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Adverse consequences of herbicide residues on legumes in dryland agriculture

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ABSTRACT

Context or problem: Selective herbicides control weeds in cereal crops and break down over time, allowing safe planting of legumes in the following years. However due to climatic inconsistencies and changing farming practices, this is not always the case, and residues can inhibit formation of legume/rhizobia symbioses.
Objective or research question: The objectives were to determine whether: i) exposure to triasulfuron, even at extremely low levels, reduces shoot and root growth and nodulation of five diverse and widely sown legume pasture cultivars in Australian farming systems; and ii) sowing legumes prior to recommended plant-back criteria being met for chlorsulfuron, triasulfuron herbicide, clopyralid, and pyroxasulfone herbicides results in unacceptable damage to subsequently sown pasture and crop legumes, causing reduced root and shoot growth, nodulation and N fixation.
Methods: A series of glasshouse and field experiments explored herbicide residue impact on commonly used legumes in dryland farming systems.
Results: A glasshouse study determined triasulfuron at concentration 0.000225 g a.i/ha, a (1/100,000) dilution of the label rate caused significant ($p < 0.001$) decrease in nodule count, root length, root, shoot weight for *Trifolium spumosum* cv. Bartolo and *T. subterraneum* cv. Dalkeith, and at 0.225 g a.i/ha and 2.25 g a.i/ha for all five cultivars tested. A bioassay assessed *T. subterraneum* cv. Dalkeith health when grown in field soil-cores taken 4, 7 and 10 months after herbicide application (chlorsulfuron, triasulfuron, clopyralid and pyroxasulfone) to a wheat crop. For all three, herbicide residues significantly decreased ($p < 0.001$) nodule number, shoot weight, root length and whole plant weight of *T. subterraneum* cv. Dalkeith compared to control. A field experiment assessed nodulation of five pasture and two crop legumes sown dry (dormant summer sowing), or following rainfall 10.5 months after initial herbicide application. Nodulation of all legume cultivars decreased in plots treated with clopyralid. Chlorsulfuron decreased nodulation for all cultivars except *T. glanduliferum* and *T. subterraneum*. Triasulfuron reduced nodulation for all cultivars except *Ornithopus sativus* and *T. spumosum*. Pyroxasulfone decreased nodulation *of Biserrula pelecinus* cv. Casbah and *Lupinus angustifolius* cv. Mandalup.
Conclusions: Herbicide residues from preceding cereal crops reduced fitness and symbiotically fixed N in subsequently sown pasture or crop legumes.
Implications or significance: Our study highlighted label plant-back recommendations should be strictly adhered to, despite conflict with modern farming approaches of dry or early sowing) to combat climate change. This outcome may consequently lower profitability and increase the carbon footprint of farming systems.

1. Introduction

It is estimated that crop yields must increase approximately 1.1 % per year globally to ensure adequate food supply for the human population (Crews et al., 2016; Fischer and Connor, 2018). The challenge to increased world food production is exacerbated by diminishing natural resources and climate variation, which is delivering less predictable rainfall events and higher temperatures that collectively impact

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plant growth (Porter and Semenov, 2005; Wang, 2005; Thornton et al., 2014; Trnka et al., 2014; Serdeczny et al., 2017). An essential component of sustainable agriculture in dryland farming systems is the integration of effectively nodulated legumes (Hardarson and Atkins, 2003; Howieson et al., 2008; Sprent, 2009; Nutt et al., 2021). Agricultural legumes can provide ecosystem nitrogen (N) by means of a symbiotic process with root-nodule bacteria (rhizobia), commonly termed biological N fixation (BNF) (Herridge, 2008; Dupont et al., 2012). Importantly, the incorporation of legumes into crop rotations assists in reducing inherent risk in the farming system by reducing expenditure on fertiliser N, and allows better management of weeds, pests and diseases (Howieson et al., 2008; Peoples et al., 2009; Drew et al., 2012; Giller et al., 2016; Dowling et al., 2021; Rouge et al., 2023). Therefore, a major outcome of BNF is that it potentiates an increase in farm profitability, while reducing the environmental impacts of synthetic N production and its application (Baldock and Ballard, 2004; Lüscher et al., 2014; Yates et al., 2021).

Weed control in dryland agriculture is another major issue faced by growers, with weeds accounting for 37 % of yield losses. Apart from competition for resources, weeds may carry diseases, contaminate produce, obstruct the harvesting process and ultimately reduce grain yields (Matthews et al., 1996; Oerke and Dehne, 2004; Walsh et al., 2013; Soltani et al., 2018). The widespread adoption of conservation tillage has seen herbicides replace mechanical tillage as the primary method for weed control in many farming communities (McErlich and Boydston, 2014). However, the unilateral reliance on herbicides to control weeds, combined with increased cropping intensity, has increased crop phytotoxicity and the development of widespread herbicide resistance in agricultural weeds (Délye et al., 2013; Heap, 2014; Kirkegaard et al., 2016; Meftaul et al., 2020; Rose et al., 2022). To counter herbicide resistance has required the deployment of multiple modes of action (MoA) in herbicide chemistry (Powles and Yu, 2010; Norsworthy et al., 2012; Délye et al., 2013; Harries et al., 2020).

Some groups of herbicides have been developed with prolonged activity in the soil to enable control of both weeds that are present at the time of herbicide application, as well as those which germinate in the following weeks or months. Applying herbicides with such "residual activity" is a convenient, inexpensive and effective way for the continued control of weeds from a single herbicide application (Walsh and Powles, 2007). For example, sulfonylurea (SU) (MoA group 2) (Shaner, 2014) and clopyralid (MoA group 4) are pre- and post-emergent herbicides respectively which are commonly applied to both crops and pastures and provide residual activity that is injurious to weeds months after application. Whilst convenient, a risk to this prolonged activity is that herbicide residues may unpredictably have a detrimental effect on susceptible, non-target rotational break crops, such as canola, and crop and pasture legumes sown in the next growing season (Anderson et al., 2004; Hollaway et al., 2006). In the case of agricultural legumes, residual herbicides have been reported to deleteriously effect (i); the legume directly (Hollaway et al., 2006; Siddique et al., 2012; Cornelius and Bradley, 2017; Rose et al., 2022), (ii); the rhizobia (Eberbach and Douglas, 1989; Anderson et al., 2004; Malik et al., 2017; Eberbach, 2018; Medo et al., 2020), (iii); the nodulation process (Eberbach and Douglas, 1989; Drouin et al., 2010; Eberbach, 2018) and (iv); ultimately N fixation (Sprout et al., 1992; Koopman et al., 1995; Zargar et al., 2020).

To avoid phytotoxicity from herbicide residues in soil, farmers are informed via label warnings to abide by recommended "plant-back" periods for each specific herbicide. The plant-back period is based upon an estimate of how long deleterious concentrations of each herbicide are likely to remain in the soil. The estimate is drawn from previous field research, combined with a detailed understanding of the specific chemical characteristics of the herbicide and its dissipation in soil. In reality, the residual activity of a herbicide is a complex function dependent on elapsed time, total and timing of rainfall, soil chemistry and soil biological activity required to transform the chemical to sub-lethal levels, before the desired crops or pastures can be sown (Moyer et al., 1990; Moyer, 1995; Janaki et al., 2015). Recommended plant-back periods are considered as a guide only, as the rate of break down of herbicides by biological activity (Anderson, 1984) or hydrolysis is unpredictable (Sarmah et al., 1998; Hollaway et al., 2006). The predictability may become less accurate as changing climatic patterns and the erratic nature of rainfall events intensify, to the point where generalisations about herbicide safety are considered unsatisfactory (Nguyen et al., 2016).

In the case of legume pastures, a recent innovation termed "dormant summer sowing", as described by Nutt et al. (2021) relies upon the shallow sowing of hard-seeded genotypes of annual pasture legumes into dry soil in late summer. Diurnal fluctuations in temperature and moisture provide conditions for break-down of the physical dormancy of the seed. This preconditions the hard seed-coat for germination upon rainfall events in early autumn (Howieson et al., 2021). This approach overcomes significant logistical barriers to pasture legume renovation, such as labor supply and the timing of other on-farm operations (Hogg and Davis, 2009). In seasons when early autumn rains arrive, delivering conditions to germinate the softened dry sown seeds, dormant summer sowing increases herbage production in winter in the renovated pastures by as much as 10-fold (Nutt et al., 2021). Our concern, however, is that this changed practice has the potential to reduce the effective plant-back time available for the break-down of herbicides in the soil. In southern Australia seedlings may, germinate up to two months earlier (say early April) than in conventional seeding of pastures (say mid-June). This potentially increases the exposure of the sown legumes to lethal levels of herbicide residues applied in the preceding crop.

Combined with this uncertainty, information on new herbicides and their effects on non-target agricultural plants, particularly for different environmental conditions, is often incomplete (Rose et al., 2022). In recent times there have been many confirmed reports of annual legume pastures regenerating after crops (or sown into stubbles) in which plants are necrotic, deformed and stunted, with pruned roots lacking root hairs and low nodule numbers (Yates et al., 2014). Several studies have demonstrated that establishment of annual medics (Medicago spp.) in cereal rotations has been restricted by SU-herbicide residues (Noy, 1996; Wilhelm and Hollaway, 1998). Although usually accurate for the above-ground health of the plant, plant-back recommendations for herbicides do not appear to consider their potential root system impacts, particularly the nodulation of legumes. In general there is limited published information on the impact of residual herbicides on legume pasture production and N fixation.

To assess the unintended consequences of residual herbicides applied to cereal crops on subsequent pasture legumes in contemporary Australian farming systems, a series of glasshouse and field studies were undertaken. We hypothesised that:

i) Exposure to the SU herbicide triasulfuron, even at extremely low levels, reduces shoot and root growth and nodulation of five diverse and widely sown legume pasture cultivars in Australian farming systems.
ii) Sowing legumes prior to recommended plant-back times for chlorsulfuron, triasulfuron, clopyralid, and pyroxasulfone herbicides results in reduced root and shoot growth, nodulation and N fixation in subsequently sown pasture and crop legumes.

2. Materials and methods

2.1. Experiment 1: Triasulfuron toxicity; dilution rates

This experiment determined the effect of herbicide exposure on plant fitness on commercially available legume pasture cultivars. A replicated (n = 4) complete randomised block design experiment with five herbicide dilutions was arranged in a naturally lit phytotron maintained at 22 °C. Five legumes *M. littoralis* (cv. Herald), *Ornithopus sativus* L. (cv.

Margurita), *Trifolium spumosum* L. (cv. Bartolo), *T. subterraneum* L. (cv. Dalkeith), and *Medicago littoralis* L. (cv. Angel) were used. The Angel cultivar was developed and incorporated in the experiment due to its tolerance to SU herbicide soil residues (Howieson and Ballard, 2004; Peck and Howie, 2012). Seeds were sown in 160 mm rows, 20 mm apart in a clear plastic rectangle container (115 mm width, 170 mm length, 70 mm height). Soil mixture used (1 kg/container) consisted of 1-part red sandy loam to 1-part standard potting mixture (soil $pH_{(Ca)}$ 5.5, organic carbon (chromic acid digestion) % = 1.8). Four blocks of triasulfuron herbicide were applied as Logran® at five concentrations. Concentrations represented a series of 10-fold dilutions of the label rate; 2.25 g a.i/ha, 0.225 g a.i/ha, 0.0225 g a.i/ha, 0.00225 g a.i/ha, 0.000225 g a.i/ha and control (0 g a.i/ha) for each of the five host-species.

The experiment assumed an incorporation depth of 50 mm for surface application filtration with calculations based on a bulk density of 1.2 g/cm^3 . Prior to sowing, soil in the containers was watered to field capacity with 270 ml of solution, with deionised (DI) water (control) or DI water mixed with Triasulfuron herbicide, to achieve the correct dilution. All seed was hand scarified with sand paper (Yates et al., 2016) to ensure germination. Seed was surface sterilised by immersion in 70 % (v/v) ethanol (60 s) and dried. Seed was inoculated with peat containing a minimum 1×10^9 cells/g of a legume- specific rhizobial strain. Inoculum was manufactured at Murdoch University using procedures described in Deaker et al. (2016). Recommended label rates were used for inoculation: 1 g of Group AL (RRI128) peat was applied to 100 g seed of *M. littoralis* seed, and 0.5 g of Group S (WSM471) and Group C (WSM1325) peat applied to 100 g seed of *O. sativus* and *T. spumosum/T. subterraneum*, respectively. The peat was adhered to the seed in a slurry with a 10 % (100 g/L) solution of SeedStik™ sticker (100 ml/5 kg) five hours prior to sowing. After 25 days, seedlings were carefully extracted and hand-washed to remove adhering soil and the number of nodules per plant, tap root length and root and shoot dry weights (dried for 2 days at 65 °C) recorded.

2.2. Experiment 2A: Residual herbicide activity in soil cores taken within the plant-back period

To evaluate herbicide residue effects on legume plant health in situ a factorial experiment with a randomised block design and three replicates per treatment was established. The field site near Brookton, Western Australia (32°16′52.22″S, 116°50′25.40″E) consisted of ferric chromosol soil (grey sandy-loam duplex) with gravelly clay at a depth of approximately 25–50 cm (soil 0–10 cm; $pH_{(Ca)}$ 4.6, organic carbon % = 1.09, clay % = <2, bulk density = 1.2 g/cm^3). On July 11 2012, four herbicides (chlorsulfuron, triasulfuron, clopyralid, and pyroxasulfone) (Table 1) were applied over a wheat crop cv. Mace® (established by the farmer, sown May 22 2012) at mid-tillering (Zadoks scale) (Zadoks et al., 1974), with unsprayed areas as controls (Table 1). Each plot was 15 m (length) by 6 m (width). Each herbicide was combined with wetter (BS1000 at 0.2 %v/v) and applied via a 5 m boom spray. The boom spray consisted of double overlap nozzles at a water rate of 100 L/ha spaced at 0.5 m and was mounted on a quad motor bike which sprayed down the center of each plot (at 10 km/h).

Through the following late spring, summer and autumn (on November 14 2012, February 12 2013 and May 15 2013), three core samples of soil were taken from each plot (n = 15) representing 4, 7 and 10 months since herbicide application. Cores were used to estimate the break down rate of the herbicides, via a legume plant bioassay conducted in the glasshouse. Cores were not taken in July 2013 (12 months after herbicide application) as pasture species were sown over the plots on the May 21 2013 (see experiment 2B). Total rainfall on the site (after the spray application) was 132 mm (by November 15 2012), 171 mm (by February 15 2013) and 276 mm (by May 15 2013) (Table 2). Monthly rainfall was separated into the first 15 days of the month to coincide with soil core sampling. For each sampling date, entire soil cores were carefully placed into Ziplock bags and then into pots to avoid disturbance, before transferring to a temperature-controlled phytotron and watered to field capacity (Yates, 2016).

Scarified seed of *T. subterraneum* (cv. Dalkeith) was surface-sterilised (by immersion in 70 % (v/v) ethanol (60 s) and dried. It was then coated with a commercial peat inoculant (Group C – WSM1325) at the recommended label rate (0.5 g peat containing a minimum 1×10^9 cells/g applied to 100 g seed) in a 10 % (100 g/L) solution of SeedStik™ sticker (100 ml/5 kg). Eight seeds (inoculated five hours prior) were sown into each pot at a depth of 10 mm, then covered with soil. Seedlings were harvested and soil carefully hand-washed from the root system after 28 days with the number of nodules per plant, root length, root and shoot dry weights (4 days at 65 °C) measured.

2.3. Experiment 2B: Residual herbicide activity on dry or wet sown legumes at the end of the plant-back period

In the same field experiment as Experiment 2 A we examined the impact of of seeding legumes towards the end of the plant-back period, after minimum rainfall requirements were met. The legumes were established via two methods on two occasions (Table 3). Firstly, unprocessed pod segments (*O. sativus* cv. Margurita) or unscarified seed (*T. spumosum* cv. Bartolo) were established with the dormant summer sowing technique (SS) at 1 cm depth as described by Nutt et al. (2021), into dry soil on February 20 2013. The conventionally sown (CS) treatments (at the break of season) (Table 3) were seeded on May 21 2013 into moist soil after a Glyphosate (1.5 L/ha, 450 g/L) knock-down applied to the site eight days prior. Nine legume treatments (Table 3)

Table 1
Chemical active ingredient applied, the application rate sprayed over wheat in July 2012 and the label plant-back period in terms of time (months) and minimum interim rainfall (mm) for *Trifolium subterraneum* when grown in soil $pH_{(Ca)}$ 4.6.

Herbicide treatment	Herbicide Group	Application rate (g/ha)	Plant-back period (months)	Minimum interim rainfall (mm)
control	-	-	-	-
chlorsulfuron (750 gai/kg)	2	15	12	NS[a]
triasulfuron (750 gai/kg)	2	30	12	300[b]
clopyralid (750 gai/kg)	4	115	12	150
pyroxasulfone (850 gai/kg)	15	118	9	250

[a] Not specified.
[b] Minimum rainfall requirements between application and sowing the following crop in soil < $pH_{(H20)}$6.5.

Table 2
Monthly (rainfall for the first 15 days of the month in parenthesis) and yearly (total) rainfall (mm) in Brookton, Western Australia for the years 2012 and 2013 of the field experiments.

	Brookton rainfall (mm)		
	2012	2013	Long term average
January	(2) 3	(24) 24	13
February	(24) 27	(0) 0	16
March	(0) 3	(47) 59	18
April	(5) 9	(2) 3	26
May	(36) 37	(44) 62	56
June	(45) 64	(11) 19	84
July	(7) 11	(41) 106	85
August	(39) 48	(39) 61	62
September	(37) 59	(67) 98	38
October	(1) 3	(9) 22	25
November	(18) 30	(1) 2	15
December	(1) 3	(5) 5	10
Total	**297**	**461**	**448**

Table 3
Legume treatment with seed rate sown (kg/ha), sowing treatment (CS-conventional sown, SS- dormant summer sown) and inoculant method and rate applied for field assessment of impact of residual herbicide applied to wheat in the previous year.

Legume treatment	Seed rate sown (kg/ha)	Sowing treatment	Inoculant method and rate applied
Trifolium. spumosum L. cv. Bartolo	7	CC	0.5 g peat Group C
T. spumosum cv. Bartolo	20	SS	ALOSCA Group C 10 kg/ha
Ornithopus sativus L. cv. Margurita	6	CC	0.5 g peat Group S
O. sativus cv. Margurita	25	Pod SS	ALOSCA Group S 10 kg/ha
T. glanduliferum Boiss. cv. Prima	6	CC	0.5 g peat Group C
Biserrula pelecinus L. cv. Casbah	5	CC	2.5 g peat Group Bis Special
T. subterraneum L.cv. Dalkeith	8	CC	0.5 g peat Group C
Pisum sativum L. cv. Kaspa	100	CC	0.25 g peat Group E
Lupinus angustifolius L.cv. Mandelup	70	CC	Group G to 100 g of seed adhered by a 10 % (100 g/L) solution of SeedStik™ sticker (100 ml/5 kg).

were seeded at 5–10 mm depth. An Aitchison Mini Seeder (AT-SM4020C, Reese Group, New Zealand) was used to sow through the wheat stubble remaining after harvest (approximately 20 cm high) in the year following herbicide application.

Legume seed was inoculated five hours before sowing, with the correct commercial inoculant at the recommended label rate (Table 3). The germination date around May 26 following both seeding times was thus approximately 10.5 months after the application of the herbicides to the preceding wheat crop. An immediate post-sowing application of Bifenthrin (200 ml/ha, 250 g/L) was applied over all plots. Clethodim (330 ml/ha, 360 g/L) was applied post-emergent at the 4–6 node legume growth stage to control annual grass weeds along with alpha-cypermethrin (200 ml/ha, 100 g/L) to prevent damage from insects. Plots were sown perpendicular to the original sowings in randomised 65 m strips at a width of 1.5 m resulting in three replications. Fertiliser was applied at seeding with 80 kg/ha of superphosphate: potash 2:1 (5.6 % P, 16.3 % K, 7 % S, 13.45 % Ca).

On August 15 2013, 20 plants were randomly selected and carefully excavated from the plots. Summer sown legumes demonstrate a staggered germination and therefore plants were selected for sampling with similar growth stages between the two sowing procedures. The root systems were carefully hand-washed and the number of nodules on each individual plant counted with number of nodules assigned (Yates et al., 2016). In addition, the percentage of N in the shoots derived from the atmosphere (%Ndfa) was quantified via 15 N natural abundance by sampling at peak biomass production prior to pod formation on the 30th September 2013 (Yates et al., 2021b). The %Ndfa was analysed from a combined treatment sample on each cultivar and as such was not statistically analysed.

2.4. Statistical analysis

All analyses and graphs were performed in R (Team, 2020). The data were explored for violation of assumptions (normality, heterogeneity, independence, sphericity) prior to analysis. Data were analysed by fitting response variables (dry weight, nodule number, seed yield) to linear mixed effects model with an appropriate link (Guassian or Poisson) with predictor (herbicide treatment, host species) as fixed effect and block effect as random effect in package lme4 (Bates et al., 2007; Hu and Spilke, 2009; Bates et al., 2014). The Akaike information criterion (AIC) were used in model selection where appropriate. All models were evaluated through visual assessment of the residuals. Paired comparisons were estimated using the Tukey method for p-value adjustment in package Emmeans and the degrees of freedom method used was Kenward-roger (Hu and Spilke, 2009; Lenth, 2019). Estimated marginal means, confidence intervals and comparison arrows (based on Tukey method) were extracted from the models using package Emmeans (Lenth, 2019) and graphed using Ggplot2 (Wickham, 2009). Comparison arrows represent Tukey pairwise comparisons between treatments (rhizobium strains). Where the arrows overlap, there is no significant difference between treatments (Lenth, 2019).

3. Results

3.1. Experiment 1: Triasulfuron toxicity; dilution rates

Exposure to triasulfuron herbicide at the lowest concentration (0.000225 g a.i/ha) caused a significant ($p < 0.001$) decrease in all plant fitness parameters. This included nodule count, root length, root, shoot and whole plant weight for both *T. spumosum* (cv. Bartolo) and *T. subterraneum* (cv. Dalkeith). At 0.225 g a.i/ha and 2.25 g a.i/ha nodule count, root length, root, shoot and whole plant weight was reduced for all four species (five cultivars) of pasture legumes (Figs. 1 and 2). Clear separation was noted between *M. littoralis* cultivars Angel and Herald with the former achieving greater root dry weight, number of nodules and root length at (0.00225 g a.i/ha) of triasulfuron (Fig. 1). No differences between the cultivars (Angel and Herald) were observed at the rates of 0.225 g a.i/ha or 2.25 g a.i/ha in nodule numbers, root length, nor root and shoot dry weight, indicating no differential tolerance at those concentration levels.

3.2. Experiment 2A: Residual herbicide activity in soil cores taken within the plant-back period

For the three dates of coring (4, 7 and 10 months following herbicide application), and for all herbicides applied in the wheat phase, herbicide residues significantly decreased ($p < 0.001$) the number of nodules, shoot weight, root length and whole plant weight of *T. subterraneum* (cv. Dalkeith) in comparison to the control cores (no herbicide applied) (Figs. 3 and 4). Root length was also reduced for all herbicide treatments, except for pyroxasulfone 4 months after herbicide application (Fig. 3). Clopyralid was particularly damaging in comparison to the unsprayed soil cores, reducing the number of nodules by at least 80 % after four and seven months, and by 60 % after 10 months. Chlorsulfuron and triasulfuron caused as much as 60 % reduction in nodule number in all soil cores. Pyroxasulfone residues in cores taken after 4 months caused a 25 % reduction in nodule number on *T. subterraneum,* and nodule number decreased by at least 50 % on the plants grown in the cores taken at 7 and 10 months (Fig. 3).

3.3. Experiment 2B: Residual herbicide activity on dry or wet sown legumes at the end of the plant-back period

For the legumes seeded into the field soil in situ, all nine treatments planted into the plots that had been sprayed with clopyralid 10 months earlier in the preceding wheat crop had lower nodule ratings ($p < 0.001$) than legumes growing in the unsprayed control plots (Fig. 5). Similarly, chlorsulfuron treated plots produced lower ($p < 0.05$) nodule numbers for all legumes apart from *T. glanduliferum* (cv. Prima) ($p = 0.405$) (Fig. 5). Nodule ratings were lower ($p < 0.05$) for all legumes except conventionally and summer-sown *T. spumosum* cv. Bartolo and *O. sativus* (cv. Margurita), when grown in plots sprayed with triasulfuron previously. Two legumes *B. pelecinus* cv. Casbah and *L. angustifolius* cv. Mandalup had lower ($P < 0.001$) nodulation ratings than the control when grown in plots which had received pyroxasulfone (Fig. 5). Of the applied herbicides, clopyralid and chlorsulfuron were the

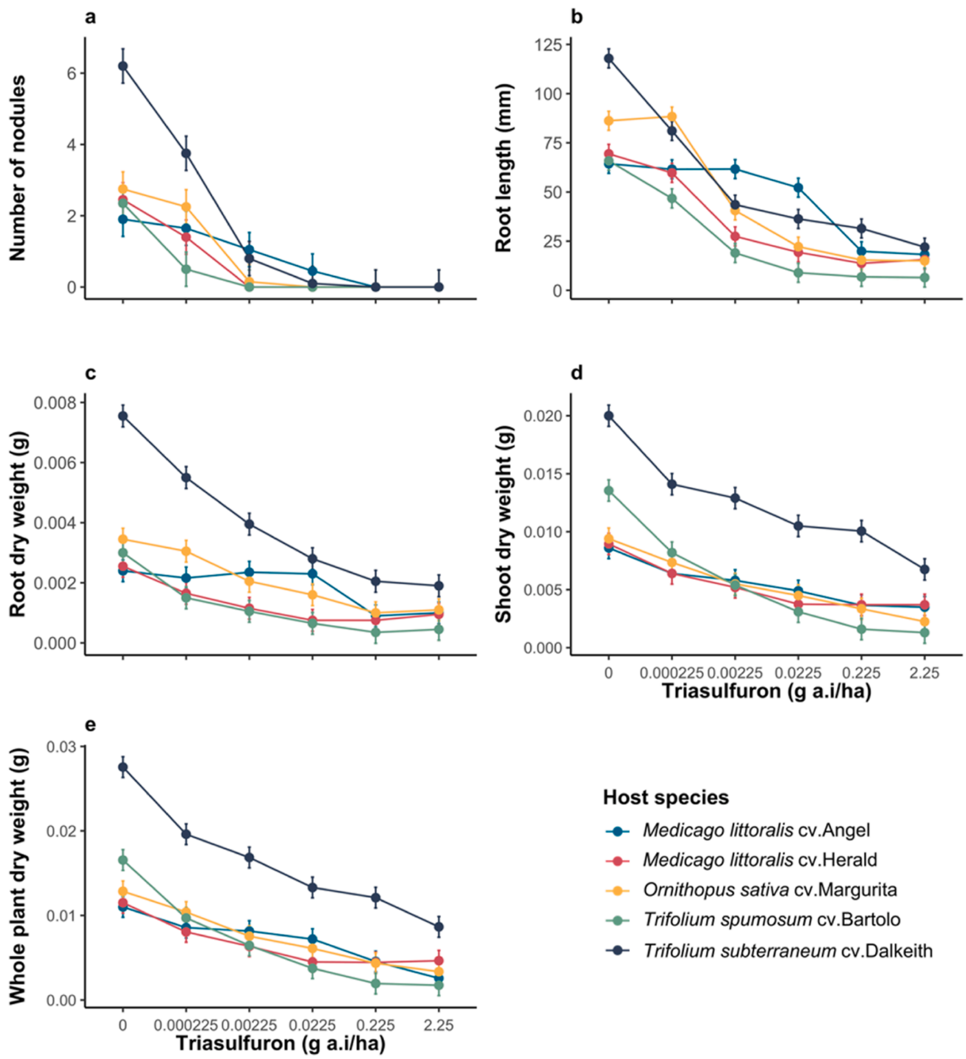

Fig. 1. Measurements of a. number of nodules per plant, b. root length per plant (mm), c. root dry weight (g), d. shoot dry weight (g) and e. whole plant dry weight (g). Glasshouse trial for triasulfuron herbicide concentrations (0.000225 g a.i/ha), (0.00225 g a.i/ha), (0.0225 g a.i/ha), (0.225 g a.i/ha), (2.25 g a.i/ha) and control (0 g a.i/ha) on host species *Medicago littoralis* (cv. Angel), *M. littoralis* (cv. Herald), *Ornithopus sativus* (cv. Margurita), *Trifolium spumosum* (cv. Bartolo) and *T. subterraneum* (cv. Dalkeith) after 25 days growth. Points are estimated marginal means and error bars are 95 % confidence intervals.

most damaging, with the former reducing the nodulation scores of all legume treatments in comparison to the controls by over 50 % (Fig. 5). Over 60 % of the interactions between the nine legume treatments and residual soil herbicides sampled 13 months after application produced significantly lower nodule ratings when compared to the plants grown in the unsprayed control plots (Fig. 5). The effects of the herbicides on dormant summer sown legumes reflected those for the same legumes when sown conventionally.

For the pasture legumes, the %Ndfa analysis indicated a reduction in the amount of fixed N when legumes were grown in soil previously sprayed with the four herbicides, in comparison to the unsprayed control plots (Table 4). This was not the case for the crop legumes *P. sativum* (cv. Kaspa) and *L. angustifolius* (cv. Mandelup) (Table 4).

4. Discussion

This study was undertaken to examine possible unintended consequences on legume growth and nodulation of herbicide residues remaining phytotoxic in the soil during the growing season following their application to a cereal crop. While understanding that some herbicides are designed to have residual effects on weeds, and that conservative "plant-back" recommendations are provided, we examined effects on non-target plants prior to the expiration of the recommended plant-back period for the chemicals. This data was sought because changing farming systems have seen an alteration to the way farmers are managing their seeding times. For example, in south-western Australia, there has been a 15 % decline in the average winter rainfall since 1970, up to 20 % in much of the wheatbelt where many regions periodically receive less than 200 mm total annual rainfall (Moyer et al. (1990) (Ludwig et al., 2009; Asseng and Pannell, 2013). South-eastern Australia has had decreasing late autumn and winter rainfall since the 1990's (Hochman et al., 2017). As a result in such modern Mediterranean dryland agriculture systems, it is increasingly evident that legumes as with other crops, must be sown early in the growing season to optimise production and profitability (Fletcher et al., 2016; Hunt et al., 2021). Under these circumstances, application of herbicides with residual activity in the year prior makes it difficult for farmers to adhere to a plant-back of 12 months.

Where legume break crops are sown into dry soil after a cereal crop, their germination may occur at 8–10 months after the final (post-emergent) herbicide application in that preceding crop, depending upon the timing of the first rainfall events. This is particularly so for pasture legumes established by the revolutionary technique of dormant summer sowing. Potentiating plant injury from plant-back noncompliance may

Fig. 2. Nodule formation in *Trifolium subterraneum* (cv. Dalkeith) grown for 25 days in the presence of triasulfuron applied to soil at concentrations of a) 2.25 g a.i/ha, b) 0.225 g a.i/ha, c) 0.0225 g a.i/ha, d) 0.00225 g a.i/ha, e) 0.000225 g a.i/ha and f) control (0 g a.i/ha).

be the drying climate in some farming regions, where minimum rainfall requirements for herbicide break down are difficult to meet. Herbicide break down is correlated with time in the soil, which can interact with soil type, pH, exposure to soil microbes and moisture (Engvild and Jensen, 1969; Anderson, 1984). The SU group herbicides triasulfuron, chlorsulfuron and the synthetic auxin herbicide clopyralid are weak acids that degrade over time primarily through chemical hydrolysis (Sarmah et al., 1998). Hydrolysis requires water as a reagent, so the rate of degradation is dependent on soil moisture (Walker and Brown, 1983). The rate of degradation of these three herbicides can also be very dependent on soil pH. Because they are weak acids, these herbicides can transition between a neutral and anionic form in the soil, through the exchange of hydrogen and hydroxyl groups (Beyer et al., 1987). The neutral form is the primary compound encountered in acidic soils and this form is more rapidly degraded through hydrolysis (Sabadie, 1996). Therefore, its chemical degradation will be significantly faster on acid soils such as those utilised in our experiments. Despite this, we demonstrated impact upon plant fitness in such soils 10.5 months after application. Somewhat instructively, we showed that one of these herbicides, triasulfuron, was phytotoxic to root function at a 1/100,000 dilution of its label rate. Where microbial or biochemical-mediated break down of herbicides in the soil is limited because of variable factors such as high clay content, low pH, reduced wet periods or low organic matter, it is feasible that the active ingredient remains present at 1/100,000 of the label rate or greater for a period in excess of 10 months.

We also demonstrated that where the plant-back period or minimum rainfall requirements were not met, significant disruption to root health was common. Over 60 % of the interactions between the nine legume treatments and residual soil herbicides in experiment 2B produced a significantly lower nodule rating when compared to the plants grown in the unsprayed control plots. Our results confirm reports that residues of soil-applied herbicides can impart injury on non-target plants in mixed farming systems prior to the expiration of the label plant-back period despite early sowing representing a pragmatic approach to including legumes in farming systems. This research captured not only the detrimental effects of herbicide residues on above ground biomass, but also quantified the damaging effects on the root system, which led to reduced nodulation of legumes. We believe these parameters are insufficiently examined in published legume field research given the importance of BNF in mixed farming systems. As it is exceedingly difficult to measure chemical concentrations at 1/100,000 dilution in soil using standard chemistry (Rose, 2022), a legume root bio-assay involving nodulation may be a more practical instrument for detection of herbicide residues, eventually leading to greater precision in label recommendations for herbicides. The legume root bioassay is an important and useful tool that could be further implemented in agricultural and horticultural industries.

These findings are significant to global crop and pasture systems. Particularly in situations where the two plant types are systematically integrated in same or subsequent year with incompatible herbicides. Such as in multiple cropping systems (Waha et al., 2020), ley-farming systems (Puckridge and French, 1983), or the recently invented perennial ley-farming systems (Edwards et al., 2019). Integrated weed management by herbicide application in multiple cropping systems provides challenges (Hulme, 2023; Rouge et al., 2023) and require innovative approaches (Colnenne-David et al., 2017). Whilst, in ley-farming systems, changing rainfall patterns may mean that there is an increasing incidence of moisture requirements for herbicide break down not being sufficiently met for safe application of the chemicals to soil. The advent of dormant summer sowing of certain pasture legumes (Nutt et al., 2021), and their patterns of hard seed break down (Howieson et al., 2021; Harrison et al., 2023) for subsequent regeneration, increases the risk that toxic concentrations of herbicide residues applied in the cropping phase. Consequently this will impact long term pasture productivity and the BNF produced by them. Under current economics, this BNF is integral to the success of this farming system, because its subsequent transformation into labile N can maintain cereal yields and proteins in the absence of fertilizer N (Harrison et al., 2023).

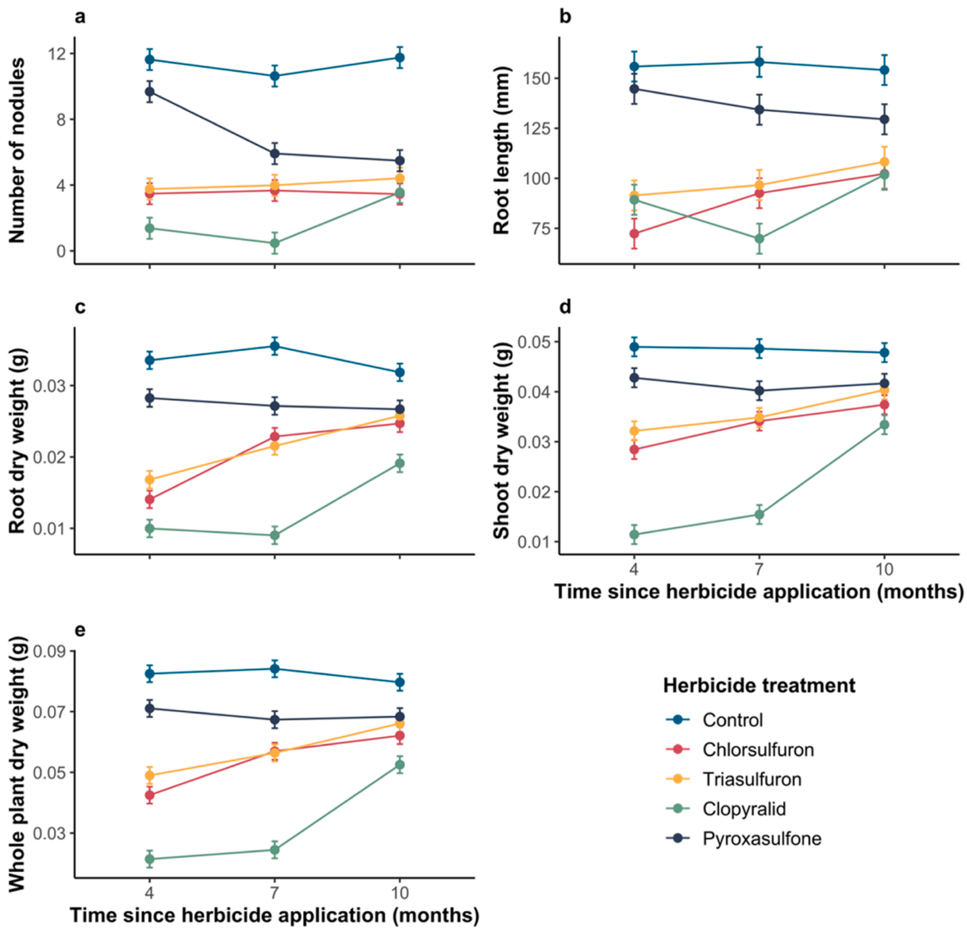

Fig. 3. Measurements of a. number of nodules per plant, b. root length per plant (mm), c. root dry weight (g), d. shoot dry weight (g) and e. whole plant dry weight (g) after 28 days growth of *Trifolium subterraneum* (cv. Dalkeith). Soil core samples taken from the field (Brookton) at (4, 7 and 10 months) after herbicide application July 11, 2012, (chlorsulfuron (750 gai/kg), triasulfuron (750 gai/kg), clopyralid (750 gai/kg), pyroxasulfone (850 gai/kg)) and control (no herbicide) and then bioassayed in a glasshouse experiment. Points are estimated marginal means and error bars are 95 % confidence intervals.

4.1. Triasulfuron toxicity

The glasshouse experiment (1) confirmed that the plant fitness of a range of agricultural annual pasture legumes was severely compromised even when exposed to extremely low levels of the SU group herbicide triasulfuron delivered to soil. Triasulfuron and other related herbicides in the SU group have been reported to reduce nodulation and N fixation of legumes (Eberbach and Douglas, 1989; Mårtensson and Nilsson, 1989; Anderson et al., 2004; Zawoznik and Tomaro, 2005; Farquharson, 2010). However, in our experiment an extreme sensitivity was demonstrated, with disruption of nodulation and a reduction in shoot and root weight recorded at concentrations that represent a 1/100,000 dilution of the recommended label rate for four of the five cultivars examined.

The exception to this was *M. littoralis* cv. Angel, which was developed via chemical mutagenesis to be tolerant to SU herbicides. Previous field experiments in the presence of triasulfuron residues have shown that Angel achieved higher biomass and seed yield compared to its parent cv. Herald (Howieson and Ballard, 2004; Peck and Howie, 2012). However, we showed decreased root length and nodulation in this cultivar when exposed to the herbicide at low dilution in the absence of an effect on shoot weight. There has been little published information on the nodulation and root growth of cultivars specifically selected to tolerate precise herbicide chemistry. It is important to note that production losses can still occur if the level of SU residue exceeds cultivar tolerance threshold. Therefore, the expected benefits from increased tolerance to SU chemistry may be clouded, and the BNF outcomes may be suboptimal, increasing the reliance on synthetically applied N within the farming system (Hackney et al., 2019).

Tap root length and number of nodules provided sensitive measurements of triasulfuron toxicity. Sulfonylurea herbicides work by inhibiting the acetohydroxyacid synthase (AHAS) enzyme which is required for the biosynthesis of the amino acids isoleucine, leucine and valine (LaRossa and Schloss, 1984). The precise mechanism that leads to cell death is not completely understood but impacted plants display severely stunted roots (Beyer et al., 1987). The AHAS enzyme is also present in soil bacteria, including rhizobial species (Nelson and Sadowsky, 2015). For this reason, previous studies have postulated that sulfonylurea residues may directly impact rhizobial populations in their free-living form (Anderson et al., 2004). For the sulfonylurea herbicide chlorsulfuron Farquharson (2010) determined that free-living *Mesorhizobium* populations were largely insensitive to this herbicide. However, Anderson et al. (2004) demonstrated that *Mesorhizobium* previously exposed to chlorsulfuron formed fewer nodules when inoculated into herbicide-free soil, indicating that exposure impacted their ability to enter into symbiosis. This could have a specific consequence in self-regenerating legume pastures, where nodulation is reliant on soil populations of rhizobia that will have been exposed to herbicides applied in the cereal phase.

It is concerning that a wide range of commonly used pasture legumes have recorded deleterious effects when exposed to such low concentrations of triasulfuron, especially *T. subterraneum*. This legume has been the cornerstone of agriculture in southern Australia for 100 years, and reports of its decline (Burnett et al., 1994) coincide with the broad uptake of herbicides. This is critical information, as triasulfuron and other

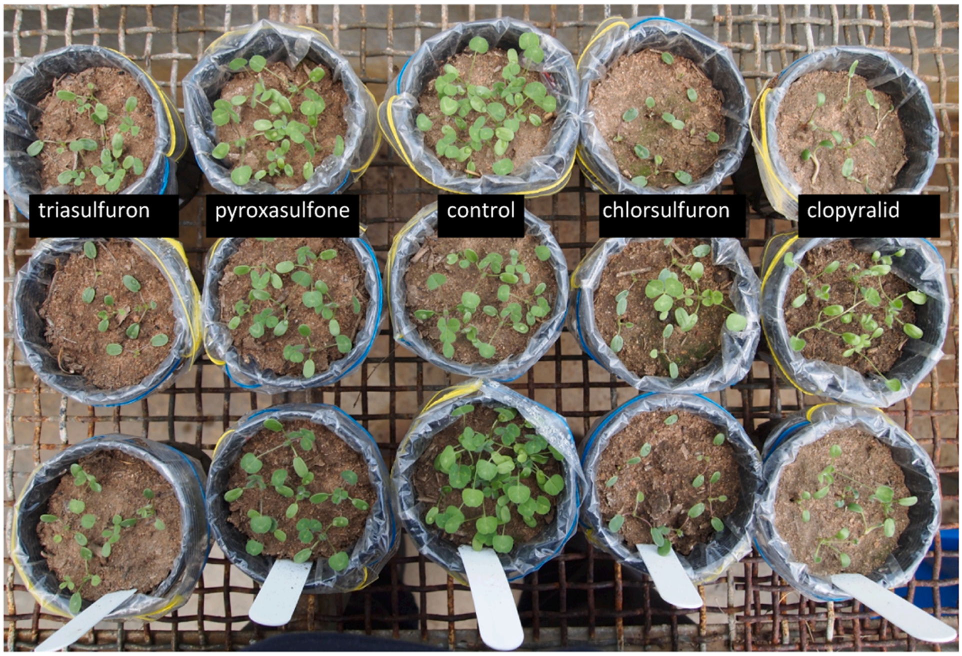

Fig. 4. Growth of *Trifolium subterraneum* cv. Dalkeith at 28 days in three replications of soil cores taken from the field at Brookton on November 2012 and tested for residues in the glasshouse four months after application (July 2012) of herbicides (experiment B) triasulfuron (750 g ai/kg), pyroxasulfone (850 g ai/kg), control: no herbicide, chlorsulfuron (750 g ai/kg), clopyralid (750 g ai/kg).

chemically related herbicides (SUs) remain widely used in contemporary mixed farming systems (Harries et al., 2020).

4.2. Residual herbicide activity

Plant-back requirements listed on herbicide labels may not be adhered to particularly in low rainfall agroecological regions which regularly experience climatic variability. For instance, in Australian dryland farming, many areas do not receive the 300 mm and 250 mm of rainfall required to meet label plant-back requirements for SU and pyroxasulfone chemistry, respectively.

As expected, residues from application of triasulfuron, chlorsulfuron and the synthetic auxin clopyralid to the preceding wheat crop had harmful effects on *T. subterraneum*, nine months after application. It was surprising however, that pyroxasulfone, which is a broad-spectrum herbicide recommended for grass control, reduced nodule number. While it has been shown to control, or suppress, some annual broadleaf weeds (Soltani et al., 2018), our data demonstrate the acute relevance of the plant-back period, even for chemicals not traditionally applied for broad leaf weed control. In our experiments, interacting factors that may have contributed to an unpredictable rate of break down of the herbicides included very low levels of clay (<5 %) and soil carbon (1.09 %), which could have inhibited their microbial decomposition (Beyer et al., 1987; Sarmah et al., 1998; Sarmah and Sadadie, 2002; Hollaway et al., 2006). In this context, complex plant-herbicide interactions have been recently reported in dryland farming systems where soil amelioration activities such as surface liming, claying and strategic deep tillage have increased the phytotoxicity of soil-applied herbicides because of a disruption to microbial influences upon their decay (Edwards et al., 2023).

If opening growing-season rains (predominately late May through to June in southern Australia) fall in late April or early May, thereby providing an early autumn break of season for regenerating legume pastures, or those newly renovated by dormant summer sowing (Howieson et al., 2021; Nutt et al., 2021), our experiments indicate that exposure to common herbicides applied in the preceding cereal crop will cause a reduction in plant fitness. With sufficient rainfall, plant-back recommendations applied to the soil type we examined specify at least a 12-month period for clopyralid, chlorsulfuron and triasulfuron, but a 9-month time-period for pyroxasulfone. Our data supports this recommendation; however, nodulation was reduced by at least 50 % by all four herbicides evaluated. In effect, the plant-back period becomes unmanageable with early season rains, confirming our second hypothesis. Such circumstances would reduce production from the legume pasture and ultimately the pool of BNF available for the entire farming system. Parenthetically, clopyralid is regularly used to control summer weeds in dryland farming systems (Beste, 1983), with applications, therefore, only several months prior to the winter growing season.

Although we investigated the impact of herbicides singularly, current farming practices use mixed herbicide applications on multiple occasions through the growing season and in fallows (Harries et al., 2020). Hence the damage reported in this paper may be lower than is experienced with contemporary practices. A study by Rose et al. (2022) lighted the need for more information on the residual carryover of multiple herbicide classes in agricultural soils, and the risks to future crops. In response to this, farmers are seeking crop genetics that provide tolerance to multiple phytotoxicity thresholds (Rose et al., 2022). There are now herbicide-resistant crop options available, including pasture and grain legume cultivars, which possess increased tolerance or resistance to various herbicide MOAs (Gaines et al., 2020; Brunharo et al., 2022). However, our data emphasises that care must be taken to observe below ground impacts of these herbicides, even when the cultivars are considered tolerant. This factor, combined with the potential that these evolving approaches to weed control could ultimately increase the number of weed species resistant to multiple herbicide MOAs, may confer rapid obsolescence on the present herbicides (Hulme, 2022).

5. Conclusion

Our research highlighted that the persistence in soil of residual herbicides impacted non-target plants, such as legumes, and that plant-back recommendations should be adhered to within the farming system. However, changed farming practices in the face of variable rainfall, which has pushed the seeding times of rotational break crops forward,

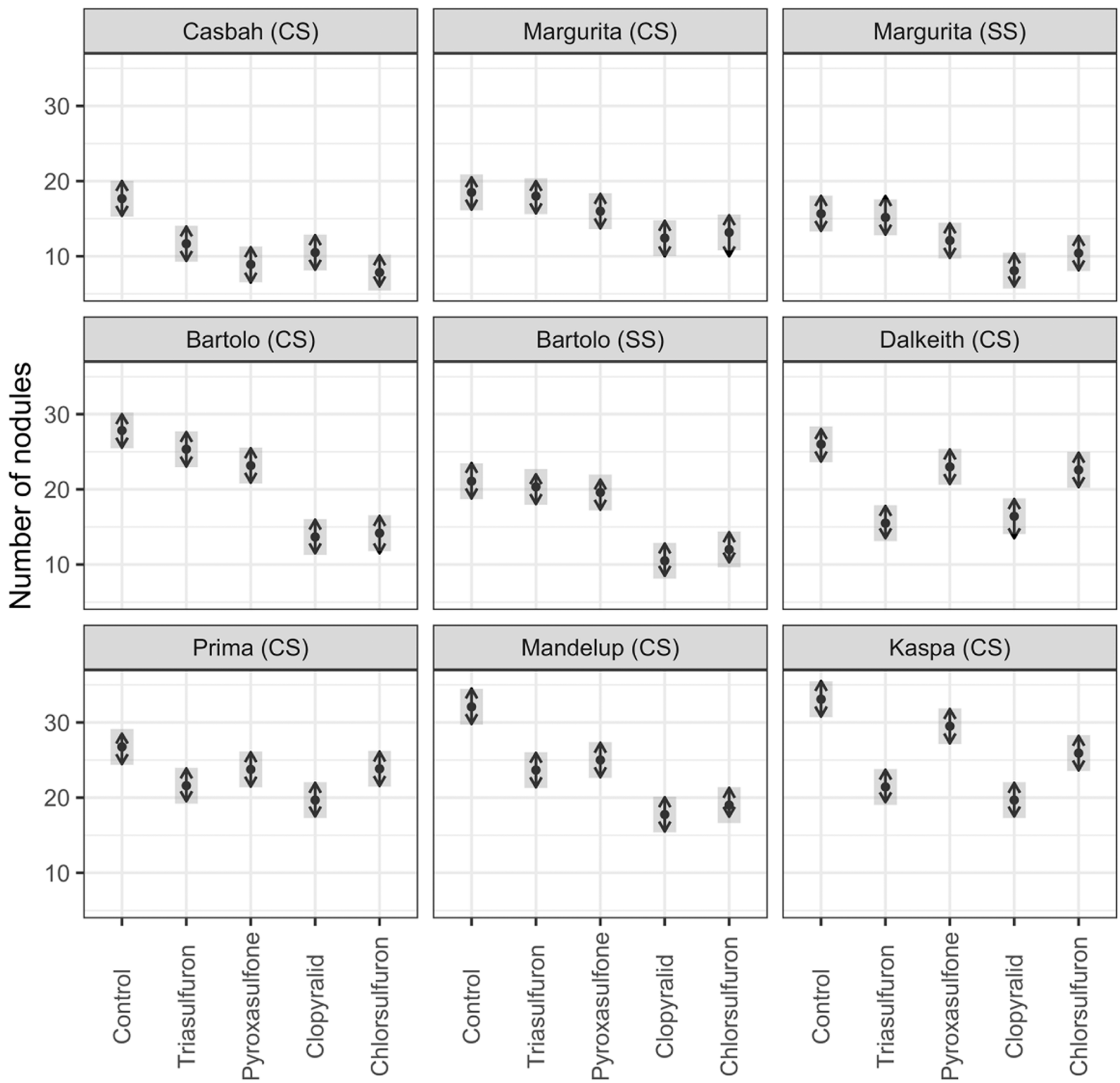

Fig. 5. Average number of nodules per plant at for nine conventionally sown (CS) and summer sown (SS) legume treatments. Legume treatments were; *Trifolium spumosum* (cv. Bartolo) (CS), *T. spumosum* (cv. Bartolo) (SS), *Ornithopus sativus* (cv. Margurita) (CS), *O. sativus*, (cv. Margurita) (SS), *T. glanduliferum* (cv. Prima) (CS), *Biserrula pelecinus* (cv. Casbah) (CS), *T. subterraneum* (cv. Dalkeith) (CS), *Pisum sativum* (cv. Kaspa) (CS) and *Lupinus angustifolius* (cv. Mandelup) (CS). The field site in Brookton, Western Australia was sown across four herbicide treatments; chlorsulfuron (750 gai/kg), triasulfuron (750 gai/kg), clopyralid (750 gai/kg), pyroxasulfone (850 gai/kg) and control (no herbicide). Herbicides were applied in the previous wheat crop approximately 10 months prior. Points are estimated marginal means, shading represents 95 % confidence intervals and arrows indicate Tukey comparison values (where lines overlap there is no significant difference).

Table 4
The percentage of nitrogen derived from atmosphere (%Ndfa) for nine legumes *Trifolium spumosum* (cv. Bartolo), *T. spumosum* (cv. Bartolo) Summer sown (SS), *Ornithopus sativus* (cv. Margurita), *O. sativus*, (cv. Margurita) Summer sown (SS), *T. glanduliferum* (cv. Prima), *Biserrula pelecinus* (cv. Casbah), *T. subterraneum* (cv. Dalkeith), *Pisum sativum* (cv. Kaspa), and *Lupinus angustifolius* (cv. Mandelup) grown in soil in which four different herbicides (chlorsulfuron, triasulfuron, clopyralid, pyroxasulfone and control: no herbicide) were applied to wheat approximately 14 months prior to data collection in Brookton, WA. Legumes were sampled at peak biomass production prior to pod formation.

Legume cultivar									
Herbicide Treatment	Bartolo	Bartolo (SS)	Margurita	Margurita (SS)	Prima	Dalkeith	Casbah	Mandelup	Kaspa
Clopyralid (115 g/ha)	40.3	34.5	45.0	34.2	72.7	24.5	47.7	86.1	81.2
Chlorsulfuron (15 g/ha)	9.5	7.4	62.8	21.7	61.4	49.3	75.4	90.3	75.7
Triasulfuron (35 g/ha)	44.5	10.1	31.9	23.9	70.7	57.4	53.2	76.0	80.1
Pyroxasulfone (118 g/ha)	40.4	15.1	41.9	28.6	49.0	31.4	65.2	79.9	78.5
Control (no herbicide)	61.9	47.2	59.7	39.6	71.5	57.5	77.5	89.9	80.2

are in conflict with this recommendation. There has been a consistent decline of plantings of grain and forage legumes in many global dryland farming systems (Magrini et al., 2016; Preissel et al., 2015), bringing an increased reliance upon synthetic N and other non-legume break crops (Harries et al., 2020). But other considerations are now pertinent to the consumer, such as legumes incorporated successfully into farming systems reduce synthetic N production and hence greenhouse gas emissions. Future farming systems may require the incorporation of legumes to meet public pressure to achieve a lower carbon footprint. In addition, increased legume use will play an important role, particularly in Australian agriculture, in producing improved quality high protein grain (Harrison et al., 2023). Selective herbicides are used to control weeds in cereal crops and are intended to break down over time, allowing the safe planting of crop and pasture legumes in the following year. However due to climatic inconsistencies and changing farming practices, herbicide residues do not always break down within a pragmatic plant-back period and thus can inhibit the formation of an effective symbioses between agricultural legumes and their associated rhizobia.

Declaration of Competing Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Data availability

The authors do not have permission to share data.

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