Collecting vegetative material of forage grasses and legumes

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N.R. Sackville Hamilton and K.H. Chorlton
Plant Science Division,
IGER, Plas Gogerddan, Aberystwyth, Dyfed SY23 3EB, UK.

Introduction

In a number of situations it is preferable – and sometimes necessary – to collect germplasm as vegetative rather than seed samples. The decision whether to collect vegetative material or seeds will affect the genetic composition of the resulting collection in a way that depends on the population biology of the species. It will also affect both optimum sampling strategy and sampling procedure. Collectors must be aware of the consequences. They must match the choice of sample type both to the biology of the species and to the objectives of the collecting expedition, and must adapt sampling strategies and procedures accordingly.

This chapter outlines the factors that contribute to the decision to collect vegetative samples of forages and describes aspects of population structure that are likely to be associated with species chosen for vegetative sampling. It then considers the consequences – both the direct consequences of sampling vegetatively and the indirect consequences associated with population structure – and describes the procedures involved in planning and executing this kind of collecting. It also covers back-at-base issues in detail, because dealing with vegetative samples is very different from processing seeds. It is mainly outbreeding, clonal, perennial, natural and seminatural forage species that are dealt with. Indeed, the examples mostly involve *Trifolium repens* and other temperate species. However, the general concepts and conclusions are equally applicable to tropical forages, although much less is known about these, and exact guidelines are therefore more difficult to provide. In some cases, the concepts will also be applicable to other kinds of plants, for example vegetatively propagated crops and woody perennials. However, specific issues relevant to collecting vegetative material of these plants are discussed in Chapters 21 and 23 respectively.

Problems associated with collecting vegetative material

The collector of vegetative plant parts is faced with problems and constraints that are entirely different from, or at any rate more severe than, those faced by the seed collector. The main ones are as follows:

- *Taxonomic identification of material.* Most published keys and other identification aids rely almost exclusively on flower and fruit characters, though exceptions exist (e.g. Hubbard, 1968). If the target species are in the vegetative state, they may be particularly difficult to find and distinguish. It may be necessary for collectors to develop their own identification aids (Chapter 11).
- *Sample size.* The size of vegetative samples, especially if composed of rooted plants in soil divots, may restrict the total number of both individuals per population and samples that can be collected during an expedition. If it does, it will affect sampling strategy, as described below, and, to the extent that the amount of genetic variation sampled increases with sample size, may cause vegetative collecting to be less effective than seed collecting in acquiring the maximum diversity of alleles.
- *Sampling speed.* Collecting one vegetative unit usually requires more time and care than collecting one seed. This affects sampling strategy and, if time is limiting, may again cause vegetative collecting to be less effective than seed collecting.
- *Storage in transit.* In contrast to the seeds of most species, many types of vegetative material have a short life in storage. If arrangements cannot be made for each sample to be quickly returned to the gene bank to be processed while the expedition continues, an appropriate form of temporary storage must be arranged, such as a cool, moist atmosphere in a refrigerator in the collecting vehicle.
- *Quarantine.* It is usually necessary to place vegetative samples under stricter quarantine than seed samples, both because of the greater variety of pathogens and pests that are liable to be moved with vegetative material and in soil, and because cleaning procedures cannot be as effective as with seed.
- *Preparation of material for the gene bank.* In whatever form the samples are finally stored (as seeds, *in vitro*, as vegetative propagules or in field gene banks), accessions must be prepared from the collected material. The time taken to do this may actually be less than for seed samples, especially when seed dormancy has to be broken. In most cases in *Lolium* and *Trifolium*, seeds can be obtained from vegetative samples within one year.

Reasons for collecting vegetative material

Because of the extra difficulties associated with collecting vegetative samples, it is normally undertaken only when it is impossible to collect

seeds or when collecting seeds introduces other problems. A range of situations in which collecting vegetative material will be an advantage are discussed below.

Vegetatively propagated crops

In some crops, the normal means of propagation is vegetative. The genetic variants produced by sexual reproduction may be of interest in a breeding programme, but the actual genotypes being grown by farmers will also need to be conserved. Collecting vegetatively propagated crops, with special reference to roots and tubers, is covered in Chapter 21.

Seed production unpredictable

In many perennial species, seed production is irregular or intermittent, varying in time and space and with genotype. Seed collecting may then be unsatisfactory, due to their poor quality and/or quantity. In many temperate pastures, for example, most plants produce no seeds in most years, largely as a result of agricultural practices. Hay and silage cuts are taken before the seeds ripen, removing immature inflorescences, because that is when feed quality is highest. In a well-grazed pasture, most inflorescences are eaten before maturity. Seeds mature in abundance only when the farmer manages a field to produce a seed crop. The absence of suitable insect pollinators can also depress seed set, as in the case of *T. repens* at the upper limits of its altitudinal range in the equatorial Andes (about 4600 m), where flowering is abundant but seed production virtually nil (pers. obs.).

Seeds mature at different times

In many populations of outbreeding species, seeds may mature over a long period of time. At any one time, only a small proportion of individuals may bear ripe seed. In temperate grasslands, for example, the seed-ripening season can extend throughout most of the growing season, with considerable differences among species. There may also be variation in seed-ripening among populations of a single species within a region.

Fast-shattering fruits

The fruit of some wild species shatter quickly after maturation, dispersing the seeds, which are therefore accessible to the collector for only a very brief period.

To avoid bias towards genotypes with ripe seeds

All of the above may introduce a genetic bias, in a seed sample, towards genotypes with ripe seeds at the time of collecting. For germplasm conservation this is unsatisfactory, and can be avoided by sampling vegetative material. For example, the two 'species' *Lolium multiflorum* and *L. perenne* are freely cross-compatible, producing fertile hybrids. In Europe, they form a hybrid swarm showing continuous variation

between the two extreme parental types. The *L. multiflorum* extreme characteristically flowers throughout the year, whereas *L. perenne* has a more seasonal flowering period. A seed sample taken from a hybrid population between July and September is likely to contain more seeds of the *L. multiflorum* type and will therefore be biased towards that parent, whereas a vegetative sample will be unbiased.

To avoid bias towards large plants

Many perennial herbaceous plants are clonal. Some forms of vegetative propagation, such as stolons or rhizomes with an indeterminate growth habit and adventitious roots, can make a genetic individual, or clone, potentially immortal. A well-established population may contain some very large, old clones. For example, Harberd (1963) recorded a clone of *T. repens* 20 m across, with an estimated minimum age of 100 years. Oinonen (1967) recorded a single clone of *Pteridium aquilinum* covering 1500 m², and probably a thousand years old. A population of such species is likely to show a skewed size distribution, with few large and many small clones.

Sampling inflorescences or cuttings at random in such populations results in non-random sampling of individuals. Sampling is biased towards large clones, and a single large clone may unwittingly be sampled several times. For some purposes, the bias may be useful. Large clones are large because they have maintained a positive growth rate over a long period of time. Small clones are small either because they are young or because, though old, they have not grown well. Thus large clones have in a sense been 'tested' by natural selection and performed well, whereas small clones are either untested or have performed badly. If the sample is intended for use in breeding for the same environment, this preliminary on-the-spot selection may be an advantage. However, for other purposes the bias is undesirable. First, it might exclude genes of value in another location or in a future environment. Secondly, for a conservation collection, systematic exclusion of any genes is undesirable. Thirdly, the bias will invalidate studies of population genetics that require a random sample of clones.

Repeated sampling of the same clone is commonly minimized by spacing sampling points (for seeds or vegetative material) far apart in relation to the expected size of clones, but this does not eliminate the bias towards large clones. It is particularly unsatisfactory in species such as *T. repens*, in which individual clones may be large and yet closely intermingled. For example, Cahn and Harper (1976) found up to ten different clones of *T. repens* in a single dm² of pasture. Sackville Hamilton (1980) found up to three overlapping clones in 1 cm² of pasture in a population where individual clones were several metres in diameter.

The only complete solution is to take large numbers of cuttings and identify clones using whatever markers may be available (e.g. major gene loci for self-incompatibility, polymorphic enzyme systems or deoxyribo-

nucleic acid (DNA) fingerprinting). However, such intensive sampling and post-sampling effort, essential for population genetics studies, cannot usually be contemplated as part of most large-scale germplasm collecting, where bias can be avoided only for species whose clones are readily distinguishable in the field. For example, the leaf markings of *T. repens* provide a useful, although not definitive, guide to the genetic identity of plants. They are expressed most strongly in the cool season, when the plants are not flowering, and so are most readily used when sampling vegetatively.

A sample may be biased both by seed presence and by plant size. For example, Table 22.1 compares seed and vegetative samples of two populations of *Festuca pratensis* in the hybrid zone between the diploid and the cross-compatible tetraploid (*F. pratensis* var. *appenina*). The two chromosome races are virtually indistinguishable morphologically in the field. Both vegetative samples contained a high proportion of triploid hybrids, which were absent from one seed sample and at low frequency in the other. The diploid was absent from the vegetative sample at site A but comprised 100% of the seed sample. At site B, the tetraploid was absent from the vegetative sample but comprised most of the seed sample. No single source of bias could explain all these results. Differences may be postulated in flowering times of diploid and tetraploid at the two sites to explain the biased compositions of the seed samples. However, to explain their absence from the vegetative samples, it is necessary to postulate size bias, i.e. that the triploid has high vegetative vigour but low fertility, producing large plants that are favoured in vegetative sampling.

Collecting outside normal ripe-seed season

It is essential that a seed collecting expedition be timed to coincide with the availability of ripe seed. This requires good local knowledge and may be particularly difficult if the targeted populations differ widely in their flowering times. The entire route of an expedition may need to be planned around flowering times, beginning with early-flowering and ending with late-flowering populations. The timing of vegetative collecting is likely to be less critical, depending on the species and the type of vegetative sample. For example, rooted cuttings of stolons of *T. repens* can be taken successfully at any time of year, even in midwinter (Sackville Hamilton, 1980). In such cases, collecting vegetative material guarantees success, and intensive collecting can be started whenever required and continued as long as necessary.

Within-population targeting

The genotypes and sizes of plants in a population reflect the outcome of natural selection acting on the gene pool of the population in the juvenile and mature vegetative phases. A seed sample represents the next generation's unselected recombination potential of a known female parent with a generally unknown male parent. The male parent may be

Table 22.1. Percentage of diploid, triploid and tetraploid plants in seed and vegetative samples taken from two populations of *Festuca pratensis* ($n = 7$) in the Carpathian mountains of Romania. The vegetative samples were taken in August 1980, and the seed samples in October of the same year.

Site	Sample type	Number of plants sampled	Percentage of samples in ploidy level		
			$2n = 14$	$3n = 21$	$4n = 28$
A	Vegetative	14	0	86	14
	Seed	42	100	0	0
B	Vegetative	16	0	100	0
	Seed	17	0	24	76

from a different microenvironment (see below) within the same site, if not from an alien site. In general, therefore, the genetic composition of a vegetative sample will more closely reflect population subdivision associated with microenvironmental variation, and may be used to target locally 'adapted' gene complexes.

Juveniles do not produce seed

Many perennials do not produce seeds in the first years of growth. If there is selective mortality before the age of first reproduction, the genotypic composition of the mother plants of a seed collection will not be the same as that of the total vegetative population including juveniles. If the population is in equilibrium, a seed sample would give rise to the same genetic composition of juveniles, and so this would not cause a genetic bias. However, if mortality before reproduction is causing a directional shift in genetic composition between successive generations, a seed sample will be biased against genotypes present only as juveniles.

Genetic structure of populations of clonal species

The genetic structure of populations will determine the amount of genetic variation recovered by a particular sampling strategy. Populations of clonal species are often very different from populations of species which do not spread appreciably by vegetative means in the number and spatial arrangement of the genotypes that comprise them.

Number of clones per population

Because the clones of some species are potentially immortal, being able to spread vegetatively indefinitely, it is theoretically possible for a population to comprise a single clone. For example, Sackville Hamilton (1980) found a number of populations of *T. repens* consisting of only one

or two clones, apparently in locations near the limits of the ecological tolerance of the species for shade (i.e. in long grass or shrubland). In contrast, populations of the species in old pastures can contain many hundreds or thousands of clones (Sackville Hamilton, 1980; Gliddon and Trathan, 1985).

Population subdivision

It has long been established that in most plant species effective population size is much smaller than the apparent physical limits of any given population. For example, the reproductive genetic neighbourhood area of *T. repens* is $2 m^2$ (Gliddon and Saleem, 1985), although a typical, apparently uniform pasture containing the species can be $10^4 m^2$ to $10^6 m^2$ or larger. This affords considerable opportunity for the development of genetically distinct subpopulations on a very small scale relative to the perceived extent of an apparently spatially continuous population.

Early work on genetic differentiation caused by strong selection pressures revealed sharp genetic boundaries between subpopulations, e.g. corresponding to differences in heavy metals levels in the soil. More recent studies have shown similar population subdivision in relation to a variety of different kinds of selection pressure. For example, Table 22.2 shows subdivision of a population of perennial ryegrass caused by a path traversing diagonally through a hay meadow. Progeny of samples from the path were more prostrate and more cold-tolerant and showed later spring growth and later flowering than those from the undisturbed meadow. A number of other examples in *Lolium* are given by Tyler and Chorlton (1976).

In another set of studies (reviewed by Sackville Hamilton, 1990), vegetative samples were taken from subpopulations of *T. repens* in four different microsites within a single old 0.75 ha pasture in the north of Wales (Turkington, 1975). The four microsites seemed to differ only in the dominant species of grass. The samples were multiplied in a glasshouse so that each could be transplanted back into all four original sites in the pasture. Each sample performed best when transplanted back into its native site, revealing a previously unexpected fineness of specialization within a single apparently uniform population. In the next stage of the study, the four samples of *T. repens* were grown in a glasshouse

Table 22.2. Characteristics of progeny of perennial ryegrass obtained from plants collected in 1967 from a hay meadow and a path traversing the same meadow in northern Italy.

Location within site	Seedling height (cm)	Date of flowering	Spring growth (kg ha^{-1} day^{-1})	Survival after freezing (%)
Hay meadow	11.4	1 May	20.5	57
Path	6.5	31 May	3.7	92

in all pairwise combinations with the four species of grass that domi-
nated the four original collecting microsites. Again, three of the four
samples gave the highest yield when grown with the grass species they
were originally found with. Using other pastures of different ages, it was
shown that this specialization takes three to ten years to develop.
Effectively the same experiment, but at a still finer level of microsite
distinction is reported by Gliddon and Trathan (1985). At each of four
microsites within a single field dominated by *L. perenne*, a cutting was
taken of *T. repens* and of the neighbouring clone of *L. perenne*. The four
pairs of clones were multiplied and grown in all 16 grass–clover combi-
nations, and again each tended to do best when grown in combination
with its original 'partner'.

These studies show that *T. repens* is phenotypically responsive to
remarkably fine microenvironmental variations. Moreover, one popu-
lation is genetically variable for this responsiveness and divided into
subpopulations specialized with respect to the fine-scale microenviron-
mental heterogeneity. This potential for substructuring is greatest in
long-lived perennials with a stoloniferous or rhizomatous habit, i.e. in the
very species most likely to be sampled vegetatively. Their growth habit
enables them to 'forage' for and exploit favourable microsites. A strati-
fied sampling strategy at each collecting site, recommended for general
application in Chapter 5, will be especially appropriate for such species.

Sampling strategy

Marshall and Brown (1975) outline optimum sampling strategies for
genetic resources conservation. This work is updated in Chapter 5. In
this section those aspects specific to vegetative sampling are considered.

Number of sites and plants per site

The problem here is to choose, within the constraints of the resources
available, how many sites should be sampled and how many plants
should be taken at each site. In collecting for conservation, the aim is
to collect maximum genetic variation. Oka (1969) estimated the propor-
tion of the total genetic variation captured from a target area as

$$G = 1 - \{1 - P + P(1 - p)^n\}^N$$

where P = proportion of total variation represented by one population;
p = proportion of one population's variation represented by one plant;
N = number of populations sampled; and n = number of plants sampled
per population.

Marshall and Brown (1975) present solutions of this equation maxi-
mizing G, the proportion of genetic variation captured, for a range of
assumptions, but do not explicitly consider vegetative collecting of
clonal plants. For outbreeding species, they suggest sampling 30–55
plants per site at six to nine sites per day (Chapter 5). This solution for

outbreeding species is based on P between 0.50 and 0.75 and $p = 0.05$. Sackville Hamilton (1980) estimated $P = 0.37$ for variation in leaf area in a collection of *T. repens* from one area in the east of England, which would suggest sampling marginally fewer plants per site and more sites.

In a large population of a clonal species $p = 0.05$ may be accurate, but where the population contains only a few clones (see below) p will be much higher. In the extreme case of a population with one clone, $p = 1$. In practice, this simply means that the suggested sampling rates are accurate for large populations but impossible for small ones, and the number of plants sampled per population must be reduced.

The calculations also assume that size of the collection is limited by the time available for collecting, and that the time required to visit a site is 25 to 100 times greater than the time required to collect one plant. Both assumptions require modification for vegetative collecting. It usually takes longer to collect one vegetative sample than one seed, so we should expect smaller ratios and a smaller optimum sample size per population. In addition, time may not be the limiting factor in vegetative collecting. There may be a limit to the total number of plants that can be collected or subsequently processed, either because of the large size of each sample and limited temporary storage space in the collecting vehicle or the collector's rucksack, or because of limited labour for subsequent processing. If total number of samples is the limiting factor, then we have the constraint $Nn = k$ in Oka's formula, where k is a constant, the total number of plants that can be handled. Under this constraint, G is maximal at $n = 1$, and $N = k$ regardless of the values of p and P. That is, to maximize the diversity sampled, just one clone should be sampled from each of as many populations as possible. In practice, however, this is undesirable, because we would then have a poor estimate of the population mean and no estimate of diversity within the population. In obligate outbreeders it would also mean that no seed production would be possible except by crossing plants from different collecting sites. In any case, optimum sample size per population is considerably smaller than in seed collecting.

If the species is outbreeding and needs to be quarantined after international shipment, another constraint may be more limiting. Each population sample must be kept in its own isolation chamber in special quarantine facilities, isolated from cross-pollination with other samples and isolated from possible contamination of other plants. The total number of population samples that can be handled post-collecting may be the limiting factor for the number of sites visited, i.e. the number of sites visited is fixed. The number of plants sampled per population should then be increased until the next constraint becomes operative, whether that is the total number of plants that can be collected, the time available for collecting, or the number of clones in the population.

Site selection

Since the early days of genecology (the study of population genetics in relation to habitats) (Clausen *et al.*, 1940), a wealth of evidence has accumulated that genetic differences among populations of a species are associated with environmental differences in the sites they occupy. The genotypic composition of populations has been shown to be influenced by every environmental variable studied, whether biotic (e.g. competitors, mates, predators, pathogens) or physical (e.g. moisture, pH, soil structure, nutrients, temperature, quantity and spectrum of light). As emphasized by Marshall and Brown (1975) and others (Chapter 5), it is thus essential to seek the greatest diversity of environments in putting together a germplasm collection. A clustered distribution of collecting sites, besides decreasing the time wasted travelling between sites and improving the value of the collection for studies on ecogeographic variation, will: (i) increase the range of types of environmental diversity sampled, and (ii) 'force the explorer to search consciously for markedly different habitats within a region' (Marshall and Brown, 1975).

An illustration is given by Sackville Hamilton *et al.* (1979). In a small collection from all locatable natural populations of *T. repens* in one region in the east of England, variation for mean leaf length was greater than that observed in a large collection covering most of the global geographic range of the species, and much greater than that in the UK component of the global collection. The difference can be attributed to the fact that the global collection was targeted on a single specific habitat – agricultural grazed pastures – whereas the east of England collection, although geographically much more restricted, represented a much greater variety of habitats.

No type of site should be systematically omitted during collecting. For example, Sackville Hamilton (1980) made a two-level collection of *T. repens* from eastern England: an extensive collection of a few clones from every wild population located, followed by an intensive collection of many clones from six selected populations. The six populations were chosen partly for their distinctness, but partly also to enable the same stratified sampling procedure from a 10×10 m plot to be applied at all six populations to facilitate sample comparisons. Although selected for distinctness, genetic variation among the six intensively sampled populations was far lower than among the extensive collection. Selecting populations with a structure sufficiently similar to allow the same sampling procedure to be applied appears to have had the negative effect of eliminating from consideration a great range of environments (e.g. paths, verges, gaps in shrubland, etc.) that support small but genetically distinct populations.

So, in collecting a species where population size can vary from one to several thousand, no attempt should be made to standardize the sampling strategy within populations, since that can be achieved only by excluding some sites. Moreover, since small populations are likely to occur in environments near the ecological limits of the species, they are

likely to comprise extreme genotypes. As much, if not more, emphasis should be placed on sampling these small populations from unusual environments.

Population sampling

No single systematic procedure should be followed for sampling within sites. Rather, the sampling procedure must be matched to the population being sampled. In a population genetically subdivided by microenvironmental heterogeneity, as all larger populations will be (see above), targeting the various microenvironments within the site will extract more variation than random sampling. The sampling bias towards large plants (see above) is likely to improve the success of such targeting. The extent of population subdivision in relation to the apparently most trivial microenvironmental heterogeneity emphasizes the importance of noting and targeting even the tiniest variants. However, logistical constraints generally dictate that only samples from the more obvious microenvironmental variants (e.g. on and off a path, under and away from a hedge, fence, roadside verge, etc.) will be retained as separate accessions in the gene bank. Finer subdivisions will have to be bulked unless required for population genetics studies.

Collecting methodology

Equipment

The collecting vehicle must be capable of carrying the collecting team with their personal equipment and collecting equipment as well as up to 200 kg of vegetative samples. The vehicle used at the Institute of Grassland and Environmental Research (IGER), UK, is specially modified to keep vegetative samples alive. It has a fitted refrigeration system, consisting of two small refrigerators. These have been adapted to run off the vehicle alternator when travelling, a 240 V external alternating current (AC) supply when the vehicle is stationary and bottled gas in the absence of a suitable electrical supply point. The freezer compartments of the refrigerators are used to maintain a constant supply of frozen gel packs, which are then used to keep collected material cool in insulated expanded polystyrene boxes. For trips of relatively short duration, or in cool regions, fitted refrigeration may not be essential, though it will always be desirable.

Table 22.3 gives a list of the specialized equipment used by IGER teams in collecting vegetative samples.

Sampling technique

Different sampling techniques are used for different species. If the species produces vegetative propagules that are self-contained survival and dispersal structures (e.g. bulbils), sampling will be much the same

Table 22.3. Specialized equipment used by IGER teams in collecting vegetative samples.

Item	Quantity
Refrigerators	2
Gas bottles for refrigerators (15 kg)	2
Insulated boxes (capacity 240 l)	3
Gel packs	25
Thermometers	4
Extension cable (30 m)	1
Electrical adaptor	1
Polythene bags (450 × 300 mm)	1000
Plastic labels	250
Trowels	2

as for seeds. If, however, it is necessary to take cuttings of actively growing tillers, stolons, shoots, etc., more care is needed to prevent death of the cutting before it is processed at the gene bank.

For species whose cuttings do not root readily, the sample must be a rooted plantlet in a divot of soil. Where adventitious roots are readily produced, as from the stolons of *T. repens*, unrooted cuttings may be taken, although even in these cases survival and establishment can be increased to nearly 100% by including roots and soil in the sample. However, the inclusion of roots and soil increases sample bulk several-fold. Taking unrooted cuttings may increase the total number of samples that can be collected by so much that more samples may ultimately be established despite a lower survival rate. Which of the two methods is used depends on:

- to what extent survival is affected by including roots and soil;
- how severely the total number of samples collected is limited by their bulk;
- whether it is more important to establish many cuttings (allele conservation) or specific cuttings (population genetics or targeted conservation);
- whether the soil itself will be useful.

Including soil provides a plant-specific sample of rooting medium that can be analysed later. In addition, for legumes, host-specific strains of *Rhizobium* can be sampled from the nodules of a rooted sample and in some cases from the soil itself (Chapter 26).

To illustrate the collecting procedure, assume a team of three collectors sampling *L. perenne* and *T. repens* from a uniform seminatural grassland pasture defined by a stock-proof boundary, within which some type of agricultural management is applied. According to the owner or manager, the pasture has never been ploughed and reseeded with

modern cultivars, at least not within living memory, and there has been a long history of agricultural management.

- The site is examined to confirm the presence or absence of the target species and to check for microenvironmental discontinuities. Detailed history and management details of the site, along with permission to collect, are obtained from the landowner. Once the presence of the target species is established and it has been confirmed that the site fits all the collecting criteria, sampling commences.
- A unique site number is allocated to the site. Each species to be collected from the site is given a different collecting number. These numbers are written on separate polythene bags (in waterproof ink) and also on plastic labels which go into the bags. These numbered bags are then carried around the site and filled with vegetative divots. Collector 1 collects stolons of *T. repens*, each with a small divot of soil. Collector 2 collects *L. perenne* divots as clumps of rooted tillers about 5 cm in diameter. Divots may contain both species, in which case both are extracted when the divot is processed on return to base. Collector 3 records general site details (Chapter 19) and then assists the other two collectors in sampling.
- Some 35–45 divots of *T. repens* and 25–35 divots of *L. perenne* are dug up using a sharpened knife or trowel. (There is a greater rate of loss of *T. repens* divots in transit.) Divots are collected over the whole area of the collecting site with due regard to spacing between points of collecting. Air is expelled from the polythene bags of divots and the bags are sealed. The filled bags are stored in a cooled, insulated box in the collecting vehicle.
- A collecting form is completed for each sample collected at the site. In this example, because there are no microenvironmental discontinuities within the site, two forms are filled in, one for each of the two species. The two forms will contain identical data for nearly all sections, except collecting number, species name, sampling details, associated samples (perhaps) and population data. The data are fully entered on site, with the help of the landowner to ensure accuracy. Information is pooled from the three collectors who have walked the site to sample it and the landowner who has managed it.
- The samples are packed into airtight polythene bags and placed into insulated boxes. Frozen gel packs from the refrigerator are packed around the bags to keep them cool and reduce plant respiration rates. Alternatives to plastic bags for storing samples include burlap sacks (kept moist) and styrofoam boxes (plant material wrapped in moist newspaper).

This process of collecting plants and data normally takes about 90 minutes for two separate vegetative samples from one site. The minimum time on site for one vegetative sample per site is about 1 hour and on a complex site with observable microenvironmental discontinuities this may increase to about 2 hours. Allowing for travelling time between

sites, the average number of sites covered per day in the manner described is four or five.

At the end of the collecting day, the ideal situation is to return to a base, where the bags of divots can be opened and the divots (with identifying labels) laid out in separate trays for each sample to be watered if dry or allowed to drain if too wet. A secure area is required where the divots will not be disturbed and where separate samples can be kept apart. When the expedition is ready to return to its home station, the moist (not wet) divots are repacked in their respective bags and placed in cooled insulated boxes in the vehicle for transit.

Post-collecting

The post-collecting handling of vegetative material is very different from seed handling. It needs specialized facilities, in particular if quarantine is involved, and must be rapid.

Quarantine

The material collected may need to be sent abroad, for example for safety duplication or because adequate facilities for the conservation of vegetative material and/or the production of seeds for storage are lacking within the country. The transfer of live plant material from one country to another requires a phytosanitary certificate from the country of origin and an import licence from the country of final destination. These documents will need to be arranged at the early planning stage, in some cases at least six months in advance of the expedition departure date. Some countries do not allow the entry of soil. In such cases, soil will have to be washed off the roots and the plants wrapped in moist paper and stored in plastic bags before shipment. There may also be restrictions on the movement of plants and soil within a country, for example if a soil-borne disease is present in some districts and not others.

For a collection arriving at IGER on 1 September, the procedure, with specific reference to *L. perenne* and *T. repens*, is summarized in Table 22.4. For dealing with a collection originating from within the country, quarantine will usually not be necessary, but the rest of the procedure will be no different.

Seed production

All samples are maintained in quarantine while flowering to generate seeds for storage in the gene bank. The aim is to produce a maximum yield of high-quality seeds from each genotype, excluding foreign pollen. Both *L. perenne* and *T. repens* are self-incompatible, the former wind-pollinated and the latter insect-pollinated. A secondary quarantine facility is used at IGER for seed production, divided into isolation chambers to prevent cross-pollination from other populations (Table 22.5). As each

Table 22.4. IGER procedure after collection arrival.

Date	Stage	Action
1 Sept	Plant collection arrives	Copy of import licence given to customs officers at port of entry. Signed by customs and forwarded to Ministry of Agriculture as proof of entry of vegetative samples
2 Sept	Place divots in quarantine	Move sealed bags of divots to quarantine house. Each population placed in a separate plastic, compartmented tray, with soil of divots in contact with capillary matting
	Divots 'recover'	Spray with systemic insecticide and fungicide if necessary
16 Sept	Extract plants and soil from divots	Each divot is broken down and cleaned. One tiller and/or stolon per divot is retained and planted in population groups in sterile compost in plastic compartmented trays. Original soil collected from the divots, setting aside two soil samples per collecting site. One soil sample sent direct to microbiology lab for extraction of *Rhizobium*, the other dried and analysed. Waste soil and vegetation put in sealed polythene sacks for sterilization and safe disposal
	Promote active regrowth	In temperate latitudes, as natural day length and temperature decrease, supplement artificially to give an equivalent 16-hour day at 20°C. Cut back and fertilize as necessary
15 Dec	Promote flowering	Remove supplementary light and heat, to allow floral induction under normal short day length and low temperatures
	Control pests and diseases	Material monitored regularly, and sprayed if necessary
1 March	Material potted on	Transplant into 15 cm diameter plastic pots of sterile compost, maintaining genotype and population identity

population sample comes into flower, all genotypes from that population are transferred to a vacant isolation chamber. Cross-pollination within each population sample is promoted by the ventilation system for *L. perenne* and by bees for *T. repens*.

Clean, high-quality seeds are obtained from each genotype within the seed island and kept separately in labelled packets. These individual

Table 22.5. IGER procedure for seed production.

Date	Operation	Comment
End March	Monitor populations for onset of flowering	Emergence likely to be over several months even for a collection from a small geographic area
End April–early July	Immediately prior to anthesis, spray population against pests and diseases and transfer each population to a separate isolation chamber ('seed island')	Pots in random block design. Isolation chamber can be either in quarantine house or in a separate isolation block of similar design
4–6 weeks later	Seed island removed from isolation chamber 15 days after peak anthesis, the heads being covered with labelled pollen-proof bags	Allows increased throughput of seed islands
2 weeks later	Heads cut off individual genotypes (still in bags) and hung up to dry	Heads (in population groups) dried slowly in glasshouse, laboratory or ideally a drying room
4–6 weeks later	Dry heads initially pounded to remove most of the seed and then mechanically threshed to remove the remainder. Seeds hand-sieved and mechanically blown in a column blower	Dry seed heads essential for efficient threshing.

mother-plant seed packets, making up the seed island, are transferred to a drying cabinet where the seeds are dried passively over silica gel to 5–7% moisture content. After drying, each seed lot is weighed and divided up as follows:

1. Approximately 0.1 g of seeds from each mother plant is stored in separate packets at −20°C in the long-term store. This is the basic seed. All seed samples are stored in sealed foil-laminate envelopes.
2. A balanced bulk for each population is made up of equal numbers of seeds from each mother plant. The number of seeds per mother plant is the lowest usable number produced by any one mother plant. The samples are stored at $0 \pm 2°C$ in medium-term store and used for research and exchange.
3. An unbalanced bulk is made up of the remainder of the seeds. These samples are stored at $0 \pm 2°C$ in medium-term store and are used for evaluation.

Conclusions

Collecting vegetative material has an important role in germplasm conservation. Although it raises some additional problems compared with seed collecting, it also has several specific advantages which make it preferable, or necessary, for some species and some purposes.

A decision to collect vegetative material has consequences for all stages of an expedition. The optimum sampling strategy changes, in terms of the optimum number of sites visited and plants sampled per site. The criteria for site selection and the method for sampling each population are also different. In general, more care must be taken, sampling fewer genotypes than in seed collecting. Different sampling techniques are required. Different equipment must be prepared to collect the samples and keep them alive. Different logistic arrangements for the temporary storage of samples must be made to keep the samples alive throughout the expedition, either by using whatever facilities are available *en route* or by frequent transfer back to the home institute during the collecting. More stringent phytosanitary regulations governing import of living material must be observed. Different post-collecting procedures for quarantine must be followed and seeds will have to be produced for storage and easier use and exchange of the germplasm.

In summary, optimum sample sizes per population are generally smaller than for seed samples. In some situations a sample size of one maximizes the diversity collected, although this extreme is not recommended because it eliminates information on diversity within populations. In many species, targeting the full diversity of microenvironments occupied by a population will extract greater diversity than a random sample. It is recommended that just two or three plants be taken from each microenvironment.

Acknowledgements

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