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Protocols for weed seed bank determination in agro-ecosystems

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INTRODUCTION

A number of reasons exist for studying weed seed banks. Perhaps Mayor and Dessaint (1998, pp. 95-96) best summarized these for agronomic purposes in the following statement:

- Seed banks are of ecological and evolutionary importance in the dynamics of weed populations and communities.
- Seed longevity and carryover of viable seeds in the soil from previous years can buffer the effects of weed control and hence maintain the weed problem. Some researchers have found that, with the exception of weeds with large seeds, the seed bank is a better indicator of long-term influences of agronomic practices on weeds than aboveground vegetation.

Seed banks are also studied for the purpose of anticipating forthcoming weed problems, assessing biodiversity and granivore food resources, and so forth. Although the goals of seed bank studies can be quite diverse, a common denominator among seed bank researchers typically involves an interest in sampling adequacy. Indeed, researchers new to the field often express anxiety regarding methodologies for sampling and quantifying seed banks. The purpose of this chapter is to provide some guidelines regarding sampling protocols for estimating weed seed banks in soil.

The recent symposium volume edited by Champion *et al.* (1998) provides one of the best available condensations of seed bank studies and methodologies. We encourage researchers who desire to begin working on seed banks to consult this book. Another very useful treatise on seed banks is Leck *et al.* (1989). The review by Roberts (1981) and chapters in Baskin and Baskin (1998) also contain much useful information. Otherwise, the first and most important guidance we can provide is that the objectives of the overall experiment should be matched to the need to have seed bank information. Will knowledge of seed bank composition and density provide insights that are more useful than those gained from knowledge of aboveground vegetation? Although seed bank analyses usually are not very expensive, they are labour-intensive. Consequently, the objectives of seed bank studies should be clear and unequivocal. Researchers must remember that the seed bank is part of a dynamic soil-plant-animal-microbe system, and the tedious work required to characterize the seed bank only provides us with a 'shapshot' in time.

The second most important guidance we can supply is that there is no universal sampling protocol applicable to all studies of soil seed banks. Each investigator has specific objectives and unique limitations in terms of labour and equipment. Moreover, each agro-ecosystem to be studied also has characteristics that may demand distinctive experimental protocols. Unique protocols are most obvious for agricultural systems with specific weeds: e.g. rare - vs common, small - vs large-seeded, and widely dispersed - vs aggregated species. However, the physical environment also plays a large role in sampling efficiencies and protocols. For instance, wet clay soils are much more difficult to sample than moist loam soils. These differences must be taken into account when designing practical protocols for assessing weed seed banks.

Seed bank researchers must devise protocols that are suitable to their objectives, equipment and labour constraints, and the agricultural system in which they work. Consequently, our goals in this report are to provide guidelines that may help researchers, especially those new to seed bank analyses and to tailor new studies on weed seed banks with a minimum of effort devoted to protocol development. This is not to suggest that new and more efficient protocols should not be devised. Instead, we urge new seed bank researchers not to duplicate and repeat weak protocols that could have been stronger with only slight modifications. These modifications often require no extra labour or expense. In some cases, only the time or type of sampling would need to be altered.

SAMPLING FIELD SOILS

Seed banks typically are confined to the surface and upper 30 cm of soil, although some perennial plants maintain seeds in aboveground seed banks (e.g. in serotinous cones of *Pinus contorta* Douglas ex Loudon). Therefore, sampling soil usually is a necessary component of seed bank studies. The most obvious questions that arise are: How many and what size soil samples should be taken?

The horizontal distribution of seeds across soil determines, in part, how many soil samples need to be taken. Weed seeds typically are not distributed randomly across a field. If they were, sampling seed banks would be much easier. Instead, weed seed banks almost always are highly aggregated in agricultural fields (Wiles and Schweizer, 1999, Chauvel *et al.* 1989). The aggregation can be the result of very limited dispersal away from parental plants, such as with early-maturing weeds in late-maturing crops (e.g. *Avena fatua* L. in soybean); or human-mediated dispersal of weeds that mature synchronously with crops, wherein seeds are spread in strips across fields by combine harvesters (e.g. *A. fatua* in wheat). Such aggregation affects the results of soil seed bank sampling.

The spatial pattern of seed banks often can be described mathematically by a negative binomial distribution (NBD). From a practical point of view, this basically means that many soil samples representative of the seed bank for any particular species will have no seeds, and a few samples will have high numbers of seeds. For instance, Jones (1998) found that at least half of her sampled cores were devoid of seeds when mean seed densities were less than 4 000 m^{-2}, and that 75 percent of cores were devoid of seeds when seed densities were less than 750 m^{-2}. Note that seed density changes the apparent level of aggregation. Typically, as the density of a species increases, the level of aggregation decreases, and the ease of adequate sampling rises, but much variation surrounds this generality. For instance, in the equation describing NBD, the level of aggregation is associated with the k coefficient, which was reported by Chauvel *et al.* (1989) for several common temperate weed species (Table 1).

Table 1. Descriptive statistics for the distribution of seeds of five of the more abundant species across a field in France, where N is the total number of seeds detected in soil cores (4.7 cm diameter and 30 cm deep), m is mean seed number per core, CV is the coefficient of variation (s/m), and k is the NBD aggregation coefficient.

Species	N	m	CV	k
Thlaspi arvense L.	1105	5.58	0.72	2.79
Sinapis arvensis L.	1079	5.45	1.01	1.20
Chenopodium album L.	881	4.45	0.96	1.42
Alopecurus myosuroides Huds.	527	2.66	1.31	0.75
Fallopia convolulus (L.) Loeve	409	2.07	0.83	4.76

The most common species (highest value of m) in Table 1 was *T. arvense*, which had the lowest CV and one of the highest k values, meaning that it was not as aggregated as most other species. The least common species was *F. convolvulus*, which might have been expected to be the most aggregated, but in this instance it was the least aggregated plant (highest k value). *A. myosuroides* was the most aggregated species (k was lowest), and its CV was highest and m almost the lowest among the species. These results suggest that the most difficult species to detect in this particular field would be *A. myosuroides*. Nevertheless, with the proper sample number and size, even the density of *A. myosuroides* could be established with some certainty.

How much soil to sample?

The initial question that needs to be answered is: "How much soil do I need to sample to get an accurate portrayal of the seed bank?" The amount of soil sampled is a product of the number of cores and the size of the cores. Core size involves core area or diameter (i.e. most soil sampling tools are tubes with circular orifices) and also core depth.

How many cores?

The absence of randomly varying seed populations in soil introduces problems for sampling seed banks. The primary question is how many soil cores should be sampled for an adequate representation of a seed bank. One of the best papers in recent years to address this issue was the synthesis of a multi-nation and multi-year study sponsored by the European Weed Research Society (Dessaint *et al.* 1996).

Abundant empirical evidence of seed bank densities from five countries showed a consistent relationship between mean seed bank density and variance. Overall, the relationship was defined as $\log_{10} s^2 = 0.45 + 1.41 \log_{10} m$, which is an adaptation of Taylor's Power Law. From this relationship, Dessaint *et al.* (1996) derived an equation that helps approximate sampling adequacy based upon differing levels of desired precision. That equation was

$$N = 10^{0.45} (m/509)^{-0.59} D^{-2} \qquad [1]$$

Where N is the estimated number of necessary samples (i.e. 5-cm diameter soil cores) to adequately represent a seed bank, and D represents the desired level of precision. D is defined as the standard error of the mean divided by the mean (SE_m / m). The value of m is divided by 509 to convert the area of a 5-cm diameter core to 1 m^2. Dessaint *et al.* (1996) indicated that a D value of 0.3 was a practical level of precision for seed bank studies. We believe that even a precision value of 0.5, which is less precise than 0.3, may be adequate depending upon the goals of the researchers. For instance, perhaps D could be set to 0.4–0.5 for species that are relatively

uncommon but easily controlled. In contrast, species that are both common and difficult to manage probably merit D values of 0.2–0.3. Thus, sampling efforts can be conditioned by the required value of the resulting information.

Table 2 represents solutions to Equation 1 for hypothetical seed densities of 10, 50, 100, 500, 1000, 5 000, and 10 000 seeds m^{-2}, each with a precision level of 0.2, 0.3, 0.4, and 0.5. These results by Dessaint *et al.* (1996) are dependent upon the use of soil cores that are 5 cm in diameter. Results would differ for cores with other diameters: higher numbers of samples for smaller diameter cores, and fewer samples for larger cores. However, as stressed below, 5-cm diameter cores are an ideal size for seed bank studies, and we advocate the use of this size of soil sampling tool.

Table 2. The number of soil cores (5 cm diameter) necessary to determine seed bank densities under four levels of desired precision assuming various seed densities.

Seed bank (seeds m^{-2})	Precision level (*D*)			
	0.2	**0.3**	**0.4**	**0.5**
10	716	318	179	115
50	277	123	69	44
100	184	82	46	29
500	71	32	18	11
1000	47	21	12	8
5000	18	8	5	3
10000	12	5	3	2

The results shown in Table 2 (Dessaint *et al.* 1996) also may be specific to the region in which the data were collected, but this region was quite broad, involved many crops and soil types, crossed several national boundaries, and spanned Mediterranean through temperate agro-ecosystems. In any event, the number of cores necessary for estimating seed bank densities is not nearly as large as other literature sources indicate, provided that densities of the species of interest are greater than 100 seeds m^{-2}. With some luck, these results will have universal application (although exceptions certainly can be expected), which will help to alleviate a formidable burden on seed bank researchers. It should be kept in mind, however, that if the goal of the study is to characterize the seed flora and density completely, as in a weed community analysis, then the number of cores required is higher because the less common species will be sampled at a lower level of precision than the more common species. In agricultural fields, over 90 percent of the seeds might be comprised of just a few of the 30 or more species represented in the seed bank. Therefore, some of the more interesting changes in weed communities – such as shifts in response to management – might be happening among the less abundant species, and higher sampling intensity is required to detect these changes.

Although seed banks range greatly in density, the median value for crop fields in Minnesota is about 1000 seeds m^{-2} (Forcella *et al.* 1993). Estimation of this density would require 21 samples for the recommended precision level of 0.3 according to Table 2. Clearly, species with very low densities (<100 seeds m^{-2}) would require so many soil cores that precise determination of their seed banks is not practical. An example of such a species is *Xanthium strumarium* L. Its seeds are dispersed in two-seeded capsules (burs), the size of which is about 1 x 2 cm. Not many of these large fruits are produced relative to other weeds with smaller seeds. Consequently, detection of *X. strumarium* in seed banks is rare, except in very dense infestations, so that an accurate estimation of the size of its seed bank is quite difficult. Despite its importance as a weed

and the desire of researchers to understand its seed bank dynamics, such a large-seeded species is not easily amenable to seed bank analysis.

What size core?

The diameter of the core usually depends upon available equipment. Most hand-held soil sampling equipment was developed for soil scientists, and much of it is about 2–3 cm in diameter. Although, theoretically, any soil core diameter is suitable for sampling weed seed banks, certain sizes are far more practical than others.

As mentioned above, fewer large-diameter cores than small-diameter cores are necessary to sample seed banks adequately. However, large diameter cores amass great quantities of soil quickly and may overwhelm the researcher. For example, a single 10 cm diameter soil core (15 cm depth) has a dry weight of about 1–2 kg. Hypothetically, even if only ten cores were exhumed per plot, the total weight (soil plus soil water) of the sample might be 20 kg. If an experiment had ten treatments and five replications, the total mass of sampled soil might be as much as 1 000 kg. Such massive amounts of soil can be unwieldy for transportation to and from the field site, as well as within the laboratory.

In contrast, the soil in narrow cores (e.g. 2 cm diameter) might weigh only 50–100 g, which makes them easy to handle and transport. However, the probability of detecting seeds in such small amounts of soil is so very low that that many cores must be sampled for compensation. There are very few studies that compare core sizes for sampling efficiency. Benoit *et al.* (1989) found that augers with diameters of 1.9, 2.7, and 3.3 cm were not different in estimating the number of *Chenopodium album* seeds when a similar volume of soil was sampled. Although the largest and smallest of these diameters differ only by a factor of 1.7, the largest and smallest volumes of sampled soil from a single core exhumed by these augers differ by a factor of 3. In other words, three times as many soil cores of 1.9 cm diameter would need to be sampled as cores of 3.3 cm diameter in order to estimate similar seed bank densities. The additional labour involved in taking more cores may not compensate for the ease of using small-diameter cores.

Our experience is that 5 cm diameter cores represent a practical solution to the problem of core sizes. This size core is large enough to detect seeds, but small enough not to burden the researcher with too much soil. We advocate their use in seed bank studies.

However, also other factors are involved in the choice of diameters of soil-coring devices. The most significant of these is soil texture and soil water content. Wet soils with high percentages of expanding clays are notoriously difficult to remove from sample tubes, especially from small-diameter tubes. Cores with diameters of up to 10 cm should be considered for such soils. Application of non-toxic oils (vegetable oils) to the coring implement helps greatly in preventing clay from sticking to the device. Orifices that are a few millimetres narrower than the diameter of the sampling tube also may aid in preventing the soil from adhering too tightly to the inside of the sample tube. However, these types of coring tools are more likely to compress soils with low bulk densities while the coring tool is being driven into the soil. This compaction of the core confounds reliability of core depths. In contrast, very dry soil can resist penetration by coring implements. In these cases, narrow cores may be more practical than the recommended 5 cm diameter cores. Researchers must be practical and balance multiple factors when choosing sampling equipment.

Vehicle-mounted soil probes are available and very useful for sampling large plots and fields. Hydraulics drive the probes into the soil, retract the probes, and remove soil from the probes,

which greatly facilitates sampling in many types of conditions. However, vehicle-mounted equipment is clumsy in small research plots. Hand-held equipment often is easier to use in these cases.

What depth of soil to sample?

The depth to which soil cores should be taken is entirely dependent upon the objectives of the research. Generally, few seedlings have the ability to emerge if their seeds are buried deeper than 10 cm. Exceptions include large-seeded species such as *A. fatua* and *X. strumarium*. Consequently, soil samples rarely need to exceed 10 cm depth. However, many seed bank researchers are interested in differing tillage systems wherein seeds are buried differentially at depths by the tillage implements. In these cases, samples to 30 cm depth may be necessary. These researchers should recall, however, that seed burial by mechanical tillage equipment is a physical process that is quite consistent (see below). In other words, the same type of plough buries seeds in the same proportions and at the same depths regardless of soil type, location, time of year, etc. This consistency means that if the tillage system is known, then the relative proportions of seeds at different depths can be estimated without ever sampling the seed bank. Consequently, only a single depth may need to be sampled to estimate the seed bank of the entire soil profile, and that depth should probably be a function of the emergence-from-depth characteristics of the species of greatest concern to the researcher.

What spatial arrangement of samples?

Whether sampling soils within a plot or an entire field, researchers must decide on the spatial distribution of the samples. Random sampling designs would be appropriate if seeds were distributed randomly. Furthermore, the amount of time spent locating random points in a field, based upon *a priori* selection of random numbers, is usually not practical. For ease of sampling, many researchers take soil cores at roughly evenly-spaced intervals along a simple W-shaped pattern within a plot or field. Others have used X-shaped patterns or a single diagonal transect. Colbach *et al.* (2000) examined various patterns for sampling accuracy in aggregated weed populations and concluded that many patterns provided equivalent results, with the diagonal transect being the simplest design to use. We recommend that any design is acceptable provided that it somehow spans the length and width of the plot or field.

When to sample seed banks?

We cannot stress too greatly the need for logic when deciding the appropriate time of year to sample seed banks. Several authors report a lack of correspondence between aboveground vegetation and seed bank composition and density. The absence of such a relationship too often reflects the illogical times that these authors chose to sample the seed bank. For the sake of camaraderie we will not cite references in this regard, but even a casual perusal of Methods and Materials sections of the seed bank literature will confirm our assertion.

Basically, if a goal of the research is to relate seed banks to forthcoming aboveground vegetation, then seed banks should be sampled at times that follow seed shed but precede seed germination. Sampling seed banks after seedling emergence has little value, in theory or in practice. Samples need to be taken at a time that makes sense for the objectives of the study, based on the phenology of seed dispersal and germination in the habitat of interest. Thus, in temperate zones, seed banks of summer annual weeds should be sampled prior to the first springtime flush of seedling emergence. Similarly, seed banks of winter annuals should be

sampled before emergence of the first seedlings in autumn. Analogous logic should be used for sampling times in semi-tropical and tropical zones with distinct wet and dry seasons. The same reasoning should apply to irrigated land regardless of season; that is, the soils should be sampled before the onset of irrigation and subsequent seed germination.

Even when soil samples are taken before seed germination, there still can be a question regarding time of sampling. For example, in northern temperate zones, seeds of many summer annual weeds are shed during August through October and germinate during the following March through June, the exact times being species-dependent. The period of time for properly sampling seed banks of these species would be late autumn through early spring (e.g. November to March). Even during this winter period of apparent quiescence, however, there is biological activity with regard to buried seeds. This activity affects seed mortality, and it may influence the times for best sampling of seed banks.

Comparisons of viable seed banks of summer annual weeds from two sampling times (immediately after seed shed in autumn and immediately before seed germination in spring) indicated an approximate 10 percent loss in viability over winter and a slight superiority of springtime samples for predicting forthcoming aboveground weed densities (Forcella, 1992). Theoretically, this is exactly what would be expected. Aboveground vegetation of annual weeds should be a better reflection of the seed bank immediately before seed germination than the seed bank several months in the past because many and varied mortality events could have occurred during the intervening period.

Although the theory of seed bank sampling design may point to sampling immediately before seed germination, the practical aspects of seed bank research may justify sampling sooner (but still only after seed shed). Researchers need to balance desired accuracy with workloads. In the above example, spring not only is the best time to sample soils, but it is the only time to sow crops and implement many weed control procedures. Consequently, little time exists in spring for the extra work involved with sampling seed banks. Earlier sampling of seed banks can be justified for this reason alone, if not in conjunction with other reasons. Researchers often have to balance good protocols with labour constraints.

An additional reason for early sampling involves the length of time necessary to process soil samples in the laboratory or glasshouse (see below). If the goal of the research is to use seed bank information to help make recommendations for weed management treatments (Schweizer *et al.* 1997) then the information must be available at the time the treatments are to be implemented. This could be as early as days or even weeks before experimental plots are sown with crops in the case of early preplant herbicide treatments. Consequently, sampling soils months before crop sowing may be necessary.

HOW SHOULD SOIL CORES BE PROCESSED?

Once cores have been exhumed from the soil, there are two primary techniques for enumerating the number of seeds in these cores. The two methods give differing results, but the results of the two methods usually correlate with one another (Ball and Miller 1989, Barberi *et al.* 1998, Cardina and Sparrow, 1996; Forcella 1992).

Direct seed extraction

The first technique can be termed "direct seed extraction," and Malone, (1967) is the author most often cited for this technique. The direct seed extraction method can be used on (a) the entire soil sample derived from entire individual cores, (b) subsamples of individual cores, or (c) subsamples of soil from aggregated cores. Clearly, labour requirements decrease from a–c, as does reliability of the resulting estimates of seed bank densities.

A typical soil core of 5 cm diameter and 10 cm depth has a dry weight of about 200–300 g. Naturally, if labour is not in short supply, extracting seed from the entire soil core is preferred. However, a shortage of labour (or associated enthusiasm) is common in seed bank studies. Thus, some understanding of what proportion of a soil core must be examined is important. Analyses of differing amounts of well-mixed soil, in 20 g increments from typical cores indicated that, in general, 100 g was necessary for an adequate representation of the entire soil core (Forcella, 1992).

Subsampling soil from aggregated cores would be recommended most often in studies where individual cores were small (< 5 cm diameter), but where many cores were exhumed. Examining the entire soil volume from small-diameter cores typically would be futile because the probability of small cores harbouring even one seed is very low (Benoit *et al.*1989; Jones, 1998).

In the direct seed extraction technique, seeds are separated from soil typically by washing or flotation. The washing method has many variations. Most simply, the soil sample is placed on a screen with a mesh size smaller than the smallest expected seed. However, sieving risks loss of seeds that might be similar in size or shape to the objects being separated, or that might adhere to them. Sieving – especially for dry samples – can damage seeds that are thin, light, and fragile, but it can aid in scarifying seeds with hard seed-coats. Several researchers have used a series of sieves with different mesh sizes to sort seeds according to size. Mesh size is a critical factor in determining the efficiency of seed separation. A mesh size of about 0.2 mm can catch most small seeds, but would not be effective for dust-like seeds of species like *Orbanche* (see below). There can also be considerable variation in seed size among seeds from a given species, even from a single plant. Therefore, the mesh size chosen to detect seeds of a given species must be small enough to catch the smallest individuals of that species.

The sample can be pre-soaked for a short time to saturate and loosen clay aggregates. Soaking the soil sample in a solution of sodium hexametaphosphate will improve dispersal of clay aggregates. The next step is to remove clay, silt, and fine sand particles from the sample. This commonly is done by shaking the sample while it is held by the screen, or by passing a jet of water over the sample. Once the fine particles have passed through the screen, the remainder of the sample includes seeds, organic debris, sand particles, and in clay-rich soils, clay aggregates that did not fully disperse. These latter clay aggregates often can be eliminated by applying gentle pressure with fingertips until the aggregates crush and pass through the screen. The seeds and organic debris that remain on the screen are separated from the sand particles by differential flotation (see below). If sand particles are not abundant, the sample can washed onto mesh (e.g. cheese cloth) and air-dried, whereupon the seeds are separated from the organic debris by hand. Air-driven seed cleaners also can be used to separate organic debris from seeds in dried samples.

An elutriator is a device that mechanically performs the same procedures as described in the preceding paragraph (Gross and Renner, 1989). The beauty of elutriators is that they can process several samples simultaneously. The equivalent of a primitive, but motorized, clothes washing

machine was used successfully for removing soil from buried seed samples by Fay (1978). These machines are a convenience, but are not essential for seed bank analyses.

The flotation method often is used after the soil sample has been washed free of clay, silt, and fine sands, but whole unprocessed samples can be used as well. Here, the goal is to affect the buoyancy of seeds and soil particles differentially. A number of differing salts can be used for this purpose. Potassium carbonate has proven to be useful in this endeavour, in that it permits separation of the seeds from the soil particles. Short exposure to it is not toxic for seeds of some species (Buhler and Maxwell, 1993), but it may have a detrimental effect on others (Luschei *et al.*1998). Some organic debris typically floats with the seeds. If large tubes are used to hold the samples and potassium carbonate solution, these can be centrifuged to affect separation of seeds from soil particles (Buhler and Maxwell, (1993). This method is most useful if a single species is of interest, and detergent and salt concentrations that are effective, but not toxic, can be determined. All direct seed extraction methods provide estimates of total seed bank densities, including densities of dead seeds (see below). Thus, this technique is especially valuable for studies involved with population dynamics of weeds. The technique may not be always appropriate for the correlation of seed banks with seedling populations, as the technique may confuse dead, dormant, and non-dormant seeds with one another. Additional and routine tests are available to determine viability in the isolated seeds (see below), but as yet there is no routine method to distinguish dormant from non-dormant seeds (but see Fennimore *et al.* 1999).

Seed identification

Once seeds are more-or-less isolated using the direct seed extraction method, the seeds must then be identified. The sample material that remains after direct seed extraction typically is not pure seed, but a mixture of seeds, other organic materials, and soil particles. Perhaps the most time-consuming portion of the direct seed extraction method is examining these mixtures under magnification, and locating and then identifying the seeds. A thoroughly experienced and keenly-sighted researcher with a catalogued seed collection is the best possible tool for seed identification, but failing that, some excellent handbooks exist. Most of these texts are regional. Delorit, (1970) for example, is an excellent resource for North American researchers.

Image analysis, that is, computerized analysis of electronic images of isolated seeds, holds some promise for identifying weed seeds. However, little effort seems to have been devoted to this topic recently (Benoit *et al.* 1992, Buhler and Maxwell, 1993), probably because the human eye still can distinguish seeds and species so much more rapidly than any machine.

Another interesting and modern tool for weed seed identification is DNA fingerprinting. This method may be most appropriate for identifying species and biotypes with seeds that are indistinguishable visually (Fennimore *et al.* 1999, Joel *et al.* 1998, Mucher, 2000).

The hand-separation and counting processes generally are performed on samples that have dried after sieving and flotation. Unfortunately, seeds of some species (e.g. *Impatiens* spp.) lose viability quickly after drying, which introduces error in estimates of viable seed densities.

Viability testing

The seeds that are isolated through direct seed extraction may be viable or dead. These seeds can be tested for viability. The simplest viability test is to probe the seeds with fine-tipped forceps, remove obviously dead seeds, and then attempt to germinate those seeds that appear firm. The

number of seeds that germinate provides an estimate of the abundance of seeds that are both viable and non-dormant. However, it may not provide information relevant to the number of viable but dormant seeds.

The viability of seeds can be determined through the well-known tetrazolium chloride (TZ) test. The TZ test is simple for the purposes of most weed researchers, but for seed technologists the test can be quite complex. Typically, seeds are soaked in a 0.1–0.2 percent TZ solution for a few hours to one week at 10–30 C, depending upon species and research objectives. The hydrogen released by dehydrogenase reactions in living tissues combines with TZ to form a red pigment. Thus, if seeds exposed to TZ eventually turn pink or red, they contain living tissue, whereas those without the red stain are presumed to be dead.

Complexity of the TZ test arises in many forms. For example, when the growing point (embryonic axis) within a seed is no longer able to grow, its cotyledons and other tissues may still contain enough dehydrogenase and produce sufficient H to elicit a pink/red response to TZ. Furthermore, microorganisms that consume dead and dying seeds also contain dehydrogeanses and produce H, which can react with TZ and produce elicit false positive results. Careful observation of seeds after exposure to TZ can eliminate these errors. Observations of red staining of the radicle and hypocotyl or coleoptile are critically important for proper determination of viability.

A publication of the Association of Official Seed Analysts (AOSA), Tetrazolium Testing Handbook (Peters, 2000) (http://www.aosaseed.com/tetra/TZcommitteemain.html) provides many excellent species-specific drawings and insights for the proper use of TZ in the commercial seed industry. For weeds, however, exact determination of seed viability is more of a research interest than an economic and industrial requirement. Consequently, some of the highly structured and species-specific AOSA guidelines may be relaxed. The following two paragraphs describe procedures that we have found to be useful for some species common to North America.

Where possible, split air-dried seeds symmetrically with a single-edged razor to bisect and expose the embryo. Thus, the cut surface of each half of the seed should show at least parts of the radicle and hypocotyl (e.g. *Abutilon theophrasti* Medik.) or the radicle and coleoptile (e.g. *Setaria faberi* Herrm.). Choose the half-seed that appears most intact, place on blotter paper saturated with 0.2 percent TZ, and incubate at 25 C. After 12 hours' incubation, viable seeds exhibit red growing points, whereas dead seeds retain their original colour. The period of incubation usually is too short for substantial growth of micro-organisms.

Seeds of some species are not amenable to symmetrical bisection along the embryonic axis because of their small size or shape (e.g. *Chenopodium album*). These types of seeds can be split in any fashion with a sharp razor so that the radicle or hypocotyl is exposed on at least one half of the seed. Because the appropriate half-seed is difficult to discern at this stage, incubate both halves of the seed in TZ. The seed is viable if either half exhibits red after 12 hours' incubation.

Verification

The value of the data obtained can be improved by testing and calibration at several steps in the direct seed extraction process. The process can be validated by adding a known number of seeds to test samples to verify that they can be separated, identified, and counted with acceptable accuracy. If accuracy is low, determining which step in the process is limiting and making

appropriate adjustments are important for correcting the protocol. Researchers need, for example, to ask the following: Are seeds lost during sieving? Can they be distinguished from other organic matter or soil mineral contaminants? Can viability be determined accurately? Do the extraction or flo tation procedures affect viability? Seed identification and counting accuracy generally decrease with increased seed number, smaller seed size, and time-related fatigue of personnel involved.

Current counting methods for determining the density of viable seeds in seed bank samples are laborious and impractical as a means of characterizing the species composition of the seed bank. Worker exposure to salt solutions and detergents, as well as hours spent peering through a hand-lens or dissecting scope, are serious limitations. A method that works for some species is likely to be inadequate for other species whose seeds differ in shape, size, durability, ease of identification, and dormancy characteristics. Agricultural seed banks in the north and central United States often contain between 20–50 species, some of which occur in very low numbers. A single method is unlikely to accurately separate, detect, and correctly identify all species. Thus, the seed separation approach is only appropriate for one – or just a few – large-seeded target species where accuracy can be verified.

GERMINATION METHOD

The second major technique for enumerating seeds in the soil seed bank is referred to as the "germination" method. This technique is primarily used to enumerate the density of non-dormant seeds in the seed bank. In this case, soil cores typically are aggregated in logical units (e.g. 20 cores from a single plot that represents an experimental treatment). To aggregate cores, they are mixed thoroughly and then inserted in trays that are placed in incubators, greenhouse (glasshouse) benches, cold frames, or nurseries depending upon the objectives of the experiment and availability of facilities. Samples must be protected from seed contamination, disturbance, and granivores and herbivores. Protection is especially important in outdoor nurseries, but even within modern greenhouses, airborne seeds of species such as dandelion (*Taraxicum officinale* Weber) are common contaminants that enter through ventilation systems.

Soil depth in the trays should be no greater than the depth from which expected species can germinate, typically less than 5 cm, with 2–3 cm best for small-seeded species. If the soil is clay-rich, it can be mixed with known volumes of clean sand or commercial potting media to improve drainage. A useful procedure to improve drainage is to line the bottom of the tray with sand, then nylon mesh, and then the soil sample. The nylon mesh allows periodic removal and stirring of the soil sample (see below) to improve germination of dormant seeds, but without unwanted contamination by the non-experimental subsoil.

Although clayey soil samples require better water drainage, sandy soil samples need better water retention. This can be affected by lining the trays with vermiculate or peat, again separated from the experimental sandy soil with nylon mesh. Clean vermiculate or peat also can be mixed with the sampled soil to increase water-holding capacity. In glasshouses, cold frames, and nurseries, shade cloth can be draped over the trays to retard evaporation and promote germination and emergence unencumbered by temporary water shortages. Trays also can be enveloped in clear plastic bags to maintain soil moisture; but this never should be done in sunlight, only in growth chambers where temperatures can be controlled precisely.

Most non-dormant seeds germinate quickly in the trays described above. Often 70 percent of the seedlings that will emerge eventually do so during the first two weeks of incubation, but this depends upon dormancy levels. All seedlings should be counted and removed as soon as most can be identified. The few seedlings that cannot be identified easily may be transplanted into pots for later identification.

After no further emergence occurs, it is common to mix the soil and begin another cycle of germination and emergence. The number of cycles varies among researchers. Roberts (1981) suggested continuing the process for two years. In our experience with summer annual species, most seeds germinate during the first cycle, about 10 percent of that number in the second cycle, and very few seeds germinate during a third or fourth cycle. There is no clear correspondence of species that germinate in one cycle but not another. Therefore, after two or three cycles we commonly stratify samples (4 C) for four or more weeks followed by alternating temperatures before returning them to the greenhouse. The purpose of this is to break dormancy in seeds that might have gone into secondary dormancy during the previous germination cycles. The intention is to mimic spring conditions that cause seeds of many summer annual species to be released from dormancy. Some workers place samples out of doors during winter for the same reason.

The time of year of sampling can influence how samples are handled. Samples taken right after seed rain might need a cold period or other stratification conditions prior to germination. Samples taken at the end of winter in temperate areas should be put in trays as soon as possible, as many seeds will have broken dormancy and are ready to germinate. Samples taken during mid-summer in temperate areas – after most weeds have emerged and before new seed rain – represent the persistent seed bank (Baskin and Baskin, 2000) which consists of mostly dormant seeds. These samples will probably require stratification and/or alternating temperatures to break dormancy and encourage germination. In the tropics, samples taken at the end of a growing season should contain freshly matured seeds that might need a dry period to break dormancy.

Some authors recommend sieving to reduce sample volume. Gravel and organic material larger than the largest anticipated seeds can easily be eliminated. If wet sieving is used, the mesh size must be very small if the smallest seeds are to be retained. For the silt loam soils we have worked with in Ohio, wet sieving results in a muddy plug of soil and organic material that is difficult to handle; therefore, this step was avoided. Nevertheless, some workers consider this step important for bulk reduction and enhancing germination of some species (Thompson *et al.* 1997).

There have been few attempts to validate the germination method because it is very difficult to do. One could add a known number of viable seeds of a given species to a volume of soil to verify that the correct number of seedlings will emerge from the sample. However, field samples contain seeds of various age and dormancy status, and the difficulty in the procedure is obtaining seedlings from all such seeds, not from readily germinable seeds. Alternatively, one can screen seeds from the soil following 'exhaustive' germination in an effort to find viable seeds that did not respond to the procedure. Any attempt to calibrate the method would have to include all species of interest. One of the values of the germination method is the ability to obtain a comprehensive assessment of species, including many that occur relatively infrequently. Thus, calibration would be impractical for such analyses.

Many workers prefer germination methods over separation because of the many limitations of the latter. Germination methods are only less laborious to a degree. Several months generally are required to obtain data, making this method impractical for prediction of potential weed

populations within a growing season. Specialized knowledge is needed to accurately identify seedlings. There is the inevitable problem of viable but dormant seeds that do not germinate during the period of the germination test in spite of efforts to provide appropriate environmental conditions for breaking dormancy and germination. Some workers have used seed separation techniques after the germination method to attempt to separate these seeds. One of the main advantages of this method over counting is its utility for detecting a wide range of species and thus for community analysis. In samples of long-term replicated experiments including tillage and crop rotation treatments, we have detected germinable seeds of 20–30 species using this method, including several species that we did not expect to find, given the composition of the aboveground community. Some of the species were represented by only a few individuals in a single plot, and it is unlikely that these would have been detected and identified properly from seed counts.

SEED BURIAL STUDIES

Some of the most valuable information regarding seed bank behaviour has arisen through experiments in which weed seeds were buried purposefully. There are three basic approaches to these types of studies, which are named: 1) inverted bottle; 2) seed packet; and 3) seeded core methods.

Inverted bottle

This approach was pioneered by Beal and others at the turn of the preceding century. The method has not been used recently. As the name implies, seeds are place in sand-filled bottles, the bottles are inverted, and then buried in soil. Bottles are retrieved at intervals, sometimes 20-year intervals, and examined for germination and viability. Because the bottles are inverted, the seeds are not exposed to the same level of hydration, drying, and rehydration as seeds in field soils. Longevity appears abnormally long for many species examined in these experiments.

Seed packet

This common approach allows seeds to be exposed to near-natural conditions after being confined to bags typically constructed with decay-resistant nylon mesh. Bags often are buried at various depths and retrieved for analysis at various times after burial. In these experiments, seed longevity typically is much less than that observed through inverted bottle experiments. If seed packets are retrieved at sufficiently short intervals, loss of viability can be ascribed to germination (through observation of seedlings or seedling remains) or simple seed death. These studies have aided greatly the recent understanding that seed longevity under natural conditions often is less than five years.

Seeded cores

This technique (e.g. Teo-Sherrell and Mortensen, 2000) involves exhuming a soil core and replacing it with soil devoid of seeds except for those purposefully added. These studies seemingly are more natural in that the added seeds are exposed to similar microclimate, microbial, and microfaunal conditions as seed packets, but also the macrofauna that would be excluded by small-mesh nylon bags. The detraction of this method is that seeded cores must be retrieved precisely so as not to include the natural seed bank inadvertently, and then processed in a manner identical to standard soil cores for seed bank studies.

VERTICAL AND HORIZONTAL SEED MOVEMENT/DISTRIBUTION

A number of seed bank studies in recent years documented and modelled seed movement. Typically, these studies involved tillage-induced movement of seeds, or coloured synthetic beads that mimic seeds. Initially, the focus of these studies was vertical movement of seeds caused by ploughs, chisels, disk, and no-till drills, with the understanding that burial depth is primarily a function of tillage implement. Deep burial was thought to be associated with a number of potentially important demographic processes, such as fatal germination and microclimate-imposed dormancy.

More recently, vertical movement of weed seeds caused by repeated tillage operations using any of a variety of tillage equipment has been studied and modelled (Cousens and Moss; 1990, Mead *et al.* 1998; Staricka *et al.* 1990). These studies all point to a consistent trend; namely, similar implements bury seeds in similar proportions at similar depths regardless of soil type and location of the experiment (Forcella *et al.* 1994). This suggests a very satisfying universality of tillage-induced seed burial models.

Additional studies also examined horizontal movement of weed seeds as a result of tillage machinery. Although movement induced by tillage equipment can be appreciable, such horizontal displacement is very little in comparison to that caused by combine harvesters.

HOW TO SAMPLE ABOVEGROUND VEGETATION?

Many textbooks in Plant Ecology list protocols for sampling aboveground vegetation, and we will not attempt to re-examine this topic. However, with respect to relating seed banks to aboveground vegetation, some of our experiences may be helpful.

Timing of plant counts is important in terms of associating results with seed bank densities. Again, plant samples need to be taken at a logical time that follows, not precedes, sampling for seed banks. Depending upon goals of the research, counts may need to be made at various times of the crop cycle; e.g. (a) immediately before crop sowing; (b) 4 weeks after sowing; (c) at maximum crop leaf area index; (d) at crop harvest, and sometimes (e) after crop harvest too. The proportion of the total plant population that emerges prior to each count can vary substantially from site to site and year to year, depending upon microclimate.

The weeds present four weeks after crop sowing usually represent the most important proportion of the total weed population, at least from the standpoint of in-crop weed control. The density represented by this proportion, however, may not correlate necessarily with seed bank density. In this case, researchers are advised to attempt a correlation between seed bank densities and weed densities at times a + b, a + b + c, b + c, and so forth. Only after these types of assessments have been made can researchers conclude that relationships exist or do not exist between seed bank densities and aboveground vegetation.

Quadrat sizes for plant counts are usually 50–5 000 times larger in surface area than soil cores. Consequently, a close correlation between seed and plant densities should not be expected (Cardina and Sparrow, 1996). For this reason and others, rank correlation may be more appropriate than regression for relating seed banks to aboveground vegetation.

Simple minimum-variance tests can determine the number of quadrats to be used quite quickly. In any event, multiple quadrats (e.g. 10 per plot, each 0.1 m^2) are much preferred to a single quadrat (e.g. 1 m^2) per plot.

Arrangement of quadrats probably is not too important provided that the placement of quadrats spans the length and width of the plot or field (Colbach *et al.* 2000). Some authors place quadrats over or adjacent to the point where soil cores were taken. Although logical, this may have little practical effect on the results given the extreme aggregation of many seed banks.

CONCLUSIONS

Recent widespread interest in weed seed banks is reflected in the abundant research reported on this topic at the Third International Weed Science Congress (Anonymous, 2001), and symposia specifically devoted to seed banks sponsored by the Association of Applied Biologists in 1998 (Champion *et al.* 1998) and 2003 (Reading, United Kingdom). With such enthusiasm for this topic, some guidance regarding sampling protocols and techniques may be useful, especially for younger scientists just beginning their research, or even older scientists with newfound interests in seed banks.

No single protocol or technique will have universal appeal, but there are a number of fine points of which seed bank researchers should be aware, and these are discussed in the report. These points are involved with sampling adequacy, sampling patterns, sampling times, seed viability testing, seed separation, seed and seedling identification, and aboveground vegetation sampling. If the guidelines provided in this report do not improve the results of future seed bank studies, we hope they at least will help alleviate some of the tedious work involved with this type of research.

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