

Intensive cropping systems select for greater seed dormancy and increased herbicide resistance levels in *Lolium rigidum* (annual ryegrass)

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Abstract

BACKGROUND: *Lolium rigidum* (annual ryegrass) is a widespread annual crop weed that has evolved high levels of resistance to selective herbicides. Anecdotal evidence suggests that intensive cropping also leads to higher seed dormancy in *L. rigidum*. This was quantified by measuring dormancy levels in *L. rigidum* populations collected from paired sites (one with nil to low cropping intensity, the other intensively cropped) located throughout the Western Australian grain belt.

RESULTS: Populations from non-cropped fields or those with low cropping intensity showed higher and faster germination than populations from fields with a medium- or high-intensity cropping regime. Resistance to selective herbicides was also higher in the medium- and high-intensity cropping fields than in the low-intensity cropping fields.

CONCLUSION: High-intensity cropping systems are likely to impose greater selection pressures for seed dormancy and selective herbicide resistance, because late-emerging seedlings avoid preplanting weed control practices (tillage and non-selective herbicide application) but are exposed to selective in-crop herbicides.

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Keywords: cropping intensity; cropping systems; evolution; herbicide resistance; seed dormancy; weed management

1 INTRODUCTION

Agricultural practices have imposed or exerted selective forces on agricultural weeds over a long period of time, allowing the weeds to escape control measures. There are now many adaptive strategies existing in weed populations that have resulted from different agricultural systems (e.g. crop mimicry to avoid hand-weeding; dwarf stature to avoid reaping: reviewed in Barrett¹), with arable fields providing a different environment to that of surrounding natural areas. Traits such as herbicide resistance or seed dormancy are selected for over time through what is often referred to as 'selection pressure'; in this case, herbicide use and (for example) soil cultivation before or during crop sowing. If the selection pressure is consistently maintained, then the selected traits become evident in the weed population. The selection for an adequate range of variation for the trait in a weed population over time is an example of adaptation to permit survival of the population under cropping conditions.

Wheat-dominated, rainfed grain cropping occurs annually on 25 million ha in temperate southern Australia. In these crop fields, the major weed is the obligate cross-pollinated *Lolium rigidum* Gaud. (annual ryegrass), which is present in high numbers and is able to persist owing to its genetic diversity.² These characteristics enable *L. rigidum* to adapt rapidly to environmental changes. For example, no-till agriculture, which conserves soil structure and moisture by minimising soil disturbance, is now a common practice in Australia.³ However, this technique relies heavily on herbicides for

weed control, and persistent herbicide use has consequently led to widespread resistance to selective in-crop herbicides such as the acetyl CoA carboxylase (ACCase) and the acetolactate synthase (ALS) inhibitors.^{4–7} In the large cropping region of Western Australia, herbicide-resistant *L. rigidum* is present in most crop fields.^{8,9}

Another factor that allows *L. rigidum* to persist in this environment is seed dormancy. Dormancy refers to a physical and/or physiological block to the germination of viable seeds when they are imbibed under ideal germination conditions.¹⁰ Like many other wild members of the Poaceae, seeds of *L. rigidum* exhibit physiological dormancy and can persist in the soil seed bank for at least 16 months.¹¹ Dormancy can be alleviated in dry seeds by extended warm periods (dry after-ripening) or in imbibed seeds by short durations of warm darkness (dark stratification).¹² Seed dormancy has both a genetic basis¹³ and strong environmental influences. Climatic conditions such as rainfall and temperature can influence the level of dormancy in an *L. rigidum* population not only during the season of seed development and maturation but also on a long-term basis.^{14,15} Factors such as soil temperature,

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moisture, nutrients and gases in the pre- and post-dispersal environments can affect the dormancy level of seeds in the soil seed bank.¹⁶ While these factors have a direct influence on the seeds, other selection pressures, for example those imposed by cropping history, may also affect the dormancy level of a population.

Agronomic practices such as crop rotation, earlier crop sowing or soil cultivation may select for more-dormant, late-emerging weed populations (reviewed in Murphy and Lemerle¹⁷). For example, *Avena fatua* populations from cropping systems with a long fallow period involving intensive herbicide use had a higher frequency of dormant and late-germinating individuals.^{18,19} *Amaranthus powellii* populations collected from perennial alfalfa crops had higher seed dormancy than those from vegetable farms with a long and intensive cropping history, apparently because the high competitive ability of an established alfalfa crop would make early germination of *A. powellii* seeds futile, exerting a selection pressure for greater seed dormancy.²⁰ In contrast, *Hordeum glaucum* populations that were collected from South Australian annual crop fields had much greater levels of seed dormancy than those collected from non-cropping situations.²¹ This and the high seed dormancy observed in many *L. rigidum* populations in the Western Australian cropping region (reviewed in Steadman *et al.*²²) could be a phenological adaptation to avoid presowing weed control in an annual crop.^{23–25} As hypothesised by Owen *et al.*,²⁶ the observed co-occurrence of high herbicide resistance and strong seed dormancy in many *L. rigidum* populations is likely to be due to the application of non-selective herbicides before crop planting to remove the early-germinating proportion of weed species, leaving the dormant seeds in the seed bank: this not only enriches the high-dormancy genes in the population but also exposes these individuals to selective herbicides, thus selecting for herbicide resistance.

To test the hypothesis that intensive cropping systems are selecting for greater dormancy in *L. rigidum* seed populations, 15 field sites were randomly selected from the Western Australian grain belt, with each containing a currently cropped field and a nearby matched field with minimal cropping history. Here, it is quantitatively established that greater cropping intensity does select for *L. rigidum* populations that (1) have higher seed dormancy, (2) are slower to germinate and (3) have a greater proportion of herbicide-resistant plants.

2 MATERIALS AND METHODS

2.1 Seed collection

Thirty *L. rigidum* populations were collected (November 2009) from 15 farms (two populations per farm) randomly selected in different cropping zones (based on climate) in the Western Australian grain belt (Fig. 1). At each farm, one field with a long cropping history (cropped regularly over a 10–20 year period or more) and a field with little to no cropping history (arable land that had not been cropped and generally had not been cultivated over the last 10–20 years or more; some fields may have been cropped once during this time) were selected and georeferenced, with mature *L. rigidum* seeds collected from each field. All samples were threshed and seeds separated from the chaff by sieving and forced-air separation.

2.2 After-ripening and germination testing

Freshly harvested seeds were placed in paper bags and stored in a ventilated glasshouse for the duration of the experiment,¹⁵ a total of 9 months. This storage method may have alleviated dormancy faster than if seeds had been after-ripened in the field; however,

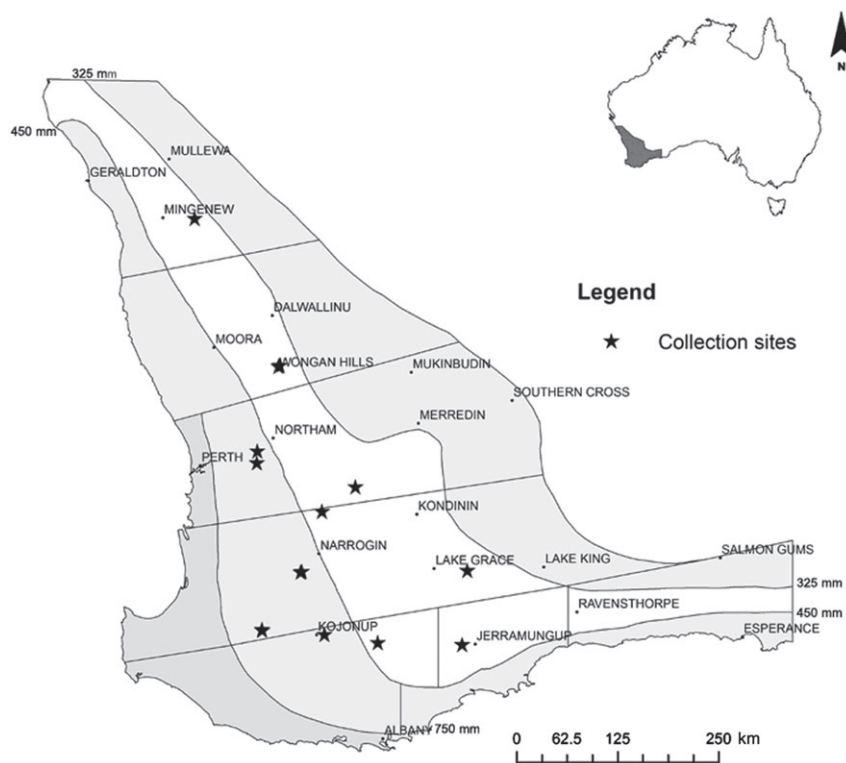


Figure 1. Map of southern Western Australia, showing where *Lolium rigidum* seeds were collected. Two samples were collected from each site (one from a cropped field, one from a non-cropped field). Agronomic zones of the grain belt are divided according to annual average rainfall and temperature zones.

as the populations were collected from different environments in the Western Australian grain belt, field storage in one location is still a somewhat artificial method of after-ripening. A Tiny Tag Transit data logger (Gemini, Chichester, UK) was located with the bags to record air temperature. Changes in seed dormancy during dry after-ripening were assessed at monthly intervals by removing subsamples from each population for germination testing. Seeds were sown in 9 cm diameter petri dishes containing 1% (w/v) solidified agar–water (50 seeds per dish, three replicates for each population) and placed in a controlled environment growth cabinet set at the optimal germination conditions for *L. rigidum*: a 12 h photoperiod of cool white fluorescent light (30 – 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$), with temperatures of 25°C during the day and 15°C at night.^{22,27,28} The number of germinated seeds was counted weekly, ending at 28 days after the start of imbibition,²² with the criterion for germination being visible radicle protrusion. Non-germinated seeds were assessed for viability by gentle pinching with forceps to determine whether they contained a turgid embryo²⁹ (pilot studies by Goggin *et al.*²⁹ have demonstrated that in *L. rigidum* this test gives the same results as tetrazolium staining), and non-viable or empty seeds were omitted from calculations. Seed viability in all 30 populations was greater than 95%.

2.3 Herbicide resistance testing

The herbicide resistance status of each population was determined by treating seedlings at the 2–3-leaf stage with diclofop-methyl (Hoegrass, 375 g AI L⁻¹; Bayer CropScience, Australia), sethoxydim (Sertin, 186 g AI L⁻¹; Bayer CropScience, Australia), clethodim (Select, 240 g AI L⁻¹; Sumitomo Chemical, Australia), sulfometuron-methyl (Oust, 750 g AI kg⁻¹; DuPont, Australia) and glyphosate (Roundup PowerMAX, 540 g AI L⁻¹; Nufarm, Australia). Screening with a range of herbicides potentially demonstrates that resistance is present across several herbicide chemistries and is thus not a random occurrence. There were three replicate treatments for each herbicide, each containing 50 seedlings. Herbicide resistance is a genetically determined trait³⁰ and does not change on the basis of the progress of after-ripening of the seed, so resistance was assessed only once. Herbicide treatments were applied between June and August, the normal growing season for this species, when the seed germination level of most populations had reached >90% (the most dormant population had a germination of ~75% at this time). Herbicides were applied at upper recommended commercial field rates for *L. rigidum* in Australia, using a custom-built spray chamber (see Owen *et al.*⁸ for spray equipment details). The rates used for diclofop-methyl, sethoxydim, clethodim, sulfometuron-methyl and glyphosate were 563, 186, 60, 15 and 540 g AI ha⁻¹ respectively. Seedling mortality was assessed 21 days after treatment, and the level of resistance to each herbicide was expressed as the percentage of the population able to survive the herbicide application. Known susceptible and resistant control populations of *L. rigidum*⁸ were used as positive and negative controls in all experiments, with 0% survival of the known susceptible population and very high survival (>90%) of the known resistant populations observed for all herbicides.

To confirm that herbicide resistance and seed dormancy are not linked in a cause-and-effect manner, seedlings germinated from very low- or very high-dormancy seed subpopulations (deliberately selected over three generations from a common dormant population³¹) were treated with diclofop, sethoxydim and sulfometuron at the same rates as described above. In order to germinate enough seedlings for herbicide screening, seeds of

the high-dormancy population were dark stratified for 42 days. Although the germination of fresh, non-stratified seeds at 28 days after the start of imbibition was very significantly different between the populations, being $65 \pm 4\%$ for the low-dormancy seeds and $8 \pm 2\%$ for the high-dormancy seeds ($P < 0.0001$), their response to herbicide treatment was identical: both populations were 100% resistant to diclofop and sethoxydim and 100% susceptible to sulfometuron (data not shown).

2.4 Cropping history

At the time of seed collection, growers were asked a series of questions relating to the fields that were sampled. Fields were classified by the number of years that they had been cropped and the use history of knockdown or presowing herbicides which kill most weeds present before crop sowing (glyphosate: >95% control), pre-emergence herbicides which are active on *L. rigidum* and other grass weeds applied before the crop has emerged (trifluralin: >95% control) and selective herbicides which are applied after the crop has emerged. Growers were also asked about weed control measures before and after planting and during the harvest operation to determine the selective forces applied by herbicides. All fields were then placed into four categories based on the level of cropping intensity: nil (N), not cropped in the last 15–20 years, with *L. rigidum* growing naturally and not controlled by tillage or herbicides; low (L), 1–3 years cropping in every 10 years; medium (M), 4–7 years; high (H), 8–10 years. Other factors such as proximity to cropping fields, crop hygiene, stocking levels and feeding regimes were also considered; for example, feeding of hay may be a source of seeds from herbicide-resistant weed populations in fields that have had very little cropping.

According to the results of the survey, N fields had not received herbicide treatment in 10 years or more, while most of the L fields had only received herbicide applications in 1–3 years out of 10 years, usually when that field was cropped (but not always). In M fields, glyphosate was applied every year that the field was cropped, while pre-emergence and in-crop herbicides were generally used less frequently (in 2–6 years out of every 10 years). H fields had experienced 8–10 years of pre-emergence and/or in-crop herbicide treatment, along with similar levels of glyphosate application, in the past 10 years.

2.5 Data analysis and statistics

Data were analysed first on a cropped (L, M and H) versus non-cropped (N) basis, and then with the four levels of cropping intensity treated separately. The time taken for each population to reach 50% germination (T_{50}) after each month of after-ripening was calculated according to Coolbear.³² For samples that did not reach 50% germination by the end of the experiment (28 days after the start of imbibition), the T_{50} was assigned a default value of 29 days. Using Gen-stat (v.15), differences between populations in terms of final germination percentage, T_{50} and herbicide resistance were assessed by ANOVA at a 5% significance level, followed by the LSD test to compare pairs of means.

3 RESULTS

Over the entire experiment, *L. rigidum* seeds from cropped fields had significantly higher levels of dormancy than those from non-cropped fields (72% final germination versus 84%; $P < 0.001$), with seeds from cropped fields maintaining significantly higher dormancy over the first 7 months of after-ripening (i.e. until July)

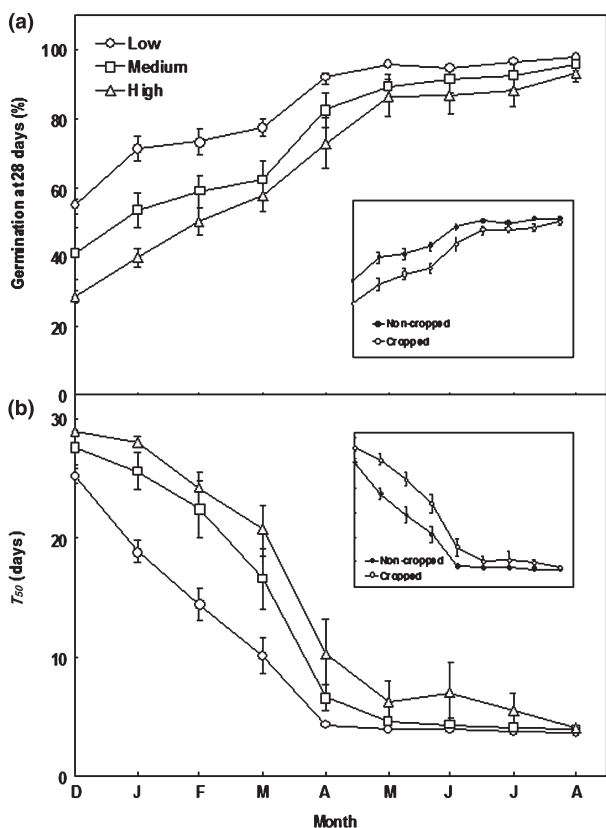


Figure 2. Dormancy release over time in seeds collected in late November 2009 from fields with low-, medium- and high-intensity cropping. After collection, seeds were stored under warm, dry conditions and sampled monthly to measure their germination level under ideal germination conditions (25/15 °C, 12 h photoperiod). (a) Germination at 28 days after the start of imbibition; (b) time to 50% germination. Values are means \pm SE ($n = 18, 4$ and 8 for populations from low-, medium- and high-intensity fields respectively). Insets show a comparison between cropped (all intensities) and non-cropped fields; axis scales are the same as for the main charts.

(Fig. 2). Populations from cropped fields were also slower to germinate (14 days versus 10 days to reach 50% germination) and had significantly higher levels of herbicide resistance than those from non-cropped fields (Fig. 3). When data were analysed on the basis of the separation of the different levels of cropping intensity, ANOVA showed no statistical differences between N and L fields; therefore, these were combined into a single 'L' category, and subsequent analyses used three categories for cropping intensity, i.e. L, M and H.

3.1 Dormancy level under different cropping intensities

From December 2009 to May 2010 there were significant ($P < 0.001$) differences in the dormancy level of *L. rigidum* seed populations collected from fields of different cropping history (Fig. 2a), with the overall mean germination being 83, 73 and 66% for the L, M and H fields respectively. As expected, the greatest differences between populations were observed after only short durations of after-ripening, i.e. in December and January. From May until July (5–7 months of after-ripening), the only significant difference in dormancy levels was between the populations from the H and L fields, and from July onwards there were no longer any differences between populations (Fig. 2a). Seed populations reached >90% germination in late April, June and August for the L, M and H fields respectively.

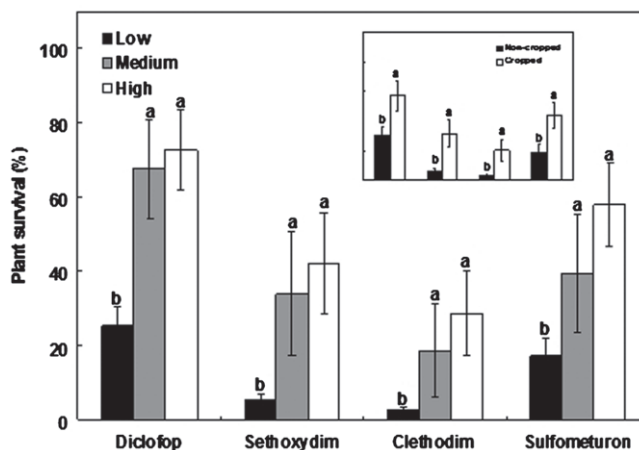


Figure 3. Herbicide resistance (percentage plant survival) for *L. rigidum* collected from low-, medium- and high-intensity cropping fields for each selective herbicide tested. Values are means \pm SE ($n = 18, 4$ and 8 for populations from low-, medium- and high-intensity fields respectively). Significant differences ($P < 0.001$) between means within each herbicide treatment are designated by different letters. The inset shows a comparison between the cropped (all intensities) and non-cropped fields. Axis scales are the same as for the main chart.

3.2 Germination rate under different cropping intensities

The rate at which the *L. rigidum* seed populations germinated was also affected by cropping intensity. Based on the time to 50% germination, seed of populations from L fields tested in the first 4 months after harvest germinated more rapidly than those from M and H fields (Fig. 2b). Between April and May, populations from the M and H fields lost dormancy more rapidly than those from the L fields, so that from May onwards there was no statistically significant difference in germination rate between the L and M populations (Fig. 2b). Populations from H fields were significantly slower to reach 50% germination until June (Fig. 2b).

3.3 Herbicide resistance under different cropping intensities

Lolium rigidum populations from the H and M fields showed similar levels of herbicide resistance, which were at least twofold ($P < 0.001$) higher than those of the populations collected from the L fields for all selective herbicides tested (Fig. 3). All populations were completely susceptible to glyphosate (data not shown).

4 DISCUSSION

Anecdotal evidence has suggested that *L. rigidum* populations in intensively cropped regions of the Western Australian grain belt exhibit higher seed dormancy than those in (for example) pasture zones, or areas where cropping is practised infrequently, owing to the fact that the dormant proportion of a seed population avoids presowing weed control measures and thus enriches the population with dormant individuals. This study has quantified the relationship between seed dormancy in *L. rigidum* and intensity of annual cropping. Populations from fields with a long history of cropping (cropped in more than 8 out of 10 years) produced a higher proportion of dormant seeds than those from fields with 0–3 out of 10 cropping years, as illustrated by their lower seed germination percentage and slower rate of germination when tested immediately after collection or after only 1 or 2 months of dry after-ripening (Fig. 2).

Although the initial dormancy level of the populations from medium-intensity (cropped in 4–7 years out of 10) cropping fields was higher than in the low-intensity fields, there was little difference between the populations in terms of germination rate after the seeds had experienced 5 months of dry after-ripening. This suggests that, while cropping practices influenced the initial level of seed dormancy of the population, there was negligible impact on factors affecting the subsequent after-ripening process (e.g. changes in the seed water potential threshold at which germination can occur³³). This relatively rapid rate of seed after-ripening means that up to 85% of the population in medium-intensity fields may have germinated in time to be exposed to the presowing use of non-selective herbicides (in April/May); but this still leaves a higher proportion of the population in the weed seed bank compared with the low-intensity fields (>95% germination). It is likely that after-ripening proceeded at a faster rate in the glasshouse (average temperature from December to May: 30 °C) than it would have in the field (average temperature of the Perth region from December to May: 23 °C),²² resulting in an overestimation in this study of the rate of depletion of the seed bank. The seed dormancy level of the *L. rigidum* populations from high-intensity cropping fields remained significantly higher than in the low-intensity fields well into the growing season (July), meaning that a large proportion of the seed bank (up to 15%) was likely to germinate long after crop sowing and weed control practices had been carried out. This highlights the importance of late-season weed control measures to prevent this highly dormant proportion of the population from replenishing the soil seed bank.

The long-term intensive crop production systems that are utilised in the Australian grain belt regularly employ herbicidal weed control strategies at the start of the growing season. However, although seed traits such as dormancy may be modified by herbicide selection history,^{23,26,34} it is unlikely that selection with herbicides directly causes dormancy,²³ or vice versa (see Section 2). Instead, the weed populations found in the fields with the longest cropping history and thus greatest herbicide use will have been enriched in genes that allow the plants to escape the unfavourable conditions associated with cropping practices. Increased seed dormancy is selected for because the late-germinating proportion of the population avoids the environmental disturbance of crop planting (involving cultivation and non-selective herbicide use). Repeated exposure of this late-germinating cohort to post-emergence herbicides eventually results in a population with high resistance to selective herbicides. These survivors cross with each other and continue to enrich the seed bank with highly dormant and herbicide-resistant individuals.

It should be noted that some of the low-intensity cropping fields did contain herbicide-resistant plants, but at significantly lower numbers than for medium- and high-intensity cropping fields. In most of these cases, the low-intensity fields, although not sprayed with herbicides themselves except during the cropping phases, were in close proximity to a cropped field. All of the low-intensity fields had sheep (a potential vector for resistant seed) grazing on them during the year, or machinery that passed through the area. Therefore, herbicide resistance in these fields could be explained by (for example) spray drift,³⁵ resistant seed brought in by livestock,³⁶ seed movement by machinery during harvest and tillage operations,^{37,38} sowing of grain contaminated with resistant weed seed³⁹ or pollen-mediated gene flow from resistant plants in neighbouring fields.^{40,41} In fact, Busi *et al.*⁴¹ found that organically farmed fields with no herbicide use history

also contained a low frequency of herbicide-resistant *L. rigidum* plants, indicating that herbicide use is not a prerequisite for the presence of resistance alleles. Other possibilities are that the pasture phase may have been treated with herbicides during the winter months, or that livestock was supplied with supplemental hay or grain, containing resistant seed, over the hot, dry months of summer. Plants germinating from these contaminating resistant seeds would freely cross with the susceptible individuals in the field, resulting in a low level of herbicide resistance in the population, but the lack of cropping activity means that there is no selection pressure for increased dormancy in these fields.

The selection for high seed dormancy in cropping situations appears to be a worldwide problem and is not confined to *L. rigidum*.^{17,19–21,25} Management programmes that delay crop sowing to allow high weed seed germination, followed by killing of the seedlings with herbicides to which resistance is rare, such as glyphosate,⁸ would allow the removal of some of these dormant weed seeds. However, delayed sowing, at least in Australian rain-fed cropping, incurs a high yield penalty (reviewed in Roper *et al.*⁴²) and would only be used for fields with high dormancy levels, on an occasional basis, for drastic reduction in weed numbers. Programmes conducted later in the season once the crop is established need to aim at reducing the amount of seed being returned to the seed bank. A number of options, such as green or brown manuring, where a crop or pasture is killed prior to maturity,⁴² and cutting crops for hay (although these will both sacrifice the crop), long fallow, which is being utilised in low rainfall zones in the northern agricultural region of Western Australia,⁴³ or applying herbicides late in the season to prevent weed seed set,⁴⁴ would help to reduce the number of seeds that enter the seed bank. End-of-season harvest operations such as narrow windrow burning, baling crops for hay and removal of chaff through ‘chaff carts’ can also minimise seed bank replenishment.⁴⁵

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