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*IBPGR Training courses: Lecture series*

Collection, characterization,  
utilization of genetic resources  
of temperate forage grasses  
and clover.



International Board for Plant Genetic Resources

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**COLLECTION, CHARACTERIZATION AND UTILIZATION OF  
GENETIC RESOURCES OF TEMPERATE FORAGE GRASS AND CLOVER**

Lectures given at the Welsh Plant Breeding Station, Aberystwyth, UK  
for an IBPGR, ECP/GR Short course held  
in October 1984

(Edited by B.F. Tyler)  
Welsh Plant Breeding Station

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The International Board for Plant Genetic Resources (IBPGR) is an autonomous international scientific organization under the aegis of the Consultative Group on International Agricultural Research (CGIAR). The IBPGR was established by the CGIAR in 1974. The basic function of the IBPGR is to promote and coordinate an international network of genetic resources centres to further the collection, conservation, documentation, evaluation and use of plant germplasm and thereby contribute to raising the standard of living and welfare of people throughout the world. Financial support for the core programme is provided by the Governments of Australia, Austria, Belgium, Canada, China, Denmark, France, Federal Republic of Germany, India, Italy, Japan, the Netherlands, Norway, Spain, Sweden, Switzerland, United Kingdom, and the USA as well as the World Bank. FAO of the United Nations provides the Headquarters.

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## INTRODUCTION

Over the past few years IBPGR has organized numerous short technical training courses on a variety of topics. Frequently the lectures given are of use to a much wider audience than the course participants and, hence, when possible, IBPGR tries to issue them in an inexpensive format.

The lectures gathered together in this publication were related to a course which was part of a major project (ECP/GR) of IBPGR organized at the behest of 26 Governments in Europe.

During a meeting of the ECP/GR Forage Working Group held in Larissa, Greece in February 1984, the WPBS was invited to organize a short course for forage genetic resources. Short courses at other institutes had been concerned mainly with the distribution of natural variation of the putative ancestors and relatives of a wide range of crop plants and methods for their collection and their conservation. In order to complement this approach this specific course concentrated on the characterization and utilization of a single crop within a plant breeding environment.

Forage breeding relies heavily on the natural variation present in wild ecotypes for basic breeding material. The selection techniques developed for breeding are often suitable as the basis for characterization procedures of genetic resources. The objective of the course was thus to illustrate those modified breeding techniques and also to discuss recent developments which might be relevant in the future.

A number of exotic wild ecotypes have been used as the basis of advanced cultivars, which are at present in use in UK and European agriculture and part of the course aimed at providing information on the various techniques used to incorporate wild germplasm into a cultivated background.



## SECTION 1: COLLECTION

Collections of genetic resources can be assembled in a number of ways; donations from institutes and seed merchants, seed exchange, and by exploration and collection in natural and seminatural vegetation. The last method is obviously basic to a primary collection. Seed exchange and donation develop from this. The value of collected material cannot be overstated, as it is in this way that specific microhabitats can be identified, environmental data obtained and the influence of management understood.

Collections of genetic resources have been made for a number of different but equally valid reasons but should all be based on the same underlying principles. However, the objectives of the collection, the breeding behaviour of the species and the resources available will all influence the techniques used. The objective of expeditions organized by the WPBS has been primarily to extend the range of genetic variation, in a predetermined direction, to form the basis of plant-breeding programmes. This determined the choice of climatic region and also the sites within regions. The majority of temperate forage species are outbreeding, and also reproduce vegetatively, so that techniques specific to forages have been developed. In the following paper, forage-collection techniques are described and discussed in relation to their applicability for both breeding material and as a basis for conservation of genetic resources.

**CHAPTER 1**  
**COLLECTION AND FIELD-SAMPLING TECHNIQUES**  
**FOR FORAGES**

B.F. Tyler, K.H. Chorlton and I.D. Thomas

At the WPBS, both breeding and gene conservation are major considerations, these dual objectives can sometimes conflict. The techniques described attempt to take this into account.

Before considering the details of on-site sampling, the collection area and site need to be chosen.

**Choice of collection area**

The choice of collection area is made by a consideration of overall climate, microclimates, topography and agricultural practice in order to forecast the region most likely to have the variation required. For example, collection areas were chosen in regions bordering the Mediterranean or near Mediterranean for populations showing winter growth potential, because populations in this region are adapted to relatively high winter light levels and temperature (Hughes et al., 1962). Considerations of climate were invoked which suggested high midsummer growth from the irrigated plains of northern Italy (Tyler and Chorlton, 1978). These forecasts are based on our studies of the biological potential of collected material and its origin.

In some instances it has been possible to make comprehensive survey collections of large areas; collecting between 60 and 100 populations. If on evaluation it has been possible to identify valuable resources related to specific habitats within these regions our aim would be to return and make more detailed collections from predetermined sites.

**Choice of collection site**

Having chosen the general area of collection the choice of sites within the area is decided by discussion with contacts in the country to be visited who not only have a knowledge of its vegetation and agriculture but also appreciate our requirements. On most occasions particular farms are selected and, after discussion with the farmer, sites that appear to have the required management are chosen.

### Sampling techniques

The actual sampling techniques now need to be considered. The main aspects are, spatial delimitation of the population, numbers of plants to be sampled, and whether to take seed samples or sample vegetatively. These will be considered separately.

The foregoing account relates specifically to temperate forage grasses but many of these considerations will apply also to outbreeding forage legumes. However, the presence of insect pollination and associated symbiotic Rhizobium bacteria will present collection and evaluation problems not associated with forage grasses. These legume features will be discussed in the relevant sections.

### Population limits

Having decided on a collection site, ideally it is necessary to attempt to ascribe physical limits to the population in a genetical sense, i.e. an interbreeding group of plants. In practice this was often found to be impossible. For example, are plants in surrounding paths and wasteground to be regarded as part of the same interbreeding group of plants as the adjacent grazed pasture? It is often possible to obtain a retrospective answer but as an immediate decision is required we decided to delimit populations in ecological terms. Thus the aim is to collect in ecologically distinct sites and confine samples to the limits of influence of the ecological factors.

Such ecological delimitation requires a consideration of the need for sampling microhabitats. An example of an actual situation will illustrate this point. During a 1967 expedition to Northern Italy a perennial ryegrass meadow in the Valtellina, regularly cut for hay, was selected as a collection site. Passing diagonally through the meadow was a heavily trampled path. The perennial ryegrass on the path was prostrate with short compact inflorescences compared with the hay meadow where the erect plants had normal inflorescences. This was an obvious example of ecological differentiation which was possibly also genetically distinct. Heavy trampling on the path and infrequent cutting in the meadow were the overriding biotic factors. The path and meadow were treated as distinct populations and sampled separately by taking random seed samples in each habitat.

On return the two samples were isolated and multiplied separately. Evaluation confirmed the previous possibility that the two populations were genetically distinct (Table 1.1). Not only were they distinct but the magnitude of differences in mean emergence date and spring growth was as great as that obtained from geographically widely distanced collections.

Table 1.1. Characteristics of perennial ryegrass progeny obtained from plants collected from a hay meadow and path traversing a meadow in northern Italy

	Seedling height (cm)	Date of flowering	Spring growth (kg/ha/day)	Survival after freezing (%)
Path	6.5	31 May	3.7	92
Hay meadow	11.4	1 May	20.5	57

Although this may be considered an extreme example of ecotypic differentiation, our experience suggests that genetic variation is often associated with similar environmental contrasts and as such considerable importance is attached to sampling microenvironments. A number of examples have been encountered and studied (Tyler and Chorlton, 1976).

In some other instances, populations from different ecological situations do not, on characterization, reveal significant genetic differences. This could occur if the selective pressures had not been sufficiently intense or had not been in operation long enough. This evidence may only be obtained by characterization; on a single visit therefore, unless we have information that the management differences are recent, we would sample in both habitats.

#### Number of plants sampled

Plant collection for breeding requires as many genetically diverse populations as possible. Genetic diversity should be maximized between, rather than within populations, so that a degree of uniformity is already present. The outbreeding forage grasses display and conceal a vast potential of intrapopulation variability so that in all breeding programmes some reduction of variation is required.

Plant collection for gene conservation, in contrast, requires that the sample collected represents as nearly as possible the adaptation of the population to the environment. Thus there is a need to cover the entire genetic range of the population. An accurate representation of a population's adaptation to the environment is essential if the collected populations are to be used in studies of biological potential in relation to origin. This is one of our objectives. It enables us to produce maps and construct graphs relating plant characteristics to environmental origin as an aid to planning subsequent expeditions.

In practice a compromise is usually possible by collecting in as many contrasting sites as possible to provide genetically diverse populations and to collect a minimum of 25-30 plants randomly distributed over the full extent of the population. As the major characters in the outbreeding forages are controlled by large numbers of additive genes, often closely linked, it is likely that relatively small collections will be sufficient to detect genetic differences in these agronomically important characters (Oka, 1975; Breese and Tyler, 1982).

#### Vegetative and seed sampling

A major consideration during collection is whether to sample vegetative units or seed. Vegetative sampling provides a sample of what is actually growing in a given environment and thus is more likely to reflect the adaptation. A seed sample is the result of hybridization of plants largely from that environment but also by possible fertilization with alien pollen. Of more concern is the possibility that in some extreme environments certain genotypes have a low flowering expression and are thus prevented from contributing to the gene pool in a seed sample.

Our experience in collecting and characterizing Lolium species has indicated that vegetative sampling is more likely than seed sampling to provide the required population structure.

Sampling Lolium populations. The most agronomically important species in the genus Lolium are L.perenne and L.multiflorum. These two species are cross-compatible and produce fertile hybrids with considerable introgression between both parental species, so that populations of Lolium in continental Europe can be regarded as a huge hybrid swarm with

L.perenne types at one extreme and L.multiflorum at the other. Selection pressure in the form of grazing or hay production will determine where on a hybrid scale any population will be located. Heavily grazed pastures will have a higher proportion of perenne types and hay meadows multiflorum types; intermediate environments, e.g. hay with spring and autumn grazing, are likely to contain a similar proportion of all types.

When this situation is considered in relation to plant collection the relative inflorescence-producing potential of the two species is important. L.multiflorum characteristically produces flowering heads throughout the year (aftermath flowering) whereas in L.perenne flowering tends to be more seasonal. Thus a seed collection made during July to September is likely to be biased and contain more seeds from the multiflorum end of the range. Comparison of characterization data from both vegetative and seed samples collected at the same site show the importance of vegetative sampling when the more persistent perenne types are required (Table 1.2).

Table 1.2. Characteristics of progeny of populations of Lolium perenne from northern Italy collected by seed and vegetative sampling. The hybrid index was constructed on diagnostic characters of L.perenne and L.multiflorum on a scale of L.perenne = 1 to L.multiflorum = 9.

	Hybrid index	Seeding year (infl./1 m drill)	Aftermath (infl./1 m drill)	Flowering time (days after 1 April)
Collection A				
Vegetative	1.3	11.2	36.1	48
Seed	2.4	22.6	49.2	49
Collection B				
Vegetative	1.2	29.5	32.8	27
Seed	3.5	71.2	52.4	33
Collection C				
Vegetative	1.9	17.1	32.8	41
Seed	3.6	31.2	36.1	44

Although this may be an extreme example due to the particular structure of the Lolium populations it is likely that seed collections will favour the more reproductively active genotypes. Vegetative sampling is thus generally favoured, but practical considerations may dictate that

seed sampling is the only method possible. In this case it is important to take, as nearly as possible, the same amount of seed from each genotype, thus avoiding bias towards more freely flowering genotypes.

### Sampling cytotypes

Sampling technique is important when different chromosome races of a species are sympatric. This was realised when we were collecting diploid and tetraploid *Festuca pratensis*. Although diploid ( $2n=14$ ) *F.pratensis* and tetraploid ( $2n=28$ ) *F.pratensis* var. *apennina* have predominantly low- and high-altitude distributions, respectively, both occur at intermediate altitudes (Tyler *et al.*, 1978). It is extremely difficult to distinguish between the cytotypes in the field (Borrill *et al.*, 1976), so their presence is not always detectable and a bulk sample should be taken. Such a sympatric site was encountered in the Carpathian Mountains in Romania. At the time of collection (August) no seed was present and vegetative samples were taken. Later in the year (October) a seed sample was taken. Both collections were examined cytologically, which indicated quite different population structures (Table 1.3). Vegetative sampling showed a very high proportion of triploids, and seed sampling showed the presence of parental cytotypes not present in the vegetative sample, presumably not sampled due to low frequency in the population. Neither method, therefore, on its own was entirely satisfactory, although vegetative sampling showed the dominance of an otherwise unsuspected hybrid.

Table 1.3. Proportion of diploid ( $2n=14$ ), triploid ( $2n=21$ ) and tetraploid ( $2n=28$ ) plants in populations of *F.pratensis* collected by seed and vegetative sampling in the Carpathian Mountains of Romania

	No. plants sampled	$2n = 14$ —(%)	$2n = 21$ —(%)	$2n = 28$ —(%)
Collection A				
Vegetative	14	0	86	14
Seed	42	100	0	0
Collection B				
Vegetative	16	0	100	0
Seed	17	0	24	76

From the genetic resources point of view this type of situation, although unusual, is of considerable interest, as natural hybridization between chromosome races of the same species could be a potent force for gene flow. Introgression of partially fertile hybrids with the parental species followed by stabilization to the parental chromosome number is a possibility (Stebbins, 1950; Humphreys, 1975). This would enable genes to flow from one chromosome race to another, with the hybrid forming the bridge. If this occurred, collections in or near such sites would be a potential source of extended genetic variation within the species.

Purely practical aspects also need to be considered. Seed samples are less time consuming, and easier to collect and conserve during collection, but timing the collection to coincide with seed maturity and the possibility of not finding inflorescences in intensively grazed situations are practical considerations in favour of vegetative sampling. Vegetative sampling, however, is considerably more time consuming and more demanding physically. The material is more difficult to conserve en route, and a quarantine period and seed regeneration are required.

All of these practical factors together with theoretical considerations, collecting objectives, reproductive strategy of the species and possible complications due to ploidy need to be considered. In practice a compromise is usually found.

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## SECTION 2: CHARACTERIZATION

It is widely recognized that characterization characters should be highly heritable, easily observed and the expression of the character be little influenced by environmental differences. In the outbreeding temperate forage grasses these conditions are seldom present. The majority of characters are polygenically inherited with relatively large numbers of genes of greater or lesser effect, often with complex linkages, and under strong environmental influence and thus of low heritability. As inheritance is quantitative the expression of characters is not easily seen or recorded. Most characters are more or less influenced by environment; population/environment interaction is the rule rather than the exception. Thus the breeding system and the quantitative nature of inheritance cause considerable difficulties in the choice and observation of characterization characters in the forage grasses.

In an attempt to select suitable characters and develop techniques for characterization, a number of biochemical and physiological characters are being studied using controlled environmental conditions. Isozymes are used to characterize genetic variation within and between populations. Two papers are presented here, Chapter 7 describes the technique Chapter 8 the use of the technique to characterize variability.

It is anticipated that physiological characters will have higher heritabilities and be more easily observed and measured than major growth features, of which they are component processes. These techniques are described in Chapter 4 and 5 and discussed in relation to characterization of genetic resources.

As has been suggested, characterization of forages in the strict sense is still at the investigative stage. However, to make full use of these resources for breeding, some form of evaluation or screening must be used to identify valuable traits. Screening for breeding presents fewer problems than characterization if the results are intended for use in environments the same as, or similar to, the screening environment. This type of evaluation has received priority since the inception of the Genetic Resources Unit and has been successful in identifying numerous 'elite' populations both for breeding and research. Chapters 2 and 3 describe the methods that have been used in both field and glasshouse for initial screening of grass and clover introductions.

## CHAPTER 2

### PRELIMINARY SCREENING OF FORAGE GRASSES

B.F. Tyler, K.H. Chorlton and I.D. Thomas

#### Introduction

Evaluation, characterization and screening are given priority in working collections associated with breeding as such information is crucial in choosing material for incorporation into breeding schemes.

There are a number of factors that must be considered in the choice of techniques and of characters to be evaluated. The techniques used must take into account the amount of seed available, manpower and land resources, as well as the relevance of the data obtained to the agronomic evaluation in the later stages of variety evaluation. Choice of characters is determined by their value to the breeder but is conditioned by the ability to obtain meaningful results without excessive commitment of resources.

Initial screening combines glasshouse and field evaluation and as such is subject to year-to-year climatic variation. Results obtained must therefore be interpreted knowing this limitation, and the screening is likely to be of value only to breeding in similar environments to the screening environment. Extrapolation to other environments is likely to be hazardous.

The characters evaluated are those primarily concerned with the seasonal growth rhythm of the populations and the reaction to stress conditions.

#### Screening methods

##### Glasshouse

The most effective way of providing single plants for field transplanting is to sow in early spring under glass. It is then possible to evaluate seedling characters on this material as a guide to establishment potential in the field. The value of screening for seedling characters, however, will be considerably influenced by seed quality, which is largely determined by environmental conditions at harvest (Bean, 1980). Thus, assuming uniform conditions at seed multiplication, seedling characters can be usefully studied.

Seed is sown at 5-cm centres in shallow boxes (52 x 40 x 9 cm) containing J.I. No. 1 compost. Provision for occasional germination failure is made either by sowing two seeds per location and thinning or by reserving one row for replacement.

Just prior to the attainment of full canopy cover an estimate of seedling habit is made; at full canopy cover the seedlings are cut and weighed. Tiller counts are best done 2-3 days after recovery. These 3 characters, dry weight, habit and tiller numbers, are the most informative for forecasting establishment potential in the field. Other seedling characters such as percentage albino, percentage coleoptile tiller, canopy height, tiller length, etc., are sometimes estimated depending on the material being screened.

#### Single plant

The seedlings are cut and after a short recovery period are taken from the glasshouse to harden outside. Individual plants are transplanted into the field from late May to late July/early August, depending on weather conditions. The populations are arranged in rows of 20 plants at 60-cm centres in 2 replicates.

Single-plant evaluation has limited value, as seasonal yields have no consistent relationship with sward yields (Lazenby and Rogers, 1964). Their main use is in determining flowering characteristics, of which mean emergence date is probably the single most important character in determining growth rhythm. In addition to mean emergence date, habit and disease are recorded at inflorescence emergence and, as an indicator to seed-production potential, abundance of inflorescences is also recorded.

It is possible to make morphological measurements or estimates on single plants for characters such as plant height, length and width of the flag leaf, height of inflorescence, etc. These are used by Registration authorities to test distinctiveness between varieties, but they are of limited value to the forage breeder (Tyler, 1985).

A variant of the single-plant layout, closely spaced single plants 23 cm apart in rows 60 cm apart, was used in the past to estimate growth attributes. This spacing was used as Lazenby and Rogers (1964) found it gave the closest agreement with sward yields. Although good agreement with sward yield was confirmed, we found it was not possible to estimate

persistence as a major sward characteristic (Tyler and Jones, 1982). As persistence is a most important breeding objective this method was replaced by swards.

### Sward

The advantage of single-plant field layouts was that an initial screening could be done with very few seeds, and often this is a limitation, particularly with genetic resources obtained via seed exchange. However, we considered that the disadvantages of inadequate interplant competition and lack of information on sward characteristics outweighed this advantage.

To obtain the amount of seed necessary for sward assessments all our own collections, both vegetative and seed, were regenerated in isolation (Tyler, 1982). The standard technique now in operation is to sow 3 replicates of plots (1.5 x 1 m) in July or August. Seeding year cuts are taken. The number depends on the amount of growth but it is usually 2. This gives an estimate of seedling establishment. In the following year the plots are subjected to a frequent cutting regime, at approximately monthly intervals during the main growing season and at 6-week intervals in spring and autumn. At each cut the growth stage and the number of flowering heads is recorded, as reproductive activity has an overriding influence on yield. Seasonal growth rates and percentage contribution at each period of the year can be calculated from the yield data and thus an estimate of the growth rhythm obtained (Tyler and Chorlton, 1974).

The following year estimates of the conservation and aftermath potential of the populations are made by cutting according to emergence dates, which have been determined the previous year, followed by a series of aftermath cuts.

In addition to yield and growth stage data, the reactions of the populations to winter conditions and drought are recorded if climatic conditions are sufficiently severe to induce stress responses. Persistence estimates (ground cover of sown species) are made throughout screening in the spring and autumn of each year, a final estimate being made in the spring of the third harvest year.

On occasions, insufficient seed is available to use this standard procedure. When this occurs seed is sown in shallow boxes

(52 x 40 x 9 cm) and after hardening the seedlings are transferred en bloc to the field. Shallow trenches are prepared and the complete 'turf' removed from the box and placed in the trench. A series of so-called 'mini-plots' are thus established which are then managed in a similar way to the standard technique.

The advantages of mini-plots over single plants are that yield estimates are likely to correlate better with swards and that observations on sward characteristics are possible (Hayward and Vivero, 1984). Mini-plots are recommended only when seed or land are limiting and not in preference to larger plots, as border plants have to be harvested due to the small size of the plots, and the sampling requires greater accuracy.

Modifications of the standard management outlined above may sometimes be necessary if preliminary observations either during collection or the initial regeneration suggest that the populations may have other attributes of considerable interest to the breeder. For instance, very early spring growth, winter growth, resistance to treading or high digestibility would necessitate some variation in the management to cater for this (Tyler and Jones, 1982).

### Screening results

#### Breeders working in environments similar to screening environment

The scheme outlined here is primarily designed for breeders working in similar environments. They will be aware of the extent of seasonal interactions and interpret the results accordingly. At the end of each preliminary evaluation all the data are presented relative to appropriate control varieties and 'elite' populations are selected to form the basis of breeding programmes (Tyler, 1985).

#### Breeders working in other environments

Preliminary screening of genetic resources should ideally evaluate those characters that show little or no interactions with environment (IPBGR, 1985) and thus have more universal applicability. However, forages in general, largely due to the outbreeding mating system, quantitative inheritance and low heritability, are notorious for showing large genotype/environment interactions (Breese, 1969).

Regression analysis and multivariate analysis such as pattern analysis are useful statistical techniques for interpretation of genetic resources data. They also have value in detecting any pattern in adaptation and hence would help in extrapolating to other environments (Hayward et al., 1982).

Thus, although preliminary screening under glasshouse and field conditions is relevant to the requirements of breeders working in similar environments, evaluation of specific physiological and biochemical attributes related to agronomic and stress related characters, may be more reliably extrapolated to other environments and form the basis of a meaningful characterization. These will be considered in the following Chapters 4 and 5.

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### CHAPTER 3

#### CHARACTERISATION OF WHITE CLOVER

Ian Rhodes

The characterization of genetic resources of white clover presents special problems, particularly in relation to their utilization in breeding programmes. The major complicating factor is that white clover is almost universally grown in mixtures with a grass. Thus the tenuous nature of relationships between easily measured spaced-plant characters, spaced-plant yield and sward performance, encountered in other forage species, is further accentuated. This is because the agronomic performance of the clover in the mixed sward is largely a function of the competitive stresses within the sward rather than a direct response to the major macroenvironmental factors (Rhodes, 1984). This implies that specific as well as general compatibility differences exist between the grass and clover populations (Rhodes, 1984; Rhodes and Mee, 1984; Evans *et al.*, 1985) which will in turn govern the procedures adopted for screening and characterization.

Several other factors contribute to the difficulties in characterization procedures. Firstly, white clover shows a high degree of intraplant plasticity and because of its stoloniferous growth habit it is 'horizontally mobile'. Furthermore, there is some evidence that genetic variation exists in the magnitude of the phenotypic plasticity (Hill, 1977).

Secondly, characterization measurements have traditionally been made on 1- to 2-year old plants, when the plant is supported by a tap root and so is physiologically very different from the older plant when the tap root has decayed and the plant is supported by a more superficial root-system.

Finally, characterization and screening procedures may be governed by the limited amounts of seed available. This can be a particular problem when the seed has to be produced from a large number of vegetative collections which have to be regenerated through insect pollination, often in quarantine conditions.

A prerequisite of the plant features used in characterization is that they are associated with some aspect of agronomic performance, and thus are of value to the plant breeder. In white clover the most easily measured characters, that is morphological characters measured on spaced plants, are mainly of use in determining the reaction to various types of defoliation, e.g. by cutting, sheep and cattle, and intensities of grazing, ranging from rotational to continuous systems. In addition, some information can be collected from spaced plants, on floral and seed-yield characteristics, and resistance to pests and diseases.

The limited scope for and value of spaced plant characteristics results in considerable emphasis being placed on the measurement of characteristics in sward conditions, with all the ensuing logistical limitations. In addition, more recent work has indicated the value of the measurement of physiological responses to stress in screening and characterization (Glendining, 1987).

Despite the caution expressed in the use of spaced plant characters, current work aimed at identifying relationships between morphophysiological characters (measurable on spaced plants) and agronomic performance (Rhodes, 1981), may ultimately provide further useful characterization features.

Taking into account the complications and limitations described above, a system of characterization and screening is operated at WPBS based on morphological, physiological and sward characteristics.

This system, which is continually evolving in relation to new physiological and agronomic information, is outlined below. Where emphasis is placed on sward characteristics, the distinction between characterization per se and screening of breeding material becomes somewhat blurred.

### Spaced plant characters

Whilst all the following characters are measured on spaced plants grown in the field, many vegetative features such as leaf size and stolon characteristics may be measured on young pot-grown plants in the glasshouse. Absolute values may differ but the discrimination between populations appears to be comparable to that obtained from field conditions.

### Vegetative characteristics

1. Leaf dimensions and shape - Leaf width and length, and area of mid-leaflet taken at flowering. The ratio of length:width provides a quantitative measure of leaf shape. This feature can also be scored on a visual basis, i.e. elongated, ovate or round.  
Recent evidence with novel breeding material suggests that categorization or ranking according to leaf size may change throughout the growing season, thus demonstrating the possible limitations of the too rigid use of this character.
2. Length of petiole, of same leaf as above.
3. Thickness of petiole - visual score.
4. Stolon thickness, measured using a gauge comprising a range of various-sized notches which can be placed around the stolon to estimate its diameter.
5. Internode length measured either on several standard internodes per plant, or by measuring the length of stolons divided by the number of nodes on them.
6. Plant height and spread.

### Flowering characteristics

1. Tendency to produce flowers in first year, i.e. in plants not exposed to short days and vernalization.
2. Flowering date. Flowering is deemed to have commenced when a plant shows petal colour development on an inflorescence. This may be expressed as date when first plant flowers or when 50% of plants have flowered.
3. Peduncle length and thickness.
4. Profuseness of flowering.

### Disease and pest damage

1. Sclerotinia infection.
2. Stem nematode (Ditylenchus dipsaci) infection.

Where possible material is grown on 'reliably infected' sites for such assessment. However, glasshouse techniques are now available for measuring stem eelworm resistance (Cook and Evans, in press).

### Percentage cyanogenic plants

Pusey (1963) described three categories of cyanogenesis, viz. cyanogenic giving a rapid yield of HCN, cyanogenic only after long incubation, and entirely acyanogenic. This chapter describes the appropriate tests.

### Leaf marks

Several types of leaf marks and degrees of expression can be identified (Davies, 1963). For simplicity the percentage of plants showing expression of the V mark is often used.

### Sward characters

White clover is grown in swards with one or preferably more companion grasses. In screening limited numbers of populations it is often of interest to use grasses collected from the same location as each clover population, in order to detect any specific compatibility relationships arising from long-term coadaptation of the grass and clover.

If it is logistically possible, monoculture plots of the clovers are also used.

Plot sizes range from a minimum of 1 x 1.5 m up to (and preferably) 2 x 2 m. Sowing rates for mixtures are 3-4 g/m<sup>2</sup> clover and 20 g/m<sup>2</sup> grass. Where clover seed is in short supply, young clover plants can be planted at up to 25 cm spacing and oversown with the grass seed. Such plots planted in May-July achieve a dense, even cover of stolon by the autumn of the planting year.

### Vegetative characters

1. Dry matter yield of grass and clover, cut to 3 cm above ground level at a frequency ranging from 3- to 6-week intervals.
2. Stolon length and weight per unit ground area, stolon weight/unit length, internode length, number of growing points. All these characters are measured on samples removed from the sward.

The measurements 1 and 2 allow estimates to be made of the degree of competitive suppression or enhancement (Rhodes and Harris, 1979) and thus compatibility of grass and clover. Loss of stolon length and weight and amounts of dead stolon during the winter may give an indication of winter hardiness.

Nondestructive measurement of stolon frequency can be obtained by counting 'contacts' of stolon with a line (i.e. string) quadrat across the plot (Harris et al., 1983).

Plot assessment will also provide realistic information on:

3. Persistency.
4. Seasonal growth patterns in both mixture and monoculture. These may often be contrasting (Rhodes, 1984).
5. Disease and pest resistance.

#### Reproductive characters and seed yield components

These measurements are usually made only on a restricted range of populations of special interest to breeders.

Monoculture plots are prepared as previously described and the following characters measured.

1. No. of heads per unit ground area;
2. No. of florets per head;
3. No. of ovules per floret;
4. Amount of nectar per floret and nectar concentration (Norris, 1985).
5. Peduncle length.

#### Physiological characterization

Particular interest has centred on the growth of white clover at low and spring temperatures. Use of a temperature-gradient tunnel (Eagles et al., 1984) has facilitated screening and characterization on this basis.

Clover plants are grown in 7.5-cm diameter pots and subjected to normal winter conditions. Following transfer of plants to a range of low temperatures in the temperature gradient tunnel, the following measurements are made.

1. Rate of leaf expansion and petiole extension;
2. Leaf and petiole dimensions;
3. Rate of stolon extension;
4. Stolon characters; internode length, stolon thickness.
5. No. of growing points.

Studies by Glendining (1987) have shown good relationships between several of these characters and field performance in mixed swards.

Two other forms of physiological characterization are cold tolerance (Chapter 4) and drought resistance (Chapter 5). The appropriate artificial screening technique described in Chapter 4 measures only one component of winter damage, viz. cold tolerance as such. The main factor in the winter damage to white clover appears to be dehydration in periods when there is a coincidence of strong cold winds and frozen soil (Harris *et al.*, 1983). However, protection of the clover plant by neighbouring grass plants does much to reduce the magnitude of winter damage in realistic mixed-sward conditions (Rhodes, 1981).

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**CHAPTER 4**  
**PHYSIOLOGICAL CHARACTERIZATION OF FORAGES**  
**FOR COLD TOLERANCE**

C.F. Eagles

Winter hardiness is a complex and variable character which is modified by environmental factors such as temperature, light intensity, soil moisture, wind, snow cover and associated low-temperature pathogens. Similarly, management practices such as intensity and time of defoliation, and fertilizer application in the autumn, can influence winter survival. The importance of these individual factors, or combinations of them, varies from year to year in one location, and between locations, in any one year. Hence, characterization of genetic resources for a breeding programme is often very difficult due to the unreliable and unpredictable nature of winters, particularly in maritime environments. To overcome this problem environmental-simulation techniques have been developed. These have potential as standardized procedures for characterization of genetic resources at an international level. Although field evaluation of winter survival may be the ultimate objective any results may be of limited use in other climatic regions since the winter environment is a unique feature of any location. The extent of this problem is demonstrated by significant interactions found between test sites, managements and varieties in field trials for winter hardiness in UK (Charles *et al.*, 1975). The requirements for a successful environmental simulation technique for assessing winter hardiness are that the character to be measured must show a close relationship with damage in a range of natural environments, and be capable of assessment with a relatively simple but reproducible technique. The most consistent factor across a range of sites is exposure to low temperature. For this reason, an artificial screening test based on resistance to freezing has been developed at Aberystwyth (Fuller and Eagles, 1978). It has been used successfully to assess cold tolerance of seedlings and tillers of grasses and winter oats, and seedlings and stolons of white clover. In order to achieve consistent results between tests it has been necessary to standardize the procedure since growth, hardening, freezing, thawing and recovery conditions can modify the results.

### Glycol freezing tank

The freezing test is conducted in an insulated stainless steel bath (1.68 x 0.86 x 0.31 m) containing 450 l of a 50% solution of ethylene glycol. Temperature is controlled by a cam programmer which regulates refrigeration and heating so that reproducible freezing cycles are achieved. The tank is designed to use either seedlings grown in cylindrical paper pots (19 mm diameter x 65 mm long) or isolated tillers which are inserted into Perspex tubes immersed into the glycol. In tiller tests it is possible to increase the capacity of the tank by using several tillers in each of the 1080 tubes.

### Freezing tests

Two types of freezing test have been used to evaluate cold tolerance of cultivars, accessions and selection lines.

First test. Employs a single freezing temperature in which all the plants in the tank are subjected to the same freezing treatment. This type of test has the advantage that multiple comparisons or a high degree of replication are possible but it often fails to separate contrasting types and is only appropriate where the plant material has a restricted range of hardiness, e.g. when selecting survivors from within a population.

Second test. Involves removal of samples of plants at successively lower temperatures, typically at 1°C intervals, to allow calculation of temperatures for a 50% killing of a population (LT<sub>50</sub>). This type of test is very flexible and gives good discrimination between contrasting types, which makes it suitable for characterization of genetic resources. The requirement for several temperature treatments (normally 6 or 7) to calculate LT<sub>50</sub> values restricts the number of comparisons in each test. This does not present any problems for seedling tests since all the stages of the assessment are conducted in controlled-environment conditions (Table 4.1) with no significant variation between tests. In contrast, tiller tests present a problem since they are normally used with naturally hardened material so that considerable variation can occur between runs due to the environmental conditions before freezing. In this type of test it is essential to include control varieties in each run.

Table 4.1. Treatments used at different stages of standard cold-tolerance tests

	Seedling test	Tiller test
Growth	15°C/16 h photoperiod for 18 days from sowing	Natural conditions (summer-autumn)
Hardening	2°C/8 h photoperiod for 14 days	Natural conditions (autumn-winter)
Freezing	LT <sub>50</sub> test with 6 or 7 temperature samples	
Recovery	15°C/16 h photoperiod for 21 days	Cool glasshouse for 28 days

Since it is not possible to separate contrasting types in an unhardened state it is necessary to harden seedlings in conditions similar to those which occur during autumn, namely low temperature with short days. For Lolium perenne, most populations have LT<sub>50</sub> values of about -6°C before hardening. After a standard hardening treatment (14 days at 2°C with 8-h photoperiods), LT<sub>50</sub> values of seedlings span a range of about 5°C between susceptible and hardy types (Table 4.2). All populations of northern temperate grasses show some ability to harden at 2°C, but discrimination between contrasting types may be less than at higher temperatures (6-8°C), which favour the hardening of hardier types.

Table 4.2. Cold-tolerance (LT<sub>50</sub>) and winter hardiness of 3 contrasting cultivars of Lolium perenne assessed in the standard seedling test presented in Table 1

Cultivar	Origin	Winter hardiness in field	LT <sub>50</sub>
Grasslands Ruanui	New Zealand	Susceptible	-10.6°C
S.23	UK	Hardy/susceptible	-13.2°C
Premo	Netherlands	Hardy	-15.8°C

Seedlings removed from the freezing conditions are allowed to thaw slowly at 2°C. Mortality is scored after recovery for 21 days at 15°C with 16-h photoperiods. In its simplest form the assessment of mortality

is based on the number of dead and alive plants in a treatment where the production of new leaves and roots are the criteria for survival. Other techniques have been used to assess damage, including subjective estimates of leaf damage, measurements of shoot growth, electrical conductivity of tissues and vital staining techniques.

In tiller tests, natural conditions are used for the growth and hardening treatments. Individual tillers separated from mature plants are frozen and after thawing they are planted in compost and maintained in a cool glasshouse for 28 days until tiller mortality is recorded.

LT<sub>50</sub> temperatures are computed from the relationships between freezing temperature and mortality by probit analysis. The data in Table 4.2 illustrate the range of LT<sub>50</sub> values in perennial ryegrass in relation to levels of hardiness under field conditions. Similar results have been found for Lolium multiflorum, Festuca arundinacea and Dactylis glomerata, with cold tolerance measured by LT<sub>50</sub> closely related to winter hardiness in field trials.

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**CHAPTER 5**  
**CHARACTERIZATION OF FORAGES FOR**  
**DROUGHT RESISTANCE**

Henry Thomas and A.P. Gay

**Introduction**

Plants have evolved a wide range of responses to drought so it is unlikely that a single test will characterize the drought resistance of a population for all the environments in which it is likely to be grown.

Drought tests carried out in the field have the advantage of being realistic or 'natural', but may be relevant only to that site in that season. Trials conducted under more controlled conditions are obviously less natural, but are likely to be repeatable (from site to site or year to year) and amenable to physiological and ecological interpretation.

Drought may be simulated in various ways: complete cessation of irrigation, partial irrigation (with a fixed volume of water or to a fixed water deficit), or addition of an osmoticum such as polyethylene glycol to the growing medium. The rate of droughting will depend on the ratio of leaf area to soil volume; large plants in small pots may dry out in a few days, whereas spaced plants in a nursery may never transpire sufficient water to cause severe soil-moisture deficits.

The plant response that is easiest to measure and interpret is herbage growth during and after drought. However, different populations may achieve drought resistance in different ways, and it will be ultimately more satisfactory to measure those physiological responses most likely to be involved.

There are three mechanisms involved in drought resistance (Turner and Kramer, 1980; Paleg and Aspinall, 1981).

1. Drought avoidance: growing only in the wet season and surviving the dry season as dormant plants or as seeds. This response has not been pursued as it is not agronomically desirable in temperate grasslands.
2. Water conservation: increasing the efficiency of water use.
3. Tolerance of dehydration: the ability to grow and survive during drought and to recover on rehydration.

The measurement is described below of plant characteristics that have been shown to be related to drought resistance in a number of plant species, and can be measured reasonably rapidly.

### Water conservation

Leaf area and leaf water conductance are of primary concern when considering characters by which water conservation of forage plants may be improved, that is those that are directly proportional to transpiration. However, it must not be forgotten that factors such as stand density, and the shape and height of the canopy may have an important consequence for water use, and need to be considered. In most forage crops it is important to maximize economic yield by increasing the height, density and leaf area of the stand, but reducing these to improve water conservation is inappropriate except in extremely arid areas. Thus, attention is focussed on reducing leaf water conductance (LWC) to reduce transpiration. Since LWC is highest and water use greatest under good growing conditions, reducing water use under good conditions will give the greatest water savings. A problem with the use of LWC as a character is that it responds rapidly to changes in environmental conditions (for example to water, light and humidity) through stomatal action. Thus characterization and selection of plants for LWC is greatly simplified if measurements are made in a controlled environment, within the range of field conditions, with continuous watering.

A selection programme at WPBS to improve water economy in Lolium perenne has utilized the LWC technique. A diffusion porometer was used because of its ability to measure LWC rapidly (about 30 s per measurement) (Gay, 1983). The plants were grown in pots on capillary benches in a controlled environment and selections for LWC were made. These selections were found to maintain differences in LWC when remeasured, and after pair- or polycrossing. Polycrossed progeny with contrasting LWC were grown under drought; the low-LWC selections continued leaf extension for longer. This was found to be a consequence of their slower water use, which left greater amounts of water in the soil, thus allowing growth for longer in the drought. Thus characterization of plants for LWC has potential for finding plants with improved water economy.

### Tolerance of dehydration

This is the ability to survive and grow during drought, and to continue growth unharmed when it rains again. Many physiological responses are involved whose net effect may be assessed by tests in the glasshouse.

Briefly, plants (40-100 per population) are grown in small pots, allowed to dry until they are severely wilted, and then cut and rewatered. Subsequent regrowth is a measure of desiccation tolerance, and is quite closely related to performance in the field during severe drought (Thomas and Norris, 1981). The detailed testing procedure is as follows:

1. The plants: a) the experimental populations; b) control plants of known response; c) monitor plants, which are used to record the progress of the drought.
2. The experimental design must be chosen to suit the facilities and to enable analysis of variance to be made.
3. The drought: water is withheld from monitor plants on 'day minus 1' and from the other plants on 'day zero', in order to obtain advance warning of the approximate LT<sub>50</sub> stage.
4. Monitoring the drought: (a) leaf extension measured daily on monitor plants; (b) when growth stops, the first batch of monitor plants are cut, watered, and examined next day for growth; (c) step (b) is repeated until only half of the plants have recovered; (d) when this occurs sufficient drought has been applied, so go on to stage 5.
5. End of drought: all experimental and control plants are cut (and herbage weighed), and then watered with fertilizer solution.
6. Within 1-2 weeks there is enough regrowth of surviving plants to be cut and weighed; relative herbage weights indicate dehydration tolerance.

Selection criteria for tolerant genotypes depend on the objectives but in selection experiments at WPBS plants have been chosen which (a) before drought exceeded the mean, and (b) after the drought were 1.5 to 2 times greater than the mean.

This character is inherited: after 3 cycles of selection and crossing ecotypic populations of Italian ryegrass, desiccation tolerance was increased by up to 31%.

These tests are useful for characterization of large numbers of populations and selection on a large scale, but give little indication of the mechanisms involved. At present 4 physiological responses are being measured, that may contribute to drought resistance.

### Osmotic potential (OP)

As a general rule, plants with more concentrated cell sap (lower osmotic potential) are more able to withstand drought. OP can be measured rapidly on single grass leaves using a vapour pressure osmometer as described below.

1. When plants are turgid (at dawn or during dull, misty weather) representative leaves are cut and put into small polythene vials.
2. As soon as possible afterwards, the vials are transferred to a deep freeze at  $-20^{\circ}\text{C}$ . Freezing destroys the cell membranes and enables the cell sap to be expressed. Samples can be stored for several months without deterioration.
3. Samples taken from the freezer are squashed, and the expressed sap (about 8 microlitres) absorbed on a filter-paper disc.
4. The disc is transferred immediately to the vapour pressure osmometer and 90 s later the OP is read.

The OP of stressed plants can be adjusted to full turgor by (a) calculating the product of (measured OP)  $\times$  (relative water content) or (b) by rehydrating the stressed plant or detached leaves overnight.

It is possible to detect OP differences of 0.5-1 bar between different ryegrass populations.

### Leaf water potential (LWP)

LWP is a measure of the degree of water stress in the plant. It has been used (e.g. Fischer and Sanchez, 1979), to identify populations which are able to avoid desiccation. LWP is most rapidly measured with a pressure bomb, using the following procedure.

1. Conditions: LWP varies according to the weather, and for selection purposes measurements for screening or selection must be made during clear weather at a fixed time of day, or in a growth-room.

2. Sampling: The leaf is wrapped in plastic film and cut off with a sharp blade. If stored in a moistened, sealed, shaded polythene bag the leaf will stay fresh for up to 30 min.
3. Measurement: The wrapped leaf is inserted into the chamber with the cut end protruding. Pressure (from a nitrogen cylinder) is increased until water (sap) is first seen to emerge from the cut end; the pressure at this point is equal in value, but opposite in sign to LWP.

Tissue elasticity. As drought progresses, plants respond to drought by forming cells which are smaller and have thicker, less elastic cell walls. Elasticity may be measured by using the pressure chamber. Pressure on the leaf is progressively increased and the amount of sap expressed measured. For a given pressure increase, the more flexible leaves lose a greater fraction of their total water.

Measurement of elasticity in pure physiological studies is very time-consuming, but this technique has been simplified, so that 30-40 leaves can be measured in a day (Thomas, 1987). Within-plant variation in elasticity is quite large, and although differences between contrasting species have been demonstrated, variation between varieties within a species is far more difficult to detect.

#### Membrane stability

When drought is severe, tissues become very desiccated, cell membranes rupture, and the cells die. The stability of membranes can be estimated by drying leaf tissue in a standard way and then measuring the ease with which solutes can be leached out of the ruptured cells. The technique described below is a modification of that used by Blum and Ebercon (1981).

1. Detached leaf laminae are washed, dried and desiccated over dry silica gel in Sterilin tubes for 24-48 h. Alternatively they may be soaked in a 40% solution of polyethylene glycol.
2. The dry laminae are then leached for 4-24 h in 20 ml deionized water in covered boiling tubes.
3. The electrical conductance of the leachate (C1) is measured using a conductivity meter reading to 1  $\mu$ s.

4. The boiling tube containing leaf and leachate is autoclaved for 15 min to release all the solutes, and allowed to cool.
5. The conductance of the leachate is again measured (C2).
6. Membrane damage is given by  $C1/C2$ . To correct for the small amount of leaching from unstressed leaves the above procedure can be followed but without desiccating the leaves in stage 1.

### Conclusion

The physiological characters that have been described above all play a part in the drought resistance of some species under some kinds of drought, but it is impossible to lay down strict guidelines for a routine testing procedure to characterize accessions.

Probably the most useful test is the relatively crude 'fast-drought' technique described previously. The value of this technique for drought characterization of forages is being assessed as present. The other techniques are more time consuming and more experimental, but both water-use characteristics and osmotic potentials have been used to screen breeding lines of cereals in dry environments (e.g. Morgan, 1983). In the eastern Mediterranean, for example, the relatively high temperatures of drought-stressed plants in nurseries can be measured very rapidly with infrared thermometers (Blum, 1986). At the other extreme, we are conducting laboratory trials to assess the growth of young grass seedlings in solutions of an osmoticum and have detected significant differences between populations. If our results prove meaningful in relation to field drought, this technique would fulfil all the criteria for a characterization test - quick, easy, cheap, repeatable and relevant.

### **Equipment**

#### Pressure chambers

Chas W. Cooke, P.O. Box 184,  
Albion Works, Albion St.,  
Birmingham B1 3D1

T. & J. Crump, Church Road,  
Ramden Bellhouse, Billemeay,  
Essex, CN11 1RR

#### Osmometer

Wescor Inc., Logan, Utah, USA

Porometer

Delta-T, 128 Low Road,  
Burwell, Cambridge, CB5 0EJ

Conductivity meters

Most laboratory suppliers distribute suitable cheap instruments.

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## CHAPTER 6

### QUALITY CHARACTERIZATION

D.I.H. Jones and G. Moseley

The nutritive quality of forage to ruminants is determined by:

- (i) The intake of metabolizable (useful) energy
- (ii) Nutrient content (protein, minerals etc.) i.e. factors affecting the utilisation of energy (Raymond, 1969).

#### Voluntary intake of forage

Forages may be defined as bulky materials of low nutrient density containing a large proportion of indigestible fibre. Consequently, as a feed source for mammalian digestion, they suffer from two major inherent limitations. An animal, in order to meet its nutritional requirements from a forage feed, must possess within its digestive tract not only a system for digesting fibre, but also a mechanism for processing very large quantities of low density material. Although these requirements are generally met by the ruminant foregut, its capacity is limited, so that its ability to obtain sufficient nutrients from a forage feed depends on its rate of throughput. The major limitation to animal production from forage is the ability of the animal to eat sufficient quantities to meet its nutrient requirements. Maximizing the digestibility of forages will improve the availability of nutrients to the animal and also increase its voluntary intake. However, because of the fibrous nature of forages they remain relatively low-quality foods. It follows, therefore, that the improvement of voluntary intake characteristics of forages is central to the improvement of their overall nutritive value.

Feeding trials have shown that different forages of similar digestibility can have markedly different voluntary intakes. A number of reasons have been put forward to explain this. These include differences in the ratio of cell wall to cell contents of plants and also the ratio of readily digestible cell wall to slowly digestible cell wall. Both these features would affect the rate of digestion, and hence the rate of throughput, without affecting the overall digestibility (Walters, 1971).

correlated with the in vivo measurement and accounts for 80% of the observed variation in intake (Moseley and Ramanathan, 1986).

### Metabolizable energy content

Metabolizable energy is defined as that proportion of the dietary energy which is available to meet the maintenance and production requirements of the animal:

$$\text{Metabolizable energy} = \text{Energy in feed} - \text{Energy in faeces} - \text{Energy in urine} + \text{Energy in methane}$$

In practice the energy losses in urine and methane (produced during fermentation) are relatively constant. Differences in metabolizable energy content are, therefore, very largely a reflection of the loss of energy in the faeces. Furthermore, the energy content of different forages is also relatively constant. Metabolizable energy content is, thus, directly related to the digestibility of dry matter or of organic matter (OM). This is termed the DOMD or D value.

An assessment of the digestibility of forages is a very important quality characteristic since it defines the useful energy that can be derived from a forage. It is also important in influencing voluntary intake (see above).

### Estimation of digestibility

Digestibility is measured in vivo by feeding a group of 3 to 6 sheep for a 20-day period on the test forage. The amount of dry matter eaten and faeces dry matter produced is determined over the last 10 days, the first 10 days being used to acclimatize the animals to the test forage. The dry matter (DM) digestibility is then calculated by:

$$\text{Digestible dry matter (\%)} = \frac{\text{DM eaten} - \text{DM excreted faeces}}{\text{DM eaten}} \times 100$$

Alternatively the organic matter digested may be calculated after determining the ash content of feed and faeces. This is usually expressed as a percentage of the dry matter eaten, i.e. DOMD or D value.

The primary and rate-limiting step in ruminant digestion is the physical breakdown of feed particles to a condition and size capable of being both efficiently digested by rumen microorganisms and passed on to the hind gut. The processes involved in this breakdown are well documented but, by far the most important, are chewing during eating and rumination. Experimental work has shown that the degree of breakdown obtained during chewing can be directly related to the voluntary intake of the forage. This suggests that the characteristics of forage plants which control intake are those physical features which affect their resistance to the shearing forces employed during eating and rumination (Moseley and Dellow, 1985).

The anatomical features of forage plants which affect their resistance to breakdown have been examined by microscopic observation of digesta from forage feeds in the rumen. These observations showed clearly that the most resistant tissues were vascular and epidermal, and suggest that such features as vascular bundle thickness, epidermal cell structure and the cuticular association with epidermal cells may play a role in affecting the toughness and elasticity of the plant. It was also clear that particle shape also varied between forages and may be important in controlling their rate of passage from the rumen. While grasses break down to form thin thread-like particles which are difficult to pass from the rumen, legumes tend to break down into blocky particles which are more rapidly passed. This characteristic appears to be controlled mainly by the geometry of vascular systems and epidermal cell shape and arrangement (Moseley, 1982).

The selection of forage plants with characteristics of improved voluntary intake depends largely on the development of a rapid predictive technique and, although the use of various forms of physical measurements have been explored, they have not been generally adopted. However, the close relationship observed between forage-particle breakdown and chewing activity suggested that some measure of the physical resistance of plant material to a shearing force could provide such a method. This has resulted in the development of a simple and fairly rapid technique based on wet maceration and particle size separation. When combined with measurements of in vitro dry matter digestibility (pepsin/cellulase) or nitrogen content, the technique predicts dry matter intake which is highly

$$\text{DOMD (\%)} = \frac{\text{OM eaten} - \text{OM excreted faeces}}{\text{DM eaten}} \times 100$$

In vivo estimation requires some 100 kg of dried forage and only applies to the evaluation of potential varieties at the later stages of a breeding programme after seed multiplication. The characterization of genetic resources and breeding programmes to improve nutritive quality require the use of laboratory screening methods.

#### Laboratory assessment of quality

An enzyme digestion method for screening forage digestibility has been developed at WPBS (Jones and Hayward, 1975). This involves the incubation of 200 mg of dried ground forage with acid pepsin for 24 h at 40°C followed by removal of the pepsin and a second incubation of the residue with buffered cellulase for 24 h at 40°C. The pepsin removes the soluble constituents i.e. sugars, protein, etc., and renders the cell wall more vulnerable to enzyme attack. The cellulase is derived from a soil fungus, Trichoderma viride and hydrolyzes the cell wall polysaccharides accessible to enzyme attack. The amount of forage solubilized in this way is closely related to the amount digested by sheep in vivo.

The technique is more precise and has advantages over detergent chemical methods of predicting digestibility, e.g. modified acid fibre, lignin, etc., since it takes account of both the fibre content of a forage and the accessibility, or degree of lignification, of the cell wall. It also has advantages over the two-stage in vitro method using rumen inoculum in that it is more reproducible, easier to manipulate and does not require the use and maintenance of surgically modified animals.

The pepsin-cellulase method has been used successfully since about 1970 at WPBS to screen breeding material and evaluate varieties.

#### Nutrient content

Apart from supplying sufficient quantities of dietary energy unsupplemented forages also need to supply sufficient protein and essential major and trace elements to meet nutritive requirements. Routine assessments of protein and minerals are, therefore, required in the later stages of selection and in the evaluation of varieties.

Attempts are also being made to improve the mineral status of forages by plant breeding. Encouraging progress has been made in improving the magnesium content of Italian ryegrass. Selection lines higher in magnesium have been developed which showed striking improvements in the intake of magnesium and magnesium availability when fed to sheep (Hides, 1986). These lines are now being further multiplied for more extensive animal evaluation. Attention is also being given to other species and to improving the availability of other elements such as copper.

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**CHAPTER 7****PROTEIN ELECTROPHORESIS: ISOENZYMES AS GENETIC MARKERS**

C.J. Pollock, J L. Stoddart, Howard Thomas and T.W.A. Jones

**Introduction**

Taxonomic and genetic studies on plants have relied upon the development of progressively more objective criteria for assessing differences between individuals and populations. Such differences, to be meaningful, must mirror the organism's genotype as closely as possible, and investigators have become more and more concerned with estimating the immediate products of gene action rather than their final morphological expression. There has been widespread acceptance of the concept that the primary structure of proteins (the unique linear sequence of amino acid residues within a polypeptide chain) bears a direct relationship to a sequence of nucleotides within the genome. Since many proteins are enzymes (specific biological catalysts), enzyme variability has been used as an indicator of genetic diversity. Variant forms of an enzyme (isoenzymes) can be defined simply as enzymes with different primary structures which can catalyze the same reaction. A variety of methods has been employed to detect the presence of isoenzymes in plant tissues but most genetic analysis has used electrophoretic separation of bulk protein extracts followed by specific chemical localization techniques to identify individual enzyme activities. Use of such techniques allows a large number of individuals to be screened non-destructively in a relatively short time, but it is important to realize the limitations imposed by such techniques and the possibility of erroneous interpretation of the data. The pitfalls of isoenzyme-based genetic analysis can be summarized in 2 statements. Firstly, not all distinct bands on electrophoretograms are true isoenzymes, and secondly, not all isoenzymes can be resolved by electrophoresis. In this chapter, we outline the ways in which such problems arise and discuss what remedies are available.

**Sources of electrophoretic variability**

For the purposes of genetic analysis it is useful to distinguish 2 classes of isoenzyme variation. The first comprises variants coded at

different loci within the genome. These forms may have different locations within the cell or the plant or may be synthesized at different times during development. They often show distinct differences in catalytic or regulatory properties allied to differences in function; differences in electrophoretic mobility are usually large. In general, all members of a population will possess such multiple forms.

The second type of variation is often called allozymic variation and is the type most frequently employed in genetic analysis. Allozymes are different forms of a polypeptide coded at a single locus and often differ in only a few amino acid residues. Catalytic and regulatory properties of allozymes are usually very similar, and within a population there may be a large number of different forms. The expression of these forms in any individual as a multiple-banding pattern on an electrophoretogram will depend upon the number of alleles and heterozygosity at the locus involved, the ploidy level, and the number of polypeptide chains in the functionally active enzyme. A few simple combinations are outlined in Table 7.1 and indicate how a relatively restricted number of allozymes in a population can be used to 'fingerprint' a large number of individuals. Since there are considered to be thousands of different functional enzymes within a plant cell at any particular time, the potential resolution of this type of analysis is very high.

Table 7.1. Simple examples of allozyme patterns in plants

Number of forms within population	Ploidy	No. of polypeptide chains in active enzyme*	Max. no. of bands for an individual	Total number of different patterns within the population
2	Diploid	1	2	3
4	Diploid	1	2	10
8	Diploid	1	2	36
4	Diploid	2	3	10
4	Tetraploid	1	4	15

\*Assumes polypeptide chains are products of expression of a single locus with no preferential associations.

However, modification of a polypeptide following transcription will not change the relationship between the primary amino acid sequence and the original nucleotide sequence but may alter the electrophoretic

mobility. Processes such as glycosylation may be vital to ensure the correct enzyme function in vivo but where such modifications show broad specificity for the size and chemical composition of the additional material then multiple electrophoretic forms can be produced from the same original polypeptide. Unless such modified forms are few in number and produced consistently regardless of other factors, such enzyme systems may be unsuitable for electrophoretic mapping.

One area where modification to protein structure is undoubtedly not under close control and where problems are encountered is when changes to proteins occur during extraction and separation. Among the many enzyme activities present in plant cells are a number which can act directly or indirectly upon other proteins in such a way as to modify their charge/mass ratio (and hence their electrophoretic mobility) without altering their catalytic properties. Proteinases can hydrolyze quite large proportions of many enzymes without affecting the catalytic centres; phenol oxidases and peroxidases may cause aggregation by covalent bonding of phenols between different polypeptides; and thiol-specific enzymes may modify the oxidation states of sulphur-containing amino acids, leading to aggregation or dissociation of multimeric enzymes. Under such circumstances the electrophoretic pattern will reflect the concerted action of such enzymes rather than the genetic constitution of the plant.

Individual enzymes vary in their sensitivity to such degradative processes, and individual plant species have widely differing amounts of the enzymes and small molecules involved. Younger tissues generally have lower activities of degradative enzymes than more mature ones. Incorporation of specific protection agents in the buffer used to extract protein may be of considerable assistance in obtaining undegraded proteins for electrophoresis. Commonly used compounds include SH-group protectants such as cysteine or dithiothreitol, chelating agents to inhibit phenolase, and phenol-binding polymers such as polyvinyl pyrrolidone. Adequate buffering in the initial stages of extraction is also important since many plant species contain significant amounts of organic acids in their vacuoles. Rapid extraction of material and initiation of electrophoresis combined with the maintenance of low temperatures until the visualization stage may also be useful. If problems are encountered in studies on new species or ecotypes it is often useful to co-extract tissue with an equal

amount of material from a species where the electrophoretic patterns are stable and well documented. Modification or disappearance of bands following co-extraction would indicate degradative activity in the new material. It must be emphasized, however, that any procedure for the extraction, electrophoresis and assay of a particular activity will not be applicable universally. The effects of manipulation of extraction conditions cannot be predicted and in some cases it may be easier to abandon studies upon a particular enzyme system rather than spend a long time optimizing the experimental techniques.

### Variation undetectable by electrophoresis

It has been established that single amino acid substitutions in a polypeptide, presumably derived from single base-pair alterations, can lead to altered electrophoretic mobility. However, not all genome alterations of this type will lead to the production of discrete allozymes. Since the genetic code is partially redundant, single base-pair modifications may still code for the same amino acid. Even where distinct polypeptides are produced, the differences may not result in differences in surface charge at the pH of the separation, either because the substitute amino acid has similar charge properties to the original or because the substitution has occurred deep in the hydrophobic core of the folded polypeptide and has not affected surface charge. Although occurrence of these types of variation do not alter the applicability of isoenzyme techniques to studies of breeding and genetic resources, it should be borne in mind that the level of variation within the population is probably substantially higher than is apparent from the use of such methods. It is important to understand the different factors which combine to generate the spectrum of protein variability within a population and to understand how to assess those components which most nearly reflect the original genomic variability.

### Protein separation using electrophoresis

Of the 20 amino acids which go to make up protein primary structure, 8 can carry an associated electrical charge which may be + or - depending on the ionic environment of the protein in which they occur. At physiological pH (normally about 7-8) the acidic amino acids aspartate,

glutamate and histidine are negatively charged and the basic amino acids lysine, hydroxylysine, arginine, tyrosine and cysteine are positively charged. The net electrical charge of a given protein reflects the relative proportions of the two groups of charged amino acids. In most soluble enzyme proteins the molar proportion of acidic amino acids exceeds that of basic amino acids. Barley leaf total protein is typical, comprising about 24% acidic and 18% basic amino acids. Thus at physiological pH most enzymes are negatively charged. If the pH of the protein solution is reduced, the balance between - and + charges becomes displaced in favour of the + charged basic amino acids (Fig. 7.1). Eventually a pH will be reached at which + and - charges balance and the protein has no net charge. This is called the isoelectric point (pI). Most enzymes have pI values in the acid range. For example, the pIs of the cytoplasmic forms of phosphoglucosomerase (PGI) from *Lolium* tissue are between 6.0 and 6.5 and those of two forms of glutamate dehydrogenase from wheat leaves between 4.8 and 5.6. Dropping the pH further results in the protein acquiring a net + charge (Fig. 7.1).

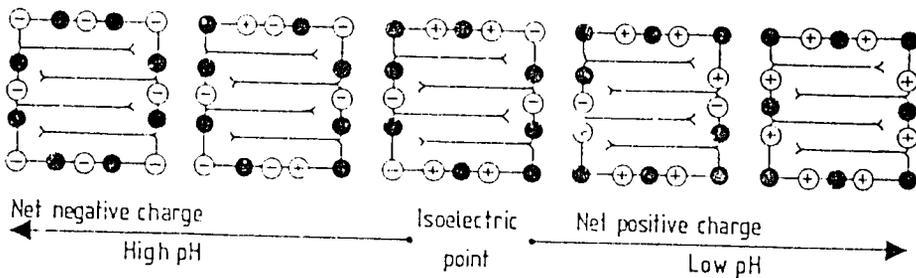


Fig. 7.1. The relationship between surface electrical charge and pH illustrated for an imaginary protein molecule.

Except at its isoelectric point, a protein in solution will move when an electric field is applied. At alkaline pH most enzymes are negatively charged and will move away from the - (cathode) region of the field and towards the anode (+). The rate of migration is determined by the charge:mass ratio of the protein. Electrophoresis is an analytical procedure which exploits differences in charge:mass ratio to fractionate proteins in a mixture by means of an electric field. Fig. 7.2a presents 2 simplified proteins with identical net negative charge (= n) but differing in size. When an electric field is applied to a mixture of proteins A and

B in alkaline solution, protein B, with the higher charge:mass ratio, migrates faster towards the anode than does A. Similarly, 3 otherwise identical proteins with unit charge differences (Fig. 7.2b) may be separated on the basis of charge:mass ratio by electrophoresis: For example, the products of 3 alleles of the same gene, varying at loci coding for 1 or 2 charged amino acids. The general principle illustrated in Fig. 2b applies equally well to enzymes made of more than 1 protein subunit. For example, each PGI enzyme molecule comprises 2 subunits of identical size. If the behaviour of protein C in Fig. 2b represents that of PGI in an individual homozygous for the fast-migrating subunit gene and the behaviour protein A that of the slow-migrating homozygote, then B shows how the hybrid slow-fast PGI from a heterozygous individual would migrate.

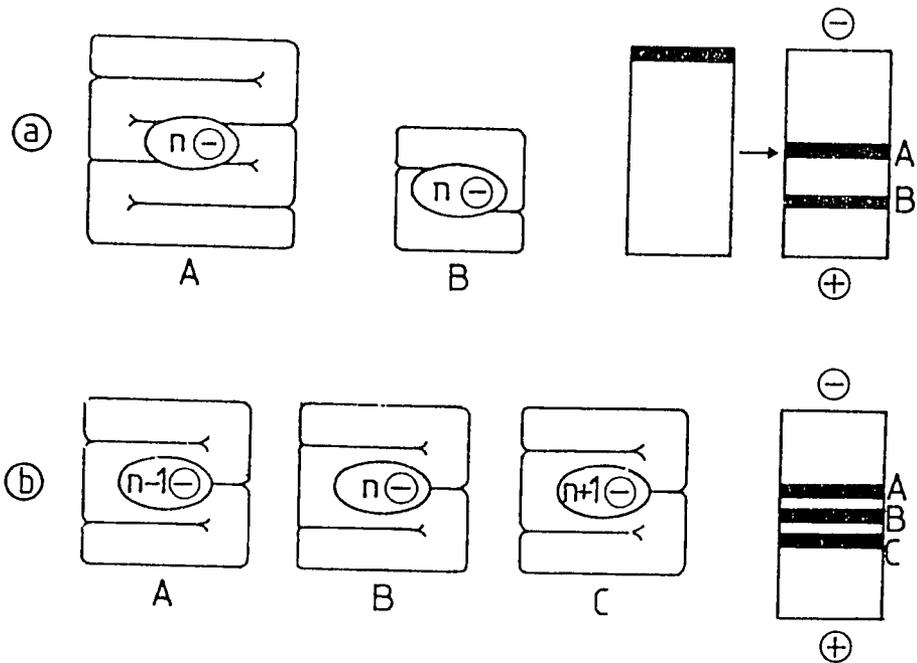


Fig. 7.2. The electrophoretic behaviour of imaginary protein molecules differing in charge:mass ratio.

During free electrophoretic migration in solution, proteins will tend to diffuse and components with incremental differences in charge:mass ratio, instead of separating as discrete bands, become indistinct, overlapping zones. This problem is aggravated by convection arising from the heating effect of the liquid medium's electrical resistance to the flow of current. By performing electrophoretic separations in an inert,

solid gel medium these difficulties are greatly reduced. Moreover, the protein pattern stabilized in a gel is easily visualized by specific staining procedures. For the routine, large-scale screening of plant enzymes by electrophoresis, starch is the gel medium of choice. It is relatively inexpensive, non-toxic and forms a gel simply by heating and allowing to cool, rather than chemical polymerization. To prepare a starch gel, a suspension of electrophoresis grade starch in buffer is heated with constant stirring to about 75°C. At this temperature the starch grains burst and there is a marked increase in the viscosity and turbidity of the mixture. The hot gel solution is degassed under vacuum and poured into a shallow trough formed by removable plastic strips sealed around the edge of a glass plate. Another plate is carefully placed on the surface of the hot gel and the gel mixture allowed to cool. The gel is cut across its entire width near to one end (the cathode end) and the protein samples, absorbed on small filter-paper wicks, are inserted into the slit at equal intervals from one side of the gel to the other. Buffer-saturated sponges connect the ends of the gel to 2 buffer compartments, one containing the platinum anode wire and the other the cathode. The electrical field is applied by means of a stabilized power supply and separation is performed at 4°C to preserve enzyme activity. At the end of the run, which may take 4-6 h, proteins are detected by staining.

It should be emphasized that a gel-separation system producing adequate separations for a given enzyme will not necessarily be acceptable for another, different enzyme, nor for the same enzyme from a different plant tissue. A number of factors need to be considered when optimizing electrophoresis conditions. The composition of the gel buffer is of great importance. For example, 6-phosphogluconate dehydrogenase from Lolium spp. gives poor separations and phosphoglucomutase activity is completely lost in a borate-based buffer system at pH 7.5, whereas a histidine-citrate system at the same pH gives excellent resolution of both enzymes. The pH of the buffer is also critical: acid phosphatase from Lolium spp., which is stable at pH 8.0, is inactivated by electrophoresis in the same buffer at pH 8.8. The molarity of the buffer is also a factor, with higher concentrations generally giving sharper but slower separations.

The nature and concentration of the gel-support medium have profound effects on protein separation patterns. The most widely used general electrophoresis medium is polyacrylamide, formed by the catalytic polymerization of acrylamide and a cross-linking monomer such as methylene bisacrylamide. By varying the composition of the polymer, gels of varying molecular pore size may be prepared. The 'particle-sieving' property of polyacrylamide allows high-resolution analysis on the basis of molecular size as well as charge:mass ratio. Isoenzymes analyzed by polyacrylamide gel electrophoresis are stained and documented in the same way as those separated on starch gels. A widely used variation of the polyacrylamide procedure is detergent-gel electrophoresis, which separates strictly by particle size and is a simple way of measuring protein molecular weights. Detergents such as sodium dodecylsulphate interact with different proteins to form complexes with essentially identical charge:mass ratios. Detergents will denature most proteins, precluding their detection by enzyme staining, but a few enzymes such as ribonuclease and peroxidase can be stained after removing the detergent from the gel. Another variation of electrophoresis is isoelectric focussing, where proteins are separated in a gradient of pH and are distributed in accordance with their pI values.

### Visualization of Separated Proteins

At the end of electrophoretic separation the protein patterns are invisible, unless associated with a chromophore such as chlorophyll or haem. To visualize them it is necessary to deposit stable, insoluble, coloured products in the gel regions occupied by the various protein species. Procedures can be divided into 2 main groupings, characterized as passive and active.

#### Passive staining

As already discussed, proteins contain varying numbers of unpaired charged groups, many of which are located in exposed regions of the molecule. Surface binding of small molecules by noncovalent or electrostatic attraction will occur and coloured dyes can be associated in this manner to render most proteins visible. The method is nonspecific and does not relate to any kinetic properties which the protein may have.

Passive staining is commonly used in polyacrylamide gel separations, particularly in denaturing systems, with amido-black and Coomessie brilliant blue being the most frequent dyes. After immersion in the dye solution, both the proteins and the background matrix are stained with equal intensity. Sequential immersion in alcohol solutions of graded strength, or in dilute acetic acid, will selectively remove colour from the background without diminishing protein staining. In taxonomic terms the complexity of the patterns obtained makes interpretation very difficult and the superimposition of proteins will frequently obscure differences. The method is usually reserved for situations where the genetic differences under study relate to nonenzymic products. Endosperm storage proteins in cereal grains are a good example.

#### Active staining

The most useful staining techniques, and those which offer the greatest selectivity, harness the catalytic activities of the separated proteins. Most frequently enzyme activity is used, although immunological interactions could also be included in this category.

With enzymes the capability exists to visualize only those proteins able to catalyze a specified reaction. This obviates many problems associated with superimposition, simplifies the pattern and allows the study of identifiable individual gene products. An additional advantage is that multiple patterns can be produced from a single gel. To visualize a specific enzyme activity the reaction catalyzed must be capable of yielding a coloured, preferably insoluble, product. In order to achieve this with certain enzymes it is necessary to link co-factor changes to secondary chromogenic reactions, or to couple with further enzymatic steps to complete the visualization. Some of the commoner techniques for isoenzymes visualization can be summarized as follows:

Diazonium salts, such as Fast Garnet GBC, can be employed in conjunction with artificial substrates to produce azo dye deposition in areas of appropriate enzyme activity. The diazotization reaction is driven by aromatic alcohols or amines which can be released from compounds such as naphthol phosphate or acetate by phosphatases or esterases. Release and, therefore, dye formation, is limited to areas with hydrolase

activity. Diazonium salts are relatively unstable in solution and sensitive to reducing agents. Appropriate precautions must be taken to maintain the effectiveness of the staining solutions.

Tetrazolium coupling is a general method for use in cases where the enzyme reaction or cofactor change can be translated into reducing power or electron donation. Reduced tetrazolium salts convert to insoluble formazans with characteristic colours. Tetrazolium salts are highly soluble and very stable in the alkaline range but they can be photoreduced and precautions must be taken to protect the solutions from light during gel staining. Apart from coupling directly to enzyme reactions which produce free electrons or superoxide ions (e.g. aldehyde oxidase), these salts can accept electrons from reduced cofactors (NADH, NADPH) through an electron carrier intermediate, such as phenazine methosulphate (PMS). The schematic coupled reaction for tetrazolium staining for malate dehydrogenase is depicted in Fig. 7.3.

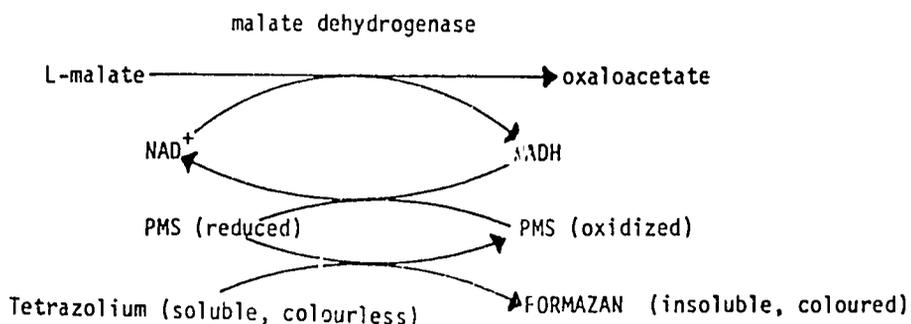


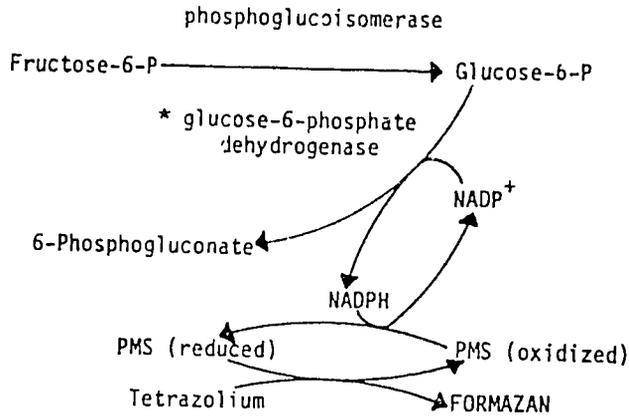
Fig. 7.3. Reaction scheme for visualizing malate dehydrogenase activity on gels.

The staining mixture contains all the components of the left-hand side of the scheme, and formazan is only deposited in those areas containing malate dehydrogenase activity. The method is generally applied to the visualization of oxidoreductases, but can be used for any enzyme capable of coupling to oxidoreductases.

Tetrazolium staining can be applied, in the negative sense, by exploiting photoreduction reactions. For example, riboflavin produces superoxide radicals in the light and these result in

general tetrazolium reduction throughout the gel. Formazan deposition is inhibited by areas containing superoxide dismutase and the isoenzyme pattern for this activity appears as clear bands on a coloured gel background.

Enzyme coupling is applied where this subject enzyme does not produce a coloured product or an exploitable change in the cofactor redox state. In these cases it may be possible to use the product of the reaction as a substrate for a second enzyme which yields a coloured product or electrons that can be coupled. This principle can be illustrated by the staining protocol for phosphoglucoisomerase (Fig. 7.4).



\* supplied as purified enzyme in the staining solution

Fig. 7.4. Reaction scheme for visualization phosphoglucoisomerase activity on gels.

Glucose 6-phosphate (colourless and diffusible) acts as a substrate for glucose-6-phosphate dehydrogenase (G6PDH) which, in turn, produces a cofactor change which can be coupled to tetrazolium. Formazan is only deposited in areas with PGI activity. In theory long chains of this sort can be constructed but, in practice, impurities and back reactions rapidly become significant.

Other methods can be used to visualize specific reactions. Amylases can be revealed as clear bands on a dark-blue background by utilizing

iodine staining of residual soluble starch in the gel. With care and temperature control this method can even be used on starch gels. Redox dyes (e.g. 5-bromoindozyl acetate), enzyme-modifiable fluorescent dyes or pH indicators have also been employed in isoenzyme detection. Full details of such systems and also working protocols for a whole range of procedures for diazo, tetrazolium and enzyme-coupled isoenzyme visualization procedures are given by Shaw and Prasad (1970) and Vallejos (1983).

#### Gel preparation for staining

Thick starch gels may be sliced using taut stainless steel wire or nylon fishing line. Normally, 2 such cuts can be made to provide 3 slices approximately 2 mm in thickness. The best staining is obtained on the cut surfaces and outside slices should be placed in staining solutions with the smooth surface downwards. Because gels are run at high pH values it is usually necessary to lower the pH to conform to the optimum for the enzymes of interest. This can be achieved by preincubation in buffer or (more often) by ensuring a sufficiently high molarity in the assay buffer. Development of enzyme patterns is hastened by incubation in warm conditions (approx. 37°C).

#### Preservation of gels

Starch gels can be preserved by overnight immersion in 50% ethanol or 50-100% glycerol. The latter is preferable because it maintains gel flexibility. Preserved gels can be stored in plastic or acetate bags and are best kept in a refrigerator. Some loss of stain intensity may occur during preservation.

#### Recording procedures

Band positions can be codified on the basis of relative migration ( $R_m$ ), which is obtained by dividing the migration distance of the marker dye front into the distance travelled by the isoenzyme band. This index is independent of run-to-run variations. Alternatively, if relatively few gene loci are expressed, and these run in well-defined regions of the gel, a simple coding system for the genes and alleles can be employed (e.g. A1, A2, A3; B1, B2, B3).

More permanent records and material for publication can be produced by photographing the gels. Illumination by transmitted light is preferable, using a diffusing light box mounted directly under the gel. Polaroid film (negative-positive Type 665) and standard 35 mm film (Kodak Panatomic-X developed in Microdol fine grain developer) are both used routinely at WPBS.

Good contrast between bands and background on the gel is essential in order to produce photographs which are suitable for publication. This requires careful optimization of both staining and photographic procedures. Panchromatic film should be used and coloured filters can be employed to advantage with some staining systems.

Computerized data acquisition is feasible using light-pen scanning of original gels or photographic negatives.

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**CHAPTER 8**  
**ISOENZYMES AS GENETIC MARKERS FOR**  
**CHARACTERIZATION OF VARIABILITY**

M.D. Hayward

In the grasses and legumes very few major gene markers are available for use in the comparative assessment of levels of variation occurring both between and within populations. The multiplicity of isozymes which can now be readily identified by the use of gel electrophoretic procedures (Chapter 7) offers the opportunity to rapidly assess the extent of genetic variation.

Isozymes are so useful as genetic markers because they have several advantages over other major gene markers such as leaf marks in Trifolium. They also however, have some limitations.

Advantages

1. The codominance of expression of allelic products at a single locus means that genotypes may be directly identified from phenotype which is free of epistatic or environmental effects.
2. The identity of individual enzymes allows attribution of allelic products to specific loci and hence comparability of loci across populations.
3. Allelic differences may be detected and the loci sampled independently of the overall level of variability.

Disadvantages

1. Post translational modification may take place due to genic or environmental effects, e.g. by a pathogen.
2. In polyploids, enzymes can arise through gene duplication which can lead to difficulties in ascribing variants to single loci.
3. Only about a quarter of the base changes which give rise to variants lead to changes in electrolytic charge which are detectable on an electrophoretic gel.
4. Only a restricted class of proteins are being assessed.

As isozymes are so easy to assay they provide useful measures of variation, particularly in outbreeding populations, in terms of 'allelic richness' and 'allelic evenness'. As such, collections may be characterized according to these two broad criteria, allowing populations to be compared and providing useful information on which strategies for plant collection may be based (Brown, 1978).

The diversity of genetic variation as measured over a range of isozyme loci may be quantified by several different measures.

#### The average number of alleles per locus

This is of limited use as it counts all alleles irrespective of their importance (frequency) and is particularly sensitive to sample size when used for purposes of comparative assessment.

#### The percentage of polymorphic loci

This again is a somewhat crude measure influenced by sample size and the number of enzymes assayed. For any individual locus an arbitrary level of gene frequency, such as 95%, is used to determine as to whether that locus is polymorphic.

#### The average heterozygosity

At an individual locus, at which several alleles may be identified, the proportion of the population which is heterozygous may be determined from:

$$h = 1 - \frac{1}{k} \sum X_{ij}$$

where  $X_{ij}$  is the frequency of the homozygous genotype for the allele;  $i$  and  $k$  are the number of alleles at that locus. Variances of this measure may be obtained according to the method of Brown and Weir (1983). This measure allows comparison at individual loci but is more meaningful when extended to several loci as an average heterozygosity or heterogeneity index.

The heterogeneity index

$$H = \frac{\sum_{k=1}^r h_k}{r}$$

where  $r$  is the number of loci and  $h_k$  the heterozygosity at the  $k$ th locus (see Nei and Roychoudhury, 1974). Here again variances of the estimates may be obtained allowing comparisons between populations to be made.

The genetic distance

The most widely used statistic for comparing the similarity of populations is the genetic distance measure ( $D$ ) of Nei (1978).

$$D = -\log_e I$$

$$I = J_{xy} / [J_x J_y]$$

where  $J_x[J_y]$  is the probability of identity of two randomly chosen alleles in  $x[y]$  and  $J_{xy}$  is the probability of identity of one gene from  $x$  with that from  $y$ . Sampling variances may be calculated and comparisons of the different populations made.

The usefulness of these various measures of genetic diversity may be shown by considering the data obtained from an isozyme survey of some populations of Lolium perenne collected in the UK and in Italy. Four enzyme systems were assayed, phosphoglucosomerase (PGI), glutamate oxaloacetate transaminase (GOT), acid phosphatase (AcP) and superoxide dismutase (SOD), which provided 5 polymorphic and 1 monomorphic loci.

On average, a greater number of alleles and heterozygous individuals or heterogeneous populations were to be found in Italy than in the UK (Hayward, 1985). Also, in the former country, the diversity between populations was very much greater, the average genetic distance being 0.1102 compared with 0.0455 for the UK. Clearly much greater genetic variation is to be found in populations of this species from Italy and as such that region should receive serious consideration for further collections where maximum diversity is desired.

Further applications of the type of data obtained from such isozyme surveys include assessment of population structure and breeding systems, measures of stability during the regeneration of plant collections and as possible identifiers of contamination.

The assessment of population structure and the breeding system, which is important in determining one's approach to the practical collection of plant genetic resources, may be obtained by consideration of the Hardy-Weinberg status of the population and the fixation index. Both of these population parameters may be easily calculated from data on the isozyme gene frequency.

The Hardy-Weinberg Law, which predicts genotype frequencies from allele frequencies under random mating, allows determination of whether such a mating system is occurring. Deviation from equilibrium may be due to selfing or possible selective advantage of one or more of the genotype classes and can indicate the extent of any variant population structure. This test should be generally applied to several loci before it can be firmly concluded that any class deficiency is valid and has not arisen as a result of sampling procedures.

The 'fixation index', based on the genic diversity and average heterozygosity, is a powerful measure of the mating structure when determined on a single locus basis. However, like its components, it is affected by the same problems of sampling and independence of loci (see Brown and Weir, 1983).

This brief consideration of the use of isozymes to look at population structure and related parameters may also be applied to the monitoring of collections during their regeneration. If initial collections are typed for a number of isozymes and genotype/gene frequency recorded as part of the descriptors of that collection, a powerful baseline may be established from which to measure any possible future genetic shift.

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### SECTION 3: UTILIZATION

The collection and characterization of genetic resources at a breeding institute can only be fully justified if these resources are to be used as the basis for varietal production. In the past, cultivated forages, in contrast to most other crops, have often been derived from wild or semi-natural ecotypes by direct exploitation unaccompanied by little hybridization and selection. Indeed, introgression between the cultivated crop and the surrounding wild ecotypes often occurs. This does not apply to the more sophisticated interspecific, intergeneric and polyploid cultivars.

However, the cross-compatibility and the high hybrid fertility between wild ecotypes and cultivated varieties have encouraged the utilization of genetic resources in forage breeding programmes. Evidence of this is shown in the fact that in the past 2 decades 12 grass and 6 forage legume cultivars, derived from introduced gene sources, have been bred by the WPBS.

The methods of utilization for breeding, or for research as a necessary prerequisite for breeding, range from direct exploitation of apparently adapted introduced ecotypes to phylogenetic studies involving wild relatives of the cultivated species. Evaluation will reveal how best a particular 'elite' introduction can be utilized. Usually a selected population is either generally well adapted with maximum expression of one or more desirable characters with no serious disadvantages in others, or adapted but with a number of disadvantageous features. Occasionally a poorly adapted population with maximum expression of one important character is identified.

The following papers describe breeding schemes utilizing material in the first 2 categories either by direct utilization, stabilizing or directional selection, or by intra- or interspecific hybridization followed by recombination and selection.

**CHAPTER 9**  
**UTILIZATION OF GENETIC RESOURCES IN BREEDING**  
**PERENNIAL RYEGRASS FOR PASTURES**

M.O. Humphreys

Initial evaluation of collected material by WPBS's Genetic Resources Unit, using information from spaced plants and small plots, provides breeders with considerable amounts of information concerning the value of accessions to particular breeding programmes. Breeders may then exploit these genetic resources in several different ways to produce new cultivars.

Direct utilization

If a particular accession/ecotype is relatively uniform, distinctive and possesses sufficient characters which meet the objectives of a breeding programme, without any seriously detrimental characters, then it may form the basis of a new cultivar immediately. Recent examples of this approach are uncommon in perennial ryegrass. However, a good illustration is provided by the heavy metal-tolerant Festuca rubra cultivar Merlin (Humphreys and Bradshaw, 1976). This was produced for reclamation, amenity and agricultural use directly from a naturally adapted ecotype found on old lead/zinc mine waste in North Wales.

Stabilizing selection

In the case of cv. Merlin, the natural selection operating on heavy metal mine waste was intense enough to strictly limit the amount of genetic variation tolerated within the ecotype. However, with ecotypes produced under rather less intensive selection pressure, the breeder is often required to do some 'tidying-up' by artificial selection to achieve the uniformity necessary for statutory trials. This is particularly critical if variation for heading date exists, as it can affect the stability of the cultivar during seed production. If additive gene effects control most of the variation expressed, simple stabilizing selection can be effective. For example, 2 generations of mass selection in a Swiss ecotype of perennial ryegrass (see Tyler and Jones, 1982 for a

description of its origin and attributes) reduced the variance of heading date from 10.7 to 4.7 with little change in mean expression (26.7 days after 1 April before selection compared to 26.1 days after selection).

### Directional selection

An ecotype may possess a particularly undesirable character which limits its immediate potential as a cultivar despite the presence of a number of other valuable characteristics. Several generations of directional selection may then be imposed to try to shift the character to a more acceptable level of expression before further cultivar development. For example, heading date in the Swiss perennial ryegrass ecotype was shifted from a very early 26 days to a more manageable 42 days after 1 April by just 3 generations of phenotypic selection for later heading. However, problems are sometimes encountered when imposing fairly intense directional selection in this way.

(1) Phenotypic variation may be limited and/or heritability small for a character. This results in a very slow response to selection. Thus, for example, crown rust susceptibility was reduced from 4.8 (scored on a 1-5 scale of increasing susceptibility) to just 4.5 after 3 generations of selection in the Swiss perennial ryegrass ecotype compared with a score of 3.0 for Frances (a more resistant control cultivar).

(2) There may be an unfavourable correlated response to selection in another character. Thus selection for later heading in the Swiss ecotype also increased winter leaf senescence from a score of 3.7 (on a 1-6 scale of increasing damage) to 4.9.

(3) Gains achieved by selection may diminish during seed multiplication under relaxed selection pressure. Examples of this with regard to yield and water-soluble carbohydrate content are given in Hayward and Abdullah (1985).

In order to avoid these problems, a more complex programme of multitrait selection is necessary using material in which useful variation is expressed for a wide range of characters. This may be achieved by using ecotypes in a more indirect way in crossing programmes prior to selection.

### Indirect utilization

In this approach, the breeder combines different genetic resources through hybridization to create totally new gene pools prior to selection. This has been used successfully in producing consistently high-yielding perennial ryegrass cultivars for pastures (Humphreys, 1985). Characters from populations differing widely in geographical origin and seasonal growth pattern are combined in this approach. For example, hybridization of the Swiss ecotype with later heading populations and cultivars of Lolium perenne has resulted in combinations of characters which have a heterotic effect on growth. Thus cut plots of a family derived from such a cross produced an increase in spring growth of 30% compared to the early Swiss parent, despite a later heading date (+25 days). In summer and autumn the hybrid-derived family exceeded the late-heading Melle parent in yield, showed considerably better rust resistance than the Swiss parent and produced an overall increase in total production of 21% and 8% compared to the early and late parent respectively.

The potential of hybrids between diverse ecotypes is evidently considerable. Unfortunately, the production of F<sub>1</sub> hybrid seed for commercial use is not feasible in grasses at the present time. However, it is possible that techniques using male sterility alleles, chemical male gametocides or the manipulation of the incompatibility system may achieve this in the future. At the present time, the breeder is forced to proceed through to the F<sub>2</sub> and subsequent generations and use some form of recurrent multitrait selection on segregating progeny to achieve a uniform and stable association of characters. On the basis that the initial F<sub>1</sub> heterosis is due to complementation of characters and dispersed dominant alleles (Jinks, 1984), and not to true overdominance at individual loci, it should be possible to retain a heterotic performance in later generations. Indeed, experience of selection lines in the F<sub>4</sub> and F<sub>5</sub> generations has shown this to be the case with the perennial ryegrass crosses described earlier. Of course it is necessary to monitor a range of different characters and use some form of selection index to do this. However, modern data collection and computing techniques now make this very feasible.

### Use of ploidy

A programme of chromosome doubling can reduce the time necessary to achieve acceptable levels of uniformity in generations derived from crosses between very diverse material. Such a programme has been used successfully to stabilize the products of interspecific hybridization between Lolium multiflorum and L. perenne (Breese et al., 1981). Essentially it reduces the rate at which potential genetic variation is released in segregating generations. Thus, it has been found that variation in heading date is reduced by 60% among tetraploid compared to corresponding diploid plants in the F<sub>2</sub> generation of a cross between the Swiss ecotype and Melle. Compared to diploids, tetraploids can also possess general qualities of increased palatability, sugar content, stress tolerance, disease resistance and compatibility with white clover. Therefore it is not surprising that their use in pastures is increasing.

By means of the various approaches outlined above, ecotypes of ryegrass collected throughout Europe are being utilized in breeding programmes at WPBS. The range of adaptations in material originating from Ireland to Hungary, Poland and Romania and from Italy to Norway is immense. As selection criteria in breeding become more precise and breeding techniques become more sophisticated, multidisciplinary research into the genetic and physiological bases of characters and their interrelationships become more necessary. If this is done the undoubted potential existing in the genetic resources available now should be realized in major advances in the cultivars of the future.

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## CHAPTER 10

USE OF THREE CONTRASTING ECOTYPES OF LOLIUM  
IN BREEDING FOR BRITISH AGRICULTURE

P. W. Wilkins

Italian ryegrasses (Lolium multiflorum Lam.)  
from the Po Valley region of Italy

Characterization of seminatural ecotypes from irrigated meadows showed extremely high regrowth, and high summer yields. Some populations from this region also have a significantly higher in vitro digestibility and lower fibrosity of the flowering stems than do existing cultivars used in Britain (Hides et al., 1983). This is likely to lead to higher levels of intake by ruminants. However, this material has a number of undesirable characteristics when grown in UK. It is winter susceptible as well as susceptible to leaf rust (Puccinia recondita) and powdery mildew (Erysiphe graminis). It is liable to shed seed before it is sufficiently mature to be harvested and, although it is very productive in summer, spring yields are relatively poor.

Two approaches were taken to overcome these problems; exploiting genetic variation between and within the north Italian ecotypes, and hybridizing with cultivars adapted to northern Europe. Some progress has been made with both methods but the former has given quicker results. Several generations of intensive selection for cold tolerance and resistance to fungal diseases were followed by spaced plant selection for a wider range of traits. A miniplot progeny test was used to select the mother plants of cultivar Tribune (Hides et al., 1980) now recommended for use in UK and France. In UK trials, it has outyielded the control cultivar RvP by 5-6% in the first harvest year, but in the second harvest year it has given similar yields. Selection has reduced its superiority over cultivar RvP in in vitro digestibility at the first conservation cut from 4 units to 2-3 units. This may be due to the increased spring growth of the cultivar relative to the ecotypes and consequent reduction in fructan accumulation. Further spaced plant selection within cultivar Tribune has resulted in a potential cultivar (Bb 2070) which gave significantly improved yields in the second harvest year at 2 UK sites

(Wilkins and Lovatt, 1985). Another potential cultivar (Bb 2042) combines high stem digestibility with good yield and winter hardiness.

**Perennial ryegrasses (*Lolium perenne* L.)**  
**resistant to ryegrass mosaic virus**

Two clones of perennial ryegrass, recovered from an old natural pasture which was heavily infested with ryegrass mosaic virus (RMV), were found to be highly resistant (Gibson and Heard, 1979). Genetic analysis showed that these plants possessed 2 independently inherited types of resistance: resistance to virus multiplication and spread within the plant, and a high level of resistance to infection (Salehuzzaman and Wilkins, 1984). The former was controlled by 2 complementary recessive genes and was strain specific, while the latter was polygenically inherited and highly effective against all known strains of the virus.

Italian ryegrasses are particularly prone to infection by RMV and no true resistance has been found within this species. Plants do vary in the severity of symptoms which they develop after infection but even the apparently most tolerant still suffer considerable reductions in yield and cold tolerance. An attempt was made to transfer resistance to infection by backcrossing to the best Italian ryegrass available at the time, cultivar Tribune. Because of the polygenic inheritance of this resistance, F<sub>1</sub> plants were intermediate and it was necessary to polycross them and select fully resistant plants with Italian ryegrass-like morphology from the F<sub>2</sub>. Four plants were selected from 6,000 F<sub>2</sub> individuals as parents for further backcrossing to cultivar Tribune. Two further cycles of backcrossing, polycrossing and selection were necessary to recover 10 genotypes which were morphologically indistinguishable from cultivar Tribune and had high levels of resistance to infection. These were polycrossed to produce the synthetic variety Bb 2113. Although morphologically similar to cultivar Tribune, Bb 2113 was significantly lower yielding. Further breeding is required before this resistance can be utilized.

**Ecotypes of perennial ryegrasses from northern Italy**

When grown in the UK, these ecotypes are extremely early to grow and flower in the spring. Hybrids with northern European cultivars often show

show marked heterosis for yield at certain times of the year. Some hybrids with early flowering cultivars such as cultivar S.24 have extremely good spring yields. They are reasonably uniform and are being developed as varieties (M.D. Hayward, pers. comm.). F<sub>1</sub> hybrids from selected winter-hardy north Italian clones crossed with late-flowering cultivars performed well all through the year, giving total annual yields over 2 harvest years 24% higher than control cultivars of similar flowering date (Wilkins, 1986). However, even these hybrids were too early flowering to be useful as general-purpose varieties. Spring management of such early flowering material is often not possible in the agricultural context, and numerous flowering stems are formed which result in poor quality pastures. An additional problem with these wide hybrids was marked segregation for flowering date among F<sub>2</sub> and F<sub>3</sub> progeny. Heterosis for yield suggested the presence of dominant genes for yield at different loci in the two parents, so a long-term programme of recombination by recurrent selection was undertaken with the intention of fixing high yield and combining it with late flowering, good persistency and resistance to fungal diseases. Four generations of intensive spaced-plant selection primarily for early spring growth and late flowering date followed by one generation of family selection primarily for plot yield and persistency resulted in a potential cultivar (Bb 10761) with 17% higher yields than cultivar Talbot under simulated grazing. Ba 10761 did not perform so well under infrequent cutting because of poor yields of reproductive material at the first conservation cut, but a further generation of family selection has shown that it is possible to combine high yields of both vegetative and reproductive growth. Initial results suggests that these high yields are due to greater nitrogen use efficiency, the essential ingredient being a low nitrogen content inherited from the north Italian ecotypes.

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