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Is the growth stimulation by low doses of glyphosate sustained over time?

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Glyphosate induced hormesis in barley is not sustained over time.

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ABSTRACT

The herbicide, glyphosate, has been shown to stimulate growth in a range of species when applied at doses of 5–60 g a.e. ha^{-1}, corresponding to realistic spray drift events. This study investigates growth of shoot parameters over time to detect whether the glyphosate induced growth increase was sustained and had a final effect on reproduction. The results showed that an actual biomass growth rate increase took place within the first week after spraying with glyphosate doses <60 g a.e. ha^{-1}. This initial growth boost kept treated plants larger than untreated plants for up to six weeks, but at harvest there was no significant difference between control plants and treated plants. Possible effects of glyphosate hormesis on the competitive ability of spray drift affected plants are discussed.

1. Introduction

Low dose growth stimulations of chemicals which are toxic at higher doses have been recorded for more than a century. The low dose stimulations have been noted for a range of chemicals and endpoints in all types of organisms from animals and plants to fungi and bacteria (Calabrese, 2005). The phenomenon was first called the Arndt–Schulz law, but in 1943, Southam and Erlich introduced the term hormesis (Southam and Erlich, 1943). Hormesis in plants have also been shown to be induced by herbicides, where several studies demonstrate that some can stimulate plant growth when applied at low doses (<50% of EC_{50}) (Cedergreen, in press; Cedergreen et al., 2007; Davies et al., 2003; Duke et al., 2006; Schabenberger et al., 1999; Velini et al., 2008). The herbicide most frequently used in the world, glyphosate, seems to be one of the herbicides which consistently induce this growth response.

When herbicide hormesis in plants has been reported, it is almost always at single time points and in individual plant traits, such as plant height, leaf area, shoot weight, etc. Studies on other organisms, mainly microorganisms and animal biomarkers, have shown a stimulatory response being induced after a transient depression of the same response (Calabrese, 2001, 2005). The same studies have, however, not been prolonged to study whether the response increase persisted over time. It has been argued that from an evolutionary perspective, the hormetic response stimulations induced by chemical stress will not lead to an increase in population fitness, but that it will happen at a cost (Forbes, 2000; Parsons, 2003). This cost could either be a decrease in another trait, as was shown for insects, where an increase in number of eggs produced was counterbalanced by lower off-spring survival (Fujiwara et al., 2002; Zanuncio et al., 2003). Several of the studies presented by Calabrese (2005) likewise showed varying degrees of hormesis and compensations in other traits where several life history parameters were measured. Alternatively to trade-off between traits, the cost could be paid over time with the hormetic response increase being followed by a subsequent decrease, where the organism "recovers" after the strain of the stress.

Wild plants growing outside field edges may receive spray drift of herbicides in the range of 1–10% field rate, corresponding to 5–60 g a.e. glyphosate ha^{-1}, depending on dose rate recommendations (Asman et al., 2003). Weeds growing underneath glyphosate resistant crops might well receive similar doses. If the short-term effects on plant growth of glyphosate at these doses are measured, they are likely to show slight stimulations, as has been shown for *Stellaria media* (Common Chickweed), *Tripleurospermum inodorum* (Scentless Mayweed), *Commelina benghalensis* (Tropical Spiderwort), *Hordeum vulgare* (Barley), *Zea mays* (maize), *Glycine max* (soybean), *Eucalyptus grandis* (Eucalyptus), *Pinus caribea* (Pine) and several species included in spray drift experiments (Cedergreen, in press; Cedergreen et al., 2007; Marrs and Frost, 1997; Marrs et al., 1991; Velini et al., 2008). The questions are whether this effect prevails in time, and how it influences the reproductive output in terms of numbers and size of seeds. An initial growth increase of

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plants sprayed with low herbicide doses could give these plants a head-start leading to a final higher biomass and corresponding seed yield. Alternatively, an initial growth increase, followed by a growth depression, might lead to a net decrease both in biomass and seed yield. The purpose of this study was to investigate the biomass growth and resource allocation pattern of glyphosate exposed plants over time until seed set.

2. Materials and methods

2.1. Plants

The annual plant *H. vulgare* was chosen as an example species, as previous work showed robust and reproducible hormesis in the used setup. Also, resource allocation to reproduction is more easily and precisely measured in a domesticated annual because it does not shed its seeds at maturation as do many small seeded wild species. Barley seeds var. Barke (Saatzucht Josef Breun, Harzogenawach, Germany) were sown in Pindstrup substrate no. 1 (Pindstrup, Denmark). Four or five seeds were sown in 7 L pots (diameter: 25 cm), which were placed in a greenhouse and connected to a drip water irrigation system. All plants were well watered with nutrient solution (Pioner NPK Makro 14-3-23 + Mg combined with Pioner Mikro with iron, Brøste, Denmark) adjusted to a pH of 5.5 and an EC of 1.3. Two experiments were run in the periods June 20th–October 16th 2006 and August 15th 2006–February 12th 2007. In these periods the mean day/night temperatures ranged from 25.0 ± 3.9 °C/20.4 ± 2.1 °C in July to 15.6 ± 1.26 °C/12.7 ± 0.44 °C in December and light periods ranged from 18 h in June/July to 7 h in December/January with an average outside light intensity of 345 ± 260 W (max: 850 W) in July and 37 ± 32 W (max: 135 W) in December. For the winter experiment, an additional light source of sodium lamps (Son-T Pia Green Power, Phillips, The Netherlands) at a density of three lamps per 8 m^{-2} were turned on from 7.30 to 20.30 from October 11th 2006 to harvest. Due to problems with aphids, *Aphidius colemani* were released among the plants every two weeks beginning August 3rd. As these predatory insects could not control the aphid population to a level below what could be expected to influence plant yield, all plants were sprayed with the insecticide imidacloprid (Confidor, Bayer, Germany) at the recommended dose of 50 g a.e. ha^{-1} on August 8th and September 9th 2006. No bias in the amphid infestation depending on treatment was observed. Approximately one month prior to harvest watering was terminated to allow the plants to mature.

2.2. Treatment

For the first experiment 10 pots with each five plants were sprayed with glyphosate at the doses: 4, 8, 16, 32, 63, 125, 250 and 500 g a.e. ha^{-1} (Glyphonova: 360 g a.e. L^{-1}/486 glyphosate isopropylamine salt formulated with fatty amine ethoxylate (Harry Teischer, Cheminova, personal communication), Cheminova, Denmark). The plants were sprayed 10 days after sowing at the two-leaf stage. The spraying was done in a spray cabin, using Hardi LD-02-110 hydraulic nozzles, a pressure of 4 bar and a spraying volume of 150 L^{-1} ha^{-1}. Twenty pots with each five plants were left for controls. The plants were placed in a randomised setup. After one week, one plant from each pot was harvested, and leaf area and dry weight, plus stalk length and dry weight were determined for control plants and plants treated with 8 and 32 g a.e. ha^{-1}, as previous experiments had shown these doses to be within the range that could induce a growth increase. For the remaining plants, total plant dry weight was determined after drying the plants at 80 °C. During the following seven weeks, five plants of each treatment and 10 control plants were harvested and treated similarly. At boosting, and the weeks thereafter, spike number and dry weight per plant were also measured for control plants and plants treated with 8 and 32 g a.e. ha^{-1}, as was the proportion of fresh and desiccated leaf area. Average daily relative growth rates (RGR) for the different time intervals were calculated as: $(\ln(DW_t) - \ln(DW_{t-1}))/7$ days, where DW_t is the average plant dry weight at harvest time t and DW_{t-1} is the average harvest dry weight one week before t. The average dry weight at the time of spraying was 9.8 ± 1.5 mg $plant^{-1}$ ($n = 10$). When the plants had matured, the remaining 10 plants per treatment and 20 controls were harvested and straw and spike dry weights were determined together with grain weight and number.

For the second experiment four seeds were sown in each pot, and 10 pots were sprayed with glyphosate at the doses: 4, 8, 16, 32, 63 and 125 g a.e. ha^{-1} as described above. Twenty-five pots were left as controls. One week after spraying, one plant was harvested from each pot, leaving three plants per pot to grow to maturity. The harvested plants were dried at 80 °C and dry weight was determined. When the plants had matured, they were harvested and straw and spike dry weights were determined together with grain weight and number.

2.3. Statistics

All data on biomass increase were described with a three parameter logistic dose–response model (Cedergreen et al., 2005):

$$y = \frac{d}{1 + (x/e)^b} \tag{1}$$

where y is the measured response, d is the upper limit of the curve corresponding to the control values, e is the dose of 50% response decrease (ED_{50}), and b is proportional to the slope of the dose–response curve around e. In addition, all data were described with a biphasic logistic dose–response model including a term for hormesis (Cedergreen et al., 2005):

$$y = \frac{d + f\exp(-1/x^{\alpha})}{1 + (x/e)^b} \tag{2}$$

In this equation f is the parameter describing the degree of hormetic increase, α is a parameter determining the slope of the hormetic increase and e determines the infliction point of the decreasing part of the lower bound of the dose of 50% response decrease (ED_{50}). As there are rarely enough data to estimate α, its value was fixed to

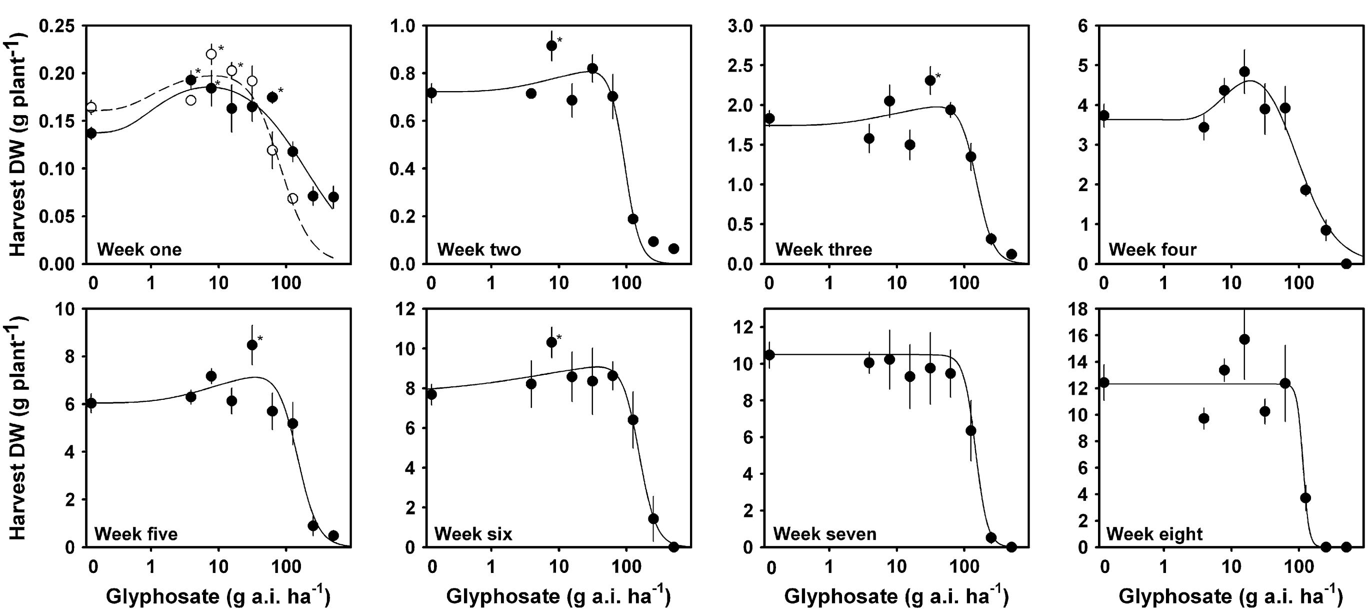

Fig. 1. The dry weight of plants harvested once a week after the time of spraying for eight weeks as a function of glyphosate dose. Data for the second experiment harvested one week after spraying are given with open symbols. Data are given as mean ± SE ($n = 5$ for treated plants and $n = 10$ for controls) and are fitted to a biphasic dose–response model in the cases where this model gave the best fit based on residual sums of squares. Fit parameters are given in Table 1. Individual treatments which were significantly higher than controls (two-tailed t-test) are denoted with *.

Table 1
Model parameters of the model with the lowest residual sums of squares for whole plant dry weight as a function of glyphosate dose

Time after harvest	b	d	e	f	α	max	Dose$_{max}$	P
Week one	0.87 ± 0.24	0.14 ± 0.01	118 ± 58	0.19 ± 0.08	1	0.19	7	<0.001
Week two	3.88 ± 1.16	0.72 ± 0.04	94 ± 9	0.17 ± 0.12	0.5	0.79	23	0.16
Week three	3.46 ± 1.00	1.74 ± 0.11	151 ± 17	0.42 ± 0.29	0.5	1.98	28	0.15
Week four	1.44 ± 0.78	3.63 ± 0.26	81 ± 49	2.62 ± 2.5	1	4.54	22	0.03
Week five	2.88 ± 1.19	6.05 ± 0.42	146 ± 23	2.03 ± 1.42	0.5	7.13	35	0.11
Week six	3.43 ± 1.41	7.83 ± 0.66	153 ± 22	2.70 ± 2.10	0.25	9.07	37	0.02
Week seven	4.97 ± 2.50	10.50 ± 0.41	147 ± 15	–	–	–	–	0.57
Week eight	9.86 ± 39	12.31 ± 0.64	115 ± 39	–	–	–	–	0.75
Week one, exp. 2	2.11 ± 0.41	0.16 ± 0.01	81 ± 9	0.05 ± 0.02	0.5	0.20	11	0.001

Parameter explanations (b, d, e, f and α) are given in Eq. (2). For curves with hormesis, the maximum response is given under max (g dry weight plant^{-1}) and the corresponding dose as dose$_{max}$ (g a.e. ha^{-1}). All parameters except α, which is fixed, and max and dose$_{max}$, which are calculated, are given ±SE. The P-value for the lack-of-fit F-test comparing the models with and without the hormesis term is also given. For $P < 0.05$ the hormesis model fits significantly better than the three parameter logistic model. The curve from the second experiment (exp. 2) is also included.

0.25, 0.5 or 1, which, from experience, covers the range of slope increase in most hormetic dose–response curves in plants. The best fitting curve of the three was chosen based on residual sums of squares. Setting f to zero removes the hormetic term $f\exp(-1/x^{\alpha})$ from the equation and reduces it to the classic three parameter log-logistic model with e equal to ED_{50}. The two non-linear models, with and without the hormetic term, were compared, both by evaluating residuals as well as by an F-test, to determine which of the models best described the data (Bates and Watts, 1988). Comparisons between single doses and controls were done with a two-tailed t-test.

To test whether there was any difference between the harvest data from the two experiments, data were fitted jointly, either to one common dose–response model (giving one d, b and e parameter), or to two separate models (giving d_1, d_2, b_1, b_2, e_1 and e_2). The two models' fits were then compared with a lack-of-fit F-test. If there was no significant difference between the models fitting the curves jointly or separately ($P > 0.05$), the curves were not significantly different. All analyses were made using the language and environment R (R Development Core Team, 2004) with the add-on package drc (Ritz and Streibig, 2005).

3. Results

In both experiments, there was a significant dry weight increase in the plants sprayed with low doses of glyphosate a week after spraying (Fig. 1, Table 1). This effect was maintained for approximately six weeks, after which no significant increases of treated plants compared to controls were seen. The dry weight increase was approximately 33 ± 6% of controls during the first six weeks after spraying, calculated from the difference between the treatment with the maximal response and the control. Calculated from the maximum of the fitted curve, which might be a more reproducible estimate due to the large variance between treatments, the dry weight increase was 20 ± 9%.

Looking at the relative growth rates of the plants within the hormetic dose-range (sprayed with <63 g a.e. ha^{-1}), the first eight weeks after spraying showed that the sprayed plants only grew faster than the control plants during the first week after spraying (except plants treated with 16 g a.e. ha^{-1}) (Fig. 2). During the following three weeks, growth rates of the treated plants were generally either lower or similar to the control growth rates (Fig. 2). Testing the average growth rates of the five treatments <63 g a.e. ha^{-1} against the control (two-tailed t-test assuming equal variance) showed no significant difference between control and treated plants ($p > 0.05$).

Evaluating different morphological traits of plants within the hormetic dose-range showed that particularly stalk parameters (stalk dry weight, length and number) increased compared to the control shortly after spraying (Fig. 3). Leaf dry weight was, however, at its maximum five to seven weeks after spraying. Treatments significantly lower than controls were only observed eight weeks after spraying for leaf dry weight and area, and spike number of plants treated with 32 g a.e. ha^{-1}.

At the time of harvest there was only little sign of stimulation in any of the measured parameters (spike, straw and grain weight and number) (Fig. 4, Table 2). On the other hand, there were no indications of a decrease in any of the harvest parameters for the plants sprayed with doses giving initial growth stimulation (4–63 g a.e. ha^{-1}) either. The winter grown barley plants (Experiment 2) had almost three times as much biomass in the straw fraction compared to the summer grown plants (Experiment 1), while there was no, or only little, difference between the two experiments when it came to spike weight and weight of the individual grains. The summer grown plants had approximately 45% more grains per plant compared to the winter grown plants, resulting in approximately 30% higher total grain yield (Fig. 3, Table 2).

4. Discussion

The actual increase in growth rates of the glyphosate treated plants primarily took place within the first week after spraying, after which it declined to levels slightly below the control plants for the following three weeks. This pattern in growth rates, though small, corresponds to the theory of trade-off over time with an initial increase being followed by a slight depression (Forbes, 2000; Parsons, 2003). Growth rates in this experiment were only measured on above ground biomass, and the increased shoot growth could therefore theoretically be a result of

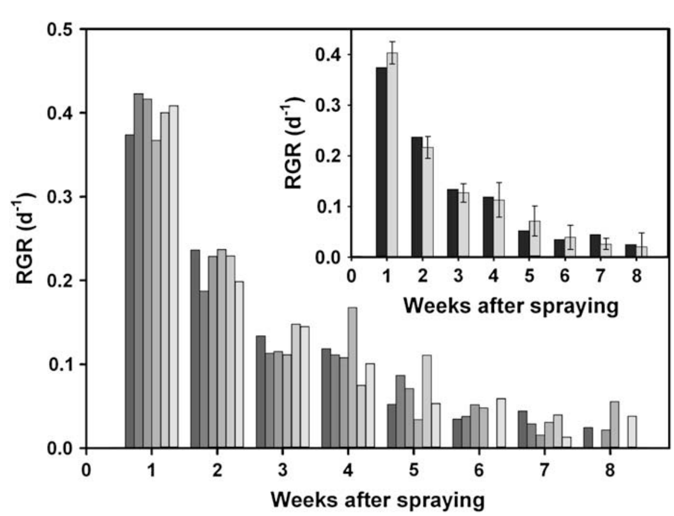

Fig. 2. Relative growth rates (RGR) calculated from the mean dry weight of the 10 control plants and five plants harvested from each treatment once a week during the first eight weeks after spraying with glyphosate. The darkest columns are the controls followed by the treatments: 4, 8, 16, 32 and 63 g a.e. ha^{-1} with decreasing shade. The inset shows the figure with the average RGR of the treated plants ± SD (shaded bar) compared to the control (black bar).

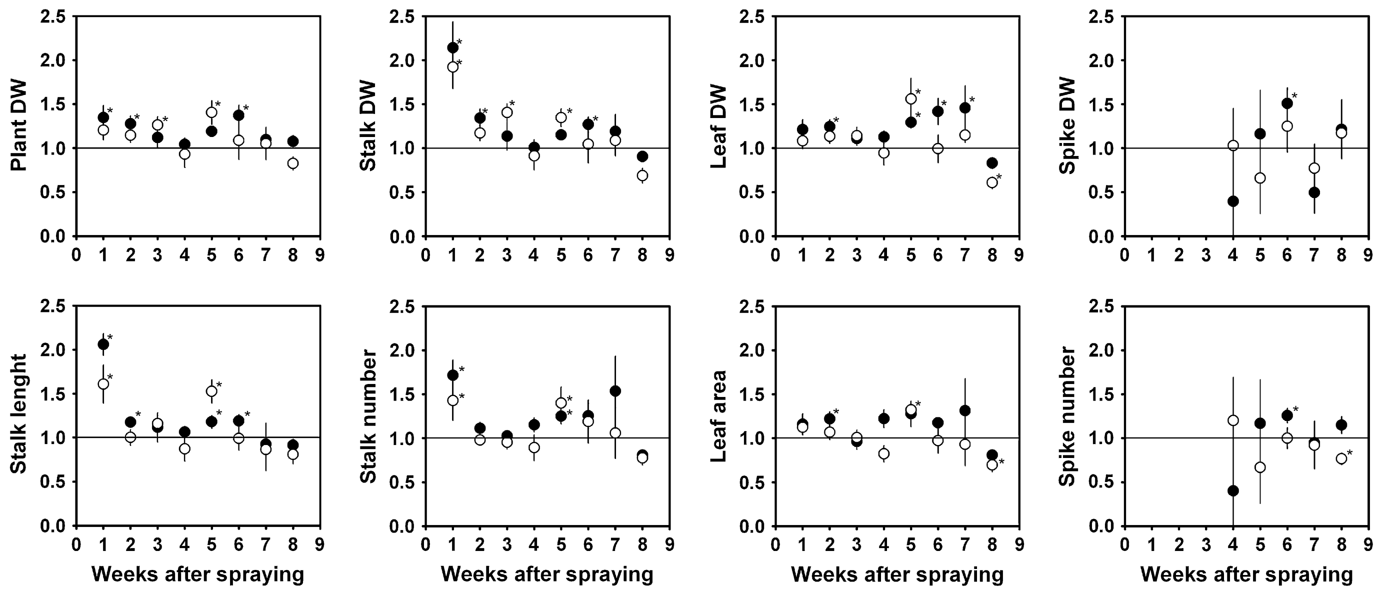

Fig. 3. The response of different morphological parameters of plants sprayed with glyphosate at either 8 g a.e. ha^{-1} (filled symbols) or 32 g a.e. ha^{-1} (open symbols) relative to the control, which is set to one, at various weeks after spraying. Differences between control ($n = 10$) and treated plants ($n = 5$) tested by a two-tailed t-test are denoted with *. Data are given as mean ± SD.

a changed resource allocation between root and shoot. Experiments on hydroponically grown barley plants have, however, shown the hormetic growth stimulation of glyphosate to take place in both root and shoot, even increasing the root:shoot ratio (Cedergreen, in press). Hence, the growth stimulations observed during the first week in this study are real, and it appears the cost is paid over time.

Even though growth rates only increased during the first week after spraying, the first week growth rates were so fast that the biomass increases of the plants treated with <63 g a.e. ha^{-1} were maintained for approximately six weeks after spraying. Such a boost in growth could give plants, receiving growth stimulating doses, a competitive advantage to species whose sensitivity differs, and for whom the applied doses therefore either have no effect or even have an adverse effect. Even though glyphosate is considered a non-selective herbicide, the sensitivity among species varies (Cerdeira and Duke, 2006; Gove et al., 2007). Spray drift experiments have shown increased growth in some species at low glyphosate doses, but as these trials were on mixed communities, it could not be concluded whether the results were caused by hormetic growth increases or from changes in competition between species due to adverse effects on competitors (Marrs and Frost, 1997; Marrs et al., 1991). In another study where wild species were treated separately in both greenhouses and in the field, the two species *Carex remota* and *Primula vulgaris* sprayed with 22 g a.e. ha^{-1} glyphosate obtained a higher biomass than the control

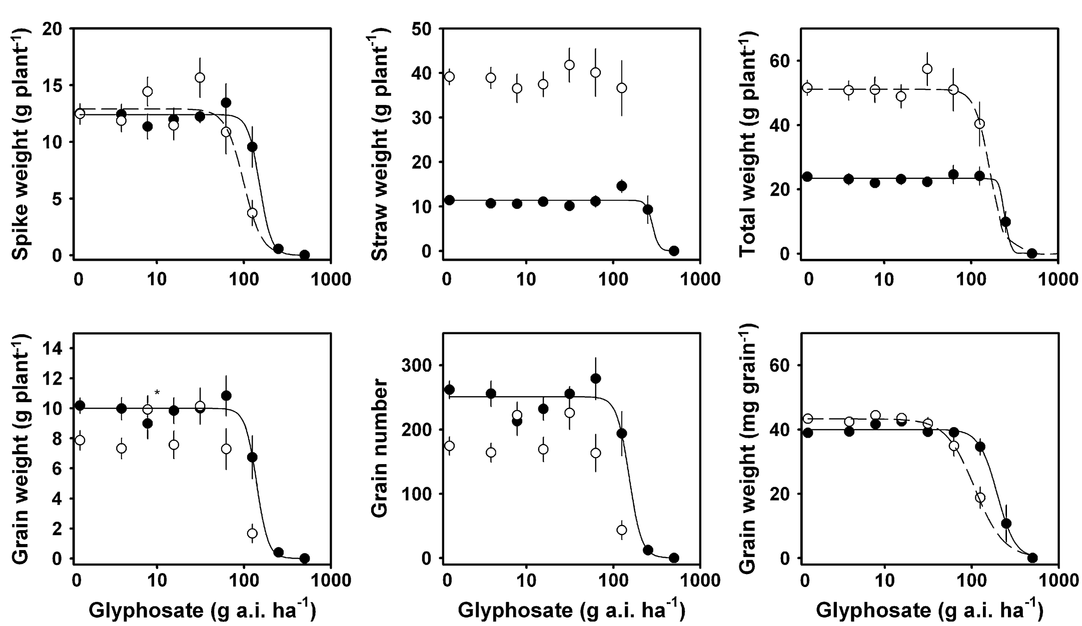

Fig. 4. Harvest data from the first (closed symbols) and the second (open symbols) experiment. Data are fitted to a log-logistic dose–response model when possible (Table 2), and are given as mean ± SD ($n = 10$; 20 and 30; 75 for treated; control treatments in the first and second experiment, respectively).

Table 2
Dose–response model parameters for the log-logistic model fitted to the harvest data

Endpoint	Exp.	d	b	EC_{50}	P
Spike (g plant^{-1})	1	12.4 ± 0.4	6.5 ± 3.2	152 ± 14	0.10
	2	12.9 ± 0.5	4.2 ± 1.4	100 ± 12	
Straw (g plant^{-1})	1	11.4 ± 0.5	12.1 ± 31.3	283 ± 91	
	2	39 ± 16	–	–	
Total (g plant^{-1})	1	23.4 ± 0.7	13.7 ± 71.5	244 ± 30	<0.001
	2	51.7 ± 1.6	5.1 ± 9.7	160 ± 76	
Grain weight (g plant^{-1})	1	10.0 ± 0.3	6.6 ± 5.8	140 ± 17	
	2	7.8 ± 5.7	–	–	
Grain number (plant^{-1})	1	251 ± 8	6.4 ± 3.2	152 ± 18	
	2	173 ± 122	–	–	
Grain weight (mg grain^{-1})	1	40.0 ± 0.8	4.2 ± 0.8	196 ± 12	<0.001
	2	43.3 ± 0.7	2.6 ± 0.4	109 ± 7	

The P-value is from the lack-of-fit F-test between the data from the two experiments fitted jointly or separately. A $P > 0.05$ indicates not significantly different curves. The upper limit d for curves that could not be fitted to a dose–response model is calculated as the average ± SD of the control plants.

plants 10 weeks after spraying in the greenhouse, and even a year after spraying the field, while the four other species surveyed either had a similar or a decreased biomass at harvest (Gove et al., 2007). Hence, it is likely that hormetic growth stimulations, as those observed in the present study, influence the competitive ability of species exposed to spray drift events.

This effect on competitive ability might be further strengthened by the fact that this study showed stalk length (dry weight and number) to be the first morphological parameter to increase, followed by an increase in leaf area and dry weight several weeks after spraying. An increase in stalk length will increase the plants' competitive ability for light. The pattern of increased stalk length and number followed by an increase in leaf growth parallels the morphological changes induced by auxin-type herbicides (Copping and Hewitt, 1998). Glyphosate interferes with the auxin pathway by blocking some of the precursors of auxin synthesis (Cobb, 1992; Crozier et al., 2000). Hence, it could be hypothesized that the morphological responses observed at low glyphosate doses were due to an increased activation of auxin. This could lead to a transient increase in auxin activity to compensate for the decreased auxin synthesis. How an increase in auxin activity should lead to an increase in biomass production is, however, unresolved and needs further study. Strobilurin fungicides have been shown to increase grain yield by depressing ethylene production, thereby keeping plants green and photosynthesising for longer (Gerhard et al., 1998; Grossmann et al., 1999). A similar greening effect was not observed for glyphosate in the present study, where the plants treated with <63 g a.e. ha^{-1} senesced at the same rate as the controls.

Seed production was not influenced by the glyphosate treatment <63 g a.e. ha^{-1}, neither in terms of seed numbers produced or in terms of individual seed weight. The study by Gove et al. (2007), where fecundity (number of flowering plants and number of flowers at harvest) in the field was measured one year after spraying with 22 g a.e. ha^{-1}, also showed no significant trend in effects on reproduction. In this study, four out of five species showed increased numbers of flowers in an unfertilised plot, while the opposite was the case in the fertilized plot (Gove et al., 2007). In the present study we found similar results, even though the two experiments were run under very different temperature and light conditions. Other recent studies have, however, found growth impairment of low glyphosate doses in micro-nutrient deprived plants due to decreased uptake of iron and manganese (Eker et al., 2006; Ozturk et al., 2008). Plant nutrient availability is therefore likely to impact the occurrence of hormesis under natural growth conditions.

5. Conclusion

The study concludes that hormesis in barley, in the form of an actual growth increase, does take place within the first week after spraying with glyphosate doses <63 g a.e. ha^{-1}, but that the initial growth increase is followed by a slight growth rate decrease. The initial growth increase is, however, sufficient to keep treated plants larger than untreated plants for up to six weeks after spraying. It is proposed that the initial growth boost of low dose glyphosate treated plants could influence the competitive ability of affected non-target species.

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