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Compendium of branched broomrape research

Section 4. Detection

A COMPILATION OF RESEARCH REPORTS FROM THE BRANCHED BROOMRAPE ERADICATION PROGRAM SOUTH AUSTRALIA

DECEMBER 2013

PREMIUM FOOD AND WINE FROM OUR CLEAN ENVIRONMENT

SOUTH AUSTRALIA

Government of South Australia
Primary Industries and Regions SA

Compendium of branched broomrape research

Information current as of 4 December 2013

Disclaimer

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See also the following publications:

Correll R., Marvanek S. (2006) Sampling for detection of branched broomrape. In Fifteenth Australian Weeds Conference Papers and Proceedings (Eds C. Preston, J.H. Watts, N.D. Crossman), Weed Management Society of South Australia, Adelaide, pp. 618-621.

Prider J., Ophel Keller K., McKay A. (2013) Molecular diagnosis of parasite seed banks. In Parasitic Orobanchaceae: Parasitic Mechanisms and Control Strategies. (Eds D.M. Joel, J. Gressel, L.J. Musselman), Springer Verlag, Heidelberg Berlin, 357-368.

1. DNA detection of broomrape in seed and soil

Alan McKay[1], Kathy Ophel Keller[1], Di Hartley[2], Wolf Wanjura[2] and John Curran[2]

[1]SARDI Field Crop Pathology Unit

[2]CSIRO Entomology

2004

Summary

A DNA test to quantify branched broomrape (*Orobanche ramosa*; BBR) in soil has been developed by SARDI and CSIRO Entomology. The assay is based on methodology developed to measure levels of soilborne pathogens of broadacre crops using soil samples ranging up to 550 g. Technology treated as "Commercial and In-Confidence" is used to extract DNA from the soil, and the BBR seedbank is measured using a quantitative PCR.

The assay designed by CSIRO Entomology, is based on DNA specific sequences in the internal transcribed spacer region (ITS) of the ribosomal gene. The sequencing was done using DNA extracted from seeds from a number of O. *ramosa* populations, O. *cernua*, and O. *australis*. This revealed distinct differences between O. *ramosa*, O. *cernua* and O. *australis*. The latter two were more closely related, which is consistent with international studies on taxonomy of broomrape.

A specific DNA probe was developed and tested at SARDI on plant samples of all three species. This work confirmed the DNA assay conditions for polymerase chain reaction (PCR), which gave specific amplification of only O. *ramosa* DNA.

Soil samples were 'spiked' with *O. ramosa* seeds to develop a quantitative assay. Initial results demonstrated that the CSIRO-SARDI system of soil DNA extraction followed by quantitative PCR was capable of detecting broomrape seed in soil, but the sensitivity of the test was poor. Primer redesign and further optimisation of PCR conditions increased the detection limit to 40 seeds / 400 g soil. By comparison, a known naturally-infected field site had BBR levels of 2,000 to 3,000 seeds / 400 g soil.

Refinements to the assay have increased assay sensitivity to one seed per 200 g soil, which is equivalent to about 700 seeds m^{-2} to a depth of 10 cm.

Test sensitivity was also examined in sheep dung and grain samples spiked with *O. ramosa* seed. Initial studies revealed that although seed could be detected in both types of samples, the sensitivity was poor as there were considerable issues relating to DNA extraction. Subsequent work focussed on the sheep dung samples and the assay now appears comparable to the soil assay.

Calibration of the test was initially complicated by variation in broomrape seed types. Four maturity types were observed- gold shrivelled, black shrivelled, gold full and black full. The assay could not detect significant levels of DNA in the shrivelled seeds, and the full gold coloured seeds had 60% less DNA than the full black seeds. Germination tests indicated that the shrivelled seeds were not viable and the gold seeds were immature seeds with low viability. Further work is required to examine variation in longevity.

The soil and sheep dung tests are available direct from SARDI on a cost recovery basis of $30 / sample Tests are used by the Broomrape Eradication Program, GRDC projects, and farming system projects including the Mallee Sustainable Farming System project (MSFP). If necessary, the *O. ramosa* test can

be combined with other disease tests. During 2002 /03, 461 research samples were processed for different projects.

SARDI will continue to provide this testing service to research projects and to the eradication program.

Conclusions and Recommendations

A DNA-based assay is now available to specifically detect and quantify *O. ramosa* in soil samples. The assay has a detection limit of one seed per 200 g soil. Preliminary results also indicate a similar level of detection can be achieved in samples of sheep dung. This test is now available to research programs and to the broomrape eradication program on a cost-recovery basis, as part of SARDI's Diagnostic Services. For this tool to be useful, more work needs to be done on sampling strategies in soil.

2. Improvements in DNA assay for measuring branched broomrape seed reserves

Alan Mckay

SARDI Field Crop Pathology Unit

October 2004

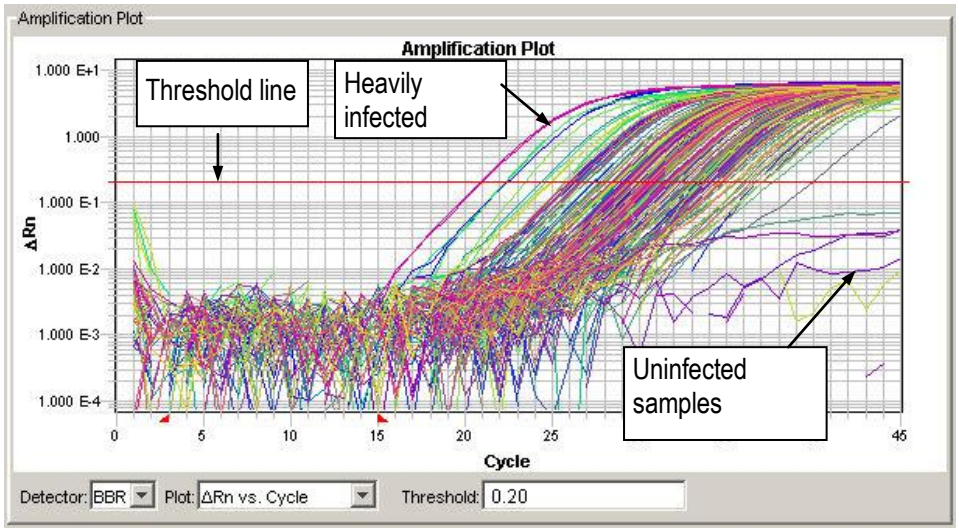
SARDI Crop Pathology and CSIRO Entomology with support from GRDC developed a quantitative PCR based assay to assess branched broomrape (BBR) seeds in 500 g soil samples based on Root Disease Testing Service (RDTS) technology. The DNA assay was designed to detect the BBR in the mallee (putatively *Orobanche mutelli*) and not the clover (*O. minor*) or native (*O. cernua* var. *australis*) broomrapes that occur in Australia. Based on published sequences the BBR assay will detect the following species in the Trionychon group; *O. ramosa*, *O.* cf. *rosmarina*, *O. nana*, *O.* cf. *nana*, *O. oxyloba*, *O.* cf. *oxyloba*, *O. tunetana*, *O. pulchella*, *O.* cf. *aegyptiaca*, *O. bungeana*, *O.* cf. *graciosa*, *O.* cf. *coelestis*, *O.* cf. *heidreichii*, *O. lavandulacea*. Other species in the Trionychon group the assay will probably not detect include: *O. purpurea*, *O. purpurea* var. *purpurea*, *O. purpurea* var. *bohemica*, *O. nowackiana*, *O. arenaria*, *O. caesia*.

With the original assay, PCR was used to amplify the BBR DNA and then fluorescent probes were used to measure how much product was produced. This process took 3 days to produce results. Recently SARDI has invested in new equipment and converted the BBR assay to a real-time (Taqman) format, enabling results to be produced in as little as 2 days.

The real-time platform measures the amplified target DNA directly in each well during the PCR process. The target DNA level doubles with each PCR cycle producing characteristic amplification curves as shown in the chart below.

The original assay could detect 1 BBR seed per 200 g of soil and may saturate at high BBR levels. The real-time assay can detect at least 1 BBR seed / 200g and possibly down to 1 seed / 500 g soil. The real-time assay is also better at measuring high BBR levels, at least 1,000,000 seeds / 200 g soil.

The fluorescent signals are converted to equivalent numbers of BBR seeds per 200 g of soil via comparison to standards run on the plate. In heavily infected samples the fluorescence rises after relatively few cycles, while in uninfected samples the fluorescent signal does not cross the threshold line. Plate controls are used to check that no contamination occurs. This is important, as PCR assays are very sensitive. If contamination is detected in the plate controls, the whole batch is retested.



3. Analysis of precision and sensitivity of DNA assay for detection of broomrape seed in soil samples

Ray Correll

Rho Environmetrics

June 2004

Summary

The enhanced DNA probe technique is reliable for seed densities exceeding 10 seeds per 200 g sample. Below that level there is high laboratory variation – for lower levels accuracy can be enhanced by repeating the laboratory assay.

- The enhanced DNA probe technique will be useful in detecting seeds with the following likelihoods for different seed densities
- 50% chance of detection at 0.83 seeds per 200 g
- 80% chance at 1.6 seeds / 200 g
- 90% chance at 2.4 seeds / 200 g
- 95% chance at 3.3 seeds / 200 g
- 99.9% chance at 21 seeds / 200 g.

Introduction

Branched Broomrape is a serious parasitic weed. It spends much of its life cycle under the ground where it parasitizes roots of a broad range of hosts. The plant produces numerous (about 30,000) seeds per plant. The Branched Broomrape program aims at eradicating this weed from the mallee district of the Lower Murray.

Clearly one of the challenges is to understand the movement of seeds. The use of a DNA test has been developed by SARDI (Dr Alan Mackay). Field trials were conducted by the University of Adelaide by Dr John Virtue.

Previous studies have indicated that a less sensitive DNA technique were not good for detection. The DNA technique has since been enhanced. This study was intended to explore the efficacy of the new technique.

Motivation for good seed sampling

Good seed sampling techniques are needed for both research and management. Seed studies are necessary for our understanding of the spatial distribution (clustering, vertical, distance from source, effectiveness of treatments etc) as well as rates of decline of the seed capital.

Experimental

Data were available from 15 plots with a range of seed densities in a field trial.

20 composite samples were collected from each plot, consisting of 25 small cores taken in a regular grid pattern. Each sample of approximately 500 g of soil was analysed for branched broomrape seeds by SARDI using the enhanced DNA probe technique.

Calibration data

The calibration data supplied are shown in Table 1. A summary of those data is shown in Table 2.

Table 1 Calibration data for enhanced DNA probe technique

Replicate	Dose	Sample	Count
1	High	S1	37
1	High	S2	24
1	Low	S3	44
1	Low	S4	18
2	High	S1	37
2	High	S2	26
2	Low	S3	24
2	Low	S4	11

Table 2 Summary of calibration data for enhanced DNA test

Dose	Description	No. of seeds	Soil Weight	Seed 200 g^{-1}	Mean count
High	50 seeds in approx 400g pure sand	50	400	25	31
Low	10 seeds in exactly 200g pure sand	10	200	10	24.2

There was wide variation within each treatment. Despite the difference in the seeds 200 g^{-1}, there was no significant difference between the estimated seed densities as measured by the DNA probe.

It is recommended that more calibration data – perhaps ten times more would be appropriate.

Precision of seed density estimates

The relationship between plot mean and the standard deviation is shown in Figure 1. The the plot mean is moderately reliable – for a plot mean of 100, the standard deviation is so the standard error is approximately 6, giving a coefficient of variation of 6 %. The standard approximate coefficient of variation of 16 %. From

Figure 1 it is clear that there is an almost linear relationship between the standard deviation and the mean. There was one apparent outlier in the data set.

Duplicate laboratory data were available for each sample. These data enabled the relationship between the laboratory coefficient of variation and the sample mean to be estimated. The laboratory coefficient of variation was approximately 20 % for samples where the mean exceeded 20 seeds per 200 g soil (Figure

2). Below that seed concentration the coefficient of variation was very large, reaching 100% when the seed density was 5 seeds per 200 g, and attaining much higher values for very low seed densities.

Laboratory variation clearly contributes a significant proportion of the overall variation. The overall variation can therefore be reduced by undertaking duplicate laboratory analyses. Furthermore, at low seed levels, laboratory variation is a very important component, and several laboratory runs should be [illegible] less that 5 per 200 g.

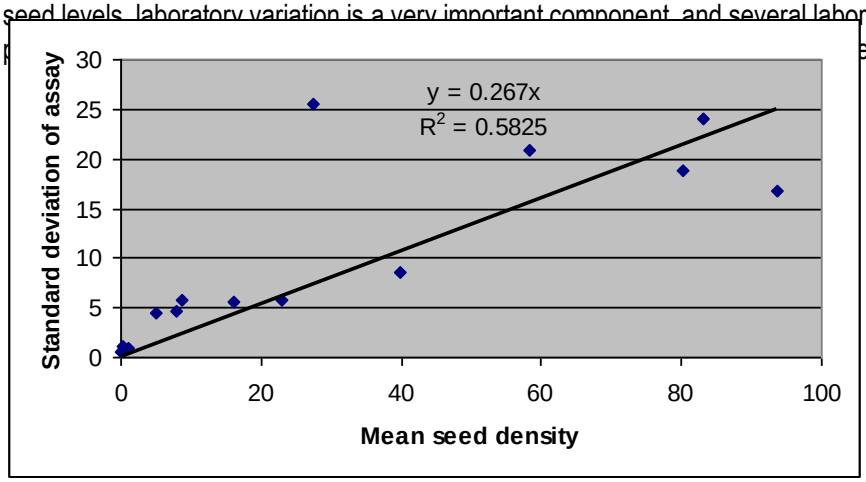


Figure 1. Relationship between laboratory variation and sample mean

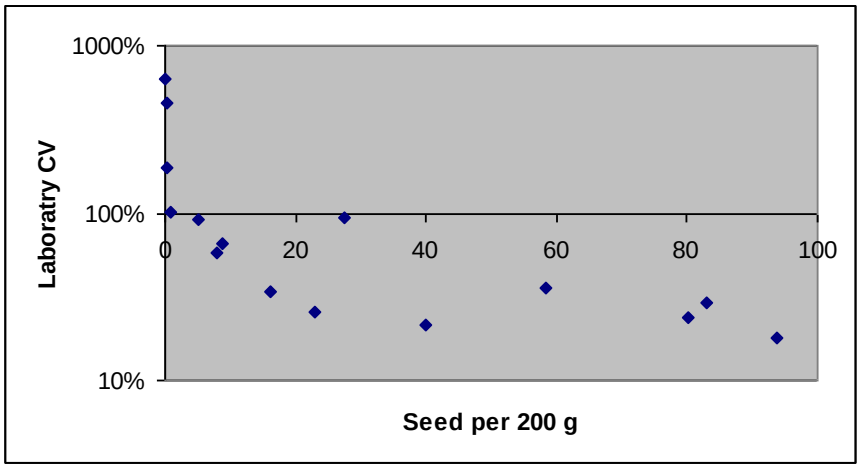


Figure 2. Relationship between CV and seed density (y axis is log scale)

Typically within a plot there was a good correlation between the two laboratory analyses (

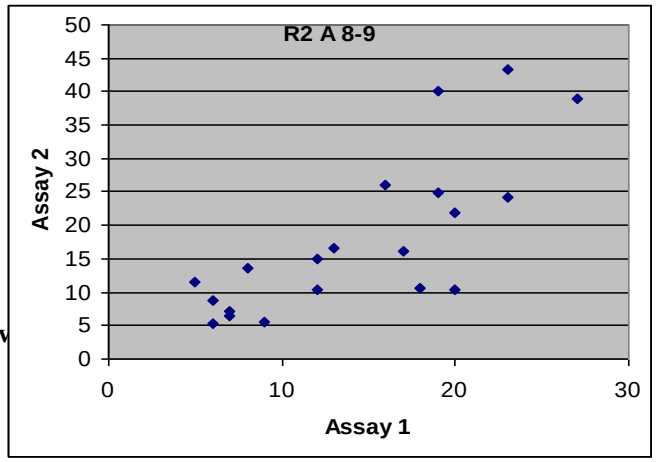


Figure 3). Hov

Figure 4). This was particularly true when the sample mean was less than 10 seeds per 200 g. The correlation was at times induced by an outlier as shown in

Figure 5.

Figure 3. Typical correlation between assays within a plot (r^2 = 0.774)

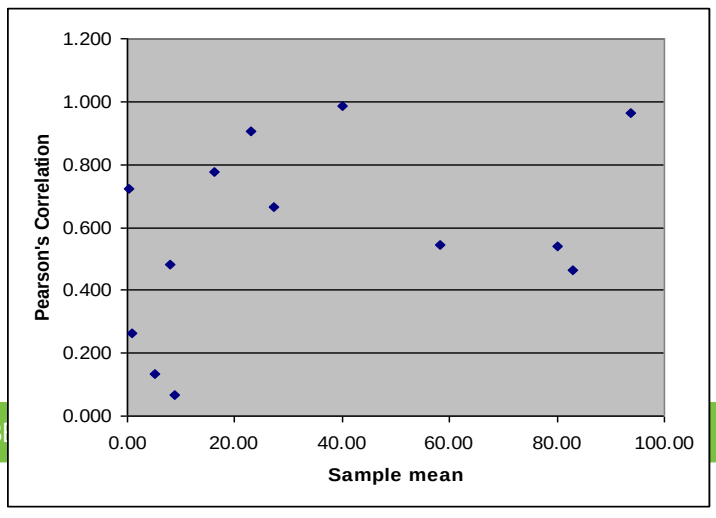


Figure 4. Effect of sample mean on correlation between assays on same field sample

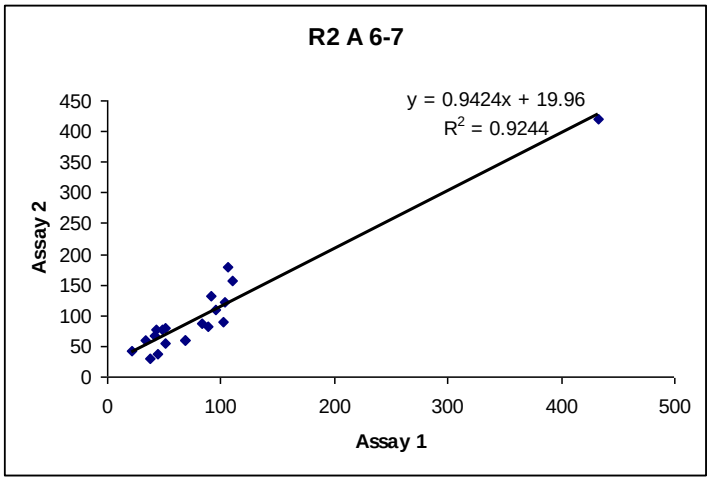


Figure 5. Effect of outlier on correlation

In general the following points can be made concerning the correlations between the two assays

- There is a low correlation when the sample mean is less than 10 seeds per 200 g soil
- The correlation is about 0.8 when the sample mean is greater than 10 seeds per 200 g soil
- The expected scatter of the correlation ranges from 0.55 to 0.91 when correlation is 0.8. This is based on the transform z = 0.5exp ((1+r)/(1-r) is normally distributed.
- Some samples outside expected range – perhaps due to outliers

Likelihood of detection

A key use of the DNA probe is to detect samples that are positive for branched broomrape seed. The proportion of samples that are positive is referred to as frequency.

Modelling the frequency data

It is intuitively obvious that the frequency of detection is a function of the seed density. This is modelled by the logistic function

$$f = \frac{1}{1+\exp(b(d-m))}$$

where *m* is the seed density where there is a 50% chance of detection.

The above model was fitted in Excel using plot estimates of mean and frequency to give initial values for *m* and *b* followed by the solver routine in tools.

The fit to the data is shown in

Figure 6, with a central value of 0.83. The fit is excellent.

Implications of the model

The implications that can be drawn from the model are

- 50% chance of detection at 0.83 seeds per 200 g
- 80% chance at 1.6 seeds / 200 g
- 90% chance at 2.4 seeds / 200 g
- 95% chance at 3.3 seeds / 200 g
- 99.9% chance at 21 seeds / 200 g

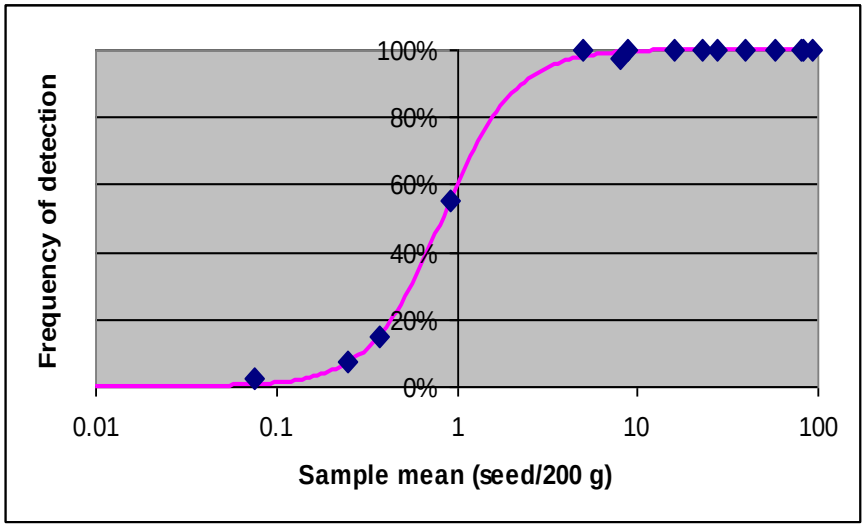


Figure 6. Effect of increasing seed density on probability of detection

4. Time for DNA decomposition of broomrape seed

Che DeDear and John Virtue

Department of Water, Land and Biodiversity Conservation

July 2003

Aim

An assay is used to detect and quantify branched broomrape DNA in a soil sample. The use of the DNA assay to evaluate treated broomrape seed banks, e.g. following fumigation, requires the DNA of any seed that is killed by the treatment to decay. This is usually the result of the active of soil micro-organisms in a biologically active soil. DNA can be detected in non-viable seed at low levels (Alan McKay, pers. comm.). The aim of this study was to determine the post-treatment time required in the field prior to sampling for comparative broomrape seed bank decline assessed using a DNA assay.

Methods

Broomrape seeds were subject to three treatments:

1. 1% Niproquat solution
2. 80% ethanol solution
3. Water

Batches of seeds were soaked in these solutions for several days. Three replicates of twenty seeds from each treatment were placed between two 20 mm filter papers and placed in 200 g of Tailem Bend sand. The number of seeds in these samples was assessed using the DNA assay.
Samples of the remaining seeds were assessed for viability using germination and tetrazolium tests.

Results

There were no viable seeds in the Niproquat or ethanol-treated samples but control seeds were viable. DNA was detected in all samples. The number of broomrape seeds estimated using the DNA assay was less in Niproquat and ethanol treated seeds. Fewer seeds were estimated in the ethanol-treated samples as amplification of DNA from organisms stored or treated with ethanol has been observed to be poor (Alan McKay, pers. comm.). The decline in DNA detected in these samples is therefore probably not due to decay but the interference of the treatment-chemical with the PCR process during the DNA assay.

Table 1. Estimates of broomrape seed numbers in soil samples following treatment of seed. Value for each sample is average of two assays. Values are means ± 1 SE, *n* = 3.

Treatment	Number of seeds added to sample	Number of seeds detected by DNA bioassay
control	20	16 ± 6.17
1% Niproquat	20	11 ± 0.87
80% ethanol	20	4 ± 0.5

Further experiments to assess the time required for DNA decay to occur in biologically active soils was not done.

5. DNA assay: Sampling protocol for experimental plots

Ray Correll

Rho Environmetrics

September 2003

Introduction

When sampling, the steps are firstly to define the purpose of the sampling, then the population and then the sampling unit, sampling frame and sample number. On reading the proposal (fumigation trial) there seems to be several explicit and implicit aims. At this stage I am unsure whether the proposal addresses these, and in particular whether it addresses the most important aims as effectively as it does the subsidiary aims.

Purpose of sampling

The purposes of the sampling include (in approximate order of importance)

- Determining the effect of the field treatments
- Determining the number (or density) of branched broomrape seeds in each plot to enable a comparison of the treatments
- Determine the reliability of the sampling scheme that was used to determine the plot means
- Assess the effect of different core sizes on the accuracy of a the resultant composite sample
- Assess the effectiveness of using a gravity table to concentrate the seeds
- Study the spatial variability of the seeds
- Determine a suitable plot size
- Determine the number of cores (samples required to obtain a stable mean)

Some of these aims can be achieved readily, while in other cases the aims are in conflict. Comments on how best to achieve these aims are given below.

Number of cores per composite

If a 25mm diameter core is used to a depth of 100 mm, the volume of each core is about 50 ml, or about 65 g. Seven such cores could be bulked to form a single composite sample.

Use of Latin square sampling

A useful strategy to obtain a suitable composite sample is to first grid the area into a 7 by 7 pattern, so that it can be treated as a Latin square. A sample is then taken from each section that has the same letter (e.g. the 7 areas labeled 'C'). Such a sampling ensures that the cores are widely spaced and that the within plot variation is included within the sample. Two other samples, perhaps based on 'E' and 'G' could also be taken. All three samples would give a good representation of the entire plot, and the variation between samples gives a good measure of the between sample variability. Such a scheme will give a good representation of the mean and a measure of its reliability. Up to 7 such composites could be taken in this manner.

If further samples are required, other alphabets (i.e. Graeco-Latin squares) can be used to ensure further samples are also well separated.

Table 3 Sampling pattern for subsamples

A	B	C	D	E	F	G
F	G	A	B	C	D	E
D	E	F	G	A	B	C
B	C	D	E	F	G	A
G	A	B	C	D	E	F
E	F	G	A	B	C	D
C	D	E	F	G	A	B

Determining the effect of the field treatments

Determining the effect of the field treatments requires an accurate estimation of the plot mean. The Latin square sampling will achieve the requirement for an accurate estimate of the mean for each plot. It also gives a good estimate of the reliability of that estimate. As such it answers questions 1 and 2.

Effect of different core sizes

There are two separate questions to be considered. The first is whether changing core sizes affects the mean. A more difficult question is whether changing the core size affects the variance.

We will assume that the cores are bulked into composite samples of approximately 400 g, or about 300 ml. For simplicity we assume that core depth is about 100 mm. Some options for core diameters are shown Table 4.

Table 4 Possible core diameters for sampling

Number of cores	1	6	10
Core diameter	6.2	2.5	2.0

The effect of different core sizes can be assessed by considering only a few core sizes. It may not be feasible to use a core size less that 20 mm, and there is an upper limit of 6.2 mm.

The number of replicates to test for a change in the variance may be quite high. For example, as shown in Table 5, 10 replicates are required to have a 50% chance of detecting a 3-fold difference in the variance between two core diameters.

Table 5 Replicates required to detect minimum change in the variance ratio

Number of replicates	Minimum ratio
5	5.1
10	3.0
20	2.1
50	1.6
100	1.4

It may not be feasible to detect a change of less than 50% in the variance of the samples. It may not be worthwhile therefore to undertake sampling to test for the effect of using different core sizes.

Effectiveness of using a gravity table

The effectiveness of using a gravity table may be required to be tested. In particular, the concentration of the bbr seeds must be quantified. Quantification of the effectiveness of the device can be determined by comparing the concentrated soil with the original soil.

There is no requirement for all the samples to come from the same soil population. There may be advantages in sampling only enough soil to provide 2 kg of soil for the gravity table and 2 kg for 5 400 kg samples. A number, perhaps 10, such samplings should be compared.

Initially the samples should all be taken from similar soil. However, it may be necessary to use a range of soil types to see whether the effectiveness of the gravity table is similar for all soils.

The cost of using the gravity table must be compared with the reductions in the laboratory costs.

Spatial variability of the seeds

Spatial variability of the branched boomraper seeds cannot be determined using composite sampling. Theoretical calculations show that the probability of detecting a seed further than 3 m from a plant is low.

Determine a suitable plot size

Suitable plot sizes are typically small, but are generally limited by the feasible size of the treated areas. The use of smaller plots enables smaller block size and hence less heterogeneity.

Where the area of the plots has been determined by field treatments, a larger sample should be used. A feasible sampling area may be to sample bands of perhaps 0.5 m wide that cut across the plots.

Determine the number of cores

The number of cores can be determined from an estimate of the variance between samples. It is anticipated that the variance can be reduced by using a composite sampling technique as described earlier in this report.

The distribution of the number of seeds per sample should be carefully studied. Outliers may induce a large fourth moment, which may lead to unstable estimates of the variance.

6. Sample size for collecting soil cores from experimental plots for DNA bioassay

John Virtue[1] and John Heap[2]

[1] Department of Water Land and Biodiversity Conservation

[2] SARDI

March 2004

Aim and methods

The aim of this study was to determine the number of soil cores that would give the most precise estimate of the number of broomrape seeds in the seed bank using the DNA bioassay. An auger 13 mm in diameter and 100 mm long was used to sample soil by pushing it vertically into the soil. This corer was developed by SARDI for the collection of soil samples for the analysis of soil pathogens using a DNA bioassay. A composite of 25 such cores yielded a sample size of approximately 500 g soil to be used for the DNA bioassay. 20 such composite core samples were collected from each of 15 plots at the Mannum Trial Site that differed in seed density.

For the DNA bioassay the sample was ground and then subsampled for the detection test. Two subsamples were assayed from each composite soil sample.

A bootstrap analysis was used to resample from the 20 samples in each plot in order to calculate the coefficient of variation for different core numbers.

Results

There was a correlation between the results for the two subsamples assayed from the same composite soil sample (Fig. 1). For subsequent analyses we use the average seed number from the two subsamples.

The distribution of seed numbers was strongly right-skewed with most cores comprising small numbers of seeds; the majority of observations were between 0 and 25 seeds per 500 g soil, with two outliers with more than 300 seeds (326 and 456 seeds per 500 g soil) (Fig. 2).

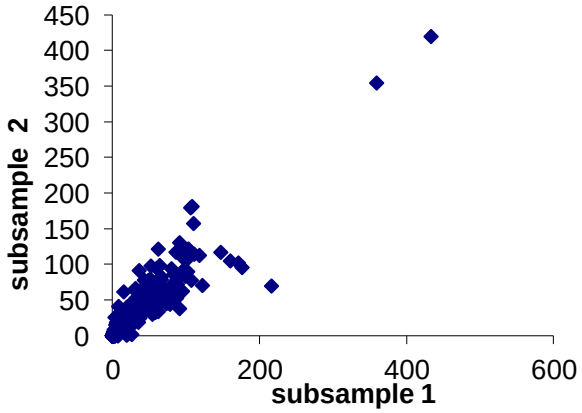


Figure 1. Broomrape seed numbers estimated from two subsamples of each composite soil core using DNA bioassay.

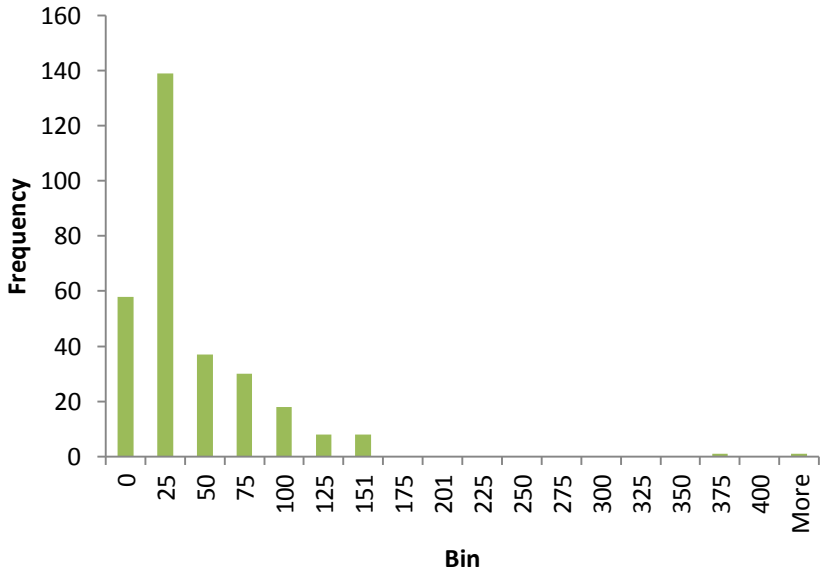


Figure 2. Histogram of number of broomrape seeds per 500 g soil sample pooled across the 15 plots.

Four plots had an average of less than 1 seed per sample. As the detection limit for the DNA bioassay is 2 seeds per sample, we omitted these samples from further analyses. This left 11 plots with average seed numbers ranging from 5 – 86 seeds per sample (Fig. 3).

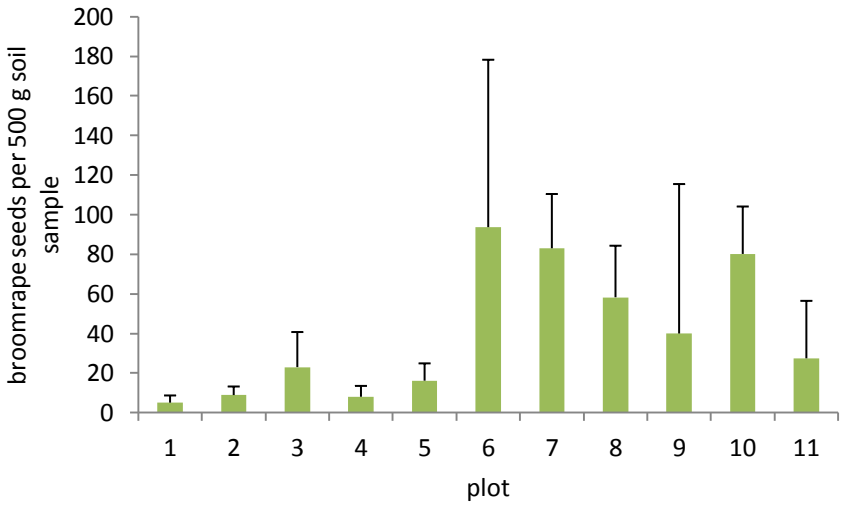


Figure 3. Broomrape seeds estimated within a 500 g soil sample from each sampled plot (plots with less than 1 seed per 500 g soil not included). Bars are means + 1 standard deviation, n = 20.

The mean value of the coefficient of variation was 35% with four composite samples per plot and decreased to 23% at ten composite samples per plot. The extra effort in collecting more than 15 composite cores per plot does not substantially decrease the variability in the estimate of seed number.

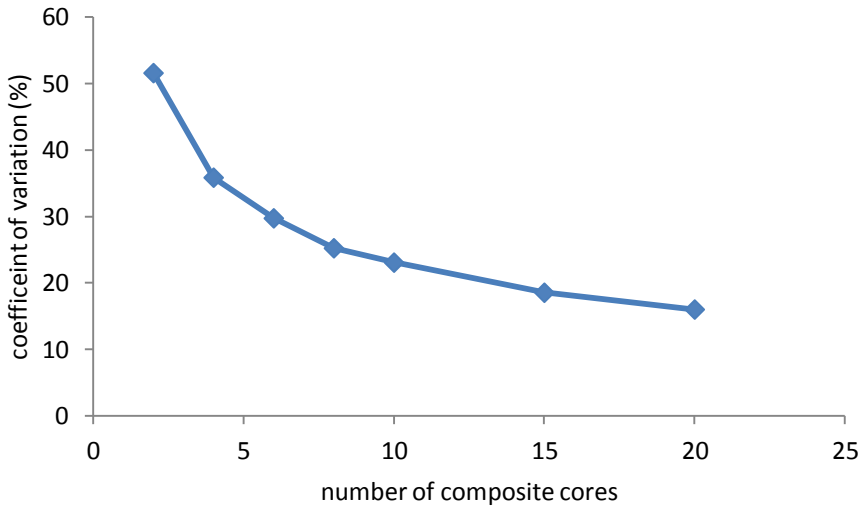


Figure 4. The effect of increased sample size on variability in broomrape seeds detected in samples using a DNA bioassay. Each point is the mean of 11 plots.

There was variability between plots (Fig. 5). The coefficient of variation in plot 9 was 20% or more greater than in other plots. Cores from this plot ranged between 3 and 356 seeds per 500 g and are an example of the extreme spatial variability in the broomrape seed bank.

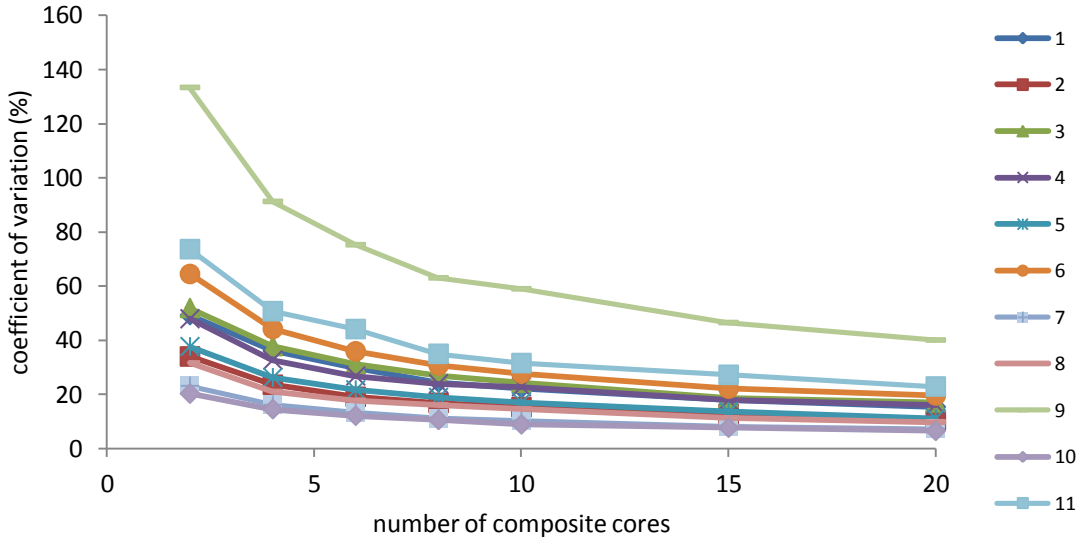


Figure 5. The effect of increased sample size on variability in broomrape seeds detected in samples using a DNA bioassay. Each line represents a separate plot.

This analysis can be used to determine sample sizes for soil cores to detect broomrape seeds in experimental plots. Smaller numbers of samples could be collected when there is likely to be a large difference between treatments, e.g. a fumigation study. If smaller treatment effects are expected, then larger sample sizes would be required.

7. Comparison of soil sampling methods for collection of samples for DNA assay

Anna Williams and Darryl Miegel

Branched Broomrape Eradication Program

August 2007

Aim

To compare two currently used soil sampling techniques to the linear soil sampler, with the aim of determining which method has the lowest variation between samples.

Methods

Six plots at the Mannum Trial Site were used for sampling. Three were control plots and three had been treated with pine oil in 2005. A 5% solution of pine oil was applied with water at the rate of 20, 000 L ha^{-1}. a previous experiment. Sampling was done in 2006.

JV cores

Soils were sampled using a 13 mm diameter and 100 mm long soil corer that was pushed vertically into the soil. 25 such cores were combined to produce one composite sample. Four such samples were collected from each plot, adjacent to the linear sample trench.

JM cores

A soil auger 50 mm in diameter and 100 mm deep was used to collect five cores from each plot adjacent to the linear trench. The soil from the cores was combined, sieved to remove stones and other debris, mixed, and then subsampled to create four subsamples of 200-300 g for each plot.

Linear sampler

The linear soil sampler was a device that collected a soil sample from a trench 2 cm in width. We collected 4 x 3m long linear soil samples from each plot. The large quantity of soil was divided into two large bowls, weighed and then sieved to remove rocks, sticks and other debris. The removed material was weighed. The soil form each bowl was passed separately through a subsampler that divided the sample into four subsamples. The subsamples from each bowl were combined to give four subsamples. The heaviest sample was kept for further subsampling and the other three subsamples were discarded. Four further subsamples were created, one for DNA assay and the other three combined and then subsampled again to create three more DNA assay samples with the fourth sample discarded. Each linear sample therefore yielded four subsamples for DNA assay.

Analysis

A Generalised Linear Model was used to test for differences in estimates of broomrape seed numbers between treatments and sampling methods and their interaction. We used a negative binomial distribution with a logratio link function. An iterative fitting procedure was used, removing model terms at each step and testing the change in residual deviance for each subsequent model with a chi-square test.

Results

The majority of 200 g soil samples contained an estimate of from 10-50 broomrape seeds (Figure 1). The distribution of values was right-skewed, with a few samples having very high seed numbers and most samples less than 200 seeds. Using the JV core method, a significant difference was detected between treated and untreated plots, but this difference was not detected using the other soil sampling methods. (χ^2, p = 0.04). Control plots had more seeds than plots treated with pine oil the year before (Figure 2). Regardless of treatment effects, there was a significant difference in estimates of seed numbers using the three seed sampling methods (χ^2, p <0.001).

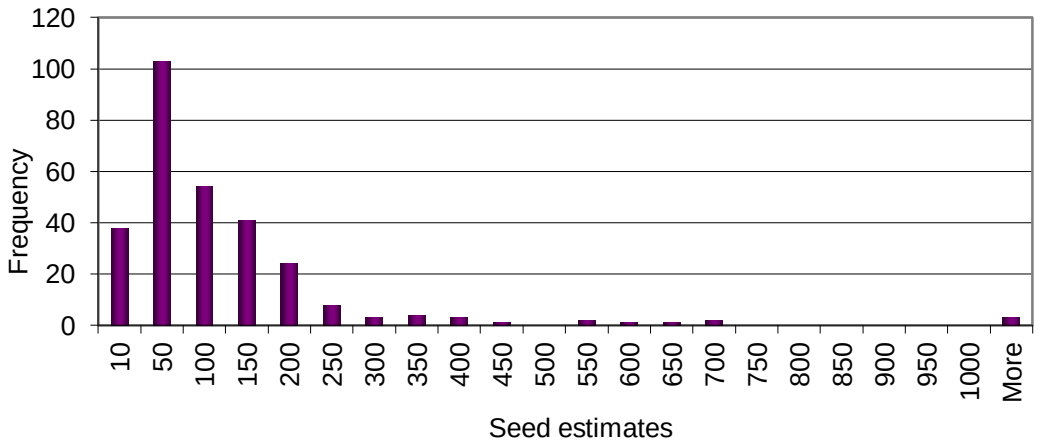


Figure 1. Histogram of number of broomrape seeds per 200 g sample estimated using a DNA assay across all four plots using three different sampling methods.

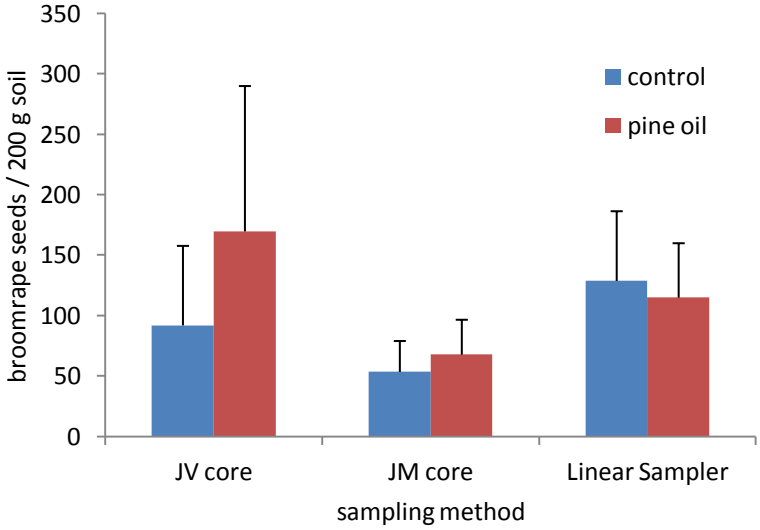


Figure 2. Estimates of broomrape seed numbers in 200 g soil samples collected using three methods form plots treated with pine oil or untreated controls. Bars are means + 1 SE, *n* = 4.

There were differences in estimates of seed numbers and variance from DNA assays using the three methods (Figure 3). Although taking many small samples (JV cores) gave a similar mean number of seeds as a single very large sample (linear sampler), there was very high variability. Fewer larger cores (JM cores) were less variable than many smaller cores but these larger cores may have underestimated seed bank size. The very large samples were the most accurate means of sampling but they were time consuming to process. The sample collection using the linear sampler took 2 hours but samples took 16 hours to process. JM cores took 2 hours to collect and 6 hours to subsample. JV cores took one day to collect with no further processing. All time estimates were for two people.

The JV cores were the only technique that did not have a sub-sampling step. This may have been the reason for the high variability using this method. The samples were not sieved therefore they may have included portions of broomrape vegetative material that would have inflated the DNA present and thus amplified. Field samples should be sieved to remove this material.

Although samples from the linear sampler gave precise estimates of seed numbers, this method has a number of disadvantages. The sub-sampler cannot process damp soil so can only be used in dry conditions or if soil has been pre-dried, adding another processing step. The method requires machinery so is less portable than soil corers. Machinery would require decontamination if taken into infected paddocks. There is also a higher risk of cross-contamination of samples with the larger amount of sample handling.

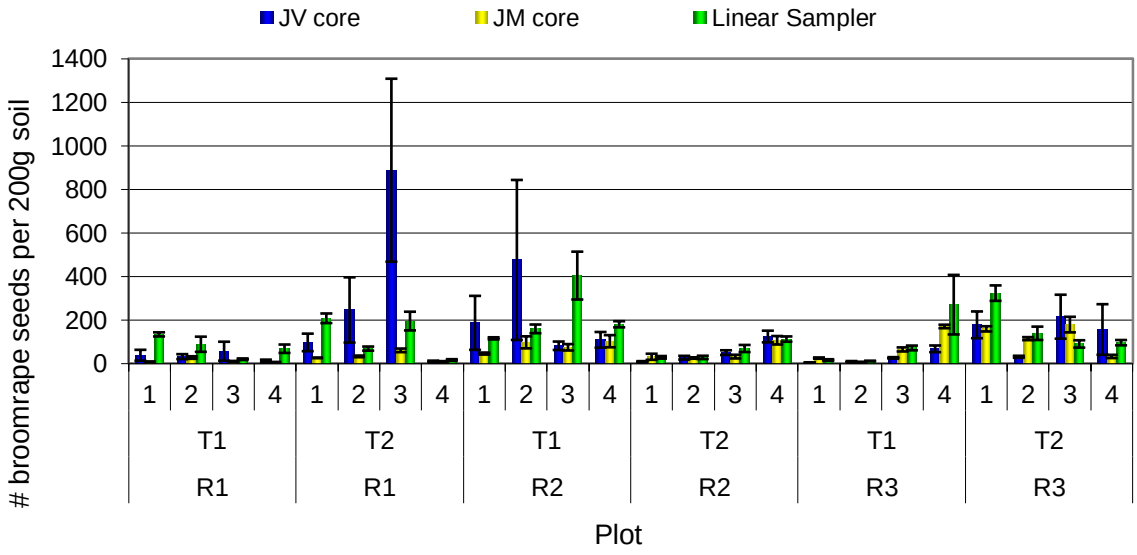


Figure 3. Branched broomrape seeds estimated by DNA assay from soil cores in each plot collected using three different sampling techniques. Bars are means ± 1SE, *n* = 4. T1 = control, T 2 = pine oil, R = replicate.

Conclusions

Due to the high degree of spatial variance in the broomrape seed bank there is the potential for the estimates of seed number to vary substantially between samples. This study has shown that this can affect the conclusions drawn about treatment effects. Careful consideration of the sampling strategy needs to be taken into account when evaluating the broomrape seed bank.

8. Detecting branched broomrape (*Orobanche ramosa*) DNA in sheep manure

Nick Secomb

Branched Broomrape Eradication Program

2003

Abstract

Branched Broomrape (*Orobanche ramosa*) seeds can be identified in sheep dung using the DNA probe developed by the South Australian Research and Development Institute (SARDI). Testing showed that as few as 2 seeds per 400g sample can be detected by the DNA probe. The DNA probe can also accurately confirm that no branched broomrape seeds are present in a sample.

Introduction

Branched broomrape is a parasitic weed of a wide range of broadleaf crops in the Mediterranean, Europe, central Asia, the Middle East, South Africa and North and South America. Broomrapes are root parasites that extract all of their nutrient requirements from their host plant.

The only known population of branched broomrape in Australia was discovered in 1992 in the Bowhill area (approximately 100km east of Adelaide). The detected plants were eradicated by fumigation. Between 1993 and 1997, plants were found at six more sites on the original property and an adjoining property. These plants were either fumigated or hand-picked. Between 1999 and 2002, an additional 381 paddocks were found to contain infestations of branched broomrape and a quarantine area was gazetted (Fig. 1).

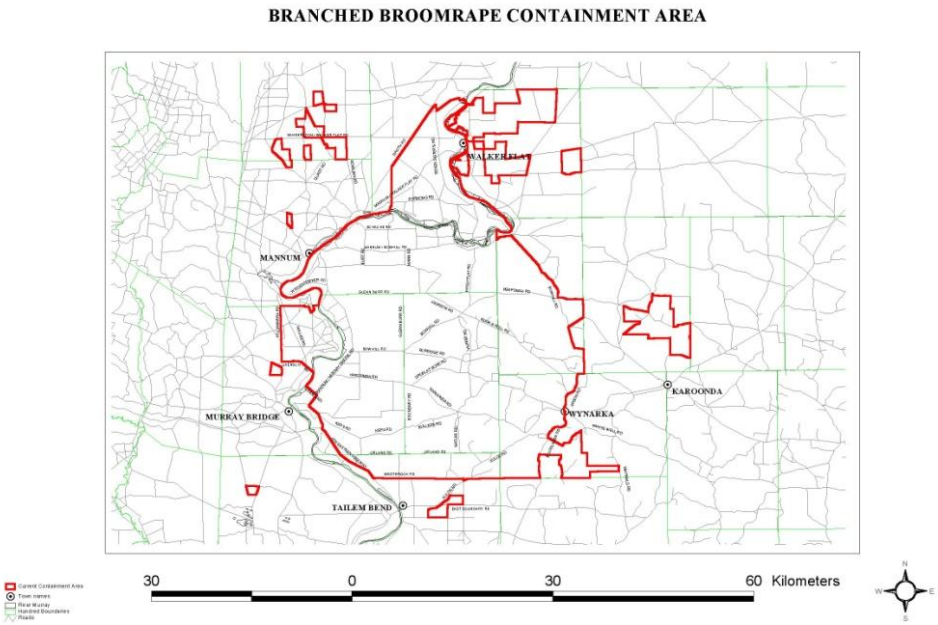


Figure 1. The branched broomrape quarantine area in South Australia

The methods of dispersal for branched broomrape seed in Australia are largely unknown although livestock have been observed grazing branched broomrape plants in the field. It's unclear whether or not viable seed can pass through the gastro-intestinal tract of a sheep and, if so, how long they take to pass.

A DNA probe, which is capable of determining whether or not branched broomrape seeds are present in a soil sample, has been developed. It's uncertain whether or not the large amounts of organic matter (like that found in manure) can mask the presence of branched broomrape DNA.

Testing was required to see if the DNA probe is capable of finding seeds of branched broomrape in sheep manure.

Two different tests can be conducted using the DNA probe. The first test can very accurately (down to 1 seed per 200g sample) determine whether or not branched broomrape seeds are present in a sample. The second test cannot determine the presence/absence of branched broomrape seeds with the same accuracy but can accurately estimate the number of seeds present down to 5 seeds per 200g sample.

Methodology

9.6 kilograms of fresh sheep dung was collected from sheep delivered for sale at the Dublin sheep saleyards. The sheep had been delivered from the Yorke Peninsula of South Australia (approximately 200 km from any known infestations of branched broomrape). The dung was placed into a bag and transported to the Waite campus for processing. The dung was divided into four replicates of six 400g samples (24 samples in total). Each sample was placed in a cotton bag. A 400g sample was recognised as an average daily output from a single sheep.

Samples were spiked with known numbers of branched broomrape seed. Each sample was inoculated with one of six different amounts of branched broomrape seed (see Table 1). Four replicates were repeated for each amount.

Table 1. Numbers of branched broomrape seeds used to spike sheep dung samples

Number of branched broomrape seeds added per 400g sample	Rep. 1	Rep. 2	Rep. 3	Rep. 4
	10 (10S1)	10 (10S2)	10 (10S3)	10 (10S4)
	8 (8S1)	8 (8S2)	8 (8S3)	8 (8S4)
	6 (6S1)	6 (6S2)	6 (6S3)	6(6S4)
	4 (4S1)	4 (4S2)	4 (4S3)	4 (4S4)
	2 (2S1)	2 (2S2)	2 (2S3)	2 (2S4)
	0 (0S1)	0 (0S2)	0 (0S3)	0 (0S4)

The spiking method involved placing a single, viable branched broomrape seed inside a pellet of dung using a metal probe. Once a sufficient number of pellets from a sample had been inoculated, the spiked pellets were placed in a cotton bag with the un-spiked pellets and shaken to distribute them evenly. Each 400g sample was dried in a glasshouse for 48 hours. After drying, each sample was weighed and divided into four sub-samples, each of equal weight. Each sub-sample was placed in a cotton bag and labelled for processing. It was hoped that sub-sampling would increase the probability that the DNA test could identify low numbers of branched broomrape seed.

Samples were collected and tested for branched broomrape DNA using testing method number 1.

Results

Once tested, sub-samples were pooled to determine the DNA probe's ability to find seed in a potential daily sample from a single sheep (see Table 3).

Table 3. Difference between number of seeds detected by DNA assay and number of seeds in spiked manure sample.

	Actual Seed Numbers	DNA probe estimate	Variation
0S1	0	0	0
0S2	0	0	0
0S3	0	0	0
0S4	0	0	0
2S1	2	1	-1
2S2	2	1	-1
2S3	2	1	-1
2S4	2	4	2
4S1	4	4	0
4S2	4	3	-1
4S3	4	3	-1
4S4	4	6	2
6S1	6	1	-5
6S2	6	4	-2
6S3	6	7	1
6S4	6	6	0
8S1	8	4	-4
8S2	8	6	-2
8S3	8	5	-3
8S4	8	2	-6
10S1	10	9	-1
10S2	10	7	-3
10S3	10	6	-4
10S4	10	6	-4

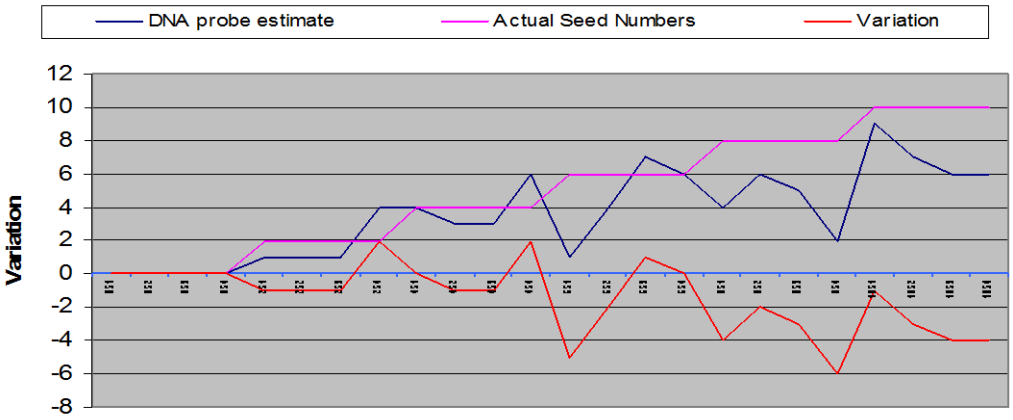


Figure 2. Variation between DNA seed estimate and actual seed numbers

Discussion

As predicted, test 1 did not give an accurate estimate on the actual number of seeds present in each sample. Estimates were up to 83% different to the actual number of seeds present in each sample.

While the test did not give an accurate estimate on the number of seeds present, it was able to accurately detect the presence or absence of branched broomrape seed in each sample. No branched broomrape DNA was recorded in any of the Control samples. Branched broomrape DNA was found in all spiked samples.

From this we can assume that test 1 is a useful tool to determine the presence and or absence of branched broomrape seeds in sheep manure. As few as two branched broomrape seeds can be detected in a 400g sample. Manure samples that do not contain branched broomrape seeds can be detected with some confidence.

These results suggest that the DNA probe can be used to successfully assess the retention time for branched broomrape seed in a sheep's gastro-intestinal tract. Further trials should focus on inoculating sheep with a known number of branched broomrape seeds and collecting manure for DNA testing to determine the time at which no more seeds are excreted.

9. Efficacy of survey teams in detecting branched broomrape in paddocks

Ray Correll

Rho Environmetrics

August 2004

Introduction

The eradication of branched broomrape is very dependent on the ability of the eradication team to detect branched broomrape in the field. The most important tool for assessing this are the spring surveys when the flower spikes are visible.

The surveys are costly and part of the motivation for this report is to assess the effectiveness of the current practice. Following on from that assessment it may be feasible to increase their efficacy.

Currently line transects are used in the field. There are two types of transects

1. Perimeter surveys (P), where an observer walks the perimeter of the paddock looking for broomrape in the paddock
2. Internal paddock surveys, which traverse the paddock three times in the form of a Z.

A comparison of the efficacy of these two strategies is available from these data.

A further concern is the fraction of the paddock surveyed. Typically the area viewed is less than 5%. The distance from the transect line to the observed branched broomrape plant offers an estimate of this. Furthermore, the visibility distance from the line will vary depending on the land use; variation between land uses can be confused with different incidences that are attributable to crop type. Assessing the visibility differences was another aim of this study.

An additional strategy was to assess the variability of observers. Some hint of the size of the variability can be gauge through the 'Broomrape team of the year' contest. It would appear that some observers are better than others; this affects the efficiency of the survey. In quantitative terms, if one observer is only seeing half the plants that another is seeing, then he is effectively missing half. A comparison of the observers will set an upper bound on their efficacy.

This report discusses results from the field sheets obtained from the 2003 survey.

Data

The data were obtained as field sheets. Data quality was variable, with a variety of problems. In many cases data were not entered at all. Some distances were not feasible – e.g. a distance of 400 m clearly did not refer to the distance from the transect.

The person who first sighted the broomrape was not usually recorded; when it was recorded it was frequently not by an alleged team member.

It is hoped that this can be improved for the 2004 survey.

Results

Land use and transect location

From all the (317) observations of branched broomrape in 2003, only 117 observations had sufficient data to be included in this report. A summary of the observations is given in Table 6.

Well over half the observations were in pasture, with the next highest contribution with no paddock use recorded. Pasture/almonds had 13 observations. Crop had only 5, with another three as scrub/cereal. There was an additional observation in almonds.

Table 6 Summary of observations of branched broomrape in paddock perimeter (P) and interior (Z) transects

Land use	*P*	*P & Z*	*Z*	*Blank*	*Grand Total*
Almonds			1		1
Cereals	5				5
Cereals/Scrub			3		3
Pasture	35		31		66
Pasture/Almonds			13		13
Pasture/Scrub			1		1
Scrub		1			1
Not recorded	14		6	4	24
Grand Total	54	1	55	4	114

There were almost the same number of observations on the paddock perimeter transect (P) as on the interior Z transect. However their distribution differed across the land uses. For example there were no Z observations in cereal and only Z observations in almonds or almond/pasture.

In pasture, there were almost equal numbers in the P as the Z; the difference could be due to chance or perhaps the longer distance of the perimeter compared to the Z. (For the mathematically minded, for a square paddock the ratio is squareroot of 10 v 4 or 80% of the perimeter).

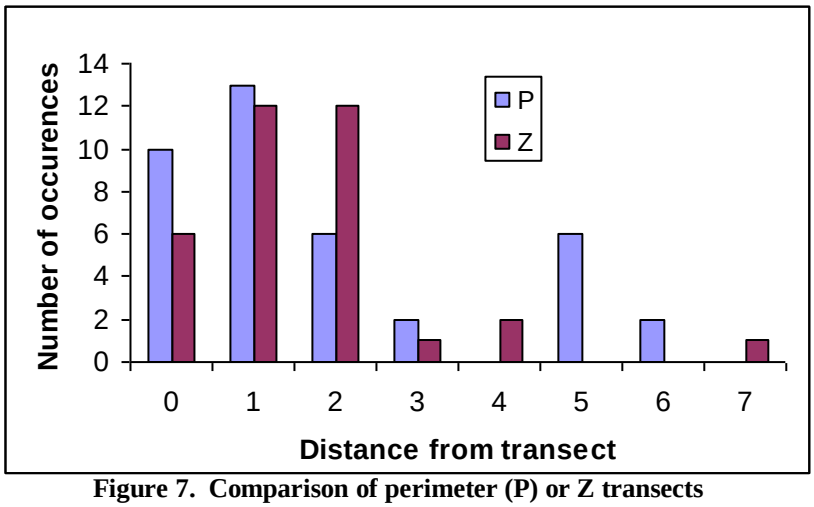


Figure 7. Comparison of perimeter (P) or Z transects

Distance from transect

A comparison of the sighting distance for pastures is shown in

Figure 7. There would appear to have been a greater distance from the perimeter than from the Z. This may be because the observer concentrated on looking in a single direction, compared to the observer n the Z who had to look both ways.

The sighting distances in the cereal were consistently low, with one about 0.3 m, two at 1 m and one at 2 m, giving a mean of just over 1 m. This is about half that observed for cereals.

Discussion

Data quality

There are numerous data deficiencies in the data. It is important to stress to the observers that there is a purpose for requesting theses data. A quality control should be run by the supervising staff early in the survey period to ensure the data quality is satisfactory.

Outliers in the data should be investigated promptly and the observer informed accordingly.

Concentration on perimeter

There was no evidence of concentration of the branched broomrape near the perimeter as evidenced by the near equal numbers in P as Z. This observation may have implications on the mode of spread of branched broomrape at least in pasture.

Comparison of observers

A comparison among observers was not feasible because of the low data quality. The method of assessing this from the team data has been developed and could be readily undertaken given appropriate data.

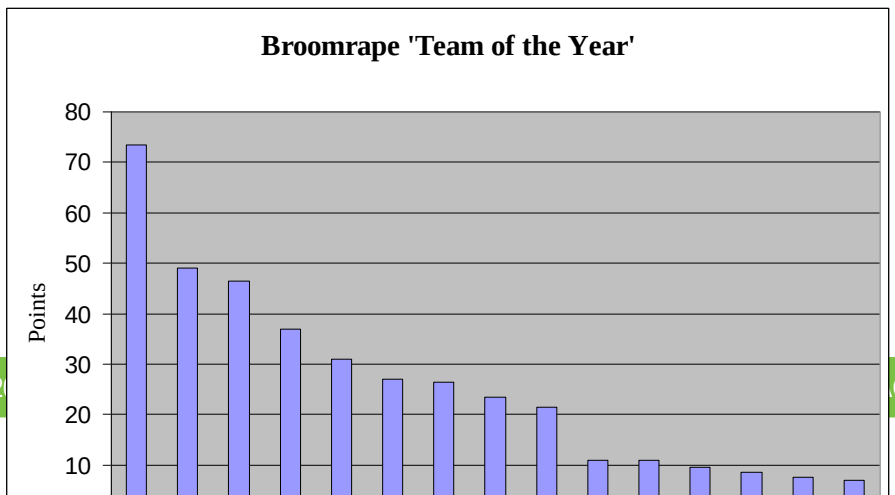


Figure 8. Comparison of team performance

As a surrogate, the relative performance of teams is shown in

Figure 8. This shows a 5 fold difference between teams; an informal analysis would indicate that the difference between observers must be causing a large underestimate of the number of broomrapes – perhaps by a factor of two.

10. Volatiles for the detection of broomrape by sniffer dogs

Che DeDear

Animal and Plant Control Commission

September 2001

Aim

As part of the investigation into the use of sniffer dogs for the detection of branched broomrape plants, sampling of the volatiles released by broomrape plants at various stages of development was conducted and compared to the volatiles released by carrot.

Methods and materials

A stem of each plant was enclosed in a two-piece vessel enclosing the stems and over the course of several hours, the headspace was sampled by means of a low-flow vacuum pump (Fig. 1). The volatile components were trapped on an adsorbent resin (Tenax or equivalent), which was packed within a desorption tube attached to the pump. Subsequently, these trapped components were desorbed by means of a short path thermal desorption unit(10) into a gas chromatograph equipped with a cryo trap (10). A blank is purged and analyzed to determine artefacts from the system.

The GC/MS (Hewlett Packard 5890/5971) was configured with two matched (50 meter, methyl silicone) capillary columns, one inlet, and two detectors (MSD and FID). The inlet was modified to accept the thermal desorption unit. The cryo trap was installed at the head of the columns and cooled to -50^{o}C by carbon dioxide. Desorption took place at 220^{o}C for 4 minutes. After desorption was complete, the cryo trap was heated to match the inlet temperature, while the GC temperature was programmed from 35^{0}C to 240^{0}C at 3^{o}C per minute.

Results

The gas chromatograph profiles for each sample are presented in Table 1.

	Area under curve				
volatile	Carrot 1	Carrot 2	Broomrape bud	Broomrape flower 1	Broomrape flower 2
Hexanal	846938		2662068	2680839	2475774
2-Hexenal, (E)	1981099	468189	12372541	4144749	1479905
1-Hexanol	281924		2397532	526324	598341
1-Hexanol, 2-ethyl	1725523	3504977			
2-Hexen-1-ol, (E)			2937532	420802	590684
1-Octen-3-ol		795863	500173		197749
Benzeneacetaldehyde	903925	690498		1512679	1112955

In comparison with carrot, the volatiles signature for broomrape was unique for the presence of 2-Hexen-1-ol, (E) and the absence of 1-Hexanol, 2-ethyl. Benzeneacetaldehyde was not detected in broomrape buds but was detected in flowering broomrape stems and carrot.

Figure 1. Apparatus for collecting samples

11. Changes in new discovery rates with increasing number of surveys

Ray Correll

Rho Environmetrics

August 2011

Introduction

Previous data have indicated that the number of new discoveries of branched broomrape has been decreasing, especially within the quarantine area. Part of the effect is due to the decreased number of potential paddocks where a new discovery can be made because by definition a new discovery cannot occur in a paddock where it has previously been seen. This study quantifies the rate of discovery as a function of the number of times a paddock has been inspected.

Data

The data used were based on the annual surveys' data base. These data have some limitations:

Currently poor information is available about paddocks that were surveyed in 1999 but were considered free of broomrape;

Most paddocks outside the quarantine area have only been surveyed a few times –typically the surveys were on a 3- year cycle. For this reason there were few paddocks outside the quarantine area that had been surveyed more than 5 times.

Statistical methods

The data were arranged so that each paddock could be classified by the number of times it had been surveyed. Paddocks were excluded following a positive detection. Every paddock in the data base had been survey at least once, so following the first survey paddocks were classified as 'non-detected' or detected.

In the next year, paddocks may not have been eligible because there had previously been a detection or they had not been surveyed twice. In practice, each paddock was considered for each year and classified accordingly.

Two classifications were used – initially all surveyed paddocks were considered and this produced lower initial detection rates than when the analysis was confined to paddocks within the quarantine area. The resulting tabulations were in effect life tables, where death is considered as a detection. In common with life tables there is incomplete data because many paddocks had not been exposed to all the surveys (in life tables this is referred to as left censoring).

The proportion of paddocks where there was a detection was plotted against the number of surveys for that paddock. The data were modelled using a generalised linear regression with binomial errors – the observations are not independent so it would be inappropriate to quote standard errors from that analysis.

Results

A summary of the data is given in Table 7. A plot of the proportions is shown in

Figure 9. The detection rates are in effect identical for the two approaches after 6 years – this is probably an artefact as the two data sets were almost identical after year 5.

The binomial mode was used to generate predicted proportions for future years (Table 8).

Table 7 Paddocks classified by number of times surveyed and whether a new discovery was made

Number of years surveyed	All paddocks		Paddocks within the quarantine area	
	Detected	Non-Detected	Detected	Non-Detected
1	412	16679	412	4689
2	152	12261	152	4510
3	71	8537	71	4323
4	94	5920	93	4143
5	18	4374	18	4005
6	27	3987	27	3895
7	35	3819	35	3760
8	10	3716	10	3699
9	3	3583	3	3574
10	3	3447	3	3443
11	4	2083	4	2081
12	0	8	0	8

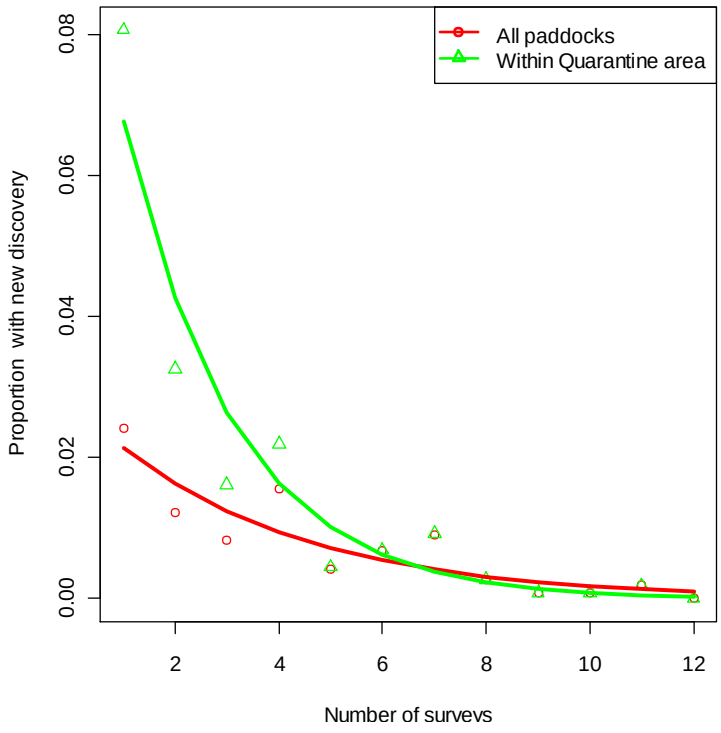


Figure 9. Effect of number of surveys on the probability of a new detection of broomrape

Table 8. Fitted values for probabilities of detection

Years of inspection	Probability of detection in the inspection area	Probability of detection within the quarantine area
1	2.10%	6.80%
2	1.60%	4.30%
3	1.20%	2.70%
4	0.94%	1.60%
5	0.72%	1.00%
6	0.55%	0.62%
7	0.42%	0.38%
8	0.32%	0.23%
9	0.24%	0.14%
10	0.180%	0.088%
11	0.140%	0.054%
12	0.100%	0.033%
13	0.080%	0.020%
14	0.060%	0.012%
15	0.0460%	0.0075%
16	0.0350%	0.0046%
17	0.0260%	0.0028%
18	0.0200%	0.0017%
19	0.0150%	0.0011%
20	0.0120%	0.0007%