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Compendium of branched broomrape research

Section 5. Seed bank decline

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

MAY 2014

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 16 May 2014

Disclaimer

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Prider J., Correll R., Warren P. (2013) A model for risk-based assessment of *Phelipanche mutelii* eradication in fields. Weed Research 52, 526-534.

Matthews J., Miegel D., Hayton D.. (2006) Seed bank and seed bank reduction of *Orobanche ramosa* in South Australia. 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. 626-628.

1. Determining *Orobanche ramosa* seed longevity by accelerated aging

Jane Prider and Anna Williams

Branched Broomrape Eradication Program

March 2010

Summary

An estimate of the persistence of *Orobanche ramosa* seed in the soil seed bank is an essential requirement of the Branched Broomrape Eradication Program. Although two seed burial trials are currently underway to estimate seed longevity, the final results will not be available for some time. We used an accelerated or controlled aging test to rapidly determine the longevity of *O. ramosa* seed. The test showed that *O.ramosa* had a gradual loss in seed viability over time with a 50% decline in seed viability after 39 days. There is currently no accurate method of converting the aging time to a time in years that seeds will persist under field conditions. A published study suggests that this decline equates with a seed persistence of less than 3 years in the soil (Long *et al.* 2008). The results of our own seed burial trials do not support this result, with more than 50% of seeds remaining viable after 3 years. The controlled aging test does not accurately given an estimate of *O. ramosa* seed longevity.

Introduction

Prediction of seed longevity is important for any weed eradication program and this often needs to be determined when the program commences. Inadequate information about seed bank longevity can extend the duration of an eradication program and may underestimate the effort required for weed control (Panetta 2007). It is also essential for deciding whether eradication has been achieved or if monitoring should continue (Regan *et al.* 2006).

Reliable information about the longevity of *Orobanche ramosa* seed was lacking when the Branched Broomrape Eradication Program was instigated in 1999. Non-sourced data suggested that *O. ramosa* seed longevity was 13 years in the field (Parker and Riches 1993) and data for other *Orobanche* species is similarly anecdotal (Holm *et al.* 1997; Parker and Riches 1993). There is only one long-term field study of *Orobanche* seed longevity, which estimated that the viability of *O. crenata* seed was reduced to less than 1% after 12 years (Lopez-Granados and Garcia-Torres 1999). A shorter term study over six months indicates that viability loss in *O. crenata* is similar to *O. ramosa*, at least during the early stages of burial (Grenz and Sauerborn 2007). Laboratory stored *Orobanche* seed lost viability after 15 years (Linke and Saxena 1991).

Real-time trials of seed bank persistence in the field give the most informative estimates of seed persistence but for long-lived seeds there may be a considerable time delay before seed persistence can be estimated. Controlled or accelerated aging tests provide a means of estimating loss of seed viability due to natural aging processes (Probert *et al.* 2009). In this technique, seeds are subject to temperature and moisture conditions that accelerate seed aging. Enzymatic degradation and free radical production are aging processes that are favoured by moist, warm conditions. The aging tests therefore measure the inherent resilience of seeds to moisture and temperature stresses.

The aim of this project was to use a controlled aging test to rapidly estimate the seed longevity of *Orobanche ramosa*.

Method

Controlled aging test

The aging tests, conducted in 2008, compared *Orobanche ramosa* with a control species, *Brassica rapus*. The testing procedure is a standard developed by the Royal Botanic Gardens, Kew, that is used by the Adelaide Botanic Gardens in their Millenium Seedbank Project (Davies and Probert 2004). The facilities of the Adelaide Botanic Gardens were used for this study.

Samples of seed were initially hydrated by incubating at 20 °C and 47% relative humidity for two weeks. After hydration the seeds were transferred to the aging chamber set at 45 °C and 60% relative humidity (Fig. 1). Subsamples were retrieved from the chamber at set times (1, 2, 5, 10, 20, 30, 75, 100, and 125 days) to conduct germination tests.

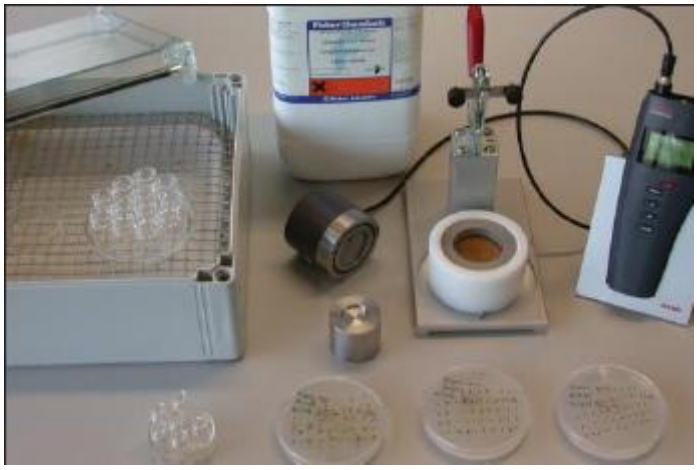

Figure 1. Accelerated aging chamber

Brassica seeds were divided into two replicates of 50 seeds each, placed on 0.7% (w/v) agar plates and incubated at 10/20 °C alternating temperature/light cycle (to mimic autumn/spring conditions). Germination was scored twice weekly until no further germination was observed over a two week period.

Two replicates of 100 *Orobanche* seeds were placed on glass fibre filter papers, germination stimulant (GR24) was added and plates were incubated at 20 ºC. Germination was scored after two weeks and at weekly intervals thereafter until no further germination was observed. Ungerminated *Orobanche* seed was tested for viability using a tetrazolium test. Seeds were bleached in 1% NaOCl for 5 minutes and then thoroughly rinsed. Seeds were then immersed in a 1% tetrazolium solution for two weeks. Viable seeds that had reacted to the tetrazolium by staining red were scored.

The tests were repeated three times and the results presented are the results of the first test for *B. napus* and the second test for *O. ramosa.* The other tests for *O.ramosa* gave very poor results but the reasons for this are not known.

Analysis

All viable *Brassica* seeds germinated, so germination was equivalent to viable seed. *Orobanche* viability was calculated as the sum of the germinated seed plus viable ungerminated seed. A seed viability loss curve was constructed and the time taken for seeds to decline to 50% viability (P_{50}) was calculated. Data for each species was subject to probit analysis using Genstat Version 9.1. This analysis gives an estimate of P_{50} through fitting the viability equation:

Viability = Initial viability – (days/σ)

where σ is the standard deviation of the normal distribution of seed deaths in time.

Seed destruction of aged seeds

An experiment was also conducted to examine whether aged seeds were more susceptible to seed destruction products. The products tested were Pine Oil, Niproquat and Basamid. There may have been problems with the application of the products (which were applied *in vitro* after seeds had been aged) as several products which have been shown to be effective in other studies did not kill seeds in this experiment. These results are therefore not presented.

Results

Viability loss in *Orobanche* seeds was initially very variable (Fig. 2). There was a gradual loss in viability over time. The P_{50} value (decline to 50% viability) for *Orobanche* was 39.23 days. In contrast, there was no marked decline in *Brassica* seed viability until day 20, after which there was a rapid decline (Fig. 3). The P_{50} for *Brassica* was 48.15 days.

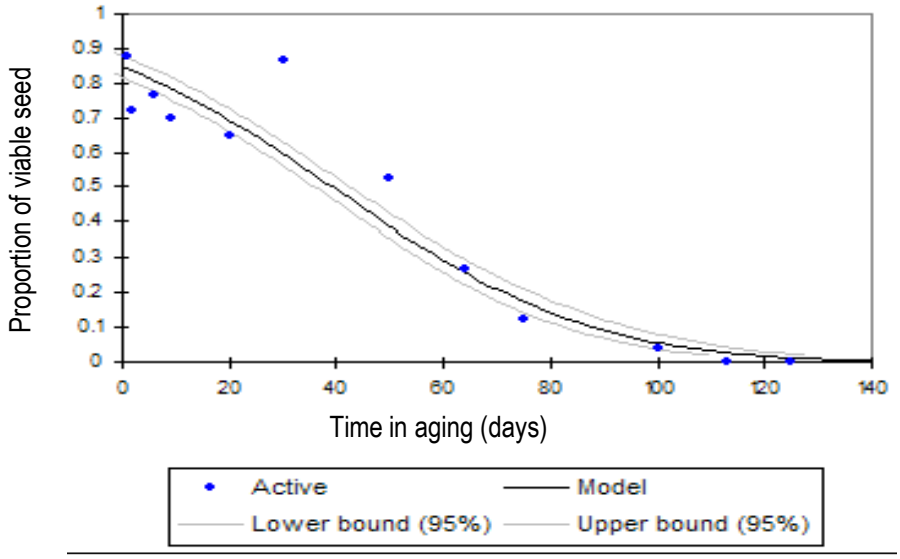

Figure 2. Response of *Orobanche ramosa* to controlled aging. The curve is cumulative percentage normal germination.

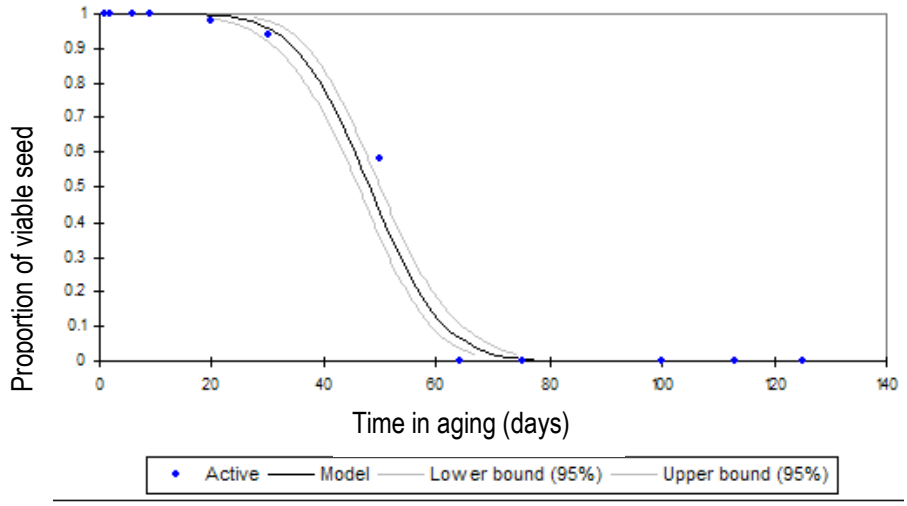

Figure 3. Response of *Brassica napus* to controlled aging. The curve is cumulative percentage normal germination.

Discussion

The results of the aging test (P_{50} values) indicate that *Brassica* seed has greater longevity than *Orobanche* seed, although more accurately, the curves indicate that viability loss in *Orobanche* is at least initially more rapid than *Brassica*. There is no accurate means of converting the time in aging to a time scale of persistence under field conditions. Long *et al.* (2008) found a positive correlation between P_{50} values and seed persistence in several European species but their data set was geographically and taxonomically limited. They also compared results of aging tests with field burial trials in some species from Queensland and suggested that species with a P_{50} between 20 and 50 days have short-lived seed banks, persisting from 1-3 years. However the longest field studies they cited were only of four years duration and the majority only ran for up to two years.

From field studies of *O. ramosa* and *B. napus*, a seed persistence of from 1-3 years appears to be an underestimate. Studies of volunteer canola emergence indicate that *B. napus* seed can persist for at least 9 to 10 years (D'Hertefeldt *et al.* 2008; Lutman *et al.* 2005) and our seed longevity experiment demonstrates a decline of less than 50% viability for *O.ramosa* seeds after 3 years. The controlled aging tests do not give an accurate prediction of *O. ramosa* seed longevity.

The shape of the viability curves is of some interest. It shows that *O. ramosa* seeds have no initial resilience against aging as appears to be the case for the *B. napus* seeds. The lack of a hard seed coat may be one reason for this. The P_{50} value is not very revealing as under field conditions there will be considerable variation in seed persistence in relation to seed burial depth, soil temperature and moisture status, seed lot and disturbance regimes. Kebreab and Murdoch (1999) used the results of aging tests to predict the decline of *O. crenata* under field conditions but they tested aging under a broad range of temperature and humidity conditions. The single aging test we used here has limited use for this type of prediction.

Controlled aging tests were developed by Kew to test the longevity of seed in storage and have more recently been used to test the vigour of different seed lots (Probert *et al.* 2009). The greatest utility of the method is by using the P_{50} values as relative scores to make comparisons between different species or seed lots. For the broomrape program the technique may be useful for making comparisons of the vigour of *O.ramosa* seeds from different hosts or in different years.

Conclusions

A controlled aging test was not able to accurately predict the longevity of *O. ramosa* seed. This may change with the publication of further data comparing the results of controlled aging tests with long-term seed longevity trials. At best this will only give a range (in years) for seed persistence and is not likely to give a precise estimate.

The method may provide a useful tool for making relative comparisons of the vigour of different seed lots.

Acknowledgments

We thank Dr Phil Ainsley and the staff of the Millenium Seed Bank Project at the Adelaide Botanic Gardens for their assistance in the design of this project, data collection and data analysis and for the use of equipment.

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2. Broomrape seed bank studies, 2003-2008

John Matthews, Darryl Meigel and Dorothee Hayton

The University of Adelaide

Background

Orobanche ramosa is a species that can produce seeds in profusion and the seeds are reputed to persist in the soil for many years. Management of the species and commitment to eradication requires a sound appreciation of the period of retained seed viability or the seedbank life. Documented seedbank life values from overseas could not be relevant here due to Australian conditions and so seed bank studies were begun in summer 2003. A good appreciation of the seedbank life is essential to understand the period of time the eradication effort has to last and how long the farmers and managers within the quarantine area will be affected.

General methods

Seed bank studies were begun in summer 2003 and 6 monthly assessments 11 data points in 5.5 years. *Orobanche* seeds were collected from a single site of about 2.4ha in size in the previous summer, dried, cleaned and mixed with 5g of sieved sandy soil and were placed in stainless steel sachets. The sand was sieved to pass through a 110µ sieve to remove all fines and vegetable matter. Seeds number for burial was assessed by volume, about 1200 seeds were installed at site 1 and 2800 at site 2. Seed numbers were expected to show small variability due to the enumeration method, but it was anticipated that small variations would not influence the results. Sachets were buried at 2 sites; at depths of 2 and 5 cm on 1 site and 5 and 10cm at site 2. Sachets were retrieved on a six monthly schedule for the first 5.5 years and visibly intact seeds counted and the viability determined by tetrazolium staining after germination studies.[1]

Seedbank decline

The number of germinable seeds is a combination of the number of intact seeds and the viability of those seeds. Each parameter has been assessed separately and the resultant population of viable seeds was calculated. The pattern of decline and visual assessment of the contents of the packets, the seedbank decline is shown in Figure 1.

The projections on Figure 1 are for illustration only and the anticipated seedbank life is shown in Table 1. The table shows the number of years since the start of the trial to elapse for various calculated proportions of viable seed remaining.

The viability of seeds in the sachets is the most influential parameter of the decline of viable seed number. The loss of viability contributes about 80% of the decline of viable seed bank. The loss of viability is shown in Figure 2. The number of intact seeds retrieved from the sachets has shown a slight decline indicating that seed coat integrity is robust and supporting the observation that loss of viability is of greater importance in the loss of germinable seeds from a seed bank. Intact seed number has declined by an average of 20% in the sampling period, data shown in Figure 3.

The number of intact seeds has not exhibited much decline over time but it has shown that seed viability is of critical importance in reducing the potentially active seedbank.

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[1] Later analysis of this data set (see Section 5.4) indicates that tetrazolium tests were only used after 2006 therefore this paper describes the results of germination and not viability

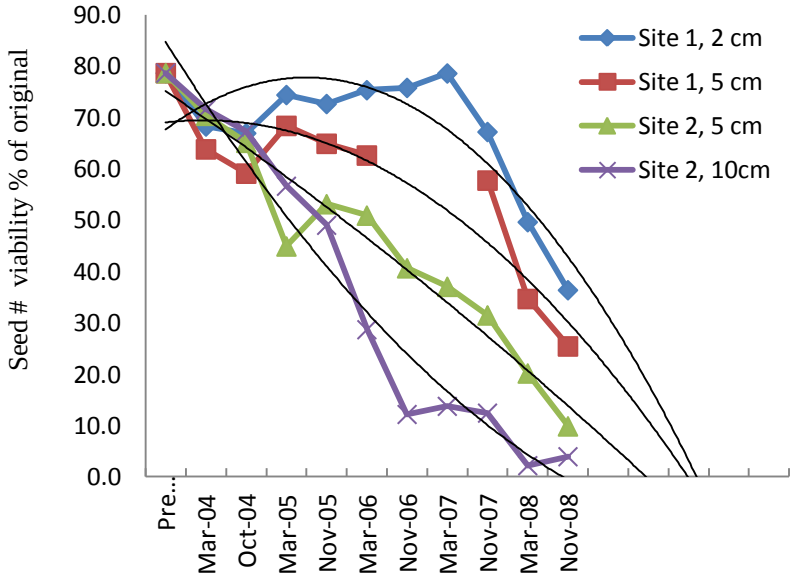

Figure 1. Germinable seed bank

Table 1. Projections of the number of years before the seed bank declines to a specified proportion

	Years to specified proportion of viable seeds			
Proportion of seeds	Site 1 2cm	Site 1 5cm	Site 2 5 cm	Site 2 10cm
0.5	4.6	4.3	2.2	1.8
0.05	6.1	6.1	7.0	4.1
0.01	6.8	7.0	9.5	5.2
0.001	7.6	8.1	12.9	6.8
0.0001	8.5	9.2	16.3	8.4

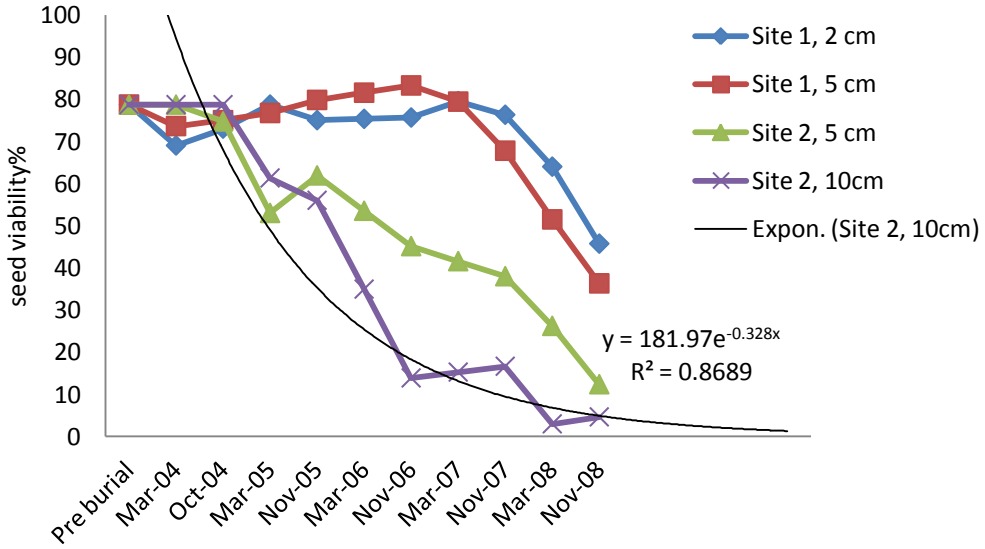

Figure 2. Viability of seeds from seed sachets over time.

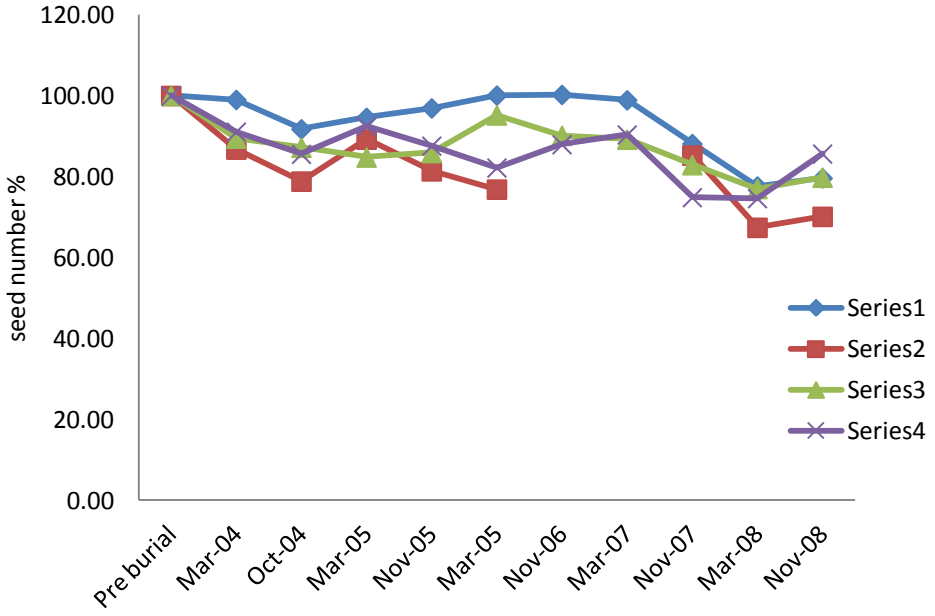

Figure 3. Number of intact seeds from seed sachets over time.

Discussion

The decline in the number of viable seed in the quarantine area as indicated by the combined loss of viability and the loss of intact seeds from seed sachets was shown to be quite substantial. The rate of decline illustrated in figure 1 and caculated by regression in table 1 suggests that viable seed may be at 1/1000th of the initial level between 7 and 13 years from the start depending on the site and the depth. There is more analysis to be done to determine the range of variability around this single mean estimate. On a more practical issue, the role of loss of seed viability in overall decline is of great importance and any improvement in our understanding of the factors affecting seed decline could be of help. Conversely any management practice that reduces seed decline could be considered to be reducing the rate of eradication.

The sensitivity of the calculated rate of decline to the last few datum needs to be noted. The last three data points in all series has produced the declining trend. More data will always reinforce the veracity of the trend; that may be viewed with scepticism, but the truth of it cannot be disputed. This is the first of this type of experiment undertaken in this novel programme with much riding on the trend. Caution is advised until the trend is established.

There will be a need preserve the integrity of the data gathered so far and also for new data as it is generated. Also it will be important to maintain some consistency in gathering seed samples, storing of seed in the laboratory and processing the seed. Long term experiments can quickly fail if the impetus and the standard is not maintained.

There is the possibility that clusters of seed may degenerate more rapidly than isolated seeds in the soil, this has been canvassed in the literature. That may be the case but the total loss of viability within sachets is not rapid, certainly not within 1 or even 2 sampling periods, indicating a slow decline of viability more consistent with loss of integrity of the seed coat and subsequent infection. Factors affecting the loss of integrity of the seed coat and the potential rate of fungal infection are unknown, references in the literature suggest *Fusarium* sp. are frequently isolated from infected seed and plants.

In many cases within the quarantine area, if this experiment has relevance, the seedbank life of paddocks without fresh seed return is approaching a low level. The threat of large numbers of broomrape emerging in these areas is reducing but isolated plants may be expected to still be germinable. Thus the prevention

effort needs to be maintained or increased on areas approaching the 10+ years of freedom. An accurate assessment of the real seedbank in these paddocks has not been undertaken to date.

Acknowledgements

Rho Environmetrics Pty Ltd PO 366 Highgate South Australia 5064

Dr Chris Preston, University of Adelaide

Appendix

Seed viability Figure 2

Intercept equations and R2

Site 1 2 cm $Y=0 + (78.7)/(1+10^{((1.880-X)* -12.18)})$ $r^2 = 0.4511$

Site 1 5 cm $Y=0 + (78.7)/(1+10^{((1.858-X)* -10.62)})$ $r^2 = 0.6570$

Site 2 5 cm $Y=0 + (78.7)/(1+10^{((1.790-X)* -2.848)})$ $r^2 = 0.3870$

Site 2 10 cm $Y=0 + (78.7)/(1+10^{((1.646-X)* -3.981)})$ $r^2 = 0.5219$

3. Analysis of branched broomrape seedbank data

Ray Correll

Rho Environmetrics Pty Ltd

Prepared for Dr John Matthews

University of Adelaide

June 2009

Introduction

This report describes long-term seed viability trial conducted under Branched Broomrape Eradication Program. The available data are described and included in this report.

The request from the client was for diagrams with fitted lines. In view of the importance of the data to branched broomrape management, a full report has been written to describe the analyses.

Data

The data consisted of tables of percent of viable seed, percent recovery of seeds, and percent of recovered and viable seeds. The actual data are shown in Appendix A.

The raw data were not available at the time of analysis, but means and standard deviations were available. Thos data are shown in Appendix B.

Statistical Methods

Change in viability

The viability of seeds for the first four years showed little change, but then declined rapidly. An exponential decay model was not adequate for describing the change in viability with time. Other models such as

$$L_{\mathrm{w}} = \left(1 - 0.9975^{D_w}\right) \times S_{\mathrm{t}}$$

as used by Grenz (2005) also do not allow for a period when there is little or no decline ii seed viability.

A simple alternative model for the proportion of viable seeds *y* at age *t* is a logistic curve which has the form

$$y_t = \frac{y_0}{1 + \exp\left(-k(m - t)\right)}$$

where y_0 is the viability of the seed at the time of burial, *k* controls the rate of breakdown and *m* the time the viability of the seeds has been reduced to half the initial value.

In fitting the model, the variability of the counting was assumed to be constant over the trial. For low counts this approximation will breakdown because when there are no more viable seeds remaining, there will be no (or little) measurement error. A weighted model should be used at that time.

An enhancement to the current model would be to force all three series to have a common intercept; that should be done before these results are published.

Change in seed recovery

Two approaches were used to assess seed recovery.

Analysis of variance

An analysis of variance was made on each series based on the recovery means and their standard deviations as supplied. This approach does not allow an investigation of individual data points. The test is not very powerful because it uses a global test for differences between time periods. The approach is report for completeness

Decay rate approach

This approach assumes that there is some decay rate that remains constant over the measurement period. Formally the number of seeds recovered is expressed as

$$x_t = x_0 \exp(-kt) + \text{error}$$

which can be reformulated as

$$\ln(x_t) = \ln(x_0) + kt + \text{error}$$

This is a simple model which has some useful properties

> x_o should be approximately 100% and should be constant across all the series
> k is a measure of the decay rate, and can be used to estimate a half life via the relationship

$$t\_0.5 = \ln 2 / k$$

Furthermore an estimate of the confidence interval of k is available and that can be used to give an estimate of the confidence interval of $t_{0.5}$. In practice we care concerned with only the upper confidence interval of $t_{0.5}$ so a one-side interval has been used.

Change in recovery and viability

The change in recovery and viability (the product of the two variables) gives a useful measure of the decline of the seedbank. The changes were dominated by changes in viability. There was also a loss even from the beginning due to seed loss even though seed viability was maintained. It is noted that if the decline in viability is logistic, the product of recovery and viability cannot be logistic.

The model used for recovery and viability was therefore a product of the models for viability and seed recovery (rescaled to account for both components being on a percentage scale).

Results

Seed viability

Data from the series for seed viability were found to fit a logistic curve as shown in

Figure 1. All four series at the most recent measurement had a viability of less than 50%. The samples at Site 1 showed little change in viability followed by a rapid decline (reflected in the high *k* parameter in Table 1. By contrast there was an earlier start to reduction in viability at Site 2, but the rate was then less, particularly in the 2.5 cm series.

The projection of the model beyond the current data is shown in

Figure 2. Projection of curves beyond the data range is uncertain, but it provides the best estimate available and has therefore been included with the caveat that there is no guarantee that the logistic model will be appropriate beyond the current data.

The data from Site 1 show an upper asymptote of 75.8%. By contrast, the Site 2 data do not come off an asymptote, so the model is pragmatic rather than mechanistic so for Site 2 the y_0 parameter is not the %viability at time 0, so y_0 has no ecological interpretation at Site 2.

Table 1. Parameter estimates of logistic model fitted to viability data

	Site.1.2cm		Site.1.5cm		Site.2.5.cm		Site.2.10cm	
	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE
Y_0	75.8	1.4	79.0	1.8	88.9	14.3	84.2	6.4
m	5.23	0.10	4.93	0.10	3.10	0.63	2.31	0.19
k	2.61	0.78	1.98	0.42	0.64	0.19	1.43	0.28

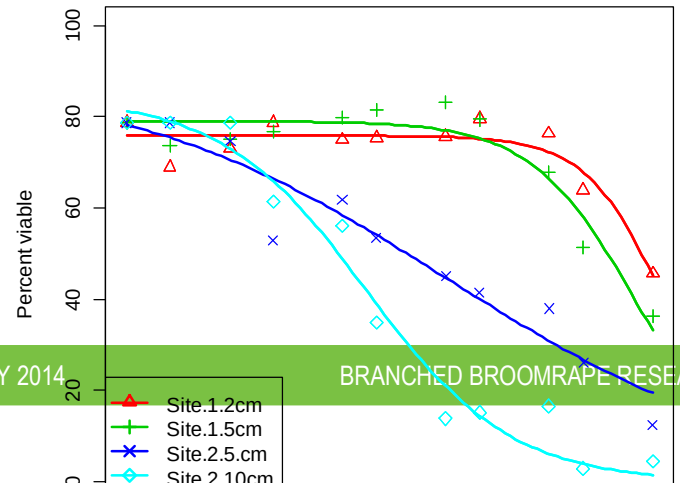

Figure 1. Details of change of viability with time over current data range

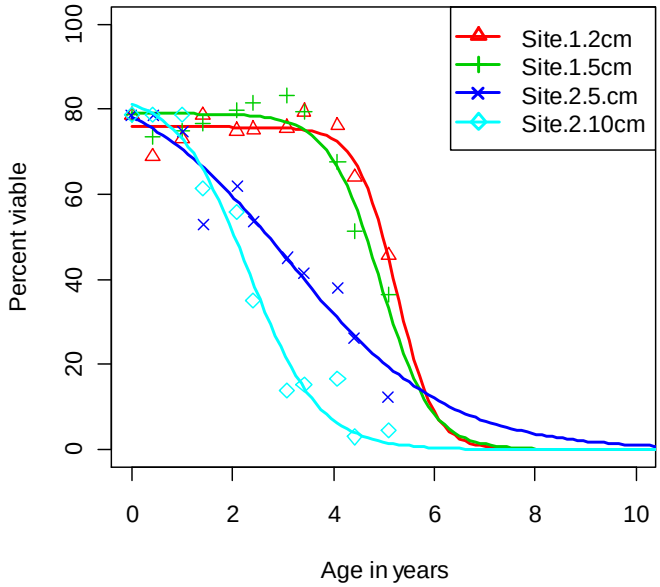

Figure 2. Details of change of viability with time over extended range

Seed recovery

The rate of seed recovery loss is shown in

Figure 3 and described in

Table **2. Details of estimates of loss rates in branched broomrape from four series**

Series	k (loss rate)				Half Life		R^2	Adjusted R^2
	Estimate	SE	't'	Probability	Estimate	Upper limit		
Site 1 2cm	-0.031	0.015	-2.076	0.068	22.6	193	0.324	0.249
Site 1 5cm	-0.039	0.022	-1.751	0.114	17.78	undefined	0.254	0.171
Site 2 5 cm	-0.031	0.011	-2.921	0.017	22.39	60	0.487	0.43
Site 2 10cm	-0.036	0.012	-2.886	0.018	19.29	533	0.481	0.423

. The model fit was better for Site 2 than for Site 1 as shown by the adjusted R^2. The model was in fact not significant for Site 1 2 cm series. The half life for each series were all approximately 20 years, but the upper bound (one sided) was much higher. Site 1 5 cm series did not have a defined upper limit.

Table 2. Details of estimates of loss rates in branched broomrape from four series

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	Estimate	SE	't'	Probability	Estimate	Upper limit		
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Site 2 5 cm	-0.031	0.011	-2.921	0.017	22.39	60	0.487	0.43
Site 2 10cm	-0.036	0.012	-2.886	0.018	19.29	533	0.481	0.423

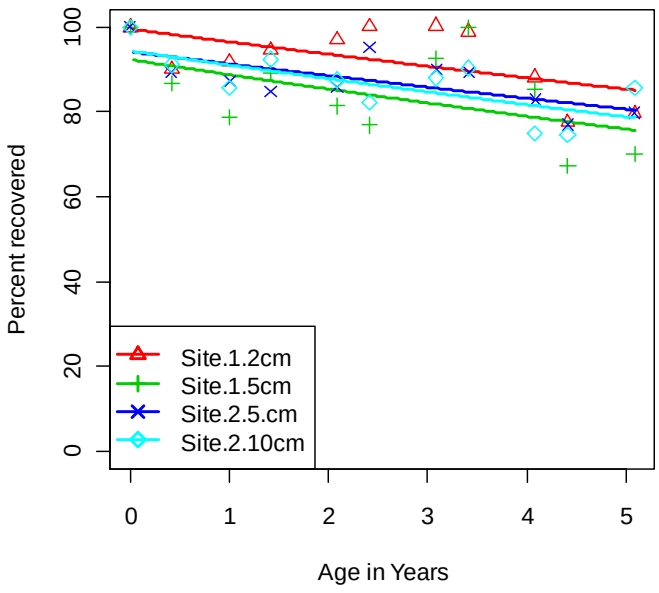

Figure 3. Recovery rates of branched broomrape in four trials. The fitted lines are negative exponentials

An alternative model recognises that the same seed numbers were meant to be buried in each packet, so the fitted values at time zero should all be the same. A summary of that regression is given in Table 3.

A further simplification is to consider that each series has both a common intercept and a common slope. That regression is shown in

Figure 4. Effect of time on percent recovery of seeds. Two points above 100% recovery were excluded from the regrsssions.

Table 4. A comparison of these two regression was tested and yielded an $F_{(3,37)} = 1.25$ ($p = 0.30$). Because there was no significant difference between the slopes the pooled slope of -0.035 shown in

Figure 4. Effect of time on percent recovery of seeds. Two points above 100% recovery were excluded from the regrsssions.

Table 4 was therefore used.

Table 3. Coefficients of regression of log recovery against time with a common intercept and separate slope for each series

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	4.551	0.022	203.735	0.000
Site 1 2cm	-0.023	0.011	-2.192	0.035
Site 1 5cm	-0.046	0.010	-4.486	0.000
Site 2 5 cm	-0.032	0.010	-3.152	0.003
Site 2 10cm	-0.037	0.010	-3.583	0.001

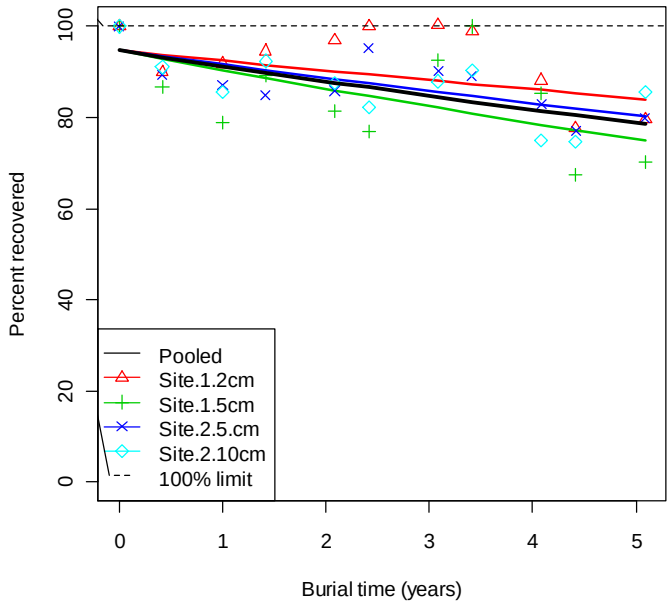

Figure 4. Effect of time on percent recovery of seeds. Two points above 100% recovery were excluded from the regrsssions.

Table 4. Coefficients of regression of log recovery against time with a common intercept and a pooled slope

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	4.549	0.023	201.9	0.000

Time	-0.035	0.008	-4.605	0.000

Combined seed viability and seed recovery

Data on the combined recovery and viability are shown in Figure 5 and Figure 6 and summarised in Table 5. The proportions shown are lower than those shown in Figure 1 over the time of the data recording. For completeness, a projection is also provided in Figure 6.

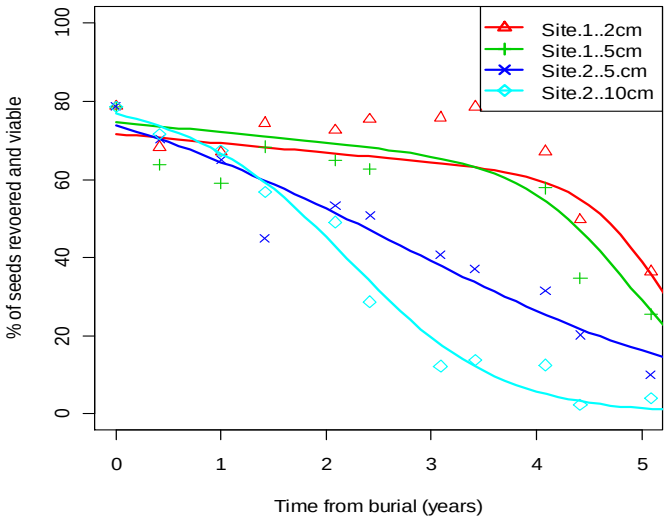

Figure 5. Changes in viable seed numbers over time in four trials. The fitted lines are logistic curves.

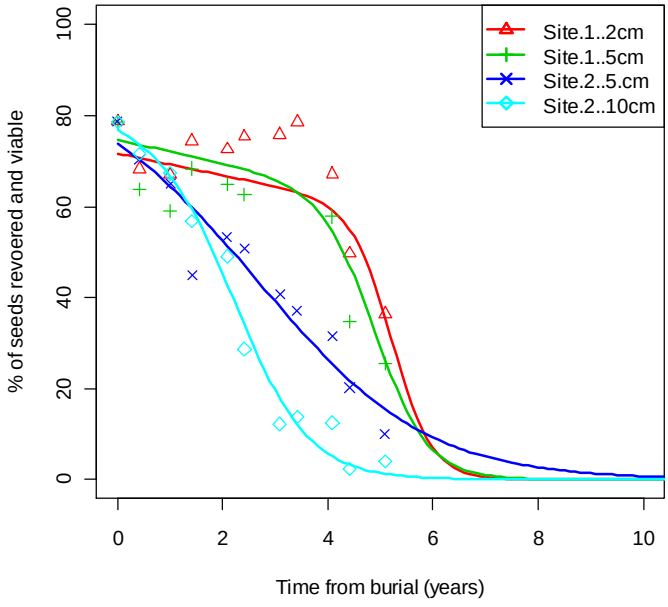

Figure 6. Changes in viable seed numbers over time in four trials over an extended range. The fitted lines are logistic curves

Estimated time required for seed bank reduction

An estimate of the time required to decrease viability to 50% is shown in

Table 5. Estimates of required time for lower proportions are provided by extrapolating. Estimates of the combined estimates are shown in Table 6. The time required for a 50% reduction is less in the combined model, but this is not always so for the lower probabilities. The *m* or mid parameter is consistently lower in the combined data but the estimated of *k* are lower in the estimates of the combined data.

Table 5. Years required to achieve a given proportion of seed viability based on logistic model

Proportion of seeds	Site 1 2cm	Site 1 5cm	Site 2 5 cm	Site 2 10cm
0.5	5.0	4.7	2.7	2.0
0.05	6.2	6.3	7.5	4.2
0.01	6.9	7.1	10.1	5.4
0.001	7.7	8.3	13.7	7.0
0.0001	8.6	9.4	17.3	8.6

Table 6. Years required to achieve a given proportion of seed viability and recovery based on logistic model

Proportion of seeds	Site 1 2cm	Site 1 5cm	Site 2 5 cm	Site 2 10cm
0.5	4.6	4.3	2.2	1.8
0.05	6.1	6.1	7.0	4.1
0.01	6.8	7.0	9.5	5.2
0.001	7.6	8.1	12.9	6.8
0.0001	8.5	9.2	16.3	8.4

Discussion

The information obtained from this trial will be critical in the management of release from quarantine, and provides estimates of seed viability under field conditions. However the range of field conditions assessed, although useful within the available resources, cannot be considered typical of all soils in the quarantine area. While the analysed in this report are the best available, an extrapolation to other soil types will be required.

The projections to times when seed viability has been reduced to 1%, 0.1% and 0.01% are very model dependent. There was evidence of model failure at Site 2. While the models are currently useful, they will be enhanced by the incorporation of future data.

The requirement for future data points raises several issues.

- There are only a limited seeds still to be harvested – thought needs to be given as to when those additional seed sets are recovered.
- The trial is long-term and ongoing. There needs to be careful archiving of the raw data so it is not dependent on any single person.

Conclusions

Seed loss from the system is slow, with a half-life of approximately 22 years.
Loss of seed viability can be describes using a logistic model.
There would appear to be little loss of viability of seeds for several years (depending on the Site) followed by a rapid decay. It is projected that after 10 years the proportion of viable seeds will be less than 1%.

Recommendations

The projections of decay rates should be reviewed following as each new set of data become available.
A robust archival system should be established for the data
Careful attention should be given as to when future bags of seeds should be removed.

References

Jan H. Grenz, Ahmad M. Manschadi, Peter DeVoil, Holger Meinke and Joachim Sauerborn (2005) Assessing Strategies for *Orobanche* sp. Control Using a Combined Seedbank and Competition Model Agron J 97:1551-1559 (2005)

Appendix A Data used in analyses as supplied.

Seed number expressed as a % of amount at beginning

Seed viability expressed as a % of seed recovered.

	Site 1 2cm	Site 1 5cm	Site 2 5 cm	Site 2 10cm	Site 1 2cm	Site 1 5cm	Site 2 5 cm	Site 2 10cm
Pre burial	100	100	100	100	78.7	78.7	78.7	78.7
Mar-04	89.96	86.75	89.45	91.07	69	73.6	78.7	78.7
Oct-04	91.75	78.83	87.20	85.57	73	75	74.7	78.7
Mar-05	94.58	89.21	84.79	92.43	78.7	76.7	53	61.3
Nov-05	96.92	81.38	85.89	87.59	75	79.8	61.9	56
Mar-06	100.08	76.85	95.19	82.20	75.32	81.52	53.52	34.94
Nov-06	100.25	92.60	90.13	87.95	75.63	83.23	45.13	13.87
Mar-07	98.92	100.00	89.20	90.36	79.5	79.4	41.57	15.26
Nov-07	88.13	85.25	82.89	74.86	76.29	67.76	38	16.64
Mar-08	77.63	67.42	77.00	74.63	63.98	51.46	26.22	2.98
Nov-08	79.63	70.13	79.80	85.64	45.72	36.3	12.38	4.63

Calculated number of viable seeds, obtained by multiplying both data sets together. Note that two data points were excluded because seed recovery rate was apparently too high to be credible

	Site 1, 2 cm	Site 1, 5 cm	Site 2, 5 cm	Site 2, 10cm
Pre burial	78.7	78.7	78.7	78.7
Mar-04	68.3	63.8	70.4	71.7

Oct-04	67.0	59.1	65.1	67.3
Mar-05	74.4	68.4	44.9	56.7
Nov-05	72.7	64.9	53.2	49.1
Mar-06	75.4	62.6	50.9	28.7
Nov-06	75.8	omitted	40.7	12.2
Mar-07	78.6	omitted	37.1	13.8
Nov-07	67.2	57.8	31.5	12.5
Mar-08	49.7	34.7	20.2	2.2
Dec-08	36.4	25.5	9.9	4.0

Appendix B Raw means and supplied standard deviations

Seed viability of seeds retrieved from buried packets as a % of seeds recovered

Sampling times	Site 1 2 cm Mean	Site 1 2 cm SD	Site 1 5 cm Mean	Site 1 5 cm SD	Site 2 5 cm Mean	Site 2 5 cm SD	Site 2 10cm Mean	Site 2 10cm
Pre burial	78.7	4.8	78.7	4.8	78.7	4.8	78.7	SD
Mar-03	69	4.9	73.6	10.5	n/a		n/a	
Oct-03	*43.0	22.5	*32.3	28.4	*22.3	12	*30.33	84.3
Mar-04	*59.0	12.4	*50.0	8.6	*35.3	29.9	*68.7	70.6
Oct-04	73.3	14.4	86	1.7	74.7	7.2	n/a	96
Mar-05	78.7	14.7		18.9	53	21	61.3	752.4
Nov-05	75	7.9	79.8	2.6	61.9	7.7	56.1	
Mar-06								772.46
Nov-06	75.63	4.62	83.23	7.71	45.13	23.03	13.87	264
Mar-07	79.47	12.2	79.43	15.1	69.40	4.1	52.53	298
Nov-07	76.3	4.12	67.8	15.4	56.9	16.2	16.64	399
Mar-08	63.98	15.46	51.64	9.11	26.22	12.64	2.98	97
Dec-08	45.72	5.85	36.3	8.6	12.38	4.67	4.63	

Number of intact seeds retrieved from buried seed packets

Sampling times	Site 1 2 cm Mean	Site 1 2 cm SD	Site 1 5 cm Mean	Site 1 5 cm SD	Site 2 5 cm Mean	Site 2 5 cm SD	Site 2 10cm Mean	Site 2 10cm SD
Pre burial	1200		1200		2800		2800	4.8
Mar-04	959	300.1	882.7	303.5	2209	156	2301.3	
Oct-04	1144.33	415.1	1010.3	211.6	2674.3	464.8	2492.7	27.5
Mar-05	1126.33	296.4	1131.3	169.9	2074	97	2684.6	23.29
Nov-05	1241.3	38.07	822.3	398.5	2736	333.9	2221.33	
Mar-06								18.18
Nov-06	1293.67	212.5	1223.33	369.05	2453.33	750.85	2543	13.51
Mar-07	1170	298	1398	470	2542	197	2517	
Nov-07	945	392	818	24	2100	428	1675	7.95
Mar-08	918	338	772	297	2212	495	2504	29.9
Dec-08	993	176	1191	258	2257	260	2292	14.3

4. Analysis of 2010 branched broomrape seed viability data

Ray Correll

Rho Environmetrics Pty Ltd

January 2011

Introduction

This is an updated analysis of John Matthew's seed longevity trial. The previous analysis by Correll (2009) used germination data and a combination of viability and germination data. This report has used only the viability data and gives a complete description of the study methods.

Site Description

Site 1

Seeds were buried in sachets of approximately 100 mm by 100 mm of stainless steel mesh.

The experimental site was approximately 10 m from some mallee, and consists of three adjacent plots each being approximately 1.2 m square. The plots are defined by CCA treated pine planks. The plots were covered with chicken wire to prevent interference from animals such as rabbits.

The plots have remained almost weed free throughout the trial. The soil is a loamy sand. Samples at this site were buried at 25 mm with black labels or 50 mm depth with red labels.

Site 2

Seeds were buried in sachets of approximately 50 mm by 35 mm of stainless steel mesh.

The experimental site was in grassland that was dominated by grass (*Stipa variabilis*?) with occasional broad leafed weeds including capeweed. The site consists of three adjacent plots each being approximately 1.2 m square. The plots are defined by CCA treated pine planks. The plots were covered by

a wire cage some 600 mm above the ground to prevent interference from animals such as rabbits. The plots contained weeds including *Stipa* (about 5 clumps per plot) with an occasional plant of cape weed. The soil is a loamy sand. Samples at this site were buried at 100 mm with white labels or 50 mm depth with red labels.

Laboratory Methods

2008 method

In 2008 seeds were separated from the sand in the sachets under a microscope. The recovered seeds were counted and a subsample of these seeds was tested for germination as below (subsample was divided between 5 petri dishes). The subsampling method is unknown. Ungerminated seeds were tested for viability as below.

2009 method

We counted germinable seed and viable seed in each replicate as follows:

Seeds were separated from sand in the sachets by floating out in 40% w/w solution of calcium chloride. The seeds were surface sterilised in a 1% solution of sodium hypochlorite and rinsed in RO water. All recovered seeds were placed onto moistened 2.5 cm glass fibre filter papers in 6 cm petri dishes. Each replicate was divided between 4 dishes for ease of counting and to minimise risk of loss of entire replicate due to fungal infection in a dish. The dishes were sealed and incubated at 20 ºC for two weeks to condition. 200 µl of GR24 was added to each filter paper and they were resealed and incubated at 20 ºC for a further two weeks. Germinated seeds were counted under a dissecting microscope. Ungerminated seeds were counted and then placed in eppendorf tubes with approx. 200 µl of 1% tetrazolium salt solution to assess viability of ungerminated seed. Tubes were maintained in the dark at 30 ºC for two weeks. Viable seeds were counted under a dissecting microscope as those seeds with the embryo stained either red or pink. Unviable or unstained seeds were also counted. Ideally the number of unviable and viable seeds should equal the number of ungerminated seeds (that were put in the tubes). There are some minor seed losses or miscounts during processing so these numbers are not exactly the same.

2010 method

Final sachets were collected from Site 1. Only two sachets were retrieved at the shallow depth and one of these had very few seeds. The Site 1 seed samples from 5 cm depth were subdivided after retrieval into three portions to provide further replicates for the next two years. One subsample was processed to provide 2010 data. The other two subsamples were reburied. Seed processing method as 2009.

Statistical Methods

The proportion of viable seeds P_o was calculated as

$$P_o = P_g + (1 - P_g) \times P_v$$

where P_g is the proportion that germinated and P_v is the proportion of non-germinated seeds that were found to be viable.

Typically a logistic equation is fitted to seed viability data. The proportion P is modelled as

$$P = \frac{a}{1 + \exp\,(-k(t - m))}$$

where *a* is some constant nominally indicating an initial viability, *t* is the burial time in years, *m* time until viability has been reduced to 50% and *k* a shape parameter. In the current situation the seeds for the shallow and deep series came from the same source so they would have had the same initial viability. Both series were fitted together using the equation

$$P = \frac{a}{1 + g_1 \exp\big(-k_1(t - m_1)\big) + g_2 \exp(-k_2(t - m_2))}$$

Where g_1 and g_2 are an indicator variable with the value of 1 or 0 to indicate whether it is shallow or deep series; similarly the subscripts on k and m indicate the series.

Data

Crucial to the estimates of change in viability is the estimate of the initial viability. The original records were reviewed by Correll and Prider (2009). A germination rate of 67% was originally recorded but data from more recent trials has had germination rates of 85%. The compromise figure for viability accepted by Correll and Prider was 90%. This value has been inserted into the viability records for this trial.

New data obtained from the 2010 exhumation is shown in Table 7. These data were used to extend the data used by Correll and Prider (2009) to create the set shown in

Table 8.

Table 7. Data from 2010 exhumation. (dp = deep; sh = shallow)

Site	Depth	Rep	Number germinated	Number ungerminated	Total seeds	number viable	number unviable	%viable
2	sh	1	16	2531	2547	446	2085	18.14%
2	sh	2	4	2519	2523	39	2471	1.71%
2	sh	3	21	2398	2419	525	1848	22.80%
2	dp	1	12	2126	2138	51	2046	2.98%
2	dp	2	1	2619	2620	6	2613	0.27%
2	dp	3	19	2181	2200	245	1907	12.15%
1	sh	1	0	2	2	1	1	
1	sh	2	no sample					
1	sh	3	156	35	191	6	27	85.01%
1	dp	1	88	87	175	26	59	65.49%
1	dp	2	53	115	168	81	23	84.86%
1	dp	3	31	371	402	295	76	81.09%

Table 8. Viability data for the seed bank data as at December 2010

Site	1		2	
Date	Shallow	Deep	Shallow	Deep
Dec-02	90	90	90	90
Mar-03	NA	NA	NA	NA
Oct-03	NA	NA	NA	NA
Mar-04	NA	NA	NA	NA
Oct-04	NA	NA	NA	NA
Mar-05	NA	NA	NA	NA
Nov-05	NA	NA	NA	NA
Mar-06	NA	NA	NA	NA
Nov-06	NA	NA	NA	NA
Mar-07	NA	NA	NA	NA
Nov-07	78.4	79.9	34.5	16.9
Mar-08	78.7	81.4	49.6	9.2
Dec-08	71.1	71.3	40.3	18.4
Nov-09	70	65.8	26	2.4
Nov-10	85.85	77.9	14.6	5.96

Results

The data is shown in

Figure 7. There was no simple trend apparent with the Site 1 series – in fact the viability recorded for the 2010 data was higher than any previous recorded viability. The observed data were however less than the inserted value of 90% viability, but because that datum was inferred, the line connecting it to the observed data is shown as a dotted line. There was no significant difference between the shallow and deep series at Site 1.

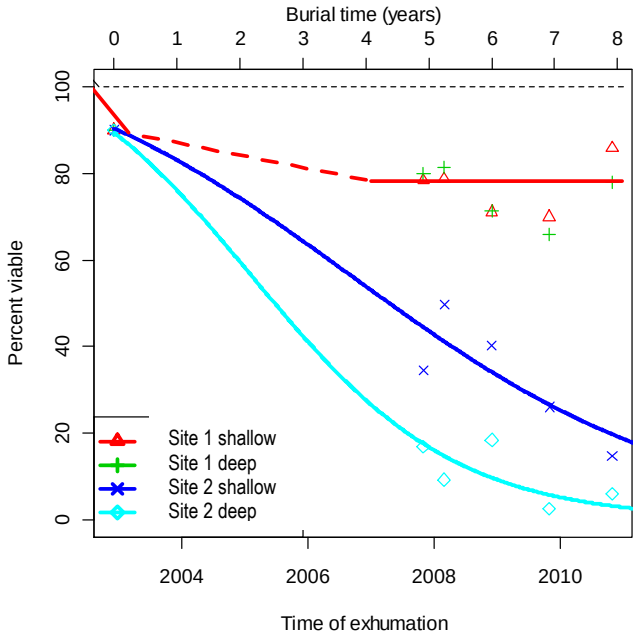

Figure 7. Effect of burial time on viability of branched broomrape seeds

The parameter estimated for the curves fitted to the Site 2 data series are shown in Table 9. The shallow and deep series differed from each other with the deep series showing a faster death rate than the shallow series.

Table 9. Parameters used in fitting logistic curve to the Site 2 viability data; m refers to time until viability is reduced to 50% and k is a shape parameter. The subscripts 1 and 2 refer to the shallow or deep series

	Estimate	Std. Error	t	Pr(>|t|)
a	112.34	35.5459	3.16	0.0159
m_1	3.7844	2.2655	1.67	0.1387
m_2	2.194	1.759	1.247	0.2524
k_1	0.3746	0.1786	2.097	0.0742
k_2	0.6208	0.2336	2.657	0.0326

The parameters shown in Table 9 were used to project likely viability rates for the data series at Site 2. These projections are shown in Table 10. After 10 years the there would be less than a 1% viability in the deep series and after 17 years for the shallow series.

Table 10. Projected percent viability of seeds based on the equation fitted to the Site 2 data series

Years since burial	Shallow	Deep
0	90.43%	89.43%
5	43.60%	16.75%
9	13.95%	1.62%
10	9.98%	0.88%
16	1.14%	0.02%
17	0.79%	0.01%
20	0.26%	0.00%

30	0.01%	0.00%
50	0.00%	0.00%

Discussion

Current results

Differences between sites – Site 1 was kept weed free as compared to Site 2.

The seed burial plots at the two experimental sites were set up separately. There are physical differences between the two sites but there is also inconsistency in the preparation of seed sachets for the sites and site maintenance:

- Seed sources for Site 2 unknown but could differ from Site 1 – there may be differences in seed lots and also in the way these seeds were collected
- Different sized sachets used
- Different amounts of seeds and sand put into sachets
- Seeds sachets buried at different times (times unknown)
- Mismatch of burial depths
- Weed control at Site 1 plots but not at Site 2

For this reason, the two sites are not directly comparable. However, even given the difference in the experimental set up, the lack of seed decay at Site 1 requires further explanation if we are to be confident in extrapolating the seed decay results across the entire quarantine area.

As there has been no decline in the number of seeds that have been recovered each year, it would appear that seeds are not being lost because they have germinated, so the hosts present at Site 2 are not having an effect on germination. Good recovery of seeds also indicates seeds have not been eaten; the mesh size would exclude small invertebrates but saprophytic micro-organisms seem also to be ineffective. One of the explanations could be the decay of seed through pathogenic microbial processes. There may be inter-site differences in the presence or activity of these organisms.

Projections are model dependent.

There was a dip in viability in the 2008 and 2009 data – this must have been an artefact because viability in the 2010 samples is higher than in any previous year where viability had been recorded.

Samples remaining

Because there has been little change in viability noted at Site 1, it was agreed that after a laboratory examination two thirds of the seeds would be reinterred for at least another year.

It is recommended that no exhumation be made of the Site 1 seeds in 2011 to prolong the life of the trial.

The number of samples remaining after the November 8th 2010 exhumation is shown below.

Plot	Site 1		Site 2	
	Black (25 mm)	Red (50 mm)	Red (50 mm)	White (100 mm)
North			9	8
Middle		2	8	8
South			10	7

Recommendations

Research plans for 2011 include an investigation of factors that may be affecting seed decay and whether this differs between the two sites.

5. Seed burial experiment – Haby Hut Road

Anna Williams, Jane Prider, Darryl Miegel and Andrew Craig

Branched Broomrape Eradication Program

February 2014

Background

Understanding the rate of seed mortality in field conditions is currently a missing link in the branched broomrape lifecycle and essential for getting an answer for the elusive question "where do all the seeds go?" The key feature of this trial is that there was a known number of seeds buried. Therefore, the fate of all the seeds originally buried could be determined. This information was not available for the other seed burial trial (Sections 5.2- 5.4).

The aim of this trial was to determine the rate of natural seed decay/attrition in field situations subject to prevailing environmental conditions, in the absence of host plants or larger seed predators.

Methods and materials

Experiment set up

A single site was selected for the field burial trial, at the edge of a cropped field, adjacent to the road boundary of Haby Hut Road (Fig. 1). The site has an easterly aspect with a sandy loam soil. Branched broomrape has been found previously in the field.

A plot measuring 15 m by 1 m was fenced with mesh that continued underground and into the plot to exclude rabbits and other animals from the plot. A data logger (WeatherMate, Environdata Australia Pty Ltd , Warwick Qld) was placed in the end of the plot. The logger was fitted with soil temperature and soil moisture sensors.

Sachets for containing seeds were constructed from Nylon Tarpee® mesh, sewn into a bag measuring approximately 5 cm by 5 cm. A total of 200 sachets were prepared.

Lots of approximately 300-500 branched broomrape seeds, collected from the Mannum Trial Site in November 2007, were placed on filter papers so that they could be photographed and then counted before placing in the sachets with 5 ml of Burdett sand. The tops of the sachets were folded over and secured with staples. An aluminium label was attached to each sachet with wire (Fig. 2).

In April 2008, the sachets were buried in the plot in 5 linear groups of 40 sachets at a depth of 5 cm. The label was left unburied. The ground was then covered by a layer of mesh (Fig. 3).

The plot was maintained weed-free by hand weeding when necessary. Herbicide (glyphosate) was used around the edge of the plot as part of the fence line weed control program for the cropped field.

Sample collection

For the first three years of the study, between July 2008 and October 2011, five sachets were collected four times during the year, corresponding to winter (July), October (spring), January (summer) and autumn (April). These collections were made to examine patterns of dormancy and the results are reported in Section 3.10).

From April 2012, five sachets were collected once annually in the second week of April.

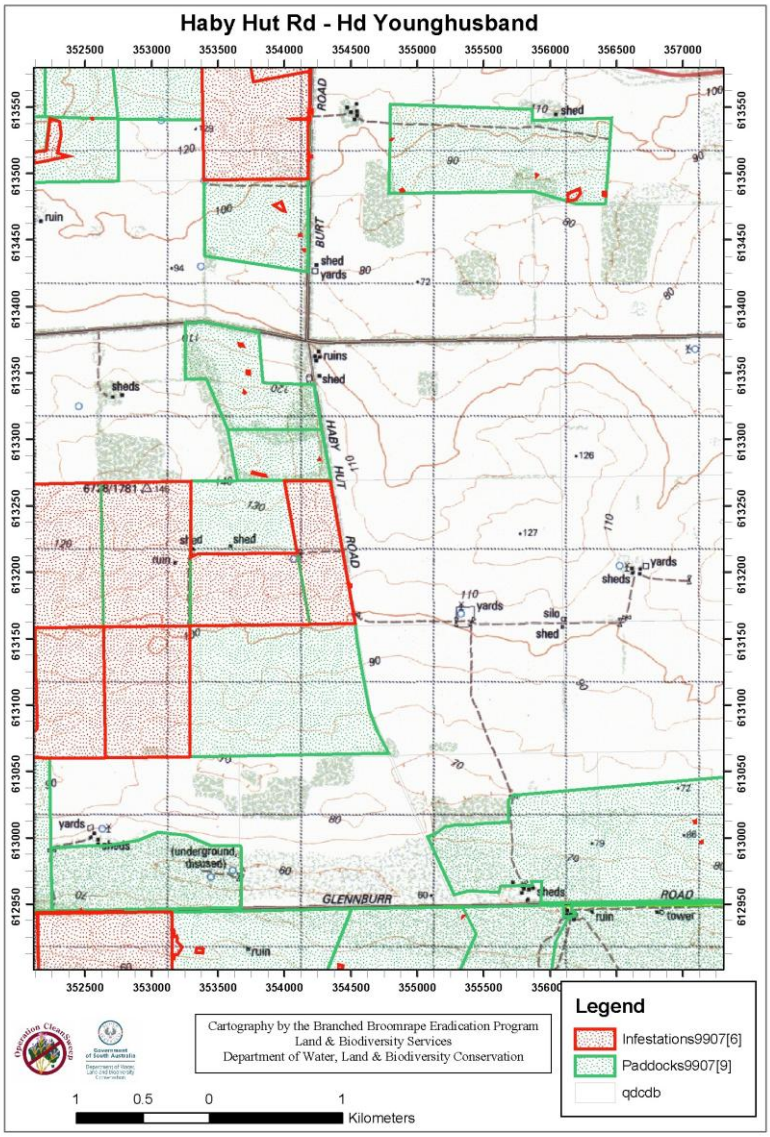

Figure 1. Site location on Haby Hut Road near Mannum.

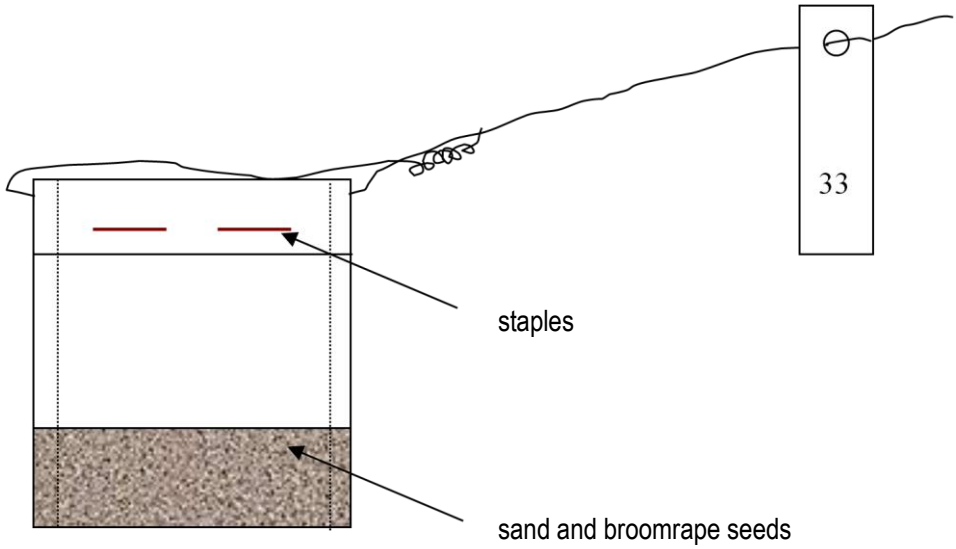

Figure 2. Sachet construction

Figure 3. The buried sachets in place in the fenced plot on Haby Hut Road.

Assessment of viability

In the laboratory, the sachets were left to air dry. Seeds were separated from sand in the sachets by floating out in 40% w/w solution of calcium chloride. The seeds were surface sterilised in a 1% solution of sodium hypochlorite and rinsed in RO water. All recovered seeds were placed onto moistened 2.5 cm glass fibre filter papers in 6 cm petri dishes. Each replicate was divided between 4 dishes for ease of counting and to minimise risk of loss of entire replicate due to fungal infection in a dish. The dishes were sealed and incubated at 20 ^{0}C for two weeks to condition. 200 µl of GR24 was added to each filter paper and they were resealed and incubated at 20 ^{0}C for a further two weeks. Germinated seeds were counted under a dissecting microscope. Ungerminated seeds were counted and then placed in eppendorf tubes with approx. 200 µl of 1% tetrazolium salt solution to assess viability of ungerminated seed. Tubes were maintained in the dark at 30 ^{0}C for two weeks. Viable seeds were counted under a dissecting microscope as those seeds with the embryo stained either red or pink. Unviable or unstained seeds were also counted. Ideally the number of unviable and viable seeds should equal the number of ungerminated seeds (that were put in the tubes). There are some minor seed losses or miscounts during processing so these numbers are not exactly the same.

At the same time, four replicates of the 2007 seed lot that had been stored in the lab at room temperature was also processed in the same manner. These samples were used a s controls to check for any discrepancies in the processing method and also to assess viability loss in lab-stored seed (see also Section 5.7)

The proportion of viable seeds (*P*) on each sampling date (*t*) was calculated according to the formula:

$$P_t = \frac{number\ of\ germinated\ seeds + \left(ungerminated\ seeds \times \frac{number\ of\ stained\ seeds}{total\ seeds\ in\ tetrazolium\ test}\right)}{total\ seeds\ retrieved}$$

The proportion of recovered seeds was calculated from the number of seeds counted prior to placement in sachets and the total number of seeds retrieved.

Results

Recovery

There has not been any trend in the number of seeds that are recovered at each sampling date. More than 85% of the original number of seeds placed in sachets have been recovered (Fig. 4). If a nominal 5% is added to that figure for processing error, e.g. seeds that remain in sand and not floated out, then at least 90% or more seeds are being recovered. There have thus been only minor or no losses of seeds to predation or germination.

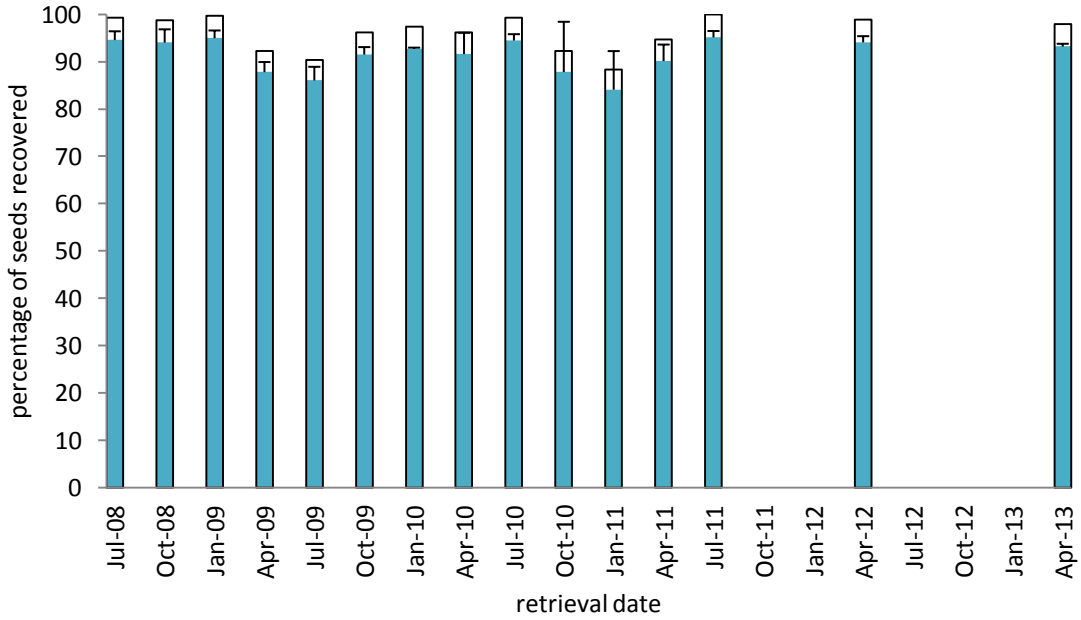

Figure 4. Percentage of seeds recovered from sachets on each sampling data. The white bars include a 5% correction inferred as losses during processing. Bars are means + 1 SE, n = 5.

Viability

With the exception of a very low viability measure in October 2008, seed viability did not decline between July 2008 and April 2011 (Fig. 5). Seed maintained similar viability to lab-stored seed over this time period. Seed viability from July 2011 has fluctuated but there has been a sustained decline in seed viability in comparison to earlier values. Some of this variability could be the result of seed processing. The tetrazolium test does not always provide an accurate assessment of viability and as germination is low in winter to spring months (Section 3.10) this test is relied upon to assess seed viability. Retrievals in April are preferred as seed is not dormant at this time and germination tests can be used to assess viability. This provides a more reliable estimate than the tetrazolium test. From April 2012, seed sachets were only retrieved in April. This will extend the duration of the experiment and provide the most reliable measurement of seed viability.

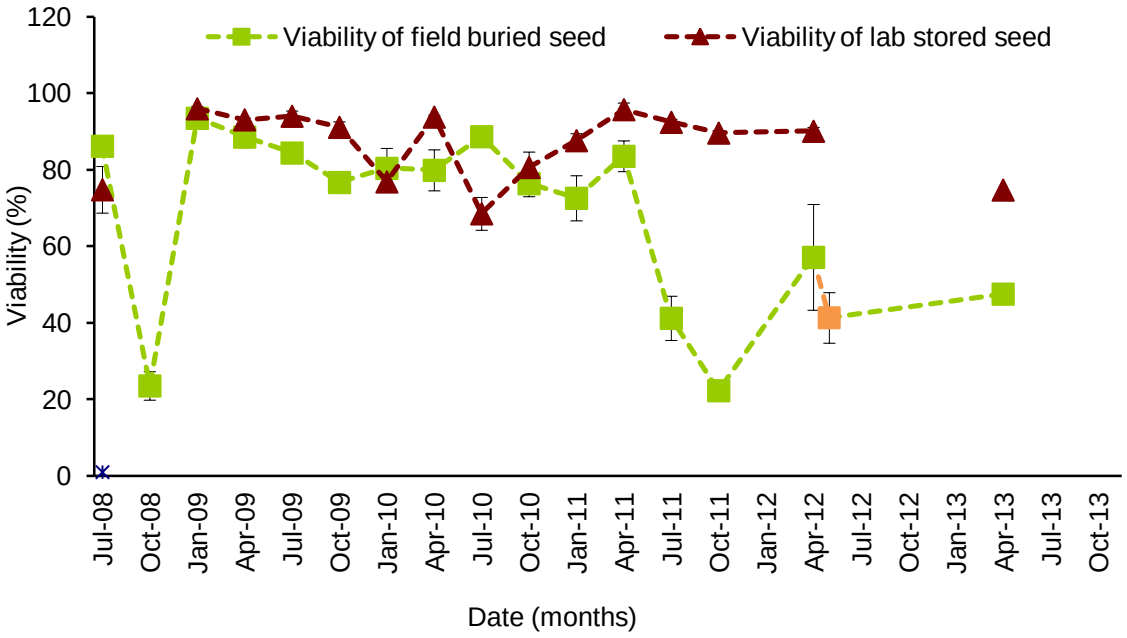

Figure 5. Viability of soil buried seed and lab-stored seed over time. Seed was buried in April 2008. The orange marker is the viability from sachets that were retrieved and then buried at the Mann site 12 months previously. Points are means ± 1 SE, n = 5.

Another seed burial study (see Sections 5.2-5.4) had found a marked decline in seed viability at Site 2. In order to test whether there was a site-specific factor that had an important effect on viability, some sachets were transferred to this site in April 2011 and retrieved 12 months later in April 2012. This seed had similar viability to seed that remained in the site (Fig. 5). The decline in viability at this site is comparable with the loss of seed viability at Site 2 at shallow depth (Section 5.4).

There was a delay in the decline of viability of lab-stored seed in comparison with field-buried seed. There has been a decline in viability of approximately 10% over five years. Over the same period there has been a decline in viability of field-stored seed of approximately 50%.

6. Soil water repellency at seed burial trial sites

Jane Prider and Andrew Craig

Branched Broomrape Eradication Program

September 2012

Introduction

Soil water repellency is a major problem on the sandy soils in southern Australia (King 1981, Cann 2000). Repellence can be caused by hydrophobic organic substances including fungal hyphae (Bond and Harris 1964), humic acids and decomposing litter (King 1981).Water repellence slows the rate of infiltration of water into the soil profile. Repellent soils may remain dry even after large rainfall events and areas of lower repellence in the soil profile create bands of varying moisture (King 1981).

Soil water repellency can result in poor germination of crops and pastures but may also affect seed bank persistence. Aging of seeds in controlled environments and whilst buried in soil seed banks is influenced by temperature and moisture content (Walters 1998, Long et al. 2008, Long et al. 2009). Drier and cooler conditions promote seed persistence whereas loss of seed vigour is more rapid under warmer and wetter conditions (Long et al. 2009). Poor infiltration of water into the soil profile, particularly during the cooler months, could therefore increase seed bank persistence.

Persistence of buried *Orobanche ramosa* seed varies with depth and among three field sites in the Quarantine Area. Although soil type differences may partly explain these results, Long et al (2009) found that soil type had no effect on seed persistence if soil moisture and temperature were controlled. The mechanism resulting in broad variation in viability loss at our sites is not known but differences in the infiltration of water after rainfall events could be one explanation. If this is important, further understanding of how soil water repellence varies across the distribution of *O. ramosa* will enable us to determine which sites are likely to support persistent *O. ramosa* soil seed banks.

Aims

This study relates to the Research Project objective;

Determine the rate of broomrape seed decay in the soil and factors that affect the rate of seed bank decline.

We have observed a slower rate of viability loss for seed buried at the Mannum Trial site (Site 1, MTS) than the Glen Burr Rd site (Site 2, GBR) or Haby Hut Rd site (HHR). This study will test the hypothesis that there is greater soil water repellence at MTS than the GBR or HHR sites.

Methods

Soil was collected from depth increments of 2.5 cm from each of the seed burial sites (total 60 samples):

- 0 – 2.5 cm
- 2.5 – 5 cm
- 5 – 7.5 cm
- 7.5 – 10 cm

Approximately 250 gm of soil was collected from each depth at five locations at each of the sites. Samples were oven-dried at 45 °C for 24 hours and then lightly passed through a 1mm sieve.

There are three lab methods to determine relative soil water repellence. Samples were divided into three parts and one test applied to each part.

A) Water droplet penetration time (WD)

   Soil was placed to 1 cm depth in a 9 cm glass petri dish. 10 X droplets were added one at a time to the soil surface and the time taken for the droplet to enter the soil was recorded. The mean time for the ten droplets provided a single value for that sample.

B) Aqueous ethanol droplet penetration time (MED)

   Ethanol solutions were prepared at concentrations of 0.2 M intervals from 0 – 5 M. Soil was placed to 1 cm depth in a 9 cm petri dish. Droplets of 40 µl of the ethanol solution were added to the soil surface. The repellence of the soil was represented by the molarity of ethanol that penetrated the soil in 10 secs. This method is useful in a very repellent soil where the penetration of a water droplet is too slow (i.e. longer than 4 minutes).

C) Infiltration rate (SRI)

   Soil was placed in a 10 cm glass funnel end stoppered with filter paper. A 25 mm X 30 mm deep ring of PVC was imbedded in the soil to 5 mm depth. 5 ml of water was added to the ring (100 mm depth of water) and the time taken for the water to completely enter the ring was recorded.

These tests were conducted in the lab at a temperature of 20 °C. Values were compared to King's values (1981) who classified soils using these methods from non-significant soil water repellence to very severe.

Results

The infiltration technique was the only method that enabled us to make measurements across the range of depths and sites sampled (Fig. 1). The water droplet method was the easiest to apply but as penetration of water was rapid in the MTS top soil layer a water droplet penetrated in less than 1 second so no measure could be recorded (Fig. 2). However, the infiltration method was very slow in the more water-repellent GBR soil collected from 0-2.5 cm. The ethanol method was useful for the more water-repellent soils (Fig. 3).

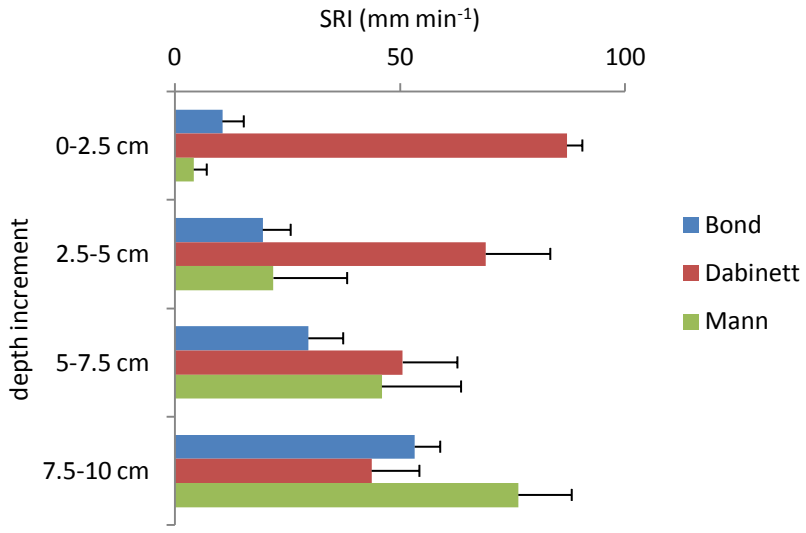

Figure 1. Infiltration of water in soils collected from the three seed burial sites using the infiltration method. Bars are means + 1SE, *n* = 5.

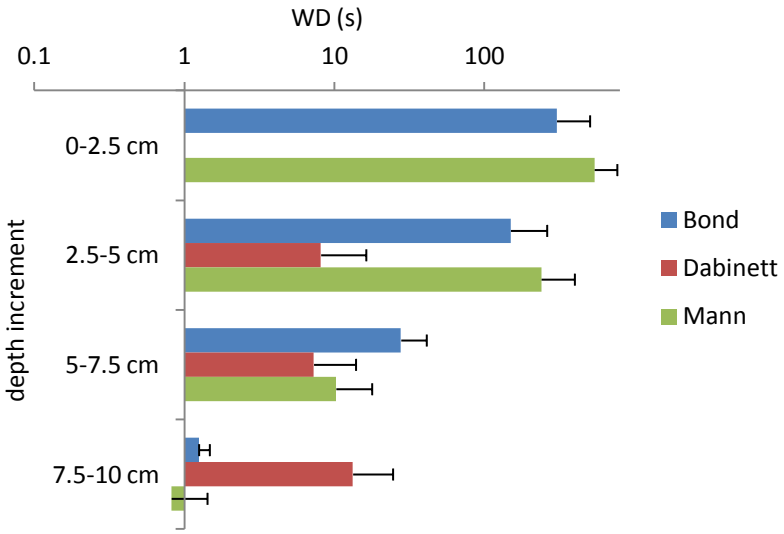

Figure 2. Time taken for a droplet of water to penetrate into soil collected from the three seed burial sites using the water droplet method. Bars are means + 1SE, *n* = 5.

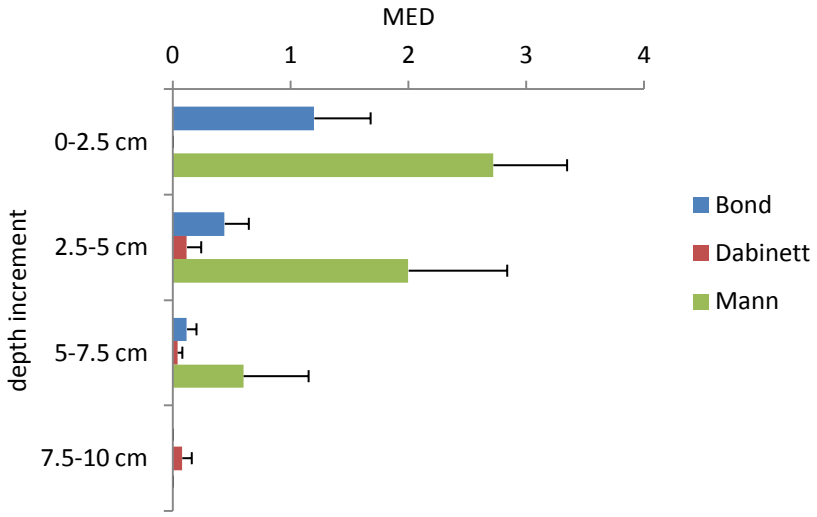

Figure 3. Molarity of a droplet of ethanol solution that penetrates after 10 seconds into soil collected from the three seed burial sites using the aqueous ethanol droplet method. Bars are means + 1SE, *n* = 5.

Contrary to expectations based on the results of our seed burial trials, the most water-repellent soil was the soil from GBR and the least repellent was from MTS.

Soil from the MTS site was more water-repellent at deeper layers than the top 0-2.5 cm but in the HHR and GBR soils there was trend for repellency to decrease down the soil profile. This pattern was most clearly seen using the infiltration method.

According to King's (1981) results, the soil from 0-5 cm depth at GBR has moderate-severe water repellency. Soil from 5- 10 cm has low water repellency at this site. The soil from the HHR site approaches moderate repellency at depths of 7.5 – 10 cm and has low repellence at shallower depths. The soil from MTS has only very low or insignificant soil water repellency.

Discussion

Although we expected that the soils from MTS would be the most water repellent as seeds had lost very little viability at this site, we found that the opposite was the case. Soils from depths where seed had been buried (2.5 and 5 cm) had very low or no repellency.

The HHR and particularly the GBR sites occur on dune tops where the soil is very sandy. It is therefore not unexpected that these soils would n be more water-repellent than the soil from the more loamy soils at MTS.

All sites have a low litter content so it feasible that the presence of soil microfungi may be responsible for the production of the hydrophobic substances within the soil profile. The deeper parts of the soil profile may provide more suitable conditions for the development of fungal communities, resulting in the increase in repellency with depth at the HHR and GBR sites.

The influence of soil microbial communities on seed bank mortality is the focus of a separate study (Section 5.11).

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1. Longevity of lab stored seed

Jane Prider and Andrew Craig

Biosecurity SA

December 2013

Summary

Seed stored in the laboratory under uncontrolled temperature conditions retains full viability for at least 7-8 years. After this time there is a rapid decline in viability such that no seed germinates and viability is less than 10% after eleven years.

Introduction

Orobanche seed is desiccation tolerant; longevity increases with a decrease in seed moisture content (Kebreab and Murdoch 1999). Reports on *Orobanche* seed survival in storage and during burial in soil differ widely. Some of these differences can be attributed to temperature and humidity conditions, variability between seed lots and to intrinsic differences in seed longevity among *Orobanche* species (Kebreab and Murdoch 1999). Linke and Saxena (1991) report an average seed longevity of 15 years for *Orobanche* seed in dry storage but for some species it was little as 3- 5 years (Linke and Saxena 1991, cited by Parker and Riches 1993). Models of longevity predictions for *Orobanche* predicted that at storage temperatures of 5 °C and a relative humidity of 50% *O. crenata* seed would only lose 5% viability after 21 years (Kebreab and Murdoch 1999). However, *O. cernua* seed in India lost viability after 6 years storage at 10 °C (Krishnamurthy et al. 1987) and *O. crenata* seed was predicted to have approximately 10% viability after 12 years storage at room temperature (Lopez-Granados and Garcia-Torres 1999).

There appears to be little clarity and consistency about predictions of seed longevity of *Orobanche* in the published literature. Collections of broomrape seed from South Australia almost annually since 1999 enabled an assessment to be made of seed longevity of *Orobanche ramosa* subsp. *mutelii* under storage. This information was important for assessing the quality of seed stored for research projects and was also useful for comparisons with soil-burial experiments.

Methods

Fruiting stems were collected from the field each year in October to December. Seeds were removed from seed capsules after collection by dry sieving and stored in air-tight glass Schott bottles. The bottles were stored in the laboratory in the light where the ambient temperature typically ranged from 15 – 25 °C.

Trial 1

In June 2013 we tested individual seed lots for each year from 1999 – 2012, with the exception of 2000 when no collection was made. We used seed collected from the Mannum Trial Site when possible but seed from other sites was also used.

Seed was initially sterilized by immersing in a 1% sodium hypochlorite solution followed by rinsing in RO water. After drying, 100-200 seeds were placed on 2 cm GF/A filter paper in a 5 cm petri dish, 200 µl of water added, and the dish sealed with parafilm. Three replicates were prepared for each seed lot. The dishes were incubated at 20 °C for 14 days to condition seeds. Dishes were then dried before 200 µl of a 1 ppm GR24 solution was added to the filter paper. The resealed dishes were incubated at 20 °C for a further 14 days and then germination was scored. To test whether the ungerminated seeds were viable, they were placed in 1.5 ml eppendorf tubes with approximately 0.5 ml of 1% 2,3,5-triphenyltetrazolium

chloride (TZ) solution. Prepared tubes were wrapped in aluminium foil to exclude light and incubated at 30 °C for 14 days. Seeds were scored viable if they were stained red.

Trial 2

In the second trail in July 2013 we tested seed from multiple seed lots collected from different sites the same year. Collection years were selected from the results of Trial 1, to either confirm these results or to test further points on the curve of interest.

Replicates were prepared as described above.

Analysis

We calculated the proportion of viable seed as the sum of the germinated and viable seed divided by the total number of seeds in the test. The data for each trial were fitted to three-parameter logistic models using the software package drc (Ritz and Streibig 2005) in the R platform (version 3.0.2, R Core Team 2013). The model was used to estimate the number of years for the proportion of viable seeds to decline by 10, 50 and 90%.

Results

Trial 1

The seed lot collected in 2012 had poor viability. However, for other seed lots there was no loss of viability over the first 7 years of storage. A logisitic model fitted to the data estimated that 10% viability loss would occur after 7.4 ± 0.4 years, 50% viability loss after 9.1 ± 0.2 years and 90% viability loss after 11.4 ± 0.5 years (Fig. 1A). Very few seeds were viable from the 1999 seed lot, or after 14 years (0.4%).

Seed stored for 11 years or more failed to germinate (Fig. 2) although a low proportion of seed was still viable (Fig. 1A). The 2012 seed lot had poor germination and the seed lot from 2007 had variable germination.

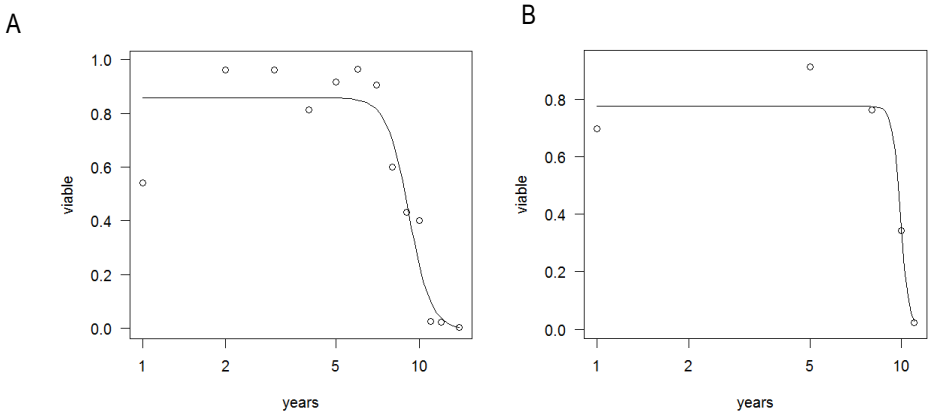

Figure 1. Loss of viability of lab stored seed. A three-parameter logistic model has been fitted to the data for A) Trial 1, and B) Trial 2.

Trial 2

Trial 2 with more seeds lots confirmed the results of Trial 1. There was minor loss of viability in the seed lots stored for 8 years. Fewer data points around the critical time of viability loss at about this time, increased the estimate for 10 % of viability loss to 9.3 ± 0.6 years. The estimate for 50% viability loss was 9.9 ± 0.1 years and 90% loss, 10.6 ± 0.5 years (Fig. 1B).

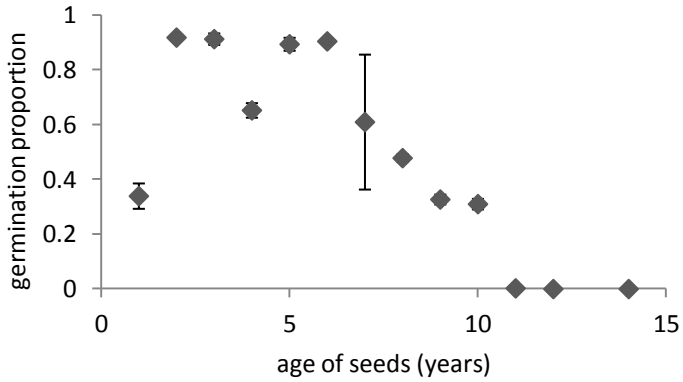

Figure 2. Proportion of germinating seeds of increasing age under laboratory storage. Each point is mean ± 1 SE, *n* = 3 for Trial 1.

Discussion

The results of our trial suggest that seed retains high viability under regular storage in the laboratory for approximately 7-8 years. After this time there is rapid loss in viability and seed more than 10 years old is likely to have a low proportion of viable seeds and very poor germination.

These data contradict some of the other published findings although the storage and experimental conditions details of many of these studies is not known. Gold et al (1978) reported that *O. ramosa* seed survived for 20 years in laboratory storage but the proportion of seeds surviving for this period is not known. Linke and Saxena 1991 (cited by Press and Riches 1993) found that *O. crenata* and *O. aegyptiaca* seed remained viable for 8 – 10 years under laboratory storage, a similar finding to our study.

Results from our seed burial experiments indicate that viability loss occurs after about 3 years, with approximately 40% seed remaining viable after 5 years (Section 5.5). A longer term study has found that a small proportion of seed remains viable after 10 years at shallow depths (Section 5.4). Model predictions estimate that seed persistence may be as long as 30 years at shallow depth, considerably longer than lab-stored seed. However model predictions may over-estimate seed longevity as our model for lab-stored seed found a rapid loss of viability after 9 years.

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2. Vertical distribution of the branched broomrape seed bank

Jane Prider and Andrew Craig

Branched Broomrape Eradication Program

June 2011

Summary

The eradication program has limited data on the vertical distribution of the branched broomrape seed bank. This information is required to determine what proportion of seed occurs at depths where loss of viability is likely to be faster and the proportion of seed that remains near the soil surface where there is a greater risk of dispersal by natural vectors. We sampled the seed bank at depth increments of 0 - 2.5 cm, 2.5 - 5cm, 5 - 10 cm and 10 - 15 cm at a fallow and cropped area at the Brinkley and Mannum trial sites and at a pasture site at Bowhill. Seed numbers were estimated with a DNA bioassay. There was a ten-fold difference in seed bank size between the Brinkley crop site and the Bowhill pasture site (both ~ 10 seeds kg^{-1} soil) and the seed bank at both Mannum sites (~200 seeds kg^{-1} soil). There was another ten-fold difference in seed bank size between the Mannum sites and the Brinkley fallow site (~1,000 seeds kg^{-1} soil). Very few seeds occurred in the depth interval 10 - 15 cm at all sites. In the cropped sites there was a relatively even distribution of seeds throughout the soil profile to depths of 10 cm. In the fallow sites there was a concentration of seeds in the top 2.5 cm. Most of this seed may have been released the previous season as it was only at the fallow sites that senescent broomrape plants were observed in the sampled plots. The exponential shape of the depth distribution curves for fallow sites indicates that not all seed released becomes buried and there is a considerable proportion of seed that is lost from the top soil layer. In comparison with data collected in other years, it appears that the broomrape seed bank at the Mannum site is declining over time in the absence of targeted control measures. Repeated sampling at the Brinkley site demonstrates the spatial and temporal variation inherent in sampling the broomrape seed bank.

Introduction

The vertical distribution of seeds in the soil profile is dependent on activities that disturb or mix the soil profile, including tillage (Starica et al. 1990) or burial by earthworms or other soil animals (Bekker et al. 1998). In the absence of soil disturbance, seed characteristics and the soil matrix can have a significant effect on seed burial. Smaller, more spherical seeds and lighter textured soils favour the natural vertical movement of seeds (Bekker et al. 1998, Benvenuti 2007).

Studies on the vertical distribution of the *Orobanche* seed bank are lacking although there are a limited number of studies on *Striga* species, which have similar size seeds. These studies revealed a very high concentration of seeds in at least the upper 15 cm of the soil profile (Robinson and Kust 1962, Van Delft et al. 1997) and differences in the seed depth profile following tillage and on different soil types (Van Delft et al. 1997). Modelling studies suggest that where tillage buries *Orobanche* seed below 15 cm it is likely to lose viability more slowly (Grenz et al. 2005).

Our seed burial experiment has shown that seed loses viability at a faster rate when buried at 10 cm depth than at 5 cm. Very slow rates of viability loss have occurred at the Mannum trial site at burial depths of 2 cm. Samples from different depths have been collected from the seed bank at the Mannum field site. No seeds were detected in samples from the surface to 2.5 cm depth with the highest concentration of seeds

at 2.5 to 5 cm and at 7.5 to 10 cm. These data have limited usefulness as no seeds were detected in many of the samples. Increasing the sample size could improve the precision of these estimates. Seeds that remain on or close to the soil surface pose a greater risk for dispersal and loss of viability is slow. Cultivation could bury seed to depths where viability loss may occur. Predictions of seed viability loss may be different for arable and non-arable sites if the distribution of seeds in the profile differs. This study will also enable us to estimate the size of the seed bank, or the detectability of the seed bank, in order to assess risk of broomrape spread.

Aims

The objective of this project is to determine the vertical distribution of broomrape seeds with depth in the soil profile in cropped and fallow paddocks.

The outcomes from this project will enable the eradication program to:

- Evaluate seed bank risk by quantifying the proportion of seed that is buried to depths where loss of viability occurs at faster rates and the proportion of seed that remains near the surface where it is more susceptible to spread
- Evaluate seed bank risk by estimating the size of the seed bank or the ability to detect seeds in the seed bank

Methods

A number of sites were examined for suitability for collection of samples. At inspection time, with the exception of the non-arable parts of the trial sites, there was no broomrape visible. We selected one site where no broomrape plants were visible and collected for the two trial sites at Brinkley and Mannum. At the trial sites we collected from cropped and fallow areas. Each site had a different management history (Table **11**). Although a comparison of seed bank depth between cropped and fallow sites was planned, the paucity of seeds sampled from sites where broomrape was not observed, limited the study largely to the trial sites.

Table 11. Management history of sample collection sites

Site	Management	Broomrape detected	Broomrape in sampled plot	Site features
Mannum - crop	Regular cropping to 5-10 cm 2002-2009, fallow 2010, but herbicide applied and slashed prior to sampling	Annual 2002-2010	0	Sandy loam above a clay layer at 10 cm depth
Brinkley - crop	Regular cropping 2002-2007?, Fallow 2008?, Cropped 2009,2010	2008,2009,2010	0	Sandy clay – limestone at 10 cm depth
Bowhill – pasture	Fallow 2010	2003, 2006	0	Deep sand at top of dune, perennial veldt grass pasture, grazed
Mannum - fallow	Fallow 2002-2010	Annual 2002-2010	5	Sandy loam, summer weeds present
Brinkley-fallow	Regular cropping 2002-2007?, Fallow 2009,2010	2008,2009,2010	45	Sandy clay – limestone at 10 cm depth, summer weeds present

At each site a 4 m by 4 m plot was placed in the broomrape infected area. The plot was divided into a 1 m by 1 m grid. The corners of the grid formed the upper left corner of each of our sampling areas, for a total of 15 samples collected in each plot. Each sampling area comprised a 20 cm by 20 cm core. A metal

frame was pushed or hammered into the soil to 15 cm deep. Soil was collected from the core with a trowel in depth increments of 0-2.5 cm, 2.5- 5 cm, 5-10 cm, and 10-15 cm. Soil from each layer was thoroughly mixed and passed through a 1 mm sieve. Approximately 3-400 g of soil was transferred into a plastic bag and the remainder discarded. Soil samples were sent to the SARDI Molecular Diagnostics Laboratory for broomrape DNA detection. Samples were collected from 15 March to 7 April 2011.

Analysis

The DNA analysis results give an estimate of seed numbers per 200 g soil sample. This was converted to the number of seeds per square metre (to a depth of 1 cm) by assuming that soil density was 1.4 g cm^{-3}. Results are also expressed as numbers of seeds kg^{-1}.

Curves were fitted to the data for seed distribution with depth using a logistic three –parameter model. Model fits were tested against a linear model using ANOVA and all non-linear models gave a better fit to the data than a linear model.

Results

Broomrape seed was detected at all sites although plants from last season were not present in all sampling areas. We sampled the largest number of seeds from the fallow site at Brinkley which supported the highest density of plants (

Figure 8). The cropped site at Brinkley, although adjacent to the fallow site and within the area where high densities of broomrape were detected in 2008, had much lower numbers of seeds. Seeds detected at this site were similar in number to those detected at the pasture site at Bowhill, where no plants were observed. Total seed numbers at Mannum were similar for fallow and cropped areas and are indicative of a large residual broomrape seed bank at this site. Seed numbers for other soil measurement units are in Appendix A.

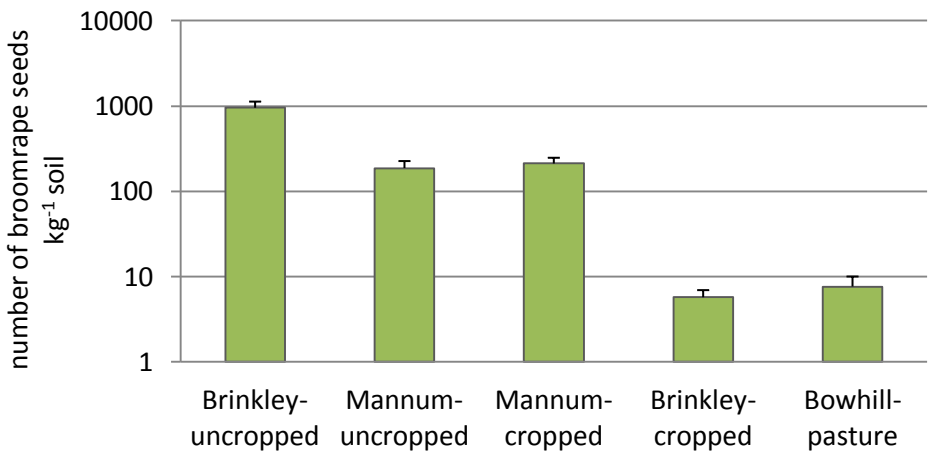
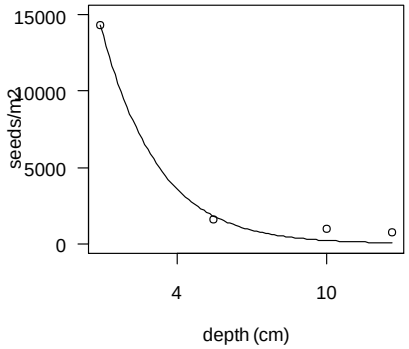
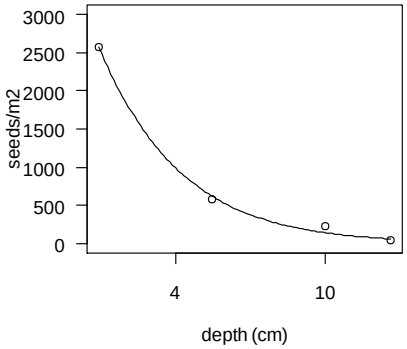


Figure 8. Total broomrape seeds kg^{-1} estimated from soil samples collected to a depth of 15 cm. Note that the y-axis scale is logarithmic. Bars are mean + 1SE (n = 15).

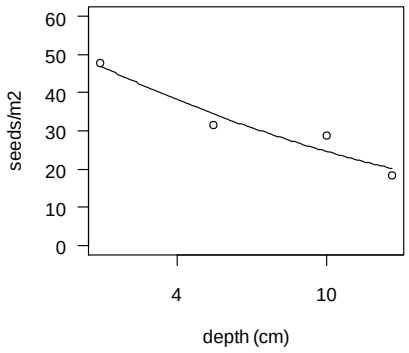
The shape of the curves for the vertical seed distributions differed between sites (Figure 9). For the two fallow sites, where senescent broomrape plants were observed, there were high numbers of seeds detected at shallow depths (0 – 2.5 cm) with a steep exponential decline in seed numbers down the soil profile. In the cropped sites, there was no concentration of seeds on the soil surface. At the Brinkley site there was a gradual decline in detected seeds down the soil profile. At the Mannum site seeds were relatively evenly distributed in the top 10 cm of the soil profile and declined such that few seeds were found at 15 cm depth. A similar pattern occurred at the Bowhill site, although the decline was at less than 10 cm.



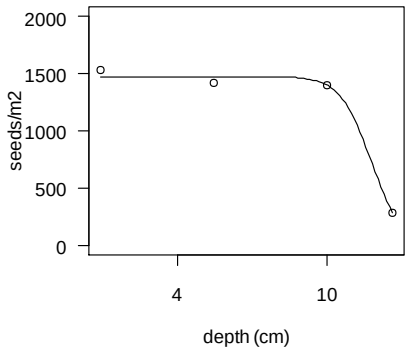
Brinkley - fallow



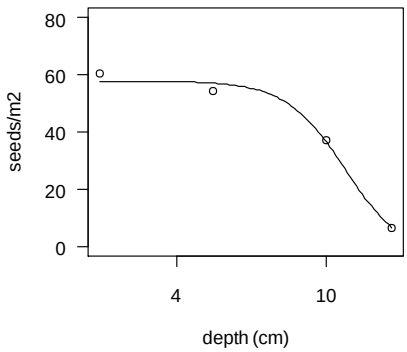
Mannum - fallow



Brinkley - cropped



Mannum - cropped



Bowhill - pasture

Figure 9. Three –parameter logistic curves fitted to seed numbers (per square metre) for each site. Note that the y axis scale differs for each plot. Points represent the mean for each depth increment (*n* = 15).

The proportion of seeds occurring at each depth interval differed according to the management of the site. The fallow sites had shallow seed banks, with approximately 80% of the sampled seeds occurring within 2.5 cm of the soil surface (Figure 3). The distribution of seeds through the soil profile was more uniform in the cropped site than the pasture site. Sampling revealed a low proportion of seeds at the 10 -15 cm depth increment at all sites, therefore it is expected that few seeds occur below 15 cm and we sampled the majority of the profile where seeds could be expected.

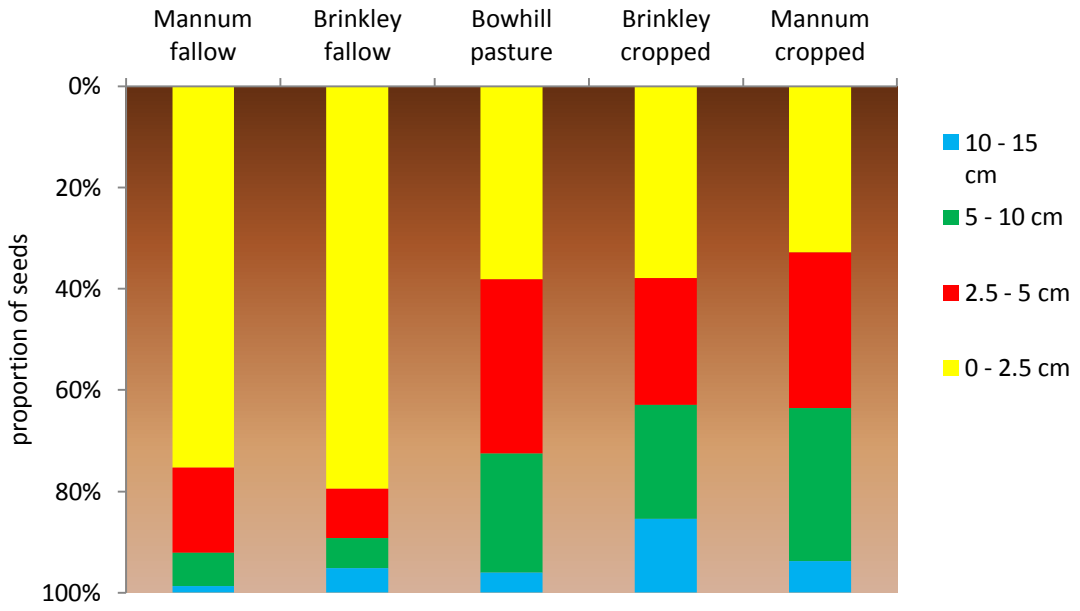


Figure 10. Proportions of seeds sampled with depth down the soil profile, based on estimates of seed number m^{-2} (*n* = 15).

Discussion

The temporal and spatial variability associated with broomrape seed bank sampling makes it difficult to quantify or to assess changes in seed bank size over time. For this study we used large sampling cores and found broomrape seeds at all sites although there was high variation between sites. Sites that were expected to yield large seed banks such as the cropped site at Brinkley had very few seeds. Conversely, there were high seed numbers in the cropped area at the Mannum trial site where large broomrape populations have not occurred for several years. This provides evidence for seed bank persistence.

Samples from the Mannum trial site have varied within and among years. Across all sampling occasions the mean number of seeds detected has been 364 kg^{-1}, but has ranged from 5 to 654 seeds kg^{-1}. Our measure this year of 254 seeds kg^{-1} is within this range but less than the overall average, however this could be influenced by the sampling method used and ignores spatial variation across the site. Previous sampling at this site has used a variety of methods but our core size is closest to the linear sampling method which in 2006 estimated a seed bank size of approximately 600 seeds kg^{-1}. We can tentatively assume there has been some natural decline in the seed bank over time at this site.

Sampling of the Brinkley Trial Site seed bank in 2009 gave estimates of 20-30 seeds kg^{-1}. This was much lower than expected given the high density broomrape population observed in 2008. For this season, even

ignoring recent seed input in the 0 - 2.5 depth increment, we recorded 790 seeds kg^{-1}. At a population density of 5 plants m^{-2} as measured in our sampling area, and assuming 11,000 seeds per plant, we should expect to sample 380 seeds kg^{-1}. Our estimates of greater than 1,000 seeds kg^{-1} indicated a residual seed bank from previous years, and errors possibly due to spatial variation in our estimates for 2009.

It appears that cultivation results in a uniform distribution of seed through the soil profile. However the lack of visible senescent plants in cropped sites prevented the selection of sampling sites where there was a known seed input immediately prior to sampling, unlike the fallow sites. Without recent seed input, there is an even distribution of seeds to depths of 10 cm in cropped sites. Newly released seed appears to remain on or near the soil surface for at least the first few months after release. Seeds on the soil surface would be a risk for dispersal by wind and this may already have removed seeds at the time of sampling. Surface cover is important for reducing wind dispersal (Ginman 2009) and the high weed cover in fallow plots may have helped concentrate seeds on the soil surface this season. Maintaining vegetative cover until the following growing season would therefore be important for reducing seed dispersal and cultivation may also reduce the risk of wind dispersal of newly released seed by burying seed.

The only study that has examined natural burial of *Orobanche* seeds found that seed moved 9.4 mm in a sandy soil in 5 months but did not move any further during the following year (Benvenuti 2007). Our results suggest that cultivation or other forms of disturbance have contributed to burial of broomrape seeds. Seeds collected to within 2.5 cm of the soil surface at the fallow sites may represent more than the current season's seed input. Although it is assumed that very small seeds such as those of *Striga* and *Orobanche* may be readily buried, we found very few seeds at depths between 10-15 cm. Robinson and Kust (1962), who examined soil layers to depths of 60 inches, found *Striga* seeds at that depth although most seeds were concentrated on the soil surface. Site conditions may prevent or slow the vertical movement of seeds at our sites. A solid rock layer was encountered at the Brinkley site at depths of 15 cm and heavy clay layers below 10 cm would slow the downward movement of seeds at Mannum. Natural burial of *Striga* or *Orobanche* seeds is greater in sandy soil than clay soil (Van Delft et al. 1997, Benvenuti 2007). There have been observations of broomrape plants in the Quarantine Area at depths of 1 m (D. Miegel, pers. comm.) however these plants were observed in areas of sand drift and the seeds may not have been buried to this depth. It is believed that seeds that become deeply buried may remain viable due to the anoxic conditions that prevent decay by soil micro-organisms. In this case regular tillage may bring the seeds to the soil surface where they may encounter suitable conditions for germination.

Our seed burial experiments have found no evidence that broomrape seeds rapidly lose viability at shallow burial depths as has been found for *Striga* (Robinson and Kust 1962). At the Mann site on deep sand, viability loss has been more rapid at 10 cm depth than at 5 cm depth. At the Mannum site viability loss has not been significantly greater at 5 cm depth than at 2 cm depth. It would seem that burial at least greater than 5 cm is required for optimal loss of seed viability. Our data suggest that only a small proportion of seed is buried. The number of seeds at the Brinkley fallow site at depths greater than 2.5 cm is similar to the Mannum site where recent broomrape population density has not been observed as high as at the Brinkley site. The exponential shape of the depth distribution curves for fallow sites indicates that not all seed released becomes buried and there is a considerable proportion of seed that is lost from the top soil layers. There may be several fates for newly released seed; burial, dispersal, germination, predation or mortality. Thus viability loss after burial may account for only minor losses of seed.

Summary of findings

- Sites that have supported large populations of broomrape (e.g. Mannum crop and Brinkley fallow) do not have large broomrape seed banks as would be expected from the large seed inputs detected in the uppermost soil layers.
- Newly released seed remains in the uppermost soil layers for at least five months after release and poses a risk for dispersal by wind. Retention of vegetative cover would minimise the risk of dispersal by this vector.

- Cultivation results in a uniform distribution of broomrape seed through the soil profile and burial to depths where it may lose viability more rapidly.
- The absence of any control methods at the Mannum trial site indicates that there has been a natural decline in the broomrape seed bank.

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Appendix

Number of seeds (pooled over depth intervals)

Site	Seeds 200 g^{-1} soil	Seeds kg^{-1} soil	Seeds m^{-2} to 15 cm depth
Brinkley fallow	238	1144	160,191
Mannum fallow	47	225	31,464
Mannum cropped	53	254	35,571
Brinkley cropped	1	7	963
Frahn pasture	2	9	1,286

3. Seed bank decline under different land uses

Jane Prider and Andrew Craig

Branched Broomrape Eradication Program

January 2011

Introduction

In the absence of host plants and seed predators, our seed burial experiments have measured no decline in the *Orobanche ramosa* (broomrape) seed bank in some sites after 8 years (Section 5.4). Modelling based on these data estimate that in the absence of hosts, loss of seed viability could take from 12 - 25 years or more (Section 5.4). Although host denial management options can prevent broomrape emergence and the addition of further seeds to the seed bank, a long-lived seed bank increases the risk of paddocks returning broomrape infections when host control fails, even after a long period of time. A persistent seed bank remains one of the major obstacles to broomrape eradication.

Interactions of plant species with *Orobanche* can be classified into three distinct categories. Host plants stimulate parasitic seed germination, tubercle development and seed production; false-host plants stimulate parasitic seed germination without tubercle formation; and non-host plants do not stimulate parasitic seed germination or attachment (Lins et al. 2006). Amongst hosts and false hosts, plants differ in their susceptibility to *Orobanche* infection and their stimulation of germination (Ross et al. 2004, Qasem and Foy 2007, Fernandez-Aparicio et al. 2009). Therefore, different land uses are expected to have different impacts on the broomrape soil seed bank depending on the presence and identity of hosts, false-hosts or non-hosts.

Land use practices provide a means of reducing the seed bank in the absence of chemical use. Crops that promote the germination of broomrape seeds but provide resistance to broomrape infection and/or development to reproductive maturity offer some scope for seed bank reduction. These so called trap crops have been used for the control of broomrape overseas, usually on a crop rotation basis (Linke et al. 1991, Kleifeld et al. 1994, Schnell et al. 1994). Catch crops comprise hosts that must be harvested prior to maturity as broomrape is able reach reproductive maturity on these hosts (Acharya et al. 2002). Catch and trap crops are not likely to be a tool for eradication of broomrape but such crops may be good choices for reducing the broomrape seed bank. Knowledge of the relative impacts of different land uses on the soil seed bank would enable landholders to weigh up the risks and benefits of different cropping options. This may also be useful for evaluating the progress of a paddock towards eradication, by providing estimates of seed bank decline based on paddock history.

The goal of this series of experiments is to assess the impact of land use on branched broomrape soil seed bank decline by:

- quantifying germination stimulation of different crop varieties;
- assessing the hosting ability of different crop varieties; and
- providing estimates of seed bank decline in the soil through glasshouse and field studies.

The outcomes from this project will enable the eradication program to evaluate the use of trap crops as a tool for controlling broomrape and estimate seed bank decline under different land uses for risk assessment purposes.

Methods

Germination

The ability of different hosts to stimulate broomrape germination was assessed using the polybag culture method in 2010. After bags were prepared (see Laboratory Protocol document), approximately 400-600 broomrape seeds were sprinkled on the lower part of the top half of the wet filter paper. Bags were suspended vertically in the dark for two weeks for seed to condition. Five seeds of each host type (see Table 1) were placed along the top of the filter paper and covered with a strip of wet filter paper to keep them in place. Bags were maintained under artificial light in the lab and kept moist by adding a half-strength nutrient solution. After approximately four weeks, when host roots had grown, bags were checked under a microscope for broomrape germination. Seeds that had germinated were marked on the outside of the bag so they were not counted twice. Germination counts were made weekly for the next three months. Bags were also monitored for the development of broomrape tubercles.

Pot trials

Drawdown of the broomrape soil seed bank was assessed by growing crop varieties in pots of broomrape seed-inoculated soil, destroying these hosts, and then replanting the pots with a known host as an indicator species. We measured broomrape infections on indicator hosts as a relative measure of the broomrape seed bank.

Pot trials were conducted in the glasshouse. We prepared 0.8 L pots by filling with a mixture of sterilised Burdett sand and 0.1 ml of broomrape seed. Pots were watered and left for two weeks for broomrape seed to condition before crop seeds were planted. We planted several seeds in each pot (depending on seed size) and later thinned to one plant per pot. Twenty replicate pots were prepared for each crop, including a control without a host. Host varieties differed slightly in each year of the experiment (Table 1).

Table 1. Land use or crops used in the trials and their suspected host type.

Land use or crop	Host type	Varieties trialled 2008	Varieties trialled 2009/10
Bare ground	Control		
Cretan weed	Host		
Canola		Surpass	Boomer TT
		Tanami	Clearfield 43C80
Brassica	Host	Winnifred Grazing	Winnifred Grazing
Mustard	Host		
Vetch	Host	Blanchefleur	Blanchefleur
Safflower	Host		
Medic	Host	Angel	Angel
		Herald	Herald
Turnip	Host	Purple Top	Purple Top
Barley	Non-host	Schooner	Schooner
		Maritime	Maritime
			Buloke
Oat	Non-host	Euro	Euro
		Wintaroo	Wintaroo
Wheat	Non-host	Frame	Brennan Red
		Peake	Gladius
		Yipti	Peake
			Yitpi

In 2008, host growth was terminated at 1000 GDD by cutting the host plants at soil level. One month later, four safflower seeds were sown in each pot, later thinned to one plant per pot. Safflower was grown for 1000 GDD and then the soil was washed from the roots. Broomrape tubercles were counted on safflower plants. Tubercles were observed on the old host roots in the pot so these were also counted.

In 2009, we terminated host growth at 1500 GDD by cutting host plants at the soil surface. For half of the pots, we washed the soil off the roots and counted broomrape tubercles. The other pots were sown in 2010 with Blanchefleur vetch. This provided sufficient time for the original hosts to die and more closely mimics what would happen in the field with a single crop each year. In 2010, broomrape tubercles were collected from vetch roots after 1500 GDD.

Field seed bank

Plots were prepared at the Mannum field site in 2008 and the Brinkley field site in 2009 to assess the drawdown of the natural seed bank. Crops failed in the 2008 year so that is not described further. In 2009, we selected a site at Brinkley that supported a dense population of broomrape plants in the previous year (up to 200 plants m^{-2}). We tested crop varieties based on the results of the 2008 pot trials.

Table 2. Treatments for field plots at Brinkley

Land use treatment	Crop variety	Seeding rate (kg ha^{-1})
Bare ground		
Weedy pasture		
Medic	Angel	20
Canola x1	Clearfield 43C80	5
Canola x2	Clearfield 43C80	5
	BoomerTT	5
Wheat	Gladius	60
Barley	Buloke	50
Oat + vetch	Wintaroo Oat	30
	Blanchefleur Vetch	30

We cultivated 9 treatment plots as listed in Table 2, 2 m by 15 m, in four blocks or replicates, with 1 m spacing between plots. In April 2009, we collected four replicate soil samples from each plot, each comprising 25, 10 cm deep soil cores from random locations within each plot. The number of broomrape seeds in a 200 g subsample of each soil sample was assessed using a DNA assay by the SARDI Molecular Diagnostics laboratory.

Early season weeds were sprayed with 1 L ha^{-1} glyphosate following the break of season rains in May. Crops were planted in each plot at the rates shown in Table 2. DAP fertiliser was applied at the rate of 60 kg ha^{-1} to the cereals and medic. The canola and safflower plots received DAP at 80 kg ha^{-1}. Pesticides were applied in July 2009 to control weeds and insect pests. An application of Intervix at 400 ml ha^{-1} was sprayed on canola. This destroyed the Boomer TT variety but not the Clearfield variety. The bare soil plots were kept clear by spraying with glyphosate. The weedy pasture treatments remained untreated.

At 1000 GDD, ten plants were collected from each plot and we counted the number of broomrape tubercles under a microscope. All hosts were destroyed in plots by spraying with glyphosate on 30th September (approximately 1250 GDD).

Further soil samples were collected one month later from the same locations as the previous soil cores. Samples of 200 g of soil from the bare ground, oat + vetch and barley plot were assessed for broomrape seed by DNA assay. In 2010, the four replicate soil samples from each plot were placed in 0.4 L pots and four Blanchefleur vetch seeds were planted, later thinned to one plant per pot. These plants were

harvested at 1500 GDD, the soil washed from roots and broomrape tubercles counted under the microscope.

Data analysis

Where data analysis was considered to be warranted, a non-parametric Kruskal Wallis (KW) test was used to test if there was a difference between crop types. There is no assumption of a normal distribution of values for this test although variances must be homogeneous. Some variables were square-root transformed to meet these assumptions. To test which of the crops differed, *post hoc* pairwise Behrens-Fisher tests were used. We used R 2.12.1 software for all analyses (R Development Core Team 2010). Tests were considered significant at $\alpha < 0.05$.

Results

Germination

The tested crops differed significantly in their stimulation of broomrape germination (KW, $\chi^2 = 26.69$, $p < 0.001$). There were three broad groupings of crops according to their stimulatory activity. The brassicas and vetch formed one group with high broomrape germination stimulation, the wheat varieties Peake, Brennan Red and Yitpi had low germination stimulation and the oat and barley cereals Euro, Wintaroo, Maritime and Schooner had some minor activity (Table 3). Few seeds germinated with safflower but this host had fungal infections in the polybags. There was poor germination of cretan weed, Herald and Angel medic in polybags so no data could be collected for those plants. Germinated broomrape seeds were found amongst roots of Gladius wheat and Buloke barley in pot experiments but not in the polybags. Thus, all the cereals are false hosts and not non-hosts (see Table 1) as they are able to stimulate broomrape germination but no haustorial attachment occurs.

Table 3. Percentage germination of broomrape seed grown in polybags with crops. For germination values >1%, values labelled with a different letter are significantly different (Behrens-Fisher test $\alpha < 0.05$). The dashed lines separate groups according to their germination stimulatory activity.

Crop variety	Germination % (mean ± 1 SE)
Canola - Clearfield 43C80	51.02 ± 10.12[a]
Canola - Boomer TT	47.00 ± 7.66[a]
Brassica - Winfred Grazing	35.41 ± 11.46[a]
Turnip - Purple Top	32.70 ± 12.29[a]
Vetch - Blanchefleur	25.61 ± 7.52[a]
Mustard	16.77 ± 4.61[ab]
Wheat - Peake	7.32 ± 0.82[b]
Wheat - Yitpi	5.44 ± 3.76[bc]
Wheat - Brennan Red	1.17 ± 0.91[c]
Safflower	0.64 ± 0.45
Oat - Euro	0.32 ± 0.09
Oat - Wintaroo	0.10 ± 0.10
Barley - Maritime	0.08 ± 0.08
Barley - Schooner	0.05 ± 0.05
Barley - Buloke	0 ± 0
Wheat - Gladius	0 ± 0
Control (no host)	0 ± 0

Pot trials

Broomrape infection in pots was very poor in 2008. Very few safflower plants became infected and where the original crop in the pot had not hosted any broomrape there was no later infection of safflower (Fig 1). Of the original crops, cretan weed and vetch had the most broomrape tubercles but there was no indication that there was a drawdown of the seed bank as safflower in these pots also had high infection.

Infection of the original crops was improved in the following year when the experiment was repeated (Fig 2). Some of the cereals; Wintaroo and Euro Oat, Brennan Red Wheat, had early stage attachments of broomrape. Although the radicle had adhered to the root this did not swell, indicating that no penetration of host tissue had occurred and no haustoria formed. Germinated broomrape seeds were observed amongst the roots of many of the cereal varieties.

There was very good infection of vetch by broomrape in the following year but the number of tubercles did not differ between original host types (Fig 2) (KW $\chi^2 = 18.69$ $p = 0.48$). Pots that grew the most susceptible original host, cretan weed, had the most broomrape tubercles the following year.

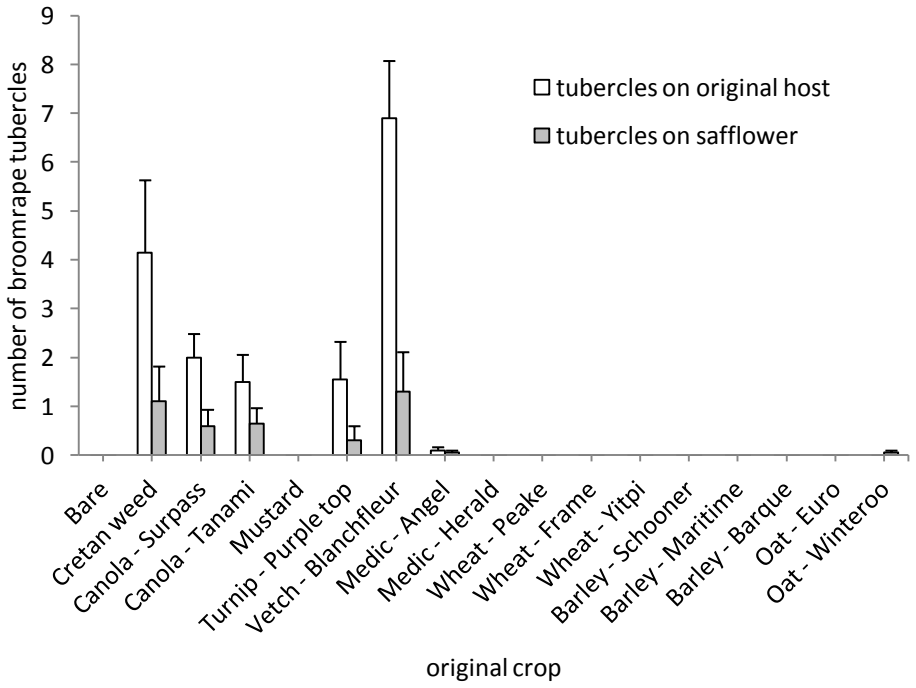

Figure 1. Broomrape tubercles retrieved from roots of original crops and after from the roots of safflower transplanted into the same pots in 2008.

Field Seed bank

Before crops were planted at the Brinkley field site, the average number of broomrape seeds per 200 g soil was 3.86 ± 0.34 ($n = 144$). The seeds were patchily distributed throughout the trial site and many of the plots had no seeds. This is much less than the expected number of seeds given the high density of emerged broomrape observed at the site the previous year. At an estimated broomrape population density of 200 m^{-2}, at least some samples were expected to have up to 3,000 seeds 200 g^{-1} soil, even with only 1 plant m^{-2} there should be 15 seeds 200 g^{-1} soil. This is based on seed production of 11,000 seeds $plant^{-1}$ and a soil bulk density of 1.44 g. Our measured seed densities give an estimate of about 1 plant 5 m^{-2} and this is only taking into account seeds that may have been released the previous year. For

the post-treatment seed collections, we restricted seed collection to three land use types and collected from the bare, barley and oat + vetch plots. There was no change in the number of seeds sampled from the seed bank or differences between the land use types (Fig 3).

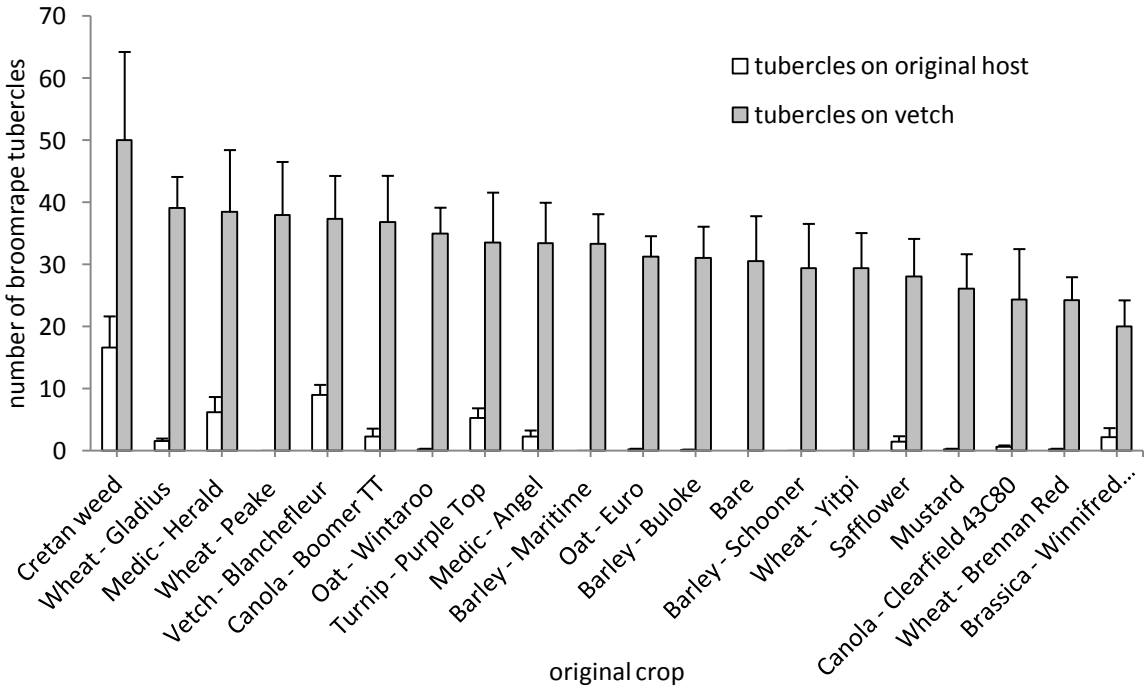

Figure 2. Broomrape tubercles retrieved from roots of original crops in 2009 and after from the roots of Blanchefleur vetch transplanted into the same pots in 2010.

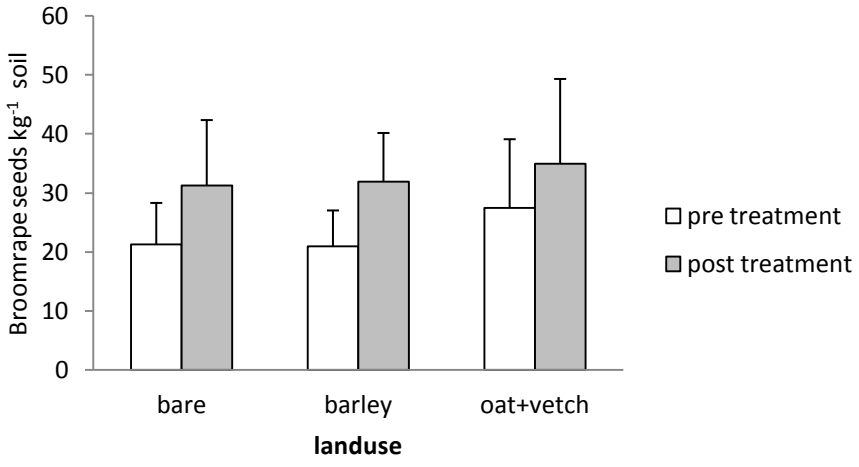

Figure 3. Broomrape seed in soil from plots before crop planting and following destruction of the crop (Mean ± 1 SE, *n* = 4)

Only one broomrape tubercle was found on a vetch plant in the field samples.

For vetch transplanted into soil collected from the field plots, very few plants were infected by broomrape. There was not sufficient broomrape infection to make meaningful treatment comparisons.

Discussion

We have demonstrated that of the plants tested, which are common crops in the Quarantine Area, all have some stimulation of broomrape germination. There is potentially some drawdown of the broomrape seed bank by germination where there is plant cover present and broomrape emergence is prevented. Studies overseas have suggested that many cereals could be suitable trap crops for weedy *Orobanche* species (Ross et al. 2004, Lins et al. 2006, Fernandez-Aparicio et al. 2009). This is mostly based on laboratory germination assays and the effectiveness in the field may not be as great. The germination percentages that we recorded from the polybags cannot be used for absolute estimates of seed bank decline as seeds in polybags are in close proximity to the host roots, which would not occur in soils. López-Granados and Garcia-Torres (1993) estimated that approximately 30-40% of *O. crenata* seeds in the top 20 cm arable layer germinate in the presence of cultivated faba bean hosts. This figure was based on measures of 50% germination of field –buried seed after artificial stimulation of viable seeds so is likely to be an over-estimate. More reliable data comes from a study by Acharya *et al.* (2002). They estimated *O. aegyptiaca* seed bank drawdown maxima of from 20-30% in *Brassica campestris* crops, compared to a background decline under no cropping of 5%.

Our measures of seed bank drawdown under natural conditions for different land uses at Brinkley provided no evidence of a decline in the seed bank. The estimates of seed bank numbers prior to crop planting were very much lower than would be expected given the population of broomrape observed at the site the previous year. The seed bank had not declined as although in 2009 there were very few broomrape observed at the site, in 2010 there was a high density population present. It is most likely that due to the very uneven distribution of broomrape seed in the soil profile, the soil coring is not providing an accurate estimate of seed numbers.

In a study of broomrape seed bank density in faba bean fields, seed densities ranged from 800 to 4,000 seed 200 g^{-1} soil where the number of emerged plants was 11 – 45 m^{-2} (López-Granados and Garcia-Torres 1993) These data are variable with many zero values but the estimates of seed bank density are within our estimates for what should have been detected but not what was detected. Measures of *Striga* seed bank density show similar spatial variation with no relationship between seed bank size and density of emerged plants (Van Delft et al. 1997).

The pot trials also lacked the sensitivity to pick up changes in the broomrape seed bank. The results would indicate that there has been no substantial decline in the seed bank, given the high rates of infection of indicator plants grown in the soil after growing different crops. There may have been too many seeds in the original pots and that even with considerable seed losses there were still ample seeds remaining for infection. The results are unexpected as other researchers using the same glasshouse growth technique found substantial reductions in infections of indicator hosts following growth of trap crops in the same soils (Lins et al. 2006, Babaei et al. 2010). However, in Lins et al's study field results were not significant. As Van Delft et al. (1997) found with *Striga*, parasite emergence may not show a direct linear relationship with seed bank size, perhaps due to competition between parasite tubercles.

It is not certain that the lack of broomrape infection on safflower hosts in soils that had previously grown cereals in 2008 had any biological basis. Cereals can have allelopathic effects on *O. cernua* germination (Fernandez-Aparicio et al. 2007) but the chemicals are not likely to have long-term stability such that subsequent broomrape germination is affected when the cereal hosts have died. These allelopathic effects would result in lower infection of the indicator host but not seed bank decline. The tubercles retrieved from these pots could have all been on the old hosts and there were no infections of safflower. As safflower was such a poor host we used vetch in the second pot trail and got much better infection.

In conclusion, germination tests show that potentially most common crops in use in the quarantine area could facilitate some seed bank decline through germination of broomrape seed. Unfortunately our experimental methods have lacked the sensitivity to detect broomrape seed decline in pots or in field plots if it does occur.

Recommendations

The results of this study do not give us a usable estimation of soil seed bank decline under different land uses. If this information is still required by the eradication program, the experiment should be repeated using measures that can more accurately quantify seed bank decline. Counts of seed numbers in pots would provide the most accurate means of measurement although there will be loss of applicability to field conditions using this method. It is suggested that fewer crop types are trialled and host and broomrape seed density are manipulated (see results for canola in Section 2.2). A pilot study could be first initiated to test whether the DNA assay or manual counting of seeds in soil samples gives the most accurate count of seed numbers.

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4. Seed bank decline under different crops

Jane Prider and Andrew Craig

Branched Broomrape Eradication Program

January 2013

Introduction

This study follows up on the work presented in the recommendations of the previous section (Section 5.9). We test whether three crop types grown in pots of soil inoculated with broomrape seed, result in a reduction in seed number. For this experiment, we count the seeds that are placed in each pot so that we can more readily detect any decline in seed numbers.

Methods

For the experiments we used 0.8 L square pots that hold approximately 1300 g of Burdett sand. The range of seed numbers introduced to pots was based on seed densities measured in the field and the detection limit of the DNA bioassay that we were planning on using to count seeds in soil. The DNA bioassay has a detection limit of 0.005 seeds per gram of soil. The average number of broomrape seeds occurring in field-sampled soils is 0.3 seeds g^{-1} soil and the maximum number is 10 seeds g^{-1} soil. This equates to the following number of seeds per 1300 g of soil in pots and expected in a 200 g subsample:

Seeds g-1 soil	Seeds in 1300 g^{-1} soil	Seeds in 200 g^{-1} soil
0.005	6	
0.05	65	10
0.3	390	60
3	3900	600
10	13000	2000

As it is unlikely that as few as 6 seeds would be retrievable from pots, we used spikes of 65, 390, 3900 and 1300 seeds per pot in our experiments.

Pilot study

To establish whether we could accurately assess seed numbers in 200 g subsamples of soil taken from spiked pots we prepared three pots of soil for each seed density. Broomrape seeds were counted out and then mixed thoroughly into the soil before placing in pots. The pots were placed on moisture wicking mats in trays of water and left for one week. After air drying the soil was emptied from pots into a tray and thoroughly mixed. Three 200 g subsamples were collected from each pot.

We compared two methods for seed counts: 1) manual counting under a microscope after floating the seeds out of the soil in a calcium chloride solution, 2) DNA bioassay.

Experiment with crops

We examined six crop/host species; Clearfield canola, vetch cv. Blanchefleur, cretan weed, wheat cv. Peake, barley cv. Buloke, and oat cv. Euro.

Polybags were prepared as described in Section 5.8 to quantify germination stimulation by each species. Five host seeds were placed in each polybag and approximately 1000 broomrape seeds were added. Germinated broomrape seeds were counted three times at approximately 10 day intervals following the observation of the first germinated seeds.

In pot experiments, we used three broomrape seed densities; 0.05, 0.3, 5 seeds g^{-1} soil or 65, 390 or 3900 seeds per pot. Pots were prepared by thoroughly mixing the pre-counted seeds into the soil and then filling pots. Several host/crop seeds were sown into each pot and later thinned to one host plant per pot. We also included an unplanted control. We prepared 15 replicate pots for the 0.05 and 0.3 seed densities and 10 replicate pots for the 5 seed density. Replicate numbers were based on the results of our pilot study (see Results - pilot study).

Pots were placed on moisture wicking mats on trays of water so that seeds and soil would not be lost whilst plants were watered. However as pots became too wet using this method we had to water pots by spraying from above.

Broomrape plants were removed from pots as they emerged. When host plants had matured the pots were left to dry out and the soil emptied into trays. The soil was passed through a sieve and thoroughly mixed. Any broomrape tubercles were removed. The weight of soil was recorded. Broomrape seed counts from a 200 g subsample of soil from each pot were made using a DNA bioassay. Subsamples of 200 g of soil were also collected from a random selection of 5 replicates from the canola and control treatments and seed number counted manually after floating the seeds from the soil.

Using the weight of the soil in the pot and the number of seeds in the 200 g sample, we calculated the number of seeds in the soil at the end of the experiment. This could be compared to the known number of seeds added to the pot at the start of the experiment.

Results

Pilot study

Both seed counting methods gave a reasonable estimate of the number of seeds in a sample (Table 1). The DNA method tended to underestimate the mean of larger samples. The float method gave closer estimates, particularly for larger seed numbers. The float method had smaller deviations than the DNA method.

Table 1. Seed counts from soil using two methods. Values are from 3 replicate pots.

Seeds per pot	Seeds in 200 g subsamples				
	Expected mean	Mean - float method	St Dev – float method	Mean – DNA method	St Dev – DNA method
65	10	12.66	0.58	10*	1.41
390	60	53	5.29	77	20.42
3900	600	595	3.21	579	16.82
13000	2000	2006	94.88	1913	101.04

*This does not include one sample which had an estimate of 58 seeds

Table 2. Power analysis to determine sample size required to detect a potential 5% or 10% decline in seed number in soils using two methods.

Seeds/pot	Sample size (number of pots)			
	5% decline Float method	5% decline DNA method	10% decline Float method	10% decline DNA method
65	14	125	5	32
390	63	442	17	111
3900	1	6	1	3
13000	15	19	5	5

A power t-test was used to calculate sample sizes required to detect a 5% or 10% difference from the mean at $\alpha < 0.05$ (Table 2). From our previous work it is predicted that a 10% difference between means

could be expected with different treatments. Due to the time constraints, both in counting out seeds to put in pots and floating out seeds afterwards, we used the DNA method. We also excluded the largest seed counts to reduce pot preparation time. We used replicates of 15 pots for the 65 and 390 seed amounts and 10 replicates for the 3900 seeds.

Crops

The polybag trial confirmed that all trial species stimulated germination of broomrape (Table 3). Results for cretan weed were lower than expected due to the poor growth of cretan weed in the polybag system. The results for Clearfield canola were also lower than found in other trials. As in previous trials, the cereals were able to stimulate some broomrape germination.

Table 3. Germination of broomrape seeds in presence of roots of crop species, cretan weed and controls (no plant present). Values are mean and standard error, n = 5.

Species	Germination (%)
vetch cv. Blanchefleur	36.78 ± 7.19
Clearfield canola	5.05 ± 3.12
wheat cv. Peake	4.15 ± 1.56
oat cv. Euro	3.00 ± 0.46
barley cv. Peake	1.36 ± 0.81
cretan weed	0.02 ± 0.02
control	0

The DNA bioassay detected broomrape seeds in all samples of soil from pots in which hosts had been grown. In all treatments, including controls where no host plants had been grown, we calculated from 50 – 70 % of the original number of seeds added to the pot were present at the end of the experiment. There were no differences between treatments or seed densities in the number of seeds remaining (Fig. 1).

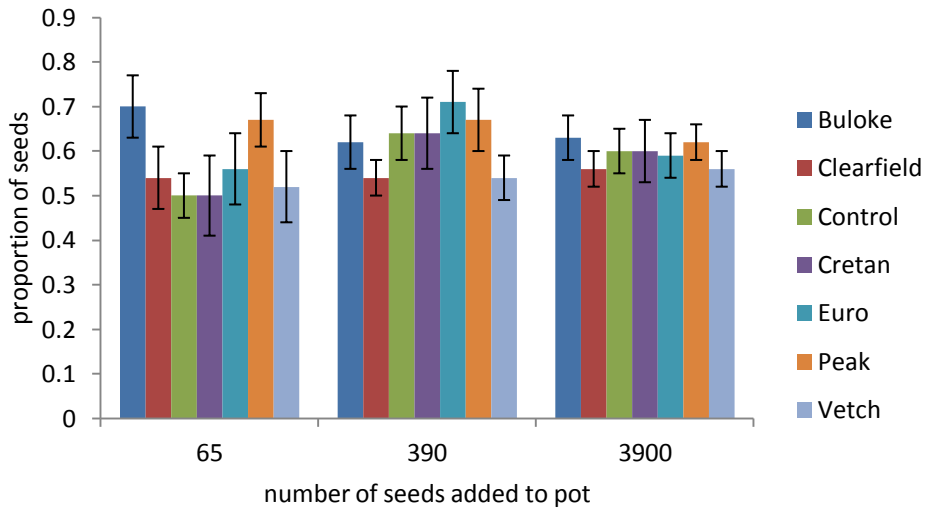

Figure 1. Calculated proportion of broomrape seeds remaining in pots in which crop species had been grown at the end of the experiment. Bars are means + 1SE, n = 15 (65 and 390 seeds per pot), n = 10, (3900 seeds/pot).

The poor estimates of seed numbers in control pots where no plants had been grown suggested that the DNA bioassay method did not provide a good estimate of seed numbers. Alternatively, seeds may have

been lost from the pots during the experiment or the soil from pots was not well mixed prior to subsampling. The float method for counting seeds was used to check the canola treatments.

Pots that were growing canola had fewer seeds than unplanted control plots at the two lower seed densities but not at the highest seed density (Fig. 2).

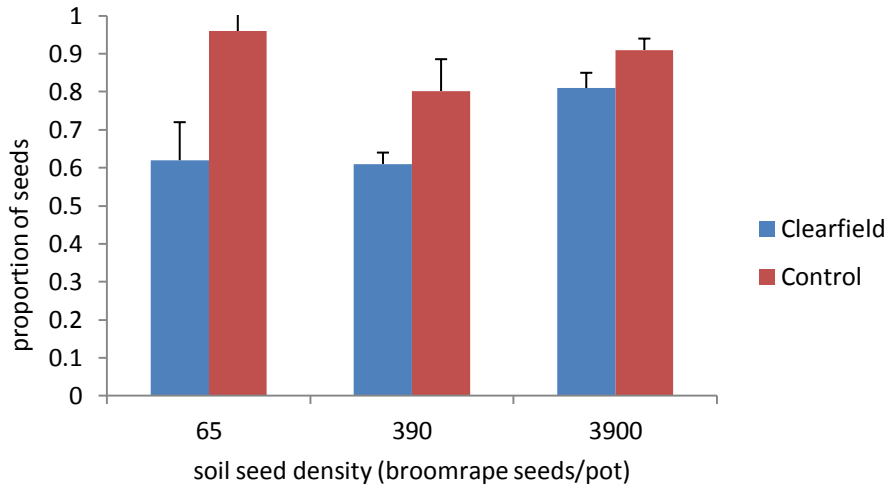

Figure 2. Proportion of broomrape seeds retrieved from pots sown with broomrape seeds of increasing density . Seeds counted using the flotation method. Bars are means + 1 SE, n = 5.

Conclusions

Although the method used in this study was more effective than using host infection as an indicator of seed bank drawdown, we were still not able to satisfactorily demonstrate seed bank drawdown as a result of germination of broomrape seed in response to stimulants released by plants into the soil. There is some evidence to suggest that host species such as canola can result in a decrease in broomrape seed numbers within the confines of a pot, but we were not able to demonstrate this for other species. The DNA bioassay method lacked the precision to detect differences in seed numbers between unplanted controls and planted treatments. Given more time we may have been able to further sample the other species using the flotation method.

The canola data suggests that a drawdown in seed numbers may occur where seeds are at lower density. However, given the poor recovery of seeds from the 390 seed pot^{-1} treatment these results are not conclusive.

11. Influence of soil micro-organisms on branched broomrape seed bank mortality

Jane Prider and Andrew Craig

Biosecurity SA

May 2014

Introduction

Reduction of seed bank persistence is an important goal of weed management. A persistent seed bank is composed of seeds that enter and survive in the soil for longer than one year. A long-term, persistent seed bank can last longer than 5 years with seeds cycling between dormancy and non-dormancy, contributing to continual weed problems (Kremer 1993). Seed persistence is determined by heritable traits but the maternal environment and soil physical, biological and chemical factors can also affect seed longevity (De Cauwer et al. 2011). Sources of seed mortality include natural physiological aging processes, predation, stimulation of germination in conditions unfavourable for establishment and attack by fungal and bacterial micro-organisms. The relative importance of these factors is species-specific but is also influenced by environmental conditions.

Soil microbial populations are influenced by soil physical properties such as particle size, soil depth, temperature and moisture status as well as chemical characteristics including nutrients, pH and oxygen (Chee-Sanford et al. 2006). As these conditions are likely to differ among sites, there will be differences in seed mortality resulting in variation in seed bank persistence across a species range (Wagner and Mitschunas 2008). Bacteria and fungi can attack seeds and may be either saprophytic when they use the seeds as a food source, or pathogenic, where the seed is killed but not consumed.

In our broomrape seed burial experiments, we have found differences in the rate of seed viability loss among sites and with depth of burial in the soil profile. There is no significant decline in the number of seeds that are retrieved over time at two sites, so seed losses are not the result of predation. Loss of viability could be due to natural aging processes with site and depth differences related to differences in soil temperature and moisture. However, there may also be differences in the activity of pathogenic soil microbes with site and soil depth.

The objective of this project was to investigate the sources of variation in the rate of seed viability loss among sites where seed burial experiments are located. We used experiments to investigate the potential sources of seed mortality that confer variability in the rate of seed viability loss among sites. We addressed the following questions:

1. Does the abundance/composition of pathogenic soil micro-organisms differ in soils from different sites?
2. Are there differences in microbial abundance/composition and/or seed viability loss with soil moisture?

We concentrated on differences in soil moisture as this has been shown to have a more marked effect on loss of seed viability (Schutte et al. 2008) than soil temperature, which we assume does differ substantially between sites, particularly for seeds at the same depth in the soil profile.

The outcomes from this project will enable the eradication program to:

1. Evaluate whether the differences in seed viability loss at sites with seed burial trials can be attributed to soil biological factors or soil moisture characteristics
2. Use the results of the experiments to develop more representative predictive models of seed bank persistence across the quarantine area
3. Evaluate the potential of soil amendments to enhance the rate of seed bank decay by micro-organisms

Methods

Experiment design

The experiment was conducted in a glasshouse using soils collected from the field. It was a full factorial experiment design testing three factors with the following levels:

1. Soil source - soil collected from the top 10 cm at each site adjacent to the seed burial trials at:
    i. Mannum Trial Site (Site 1 in field burial trials)
    ii. Glenn Burr Road (Site 2 in field burial trials)
    iii. Haby Hut Road

    Soils were sieved after collection.

2. Soil treatment - soils were treated to change the potential abundance of soil micro-organisms:
    i. none (soil sterilised in autoclave)
    ii. control (no treatment)
    iii. enhanced (soil amended with stubble to act as carbon source for microbes)

    Barley stubble was collected from the Brinkley Trial Site, air-dried and then cut into 1-2 cm pieces and sterilised in the autoclave.

    After 6 months (the first seed sachet retrieval) the sterilised treatment was discarded as it was found that the autoclave treatment had not been sufficient to kill soil micro-organisms.

3. Soil moisture – water was added to maintain soil moisture at:
    i. field capacity (15-20%)
    ii. wet and dry cycles – wet to field capacity and then left to dry out and rewet to field capacity (cycle from 5% - 20%)
    iii. dry (5%)

    After 6 months (the first seed sachet retrieval) these levels were amended to 10%, 2 – 10%, and 2% as the initial soil moisture levels were considered to be too high.

The prepared soil samples were placed in one litre rectangular plastic tubs, with five replicate tubs for each combination of soil source, soil treatment and soil moisture (see Table A1). Two seed sachets containing 5 ml of sterilised sand and approximately 500 unsterilised broomrape seeds were buried in each tub. Tubs were kept in an air-conditioned glasshouse out of direct sun. Soil water content was maintained in tubs by adding water to adjust to starting weight. Tubs were checked for weight 2 – 3 times per week.

Microbial abundance

One set of seed sachets was retrieved from the tubs after 6 months and the other set after 18 months. A subsample of 100 seeds was tested for the presence of fungal and bacterial populations on agar cultures. Seeds were removed from the sand in the sachet manually under a microscope.

Seeds from each sachet were placed in 1.5 ml eppendorf tubes and 350 µl of 1 x PBS (Phosphate Buffered Saline) was added. Preliminary testing found that countable numbers of microbial colonies could be obtained from the neat solution and 1/ 5 and 1/25 dilutions. To make the dilutions, 70 µl of neat

solution was added to 280 µl of 1 x PBS to make the 1/5 solution. To make a 1/25 solution, 70 µl of the 1/5 solution was added to 280 µl of 1 x PBS.

For plating fungal colonies, 100 µl of each dilution was added to a Potato Dextrose Agar (PDA) plate, spread, allowed to air dry, sealed with parafilm and incubated at 25 °C. Two plates were prepared for each dilution.

For plating bacterial colonies, 20 µl spots of each dilution were added to a TSA plate in triplicate. Plates were sealed with parafilm and incubated at 25 °C.

The number of bacterial colony-forming units (CFUs) on each spot on each TSA plate were counted after 1, 2 and 3 days of incubation. The number of fungal CFUs on PDA plates were counted after 2, 3 and 4 days of incubation. On day 4 the number of different CFU types on full solution plates was recorded.

Seed viability

The seeds remaining in the sachet were separated from the sand by floating out in a 40% w/w solution of calcium chloride. Seeds were sterilised in 2% sodium hypochlorite and assessed for germination and viability using the usual protocols. A control set of sachets remained dry and were kept in the laboratory. They were assessed for seed viability after 6 months.

Identification of fungal isolates

Following the second sachet retrieval and assessment of fungal colony abundance, isolates of different fungi colony types were selected for identification from genomic DNA sequences. Fungal isolates were grown on PDA plates at 25 °C for 10 -14 days prior to harvesting. DNA extractions were done using the MasterPure™ Yeast DNA Purification Kit with some slight modifications. Fungal mycelium and or colonies were scraped from the surface of the PDA plate and put into a 1.5ml Eppendorf tube containing 300 µl of Yeast Cell Lysis Solution and 1 µl RNAse. The fungi were broken up with a stainless steel pestle in the microcentrifuge tube before incubating at 65 °C for 15 minutes in a water bath. The samples were then put on ice for five minutes before 150 µl of MPC Precipitation Reagent was added and the solution was thoroughly vortex mixed for 10 seconds.

Cellular debris was pelleted by centrifugation in a microcentrifuge for 10 minutes at 12,500RPM. The supernatant was transferred to a fresh microcentrifuge tube and the DNA was precipitated with the addition of 500 µl of isopropanol and gently mixed by inversion. Centrifugation for 10 minutes at 12,500RPM was done to pellet the DNA and the supernatant was discarded. The pellet was washed in 500 µl of 70% ethanol and the ethanol discarded. The DNA pellet was air dried before resuspending in 20 -35 µl of TE buffer and stored at 4 °C for 24 hours and then at -20 °C.

DNA quality and concentration was determined by agarose gel electrophoresis. 5 µl of each sample was run on a 1.2% agarose gel at 110 volts for 30 minutes. 5 µl of MassRuler DNA Ladder (Thermo-Fisher Scientific) was run alongside samples for DNA concentration estimates.

For each sample, 20 µl of DNA, at 10 ng/µl, was sent to the Australian Genome Research Facility (AGRF) for sequencing using the Internal Transcribed Spacer primers:

ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), and

ITS4 (5'-TCCTCCGCTTATTGATATGC-3').

The majority of sequences returned were between 400 and 600 bp long. A Basic Local Alignment Search Tool (BLAST), blastn, was used to compare the sequences against the Nucleotide collection (nr/nt) database to identify the fungal colonies. Where multiple taxa were returned showing equal blastn scores, the species with the most number of alignments was selected.

Statistical analyses

We used analysis of variance models to test for the differences between levels of the factors site of soil source, soil treatment and soil moisture, on seed viability, number of fungal CFUs, number of bacterial CFUs and number of fungal CFU types. Seed viability data were arc-sine transformed, fungal colony data were log-transformed before model fitting. We omitted the sterile treatments from these analyses as the sterilisation process failed to reduce micro-organism populations as planned. Fungal colony counts for the 18 month retrieval could not be satisfactorily transformed so a Generalised Linear Model with a negative binomial distribution and a logratio link was used to test for differences between treatment effects. An iterative procedure was used fitting each factor sequentially and testing for the difference between each subsequent model with a chi-square test. We report the *p*-value from these tests.

We used the average fungal CFU count from the two replicate plates of the undiluted solution counted on day 4. We used bacterial CFU counts mostly from the undiluted solution or whichever solution gave a precise count of colony number. Where a diluted solution was used, the counts were converted to the number of CFUs per 350 µl. The number of CFUs was calculated as the average maximum number of CFUs for the three 'spots' on each plate across the three sample days.

For the 6 month fungal data we scored the presence/absence of CFUs of similar morphology on each plate and used these numbers to construct a Bray-Curtis distance matrix. We used the platform Adonis (Permutational Multivariate Analysis of variance Using Distance Matrices) to test the effects of soil moisture, soil treatment and soil source on fungal composition. We constructed canonical variates plots to examine the differences between treatment levels.

Canonical variates plots were also used to examine differences between treatment levels of fungi identified after 18 months. For these plots we only included taxa that of which there were at least five isolates.

Results

Seed viability

Viability of soil-buried seed after 6 months was less than seed that was stored dry in sachets in the lab. There was 4 - 8 % viability loss across the soils from different sites as compared to the lab-stored seeds (95% viability).

Seed viability loss after six months did not differ between soil types ($p = 0.13$) or between untreated and stubble-amended soils ($p = 0.17$). Seed viability loss was higher in the wetter soil treatments than the dry soil ($p = 0.006$, Fig. 1).

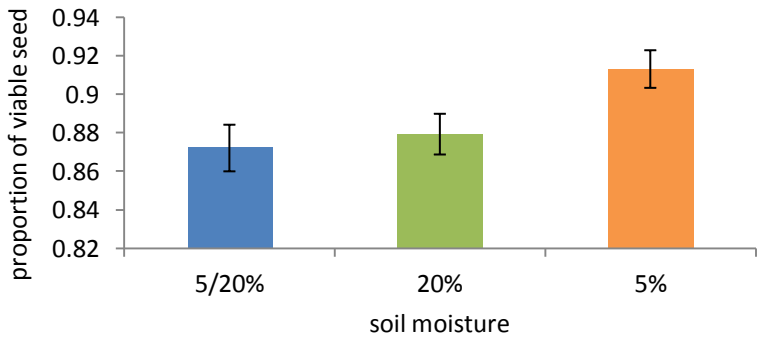

Figure 1. Effect of soil moisture on the proportion of viable broomrape seeds retrieved from sachets after 6 months. Bars are means ± 1 SE, n = 30.

There was little further loss of viability over the following 12 months of burial. Average viability across all treatments was 87%. Variation was much greater and some samples were obviously infected by fungi. Within replicates, viability ranged between 37% and 97%.

All factors had significant effects on seed viability but differences between soil types depended on soil treatment (soil source X soil treatment interaction, $p = 0.003$) and differences between treatments depended on soil water content (soil source X soil moisture interaction, $p = 0.049$). Seed viability was lowest in seeds buried in the Haby Hut Road soils that had not been amended with stubble (controls). In the stubble-amended soils, seed viability was lowest in seeds buried in the Mannum Trial Site soils (Fig.2). When treatments were combined across soil types, there was no difference in seed viability loss with soil moisture level in untreated control soils. In the stubble-amended soils greater viability loss occurred in the dry soils than the wetter soils (Fig. 3).

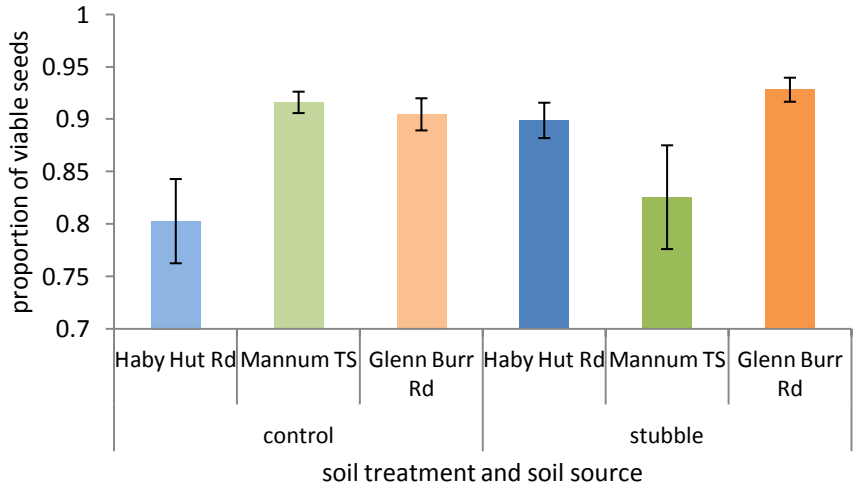

Figure 2. Proportion of viable broomrape seeds retrieved from sachets after 18 months buried in tubs of soil from different sites with/without stubble amendment. Bars are means ± 1 SE, n = 5.

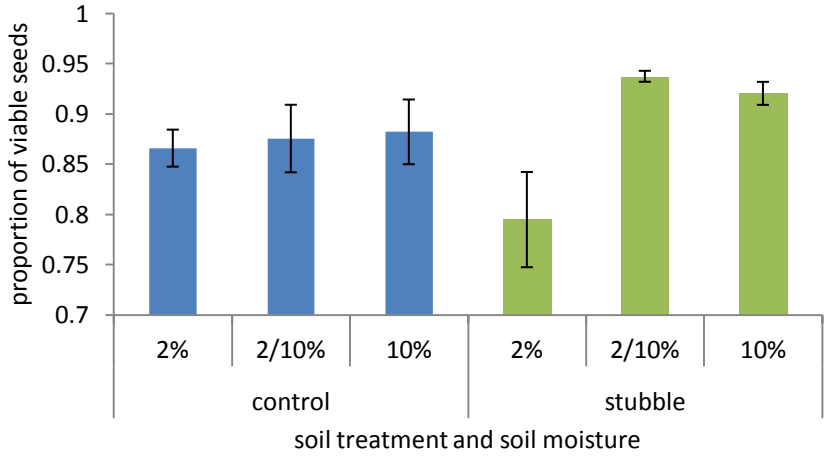

Figure 3. Proportion of viable broomrape seeds retrieved from sachets after 18 months buried in tubs of soil with/without stubble amendment and then maintained at different soil moisture levels. Bars are means ± 1 SE, n = 5.

Bacterial CFUs

After 6 months, the number of bacterial CFUs differed between treatments and there were complex interactions between treatment factors (three way interaction, p <0.001). The most CFUs were sampled from the surface of seeds retrieved from containers of dry, untreated Haby Hut Road soil (Fig. 4). On average, fewer than 100 CFUs were sampled from the wetter treatments with more CFUs occurring in the 5% soil moisture treatments.

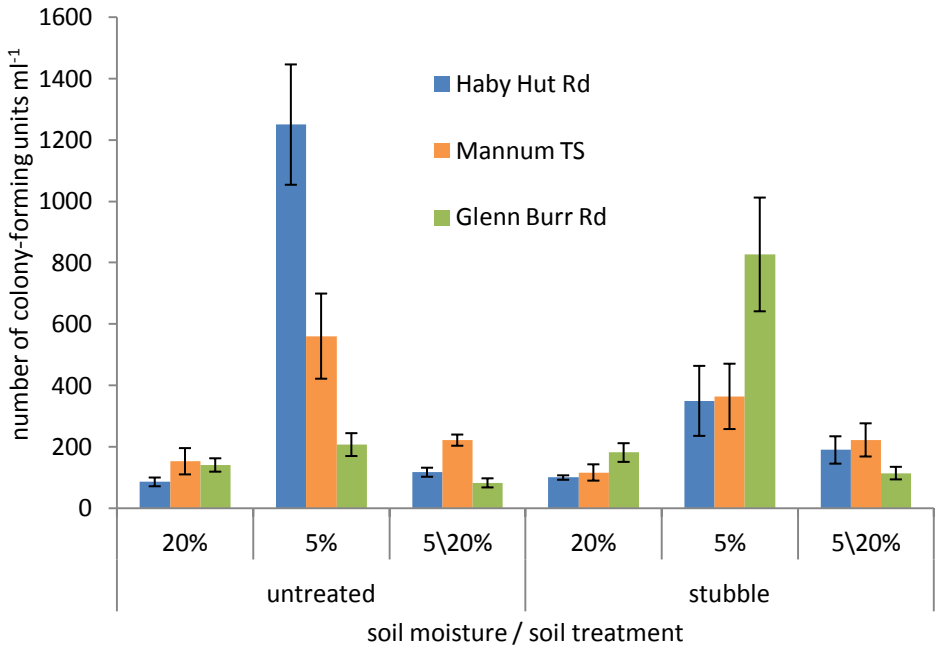

Figure 4. Number of bacterial CFUs cultured from surface of broomrape seeds buried for six months in soils with different treatments. Each bar is mean ± 1SE, n = 5.

Following a further 12 months of burial, cultured bacterial CFU counts were fewer (Fig. 5). Although soil moisture levels were decreased across all treatments, higher numbers of bacterial colonies were sampled from the surface of seeds buried in the driest soils (2% soil moisture) and this was consistent between soils of different origin. In soils from Haby Hut Road and the Mannum Trial Site, there were more bacterial colonies in soils that cycled between wet and dry than in the soils that were kept constantly at 10% soil moisture (soil moisture X soil source interaction, p = 0.029). There was no difference between untreated control soils and stubble-amended soils (p = 0.14).

Fungal CFUs

At 6 months, there were differences in the number of fungal CFUs for all treatment factors but the effects of each factor were affected by levels of the other factors. Although the most CFUs occurred in the driest treatments (Fig. 6), the stubble-amended treatments had more fungal CFUs than the wetter control treatments (soil treatment X soil moisture interaction, p = 0.049). There were other differences between stubble-amended and control treatments but only in the soil from Glenn Burr Road (soil treatment X soil source interaction, p = 0.003). Other soil source differences were not significant.

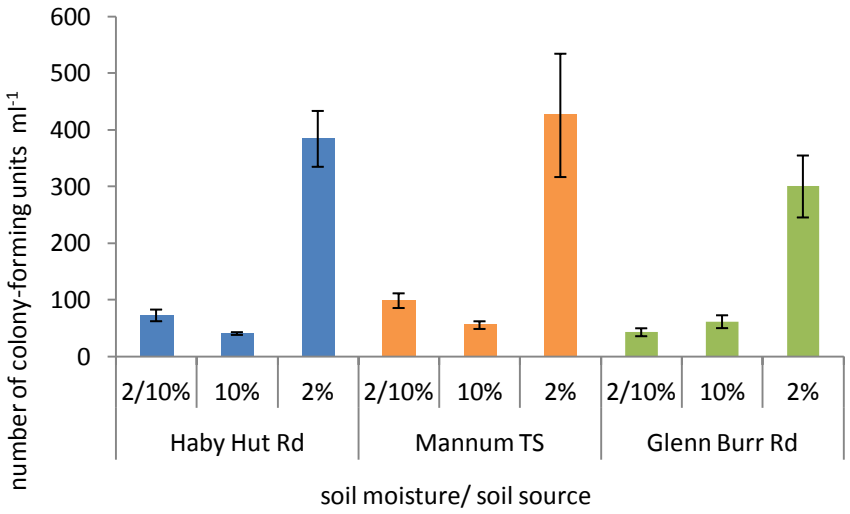

Figure 5. Influence of soil origin on the number of bacterial CFUs cultured from surface of broomrape seeds buried for 18 months in soils with different moisture levels. Each bar is mean ± 1SE, n = 10.

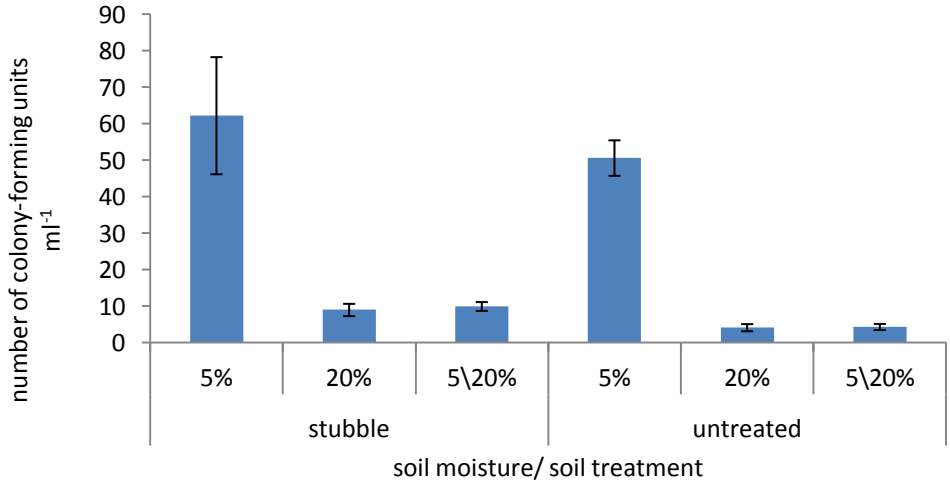

Figure 6. Influence of soil moisture on the number of fungal CFUs cultured from surface of broomrape seeds buried for 6 months in either untreated soil or soil amended with cereal stubble. Each bar is mean ± 1SE, n = 15.

After 18 months, stubble-amended treatments had an average of 23 fungal CFUs per ml and untreated control treatments, 11 fungal CFUs per ml ($p = 0.003$). Soil moisture had significant effects on CFU counts ($p < 0.001$). The dry soil treatment had more than ten times the number of CFUs as the wetter treatment (Fig. 7). Soils that cycled between wet and dry had approximately double the number of CFUs of the wetter treatment.

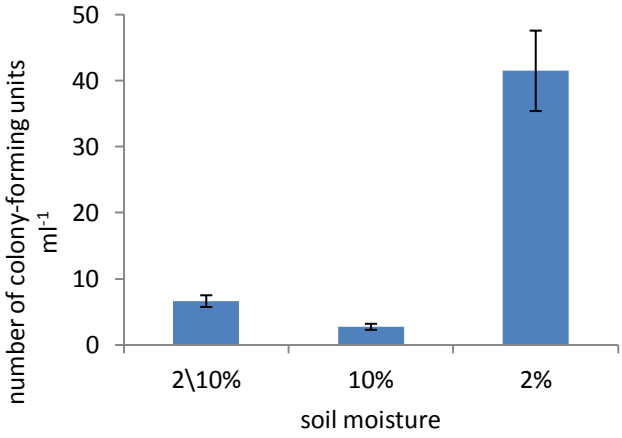

Figure 7. Influence of soil moisture on the number of fungal CFUs cultured from the surface of broomrape seeds buried for 18 months. Each bar is mean ± 1SE, n = 30.

Fungal types

Following the first retrieval, we identified 39 unique fungal CFU types based on broad physical characteristics. On average, dry treatments had from 5-6 CFU types and the wetter treatments 1-3 CFU types ($p < 0.001$). Stubble-amended treatments had more diverse assemblages than untreated soils ($p < 0.001$). Soil source, soil treatment and soil moisture level had significant effects on fungal composition although there were interactions between all these factors (three way interaction, $p = 0.007$). The canonical variates plot for the three factors highlights treatment combinations that had unique fungal composition. These were samples from dry, stubble-amended Glenn Burr Road soils and dry untreated Mannum Trial Site soils (Fig. 8).

Fungi identification

After the second retrieval, a total of 53 fungal taxa were identified from isolates (Table 1). There were only four identified species common to all sites; *Chaetomium globosum* , *Fusarium equiseti, Penicillium canescens* and *Podospora curvispora* (syn *Schizothecium curvisporum*). Four species, *Chaetomium globosum*, *Fusarium equiseti , Penicillium canescens* and *Fusarium oxysporum*, were the most frequently occurring taxa.

Groupings of fungal taxa differed between watering treatments. The low water treatments had different fungal assemblages to the high and cycled water treatments, which were similar (Fig. 9). Fungal composition also differed between soil sources. The Glen Burr Road site samples were separated from the Haby Hut Road and Mannum Trial Site samples (Fig. 10).

Of the 15 species included in this analysis there were 4 taxa that were unique to the Glen Burr Rd site: *Myrothecium verrucaria, Mortierella alpina, Fusarium tricinctum,* and *Bahusakala australiensis.* There were two taxa that were found in samples from Haby Hut Rd and the Mannum Trial Site but not Glen Burr Rd: *Geomyces* species and *Fusarium solani.*

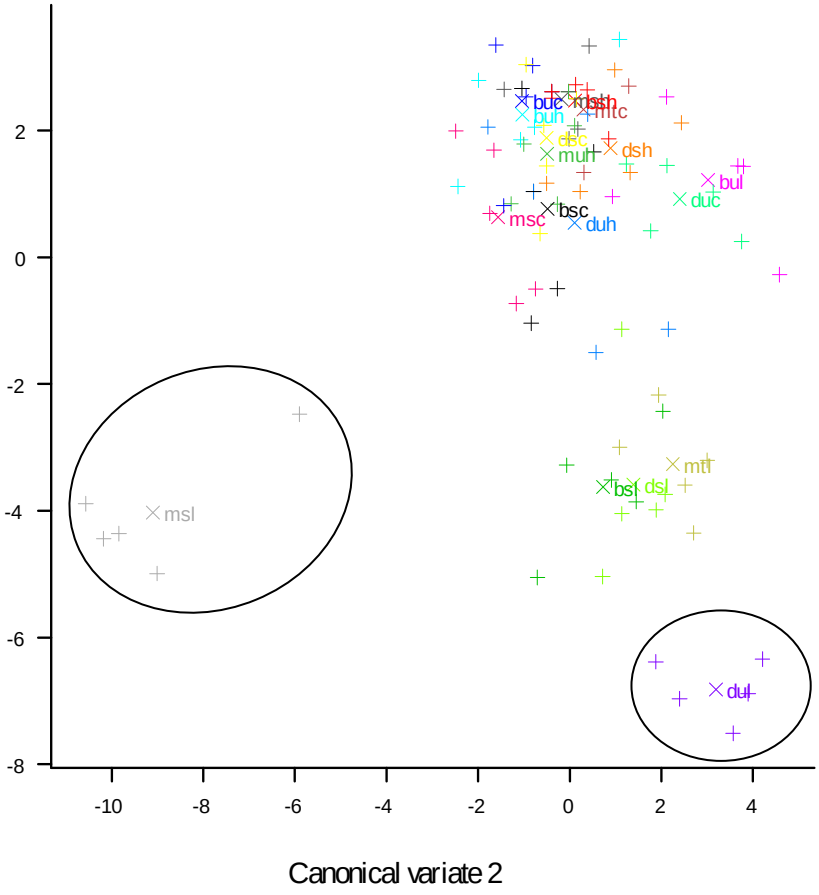

Figure 8. Canonical variate plot of fungal composition. Each cross represents the fungal composition of a sample plate and different treatment combinations are represented by different colours. Plates that have similar fungal composition occur closer together on the plot. Unique fungal communities are shown by sets of plates that are separated from others (circled). msl = dry, stubble-amended Glenn Burr Road soil, dul = dry untreated Mannum Trial Site soil.

Table 1. Fungi isolates identified from seeds buried in sachets for 18 months in soil from three sites. The final column shows taxa (some species were combined) that were used in the Canonical Variates Plots (see Figs 9, 10).

Species	Haby Hut Rd	Mannum TS	Glenn Burr Rd	analysis
Acremonium persicinum		X		
Acremonium sp.		X		
Alternaria arborescens		X		
Aspergillus fumigatus	X			
Aspergillus parasiticus		X		
Aspergillus versicolor	X			
Aureobasidium pullulans			X	
Auxarthron alboluteum			X	
Bahusakala australiensis			X	✓
Bionectria ochroleuca		X	X	✓
Ceratobasidium sp.	X			
Chaetomium globosum	X	X	X	✓
Chaetomium sp.			X	

Species	Haby Hut Rd	Mannum TS	Glenn Burr Rd	analysis
Chaetomium spirochaete		X		
Chaetomium subspirilliferum		X	X	
Cladophialophora sp.			X	
Cladorrhinum samala	X			
Cladosporium cladosporioides	X	X		
Cladosporium velox			X	
Curvularia inaequalis			X	
Embellisia allii		X		
Embellisia chlamydospora	X			
Emmonsia parva		X	X	
Eucasphaeria capensis			X	
Fusarium acuminatum	X	X		*F. avenaceum/ acuminatum*
Fusarium avenaceum (syn *Gibberella avenacea*)		X	X	
Fusarium equiseti	X	X	X	✓
Fusarium oxysporum		X	X	✓
Fusarium redolens		X		
Fusarium solani	X	X		✓
Fusarium tricinctum			X	✓
Geomyces sp. / *Geomyces vinaceus*	X			*Geomyces* sp.
Geomyces sp. / *Gymnostellatospora alpina*		X		
Ilyonectria radicicola	X			
Mortierella alpina			X	✓
Mortierella sp.		X	X	✓
Myrothecium verrucaria			X	✓
Neosartorya hiratsukae			X	
Oidiodendron cerealis			X	
Paraphoma chrysanthemicola / c.f. *chrysanthemicola*	X		X	✓
Penicillium canescens	X	X	X	✓
Penicillium chrysogenum	X			
Penicillium restrictum			X	
Penicillium sanguifluum		X		
Podospora curvispora (syn *Schizothecium curvisporum*)	X	X	X	*Podospora* sp.
Podospora glutinans	X		X	
Pseudogymnoascus pannorum	X			
Stemphylium sp.		X		
Talaromyces purpurogenus		X	X	
Thanatephorus cucumeris (*Rhizoctonia solani telemorph*)		X		
Thielavia microspora		X		
Ulocladium chartarum			X	*Ulocladium* sp.
Ulocladium consortiale		X		

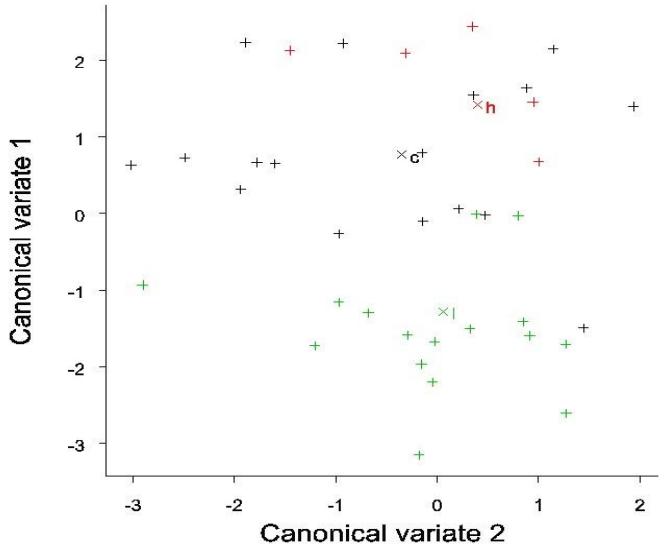

Figure 9. Canonical variate plot of fungal composition from 18 month retrieval. Each cross represents the fungal composition of a sample plate and different watering treatment combinations are represented by different colours. Plates that have similar fungal composition occur closer together on the plot. Unique fungal communities are shown by sets of plates that are separated from others. h = high water treatment (10% soil moisture), c = cycled water treatment (2/10% soil moisture), l = low water treatment (2% soil moisture).

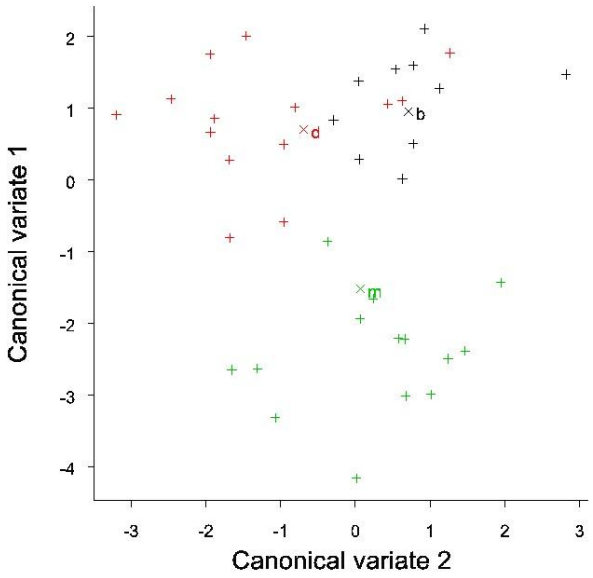

Figure 10. Canonical variate plot of fungal composition from 18 month retrieval. Each cross represents the fungal composition of a sample plate and different site treatment combinations are represented by different colours. Plates that have similar fungal composition occur closer together on the plot. Unique fungal communities are shown by sets of plates that are separated from others. m = Glen Burr Rd, d = Mannum Trial Site, b = Haby Hut Rd.

Discussion

The conclusions that can be drawn from our study are limited by problems with our experiment treatments. A single autoclave treatment failed to destroy soil microbes therefore we had no treatment that was microbe-free. The containers used for the experiment were not free-draining therefore even small amounts of added water created very moist conditions. These conditions may have not been ideal for the growth of many soil microbes, hence the greatest abundance and variety of soil microbes was found in our driest treatments.

After 6 months of burial we found that loss of seed viability was higher in the wetter soil treatments than the dry soil treatment. Seed aging is correlated with increasing soil moisture so this result was as expected. However after 18 months we found the greatest loss of seed viability occurred in the driest treatment, but only in the stubble-amended soils. At this time, fungal colony abundance was higher in stubble-amended soils and in drier treatments hence there was a correlation between loss of viability and fungal abundance. However, without a fungi-free treatment we cannot establish causation with our experiment.

Several studies have examined the use of microbes and/or their toxic metabolites for the biocontrol of parasitic plants and their seed banks (Zonno and Vurro 2002, Abouzeid et al. 2004, Vurro et al. 2009, Evidente et al. 2013, Watson 2013). Parasitic plants appear to be amenable to the use of such bio-herbicides as often they are susceptible but their hosts are not.

Bacteria such as *Pseudomonus, Azospirillium* and *Rhizobium*, can inhibit seed germination or radicle elongation of several broomrape species (Dadon et al. 2004, Mabrouk et al. 2007, Barghouthi and Salman 2010). In this experiment, the number of bacterial CFUss did not differ between our sites. The effect of bacteria on the seed bank could be consistent across sites and bacterial abundance does not explain differences in viability loss among sites. We did not identify bacteria so there may be differences in taxa among sites that could contribute to differences in seed bank decline.

We found unique assemblages of fungi in soils from our three seed burial sites. Infection of seeds by fungi is one explanation for the differences observed in seed bank decline among the sites. Several of the fungi that we isolated and identified have documented pathogenic effects on *Orobanche* seeds.

The fungi, *Myrothecium verrucaria*, isolated in our study only from seeds in the Glen Burr Rd soil, has been shown *in vitro* to inhibit the germination of *Orobanche ramosa* (Andolfi et al. 2005) and *O. crenata* (El-Kassan et al 2005). This fungus produces a number of metabolites that prevented *Orobanche* germination and are also toxic to plants and animals at very low concentrations. *Fusarium* strains were the most common isolates from our samples. The fungus, *Fusarium oxysporum* f. sp. *orthoceras* killed germinating seeds of several *Orobanche* species including *O. ramosa* (Dor et al. 2007). The toxic metabolite fusaric acid, is produced by several *Fusarium* species and is toxic to *Orobanche* at concentrations that do not affect the host (Bouizgarne et al. 2006). Other *Fusarium* species are pathogenic to germinating *Orobanche* seeds (Dor et al. 2009). Several toxins that have been isolated from fungi prevent germination but it is not known whether they are pathogenic to seeds (Zonno and Vurro 2002). Formulations of *F. oxysporum* have been developed the furthest for their application in *Orobanche* control (Sauerborn et al. 1994, Kohlschmid et al. 2009).

Several species or genera in our study have also been isolated from other *Orobanche* species. Linke et al (1992) recorded *Alternaria, Cladosporium, Fusarium oxysporum, F. solani, F. equiseti, Stemphylium* and *Ulocladium* from *O. crenata* plants from Syria, France and Morocco. Some of these species were pathogenic to tubercles and shoots but seeds were not tested. *Fusarium* species were the most common strains isolated from *O. aegyptiaca* plants in Nepal (Thomas et al. 1999)(Thomas et al 1999. Other strains isolated in common with our study were *Acremonium, Alternaria, Cladosporium cladosporioides* and *Mortierella alpina*. *Alternaria* has been found to attack *Orobanche* seeds, resulting in maceration of ungerminated seed (Hameed et al. 2001).

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