable for further evaluation of promising genotypes or accessions identified in the initial evaluation in larger plots and on-farm trials. Chapter 6 describes appropriate experimental designs and analysis methods for the estimation of forage performance, the effects of grazing on plant growth, evaluation of mixtures of accessions and species, and estimation of seed multiplication capacity. Chapter 7 focuses on the use and application of genomic tools in accelerating forage variety development. The final chapter highlights the challenges faced when running field trials and how to address these. Thus, this manual is aimed at guiding researchers to select suitable methodologies for the evaluation of forages for specific farming systems.

1.1 Research

Research can be defined as a systematic way of obtaining new or additional information. The main scientific disciplines (physical, earth and life sciences) have different methods and approaches to obtaining and evaluating data. Within the life sciences, two types of research can be accommodated:

- Experimental research
- Descriptive research (also known as observational research).

Using an experimental approach, the researcher applies various treatments to the object under investigation. All factors except the treatments should ideally be kept uniform, so that differences in the various groups can be attributed to the treatment.

In descriptive research, treatments are not applied to the object under investigation. Instead, the researcher studies the existing conditions that occur, for example, in a population, in a geographical area or in a field. This area of research often studies the differences that exist within or between the investigated groups or populations. These may be differences between groups occurring at the same time, or differences that occur over time in the same group. Surveys are examples of descriptive research.

Certain types of investigations are suited for experimental research while others for descriptive research. To solve a particular problem, it is often useful to carry out both descriptive and experimental investigations. For example, to sustain forage availability in the dry season we may initially undertake some observational research or search previous reports and publications on the available developed technologies to:

- Identify widely adaptable forage species for a given agro-ecology in livestock-producing communities.
- Collect and assess data on germplasm performance of the forage crop where the species is growing.
- Seek information from forage-producing farmers about the history of forage and cultivation of related crops in relation to productivity in the dry season.

We may then go on to conduct some experimental research. For example:

- Screen forage genotypes for performance, in terms of quantity and quality, in the dry season.
- Undertake multilocation studies on the performance of promising forage genotypes.

1.2 Principles in field trials

Field trial research is a form of experimental research. It is a systematic way of comparing plant production and performance on a small piece of land. Different methods are used to compare treatments to be applied to the experimental units (individual accessions or genotypes). In a field experiment, except for the different treatments, all factors are kept uniform in order to minimize their effect/influence on the quantity or quality of plant production data. Examples of treatments include different: varieties of forage genotypes; forage crop species; planting dates; amounts of fertilizer; rates of application; crop rotations and other cropping patterns.

The area where a treatment is applied is known as a plot. The trial therefore consists of several plots that together form the trial/experimental area.

Treatments are usually repeated several times in the research field. One full set of treatments is known as a replication. Normally, three to six replications are used in a trial except when screening larger numbers of entries, where two replications are commonly used in a simple lattice or incomplete block design (Yates 1936). If only one replication were used, we would not be able to say whether differences in measured traits such as yield from the various plots were caused by the treatment or heterogeneity between plots (such as differences in soil fertility, slope, border effect), or by errors during implementation. By using several replications and applying statistical procedures we can get an impression of what caused the differences between treatments and between plots. Where soil conditions are very heterogeneous or the production conditions are difficult to control, a high number of replications is desirable. In an experimental field on a slope, four or more replications should be used to reduce experimental error (Gomez and Gomez 1984).

Figure 1: Example of a trial area consisting of five treatments and four replications = 20 plots.

Replication A	1	3	2	5	4
Replication B	4	1	2	3	5
Replication C	5	4	3	1	2
Replication D	2	5	3	4	1

1.3 Types of field trials

Field trials can be characterized in different ways depending on the purpose, target group and location. Field trials can be adaptive, where resources may be redirected depending on initial findings, or research trials which generate new knowledge or technologies. Examples of adaptive research trials include studies that aim to test known technologies and adapt the technologies to local conditions. This kind of research is therefore, also sometimes known as technology verification. Adaptive agricultural research would normally be expected to produce useful results within a relatively short time, usually 2 to 4 years. While research trials are carefully controlled to support the generation of new knowledge or technologies, such as developing new crop varieties or new cropping systems, in accession and variety screening/evaluation trials, the performance of different crops and varieties are compared under specific and distinct locations or agro-ecologies. In cropping system trials, plant performance is compared under different kinds of cultural management practices, such as different:

- Types of tillage
- Planting times
- Planting methods
- Plant spacing
- Crop rotations
- Types of intercropping
- Pest protection methods
- Types or quantities of fertilizers

In cropping system trials, the varieties to be used are adapted/tested for local adaptation to ensure that they are suitable to be grown under the given climatic and edaphic conditions. Untested varieties or species should not be used because there is a risk that they are unsuitable to the local conditions. Thus, we would not know if the failure of a cropping system is caused by the cropping system or by the species or varieties used in the trial. Variety and species trials are therefore normally necessary when starting trials in a new area or in a defined agro-ecological zone.

Field trials can be carried out both as on-station and as on-farm trials. On-station trials are often used to test new technologies and to screen innovations before introduction to farmers. These are normally easy to manage and to access, and a great degree of control can be maintained over the research conditions. The on-station trials may therefore be monitored more closely than on-farm trials; it is, therefore, feasible to use a greater number of observations and treatments than in on-farm trials.

However, the conditions in research stations are often very different from those in farmers' fields. For example, soil conditions may represent only a few of the soil types encountered on farms. Furthermore, management inputs may be unrealistically high compared to what farmers apply, e.g. regarding weeding and pest control. The timing of operation is also often better at the research station. Crop responses such as yields obtained at stations are therefore frequently much higher than what can be achieved by farmers. Many technologies that appear promising in on-station field trials failed when introduced to farmers because the conditions and management at the station were unrealistic for farmers. This has been the case with many soil conservation measures that were effective in reducing erosion in small trials but were impossible for farmers to maintain when implemented on an entire field.

2 Field trial methods for herbaceous legumes and grasses

2.1 Principles in field trials

Selecting a field site representative of the target environment is the primary and critical step of running a field trial. This may include climate, altitude, soil properties, slope, vegetation and past management practices. The selected site needs to be uniform in slope, soil, sun exposure, past management, etc. The location should be easily accessible for timely monitoring and data collection and protected from domestic and wild animals. On-station trial sites are usually fenced. It is often impossible to find trial areas that fulfil all the criteria listed above, thus compromises must be made. In many cases, conditions at research stations are not typical of the target area or only cover a small part of the target area. This is one reason why on-station research often needs to be verified by on-farm trial results.

The selected site for the field trial should be cleared and soil prepared to a fine tilth before demarcating plots and planting. A composite soil sample can be collected over the experimental area using a soil auger to collect samples to a depth of 15–30 cm. At least one sample per plot should be collected and all the cores bulked if the soil appears similar. If there are obvious differences, only soils of the same appearance should be bulked. The sample(s) can be analysed for nitrogen, phosphorus, soil pH, cation exchange capacity (CEC) and texture to quantify general characteristics. Rainfall and temperature (maximum and minimum) records should be kept throughout the experiment.

2.2 Experimental treatments

Field trial experiments compare two or more treatments. Treatments could be different forage species, accessions of a given forage species, different levels of fertilizer, different frequencies of weeding, intercropping of grass species with legume forages or browse species, and so on. The choice of treatment depends on the objective of the trial. It is important to ensure that the results from the trial meet the objectives of the experiment and help to solve the identified problem. Thus, based on site characteristics and research objectives, for example, accessions may be pre-selected using information available in genebank and public databases such as ILRI (Addis Ababa, Ethiopia), Alliance of Bioversity International and CIAT (Cali, Colombia), APG (Adelaide, Australia), IITA (Ibadan, Nigeria) and CIFOR-ICRAF (Nairobi, Kenya), or by referring to published literature. Other databases such as the Tropical Forages selection tool (www.tropicalforages.info) and the Genesys plant genetic resources database (https://www.genesys-pgr.org/) could also help in identifying forage species and accessions for a given agro-ecology, crop management and production system.

In a field trial, a control treatment should be included. There are two types of control treatments: (i) Local control (e.g. a local variety in a variety trial, the plant spacing used by most farmers, local tillage methods, local weeding practices); (ii) Standard control (e.g. standard check, no weeding in a weeding trial, no fertilizer in a fertilizer trial). If a control treatment is not included, it will be difficult to evaluate the potential and benefits of new technologies

because we do not know if they are better than the local methods or the none-use of production inputs. For example, in a variety evaluation, widely adapted commercial cultivars such as *Stylosanthes hamata* cultivar Verano and for *Lablab purpureus,* the cultivar Highworth.

2.3 Seed preparation and multiplication

Before implementing a trial, the seeds of the tested genotypes should be cleaned of straw, gravel, weed seed and other impurities as well as sorted to eliminate small, deformed and visually different grains. Testing the germination percentage is carried out to help calculate the number of seeds that need to be planted and to see if the seed batch is suitable for the planned trial. A germination/viability test may be advisable if seeds are sufficient. Seed dormancy can be a problem in grasses which may need to be planted at a higher rate because the viability is often low. For some species it may also be necessary to treat the seed to help them germinate. The kind of treatment depends on the species and variety. Hard-seeded legumes require scarification before sowing (see Appendix I).

To improve establishment in areas where labour is not a limiting factor, it is possible to germinate seeds in petri dishes in the laboratory (Figure 2) and raise seedling in pots for transplanting. Similarly, for small-seeded legumes, pre-germination of seeds in petri dishes/trays and raising seedlings in pots is preferable to obtain the required stand count. The trial can be carried out for one or two years, during which time seeds can be collected from known mother plants (of bagged flower heads) for use in larger replicated trials.

Figure 2: Pre-germination of cow pea seeds on trays.

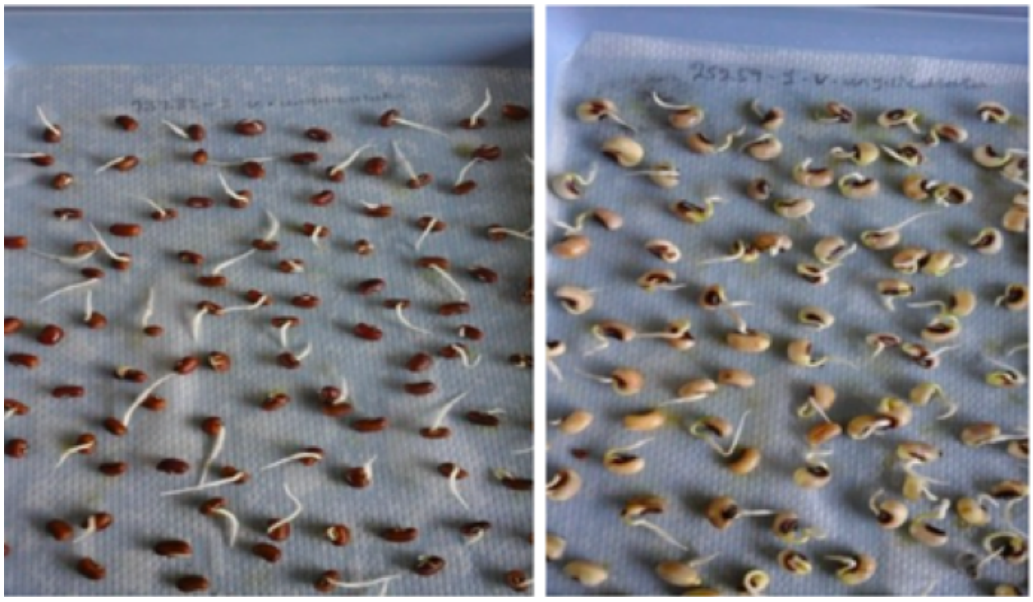


Photo credit: ILRI/Yirsaw Wubete.

2.4 Small plot observational trials

The number of seeds available for research is often a limiting factor and determines the experimental design. Small-plot observation trials are useful for evaluation of germplasm represented by quantities of seed too small to be sufficient for screening large numbers of plants (at least 20 plants). Alternatively, the researcher could carry out seed multiplication ahead of the main trial when the received seeds are low in quantity.

The small-plot observation trial can be carried out with very small amounts of seeds. For small-seeded legume species, such as *Stylosanthes* spp. or *Desmodium* spp., use 1 g of seeds on a 1 m^2 plot, giving a maximum of 100–150 plants per plot or row. Larger seeds such as *Centrosema* spp. or *L. purpureus* require a greater quantity of seed to

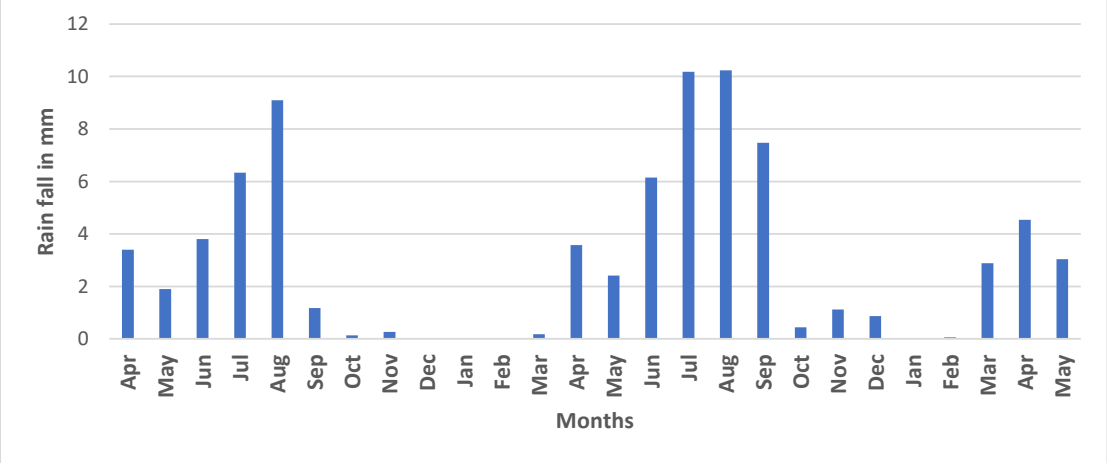
obtain an equivalent plant density per plot. Smaller amounts can be accommodated, right down to a few seeds which may also be raised in pots and transplanted to the field as spaced plants. The productive genotypes selected from these small plot trials could then be used for subsequent variety trials. Planting materials from those selected genotypes would be harvested for that purpose.

Figure 3: Field plots for a small-plot observation trial (left) and variety test experiment in larger plots (right).

Photo credit: ILRI/Ermias Habte.

For screening large numbers of accessions, a simple lattice design or augmented design, such as a partially replicated design (p-rep design) using row by column (Habte et al. 2022), are preferable to minimize spatial error. For the evaluation of a few promising accessions (Figure 3), a complete block design is common. For annual forage crops, evaluation is conducted during the main rainy season while for perennials, evaluation is done during the wet season (maximum precipitation or main rainy season) and dry season (minimum precipitation) of the year to assist in the identification of productive lines that provide feed to livestock during the dry season. For example, the ILRI field site in Bishoftu, Ethiopia (2018-2020) in Figure 4 presents the periods of maximum and minimum precipitation in the area.

Figure 4: Rainfall in Bishoftu, Ethiopia (2018–2020).



For better plant establishment, planting a field trial is commonly practised at the beginning of the main rainy season. For perennial forage crops, the first 12 weeks make up the establishment period during which data for germination and soil cover is collected. The second period of evaluation will be during the period of minimum precipitation (dry season), commencing at the end of the wet season for 12 weeks. The third period is the period of maximum precipitation (wet season) which commences near the beginning of the rains (at least when the rain is well established to include the wettest months) and lasts for approximately 15 weeks depending on the field trial

site climatic conditions. The second and third periods of evaluation commence with a standardization cut (all plots, including the sampling area and border rows, are cut down to a standard height as defined below) followed by productivity measurements as detailed below. For perennial forage species, two seasons, each of maximum and minimum precipitation, should be recorded.

2.5 Selection of experimental designs

The assignment of treatments to different plots in the trial area depends on the experimental design. It is very important to follow the principles of the experimental design because it will facilitate the correct assessment of the trial. The major components in experimental design comprise replication, randomization, blocking and experimental units. Thus, proper consideration must be given to these components when selecting the experimental design. Factors such as soil heterogeneity, type of treatments, number of treatments and the degree of precision needed also determine the number of replications, number and size of blocks, and number and size of plots.

The most common design used for evaluation of crops under field trial is discussed in Chapter 6.

2.5.1 Plot establishment

1. Plots are developed; either single row or more than a row can be used depending on the available land size.
2. For single row, depending on plant spacing, a row length of up to 3 m can be used or single plot with 0.5 x 2.0 m for the selected design with two replicates with 0.75 m to 1 m between plots. Increasing the number of replicates to three would increase precision if there is enough seed and experimental land.
3. Plant the seeds in the centre of each plot.
4. Space small numbers of seeds along the row and mark their planting sites with small sticks.
5. Plant a border row around blocks.
6. Apply fertilizer based on the need of the forage species. For legume species starter fertilizer such as Di Ammonium Phosphate (DAP) and inoculation[1] are recommended.

2.5.2 Data collection

1. Establishment

   Seedling counts should be made two to eight weeks after planting, depending on the species, to provide an estimation of emergence.

   - Count the total number of seedlings in the row to determine plant density per square metre.
   - Estimate the soil cover (percentage of the plot covered by the plants).
   - Re-fill missing seedlings by transplanting/sowing.

2. Disease and pest incidence
   - Note any obvious disease or pest problems by recording the incidence and severity[2].

1. Inoculation can be carried out during sowing by mixing the inoculant in peat with the seeds. This method cannot be used for seeds pre-treated with chemicals and so in these instances, the inoculant in peat should be mixed with water and sprinkled on the rows once the seedlings have germinated (alternative methods of inoculation are highlighted in Appendix II).

2. Incidence is the percentage of the plot infected and severity is the percentage infection on those plants showing symptoms. Both should be scored on the scale 0, 5, 10, 25, 50, 75 and 100%.

- Score these problems at regular intervals beginning from establishment and every eight weeks thereafter.
- Send diseased specimens to the germplasm health unit (GHU), or equivalent, for disease/pathogen identification.

3. Flowering and seed production
   - Monitor the plots for flowering and seed production by recording the month in which 50% of the plant canopy flowers produce seed.
   - Collect ripe seeds every two to three days; weigh and record the total for each month. It may be necessary to protect grass seeds from bird or rodent damage.

4. Biomass production
   - At the end of the growing season count the number of plants, and then cut half of each plot at 5 cm above the ground for perennial grasses and legumes which readily tiller from the crowns, up to 15 cm above the ground where two nodes are required to enhance regrowth.
   - Fresh weigh the plant material collected immediately after plant harvest. For dry matter estimation either the whole sample is oven dried or estimated from a subsample taken from the harvested samples and dried in a drying oven to determine the dry matter yield. This can then be converted to grams (g) dry matter/plant or kg/ha (if the number of plants varies tremendously) for comparison. For grasses such as Napier grass, an additional subsample will be separated into leaf and stem, to weigh the fresh and dry weight of leaf and stem tissue thus the leaf to stem ratio can be calculated by dividing leaf dry weight by stem dry weight.

5. Drought tolerance
   - During the dry season, score the plots for drought tolerance by recording the percentage of plants in each plot that remain green or retain their leaves every six weeks. In addition, physiological measurements such as water use efficiency (WUE) and photosynthetic efficiency are also good indicators for screening drought tolerance.

2.6 Procedure for cropping system trials

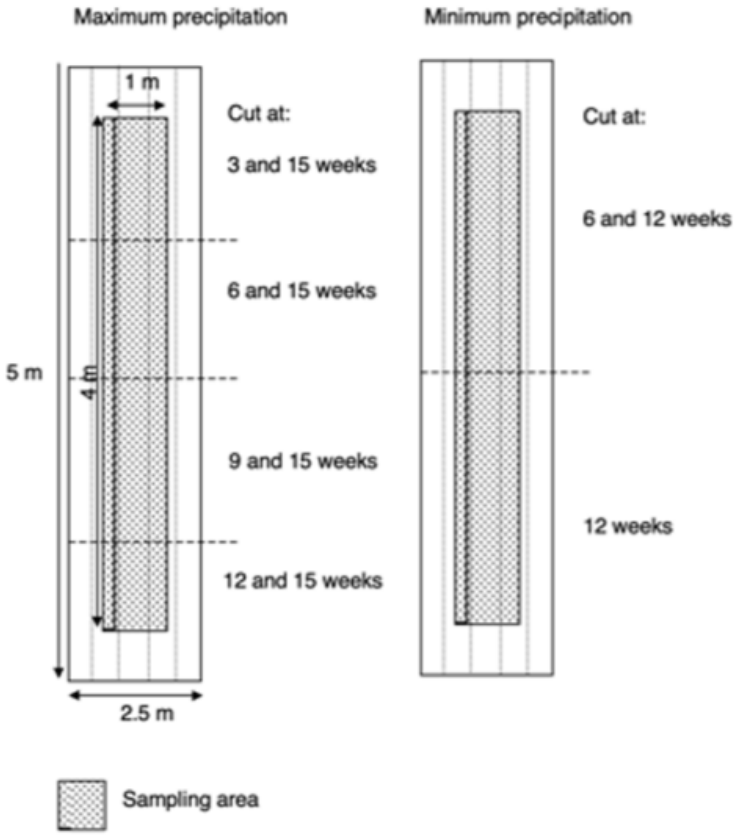
2.6.1 Plot establishment

1. Lay out 2.5 x 4 m plots with four rows of 5 m length spaced 0.5 m apart in a randomized complete block design with a split-plot arrangement (main plot = accession; subplot = harvest time) with three replicates.
2. The two central rows form the sampling area. Divide them into two or four subplots for evaluation in the dry and wet seasons, respectively (Figure 5).
3. Establish grasses and herbaceous legumes separately within the blocks and treat them as separate experiments.
4. Plant seeds evenly along the rows.
5. Apply fertilizer to both grasses and legumes at a rate of 20 kg/ha phosphorus (P) as single superphosphate (SSP) in a 20 cm band on each row at the time of planting.

6. Apply nitrogen (23 g urea in each 20 cm x 5 m band) to the grass plots eight weeks after sowing.

7. Hand weed the trial plots throughout the experiment.

Figure 5: Plot layout for standard evaluation procedure.



Note: Cutting times are times after the standardization cut at the beginning of each period.

2.6.2 Data collection

1. Establishment period
   The evaluation should be done on an area of 1 m^2 within the two central rows (sampling area) of each plot.

2. Germination and soil cover
   - Count the plants.
   - Estimate the soil cover by dividing the 1 m^2 quadrat into 25 squares of 20 x 20 cm using string[3].

3. Disease and pest incidence
   - Estimate the extent of any signs of insect attack or disease incidence as described for the small-plot observation experiment.

4. Biomass production
   - Cut 1 m^2 within the sampling area at the end of the 12-week establishment period[4].
   - Cut prostrate species at a height of 5–10 cm and erect or semi-erect species 10–15 cm above the soil.

3. This makes the estimation of soil cover easier in the early stages of growth. Once the plants are larger and the observers more experienced in making cover estimates, the whole 1 m^2 quadrat can be used again.

4. In some regions this cut will coincide with the standardization cut at the end of the wet season covering the period of minimum precipitation, whilst in other regions there may be time for an eight-week period before the rain stops and the standardization cut is made.

- Weigh the fresh material immediately (Tot FW).
- Accurately weigh a sample of 150–200 g fresh material (FW ss), oven dry it at 65°C and reweigh (DW ss) to give an estimate of the dry matter production during this period.
  Dry matter production is calculated as:
  (Tot FW x [DW ss/FW ss]) x 10 = dry matter kg/ha
  where:
  Tot FW = total fresh weight from 1 m^2 in g
  DW ss = dry weight of the subsample in g
  FW ss = fresh weight of the subsample in g

5. Feed nutrition composition
   Depending on the availability of laboratory facilities, feed quality analysis can be conducted using conventional wet chemistry procedures or near infrared spectroscopy (NIRS). Although NIRS analysis is faster and cheaper, the instrument needs to be carefully calibrated. In addition, the value of traits is predicted from the established equations that are standardized against wet chemistry analysis. For analysis, oven dried ground samples are used. The quantified feed quality traits will be presented as a percentage of dry matter (DM). The important feed quality indicators include fibre components (such as neutral detergent fibre, acid detergent fibre and lignin), crude-protein and digestibility. Generally, forages harvested at an early vegetative stage will have relatively higher digestibility and lower fibre components compared to more mature forages.

6. Fibre components
   Neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin contents of forages are key indicators used to predict forage intake and measure energy levels and livestock performance. NDF in forage materials is measured by boiling the forage in a neutral detergent solution. Similarly, ADF is determined gravimetrically as the residue remaining after acid detergent extraction, while lignin is quantified gravimetrically after the ADF residue is extracted with 72% H_2SO4 and ashed.

7. Crude-protein content
   To determine the crude-protein content, dried and milled samples are used for analysis using the Kjeldahl method[5]. Bulk replications to reduce the number of samples for analysis.

8. Digestibility
   Determination of the digestibility parameter provides an indication of the potential contribution of the plant material towards animal nutrition (Ørskov and McDonald 1979). Use an *in vivo* estimation using the nylon-bag technique (Osuji et al. 1993) with one incubation period of 48 hours for each experimental treatment. Alternatively, if the facilities are available, *in vitro* dry matter digestibility may be measured (Tilley and Terry 1963).

2.6.3 Period of minimum precipitation

Whole plots (including the border rows) should be cut at the end of the wet season at the recommended cutting heights. This is the standardization cut for the period of minimum precipitation. Half of each plot should be cut after six weeks regrowth and both halves of the plots should be cut after another six weeks (Figure 5).

1. Harvest a 1 m^2 quadrat within the sampling area at each cut as described above (yield estimation) to determine dry matter yields.
2. Cut down the remainder of the subplot.
3. Record soil cover, % green leaf and disease/pest incidence and severity at each evaluation.
4. If resources are available, analyse for feed digestibility.

5. Some indication of crude-protein content is useful when designing further evaluation trials where one may need to know how much crude-protein can be provided by the various accessions.

Period of maximum precipitation
For this period, the sampling area is divided into four subplots which should be cut 3, 6, 9 and 12 weeks after the standardization cut at the beginning of the period of maximum precipitation.

1. Weigh the material harvested from the 1 m^2 subplot (total fresh weight).
2. Take a subsample of 200–300 g fresh weight, oven dry it at 65°C to a constant weight and reweigh to determine dry matter production.
3. Analyse feed quality and digestibility of the dried samples if facilities are available.
4. Cut the border rows of the subplot after harvesting the quadrat.
5. Harvest all four subplots 15 weeks after the standardization cut, thus giving another set of regrowth ages depending on the nature of the forage species.
6. Record soil cover and insect/disease incidence and severity at each harvest.

2.6.4 Flowering and seed production

In addition to the three replications used to monitor productivity, a fourth single-row replication should be planted to enable flowering and seed production to be observed.

1. Plant a single 5 m long row of each accession, with 10 plants spaced evenly along the row. Provide climbing accessions with trellises.
2. Monitor flower development to control out crossing. In cross-pollinated species, cover the flower head/stem with a pollination bag before the initiation of flowering or pollen shedding (Appendix III).
3. Collect ripe seeds every three days if the species is self-pollinated. For cross-pollinated species, seeds can be collected if the minimum isolation distance is kept between genotypes of the same species.
4. Weigh the seeds as collected to establish the period of peak seed production.

The trial described above will enable the identification of promising material for wet and dry season use. Such material can then be incorporated into further trials as appropriate to the farming system of the target area (Chapter 4).

3 Field trial methods for fodder trees

A scheme for screening fodder trees is proposed based on the experiences from the ILRI/ICRAF/IITA collaborative multi-purpose tree evaluation research activities in southern Nigeria (Ibadan and Onne). Two stages of field trials are described within the initial evaluation section. In Stage I, a large number of accessions may be screened for edaphic and climatic adaptation. In Stage II, elite genotypes from Stage I are screened for response to different management techniques and forage quality. A few selected accessions may be subjected to more detailed evaluation as described in Chapter 4.

3.1 Stage I: Screening large numbers of genotypes

3.1.1 Establishment

1. Scarify the seeds (see Appendix I), inoculate if necessary (Appendix II) and plant in well-drained nursery bags or beds about 10 weeks before the major rains.
2. Transplant seedlings into the field when the rains have stabilised. The minimum height for transplanting should be 40–50 cm.
3. During planting, apply 15–20 g of 15:15:15 N:P:K compound fertilizer around each plant. This may not be required on fertile soils.
4. Plant seedlings of each accession in 6 m long single rows, with 3 m between rows and 0.5 m between plants in a row[6] using a completely randomized design with three to four replicates, depending on the availability of seedlings.
5. Slash inter-row spaces to reduce weed competition.
6. Replace seedlings which fail to germinate properly in the first eight weeks after transplanting with spare plants from the nursery.

3.1.2 Data collection

1. Plant height
   Measure the height of all the individual plants in each replicate with a metre stick every eight weeks and record the average for each replicate.
2. Seedling survival
   Count seedlings 3 (SN_3) and 12 (SN_{12}) months after transplanting.

6. This spacing is recommended to accommodate expected variation between species and determine yield per plant rather than yield per unit area.

Calculate the percentage of seedling survival (%SS) during the establishment year as:

$$\%SS = \frac{SN_3 - SN_{12}}{SN_3} \times 100$$

3. Disease and pest incidence
   - Score disease and pest incidence using the same system as described for herbaceous legumes and grasses (Chapter 2), based on percentages of incidence and severity.
   - Record observations every four weeks during the first year of establishment.

4. Green-leaf retention
   - Determine green-leaf retention each year during the dry season from individual plants that are not pruned.
   - Estimate the percentage of green leaf by comparing with a plant with 100% green leaves.

5. Biomass production
   - Take the first harvest when plants are 12 months old. Subsequent harvest periods should be based on phenology and climatic conditions[7].
   - At each harvest, cut the plants in the middle of the row to 0.5 m above the ground.
   - Sort materials into leaf (leaf plus fine stem up to 6 mm in diameter) and stem (greater than 6 mm in diameter).
   - Fresh weigh this material in the field, subsample, and oven dry at 65°C to a constant dry weight for the determination of dry matter.

6. Dry-season nitrogen
   If facilities are available, grind the dried biomass samples and analyse for nitrogen using the conventional Kjeldahl method.

7. Dry matter degradability
   Analysis of dry-matter degradability provides an indication of the potential contribution of the plant material towards animal nutrition (Ørskov and McDonald 1979).

3.2 Stage II: Forage value evaluation and management trial

Based on the biomass and quality-related attributes assessed in the initial screening trial, promising genotypes can be selected for further screening. At this level, dry-matter production and feed quality can be assessed under various management regimes, such as cutting height or frequency.

3.2.1 Establishment

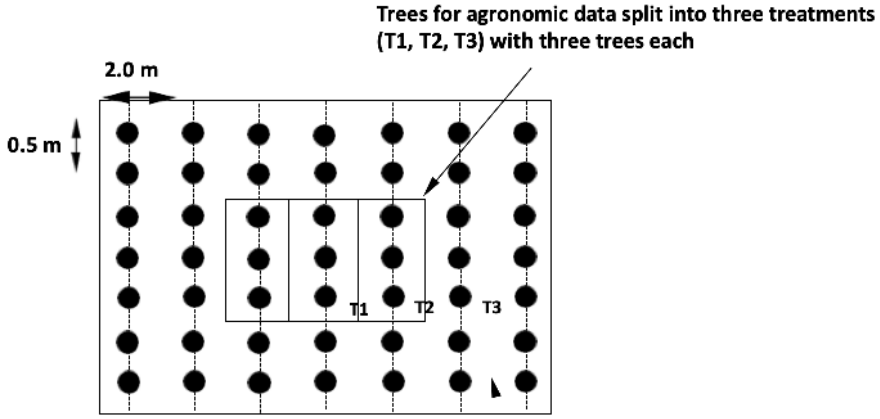
Establishment methods are similar to those described in Chapter 2.

- Plant each accession in a plot of five or seven single rows, with 2 m between rows and 0.5 m between plants in a row. Each accession should be replicated three or four times (Figure 6).
- Impose management treatments (defoliation heights or defoliation frequencies) within the plot to give a split-plot design.

7. For example, in Ibadan, harvests are taken at the end of the main wet, minor wet and dry seasons.

- Determine the agronomic data (biomass production) and nutrient content from the central rows (Figure 4). Use the outer rows for animal feeding trials if facilities are available.

Figure 6: Plot layout for small-plot management trial fodder trees



The diagram shows one plot of one accession, each plot will be replicated three or four times and the experiment set out in randomized complete block design. Border rows can be harvested for animal trials. Some trees may be left to monitor dry-season performance.

3.2.2 Data collection

1. Biomass production
    - At each harvest, cut plants in the central row according to the agronomic treatments imposed and partition into leaf (leaf plus fine stem up to 6 mm in diameter) and stem (greater than 6 mm diameter).
    - Fresh weigh this material in the field, subsample and oven dry at 65°C to a constant weight for the determination of dry matter.
    - Cut plants outside the sampling area to 0.5 m above the ground, bulk by accession and partition into leaf and stem.
    - Use the leaf portion for assessment of forage quality (see digestibility below).
2. Chemical composition
    - Analyse feed quality of the dried and ground biomass samples (leaf and stem separately) using NIRS and specifically developed calibrations.
3. Intake and animal performance
    - If facilities allow, and sufficient material is available, bulk the herbage cut from the outer rows by replication and use to feed animals for intake, preference and animal performance assessment.

4 Schemes for further evaluation

The initial evaluation identifies accessions with high biomass productivity and potential for use as fodder. However, these accessions still need further evaluation to measure the response to defoliation under grazing or cutting, seed multiplication potential and performance in on-farm situations.

The methods described in this section are used to evaluate smaller numbers of selected accessions in trials related to their use and management in farming systems. The appropriate trial design should be selected based on the end use of the forage, farming system and agro-ecological zone. Only accessions identified as promising during the initial screening (sections 2.4 and 3.1) should be used for these trials.

4.1 Small plot grazing trial

The aim of this trial is to research the performance of selected forage material under grazing. The experiment is designed for cattle, but small ruminants could be used equally well. If facilities allow, oesophageal-fistulated animals could be used to monitor diet selection and rumen-fistulated animals used for nylon-bag digestibility studies (Osuji et al. 1993).

4.1.1 Trial establishment

The recommended trial design is suitable for the evaluation of six to eight selected accessions. Plots of 4 x 5 m with 2 m between plots are a suitable size for grazing.

1. Plant accessions at recommended sowing rates in four replications using a randomized complete block design.
2. Scarify legume seeds using sandpaper before planting (Appendix I).
3. Mix seeds for each plot with 300 g single superphosphate (SSP; 18% P_2O_5 = 27 kg/ha P_2O_5).
4. If grass accessions are being evaluated, apply nitrogen as urea at the rate of 400 g urea per plot (= 42 kg/ha N).

4.1.2 Maintenance

1. Keep the inter-plot areas slashed down low but do not mound up the sides of the plots.
2. Do not weed the plots, but keep shrubs slashed and destroy termite mounds if necessary.

4.1.3 Grazing

1. If legumes are being evaluated, use four young cattle to periodically graze down the plots, starting with wet season grazing to control grasses.

2. Following establishment, observe the plots regularly. When grasses begin to dominate and are at least 10 cm higher than legume seedlings, introduce cattle into the plots to graze down the grasses.
3. Carefully monitor grazing and remove the cattle from the plots before they damage the legumes. Cut down any remaining grass so all the plots are equal.
4. Introduce the cattle every six weeks after this initial grazing, through the remainder of the wet season and into the dry season.
5. If grass accessions are being evaluated, an initial grazing is not necessary. Begin evaluation of grazing about four weeks before the end of the wet season and continue at six-weekly intervals.

4.1.4 Data collection

1. Establishment
   - Make replicated emergence counts on two quadrats of 1 m^2 per plot, four and eight weeks after establishment.
   - Estimate plant cover (sown accession/other legume/grass) using the same quadrats.
2. Sampling and biomass estimation
   - Cut one 1 m^2 quadrat at about 15 cm above the ground from each plot before each introduction of the animals.
   - Count the number of sown accession plants and divide the cut sample into sown accessions, other grasses and other forbs.
   - Record the fresh weight of each, oven dry the samples at 65°C and reweigh until you attain a constant weight.
   - Immediately after the animals have stopped grazing, cut another 1 m^2 quadrat from a different place, divide, weigh and dry as above.
3. Acceptability/palatability
   - Acceptability/palatability may be monitored in this experiment by estimating the time spent by animals on each plot. Oesophageal-fistulated animals could also be used to identify species preferences (Section 4.2).
4. Flowering
   - Record the date(s) when 50% of the plot had flowers.
5. Disease and pest incidence
   - The incidence and severity of pests and diseases on the sown accession should be recorded as described in Chapter 2.

4.2 Cut-and-carry trials

Cut-and-carry trials are suitable for estimating productivity of material used for zero grazing, or stall-feeding cattle and small ruminants. This is a common practice in many parts of sub-Saharan Africa. In these areas, population density is high and the demand for crop land leaves very little for livestock grazing. Cut-and-carry feeding is popular with smallholder dairy schemes in parts of Kenya and Tanzania. The usual practice is to cultivate high yielding grass and legume species, which are then cut in rotation on a daily basis and fed to animals maintained in enclosures. If shrubs or trees are used as fodder, these may also be cut daily and used as protein supplements to low-quality feed.

4.2.1 Trial establishment

The suggested trial design is suitable for evaluating three to five selected accessions. An estimate of how much fodder will be required per animal can be obtained on the basis that an animal will consume approximately 3% of its body weight (on a dry-weight basis) per day. If legumes are to be used as supplements with grass or as a basal diet, the legume should form about one-third of the feed on offer.

1. Prepare the land as described earlier (under initial screening methods). For cattle, 0.25 to 0.5 ha plots are necessary to meet the feed requirements of one animal for two to three months. Smaller plots of 0.10 to 0.25 ha per animal should be sufficient for small ruminants.
2. Leave a 2 m pathway around the plots to allow easy access for cutting.
3. Scarify legume seeds (Appendix I) before sowing.
4. To stimulate early development of legumes, apply 150 kg/ha single superphosphate at planting. Apply farmyard manure to promote growth of grasses.

4.2.2 Maintenance

Weeding during the initial establishment phase suppresses competition from weeds, but once the sown material is established this should no longer be necessary. Subsequent elimination of shrubs or very persistent/vigorous weeds may continue for a while.

Animals selected for the trial should undergo routine veterinary treatment such as deworming and vaccination before the trial.

4.2.3 Cutting and feeding forage

For feeding trials involving several accessions, use at least five cows or six to eight small ruminants per treatment. If the forage has a moisture content of over 80%, the material may be cut the day before feeding because wilting of such material can improve dry-matter intake. Material with a lower moisture content should be cut and fed on the same day. Chopping forage before offering it to the animals improves feed intake and limits selectivity.

1. Feed the animals 50% of their daily requirement in the morning and 50% in the evening[8].
2. Record the weight of forage offered and residue remaining at each feeding time[9].
3. Monitor the dry matter content of the forage offered and the residue to accurately estimate dry matter intake[10].
4. Weigh animals weekly or fortnightly. If lactation is also being monitored, record daily milk production and growth of suckling animals.

Accessions that are suitable for use in cut-and-carry farming systems are those which exhibit good regrowth in response to cutting, have a high biomass and produce good liveweight gains from the experimental animals.

8. Feeding twice a day improves intake. The first two weeks of the feeding regime should be regarded as the adaptation period during which the microbial population of the rumen and the whole digestive tract of the animals should adjust. Usually, at the beginning of the trial, animals tend to eat small quantities of the feed, but this increases as they become more familiar with the material. After about 10 days it is possible to predict the daily feed intake of the animals.

9. An animal is satiated from eating a forage if part of the daily ration (not the inedible fraction) remains. To minimize wastage, it is advisable to provide the estimated daily feed requirement plus 15% to ensure the animal is satiated.

10. Subsequent chemical analysis of the forage offered, plus the residue should provide a good estimate of nutrients consumed.

4.3 Seed multiplication trial

Seed multiplication potential of promising accessions should be monitored because planting material is essential for widespread use as a forage. Seeds are also required for further trials and on-farm testing. Accessions with poor seed-production capacity are not easily disseminated or accepted. The following experimental design may be used to assess the seed-production capacity of promising accessions. Seed-production plots can also be established to produce large amounts of seeds for further trials and release to farmers (Kachelriess and Tarawali 1994).

4.3.1 Trial establishment

Isolation is necessary when growing accessions of outcrossing species in the same area to ensure genetic purity. In the case of outcrossing and vegetatively propagated species such as Napier grass, bagging flower buds is essential to prevent cross pollination. Timely and properly bagged flower heads can also be used for targeted crossings to harvest hybrid seeds (Appendix III).

An isolation distance of 100 m is required when pollination is by insects and more than 400 m or temporal isolation is recommended when pollination is by wind. Separate apomicts and self-pollinating species by about 5 m between accessions to avoid mixing. Isolation may also be necessary to prevent the spread of insect pests and diseases between accessions. In this case, a distance of 100 m is recommended.

1. Lay out 4 x 5 m (or larger) plots in a randomized complete block design with four replicates of each accession with 2 m between plots for estimation of seed-production capacity.
2. Scarify legume seeds using sandpaper before planting (Appendix I).
3. Mix seeds for each plot with 300 g single superphosphate (SSP; 18% P_2O_5 = 27 kg/ha P_2O_5). If grass accessions are being evaluated, apply nitrogen as urea at a rate of 400 g urea per plot (= 42 kg/ha N).

Plots established for other trials can also be maintained and allowed to grow without further cutting or grazing to estimate seed-production potential.

Plant the seeds in rows to allow easier management at weeding, rogueing and harvest. Closer planting distances between rows ensures a good stand, reduces weeding and increases seed yield per hectare. As a general guide, sowing rates for seed-production evaluation are usually double those used for estimates of forage production. Legume accessions with creeping growth habits usually produce more seeds when grown on trellises. Wider spacing is required if seeds are produced on trellises.

4.3.2 Maintenance

Keep the plots well weeded to reduce both competition and the quantity of weed seeds in the final seed harvest. Spraying for insect pests and diseases may also be necessary to produce high quality clean seeds.

4.3.3 Data collection

Plants should be monitored for the onset of flowering, 50% flowering and the date of peak flowering.

There are two common methods for estimation of seed-production capacity.

Method I

1. Harvest ripe seeds every two or three days.
2. Determine the total seed-production capacity by measuring the weight of seeds produced per plot monthly.
3. Multiply the results of (2) to seed yield per hectare per year.

Method II[11]

1. Estimate the period of peak seed maturity and harvest the whole plot by cutting back.
2. Leave the foliage to dry then thresh it.
3. Calculate the seed yield per plot per unit time.

4.4 Evaluation of mixtures of accessions

In many cases smallholder farmers grow forages as mixtures and not as pure stands. It is therefore important to test the combining ability and yields of promising accessions in mixtures. Three types of mixtures can be considered for evaluation, namely grass–legume, legume–legume and fodder tree–herbaceous forage. Extensive research has been conducted on the assessment of productivity of mixtures of grasses and legumes and on alley cropping of fodder trees with herbaceous materials. However, evaluation of legume–legume mixtures is a relatively new research area in tropical countries. This approach is usually studied in temperate environments (Cupina et al. 2017).

4.4.1 Grass–legume

Further evaluation of performance in mixtures should only be done using accessions which have been identified as promising from initial trials. Evaluation is more straightforward if combinations of one grass and one legume are compared. The methodology described for the small-plot grazing trial (Section 4.1) or the cut-and-carry trial (Section 4.2) should be used.

To establish plots:

1. Plant the grass in rows 0.5 m apart, then broadcast the legume over the whole plot area.
2. Include controls of each grass accession alone and each legume accession alone.
3. If facilities are available, analyse for feed quality of the dried samples to give an idea of the feed value of the mixtures.

4.4.2 Legume–legume

Accessions of legumes identified through earlier evaluation can also be assessed in combination in a similar way. In this case, combinations of two or three accessions may be evaluated. A combination of a legume that is good in the establishment period with one that does well in subsequent wet seasons and one that does well in the dry season could give the potential for sustainable, year-round production. It is also recommended that legume–legume combinations are evaluated using the small-plot grazing trial (Section 4.1) or cut-and-carry trial (Section 4.2) methodologies already described.

11. This method is less labour intensive and therefore more economical, although a lower seed yield is obtained. In a species such as *Arachis pintoi* which produces subterranean seeds, this is the only possible method of harvest because the plants have to be cut back to collect the seeds by sieving from the soil.

4.4.3 Fodder trees–herbaceous mixtures

IITA and CIFOR-ICRAF developed screening methodologies to assess the agronomic potential of multi-purpose trees for alley farming or intensive feed gardens. Although these methodologies were developed to estimate productivity of herbaceous crop species with multi-purpose trees, they can be easily adapted for herbaceous forage species and fodder tree combinations. Accessions identified as promising from initial evaluation trials described in sections 2.4 and 3.1 should be used. Appropriate methodologies are described in Tripathi and Psychas (1992).

At this stage, an assessment of the value of promising accessions as livestock feed requires nutritional studies such as nylon-bag digestibility, if these were not conducted during the small-plot management trials. Details of these methodologies are presented in the feed evaluation manual (Osuji et al. 1993).

5 On-farm trials

The forages identified through initial evaluation and on-station research will ultimately be used on-farm. Conditions in local farms may be very different from those in research stations. For forages to be accepted, the promising accessions must also perform well under these conditions. On-farm research is necessary to test and validate technologies developed on-station and to adapt these technologies to farmers' field conditions. It is also useful for demonstration and transfer of technology. On-farm research should consider the biological performance of the forage and the socio-economic aspects of its adoption and role in the farming system.

On-farm research may take several different forms. It may be completely controlled by the researcher who lays out a replicated trial design on the farmer's field and controls management and collection of data. Farmer involvement can be increased so that the farmer and researcher take joint responsibility for the trial, with frequent visits and backup being provided by the researcher. On the other hand, the farmer may be completely responsible for the trial and therefore make decisions on the management and use of forage produced, although the researcher will make regular observations, collect data and provide relevant advice to the farmer.

The trial design for on-farm experiments must vary to fit the requirements of the farming system and individual farmer's needs and land availability. Researcher-controlled trials should use standard designs such as the grazing trial (Section 4.1) or cut-and-carry trial (Section 4.2). Simpler trial designs should be used as farmer participation increases. In experiments where farmers are used as replicates, the number of accessions, plot sizes and management should be uniform across all farms to allow for statistical analysis of results.

5.1 Planning an evaluation strategy

5.1.1 Selecting appropriate trials for farming systems and agro-ecological zones

The initial screening methods are suitable for all sites and should produce a uniform set of data which may be compared between sites. Whether legumes, grasses or shrubs are all evaluated at a site will depend on the aim of the evaluation, the farming systems, cultural practices and opportunity for intervention, along with the soil type and agro-ecological zone. The type of material required by the farmer for their livestock will therefore influence the selection of species and evaluation strategy. The researcher can then select the appropriate initial evaluation trial design from those suggested in this manual.

Subsequent evaluation needs to consider not only the type of material required, but how it will be used. For grazing in situ, evaluation using the small plot grazing trial (Section 4.1) would be most appropriate, whether for pure legumes or grasses, or combinations (Section 4.4). If zero grazing is feasible, the cut-and-carry trial (Section 4.2) could be used, again with pure or mixed introductions (Section 4.4). For shrubs, alley farming would be appropriate where crop–livestock integration is important, such as in areas of the humid zone. Intensive feed gardens could be useful in the humid zone and in drier areas (e.g. subhumid zone where no hardpan exists or semi-arid zone).

5.2 Animal feeding trials

Since the aim of forage plant evaluation is to improve livestock productivity, it is important to include animals as soon as possible in the evaluation procedure. However, this has to be balanced against the resources required to involve animals at an early stage of evaluation when many accessions are being tested. One valuable assessment of the potential of forage plants for animals can be obtained using the nylon-bag technique to assess *in vivo* rumen digestibility (Osuji et al. 1993). This method requires only a few animals with rumen fistulae and could be introduced as early as the RABAOC (Reseau de Recherche en Alimentation du Bétail en Afriqua Occidentale et Centrale) trial (Section 3.1) or the small-plot management trial (Section 2.4).

Oesophageal-fistulated animals may be used to monitor diet selection in the trials designed for further evaluation (Chapter 4). Animal preference for different forages can be assessed using either the difference or the occupancy-rate methods (Cook 1978; Owen-Smith and Cooper 1987; Koreth and Suth 1991). For the difference method, the forage on offer is sampled and weighed immediately before and after grazing to estimate the amount consumed (as described for the small plot grazing trial in Section 4.1). The occupancy-rate method uses a preference index to compare the amount of time spent on each species by recording which forage is being grazed at five-minute intervals throughout the grazing period.

Further details on methods of assessing feed value using animals are discussed in the feed evaluation manual (Osuji et al. 1993). Most of the research using animals should concentrate on the effect of the animal on the plants and the potential value of the material to the animals (crude-protein content, digestibility, presence of toxins etc.). Large-scale grazing trials in which animal productivity per se is monitored are very expensive. Such trials are usually not necessary in forage-evaluation programs since most of the material being tested is already recognized as beneficial to livestock. If a forage legume has good crude-protein content, is readily digestible, has no toxins and is readily accepted, then it should have a positive effect on animal productivity.

6 Experimental design and data analysis

6.1 Selection of appropriate experimental design

For screening large numbers of accessions, a simple lattice or augmented design, such as a partially replicated design (p-rep design) using rows by columns, are preferable in order to minimize spatial error. While for evaluation of a few promising accessions, a randomized complete block design can be applied. Initial evaluation involves screening large numbers of accessions to select the most promising ones. Although a graphical screening method exists, a method based on the analysis of variance is recommended. For initial screening of large numbers of accessions in a series of trials, a common control treatment (standard or check) is used. Separated analysis of variance and Dunnett's test will identify superior accessions from each trial. Dunnett's one-tailed test will test if any accession is significantly better (or worse) than the control. Promising accessions of interest can then be selected and evaluated at a second stage, using a randomized complete block design due to its simplicity and precision. The number of accessions to be tested in this case should not exceed 15 in one trial.

There are many different types of experimental designs: trials can be single- or multi-factor experiments, while treatments could be assigned to different plots in either a systematic or random manner. The most common trial design is the randomized complete block design (Section 6.1.1).

6.1.1 Randomized Complete Block Design

The Randomized Complete Block Design (RCBD) is used in experiments where:

- The number of treatments is small (usually <10).
- The soil fertility gradient goes in one direction.

The RCBD design is characterized by the following:

- Each block contains all treatments in the trial.
- The assignment of treatments within each block is random.

The RCBD design is easy to analyse and makes it simple to compensate for missing data. Any number of replications is possible, but the most common is eight.

Figure 7: Example of a RCBD design with five treatments in four replications.

Rep. 1	2	1	4	3	5
Rep. 2	2	3	5	4	1
Rep. 3	3	4	1	5	2
Rep. 4	4	2	1	3	5

6.2 Blocking

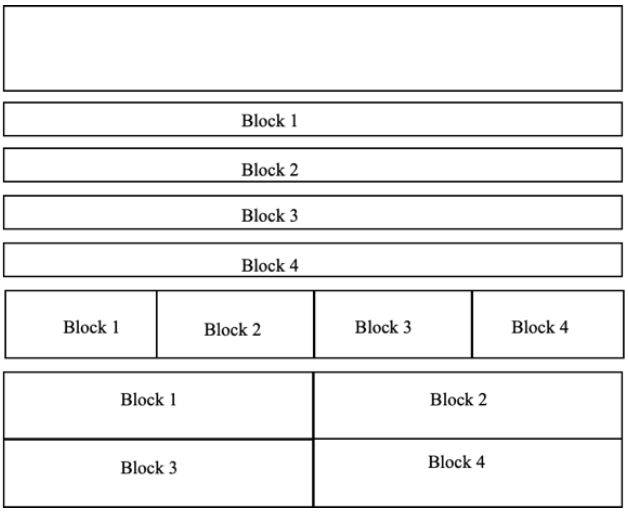
Blocking is done to help eliminate some of the problems caused by different production conditions within the trial area (especially soil fertility differences) (Figure 7). Blocking means assigning each set of treatments (replication) to a part of the trial area where conditions are relatively uniform. The aim of blocking is to minimize the difference between plots in one block, which means maximizing the difference between blocks. (Figure 8). With the help of statistical methods, differences between blocks can be distinguished from the effects of the treatments. Consequently, the comparison of treatments can be more precise. In an RCBD design, one replication (the full set of treatments) is assigned to one block (Figure 9).

Where the direction of the soil fertility gradient is known, the blocks should be placed so that the differences in soil fertility are as large as possible between the blocks. Consequently, the variation within a block is minimized. This may mean that one block has very fertile soil, another has infertile soil, and two others have intermediate soil properties.

When undertaking research on sloping land, blocks are normally long and rectangular and laid out across the slope direction. This is because erosion and drainage in the soil is likely to enhance the differences in soil fertility, moisture conditions and weed competition, especially between the top and bottom of the slope than between the 'left' and 'right' sides of the field.

Square blocks are best where the fertility gradient is not known or where it does not exist, which is often the case on flat land. A square block is likely to be best because adjacent plots are more likely to be homogeneous than plots that are further away from each other.

Figure 8: Examples of correct blocking under normal field conditions.

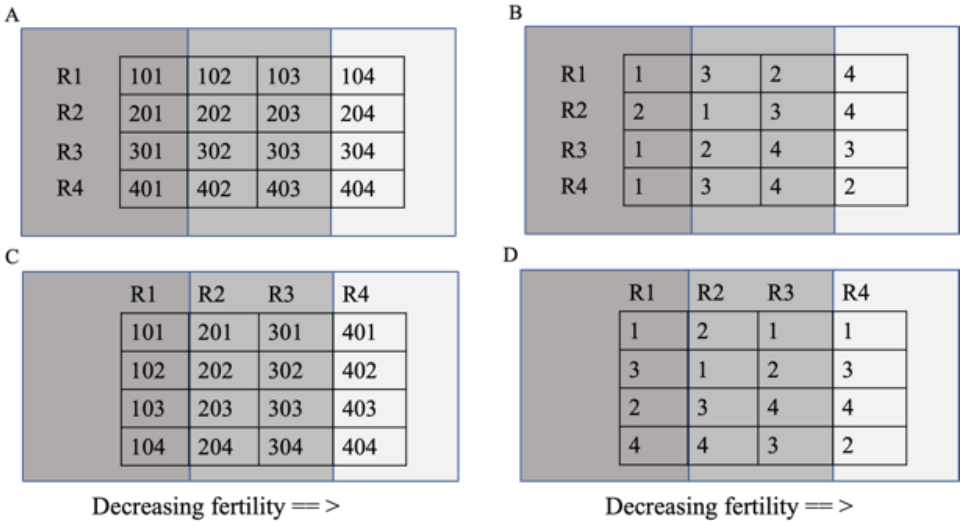


This is the correct blocking if the fertility gradient is going from the top to the bottom

This is the correct blocking if the fertility gradient is going from left to right

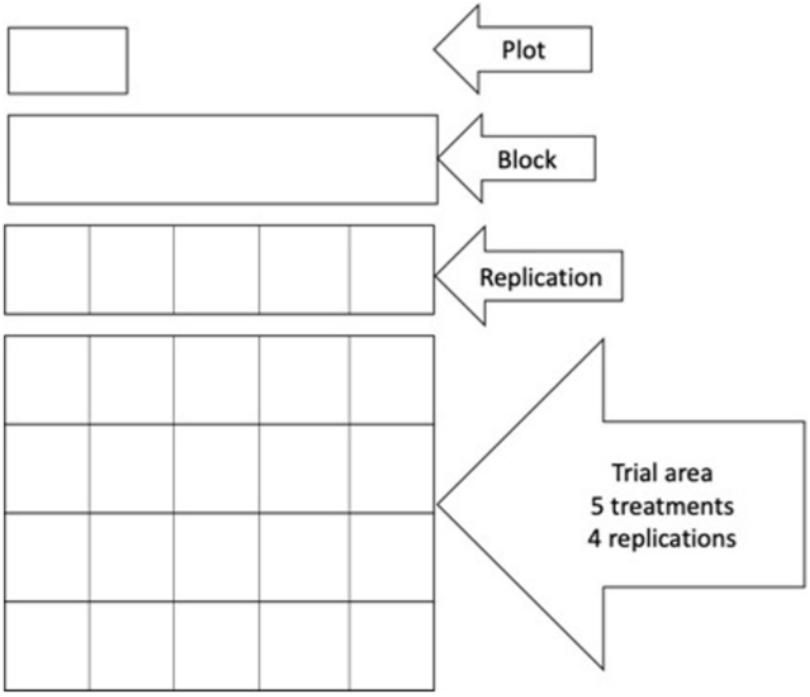
This is the correct blocking if the fertility gradient is both up and down and from left to right: or if there is no known fertility gradient

Figure 9: Example of inappropriate (A and C) and appropriate (B and D) blocking technique on land with one-way fertility.



Note: Darker areas indicate higher fertility

Figure 10: Representation of field trial entities: plot, block, replication and trial area.



When water is available, irrigation can increase yield during the dry season. If facilities are available, the drip irrigation system is preferred to the furrow irrigation system for effective water use and to uniformly irrigate plots. The irrigation interval is dependent on soil moisture content or meteorological data to measure the evapotranspiration rate.

6.3 Spatial analysis to reduce the effect of environmental factors on genotype response

Phenotype data such as biomass yield and other agro-morphological and nutritional quality traits recorded in field conditions are affected by many environmental factors that vary from plot to plot and between blocks in an experimental field. When estimating the genotypic effects and comparing genotypes, it is crucial to minimize the

environmental effect as much as possible. It is advisable to perform spatial analysis prior to data analysis and adjust the phenotypes for the environmental variability across rows and columns of the experimental field. Spatial analysis can be used to adjust the phenotype values based on environmental data acquired, such as soil moisture content, soil nutrient properties, multiple harvests, etc. The adjusted phenotype values will then be used in subsequent analyses and evaluations (Muktar et al. 2022).

6.4 Analysis of data

Analysis of data will depend on the experimental design selected and sampling method. Two distinct cases are presented below.

Case 1: Subplots are randomly allocated to each harvest time with one subplot being harvested at a time

A correct analysis for this type of sampling is to consider time of harvest as a subplot factor and analyse the data as a split-plot design with accessions as a main plot factor. In general, a format for an analysis of variance is as follows:

Source	**df**
Main plot comparisons	
Accessions (T).	t–1
Blocks (B)	b–1
Main plot error (T x B)	(t–1) (b–1)
Subplot comparisons	
Time of harvest (D)	d–1
Time of harvest x Accessions (D x T)	(d–1) (t–1)
Time of harvest x Blocks (D x B)	(d–1) (b–1)
Time of harvest x Accessions x Blocks (D x T x B)	(d–1) (t–1)

Often, the D x B and D x T x B sums of squares are pooled to give the subplot error. Before pooling, the size of D x B mean square and D x T x B mean square are compared. If the D x B mean square is less than the D x T x B mean square, then pooling is necessary. Correct standard errors for different comparisons should be derived accordingly.

Case 2: A single plot is repeatedly harvested

The methods described in this manual require accessions to be repeatedly measured for a series of preselected characters at regular intervals in the same plots. It is incorrect in this case to regard time as a split unit factor. The reason for such data recording is to examine how the effects of treatments vary with time (accessions x time of harvest interaction).

Three approaches for analysing repeated measurement data are available:

1. Straightforward analysis
2. Split-plot analysis (Greenhouse and Geisser's approach)
3. Multivariate analysis

The straightforward approach is recommended because of its simplicity and validity. The other approaches will therefore not be discussed in this text. With the straightforward approach, the scientist should specify relevant contrasts for predetermined tests. Time–trend analysis using linear, quadratic or higher order polynomial contrasts can be performed. A linear contrast tests whether there is a linear trend over time of harvest. T-test or simple analysis of variance are used to perform various tests.

6.5 Gene-environment interaction

The main objective of comparison between sites is to define genotype–environment interactions. Accessions can only be compared between sites if the following requirements are met:

- use of same experimental design
- common treatments (accessions) through sites
- same plot size
- same number of replications, if possible
- randomization of treatments is not standardized.

In line with evaluation of candidate genotypes or potential cropping systems, use a randomized complete block design with four replicates. Replicates are used within sites to provide an adequate estimate of experimental error from each site. They may be reduced if the number of sites is increased. For multi-locational analysis, only common treatments should be considered. Selection of sites should take into consideration environmental characteristics whose interactions with treatments are of interest.

Steps in a combined analysis include the following:

- Step 1: Perform individual analyses of variance of trials.
- Step 2: Select trials with the same residual error (same precision) by applying a test for homogeneity of variances (Bartlett's test, Hartley's test, etc.).
- Step 3: Perform combined analyses of trials with the same variances. In the resulting analysis of variance table, first look into the interaction of treatments x sites before drawing any conclusion.

The corresponding analysis of variance table is as follows:

Source of variation	**df**
Sites (S)	s–1
Blocks within sites	s(b–1)
Treatments (T)	t–1
Sites x treatments (S x T)	(s–1) (t–1)
Error	s(b–1) (t–1)
Total	sbt–1

In this table, sites and treatment effects are tested against the mean square of the sites x treatments interaction term. The remaining terms are tested using the error mean square term. Sites are not considered a random variable.

Stability analysis can be further employed to partition the interaction effects to rank treatment performance. Generally, stability refers to phenotypical performance such as biomass yield of a treatment/genotype with respect to different environmental factors over time. Treatments/genotypes that perform well and produce better biomass yield across environments are considered stable. Thus, the identification of best performing treatment/genotypes need to employ suitable analysis methods to assess consistency over time. As there is no ideal model for the stability analysis and to rank treatments/genotypes, several biometrical models have been proposed for use in partitioning genotype by environment interaction (GEI) and stability measurement across environments. For example, the additive main effective multiplicative interaction (AMMI) is used to assess GEI, and AMMI stability value (ASV) is used to measure stability. Hence, a treatment/genotype with lowest ASV is the most stable. In addition, yield stability index (YSI) measures the yield stability, so that a treatment/genotype with the lowest YSI value is considered as most stable and high yielding. YSI sums up the ranks of yield and ranks of yield stability as follows: YSI = Rank of ASV + rank of yield (Habte et al. 2022).

Other common biometrical models utilized for stability analysis include cultivar superiority index, Eberhart and Russel's coefficient of regression and Wricke's ecovalence models (Fasahat et al. 2015).

6.6 Grouping of similar accessions

Cluster analysis is a technique for grouping objects into clusters, so that objects with similar characteristics are in the same cluster. Cluster analysis is more useful as a classification tool than a screening tool. Principal component analysis can also help examine relationships among several quantitative variables.

6.7 Managing data collected from trials

The most convenient way to handle the data for all trial designs is to collect data using tablets which utilize file formats such as excel or other files such as DBase files which may then be used for different statistical software such as SAS, GenStat and R statistical analysis software.

- Suggested format for DBase file:
- SPECIES – species
- ACCN NO – accession number; use number only, no letters
- REP – replication
- GERM_4 – plant count (1 m^2) 4 weeks after sowing; GERM_8 – plant count, (1 m^2) 8 weeks after sowing; COVER_4 – soil cover (1 m^2) 4 weeks after sowing; COVER_8 – soil cover (1 m^2) 8 weeks after sowing
- FLOW_50 – date (numeric value, e.g. July = 7) of 50% flowering
- NO_PLTS – number of plants harvested
- TOT_DW – total dry weight of harvested plants
- DM_G_PLT – dry matter g/plant (use the REPLACE command and the previous two fields)
- SDS_7 – total seed weight for July
- SDS_8 – total seed weight for August
- SDS_ etc. – total seed weights for each month
- DGHT_12 – drought tolerance score December; DGHT_2 – drought tolerance score February; DGHT_4 – drought tolerance score April
- DISMAXI – maximum disease incidence recorded (%); DISMAXS – maximum disease severity recorded (%)

A DBase file in this form is convenient to use in a SAS analysis such as Ward's pattern analysis. If the number of accessions is small, they can also be divided up by simply sorting the DBase file based on the characters of interest.

6.8 Analysis of experimental designs in this manual

The experiments described in this manual are all simple, agronomic trials requiring randomized complete block or split-plot designs. Analysis is therefore straightforward and involves the use of means, analysis of variance and least significant difference (LSD) tests. Pattern or cluster analysis may be useful in the early evaluation stages if several accessions are used.

The following analyses are recommended:

RABAOC-style trial	Analysis of variance and LSD are sufficient. Harvest times can be considered as splits and the trial analysed as a split-plot design
Initial evaluation—shrubs	Analysis of variance, LSD
Small-plot management—shrubs	Analysis of variance, LSD
Grazing trial	For each grazing/sampling time, analysis of variance for each of the components- sown accession, grass, forbs- and an LSD for each group
Cut-and-carry trial	Analysis of variance, LSD
Seed multiplication	Statistical analysis not always necessary
Evaluation of mixtures	Analysis of variance, LSD

7 Developing genomics tools

Most tropical forage breeding projects currently rely heavily on selection based on field phenotyping/evaluations, which is time- and resource-consuming and imprecise (Lukuyu et al. 2012; Muktar et al. 2019). Besides the architecture of the species, reproduction schemes (mostly outcrossing nature), and perenniality contribute to slowing down the conventional breeding process. However, with the application of advanced genomic tools, there is the potential to significantly improve farmers' choice of varieties as well as open up opportunities for full exploitation of these species as alternative forage crops. Unfortunately, genomic tools have only been amassed for a few major grass species such as maize, wheat and rice, and the role of orphan crops such as Napier, Desho, *Brachiaria* grasses and lablab subsequently diminished to the fringes of research attention. Due to the significant reduction in cost of next generation sequencing (NGS) techniques, in the past decade, genomic information such as reference genomes have now been developed for key tropical species such as *Brachiaria* grass (Worthington et al. 2020), Napier grass (Yan et al. 2020) and lablab (Njaci et al. 2023). These reference genomes will allow implementation of genomic selection and gene editing approaches to reduce breeding time and expenditure. Furthermore, NGS can be effectively used to assess and quantify genetic diversity among genebank collections of forages.

7.1 Genotyping by sequencing

Recent advances in genotyping by sequencing (GBS) approaches provide a cost-effective method of identifying genome-wide molecular markers in species with limited genomic information and have been used as an alternative to whole genome sequencing. GBS produces a large amount of high-quality genome-wide genetic markers which are suitable for diversity analysis (Cruz et al. 2013), marker-trait associations (Muktar et al. 2022) and genomic prediction (Nguyen et al. 2018). The DArTseq sequencing technology is one of these GBS methods representing a combination of genome complexity reduction using restriction enzymes and next-generation sequencing (NGS). It also produces high-density genome-wide dominant (SilicoDArT) and co-dominant (SNP) markers (Kilian et al. 2012). To date, GBS genotyping has been performed at ILRI on key tropical forages such as Napier grass (Muktar et al. 2019), Buffel grass (Negawo et al. 2020), Rhodes grass (Negawo et al. 2021), lablab (Njaci et al. 2023), *Urochloa brizantha* (Muktar et al. 2022) and *Sesbania sesban* (Negawo et al. 2023). The genomic information developed for these species was used for genetic diversity analysis and association mapping studies where significantly linked molecular markers were identified for target traits such as high biomass, drought tolerance, protein content and aluminium tolerance (Worthington et al. 2020; Muktar et al. 2022; Njaci et al. 2023). Furthermore, the genome-wide molecular markers developed for these species can be used for molecular barcoding of genebank accessions for efficient management and utilization of genebank accessions.

7.2 Whole genome sequencing

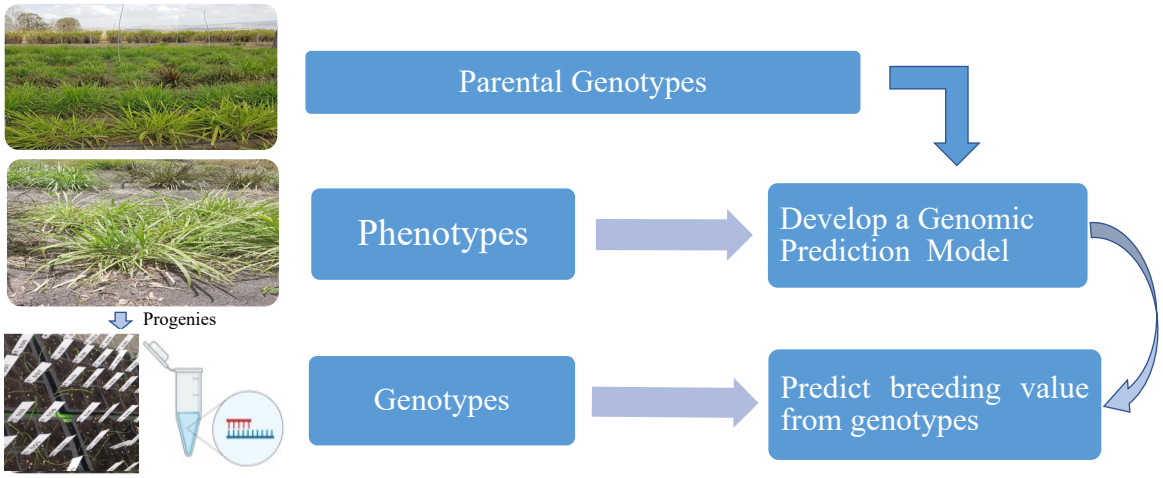
Another NGS technology is the whole genome sequencing (WGS) approach where we sequence the entire length of a genome, unlike the GBS approach where sections of the genome are genotyped. This approach is more robust and provides an overall picture of the genotyped accessions. When WGS sequence information is mapped against

a reference genome or against genotyped accessions, variant regions are detected that highlight areas of diversity which may be linked to performance and/or fitness. The WGS approach is being applied on selected forage species such as Napier grass and lablab, and millions of SNPs and indels have been detected. Developing whole genome information for these species is an ongoing project. Markers developed from these studies will also be used for diversity analysis and marker-trait association studies, and ultimately for marker-assisted breeding.

7.3 Genomic selection

The central element of developing genomic tools is to support the next generation of genomics-based breeding strategies for forages that are familiar with smallholders, require low inputs and are adaptable to different agro-ecologies. To provide smallholder farmers with superior and locally adapted varieties of forages, it is imperative to capture and preserve the widest spectrum of the species' natural genetic variation and to apply advanced plant breeding technologies for maximum exploitation of genetic diversity. Most of the stress tolerance–related and yield traits are polygenic (i.e. governed by few to many genes) and application of the traditional marker-assisted selection (MAS) approach is impractical for these quantitative traits. To overcome this challenge, a new selection tool known as genomic selection (GS) was proposed, originally on animal science (Meuwissen et al. 2001) that could facilitate selection, by using high-density single nucleotide polymorphism markers across the whole genome to predict genetic values. The main advantage of using GS is that it allows for a drastic reduction in the duration of the breeding cycle compared to traditional breeding, and minimizes the cost associated with extensive phenotyping, thereby accelerating genetic gains, and ensuring food and nutritional security (Figure 11). However, until recently, most key tropical forage species such as Napier grass, *Brachiaria* grass, Rhodes grass, etc. did not have a reference genome, thus preventing application of modern breeding tools such as genomic selection and marker-assisted breeding. The recent development of a genomic toolbox, including reference genomes for these species will pave the way for the application of GS and other breeding tools in order to develop varieties within a reduced time frame (Simeao et al. 2021).

Figure 11: Genomic selection application path for forages.



8 Challenges in field trials and how to cope with them

8.1 Soil heterogeneity

Differences in soil fertility can be a major cause of error in field trials. The soil heterogeneity may be of two kinds: a patchy distribution of soil fertility and a gradual change in fertility from one part of the field to another. In upland fields, especially recently cleared shifting cultivation fields, both types of soil fertility distribution are common. Ash deposits, termite mounds, decomposing roots, tree stumps, cow dung and intercropping are all likely to increase the patchy fertility distribution.

On sloping land, the fertility gradient usually follows the slope; fertility being higher in the lower part of the field. This is caused by erosion cum sedimentation, leaching of nutrients and internal drainage. These factors may also affect the competition from weeds and pests.

After land has been used for trials, the different treatments would often result in increased soil heterogeneity. Consequently, it may be necessary to stop further trials on that land for 1–3 years during which time the land is managed uniformly.

To reduce the interference of soil heterogeneity during trials, it is important to lay out the trials appropriately, with proper blocking and plot shape. Furthermore, statistical methodologies such as spatial analysis can be employed to adjust plot values using statistical software such as GenStat and R.

8.2 Border effect

Plants along the edge of a plot and in the centre of the plot usually perform differently (Figure 12). This so-called border effect is caused by:

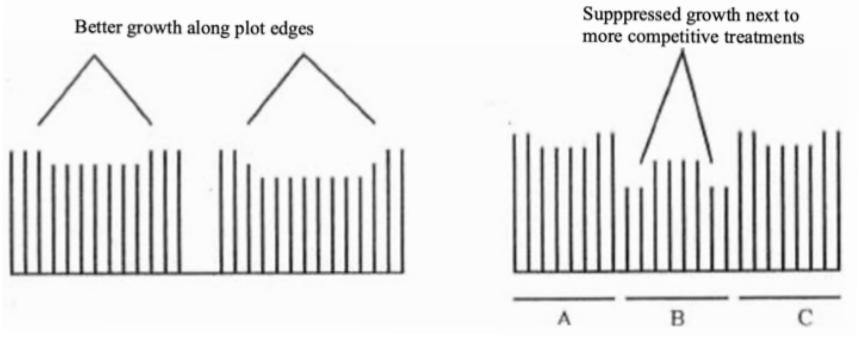
- Unplanted alleys between plots.
- Open areas surrounding the trial area.
- Adjacent treatments which vary in their competitiveness for light, water and nutrients.

It is important to minimize the border effects because the yields are not representative of the production conditions that occur in real fields. The border effect can be minimized by:

- Excluding 1–2 plant rows along the plot edges from the yield assessment and other data collection. These 1–2 rows are called the borders.
- Not using unplanted alleys between plots. If unplanted alleys are necessary to keep treatments separated and to facilitate access, the alley distance between plots can be set between 50–100 cm, depending on the availability of land and forage type under study.

- Planting 2–3 rows of plants around the edge of the experiment. These rows are known as guard rows.
- In the trial plan, extra area must be calculated for the guard rows. If unplanted alleys were used between plots, similar alleys should be put between the guard rows and adjacent plots.

Figure 12: Schematic view of border effects on plant height.



8.3 Missing plants and hills

In most field trials, uniform plant spacing and density is necessary to compare the treatments. However, gaps in the plant population often occurs because of problems with seed germination, pest attacks, careless weeding, etc.

Where there are gaps, the adjacent plants often grow much better due to more light, moisture and nutrients. The adjacent plants are therefore not representative of the real field conditions. Thus, the surrounding four hills or plants adjacent to the gap should be excluded from the yield measurement and any observation or sampling.

If more than 20% of the plants or hills are missing (including those adjacent to the gap) the results from the plot cannot be used. If 5–20% of the plants or hills are missing, it is possible to compensate for the loss by dividing the total yield by the number of harvested hills/plants and multiplying this figure by the planned number of hills/plants.

Example: If the yield was 360 from a plot where 100 hills were planted and 90 hills were actually harvested, the compensated yield would be 360 g x 100/90 = 400 g.

The risk of missing hills or plants can be reduced by:

- Testing the seed before sowing and adjusting the seed rate according to the germination percentage.
- Planting more seed than you need in the final plant stand, and thin out the surplus plants.
- Removing the least viable surplus plants.
- Re-sowing where gaps have occurred shortly after sowing.
- Transplanting plants from the guard rows.

8.4 Outlier results

When comparing the results of a treatment, the mean of one replication may be very different from the mean of the other replications. Such an unusual result will often make the whole trial non-significant in statistical terms.

However, it may be possible to eliminate the unusual result from the analysis. This requires that the unusual result be caused by an error in the trial implementation or by external factors, such as damage by domestic animals. Field notes taken during trial implementation may explain the cause of the unusual result. Elimination of the result also requires that there should be a significant difference between the unusual result and the mean of the treatment. An ‘outlier test’ is carried out to determine if a result is sufficiently different from the treatment mean to eliminate it from the statistical analysis.

An outlier test compares the standard deviation of the 'normal' results with the deviation of the possible outlier result from the mean of the 'normal' results. The unusual result can be considered an outlier if its deviation from the mean is more than four times greater than the standard deviation of the normal results. In that case, the unusual result can be eliminated from the statistical analysis. The plot result will then be considered 'missing' (Section 6.5).

Example:

In a variety trial using four replications, one variety yielded 50, 44, 47 and 22 kg per plot. The low yield in replication four may have been caused by a cow entering the plot early in the season. An outlier test is performed to see if the result is sufficiently different from the other replications.

The mean yield of replication 1, 2 and 3 is calculated: (50 + 44 + 47)/3 = 47
The standard deviation of the three replications is calculated:

$$\frac{(50-47)^2 + (44-47)^2 + (47-47)^2}{3-1}$$

$$\frac{3^2 + 3^2 + 0 = 9}{2}$$

The deviation of the possible outlier is: 47–22 = 25
Consequently, the unusual result is not an outlier, since its deviation is less than four times the standard deviation of the other replications (25/9 = 2.7).
If the unusual result had been as low as 10 kg, it would have been an outlier:

47–10 = 37
37/9 = 4.1

8.5 Missing plot data

Sometimes results are missing from one plot. This may be because of loss of data or because the result was an outlier. The missing plot data may be compensated for. In block design, this is done by using data from the other replications of the same treatment plus data from the other plots in the block where data is missing.

The following formula is used:

$$\frac{t \cdot T + r \cdot R - S}{(t-1)(r-1)}$$

t = number of treatments
T = sum of the results of treatments of the missing plot
r = number of replications
R = sum of the results of the block with the missing plot
S = the grand sum of all plots

Example:

Cowpea variety trial four treatments in four replications. Treatment 1 in block 1 is missing. Yield in kg/plot

Figure 13: Example of a cowpea variety trial with missing data.

4 **2.2**	**2** **3.2**	**1** **1.6**	**3** **1.8**
2 **3.4**	**4** **5**	**1** **1.9**	**3** **2.1**
1 **1.8**	**3** **2.3**	**2** **3.5**	**4** **2.6**
3 **2.3**	**1** **missing**	**4** **2.8**	**2** **4.0**

The missing plot data is calculated as:
(4^'*5.3+4^('*) 9.1-38)/(3*3) =2.2 kg

8.6 Residual effects

The different treatments of field trials will vary in their effect on the following crop. It is therefore sometimes best if a trial is followed by a period with no trials of 1–3 years. During those years, the land should receive uniform management to level out the differences caused by the original trial treatments. The area can be used for simple production, for propagation of seed, or it can be left fallow. The number of years the land needs to be kept out of trials depends on the impact the different treatments had on the plots. You may, for example, be interested in investigating the effect of fertilizers on the succeeding crops. In that case, all the plots that received different fertilizer treatments should be used for a single crop in the following years.

Another way of coping with a strong residual effect is by blocking the land in year 2 according to the treatments in year 1. This means that block 1 in year 2 will consist of the plots used for treatment 1 in the first year. Similarly, block 2 will consist of treatment 2 in the previous year, etc. Normally, the number of blocks in year two will be equal to the number of treatments in year 1, and the number of treatments in year 2 will be equal to the number of blocks in year 1. This kind of blocking is only suitable if the residual effect is stronger than the block difference in year 1.

Figure 14: Coping with strong residual effects.

Year 1						Year 2			
Block A	13		2	4		**Block A**			
Block B	2	1	4	3			Block A		
Block C	2	4	4	1					Block A
Block D	4	1	2	3			Block A		

Year 1						Year 2			
Block A	13		2	4		Block A	Block C	Block B	Block D
Block B	2	1	4	3		Block B	Block A	Block D	Block C
Block C	2	4	4	1		Block B	Block B	Block D	Block A
Block D	4	1	2	3		Block D	Block A	Block B	Block C

Year 2			
A3	C18	4	D2
B1	A4	D1	C3
B2	C2	D2	A2
D3	A1	B3	C4

8.7 Annual differences

Plant production varies considerably from year to year. Likewise, treatments that performed extremely poorly in one year sometimes perform very well in the second year. This is mostly caused by weather conditions and their interaction with other factors such as pests, weeds and timing of farm operations. This variation in performance is currently exacerbated by global warming leading to erratic weather conditions such as frequent droughts and flooding, every few years. It is therefore difficult to make conclusions and extension recommendations based on only one cropping season. A trial should therefore be repeated for 2–4 years, and possibly across different agro-ecological zones. Some modification can be made from year to year, but it is normally best to simply repeat the trial. This will ensure that a proper statistical analysis can be undertaken. Furthermore, it is important to keep a record of climate data during the running of the experiment so that the true genetic potential of the accessions/treatments can be estimated.

References

Cook, M.J.W. 1978. The assessment of preference. *The Journal of Animal Ecology* 805–816.

Cruz, V.M.V., Kilian, A. and Dierig, D.A. 2013. Development of DArT marker platforms and genetic diversity assessment of the US collection of the new oilseed crop *Lesquerella* and related species. *PloS One* 8(5): p.e64062.

Murphy-Bokern, D., Stoddard, F.L. and Watson, C.A. (eds). 2017. *Legumes in cropping systems*. CABI.

FAO (Food and Agriculture Organization of the United Nations). 1984. Legume inoculants and their use. A pocket manual jointly prepared by Nitrogen Fixation for Tropical Agricultural Legumes (NifTAL) Project, USA, and FAO Fertilizer and Plant Nutrition Service, Land and Water Development Division in association with FAO Crop and Grassland Production Service. FAO, Rome, Italy. 63 pp.

Fasahat, P., Rajabi, A., Mahmoudi, S.B., Bagher, M., Abdolahian, N. and Ras, J.M. 2015. An overview on the use of stability parameters in plant breeding. *Biometrics & Biostatistics International Journal* 5:149–159.

Gomez, K.A. and Gomez, A.A. 1984. *Statistical procedures for agricultural research*. New York: John Wiley & Sons.

Habte, E., Teshome, A., Muktar, M.S., Assefa, Y., Negawo, A.T., Machado, J.C., Ledo, F.J.D.S. and Jones, C.S. 2022. Productivity and feed quality performance of Napier grass (*Cenchrus purpureus*) genotypes growing under different soil moisture levels. *Plants* 11(19):2549.

Kachelriess, S. and Tarawali, S.A. 1994. Quality seed production of selected tropical pasture legumes in subhumid West Africa. *Plant Research and Development* 40:71–87.

Kilian, A., Wenzl, P., Huttner, E., Carling, J., Xia, L., Blois, H., Caig, V., Heller-Uszynska, K., Jaccoud, D., Hopper, C. and Aschenbrenner-Kilian, M. 2012. Diversity arrays technology: a generic genome profiling technology on open platforms. *Data Production and Analysis in Population Genomics: Methods and Protocols* 67–89.

Koreth, B.H. and Suth, J.W. 1991. Instantaneous intake rates of nine browse species by white deer. *Journal of Range Management* 44: 614–618.

Lukuyu, B.A., Gachuiri, C.K., Lukuyu, M.N., Lusweti, C. and Mwendia, S.W. 2012. *Feeding dairy cattle in East Africa*. Nairobi, Kenya: ILRI.

Meuwissen, T.H., Hayes, B.J. and Goddard, M. 2001. Prediction of total genetic value using genome-wide dense marker maps. *Genetics* 157(4):1819–1829.

Muktar, M.S., Debela, S., Negawo, A.T., Assefa, Y. and Jones, C.S. 2022. Genetic integrity and diversity in *Urochloa brizantha collection maintained* in the ILRI Forage Genebank. Poster prepared for the Tropentag 2022, organized by Czech University of Life Sciences, Prague, 14–16 September 2022. Nairobi, Kenya: ILRI.

Muktar, M.S., Habte, E., Teshome, A., Assefa, Y., Negawo, A.T., Lee, K.W., Zhang, J. and Jones, C.S. 2022. Insights into the genetic architecture of complex traits in Napier grass (*Cenchrus purpureus*) and QTL regions governing forage biomass yield, water use efficiency and feed quality traits. *Frontiers in Plant Science* 12:3116.

Muktar, M.S., Teshome, A., Hanson, J., Negawo, A.T., Habte, E., Domelevo Entfellner, J.B., Lee, K.W. and Jones, C.S. 2019. Genotyping by sequencing provides new insights into the diversity of Napier grass (*Cenchrus purpureus)* and reveals variation in genome-wide LD patterns between collections. *Scientific Reports* 9(1):1–15.

Negawo, A.T., Assefa, Y., Hanson, J., Abdena, A., Muktar, M.S., Habte, E., Sartie, A.M. and Jones, C.S. 2020. Genotyping-by-sequencing reveals population structure and genetic diversity of a Buffel grass (*Cenchrus ciliaris L.*) collection. *Diversity* 12(3):88.

Negawo, A.T., Akinmade, H.O., Muktar, M.S., Habte, E., Assefa, Y., Muchugi, A., Sartie, A.M. and Jones, C.S. 2022. Genetic diversity, population structure and subset development in a *Sesbania sesban* collection. *Plants* 12(1):1–13.

Negawo, A.T., Muktar, M.S., Assefa, Y., Hanson, J., Sartie, A.M., Habte, E. and Jones, C.S. 2021. Genetic diversity and population structure of a Rhodes grass (*Chloris gayana*) collection. *Genes* 12(8):1233.

Njaci, I., Waweru, B., Kamal, N. Muktar, M.S., Fisher, D., Gundlach, H., Muli, C., Muthui, L., Maranga, M., Kiambi, D., Maass, B.L., Emmrich, P.M.F., Domelevo Entfellner, J-B, Spannagl, M., Chapman, M.A., Shorinola, O. and Jones, C.S. 2023. Chromosome-level genome assembly and population genomic resource to accelerate orphan crop lablab breeding. *Nature Communications* 1:1915.

Nguyen, N.H., Premachandra, H.K.A., Kilian, A., and Knibb, W. 2018. Genomic prediction using DArT-Seq technology for yellowtail kingfish *Seriola lalandi. BMC Genomics* 19:1-9.

Ørskov, E.R. and McDonald, I. 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *The Journal of Agricultural Science* 92(2): 499–503.

Osuji, P.O., Nsahlai, I.V. and Khalili, H. 1993. *Feed evaluation* (Vol. 5). Nairobi: ILRI (aka ILCA and ILRAD).

Owen-Smith, N. and Cooper, S.M. 1987. Assessing food preferences of ungulates by acceptability indices. *The Journal of Wildlife Management* 51 (2):372–378.

Tarawali, S.A., Tarawali, G., Larbi, A. and Hanson, J. 1995. *Methods for the evaluation of legumes, grasses and fodder trees for use as livestock feed. ILRI manual 1*. ILRI (International Livestock Research Institute), Nairobi, Kenya.

Simeão, R.M., Resende, M.D.V., Alves, R.S., Pessoa-Filho, M., Azevedo, A.L.S., Jones, C.S., Pereira, J.F. and Machado, J.C. 2021. Genomic selection in tropical forage grasses: Current status and future applications. *Frontiers in Plant Science* 12:665195.

Tilley, J.M.A. and Terry, D.R. 1963. A two-stage technique for the in vitro digestion of forage crops. *Grass and Forage Science* 18(2):104–111.

Tripathi, B.R. and Psychas, P.J. 1992. *Alley farming training manual.* Ibadan: IITA

Worthington, M., Perez, J.G., Mussurova, S., Silva-Cordoba, A., Castiblanco, V., Cardoso Arango, J.A., Jones, C., Fernandez-Fuentes, N., Skot, L., Dyer, S. and Tohme, J. 2021. A new genome allows the identification of genes associated with natural variation in aluminium tolerance in *Brachiaria* grasses. *Journal of Experimental Botany* 72(2):302–319.

Yan, Q., Wu, F., Xu, P., Sun, Z., Li, J., Gao, L., Lu, L., Chen, D., Muktar, M., Jones, C. and Yi, X. 2021. The elephant grass (*Cenchrus purpureus)* genome provides insights into anthocyanidin accumulation and fast growth. *Molecular Ecology Resources* 21(2):526–542.

Yates, F. 1936. A new method of arranging variety trials involving a large number of varieties. *The Journal of Agricultural Science* 26(3):424–455.

Appendices

Appendix I: Seed scarification methods

Legumes (herbaceous and fodder trees)

Dormancy

Seed dormancy is a natural protective phenomenon which prevents premature germination of seed to ensure long-term survival of species. Dormant seeds are potentially viable seeds, but do not germinate promptly when placed under favourable conditions unless they are subjected to some special treatment.

There are two types of seed dormancy:

1. **Embryo dormancy:** The embryos are physiologically inactive due to intrinsic germination inhibitors and inactive enzyme systems. These need a period of dormancy after ripening.
2. **Dormancy due to seed coat characters:** Occurs due to a) seed coat mechanisms restrict expansion and growth of the embryo, b) prevention of the entry of gases and moisture, or c) chemicals in seed coats which inhibit germination.

Many forage legumes have seeds which are dormant because of their hard impermeable seed coats. Dormancy resulting from hard seededness is indicated by lack of imbibition. An imbibition of at least 60% (for irregular rainfall areas) and at most 90% (according to the seed trade) is expected. Hard seededness can be broken by thinning or breaking the seed coat. Three common scarification techniques are used to soften or break the seed coat. These comprise mechanical, chemical and heat scarification. Mechanical scarification is simple, affordable and safe, and is recommended for the majority of legume seeds.

Methods of breaking seed dormancy

Storage: Holding freshly harvested seed in storage will overcome dormancy problems since dormancy declines with time. Storage is often applied to grass seeds.

Scarification: Scarification treatments are employed prior to sowing to abrade the seed coat and improve permeability, e.g. most legume and some grass seeds such as *Cenchrus*, *Melinis* and *Paspalum notatum* require scarification.

Hard seededness varies between seed lots as well as within and between species. It is significantly affected by the pre- and post-harvest conditions. No one treatment can therefore be considered as a precise prescription, and it is best to test small quantities of seeds first, if possible.

Mechanical scarification

Methods of scarification

1. Physical hard-seededness (seed coat): breaking dormancy
   Mechanical scarification: Pricking, cutting or abrasing seeds results in facilitating proper gas exchange, removal of germination inhibitors or mechanical restraint using hot wire, knife, sandpaper and machine. Hot-water treatment: Immersion of seed in hot water; the temperature and duration of treatment depends on the species.
   - Physiological: breaking dormancy leaching or washing either with acid or water removes inhibitors.
   - Temperature treatments: prechilling or holding imbibed seeds in low temperature (0–100°C) for days or months. High temperature storage or pre-drying treatment at 400–500°C for several days or weeks.
   - Ageing
   - Scarify small quantities of seeds by hand using sandpaper.
   - Rub the seeds with sandpaper until one or two seeds break and damage to the seed coats is visible.
   - For larger quantities of seed, use a box lined with sandpaper. Ensure that only one layer of seed is present so that some seeds are not missed.
   - Rub the seeds in the box with a block of wood covered with sandpaper.

Manual cutting or 'nipping' of large seeds is possible but time consuming. Care should be taken to damage the seed coat at the end furthest away from the embryo to avoid killing the seed.

Larger quantities of seeds are usually more conveniently scarified using scarifiers or other machines. A speed of 500–1000 rpm is sufficient for abrasion. This can be provided by driven cylinders lined with carborundum paper or consisting of woven wire with different apertures against which nylon or wire brushes and even metal beaters revolve. The clearance is adjusted according to the size of the seeds being scarified. Commercial rice polishers can be used to abrade the seed coat but need some experimentation to determine optimum operating conditions. Hammer mills with speeds of 2,450 rpm using a screen of 4.75 mm have been used for some Medicago species. Very large amounts of seeds may be scarified by mixing with building stones and running in a cement mixer for 2–3 minutes.

Chemical treatment

The seed coat may also be damaged by immersing the seeds in concentrated sulphuric acid for 7–20 minutes (depending on the species and thickness of the seed coat) followed by washing and drying. However, this treatment can damage the seeds and different species have different degrees of resistance. Generally, this is an expensive and dangerous method and therefore considered unsuitable for field use.

Heat treatment

Some seeds, especially those with waxy or oily seed coats, can be effectively scarified using hot, but not boiling, water. Extreme temperatures can have a rapid effect (seconds to minutes) but have been found to accelerate ageing in seeds, so should only be used just before sowing. Heated revolving drums have been used in Australia, however, they are an expensive alternative to hot water. Half a 200-litre drum can accommodate one or two sacks of seed.

Seeds can be immersed in hot water of varying temperatures to ensure more uniform and predictable germination rates. A good practical way of doing this is to soak the seeds in very hot water for a few seconds, followed by overnight drying. It is important to ensure that the seeds are all fully immersed and in contact with the water.

Grasses

Grass treatments are usually carried out to facilitate seed to soil contact rather than to break specific dormancies. Deawning/debearding can be done using a cone thresher or hammer mill. A speed of 2,450 rpm with a 4.75 mm mesh screen has given satisfactory results for *Cenchrus ciliaris*, *Megathyrsus/Panicum* species, *Urochloa* species and *Chloris gayana*. Some *Brachiaria* seeds may also require scarification which is usually done using concentrated sulphuric acid for 10–15 minutes. Scarified grass seeds are more prone to attack by harvester ants and therefore require treatment with an insecticide such as Lindane before sowing.

Appendix II: Methods for inoculation of legumes

Inoculation is treating legume seeds with strains of Rhizobium bacteria, carried in a peat medium. The inoculum is usually sold along with the seed. Inoculation is recommended, particularly when introducing forage species into new areas, to ensure that the species are nodulated by the most effective and efficient *Rhizobium* bacteria strains.

Seed inoculation is the application of nodule-bacteria on the surface of legume seeds, preferably with skimmed milk, gum arabic and sugar solution as a sticking agent. Rhizobia are bacteria which are able to form a symbiotic relationship with legumes. The legume plant develops root nodules where the rhizobia fix nitrogen which then becomes available to the plant. There are different strains of Rhizobia. Some are very specific to species or even genotypes, whilst others are non-selective. Most soils already contain native Rhizobia but application of the appropriate specific Rhizobium can give substantial increases in plant growth and yield.

The appropriate Rhizobium culture is usually supplied mixed with an inert material, such as dry peat or charcoal. This is known as the inoculant. It is important to keep the inoculant cool and away from direct sunlight. The inoculant needs to be applied to the legume seeds before sowing or to the soil after sowing as a suspension. Applying the inoculant to the soil is less effective but necessary if the seeds have been treated with chemicals (fungicides, pesticides etc.) which would kill the bacteria. Recommended amounts are 6–8 g of inoculant per kg of seed.

Seed inoculation

Although this method is not as effective as other seed inoculation methods, it does have the advantage of being very simple. The inoculant may be applied dry or as a slurry. Using a slurry is a more effective method.

Methods of inoculation

- Dry (dusting) application: mix the dry inoculant with the seed immediately before planting.
- Slurry application
- Seed pelleting

Guidelines for inoculating seed

- Use peat culture.
- Ensure seed is not treated with a chemical.
- The container must not be contaminated by any toxic substance.
- Keep inoculated seed cool and away from direct sunlight.
- Sow as soon as possible after inoculation.
- Do not mix inoculated seed with acid fertilizer.
- Sow into a moist soil.
- Use only approved and tested culture.

Inoculation procedures

- Mix sticking agent and inoculant.

- Continue mixing until an even slurry is formed.
- Mix the slurry with the seed.
- Make sure every seed is coated.
- Spread seed to dry in a shaded area.
- Mix the inoculant with water to make just enough slurry to wet the seeds and coat them thoroughly without making them too wet.

Dry the seeds in a shaded area before planting; plant within 24 hours. It is possible to inoculate seeds in the evening and dry them overnight before planting early the next morning. If the soil is particularly acid, lime pellets may be used together with the inoculant. Inoculate the seeds using the slurry method as described above then roll them in finely ground limestone or powdered rock phosphate.

Soil inoculation

The inoculant can be added directly to the soil by placing it alongside the seed. This may be difficult because the quantity of inoculant is usually small. The inoculum can also be mixed with water to form a suspension then sprayed beside or beneath the seed in the early stages of germination. Further details and hints on inoculation are available in FAO (1984).

Appendix III: Napier grass crossing steps

1. Flowering in Napier grass normally occurs during short days of the season (< 11 hours of daylight). In Ethiopia (October to November) and Brazil (June to July).

2. Once flowers begin to set, protect the pistil from random pollen by covering it with a pollination bag (Appendix Figure 1).
   - The pistil should be at the stage where the sepal is yet to be opened (if a protruding pistil is observed it should be cut off ahead of bagging).
   - Bag two unopened pistils in one bag, if possible.
   - Bag as many pistils as possible to increase the chances of success (Appendix Figure 1 B).
   - Write the date of bagging at the time as cross pollination is carried out nine days after bagging.
   - Staple the pistil against one of the nearby stems in order to avoid the pollination bags falling off.
   - The best time for bagging/protecting the pistils is early morning, around 0800hrs, or late evening.
   - The pistils will be ready for fertilization, normally after nine days, and should be opened completely and with no pollen at the time of cross pollination.

Appendix Figure 1: Pistil needs to be protected/bagged before opening (A). Pistils should be bagged for at least nine days before crossing (B).

Appendix Figure 2: Pistil ready for bagging (A), pistil ready for receiving pollen (B) and pollen head ready for pollination (C).

3. Pollen head collection should be carried out far away from target mother plant/plots.
   - Label the pollination bag with the target pollen accession code. This bag will remain on the pistil after crossing.
   - Mature pollen heads will have pollinators around them such as bees. Cut the target pollen head, place it into the crossing bag and shake it (Appendix Figure 2C).
   - Remove the protection bag from the target pistil head after nine days and place the pollen bag into targeted pistil bag (make sure both the paternal and maternal codes are written on the cross-pollination bags).

4. Cross pollination:
    - Check if the pistil does not have pollen powder that can cause self-pollination, i.e. before anthesis (Appendix Figure 2).
    - Remove the sepal from the base of the pistil to avoid self-pollination at a later date.
    - Insert the pistil head into the pollen bag and shake thoroughly.
    - Remove the pollen head from the pistil bag to prevent self-pollinated seeds arising from the pollen head (sometimes you can leave the pollen head with the pistil for a day).
    - Keep the old protection bag for future recording and monitoring and write the paternal code on it.
    - Bag the cross-pollinated pistil for another 30 days for caryopses to form.
    - The best time to collect pollen and carry out pollination is between 0930hrs and 1100hrs.
5. Seed harvest is usually 30 days after crossing.

Threshing seeds

Seed threshing can be carried out with a blower machine or manually. Care should be taken as Napier grass seeds are very small (Appendix Figure 3).

Appendix Figure 3: Napier grass seeds after harvest

Germination of hybrids

1. The collected seeds (hybrids seeds) are kept for a while, ca. 30 days, before planting.
2. No need to thresh seeds ahead of regeneration.
3. Big trays can be used for seeds arising from the same crossing bag (Appendix Figure 4A).
4. Once sown, cover the seeds with fine soil as indicated below.
5. Water and keep monitoring for germination.
6. Transplant seedlings in 15 days to a new tray and into another new tray after 30 days. When the seedlings are nearly ca. 20 cm in height, they are strong enough to be transplanted in the field (Appendix Figure 4B).

Appendix Figure 4: Planting Napier grass seeds in a tray (A) and transplanting into individual pots (B)

Tools for crossing

1. Water resistant pollination bag (40 x 10 cm)
2. Stapler
3. Permanent marker
4. Cutter

Appendix Figure 5: Tools for crossing

PPE for crossing

1. Lab coat
2. Wellies (boots)
3. Protective eye goggles to be worn during crossing
4. Gloves can be useful to protect one from cutting their hands.

ISBN: 92–9146–757–x

ILRI
INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE

The International Livestock Research Institute (ILRI) works to improve food security and reduce poverty in developing countries through research for better and more sustainable use of livestock. ILRI is a CGIAR research centre. It works through a network of regional and country offices and projects in East, South and Southeast Asia, and Central, East, Southern and West Africa. ilri.org

CGIAR

CGIAR is a global agricultural research partnership for a food-secure future. Its research is carried out by 15 research centres in collaboration with hundreds of partner organizations. cgiar.org