Compendium of branched broomrape research

Section 3. Seed biology

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

DECEMBER 2013

PREMIUM FOOD AND WINE FROM OUR CLEAN ENVIRONMENT

Compendium of branched broomrape research

Information current as of 3 December 2013

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See also the following publications:

Virtue J.G., DeDear C., Potter M.J., Rieger M. (2006) Potential use of isothiocyanates in branched broomrape eradication. 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. 629-632.

Prider J., Williams A. (2014) The reproductive biology and phenology of the introduced root holoparasite, *Orobanche ramosa* subsp. *mutelii* (branched broomrape) in South Australia (in preparation)

1. Seed maturation and release

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December 2012

Introduction

Despite many studies that have examined the growth of *Orobanche* very few studies have examined the phenology of post-emergent stages of development. To assess the risk of contamination of commodities by broomrape seed, data on the timing of seed maturation and release are required.

The timing of broomrape development is influenced by a number of factors but can be reasonably estimated from soil temperatures. Maturation is also species dependent and can differ with host. One of the few studies that has examined the timing of seed release found that *O. ramosa* capsule opening (and potential seed release) occurred approximately 130 days after transplanting of tomato hosts (Diaz et al. 2006). This is equivalent to 2400-2550 GDD. In this study emergence occurred at 60 – 75 days or at the equivalent of 1100-1400 GDD, which is earlier than our 1500 GDD estimate for *O. ramosa* subsp. *mutelii* emergence in the Quarantine Area. Flowering occurred 15 days after emergence with capsule formation 15 – 20 days later. Flowering to production of mature seeds of *O. ramosa* in Texas took approximately 30 days (Sand 1983) and another estimate was just 10-14 days (Langston et al. 1985). Another study reports 6-9 days between *O. ramosa* flowering and "fruit ripening" and another 7 days before fruits dried (Gonzalez and Rodriguez 1981). However, these studies do not report the presence of mature seeds.

Orobanche ramosa subsp. *mutelii* emergence is typically 90 days after germination and results of glasshouse and field studies show that this does not vary among hosts or under different cultivation methods. These development rates are considerably longer than rates measured for *O. ramosa* in other production systems (e.g. 50-55 days, Gonzalez and Rodriguez 1981; , 65-70 days, Diaz et al. 2006) therefore we need to be cautious about adopting maturation timings from other studies. Planting date, host type, and soil type can all affect broomrape maturation times in crops (Holm et al. 1997). Collecting our own data will provide the best estimates of seed maturation and release. For example, data collected from a herbicide experiment suggests that very little mature seed is present at 1700 GDD, with only 2/10 of plants having many mature seeds or capsules at that time (Prider & Craig 2012). Broomrape seeds are held in the seed capsule until it dries out. The capsule then splits longitudinally and the seeds fall out when disturbed. Unopened capsules have been observed on *O. ramosa* subsp. *mutelii* plants during December, which depending on the initiation of development, is approximately 180 - 240 days or up to 3000 GDD.

Risk modelling of harvested commodities requires accurate estimates of the timing of seed maturation and release to determine the risk of broomrape seed presence in harvested materials. For hay production, potentially the entire broomrape stalk could be harvested but as this harvest occurs early in the season there may be no mature seed present in capsules at harvest time. Later harvests of cereal crops may occur after broomrape seeds have been released but if seeds remain in capsules long after maturation then the risk of seeds in these commodities increases.

Objectives

The objective of this project is to provide data on broomrape reproduction for models of commodity risk. The specific requirements are to quantify:

- time from emergence to production of mature seeds
- the proportion of mature seed present at different stages of capsule maturation
- timing of seed release

- seed production
- plant height

Methods

Plant height

Measurements of plant height were made on plants from three sites in the Quarantine Area; Site 1, a non-arable paddock on deep sand, Site 2, an orchard on sandy loam, and Site 3, a sandy loam adjacent to a cereal crop. Above ground plant height was measured from the natural soil level. Total stem length was measured after plants had been excavated. Plants were growing on either cretan weed or capeweed hosts.

Monitoring phenology

Detailed monitoring of flower and fruit production requires daily checks of plants so was conducted in the glasshouse. Broomrape plants were grown on cretan weed hosts in 0.8 L square pots filled with Mannum field soil and inoculated with broomrape seed. Pots were initially cultivated at the Mannum Trial Site and then transferred to the glasshouse when broomrape emerged.

Previous experiments have shown that broomrape is self-fertile, so the lack of pollinators in the glasshouse should not affect fruit and seed set. However, there may be differences in the proportion of viable capsules and seeds in self-crossed seeds compared to out-crossed seeds. This was estimated in self-compatibility experiments.

72 plants were cultivated so that there were sufficient plants for monitoring, harvesting flowers and fruits throughout the monitoring period, use in self-compatibility experiments and spare plants.

A set of 20 plants was used for collecting data on the timing (days from emergence) of reproductive events including:

- Time until buds form – i.e. differentiation of sepals obvious
- Time and order of flower opening
- Time until flowers wither
- Time when capsules start to develop
- Time for stalk to senesce
- Time when capsules start to open
- Time when capsules are fully open

On the same set of plants we counted:

- Number of branches per broomrape plant
- Number of flowers per stalk
- Number of capsules per stalk
- Number of capsules that produce mature seed, i.e. any aborted capsules (fruit set)
- Height of broomrape plants

On another set of 20 plants flowers were collected daily from bud differentiation to post-anthesis and fruits were collected weekly from formation to opening. These samples were used to determine:

- time after bud formation or anthesis that anther and stigma are receptive
- time when seeds start to mature
- number and proportion of viable seeds at various stages of capsule maturation (seed set)

Anther maturation was assessed by examining the anthers under a microscope to check for dehiscence. To check for stigma receptivity, pollen grains were removed from the stigma and examined under a microscope to check for germination. The stigmas were squashed on a microscope slide and stained with lactophenol blue (1 ml cotton blue in 100 ml lactophenol) (lactophenol = lactic acid: glycerol: phenol: distilled water, 1:1:1:1). The stigma was assumed to be receptive if germinated pollen grains were found.

Self-compatibility experiment

Five treatments were applied to individual flowers on the same broomrape plant to control for maternal effects. Flowers from different positions on the inflorescence were selected on each plant and randomly assigned to five treatments:

1. Autonomous self-pollination (autogamy) – flowers bagged
2. Self-compatibility - Hand self-pollination – bagged flowers hand pollinated after anthesis
3. Apomixis – emasculated bagged flowers
4. Hand out-crossing – bagged flowers emasculated before anthesis and pollinated with pollen of other recently opened flowers
5. Control – not bagged or emasculated

Buds were bagged before anthesis in case of bud autogamy. Capsules produced from treated flowers were collected after capsule maturation. The number of seeds was counted and the proportion of viable seed was measured by testing a subsample of seed with tetrazolium tests.

Results and discussion

Plant height

The mean above-ground height of plants collected from three sites in the field in 2012 was 9 ± 0.2 cm (n = 90). Plants growing on the sandier soils in Sites 1 and 3 had longer stems as a result of more below-ground growth (Fig. 1).

Pot grown plants had a mean above-ground height of 9 ± 0.7 cm and a mean total stem length of 14 ± 0.9 cm (n=19).

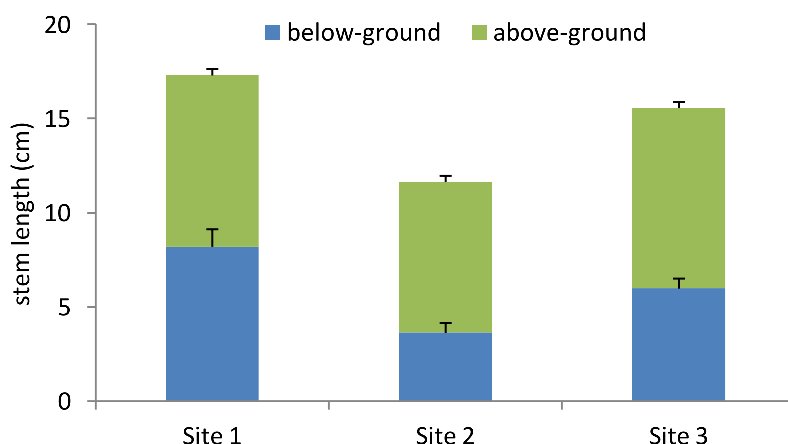

Figure 1. Stem length of branched broomrape plants collected from three sites in 2012 (Site 1 *n*= 33, Sites 2 and 3 *n* = 30).

Monitoring phenology

Results are reported for the mean of 20 monitored plants. The first flower opened, almost always at the base of the flowering stem, 8 days after stem emergence. The typical flowering period was 20 days but this depended on the number of flowers on the stem. Taller stems typically produced more flowers. Usually one flower opened each day. Flowers remained open for 6 days before they senesced. Another 22 days later the fruiting capsule dehisced. This gives an average minimum time from first emergence to seed release of 36 days.

The anthers dehisced before the flower fully opened (Fig. 2). The stigma was also receptive at this time.

All capsules examined at 14 days or later after flower senescence had mature seeds present (Fig. 3). Not all flowers examined at 13 days had mature seeds but mature seeds were frequently found in capsules 10 days after flower senescence. There was no relationship between the position on the flowering stem and the time taken for seeds to mature.

Data from annual field surveys show that the main flowering season is in October (Fig. 4).

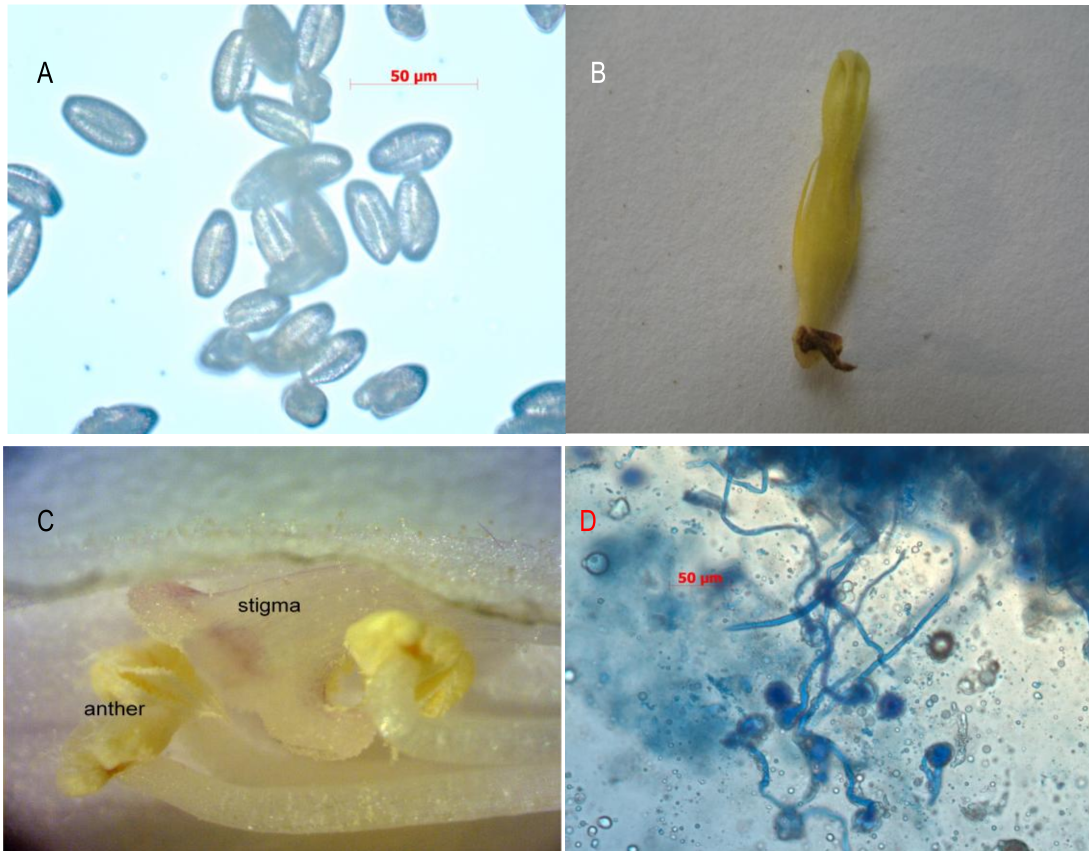

Figure 2. A) mature pollen grains, B) bud at stage when anthers dehisce before anthesis, C) dehiscent anthers inside a flower bud where the pollen has been deposited on the stigma, D) germinated pollen grains adjacent to the stigma surface.

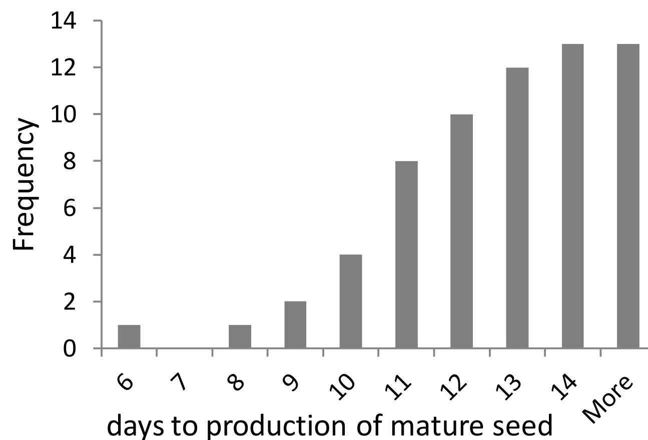

Figure 3. Histogram of frequency of capsules examined with mature seeds with days after flower senescence.

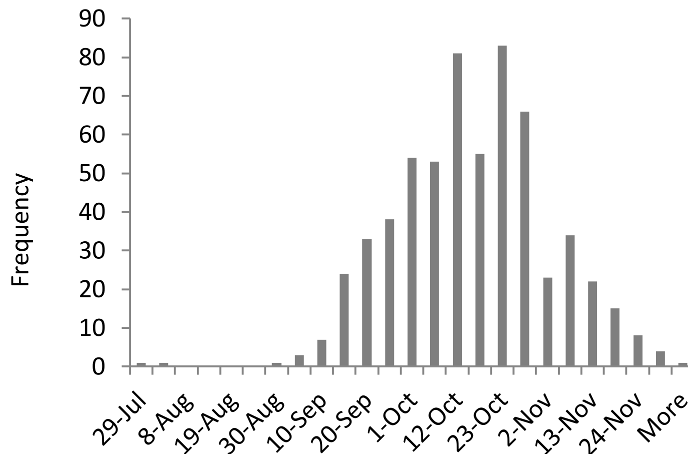

Figure 4. Data from field surveys 2003-2011 of presence of broomrape plants in flower.

Self compatability

Flowers were self-compatible. Flowers that were self-pollinated produced more seeds than outcrossed, bagged and control (unbagged) flowers (Fig. 5). The stigma of outcrossed flowers may have been damaged during emasculation which may have reduced seed production. Although some seeds were produced in the apomixis treatment this may have been the result of pollen release before emasculation. All treatments produced viable seed.

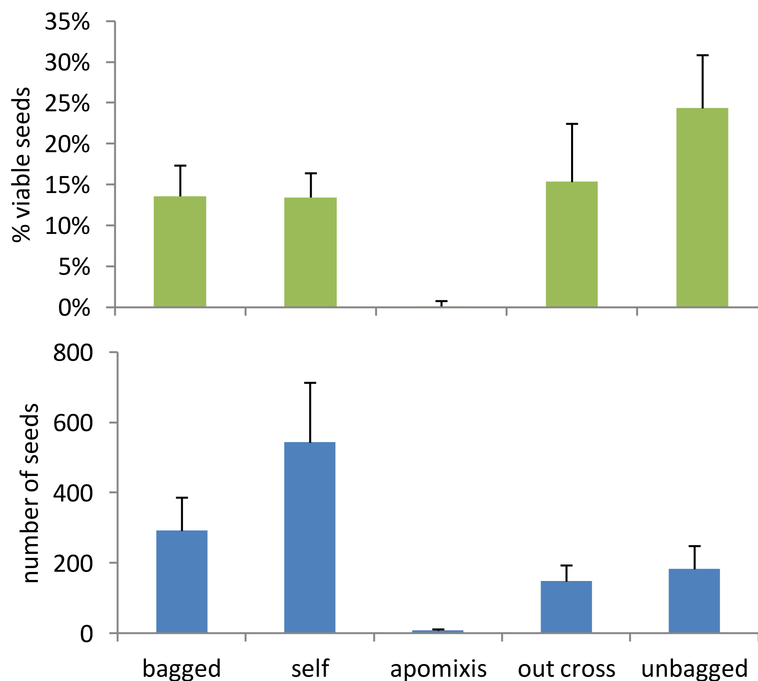

Figure 5. Seed viability and production of broomrape in experimental pollination treatments. Bars are mean + 1SE, *n* = 15.

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2. After- ripening of *Orobanche ramosa* subsp. *mutelii* seed

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Branched Broomrape Eradication Program

November 2011

Summary

Newly produced *O. ramosa* subsp. *mutelii* seed will not germinate. A brief after-ripening period of 4-8 weeks is required before seed will germinate given appropriate conditions. Germination reaches a maximum approximately 6 months after seed is produced. As the after-ripening period is short, seed produced in the spring season could germinate in the next growing season in autumn.

Introduction

Newly produced seeds are in a state of primary dormancy and require a period of after-ripening before they are physiologically able to germinate. There are reports, not supported by any data, that *Orobanche* seeds may need a period of two years or more for after-ripening (Edwards 1972, Saghir 1986) but there has been no published reliable evidence (Pieterse and Verkleij 1994). Here we report on the germination of newly harvested *O. ramosa* subsp. *mutelii* seed.

Methods

Two separate trials were conducted, the first after the collection of seed from the field in 2009 and the second in 2010. In 2009 capsules with seed were collected from two sites (Burdett and Brinkley) on November 13th. Seeds were removed from capsules using a shaker sieve. Five petri dishes, each holding 100-200 seeds were set up for germination tests on March 4th, 16 weeks after seed was picked. Seed collected from the Mannum Trial Site in 2006 and stored in the lab after collection was included as a control. Seeds were conditioned for 14 days at 20 °C, GR24 added and germination counted after 14 days at 20 °C. Ungerminated seeds were placed in tetrazolium solution for 14 days at 30 °C for 14 days and stained seeds were counted to assess viability. The germination procedure was repeated at 24 weeks after collection on April 20th.

In 2010, seed was collected from Brinkley on December 3rd. Seed was retrieved from capsules using shaker sieves and separated into three size classes:

- 106 - 150 µm
- 150 - 212 µm
- 212 - 310 µm

Germination tests started on December 15th for one month old seed, and then at 4 weekly intervals until maximum germination occurred. All seed sizes were included in the first tests but later tests used the large seed class. Tetrazolium tests for viability were conducted on ungerminated seed on the first and second test dates.

Results

2009 seed collection

Germination assessed at 16 weeks after seed collection was lower than lab-stored Mannum seed although the viability of all seed lots was similar (Fig. 1). There was a high proportion of germinated seed after 16 weeks. By 24 weeks, the Burdett seed had a germination proportion similar to controls but seed collected from the Brinkley site had not reached maximum germination. The results of this trial suggest that the after-ripening period for most seed is less than 24 weeks. In the 2010 trial seed was assessed earlier and at more frequent intervals.

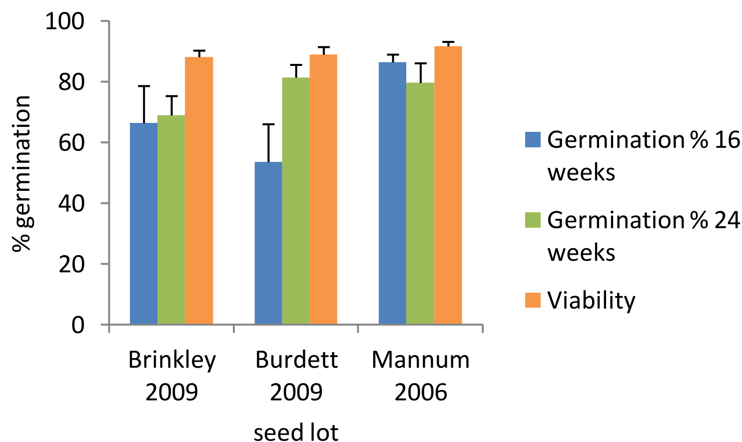

Figure 1. Germination of seed lots collected in 2009. Each bar is mean + 1 SE, n =5.

2010 seed collection

Germination of the small seed class was variable as this sieve size included many seeds that had not fully formed. The medium and large seed classes contained mostly viable seed.

A very low proportion of seeds germinated one month after collection (Fig. 2). Over the following four months germination was variable, indicating that some seed was ripe but other seed was not. By six months after collection all seed germinated.

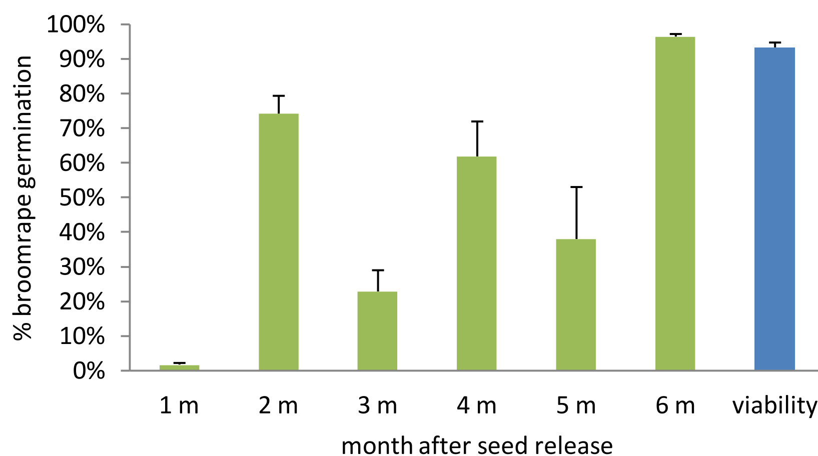

Figure 2. Germination of seeds collected in 2010. Each bar is mean + 1 SE, n =5 for the large seed size class.

Discussion

Precise estimates of after-ripening times are difficult to assess with any precision from bulked seed lots. Seed ripening occurs over a few weeks as the first seeds to mature on an infructescence are those on the base, as these are the first flowers to open (see Section 3.1). In addition, differences in emergence over a season would also result in an extended period of seed maturation in a population. For any given seed lot there would be a range of seeds at various stages of ripeness.

It is thought that the embryo of fresh seeds may not have sufficient growth for the germ tube to be able to penetrate the seed coat. Treatments that soften the seed coat can increase the germination proportion of freshly produced seed (Baskin and Baskin 1998).

We have shown that *O. ramosa* subsp. *mutelii* seed requires an after-ripening period. This is likely to be short and would occur within the first 4 - 8 weeks after seed maturation but may take up to 20 weeks. If it is assumed that on average mature seed would be present in mid-November, given peak flowering in October and a 20 day maturation period for seed (see Section 3.1), our estimate that 2010 seed was first assessed one month after maturity is a reasonable approximation.

As the after-ripening period is short, seed produced in the spring season could germinate in the next growing season in autumn.

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3. Branched broomrape seed production

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2003

Methods

These data are counts of the number of broomrape seeds from a single cohort of branched broomrape collected from the Mannum Trial Site in 2003. Plants were marked as they emerged and flowered and samples were collected when capsules matured. Plants were grouped according to the week they emerged from 18 September until 31 October.

For some samples, the capsules were removed from individual plants and then counted (individual). For other samples, the seeds were removed from 4 -5 plants collected from a plot and pooled together (bulked).

Counts were made of the number of stems, viable capsules (capsules that contained mature seed) and univiable capsules (capsules that contained no mature seed). The seeds from each plant (or bulked sample) were placed into small holes drilled into a metal plate. Each hole had a different volume used to estimate the total number of seeds. Seeds were examined under a microscope to estimate the proportion of immature or undeveloped seed, chaff or sand in the sample. Seed numbers were corrected for this proportion.

In another sample, seeds were collected from the bottom, middle and top thirds of the broomrape stem. The proportion of seeds in each portion of the stem and their viability was assessed.

Results

Counts from individual and bulked samples were similar. There was an average of 3 stems per plant and 15-18 viable capsules. Seed numbers varied considerably between plants and capsules with a range between 0 and 85,500 seeds per plant and an average of 11,500. Highest seed numbers were collected from plants with multiple stems and thus many capsules. Capsules had a range of 0 to 2,000 seeds with an average of 737. The viability of seeds was not assessed.

Table 1. Mean counts of seed production parameters collected from individual plants and 3 – 5 plants pooled to form bulked samples.

Sample type	stems	viable capsules	seeds/ plant	seeds/capsule
Individual plants	3	15	11491	515
Bulked plants	3	18	11405	960

Plants that emerged earlier in the season produced more stems (Fig. 1) although the earliest emerging plants produced fewer viable capsules than plants that emerged in late September (Fig. 2). Early emerged plants also produced more seeds than plants that emerged later in the season (Fig. 3).

More seeds were produced in the bottom third of the stem than the middle or top thirds (Fig. 4). Seeds from the bottom third were also more likely to be viable. No seeds in the top third of the stem were viable (Fig. 4). Broomrape flowers open from the bottom of the stem to the top so the bottom capsules would be the first to develop.

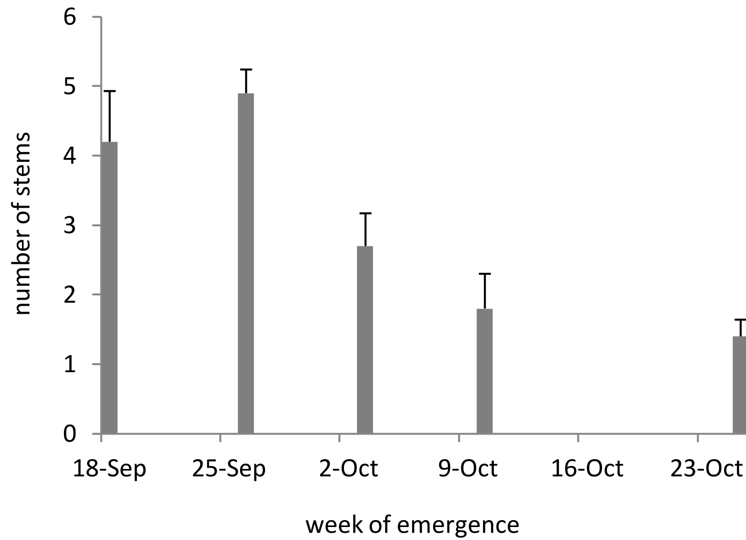


Figure 1. Number of emerged stems per plant for broomrape plants that emerged at different times of the season. Bars are means + 1 SE (*n* L to R = 5, 8, 9, 14, 13).

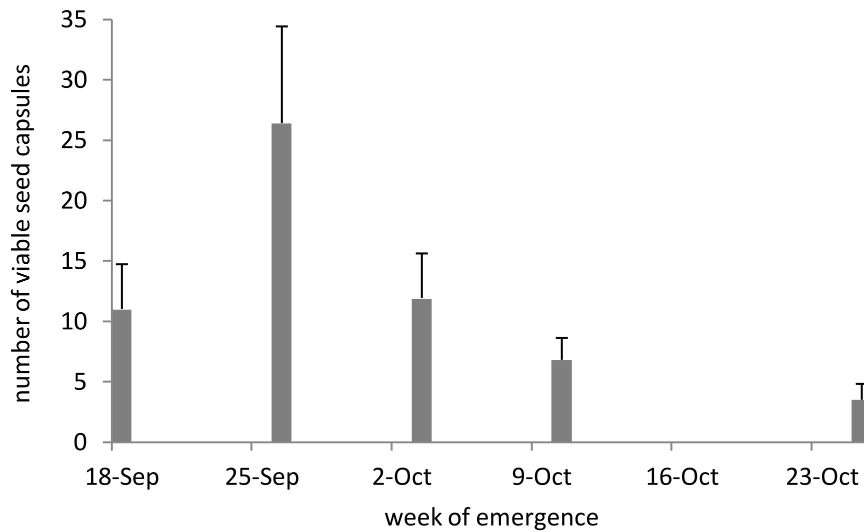


Figure 2. Number of viable capsules per plant for broomrape plants that emerged at different times of the season. Bars are means + 1 SE (*n* L to R = 5, 8, 9, 14, 13).

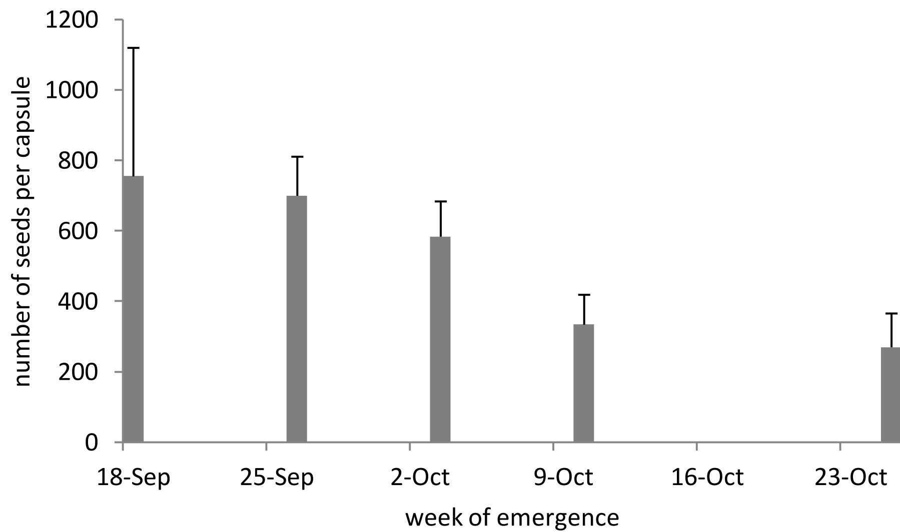


Figure 3. Mean number of mature seeds per capsule for broomrape plants that emerged at different times of the season. Bars are means + 1 SE (*n* L to R = 5, 8, 9, 14, 13).

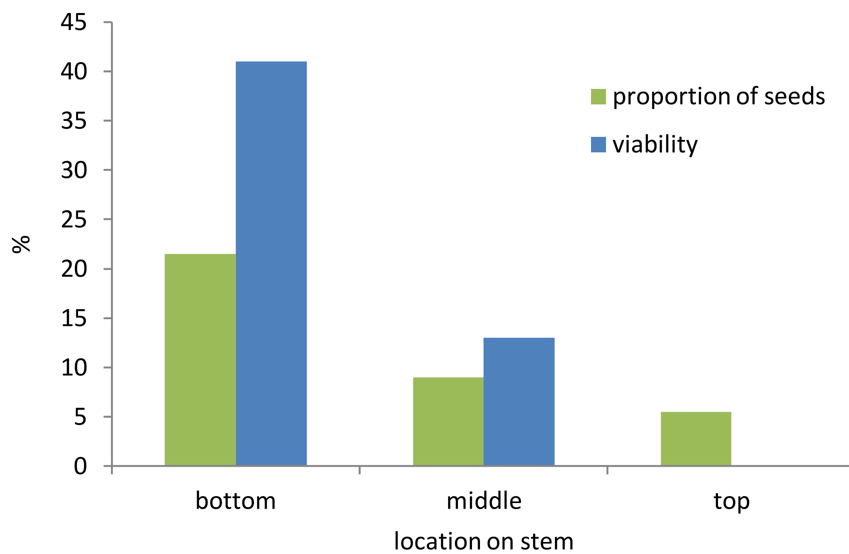


Figure 4. The percentage of seeds in different parts of the flowering stem and their viability. Sample numbers not known.

4. Broomrape mating systems

Jane Prider

Branched Broomrape Eradication Program

March 2011

Summary

In this study we assessed whether *Orobanche ramosa* is self-fertile by placing bags over flowers in sample populations at Mannum and Brinkley. We also assessed whether seed set is pollinator-limited by removing the anthers from flowers to ensure that pollen must be transferred from another flower by a pollinator. *Orobanche ramosa* was self-fertile as flowers that were bagged produced viable seed. Seed production was lower in emasculated flowers at the Mannum site. This suggests there may have been be pollinator limitation at this site where there was a small population of *O. ramosa* but not in the larger population at Brinkley.

Introduction

The mating system of a plant population refers to the occurrence and degree of out-crossing, or transfer of male gametes (pollen) between flowers. Flowers may be self-fertile (autogamous) when the pollen from a flower is able to fertilise the same flower and also self-compatible if this results in the production of viable seed (Richards 1986). Out-crossed flowers are the result of the transfer of pollen to a different flower, however most autogamous plants are also able to out-cross. Apomixis produces viable seeds in the absence of pollen transfer and fertilisation and is a form of asexual reproduction. Knowledge of a species mating system is important for determining limits to seed production and patterns of gene flow and genetic diversity in populations. For Orobanchaceae, most mating system studies have focussed on rare, native species in order to understand factors leading to rarity or the consequences of low genetic diversity in small populations (Jones 1991; Keighery 1984; Reuter 1986; Thieret 1971). Although studies of population genetics are very common for the weedy Orobanchaceae, experimental studies to determine mating systems are lacking and are instead inferred from genetic analyses.

There are a small number of studies that have examined the mating systems of *Orobanche*. *Orobanche crenata* (Musselman 1982) and *O. cernua* var. *australiana* are self-fertile (Keighery 1984) as are most species in the British flora (Jones 1991). In some self-fertile species, pollination and fertilisation occur when the mature stamens touch the stigma before the flower opens (bud autogamy, Musselman et al 1982; Keighery 1984). At least some populations of two non-weedy *Orobanche* species are apomictic (Reuter 1986, Jensen 1951). From genetic population analyses it is inferred that *O. ramosa* is an autogamous species with a low proportion of out-crossing (Satovic et al 2009).

Autogamous species have greater among population genetic diversity than within population diversity (Verkleij & Pieterse 1994), which is the case for the three published population genetic studies of *O. ramosa* (Buschmann *et al.* 2005; Roman *et al.* 2003; Vaz Patto *et al.* 2009). However our own genetic population analysis conflicts with this. We found greater genetic diversity within populations than among populations. This may indicate a primarily out-crossed mating system for our populations, long-established self-fertile or out-crossed populations, or the recent expansion of genetically-diverse populations of broomrape within the quarantine area.

The clarification of the mating system of our populations of *O. ramosa* is important for two reasons. It will enable us to define the risk that a single plant poses to the spread of broomrape and the replenishment of the broomrape seed bank. It will also enable us to interpret the results of the population genetics study.

The aims of this study are to:

- Determine the incidence and degree of self and out-crossed reproduction for several populations of *O.ramosa* in the quarantine area
- Determine whether pollination is important for seed production in *O. ramosa*

Methods

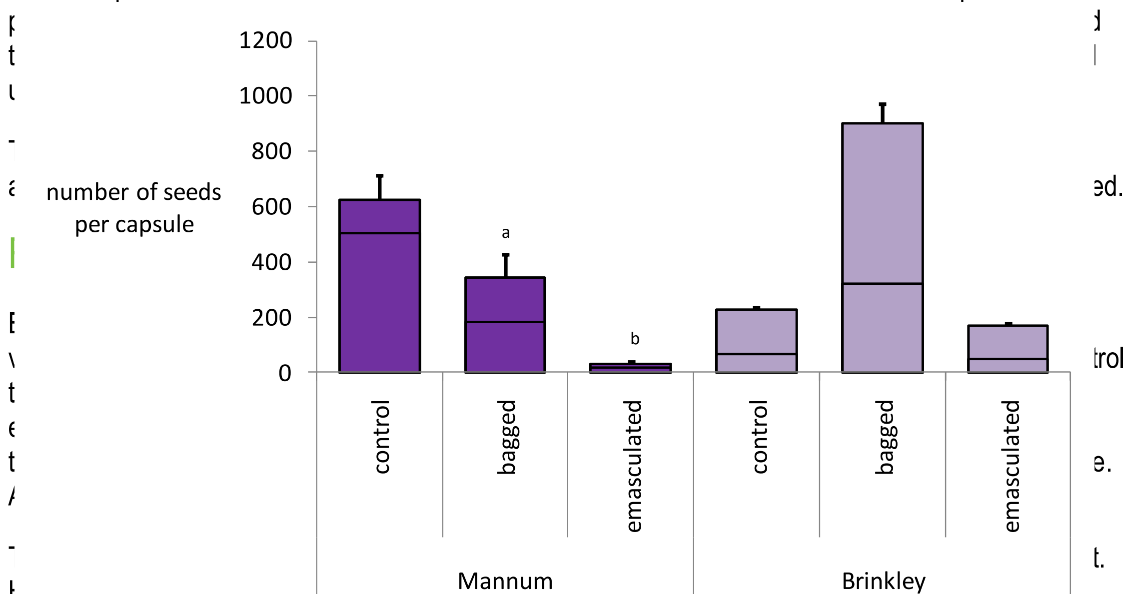
We examined the levels of natural pollination in two populations at Brinkley and Mannum in the 2010 season. We selected 10 plants at Mannum and 20 plants at Brinkley when plants had emerged and the buds had developed but in most cases before flowers had begun opening. If flowers had opened they were removed from the flower spike and not selected for treatment. Treatments were applied to unopened flowers, in case of bud autogamy, on the same flower spike to control for any maternal effects. Treatments were applied in different order on the inflorescence of each plant, selecting flowers in consecutive order from the bottom of the inflorescence.

The following treatments were applied to each inflorescence:

1. Control: free pollination – flower exposed to natural pollinator agents
2. Bagged: autonomous self-pollination – flower bagged throughout the flowering season so no access for pollinators
3. Emasculated: pollinator-mediated movement of pollen – anthers removed from flower, unbagged flowers

The emasculated treatment enabled assessment of whether pollinators are required for seed set. An estimate of natural pollination levels can be calculated from the difference between fruit set in bagged and unbagged flowers.

Mature capsules were collected before dehiscence. The seeds were removed from the capsules and



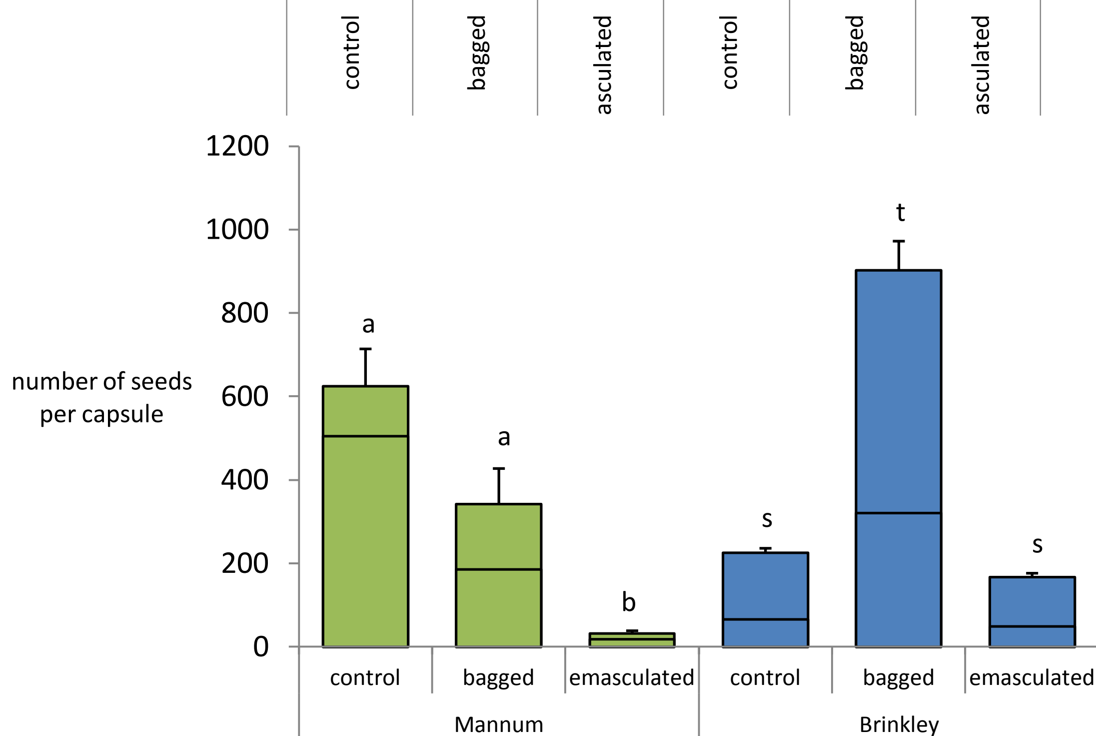


Figure 1. Number of broomrape seeds produced in capsules from outcrossed or self-crossed (control), self-crossed (bagged) or outcrossed only (emasculated) flowers at two sites. The line in each bar represents the proportion of viable seeds (below the line). Bars labelled with different letters in each site are significantly different (ANOVA, log-transformed data, p<0.001).

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5. Pollinator observations

Jane Prider

Biosecurity SA

November 2013

Introduction

There are limited pollinator observations for *Orobanche* species. Based on flower characters, it is assumed that broomrapes are insect pollinated (Kuijt 1969). Occasional visits to *O. minor* flowers by *Bombus* and *Vespula* spp. have been observed in Britain but pollen transfer has not been confirmed (Jones 1991). There is one confirmed study of pollination of *O. elatior* by bumblebees (Ollerton et al. 2007). Observations of broomrapes in Slovakia found that the weedy species of broomrapes were visited mostly by Colletid and Halictid bees and native broomrapes had a wider suite of pollinators including Vespidae wasps and Apidae bees (Toth et al. 2013).

Methods

Observations were undertaken at one field site in October 2013. The site in native vegetation had a small population of over 100 broomrape plants, hosting on capeweed and cretan weed hosts. Pan traps were used to sample the pollinator assemblage at the site. Sets of white, yellow and blue pan traps were placed amongst flowering broomrape plants in six locations. The pans were filled with water and a few drops of detergent were added. Traps remained in place for 3.5 hours on October 4, between 1030 and 1400 h and 3.5 hours on October 9, between 1000 and 1330 h.

Observations of floral visitors were made over the same time period on October 4 and for one hour (1230 to 1330 h) on October 9.

The insects trapped in the pan traps were stored in 80% ethanol and later identified to order and morphotype within each order.

Results

Conditions were dry during the study and broomrape plants were small with few flowers. Each plant had 2-6 flowers open at the time of observation. Other flowers occurring at the site included a yellow native Compositae species (see Fig. 1), *Euphorbia terracina* (false caper), *Arctotheca calendula* (capeweed), *Brachyscome* sp..

A total of 1059 insects were trapped over the 7 hours of this study. Of these, 163 were larger insects in the orders Diptera, Hymenoptera and Lepidoptera that could potentially be pollinators. This included 42 captures of bees and wasps of 14 morphotypes. There were 18 captures of syrphids, which were the most frequently observed floral visitors.

Broomrape flowers were rarely visited by insects. There was only a single observation of a visit that lasted for longer than one second. This was a visit to at least two flowers on separate plants by a syrphid hoverfly (as in Fig. 1). Foraging on each flower was for approximately 5 seconds. Pollen transfer was not confirmed.

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Figure 1. Syrphid hoverfly on native daisy. This species was also observed visiting broomrape flowers.

6. Branched broomrape seed conditioning

Jane Prider, Andrew Craig and Anna Williams

Branched Broomrape Eradication Program

December 2010

Introduction

Seeds of *Orobanche* germinate only in response to germination stimulants present in exudates from host roots. During conditioning the dormancy of the seeds is broken and they become metabolically active and progressively more responsive to germination stimulants (Matusova *et al.* 2004). Although early research suggested that all *Orobanche* species require a period of conditioning before this can occur, more recent research suggests this is not a requirement for all species (Plakhine *et al.* 2009). For example, some ecotypes of *O. aegyptiaca* and *O. cumana* do not require conditioning (Plakhine *et al*. 2009) but *O. ramosa* ecotypes do (Gibot-Leclerc *et al.* 2004; Gonzalez-Verdejo *et al.* 2005). Plakhine *et al*. (2009) also found that conditioning results in earlier optimal germination and in the case of *O. cumana*, a higher germination percentage.

After reaching maximum sensitivity, if the seeds remain moist they enter secondary dormancy and their sensitivity to germination stimulants gradually decreases (Bouwmeester *et al.* 2003) but seed viability is maintained (Van Hezewijk *et al.* 1993). Song *et al* (2005) recorded a decrease in *O. ramosa* germination after further conditioning from 7 to 28 days, which occurred at a faster rate at lower water potentials especially where temperature conditions were not optimal.

Although there is reasonable consensus in the literature that optimal conditioning periods are between 18 and 23 °C, it would seem that conditioning periods, and even the requirement for conditioning differ widely, even between seed lots of the same species. A minimum conditioning period of 7- 8 days at 20°C was found to induce optimal rates of germination in *O. ramosa* (Gonzalez-Verdejo *et al.* 2005; Song *et al*. 2005). We do not have any reliable data for declines in response to germination stimulants with prolonged conditioning (induction of secondary dormancy). This information is required for the appropriate application of some seed destruction products. For example, products that promote suicidal germination may need to be applied to conditioned seeds. Without data on secondary dormancy we do not know whether broomrape development occurs in the later part of the season if soils remain moist.

Trails in 2006 aimed to determine the optimum conditioning period for promoting maximum germination of *O. ramosa* seeds.

An experiment in 2010 addressed the following questions:

- What proportion of *O. ramosa* seeds are released from dormancy with increasing duration of conditioning at 15 and 20 °C? What is the optimal conditioning period?
- What proportion of *O. ramosa* seeds are induced into secondary dormancy with increasing duration of conditioning at 15 and 20 °C?

Methods

2006 Trials

O. ramosa seeds were surface sterilised in 5% NaOCl for 5 minutes and then rinsed 5 times in RO water. For a pilot study, 3 replicates of approximately 100 *O. ramosa* seeds were spread onto a 21 mm glass-fibre filter paper in a 5cm petri-dish. 250 µl of RO water was added to each filter paper, the petri-dishes

were sealed with parafilm and kept at 20 °C in the dark to condition for 0, 5, 8, and 20 days. After conditioning, the seeds were transferred to new filter paper and 250 µl of 10 ppm GR24 was added. The petri-dishes were sealed and kept at 20 °C in the dark. At 7 and 14 days the seeds were scored to determine percentage germination.

For a second study, two seed lots were tested. Eight replicate petri dishes of 35 seeds were conditioned for 0, 3, 6, 9, 12, 15, 18, and 21 days. After conditioning, the seeds were transferred to new filter paper and 250 µl of 10 ppm GR24 was added to 5 replicates, 250 µm of 1% TZ stain was added to the remaining 3 plates for viability testing.

The petri-dishes with GR24 were sealed and kept at 20°C in the dark. The petri-dishes with TZ were sealed and kept at 30°C in the dark. At 7 and 14 days the seeds were scored to determine percentage germination and viability. Germination was scored based on radicle emergence to the same length as the seed. Seeds stained red or pink were scored as viable.

2010 trials

Three separate seed lots were used for the trials. Two of the seed lots were from the same site collected in two different years (2006 and 2007). The third seed lot was from a different site and different year (2008). The viability of seed was > 90 % for all seed lots

We tested two conditioning temperatures –15 °C and 20 °C. Ten replicate petri dishes of about 100 broomrape seeds were prepared for each treatment combination.

Seeds were initially surface sterilised and then added to petri dishes. Seeds and filter papers in petri dishes were moistened (and kept moist) at day 0, 2, 5, 8, 12, 16, 20, 25, 30, 40, 60, 90, and 120 before the addition of germination stimulant. Dishes were regularly unsealed for aeration and then resealed.

After the addition of 10 ppm GR24 on the appropriate day, seeds were reincubated at the conditioning temperature and the germinated and ungerminated seeds were scored 7 days later. Germinated seeds were removed after counting and dishes with ungerminated seeds resealed. Counts were made again in the same way at 14 days and 21 days.

Results

2006 trial

In this trial there was 20 % germination in the absence of any conditioning period (Fig. 1). Maximum germination occurred following a conditioning period of 8 days.

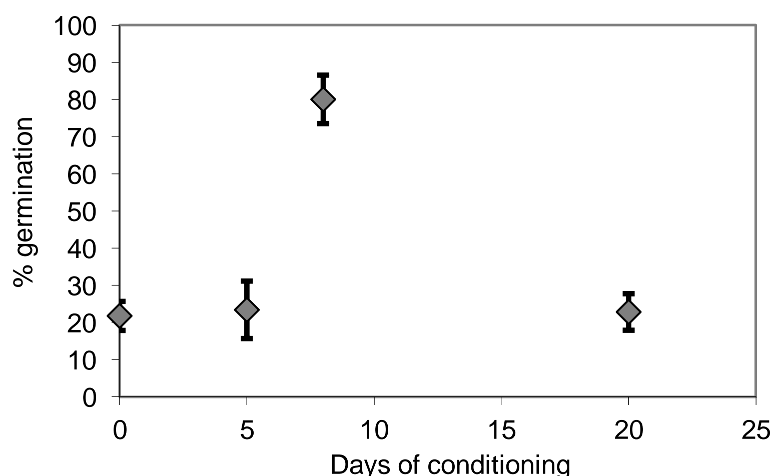

Figure 1. Results of 2006 Pilot study conditioning trial. Points are means ± 1 SE, n = 3.

In the main trial, there were differences in germination after different periods of conditioning between the two seed lots (Fig. 2). For both seed lots there was some germination without a conditioning period. In Che's seed maximum germination occurred after 3 days whilst in Dorothee's seed it did not occur until 18 days after seeds were first moistened. For both seed lots, the proportion of seed that was viable but did not germinate did not change over the time period of testing. Overall viability of both seed lots was approximately 90 %.

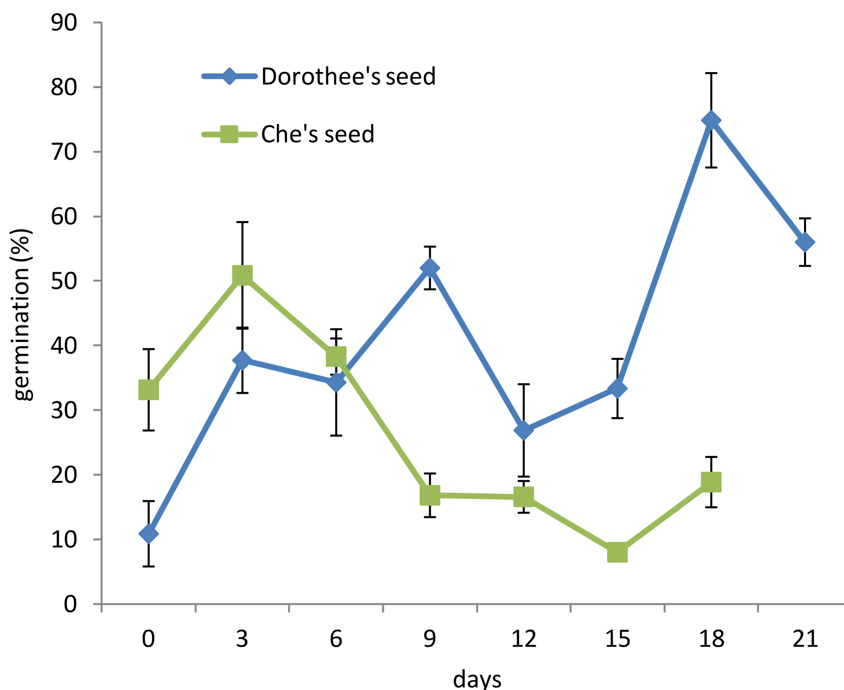

Figure 2. Germination of branched broomrape seed after different lengths of time in an imbibed state. Results are from two separate seed lots. Points are means ± 1 SE, n = 5.

In the 2010 trials, germination was greater when seeds were conditioned and germinated at 20 °C than at 15 °C (Fig. 3). At 20 °C, germination remained high over a long conditioning period, from 5 to 25 days. At 15 °C a conditioning period of 20 days induced maximum germination. Secondary dormancy was rapidly induced at 15 °C after 20 days with no germination for seeds kept moist for 60 days. At 20 °C secondary dormancy was induced after about 90 days although a small proportion of seeds still germinated after being kept moist for 120 days.

Germination occurred more rapidly for seeds conditioned and germinated at 20 °C than at 15 °C (Fig. 4). For example, after 20 days of conditioning 85 % of seeds at 20 °C had germinated 7 days after exposure to stimulant whilst at 15 °C only 12 % of seeds had germinated after the same time period.

There were minor differences between the three seed lots. In the 20 °C treatment, a lower proportion of the oldest seeds (2006) germinated without any conditioning (Fig. 5). This seed lot had maximum germination after 5 days conditioning as did the other seed lots tested.

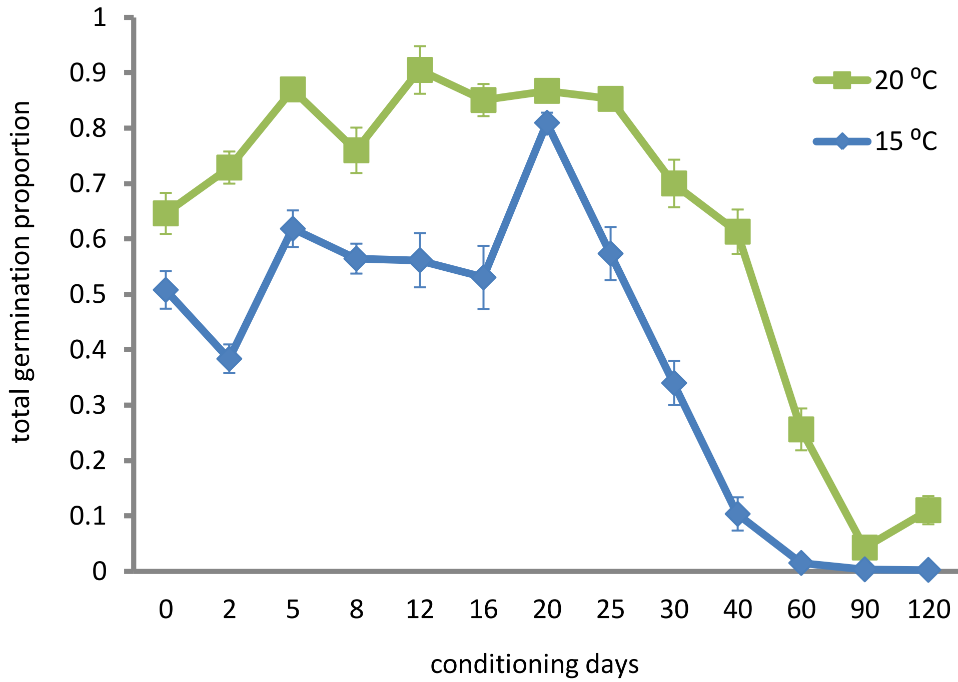

Figure 3. Results of 2010 conditioning trials. The three seed lots have been combined. Points are means ± 1 SE, n = 10.

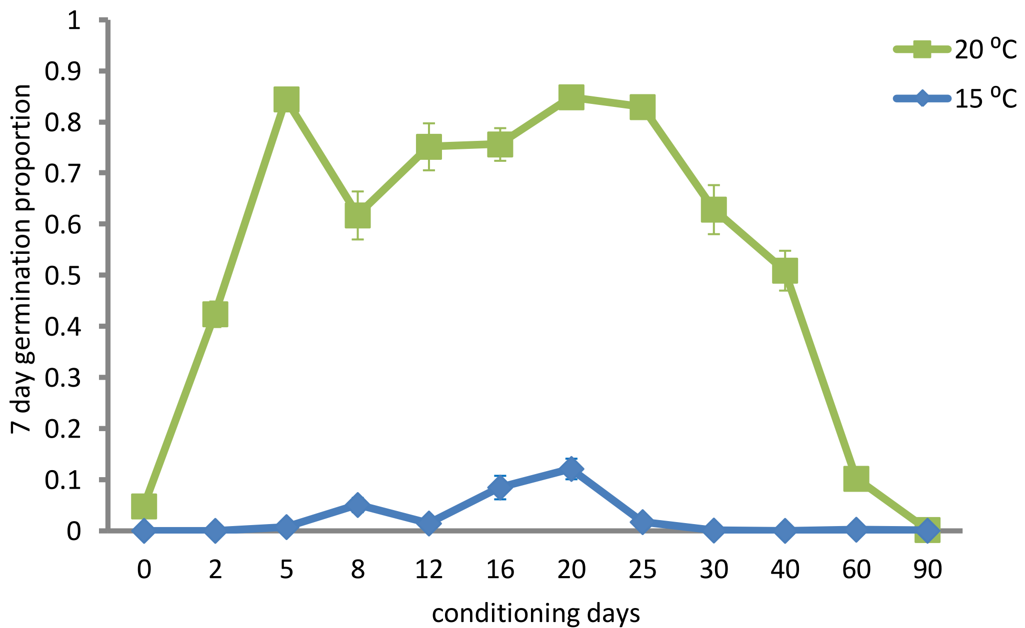

Figure 4. Germination after 7 days exposure to GR 24 stimulant following conditioning of increasing time periods at two temperatures. The three seed lots have been combined. Points are means ± 1 SE, n = 10.

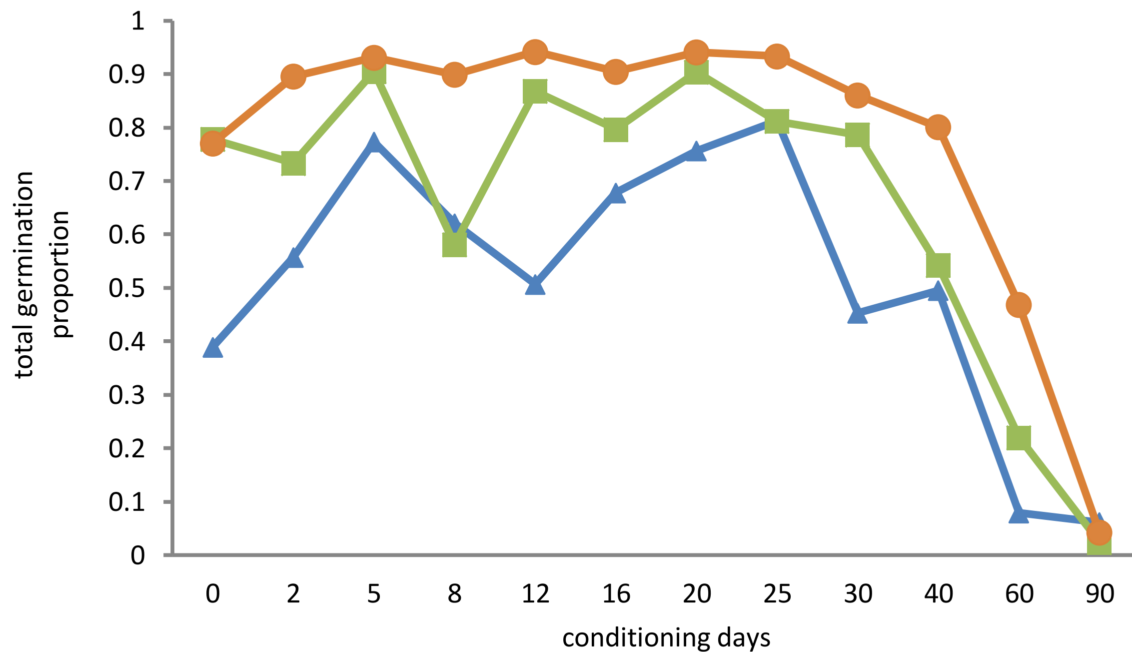

Figure 5. Germination after different lengths of conditioning period for three seed lots in the 2010 experiment at 20 °C. Points are means, n = 10. (Seed lots: blue 2006, orange 2007, green 2008)

Discussion

Our trials have consistently shown that a proportion of branched broomrape seed requires no conditioning before exposure to a stimulant to germinate although conditioning is required for optimal germination. Across the three trials, conditioning periods of approximately 5-8 days at the optimum temperature of 20 °C induce the highest germination. A few seeds remain responsive to stimulants after remaining moist for up to 120 days.

There is variability between seed lots which may in part be a result of seed age as shown in the 2010 results. Different processing was used for the two seed lots tested in 2006. The "Che" seed was washed out of the seed capsules whist for all other seeed lots the seed has been sieved dry out of the capsules. The wetted seed may have lost vigour whilst in storage. Although the viability of this seed lot did not differ from the other seed, a small proportion of this seed germinated. A longer conditioning period may have been required than the limit of 18 days that was measured.

For laboratory purposes seed is conditioned at 20 °C for 14 days.

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7. Germination temperature

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September 2012

Introduction

Knowledge of the optimum temperature for *O. ramosa* germination is important for the refinement of research methods. Many of the protocols used to assess seed responses to chemical control and environmental factors require an assessment of seed germination capacity. In addition, germination temperature optima are important for defining the limits of occurrence of broomrape. The program has always used a germination temperature of 20 °C for all testing. This is within the range that is generally optimal for broomrape species but there are no studies that have defined the germination temperature range for material assigned to *mutelii* (i.e. *O. mutelii*, *Phelipanche mutelii* or *O. ramosa* subsp. *mutelii*). For other *O. ramosa* a much broader range of temperature optima has been reported (Gibot-Leclerc et al 2004).

The aim of this experiment was to define the optimum germination for *O. ramosa* subsp. *mutelii* and the range of temperatures over which germination can occur.

Methods

For each trial, 100 sterilised broomrape seeds were added to filter paper on ten replicate petri dishes for each test temperature. The paper was moistened and the petri dishes sealed. They were placed in constant-temperature incubators for conditioning for seven days. At day 7, GR 24 was added and the dishes resealed and placed back in incubators. Germinated seeds were counted and removed at different time periods in each trial. Dishes were remoistened when necessary if they had dried out. The temperature within each incubator was measured using a T-Tech temperature logger, the germination temperature is the average logger temperature, logged at one minute intervals.

Trial 1. Temperatures: 5, 15, 20, 30 °C
Germination counts: 7, 14 and 20 days

Trial 2. Temperatures: 5, 15, 20, 34 °C
Germination counts: 4, 8, 11, 15 and 23 days

Trial 3. Temperatures: 10, 14, 21, 25, 32 °C
Germination counts: 2, 4, 7, 10, 14, 18, 21, 25 and 28 days

Trial 4. Temperatures: 18, 25, 28 °C
Germination counts: 2, 4, 7, 10, 14, 18 and 21 days.

Results

In Trial 1 and 2 there was no germination of seeds at 5 °C. In Trial 2 no seeds germinated at 34 °C. Germination proportion was greater at 20 °C than 15 °C (Fig. 1). These two trails were used to establish the upper and lower limits of germination temperature and Trials 3 and 4 were used to further refine the optimum temperature.

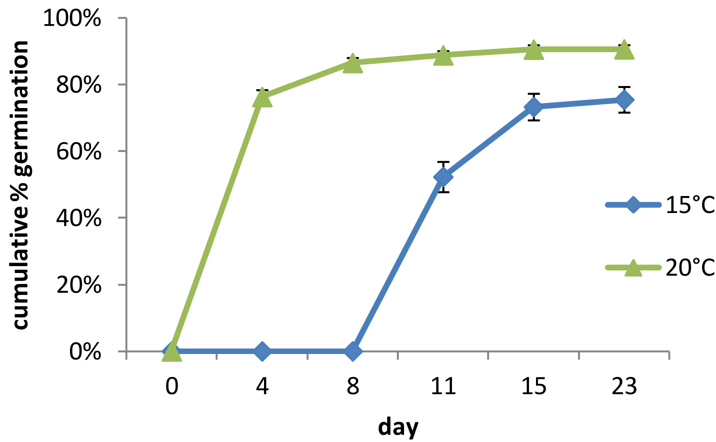

Figure 1. Results of Trial 2, values for 5 °C and 34 °C are not displayed on chart (all zero). Each point is mean ± 1 SE, n = 10.

Figure 2 shows the combined results of Trials 3 and 4. The optimum temperature for broomrape germination is 18 - 22 °C. Most germination at these temperatures occurred by Day 7. Temperatures slightly higher than this resulted in a marked decrease in germination and no germination was observed at temperatures greater than 28 °C. Some germination occurred at 10 °C but it was delayed. Germination at 14 °C reached a maximum of 40 % but during Trial 2 germination at 15 °C reached a maximum of 75% although no germination occured until 10 days after the stimulants were added.

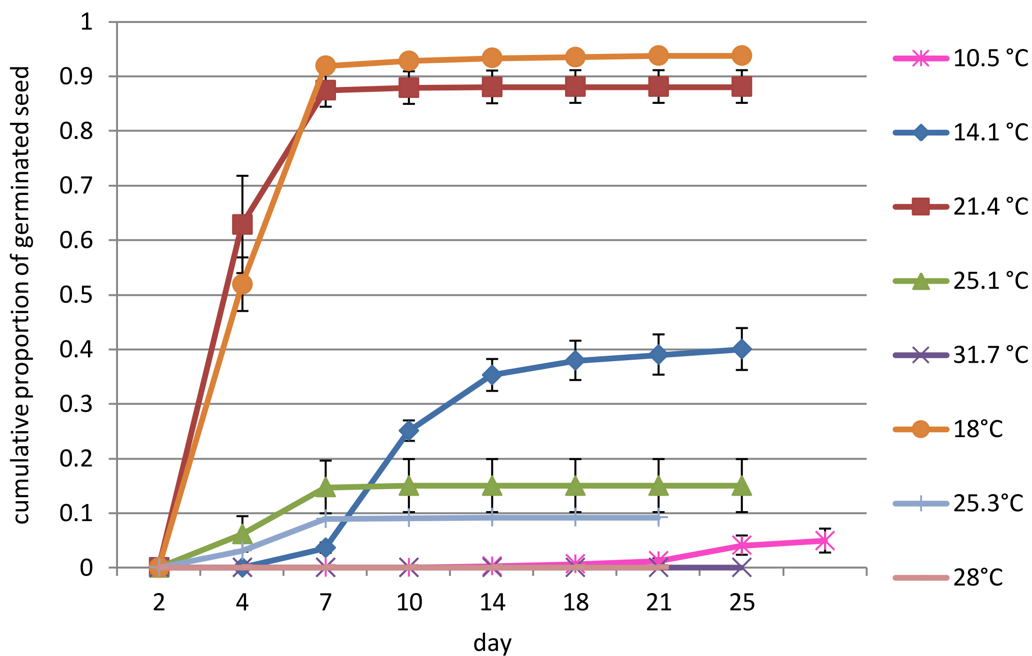

Figure 2. In vitro germination of *O. ramosa* subsp. *mutelii* at different temperatures in Trials 3 and 4.

Discussion

Although *O. ramosa* subsp. *mutelii* will germinate over a range of temperatures from 10 - 28°C the optimum temperature for germination is 18-22 °C. The proportion of seeds germinating outside these temperature optima is reduced. Seeds germinate rapidly at the optimum temperature but germination is delayed outside the optimum range.

The preferred germination temperature for *O. ramosa* subsp. *mutelii* is similar to temperatures reported for other *O. ramosa* although the range is narrower than that reported for *O. ramosa* in France. Gibot-Leclerc et al (2004) found that the lower limit of *O. ramosa* germination was 1°C and the upper limit 30 °C. The optimum temperature was 15-25 °C. Other reported temperature optima for *O. ramosa* are 18 °C (Song et al 2005), which is in agreement with our findings. Gonzalez-Verdejo et al (2005) found that *O. ramosa* germination at 24 °C occurred 3 days after stimulant addition and reached a maximum after 9 days.

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8. Conditioners and stimulants for seed germination

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Department of Water, Land and Biodiversity Conservation

November 2004

Report prepared by Jane Prider

Summary

Japanese research has indicated that the chemical norflurazon (the Group B herbicide Solicam), which inhibits carotenoid biosynthesis, can significantly reduce the conditioning period of broomrapes (Song et al 2005). Broomrape seeds normally need to be kept moist for 1-2 weeks before they will respond to chemicals from host plants which trigger germination. Laboratory experiments with branched broomrape showed that norflurazon can shorten the conditioning period and also increase overall seed germination when exposed to a germination stimulant (in this case the lab chemical GR24). Another Group F herbicide tested, diflufenican (Brodal herbicide), did not affect the conditioning period. Norflurazon is used as a soil persistent herbicide to prevent the growth of grasses and broad leaved weeds in citrus orchards. Whilst it showed promise as a tool to be applied in tandem with a germination stimulant, its persistence over several years leaves soils without vegetative cover and hence exposed to wind erosion. Hence it has not been pursued as a sustainable tool for eradicating soil seed banks of branched broomrape.

Introduction

Orobanche seeds require a period of moist stratification or conditioning at optimal temperature for several days before they are exposed to a germination stimulant. During this period seeds actively respire and various metabolic changes take place within the seed. These may include the biosynthesis of plant hormones and the formation or activation of receptors for germination stimulants (Joel et al 1995). The investigation of chemicals that may influence the germination and conditioning process has been an active area of research in the search for novel control methods for *Orobanche*.

A number of chemicals have been identified that can shorten the conditioning period or overcome suboptimal conditions of moisture availability or temperature during this period. Norflurazon and diflufenican are inhibitors of the enzyme phytoene desaturase, which catalyses the desaturation step of phytoene to phytofluene in the carotenoid biosynthesis pathway (Boger, 1996; Chae et al 2004). Carotenoids are precursors of the plant hormone abscissic acid (ABA), which at high levels inhibits seed germination. Norflurazon can enhance the germination of many plant species and can shorten the conditioning period for *Orobanche* (Chae et al 2004, Song et al 2005). Diflufenican had no effects on conditioning period of *Striga asiatica* but its effects on *Orobanche* conditioning are not known (Kusumoto et al 2006).

Orobanche seeds will only germinate in response to a germination stimulant released by the roots of plant roots. A number of these compounds have been identified and stimulants have also been synthesised that mimic them. Two synthetic stimulants, GR24 and Nijmegen 1, mimic the structure of strigolactone, a plant hormone that promotes parasitic plant germination (Johnson et al. 1976; Zwanenberg et al 2009). GR24 is mostly used for laboratory applications but Nijmegen 1 has been developed for control of parasitic weed seed banks (Nefkens et al 1997). It is designed as a suicidal germination agent, to add to the soil to stimulate parasitic weed germination before hosts are present.

Isothiocyanates are a group of allelopathic secondary metabolites produced by the roots of Brassicaceae. They have also been demonstrated to promote the germination of *Orobanche* species. The synthetic methyl isothiocyanate is used as a soil fumigant.

The aim of this series of trials was to identify products that influence the conditioning and germination process of *Orobanche ramosa* subsp. *mutelii*. A combination of approaches could then be adopted for the control of *O. ramosa* subsp. *mutelii* seed banks.

Methods

Lab trials were conducted to determine the best timing of conditioner application and the most appropriate concentrations of the chosen conditioners. A field experiment was also planned but this was not completed. Conditioners and germination stimulants as a seed bank control method therefore remain untested.

O. ramosa subsp. *mutelii* seeds were surfaced sterilized in 1% NaClO (Bleach) for approximately 3-5 minutes and rinsed several times with RO water prior to applying to glass filter disks. The seeds were then filtered and allowed to dry on the filter paper. To apply seeds to the glass filter paper in the Petri dishes, seeds were immersed in RO water and 400 µL of water seed mixture was applied to the glass filter disk. The same bleached seed lot was used for all treatments in this experiment.

A) Timing of conditioners

Two conditioners were tested and water was used as the control to determine the best timing for conditioner application.

Conditioner 1: The selective herbicide Brodal of which the active constituent is diflufenican.

Conditioner 2: The pre-emergence selective herbicide Solicam with the active ingredient norflurazon.

10^{-4} M of conditioner 1, conditioner 2 or water was applied to glass filter paper containing approximately 100 *O. ramosa* subsp. *mutelii* seed at 7, 3 and 0 days before the application of GR24. An additional water treatment was also included at 14 days before the application of GR24.

There were four replicates of each treatment, making a total of 40 glass Petri dishes. GR24 was applied on day 0 at a concentration of 5ppm ($5x10^{-5}$M) in 0.3M MES buffer.

B) Conditioner concentrations

Concentrations of 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M (or 1000ppm, 100ppm, 10ppm, 1ppm, 0.1ppm) of diflufenican and norflurazon were compared with water controls, to determine the optimal concentrations for a larger trial.

There were four replicates per treatment, with a total of 44 dishes used.

All dishes were prepared as above, using the same seed lots as above.

GR24 was applied three days after treatment.

C) Comparison of germination stimulants

One conditioner (Solicam) at a single concentration and timing determined by the preliminary experiments x 5 stimulants (Nijmegen, methyl iTC, phenyl iTC, acetone and GR24) x 5 concentrations of the stimulants: 0.01, 0.1, 0.5, 1 and 10 ppm. We also included water with all 5 stimulants and water/water and water/solicam controls. We used 4 replicates of each of these treatments. Set-up bleached seeds with

solicam or water (19/08/03) and applied the stimulants on 26/08/03, seed was counted 10 days after this on 5/09/03.

Results

Timing of conditioners

The addition of diflufenican or norflurazon during the conditioning period increased the overall percentage of broomrape seeds that germinated after GR24 was added compared to water alone (Fig. 1). Diflufenican increased germination compared with water when added 3 days before stimulant but not at 0 or 7 days. Norflurazon addition at 3 or 7 days before the addition of GR24 promoted more germination than addition on the day of GR24 application.

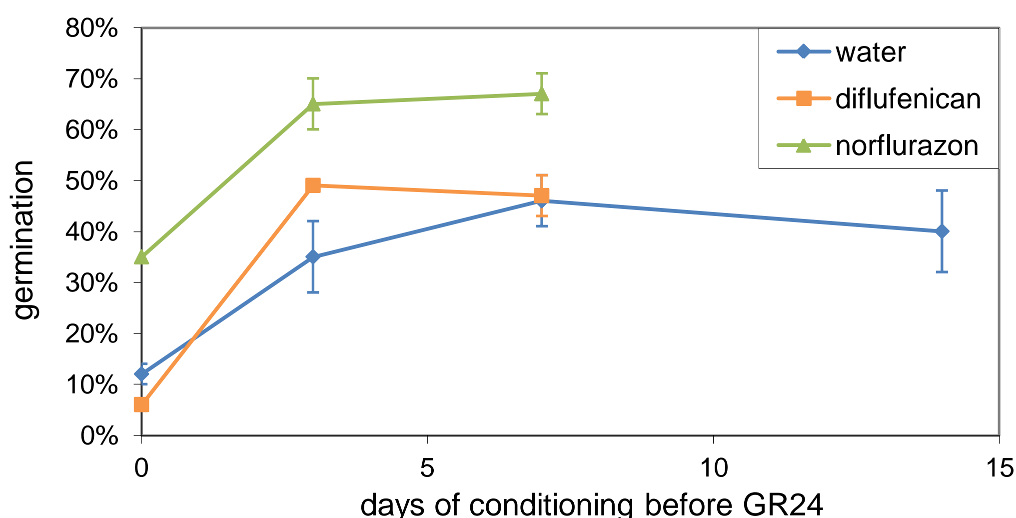

Figure 1. Influence of diflufenican and norflurazon addition on conditioning period length of *Orobanche ramosa* subsp. *mutelii*. The germination stimulant GR24 was added at Day 0. Points are means ± 1 SE, n = 4.

Conditioner concentration

Germination response to norflurazon concentration did not differ across the tested range. Very low concentrations of norflurazon added three days before GR24 addition increased the overall germination percentage (Fig. 2). In this trial the addition of diflufenican did not increase germination compared to water alone.

Comparison of germination stimulants

In the first trial, stimulants at different concentrations were tested after seeds had been conditioned with norflurazon. The highest germination was in response to the synthetic strigolactone analog GR24 and the isothiocyanate compounds (Fig. 3). The germination response to GR24 was strongly concentration dependent with highest germination at a concentration of 10 ppm. Methyl isothiocyanate inhibited germination at concentrations greater than 0.1 ppm. Phenyl isothiocyanate promoted 50-60 % germination across the range of concentrations measured. Germination was poor with the stimulants acetone and the synthetic strigolactone Nijmegen 1.

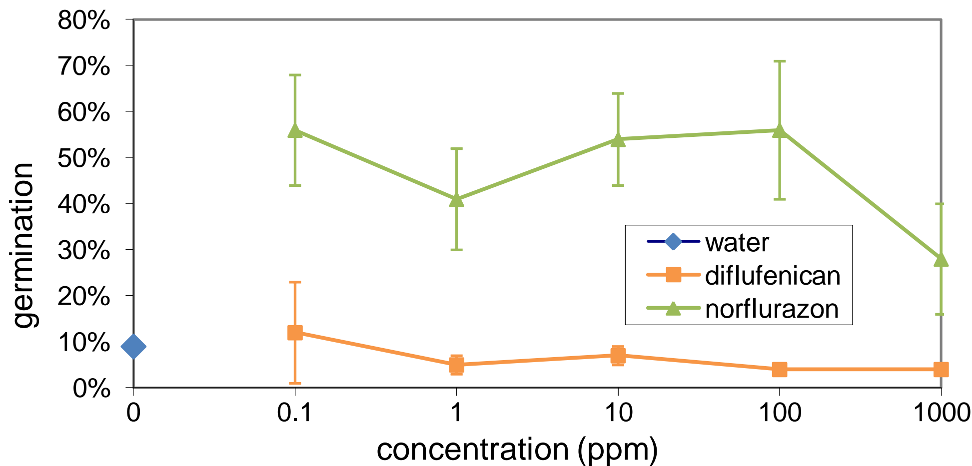

Figure 2. Germination response of *Orobanche ramosa* subsp. *mutelii* after GR24 addition for seeds conditioned for three days with water, or diflufenican and norflurazon of increasing concentration. Points are means ± 1 SE, n = 4.

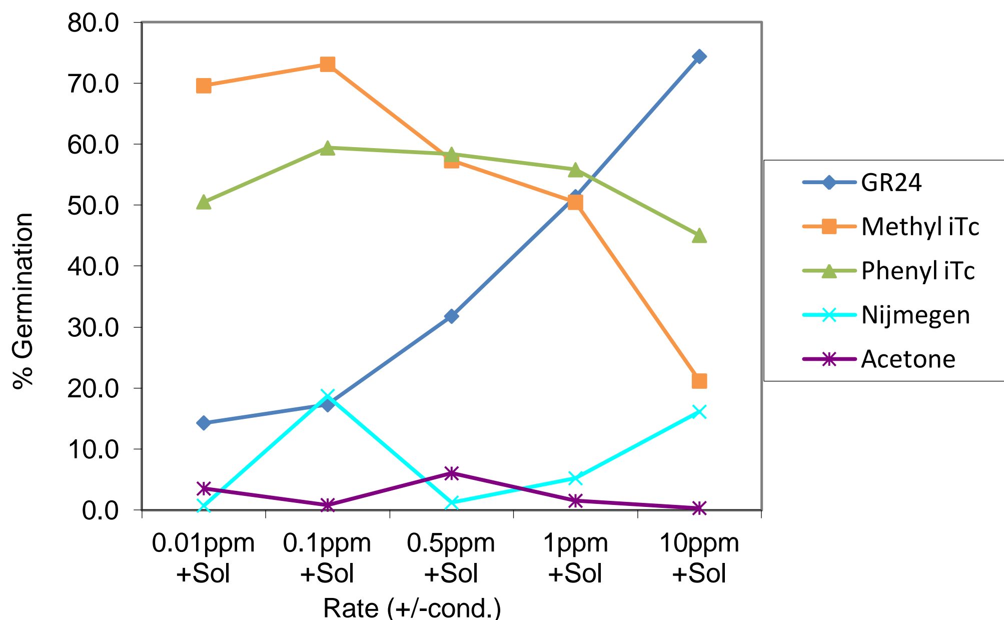

Figure 3. Germination response of *Orobanche ramosa* subsp. *mutelii* to different concentrations of germination stimulants added 7 days after conditioning with Solicam (norflurazon).

This trial also included the stimulants at a concentration of 1 ppm, conditioned without norflurazon for 3 days. A comparison of the 1 ppm treatments shows that norflurazon only had a promotive effect on germination with the poor stimulants acetone and Nijmegen 1 (Fig. 4). There was no germination in the water and Solicam alone treatments where no germination stimulant was added.

Further trails with GR24 and isothiocyanates demonstrate that the optimum concentration of GR24 for *O. ramosa* subsp. *mutelii* germination is 1 – 10 ppm (Fig. 5). The isothiocyanates are active at lower concentrations with stimulation decreasing at concentrations greater than 0.1 ppm and inhibition at the highest concentrations tested.

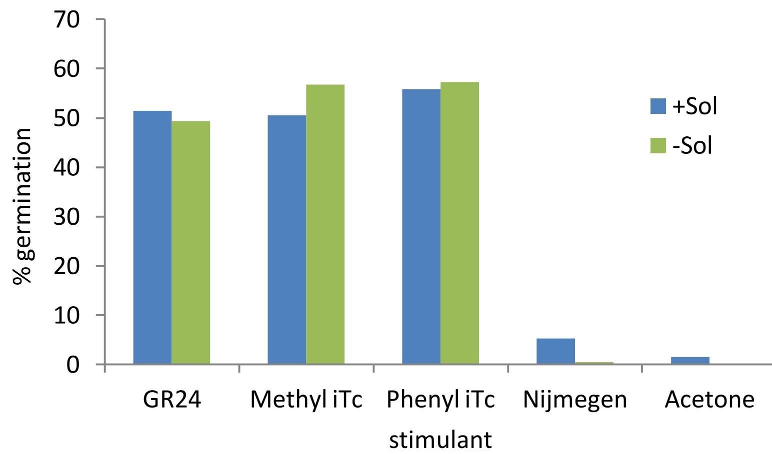

Figure 4. Effect of norflurazon applied during conditioning on germination of *O. ramosa* subsp. *mutelii* after application of different stimulants at a concentration of 1 ppm.

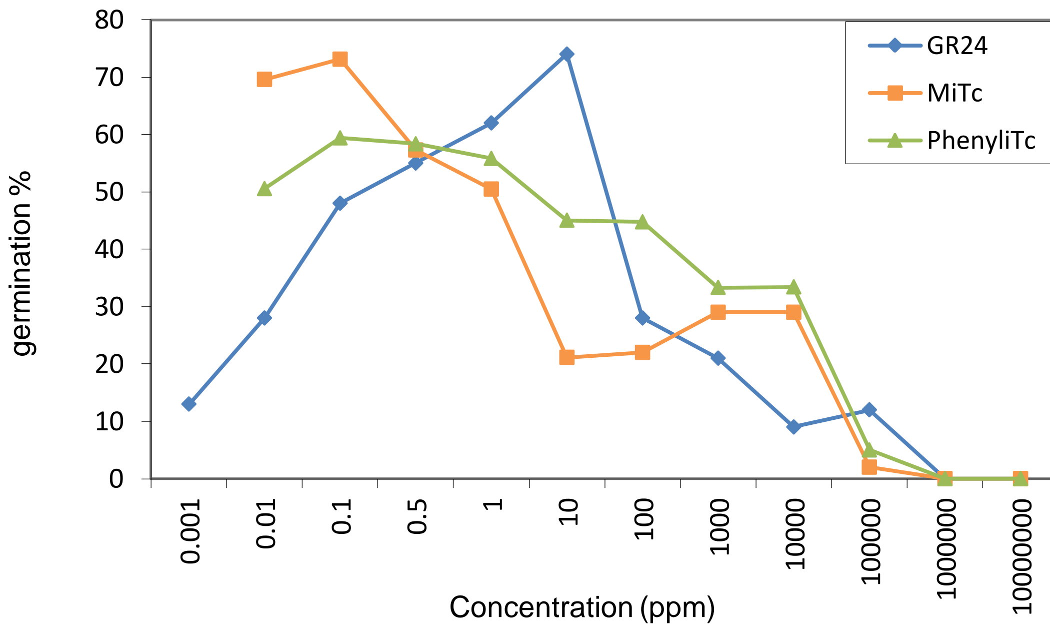

Figure 5. Comparison of germination stimulants across a broad range of concentrations on germination of *O. ramosa* subsp. *mutelii*. Each point is mean, n = 4.

Discussion

The addition of norflurazon to the conditioning medium shortened the conditioning period as a greater proportion of seds germinated following stimulant addition than when conditioned in water alone. The results were not always consistent as at times no increase in germination was observed. Diflufenican had only a limited effect on the conditioning period. Other studies have also found a decrease in the conditioning period and significantly higher germination of parasitic weed seeds following the addition of norflurazon (Chae et al 2004; Song et al. 2005; Kusumoto et al 2006) but not diflufenican (Kusumoto et al. 2006).

GR24 has long been in use as a germination stimulant of parasitic weeds (Johnson et al 1976). The compound is active at very low concentrations but inhbits germination at high concentrations (Wigchert et al 1999). This study found GR24 to be an effective stimulant for *O. ramosa* subsp. *mutelii* germination and the standard recommended concentration of 1 mg L^{-1} (Wigchert et al 1999) is used for laboratory work.

GR24 is not stable enough for use in control applications and another strigol analogue, Nijmegen 1, was developed for use in the field (Nefkens et al 1997). This compound stimulates the germination of *Orobanche crenata* (Wigchert et al 1999) but we found very limited germination stimulation of *O. ramosa* subsp. *mutelii*. No further trials of this compound for control of seed banks were done.

The isothiocyanates were also very effective stimulants of *O. ramosa* subsp. *mutelii* germination. Further work on the stimulants produced by Brassicas is presented in Section 2.2. The use of methyl isothiocynate for the control of *O. ramosa* subsp. *mutelii* is described in Section 8. The fumigation process uses high concentrations of methyl siothiocayante to kill seeds rather than low concentrations that may promote suicidal germination.

Norflurazon is used as a soil persistent herbicide to prevent the growth of grasses and broad leaved weeds in citrus orchards. Whilst it showed promise as a tool to be applied in tandem with a germination stimulant, its persistence over several years leaves soils without vegetative cover and hence exposed to wind erosion. Hence it has not been pursued as a sustainable tool for eradicating soil seed banks of branched broomrape.

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9. The influence of wet and dry cycles on dormancy release

Jane Prider and Andrew Craig

Branched Broomrape Eradication Program

February 2012

Introduction

Freshly produced broomrape seeds are in primary dormancy where they are not responsive to germination stimulants (Bista *et al.* 2004; Kebreab and Murdoch 1999). This dormancy is released over a period of conditioning, or warm stratification which occurs after the seeds are released from the mother plant (Matusova *et al.* 2004). Like other winter annual species, dormancy release in *Orobanche* occurs during summer when seeds become increasingly receptive to germination stimulants (van Hezewijk *et al.* 1994). Under continued conditioning, dormancy is induced and seeds are no longer receptive until they dry out again (Gonzalez-Verdejo *et al.* 2005). This produces cycles of dormancy release and induction, or cyclic dormancy in the seed bank (Kebreab and Murdoch 1999).

Fluctuations in seed moisture content are experienced by many water-permeable seeds during periods of sporadic rainfall. Accelerated dormancy release after a period of hydration followed by drying during the after-ripening stage, has been observed in a number of species (Baker *et al.* 2005; Gallagher *et al.* 2009; Hoyle *et al.* 2008; Lush *et al.* 1981). These wetting and drying cycles are being increasingly recognised as important for dormancy release in winter-annual or ephemerals from areas with Mediterranean climates, i.e. hot, dry summers followed by wet winters, but have not been examined in broomrape. Plakhine *et al.* (2009) suggest that following pre-season rainfall events, imbibed parasite seed enters a “stand by” state ready to respond rapidly later in the season when host roots release germination stimulants. Temperature during the after-ripening period is also important. High temperatures prior to the conditioning period reduce the moisture content of seed subsequently promoting germination (Mohamed *et al.* 1998) but higher temperatures during conditioning can also increase dormancy release in *Orobanche* (Kebreab and Murdoch 1999; Mohamed *et al.* 1998).

Although these wetting and drying cycles are important for release from primary dormancy, there is limited evidence that such processes are also important drivers of cyclic dormancy in the seed bank. Seed burial over summer promoted the germination of broomrape seed (van Hezewijk *et al.* 1994), a finding which supports our own seed burial experiments. In the Quarantine Area, investigation of the relationship between rainfall and broomrape re-occurrence at the paddock level revealed that broomrape was more likely to re-occur in a paddock after high summer rainfall. Rainfall during the growing season showed no correlation with paddock re-infection. Rainfall events that are large enough to maintain soil moisture for periods sufficient to condition seeds may result in more rapid dormancy release than dry conditions or smaller rainfall events that are insufficient for conditioning. Higher soil temperatures during these wetting and drying cycles may also increase dormancy release by decreasing seed moisture content during drying cycles, further contributing to dormancy release.

This series of laboratory experiments will replicate various summer rainfall scenarios to determine their influence on dormancy release:

1. Do single large rainfall events result in greater dormancy release than several smaller rainfall events?
2. Do higher temperatures during drying cycles increase dormancy release?

Objective

The objective of this project was to determine the effect of short-term conditioning periods on dormancy release of branched broomrape.

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

- Understand the influence of summer rainfall conditions (if any) on broomrape infection at the paddock level
- Predict broomrape seasons that are likely to have greater paddock return rates
- Evaluate the effectiveness of control methods given variations in summer rainfall (if summer rainfall is found to be an important determinant of dormancy release)

Although there have been many studies to determine the optimal conditions for broomrape seed germination, no research has actually addressed the influence of pulsed rainfall events on dormancy release. This project addressed an important gap in international research on broomrape ecology.

Method

Wet/dry cycles

We used a 16 week hydration period as that approximates the period from broomrape seed release until germination.

Seasonal correlations showed that rainfall of over 100 mm from January to the break of season was critical (see Section 7.2 and 7.3). For two of the high rainfall summers there have been very large rainfall events in February (2000 & 2003). The other high rainfall summer in 2001 had moderate rainfall events spaced out evenly. In the drier years there were no large rainfall events over 10 mm. In between years have had early large events (in January 2005, 2007) or late March in 2006. It will be assumed that the large rainfall events wet the soil profile to seed depth for a number of days but smaller rainfall events are insufficient for prolonged wetting. As Bar-Nun & Mayer (2003) found that there was a respiration peak after 3 days of conditioning, and *O. ramosa* seeds need approximately 7-8 days to condition, two hydration periods were set at 2 days and 8 days. The 8 day hydration periods occured once to simulate a single large rainfall event and at different times in the cycle, either early, mid or late season. The same hydration period was used for the 'low rainfall' scenario but split into four 2 day hydration events. A further non-hydrated treatment was a control.

Experiment design

The experiment design had two factors: hydration period and temperature. Soil temperatures in the field during summer at 10 cm depth ranges between 20 and 40 ^{o}C, although higher temperatures typically occur in early January and by break of season the maximum temperature is 20 ^{o}C. A temperature of 30 ^{o}C was tested against a control temperature of 20 ^{o}C for the experiments.

For each temperature (20 ^{o}C and 30°C) and five replicates the following hydration schedule was followed:

Table 1 . Hydration schedule

Treatment	Week 1	Week 5	Week 9	Week 13	Week 17
A	2d	2d	2d	2d	condition
B	8d	0	0	0	condition
C	0	8d	0	0	condition
D	0	0	8d	0	condition
E	0	0	0	8d	condition
F	0	0	0	0	condition

The seeds used for the experiment were collected from a site at Burdett in 2009. This seed had an average viability of 85%.

Seeds were prepared and placed in petri dishes as per the usual protocols. During the hydration period the treatments were incubated at 20 ^{0}C but the appropriate treatments were transferred back to the 30 ^{0}C incubators for drying periods.

Assessing germination

At the end of week 16 the seeds were conditioned for 14 days. With dormancy release, broomrape seeds become increasingly responsive to germination stimulant. Seeds with greater dormancy release will respond to lower concentrations of the germination stimulant GR 24 (Matusova *et al.* 2004). Our other work has found that that the optimal concentration for non-dormant seed is 10 ppm. Germination percentage of each replicate was assessed at a range of GR 24 concentrations (0.001, 0.01, 0.1, 0.5, 1, 10, 100 ppm).

Seed moisture

The moisture status of seeds was measured prior to each hydration cycle. Initially, samples of each seed lot were weighed and then reweighed after drying at 103 ^{0}C. Further replicates were maintained at each experimental temperature and weighed prior to each hydration treatment. These weights were compared to the initial weight.

Results

Seed moisture content

The seeds had an initial moisture content of 3.79 %. Seeds maintained at 30 ^{0}C had lower moisture content than seeds kept at 20 ^{0}C (ANOVA $p < 0.001$, 2.5% and 4.1% respectively). Experimental treatment had a significant effect on moisture content (ANOVA, $p = 0.01$). The seeds hydrated later (mid late – late) or non-hydrated had a lower moisture content than seeds that received the frequent 2 day wetting cycle (Fig. 1).

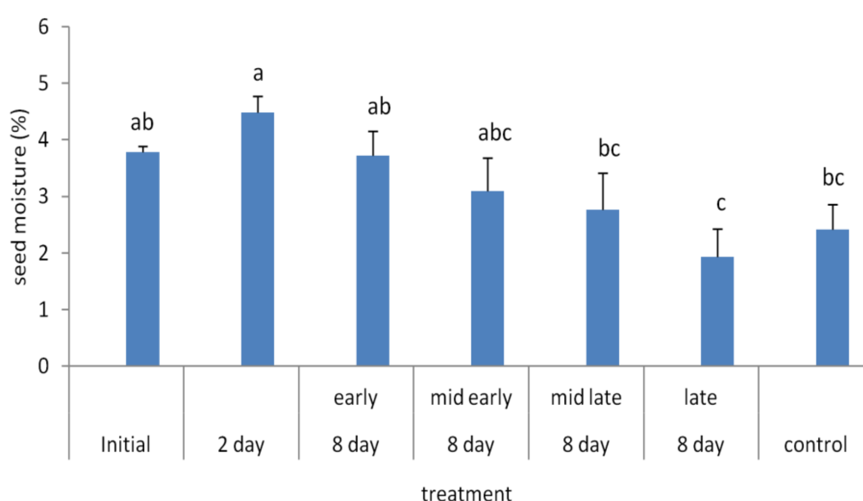

Figure 1. Moisture content of seeds subject to different wetting treatments prior to conditioning at week 17. The last wetting treatment occurred at Week 13 for the "2 day" and "8 day late" treatments. Bars are means + 1SE (n = 8, initial n = 4). Bars labelled with different letters were significantly different at α< 0.05 (LSD test).

Germination

In the 20 ^{0}C treatments, seeds that were non-hydrated or received an 8 day hydration at the start of the cycle responded to lower concentrations of GR24 and also had a higher germination percentage across the range of GR24 concentrations tested (Fig. 2). Treatments that were wet at the end of the cycle or had four short hydration periods throughout the cycle responded to higher concentrations of GR24 and had a lower overall germination percentage.

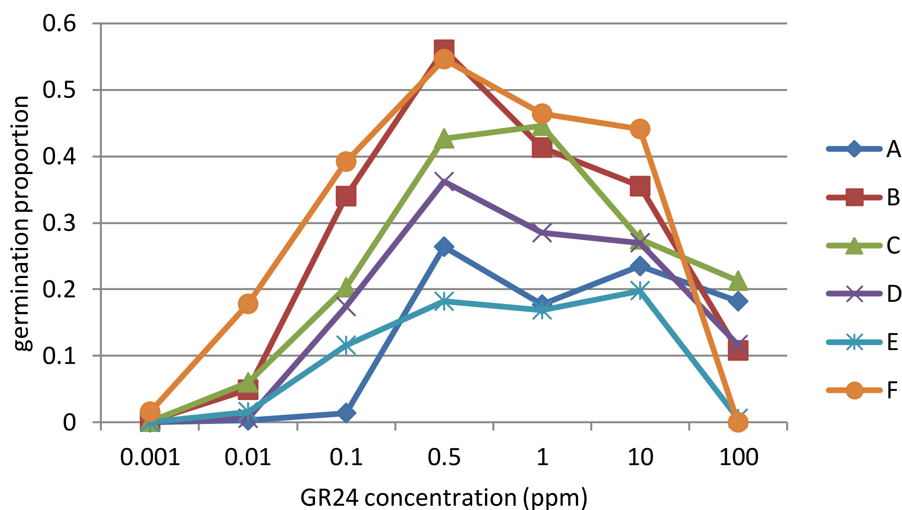

Figure 2. Germination response of broomrape seeds to increasing concentrations of GR24 after different wet/dry cycles at 20^{0}C. See Table 1 for treatment descriptions. Each point is the mean, n = 5.

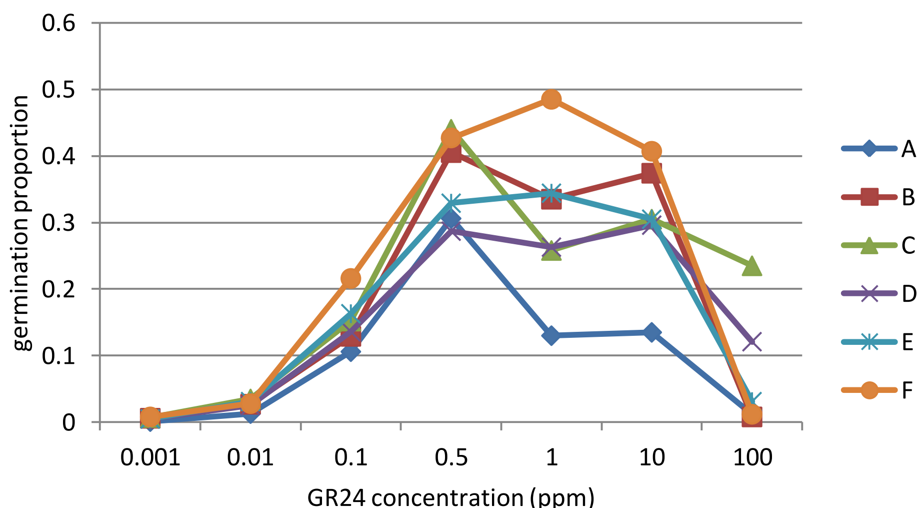

Figure 3. Germination response of broomrape seeds to increasing concentrations of GR24 after different wet/dry cycles at 30^{0}C. See Table 1 for treatment descriptions. Each point is the mean, n = 5.

In the 30 °C treatment, there was a very low germination response at GR24 concentrations of 0.001 to 0.01 ppm (Fig. 3). There was very little difference in response to GR24 below concentrations of 1 ppm. At 1 ppm GR24 the highest germination response occurred in the non-hydrated treatment. Germination was similar among the 8 day hydration treatments and lower in the repeated 2 day hydration treatment.

Discussion

Overall germination was poor for the experiment as typically maximum germination of at least 90% is observed for laboratory-stored seed. With a seed viability of 85% it was expected to observe at least 75-80% germination in our experiment. Germination was very poor in two of the replicates but was as expected in the other three replicates. However although this increased the variability, the relationships between treatments remained unchanged.

It was expected that hydration status would have a greater effect at higher temperatures but the opposite was observed with responses observed at 20 °C but not at 30 °C. In addition, the highest germination response came from the non-hydrated seeds, then by seeds that had been hydrated early followed by a long drying period. These seeds were not the driest at the time of hydration as we found that the seeds that were hydrated last had the lowest moisture content. However, mass change in very small broomrape seeds is difficult to measure with precision.

Our results support the hypothesis that large rainfall events (equivalent to the 8 day wetting periods) may release a higher proportion of broomrape seeds from dormancy than the same amount of rainfall over several shorter periods (the 4 X 2 day wetting periods). In addition, seeds were more responsive to germination stimulant (i.e. at a lower concentration) when this long wetting period occurred several weeks before. This could partly explain the correlation we found between summer rainfall and broomrape occurrence. However, in the district, recharge of soil moisture during summer increases winter crop yields, therefore improved host growth following wet summers may also promote further broomrape establishment.

10. Seasonal changes in germination response of buried branched broomrape seeds

Jane Prider and Andrew Craig

Biosecurity SA

June 2013

Summary

Field buried *Orobanche ramosa* subsp. *mutelii* seed banks undergo a period of dormancy during winter and spring when a low proportion of recovered seeds germinate even after exposure to optimal germination temperatures and stimulant. Seeds retrieved during summer and autumn germinate readily when exposed to the same conditions and have little or no dormancy. Laboratory experiments in tubs of soil demonstrated that temperature and soil moisture were critical in the induction and release of *O. ramosa* subsp. *mutelii* dormancy. All seeds maintained for 8 weeks in moist soil at alternating temperatures of 5/15 °C, similar to soils during winter, became dormant. A high proportion of seeds in moist and dry soils maintained at alternating temperatures of 15/28 °C typical of summer soils, germinated at sub-optimal and optimal germination temperatures. Seeds kept in soil at an optimal temperature of 10/22 °C, similar to autumn and spring, had a moderate proportion of dormant seeds in wet soils but not in dry soils. *O. ramosa* subsp. *mutelii* shows a distinctive winter-active annual ecology. Germination is predicted to be very responsive to rainfall events at the start of the autumn growing season. Dormancy in moist soils in late winter and spring and a lack of germination at high soil temperatures restricts germination to autumn and perhaps the month to either side of this season depending on seasonal rainfall.

Introduction

Seeds that form persistent soil seed banks are subject to seasonal fluctuations in temperature and soil moisture that can affect dormancy and cue germination. For many winter-active species, dormancy is induced during winter and spring and released in summer and autumn. This is an adaptation to avoid germination when conditions may not remain favourable for long enough to complete reproduction. Although the germination response of *Orobanche* species is cued by the release of stimulatory compounds from host roots, soil temperature and moisture also affect germination even in the presence of these compounds (Van Hezewijk et al. 1994). Secondary dormancy occurs when seeds fail to germinate under favourable conditions. In *Orobanche*, dormancy is induced as temperatures decrease in moist soils during the winter. Dormancy release occurs gradually as temperatures increase during the summer months (Van Hezewijk et al. 1994). This produces periods of dormancy induction and release, or cyclic dormancy in the seed bank (Kebreab and Murdoch 1999b).

The only published studies of germination of *Orobanche* seed following burial in the field are of *O. crenata*. This species loses dormancy in the Middle East and Spain in non-irrigated fields during autumn and seed becomes dormant again from late winter (Van Hezewijk et al. 1994, Lopez-Granados and Garcia-Torres 1999). Laboratory experiments with *O. ramosa* in France showed that seed became dormant after prolonged periods at either 5 °C or 30 °C but not at 20 °C (Gibot-Leclerc et al. 2004). Our own laboratory experiment found that *O. ramosa* subsp. *mutelii* seed does not germinate after 8 weeks at 15 °C (see Chapter 4.4), which indicates dormancy may also occur during winter.

Information about seed bank dormancy is critical for an understanding of the population dynamics of *O. ramosa* subsp. *mutelii*. This will enable predictions to be made of the risk of occurrence of the weed in

crops or other parts of Australia where growth conditions are outside the typical winter-growing season of the SA Murray mallee.

The aims of this study are to determine the influence of seasonal changes in soil moisture and temperature on the germination of *O. ramosa* subsp. *mutelii* from the soil seed bank.

Methods

Field buried seeds

Seed sachets prepared and buried for the seed longevity experiment were exhumed at 3 month intervals during the first three years of burial from April 2008 to October 2011. Details of the method are reported in Section 5.5. The data reported here are the proportion of viable seeds that germinated.

Rainfall records were recorded at the Mannum Trial site, located approximately 2 km from the seed burial site at Haby Hut Road. Soil moisture and soil temperature records at the site were obtained from 2 temperature sensors and 2 gypsum block moisture sensors buried at 5- 10 cm below the soil surface and logged hourly (Environdata WeatherMate DL40 system). Records were available from April 2008 until April 2010.

Laboratory experiment

This fully factorial experiment examined the effects of three factors on seed germination following seed burial:

- Temperature during burial
- Soil moisture during burial
- Temperature during conditioning and germination

Field collected soil was sterilized in an autoclave and then placed in 1 litre plastic tubs. Seed sachets containing 5 ml sand and approximately 500 *O. ramosa* subsp. *mutelii* seeds were buried in the centre of the tubs. The tubs were maintained at three different gravimetric moisture levels, with five replicate tubs for each moisture level:

- Dry - 5% soil moisture by weight
- Wet – 20% soil moisture by weight
- Cycle – 5/20% soil moisture by weight, soil watered to 20% and left to dry down to 5% by weight before watering again to 20%

Moisture levels in tubs were maintained by weighing and adding water when necessary, usually once or twice per week. These tubs were initially used for another experiment so tubs were maintained under these watering regimes for 6 months and left dry for 6 months before the experiment commenced.

For this experiment, tubs were stored in incubators under three different storage temperature regimes that simulated winter, summer and spring/autumn conditions:

- Low (winter) 15/5 °C
- Optimal (autumn/spring) 22/10 °C
- High (summer) 28/15 °C

Temperatures alternated between the two temperatures at twelve hour intervals.

Tubs were maintained under these soil moisture and temperature conditions for 8 weeks. Sachets were retrieved from tubs and left to dry before the seeds were separated from the sand in the sachet by floating

the contents in 40% calcium chloride solution. Seeds were sterilized in 1% sodium hypochlorite and left to dry.

The seeds from each sachet were divided equally onto three 25 mm diameter filter papers in 9 cm petri dishes and 200 µl of RO water was added. The dishes were sealed and placed in either a summer, winter or autumn/spring incubator. Seeds were conditioned for 7 days before 200 µl of GR24 was added. Dishes were returned to the incubators for a further 14 days before germination was scored. We report here the proportion of all seeds that germinated. Viability of seed was approximately 90%.

Methodology check

Applying a conditioning treatment to seeds after retrieval could affect the dormancy of seeds and hence the proportion of seeds that germinate after the addition of GR24. To check this, 5 seed sachets were exhumed from the burial site on August 1st 2013 and 5 more sachets on October 9th 2013. After the seeds had been separated from the soil, the seed from each sachet was divided into two lots. One lot of seeds received 14 days of conditioning prior to the addition of GR24. In the other lot, GR24 was added immediately. For the August retrievals there was no difference in the proportion of germinating seeds in conditioned or unconditioned treatments (ANOVA, $p = 0.34$). In the spring retrievals there was less than 0.5% germinated seeds in the unconditioned treatment but no germination in the unconditioned treatment. Germination in unconditioned seeds was significantly greater than zero (t-test, $p = 0.012$). Conditioning seeds after collection could therefore have a very minor effect on dormancy in some seasons.

Analysis

Data from the laboratory experiment was tested for significant treatment effects using an unbalanced Analysis of Variance (ANOVA) in Genstat (Ver. 9.1.0.147). Treatments with mostly zeroes were excluded from the analyses and tests were conducted on log normal transformations of the remaining data. The germination tests were run in three batches over consecutive days so batch was included as a blocking or nuisance variable in these tests. We tested for the effects of soil storage regime, soil moisture and germination temperature regime and their interactions. Non-significant interaction terms were sequentially removed from each model to produce the minimal model with best fit.

Results

Field buried seeds

There were strong seasonal cycles in soil temperatures ranging from maxima of more than 30 °C in January and February, to minima of less than 10 °C in July (Fig. 1A). Summer soil temperatures were more variable in 2009-10 than in 2008-9 which corresponded to more rainfall events in 2009-10 and a very dry summer in 2008-9 (Fig. 1B). Small rainfall events occurred throughout the year with larger rainfall events typically in summer. Soil moisture fluctuations closely correlated to rainfall events although there were some unreliable data from the gypsum block sensors when first deployed in 2008 and in the very dry soil in the summer of 2008-9 (Fig. 1B). The actual values of soil moisture are indicative only as they have not been calibrated for soil type.

A high proportion of seeds did not germinate from sachets retrieved from the field in July, particularly in the second and third years of burial (Fig. 2). Germination was also low in October but most seeds germinated form sachets retrieved in January and April, with the exception of April 2011. Large rainfall events in December 2010 and March 2011 were exceptional for this experimental period and may have affected germination patterns in 2011.

Seed viability was maintained above 80% during this period so seeds that did not germinate were viable.

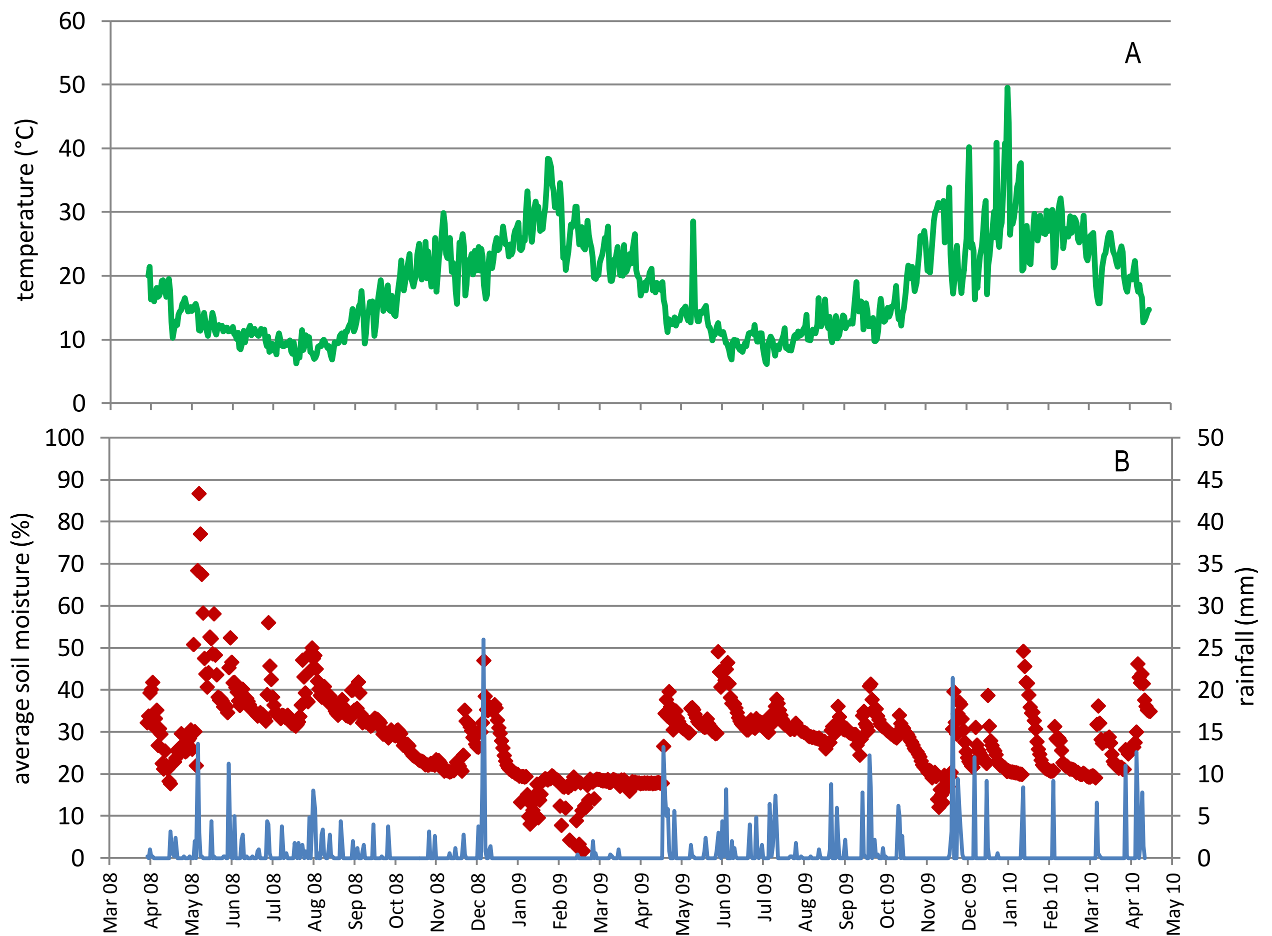

Figure 1. A) Average daily soil temperature at 5-10 cm depth and B) average daily soil moisture recorded at the field seed burial site on Haby Hut Road. The bottom chart also shows the daily rainfall records for the nearby Mannum Trial Site over the same period.

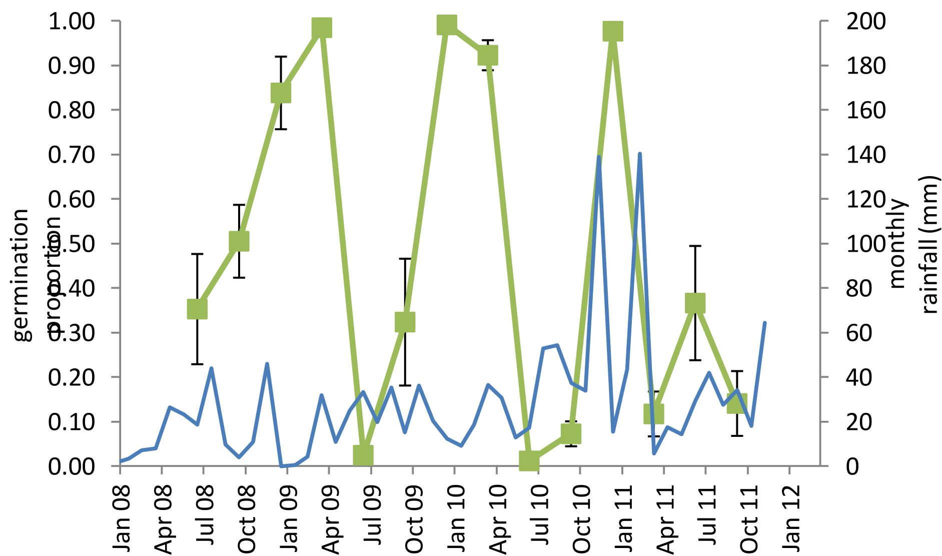

Figure 2. Germination of field-buried seeds retrieved at 3 month intervals and germinated at optimal conditioning and germinating temperatures (mean ± 1 SE, n = 5). Monthly rainfall for Mannum is shown on the second axis.

Laboratory experiment

There was a significant difference in germination of seed processed on different days (batch, Table 1) so this was taken into account in the statistical analysis.

Seed stored in the winter temperature regime in moist soils for 8 weeks became completely dormant. Seed stored in relatively dry soils of 5% soil moisture also had a very high proportion of seeds that did not germinate when subjected to optimal conditions for conditioning and germinating (Fig. 3).

Seed stored under summer temperatures did not become dormant, including seed that was stored in very moist soils although the germination response of these treatments was more variable (Fig. 3). For summer-stored seed, there was no significant difference in the germination proportion for seeds conditioned or germinated under either the winter or autumn/spring temperature regimes (germination regime, Table 1). Winter minimum temperatures of 5 °C are sub-optimal for germination (see Section 3.7) but this did not affect overall germination proportion.

Table 1. Results of ANOVA on the effects of temperature regime and soil moisture during storage and temperature regime during germination on the germination of *O. ramosa* subsp. *mutelii* seed. The Levels treatment levels of winter storage regime and the summer germination regime were omitted from the analysis as most values were zero.

Effect	df	ss	ms	vr	F pr
batch	2	1.3608	0.6804	47.48	<0.001
storage regime	1	2.11568	2.11568	147.63	<0.001
germination regime	1	0.00994	0.00994	0.69	0.409
soil moisture	2	0.13811	0.06905	4.82	0.012
storage regime X soil moisture	2	0.2365	0.11825	8.25	<0.001
Residual	51	0.73086	0.01433		
Total	59	4.59189	0.07783		

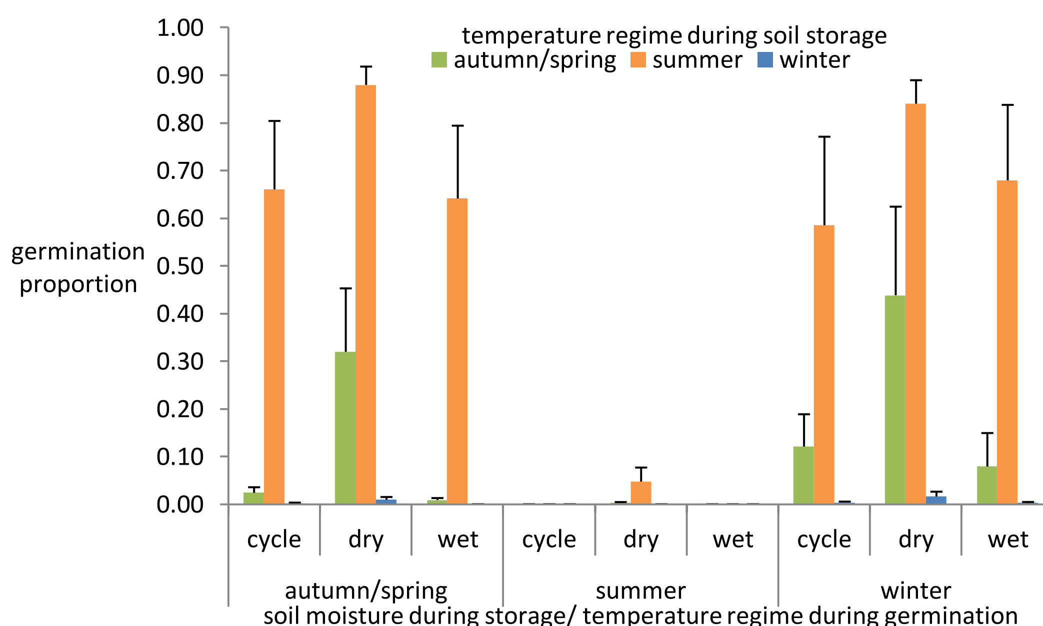

Figure 3. Germination of seed in laboratory stored for 8 weeks in three seasonal temperature regimes and soil moisture levels and later conditioned and germinated at three seasonal temperature regimes

Regardless of seed storage temperatures, seed conditioned or germinated under the summer temperature regime failed to germinate, with the exception of a small proportion of seed in the dry summer storage regime (Fig. 3).

Seed stored in the autumn/spring temperature regime had significantly higher dormancy than the summer-stored seed (storage regime, Table1). The autumn/spring stored seed also showed a significant response to soil moisture level (storage regime X soil moisture, Table 1). Seed stored under moist conditions, either constantly wet or cycling between wet and dry, had a higher proportion of dormancy than seed stored at low soil moisture levels (Fig. 3).

Discussion

The field and laboratory results show the strong influence that seasonal changes in temperature and soil moisture have on the induction and release of secondary dormancy in *O. ramosa* subsp. *mutelii*. Sustained periods of cold weather are predicted to induce maximum dormancy in seeds even if soils are not very moist. We found low levels of germination from field and experimental buried seeds under winter storage conditions. Although dormancy is released during the high temperatures of summer, even in the presence of adequate soil moisture and available hosts, broomrape seeds are not likely to germinate in the high soil temperatures of summer. These findings indicate that *O. ramosa* subsp. *mutelii* demonstrates a distinctive winter-active annual ecology. Seeds germinate in autumn after dormancy is gradually released over the warm summer months when temperatures are too hot for germination. Seeds gradually become dormant again in the cooler soils in winter so that very little germination occurs during spring even though temperatures are optimal for germination. Given the unpredictability and lack of clear seasonality in rainfall in the area where broomrape occurs, this information gives valuable insight into the risk of broomrape emergence during different seasons. Early breaking seasons are likely to provide the most risk. At this time the majority of seeds are non-dormant and if rainfall occurs when soils are still relatively warm this will provide ideal conditions for seed germination provided hosts are available. Rainfall during autumn is also significant as this can induce early dormancy in a high proportion of seeds.

The dormancy patterns of *O. ramosa* subsp. *mutelii* are different to *O. crenata*. Although *O. crenata* has peak dormancy loss in autumn like *O. ramosa* subsp. *mutelii*, *O. crenata* seed remains dormant through summer (Van Hezewijk et al. 1993, Lopez-Granados and Garcia-Torres 1999, Grenz and Sauerborn 2007). Nor does dormancy concur with that of *O. ramosa* subsp. *ramosa*, as assessed in a pot experiment (Grenz and Sauerborn 2007). They found only a small proportion of subsp. *ramosa* seed became dormant at 4 °C after 8 weeks whereas we found complete dormancy of subsp. *mutelii* in wetter soils at the higher average temperature of 10 °C in our winter treatment. Prolonged high temperatures may induce dormancy in *O. ramosa* subsp. *mutelii* as has been found with *O. ramosa* subsp. *ramosa* in France which became dormant after conditioning for 100 days at 30 °C (Gibot-Leclerc et al. 2004), but lack of regular rainfall during the summer months makes this unlikely as seeds would not remain imbibed. However, this may have occurred in March 2011 when we measured a low germination proportion of seeds retrieved from the field in April. In the same season, the high rainfall in December failed to induce dormancy for seeds retrieved in January suggesting that the period of warm wet stratification was not sufficient for dormancy to be reduced this early in the season. Dormancy induction occurs faster at lower temperatures (Van Hezewijk et al. 1993) and we found in our conditioning experiment (see Section 3.6) that although this conditioning period resulted in complete dormancy of seeds at 15 °C it did not induce full dormancy at 20 °C.

Although in this experiment we did not compare fluctuating germination temperatures with constant temperatures, comparison with our other germination temperature study shows that the alternating temperatures did not result in either a decrease or increase in overall germination. In the winter (mean 10°C) and autumn/spring (mean 16°C) germination treatments where seeds were kept at sub-optimal temperatures for 12 hours daily and optimal for 12 hours, germination was not reduced. Conversely, in the summer treatments where the temperature was optimal for 12 hours daily but super-optimal for 12 hours, no germination occurred. This is contrary to the results with other *Orobanche*, *O. crenata*, *cernua*,

aegyptiaca, *minor*, *cumana*, where alternating temperatures with a mean above or below the optima had lower germination (Foy et al. 1991, Kebreab and Murdoch 1999a) . As observed by Kebreab and Murdoch (1999), the time spent at temperature maxima has the most constraint on germination. Consequently seed near the surface is less likely to germinate, especially during the warmer months.

Grenz and Sauerborn (2007) postulated that the potential geographic range of *O. crenata* was restricted due to the narrow range of soil temperature and moisture conditions under which seed was not dormant. The lack of dormancy of *O. ramosa* subsp. *mutelii* during the summer months may extend its distribution into warmer areas where hosts and soil moisture are suitable, although temperatures below 25°C are required for germination . Unlike *O. ramosa* subsp. *ramosa*, the range would be limited by colder temperatures.

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