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UNDERSTANDING AND COMBATTING THE THREAT POSED BY RYE-GRASS (*LOLIUM MULTIFLORUM*) AS A WEED OF ARABLE CROPS

by

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ABSTRACT

Lolium multiflorum (Italian rye-grass) is an increasing problem as a weed of arable crops in the UK and many other countries. However, there exists only limited knowledge on many basic aspects of this weed's agro-ecology and how it interacts with cultural and chemical control methods. Therefore, the aim of this project was to characterise better both the agro-ecology and the basis of resistance to ACCase inhibitors in this species.

Resistance in *L. multiflorum* to at least one ACCase-inhibiting herbicide was found to be widespread in England and was detected on 70% of the 50 farms surveyed. At least one mutation in the ACCase gene (Asp-2078-Gly (in 24.5% of 384 resistant plants assayed), Ile-1781-Leu (13.3%), Ile-2041-Asn (2.1%), Cys-2088-Arg (1.8%), Trp-2027-Cys (1.0%) and Trp-1999-Cys (0.3%)) was found in 40% of the resistant plants analysed. However, at present, the most common mechanism of resistance to ACCase-inhibiting herbicides in UK *L. multiflorum* populations appears to be non-target site resistance, most likely enhanced metabolism.

Field studies showed that the majority (94%) of *L. multiflorum* plants in winter wheat fields emerged between October and December, with only 6% emerging in spring. Autumn emerging plants were much more competitive and produced on average 23 times as many seeds per plant as spring emerging cohorts. The success of *L. multiflorum* as a weed of winter cereals is due to its ability to produce high numbers of heads per plant (mean = 20) and seeds per head (mean = 295), even at low weed densities. *L. multiflorum* also had a highly detrimental effect on wheat yield with losses of up to 89%. *L. multiflorum* seed dormancy is short and determined by both genetics and weather conditions during seed maturation. By delaying the sowing date based upon predictions of dormancy status and using resistance testing to help design an effective chemical control strategy in autumn, *L. multiflorum* infestations could be significantly reduced.

INTRODUCTION

In a survey carried out throughout Europe in 2003, *Lolium* spp. were rated as the second most important herbicide-resistant weed after *A. myosuroides,* with *Papaver rhoeas* and *Avena* spp. in third and fourth place (Moss, 2004).

Within *Lolium* spp., *L. multiflorum* and *L. perenne* are distributed thoroughout the UK. However, it is *L. multiflorum* that probably represents the greater weed threat to arable crops since, although it is a common weed, *L. perenne* is not a problem in arable crops in the UK. In a survey conducted in cereal crops in Southern England after the completion of herbicide programmes in 1981, approximately 8% of fields were infested with *L. multiflorum* while less than 1% were infested with *L. perenne* (Froud-Williams & Chancellor, 1982).

The first case of herbicide resistant *L. multiflorum* populations appeared in Oregon (USA) in 1987 (Stanger & Appleby, 1989) where two populations were found to be resistant to the herbicide diclofop-methyl, an ACCase inhibitor. In 1991, just four years later, the first resistant populations appeared in the UK (Moss *et al.*, 1993). Resistance to diclofop-methyl was found in five farms in England and cross-resistance to other herbicides from the same chemical class was also demonstrated, including fenoxaprop-ethyl and fluazifop-P-butyl. There were indications of resistance to tralkoxydim and partial resistance to isoproturon, an inhibitor of photosynthesis from the group of ureas and amides, was also detected. By 2004, a total of 324 farms in England had been shown to possess herbicide resistant *L. multiflorum* populations while no reports of resistance in *L. perenne* were received (Moss *et al.*, 2005). *L. multiflorum,* as a weed of cereals, is better adapted than *L. perenne* since it can be biennial and annual, and can produce larger quantities of viable seeds than *L. perenne* which has to act as an annual species rather than act as a perennial (Moss, 2005).

In 2005, a compilation exercise for herbicide-resistant weeds in the UK was published which showed that the three major resistant grass weeds in the

UK are *A. myosuroides*, *L. multiflorum* and *Avena* spp (Moss *et al.*, 2005). Worldwide, resistance has been found in other *Lolium* spp. too, including *L. rigidum*, *L. persicum* and *L. perenne* (Heap, 2009). *L. rigidum* was the first *Lolium* sp. with confirmed resistance to herbicides in 1979, in Israel, and subsequently in Australia, in 1982. It presents cross-resistance to ten chemical groups and is present in eleven countries. According to the international survey of herbicide resistant weeds *L. rigidum* is the worst resistant grass-weed worldwide (Heap, 2009).

Nowadays, the number of cases of resistant *L. multiflorum* populations have increased and are currently present in eight countries in the world; Argentina, Brazil, Chile, France, Italy, Spain, UK and USA (Heap, 2009). The modes of action affected by resistance in *L. multiflorum* are ACCase inhibitors (30 cases reported worldwide), ALS inhibitors (16 cases), glycines (13 cases), chloroacetamide (1 case) and ureas and amides (1 case).
The threat that *L. multiflorum* poses to UK arable cropping systems is increasing. This is probably due to increased autumn cropping, especially of wheat and oilseed rape, greater use of minimum tillage, a lack of good rotations and early drilling as well as the rise of herbicide resistant varieties (Moss, 2005). Herbicides from ten different groups can be used to control *L. multiflorum* (HSE, 2008), but those most commonly used in cereal crops in the UK have traditionally been the ACCase inhibitors. Since *L. multiflorum* is a highly competitive and prolific weed, high levels of control are needed to maintain crop yields and to avoid a high seed return. The appearance of resistant populations makes it necessary to use non-chemical control methods in an integrated system. There is a lack of knowledge of *L. multiflorum* biology and how it responds to cultural control in UK conditions. In addition, the extent of this resistance and the mechanisms responsible for it need to be understood in order to develop strategies to counter this problem. Although much research has been carried out on *Lolium* spp. in other countries, these data cannot be extrapolated to UK *L. multiflorum* populations, since cropping systems and growing conditions in those countries are different to those in the UK. Therefore, there is a need for more research on the chemical and cultural control of *L. multiflorum* that is relevant to the agronomic systems in the UK.

This project aimed to use a combination of field, glasshouse and molecular studies to characterise both the agroecology and the basis of resistance to acetyl CoA inhibiting herbicides in *L. multiflorum* populations from the UK. Discovering the mechanisms of resistance in field populations and studying the agro-ecology of *L. multiflorum* should allow the development of better weed management strategies.

This project focused on the following topics:

- The seed production potential of different *L. multiflorum* populations from the UK were determined to discover how this is affected by weed density and the implications it has for population dynamics.
- The agro-ecology of *L. multiflorum* and *A. myosuroides* plants in competition with wheat was evaluated. The effects of both crop and weed densities on weed seed production and on crop yield were examined.
- Seedling emergence patterns. The proportion of *L. multiflorum* plants emerging in autumn and in winter/spring was investigated. The aim was to determine the point when the majority of plants have emerged and thus, the optimum time for herbicide applications.
- *L. multiflorum* dormancy and its implications for weed control. The relative influence of weather conditions, in particular temperature and soil moisture, during seed maturation on the initial dormancy of UK *L. multiflorum* populations was investigated, with the aim of predicting the emergence of *L. multiflorum* seedlings based on weather conditions.
- Whole plant resistance assays were carried out to provide a better understanding of the extent of resistance to ACCase-inhibiting herbicides and the cross-resistance pattern within UK *L. multiflorum* populations.
- Studies of the molecular basis of resistance in UK *L. multiflorum* populations to determine the most common mechanisms of resistance to ACCase-inhibiting herbicides and to identify the most frequent mutations responsible for target site resistance.

MATERIALS AND METHODS

Agro-ecology studies

Emergence pattern studies of five populations of Lolium multiflorum

To measure the proportion of plants emerging in autumn and spring, quadrats were placed randomly in untreated areas in four winter wheat fields in October 2006, in the counties of Buckinghamshire (High Wycombe), Essex (North Benfleet) and Hertfordshire (Hitchin and Berkhamsted07), and one field in October 2007 in Hertfordshire (Berkhamsted08).

The assessment was carried out between October and April in 24 x0.1m^2 quadrats per site. Plants were counted every four weeks in each quadrat. For a more accurate assessment of emergence, newly emerging plants were labelled every month in four quadrats per site in 2006/07 and in every quadrat in 2007/08, using plastic coffee stirrers numbered from one to seven depending on the month the plant emerged. The growth stage of all plants in the 2007/08 field and a few plants per site in the 2006/07 fields (two plants per quadrat labelled in autumn 2006 and all new emergences labelled in spring 2007) were monitored. These plants were also assessed in June to study the seed production potential and also dried and weighed, with the aim of comparing the effect of time of emergence on plant vigour and seed production potential.

Seed production potential studies of ten populations of Lolium multiflorum

In June 2006, 2007 and 2008 plants of *Lolium multiflorum* with intact inflorescences ('heads') were collected from untreated areas of winter wheat crops, in Lincolnshire (Louth), Essex (Peldon, Wickford and North Benfleet), Cambridgeshire (Chatteris 1 & 2), Buckinghamshire (High Wycombe) and Hertfordshire (Hitchin, Berkhamsted07 and Berkhamsted08). Sampling was carried out using 12 to 24 random quadrats per site (size varied from 0.05mx0.05m to 0.5mx0.5m and 0.1mx0.1m depending on the weed density) and all plants within each quadrat were collected. This system was used to ensure that a truly representative sample of plants was collected. The numbers of plants m^{-2}, tillers and heads $plant^{-1}$, head length, spikelets $head^{-1}$ and seeds $spikelet^{-1}$ were determined. A total of 1985 plants were assessed in these studies.

Dormancy studies of Lolium multiflorum seeds from field populations

Experiments were carried out in three successive years (2006-2008) to study the initial proportion of dormant seeds in a range of freshly collected *Lolium multiflorum* field populations. Mature seeds were collected from 20, 38 and 5 winter wheat fields in 22 counties in England in 2006, 2007 and 2008, respectively. Seeds were mainly collected in July during the time of peak seed shedding. Seed samples were air-dried, cleaned and tested for dormancy in Petri dishes within seven days of the collection date. Four replicates for each population and for each type of Petri dish test (in a KNO_3 solution (2 g L^{-1}) and in deionised water only) were used. Seeds lying on the soil surface are likely to be imbibed with water containing inorganic ions, thus, seeds in a KNO_3 solution would be likely to reflect field conditions. However, the use of KNO_3 could have an effect on breaking dormancy. Therefore, tests with deionised water were also carried out for comparative purposes. In 2006 and 2008 all the populations were also tested for germination two months after the collection date using the same methodology. In 2007, due to the high number of populations, a representative number only were tested for germination two months later. During the two months between the initial and subsequent tests seeds were stored in labelled envelopes at 18°C and 50% relative humidity. For comparative reasons, in 2007 two *A. myosuroides* populations were also tested for dormancy. These populations were Peldon07, from Patch C of Hams field on Peldon Hall farm, Essex and Roth07, an *A. myosuroides* sample collected from the Broadbalk experiment at Rothamsted, Hertfordshire.

Pot experiments on the effects of temperature and soil moisture on seed dormancy

Two experiments were conducted in two successive years (2007 and 2008) to investigate the effects of temperature and soil moisture conditions during spike emergence and seed maturation phase on the dormancy of *L. multiflorum* seeds produced by plants growing in pots. In the experiment carried out in 2007, plants were grown from seeds from two populations of *L. multiflorum*, Wickford and Fish (the most and least dormant populations respectively in the study carried out in 2006) and two populations of *A. myosuroides* for comparison, Peldon03 and Roth04, from Patch C of Hams field on Peldon Hall farm, Essex and the Broadbalk experiment at Rothamsted, Hertfordshire; collected in 2003 and 2004 respectively. In the experiment carried out in 2008, plants were grown only from *L. multiflorum* seeds collected in July 2007. The populations used were the two most dormant in the studies carried out in 2007, Belaugh and Dairy, collected at Litcham (Norfolk) and Bishops Stortford (Hertfordshire), respectively; and the two least dormant, Rothamsted and Cinder, collected at Geescroft field at Rothamsted (Hertfordshire) and Pauntley (Gloucestershire), respectively. In both experiments, plants were grown in 16 x 25 cm diameter pots (four plants per pot). Pots were kept in an open-sided polytunnel over winter. In June and July, when heads started to emerge, sets of 16 plants, in four pots, were subjected to four different combinations of air temperature and soil moisture: hot/wet (HW), hot/dry (HD), cool/wet (CW) and cool/dry (CD). The HW and HD treated pots were kept in a heated glasshouse with an average temperature of approximately 30°C during the day. The CW and CD treatments were kept in the open-sided polytunnel to be maintained close to ambient conditions. The 'wet' treated pots were stood inside large containers of water in order to maintain the soil close to field capacity. The 'dry' treated pots were kept drought stressed supplying only enough water to keep plants alive. Seed samples were collected when an estimated 10%, 50% and 90% had shed and were air dried, cleaned and tested for viability and dormancy within seven days of the collection date. Germination tests were carried out in Petri dishes with and without KNO_3.

Effects of crop and weed densities on the interactions between winter wheat and Lolium multiflorumand Alopecurus myosuroides

The experiment was carried out in the 2007/08 cropping year to study the seed production potential of *L. multiflorum* and *A. myosuroides* when influenced by different weed and crop plant densities to determine the weed's effects on head and seed production and on crop yield. A split-plot design was used with two blocks with two winter wheat densities in the main plots and eight *L. multiflorum* and *A. myosuroides* densities in the subplots. Two areas of 1 m^2 were marked within each subplot, one for assessment of plant and head populations, to assess weed seed production (S) and the other for crop yield assessment (Y) (Figure 1). This was done so the area would always include eight rows of wheat.

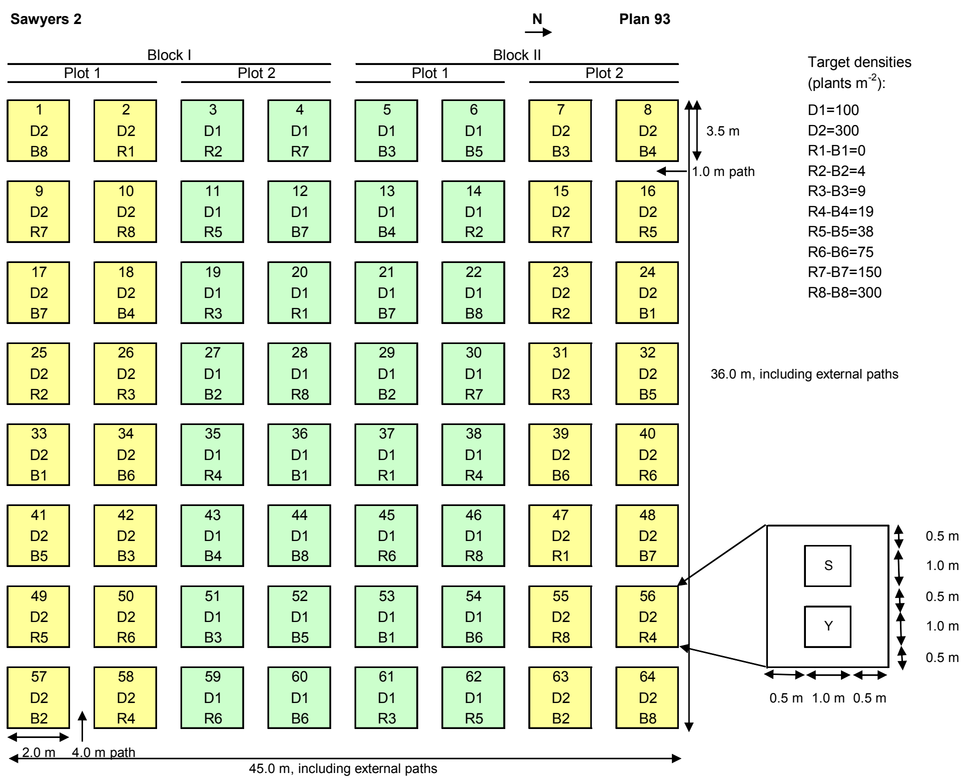

Figure 1. Split-plot design layout of the experiment plan. The experiment was divided in two blocks and each block was divided in two plots, each of them with a different wheat density (D1-D2) and subplots with eight different *L. multiflorum* (R1-R8) and *A. myosuroides* (B1-B8) densities. Each subplot included two 1m^2 quadrats for weed seed production (S) and crop yield (Y) assessments.

A commercial *L. multiflorum* cultivar (Trajan) was used in this experiment together with a susceptible *A. myosuroides* population supplied by Herbiseed (Twyford, UK). The winter wheat cultivar used was Hereward, a non-competitive variety of medium height (84 cm without PGR) (HGCA, 2009). Target plant densities for crop and weeds are listed in Figure 1.

The average wheat densities obtained were 102 plants m^{-2} (s.e. ±2.45) and 298 plants m^{-2} (s.e. ±5.46), very close to the target densities (100 and 300 plants m^{-2}). The average weed plant densities achieved for each subplot are listed in Table 1.

Table 1. Mean numbers of *A. myosuroides* and *L. multiflorum* plants m^{-2} and size and number of quadrats used for the assessment in the different subplots within the low and high wheat densities plots. Values within brackets are the standard error of the mean.

Weed density/Wheat density	*A. myosuroides*			*L. multiflorum*		
	Low	High	Quadrat	Low	High	Quadrat
1	0	0	-----	0	0	-----
2	10.0 (1)	13.0 (1)	1x 1mx1m	6.0 (1)	8.5 (4.5)	1x 1mx1m
3	15.5 (3.5)	21.5 (3.5)	1x 1mx1m	10.0 (1)	13.0 (1)	1x 1mx1m
4	40.0 (10)	40.0 (5)	2x 0.1mx0.1m	21.5 (1.5)	18.5 (2.5)	1x 1mx1m
5	65.0 (0)	55.0 (15)	2x 0.1mx0.1m	32.5 (7.5)	37.5 (2.5)	2x 0.1mx0.1m
6	135.0 (0)	112.5 (17.5)	2x 0.1mx0.1m	57.5 (7.5)	67.5 (12.5)	2x 0.1mx0.1m
7	237.5 (87.5)	287.5 (37.5)	2x 0.02mx0.02m	212.5 (12.5)	212.5 (12.5)	2x 0.02mx0.02m
8	362.5 (37.5)	412.5 (12.5)	2x 0.02mx0.02m	325.0 (75)	312.5 (37.5)	2x 0.02mx0.02m

Weed plants with intact inflorescences were collected in June from each sampling subplot. Sampling was carried out using one to two quadrats (range of sizes within 0.02m x 0.02m and 1m x 1m, depending on weed density (Table 1)) per subplot and all plants within each quadrat were collected. The numbers of plants m^{-2}, tillers and heads $plant^{-1}$, head length, spikelets $head^{-1}$ and seeds $spikelet^{-1}$ were determined in subplots with *L. multiflorum* plants. Only the numbers of plants m^{-2}, tillers and heads $plant^{-1}$ were measured in subplots with *A. myosuroides* plants. The dry weight of all *L. multiflorum* plants collected was also determined. Wheat plants within the 1 m^2 area marked within each subplot for crop yield assessment were harvested by hand in August.

Herbicide resistance studies

Response of 55 Lolium multiflorun populations to diclofop-methyl, fluazifop-P-butyl, tralkoxydim, cycloxydim and pinoxaden

In this assay 55 *Lolium multiflorum* populations collected on a semi-random basis in 2006 and 2007 from winter wheat fields from 50 farms in 22 counties of England were used. The populations were collected principally for evaluating seed dormancy and were not chosen because herbicide control had been poor. The objective of the experiment was to determine the extent of resistance to ACCase inhibiting-herbicides in England and the cross-resistance pattern.

Two single dose assays were conducted, each comprising a randomised block design with five replicate pots per herbicide and five untreated pots per population. In the first assay the response of 20 *L. multiflorum* populations collected in 2006 to the ACCase inhibiting herbicides diclofop-methyl, fluazifop-P-butyl, tralkoxydim, cycloxydim and pinoxaden was studied. In the second assay 38 populations collected in 2007 were used. In both assays the three *L. multiflorum* populations Yorks A2, Wilts B1 and Trajan were used as standard populations. The Yorks A2 population was used as target site resistant standard as it was known to carry the Ile-1781-leu point mutation (White et al., 2005). Wilts B1 was used as an enhanced metabolism standard since no key point mutation had been found so far in this population (Cocker *et al.*, 2001). Trajan was used as susceptible standard.

In both assays pre-germinated seeds were sown into nine cm pots containing peat-based compost and thinned to six plants per pot. A total of thirty pots were prepared for each population and were divided into six treatments (including nils) with five replicates. Commercial formulations of the five herbicides were applied at 1/2x field rate: 567 g.a.i diclofop-methyl ha^{-1}, 93.75 g.a.i. fluazifop-P-butyl ha^{-1}, 175 g. a.i. tralkoxydim ha^{-1}, 75 g.a.i. cycloxydim ha^{-1} and 22.5 g.a.i. pinoxaden ha^{-1}. Recommended adjuvants were used. All the herbicides were used at half of the field rate as herbicides are more active under glasshouse conditions and the herbicides previously studied, diclofop-methyl, tralkoxydim, cycloxydim and pinoxaden were shown to be effective against the susceptible standard Trajan at 1/4x field rate. After spraying, pots were returned to the glasshouse and randomised within replicates. Twenty-two days after treatment leaf samples from each surviving plant were collected and plants rated using a 1-4 injury scale prior to harvest.

In both assays, the percentage reduction in fresh weights relative to the mean weight of untreated plants was calculated for each herbicide and a one-way ANOVA test carried out using Genstat. A principal component analysis was also carried out using the percent reduction in fresh weight data and results illustrated in a principal component bi-plot.

Herbicide resistance at the molecular level

A selection of the resistant plants that resulted from the glasshouse assay carried out with 55 *L. multiflorum* populations from 50 farms described previously were screened for single nucleotide polymorphisms (SNPs) or mutations. DNA was extracted from dry leaves of a total of 384 resistant plants from 54 *L. multiflorum* populations using the "DNeasy 96 Plant Kit" (Qiagen).

The number of samples assayed per population varied depending on the number of plants surviving treatments and ranged between one and sixteen samples. Populations YorksA2 and Wilts B1 were used as target site and enhanced metabolism resistance standards, respectively. CAPS or dCAPS markers were used to screen for three SNPs, Ile-1781-Leu, Asp-2078-Gly and Cys-2088-Arg and then a high throughput SNaPshot multiplex method (Hurst *et al.*, 2009) was used to screen for these three SNPs as well as four other SNPs known to confer resistance to ACCase inhibitors.

To confirm genotypes, the CT domain of the chloroplastic ACCase gene from a selection of samples was also sequenced.

RESULTS

Agro-ecology studies

Emergence pattern studies

The majority of *Lolium multiflorum* plants in winter wheat fields in the UK emerge in autumn. On average, 94% of plants emerge in October, November and December and only 6% in spring (Figure 2).

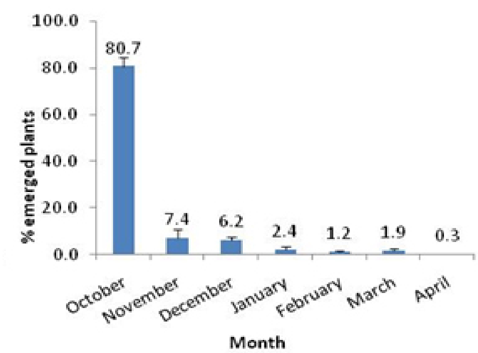

Figure 2. Average proportion of emerged *L. multiflorum* plants per month.

Plants emerged in autumn were much larger and produced on average 23 times more seeds per plant than spring emerging cohorts (Figure 3).

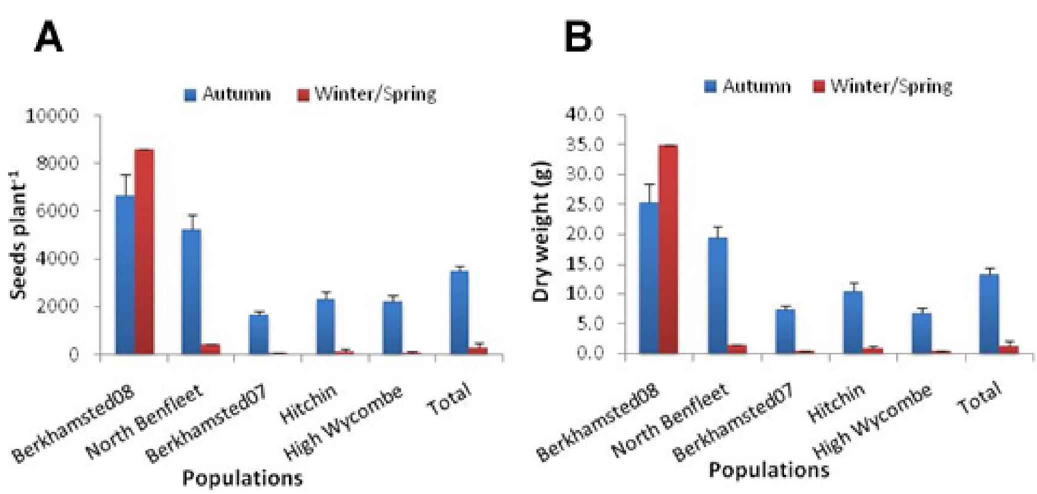

Figure 3. Mean seed production potential (A) and mean dry weight (g) (B) per plant for plants emerged in autumn and in winter/spring from the five *L. multiflorum* populations studied.

The apparently anomalous result for Berkhamsted08 was due to the presence of a single winter/spring emerging plant that was first detected in December with a growth stage of 2 leaves. This and the lack of competition because of the very low weed density in this field (3.0 plants m^{-2}) could explain why the seed production potential and dry weight for this plant is higher than the mean values for these two variables in autumn (Figure 3).

Seed production potential studies

The main determinant of seed production was the number of heads per unit area (Figure 4). The number of seeds per spikelet, spikelets per head and head length vary relatively little with plant density or between sites, and had much less influence on seed production (Figure 4). The mean number of seeds per head varied little between populations, with an average value of 295 seeds $head^{-1}$(Table 2).

Lolium multiflorum plants are highly adaptable to different weed densities and very high seed production is possible from low density populations. Populations with just 3 plants m^{-2} produced an average of 6,816 seeds $plant^{-1}$(Table 2).

Table 2. Number of seeds $head^{-1}$, seeds $plant^{-1}$ and seeds m^{-2} for the ten *L. multiflorum* populations and average value, ordered from low to high plant density.

Site	Seeds $head^{-1}$	Seeds $plant^{-1}$	Seeds m^{-2}	Plants m^{-2}
Berkhamsted08	313	6,816	18,262	3
Chat2	340	5,620	69,799	13
Peldon	328	7,220	136,006	25
Louth	266	4,467	93,958	27
North Benfleet	374	4,209	183,935	45
Berkhamsted07	276	2,050	159,587	87
Chat1	235	909	83,582	94
Hitchin	332	1,961	173,500	103
High Wycombe	241	1,568	262,131	195
Wickford	187	546	246,735	488
Average	295	3,759	143,137	

The success of *Lolium multiflorum* as a weed of winter cereals appears to be linked to its ability to produce a high number of heads, having an average of 20 heads per plant at low weed densities (

Table **3**).

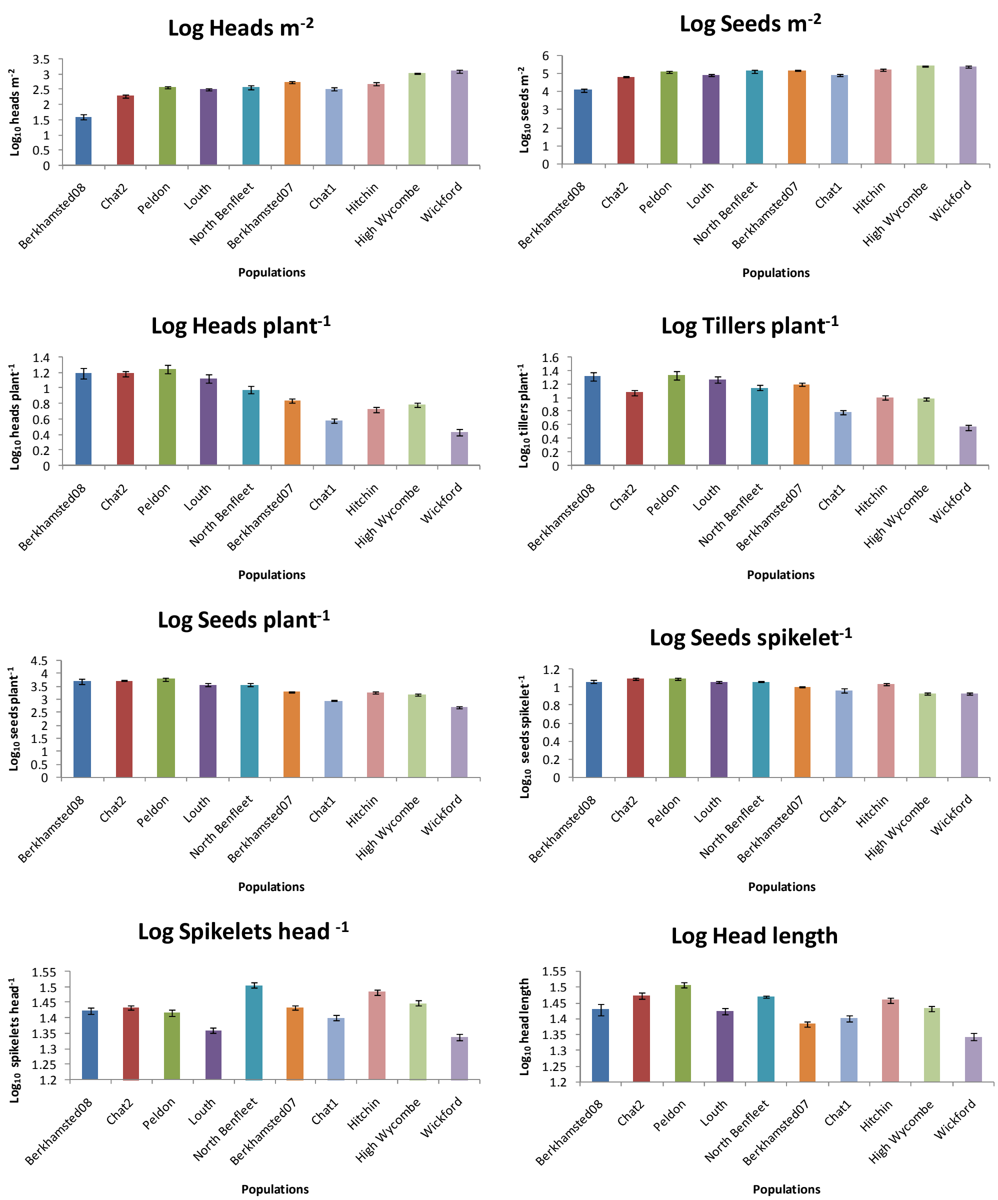

Figure 4. Mean values for the $\log_{10}$ transformed values of the number of heads m^{-2}, seeds m^{-2}, heads $plant^{-1}$, tillers $plant^{-1}$ and seeds $plant^{-1}$, seeds $spikelet^{-1}$, spikelets $head^{-1}$ and head length (g) for the ten *L. multiflorum* populations studied. Populations are arranged from low to high plant density.

These results suggest that delaying sowing date, using good crop competition and effectively controlling autumn emerging plants with herbicides should reduce *Lolium multiflorum* infestations.

Table 3. Influence of *L. multiflorum* plant density on heads m^{-2} and heads $plant^{-1}$ based on two sets of data from quadrats from populations with low (Berkhamsted08, Chat2, Peldon, Louth and North Benfleet) and high (Berkhamsted07, Chat1, Hitchin, High Wycombe and Wickford) plant density. Up to 134 plants m^{-2}, predictions should be made with the model on the left while over this value the model of the right should be used. The value where predictions change from one model to the other was chosen to be 134 plants m^{-2} because this was the point at which both models had the same prediction.

Plants m^{-2}	Below 134 plants m^{-2}		Over 134 plants m^{-2}	
	Heads m^{-2}	Heads $plant^{-1}$	Heads m^{-2}	Heads $plant^{-1}$
1	23	23.2	*9*	*9.0*
5	106	21.3	*44*	*8.8*
10	192	19.2	*87*	*8.7*
15	263	17.6	*127*	*8.5*
20	323	16.2	*166*	*8.3*
25	374	15.0	*204*	*8.1*
30	418	13.9	*240*	*8.0*
35	456	13.0	*275*	*7.8*
40	490	12.2	*308*	*7.7*
45	519	11.5	*340*	*7.6*
50	546	10.9	*371*	*7.4*
75	645	8.6	*512*	*6.8*
100	709	7.1	*631*	*6.3*
134	767	5.7	767	5.7
200	*833*	*4.2*	970	4.9
250	*863*	*3.5*	1087	4.3
500	*931*	*1.9*	1432	2.9
1000	*969*	*1.0*	1702	1.7

Dormancy studies of Lolium multiflorum seeds from field populations

Years when the months of June and July were warmer and drier than average and with fewer days of rain were associated with the production of a higher proportion of non-dormant seeds (

Figure 5, Figure 6). Thus, in these years a high proportion of *Lolium multiflorum* seeds would be expected to germinate in early autumn with favourable weather conditions.

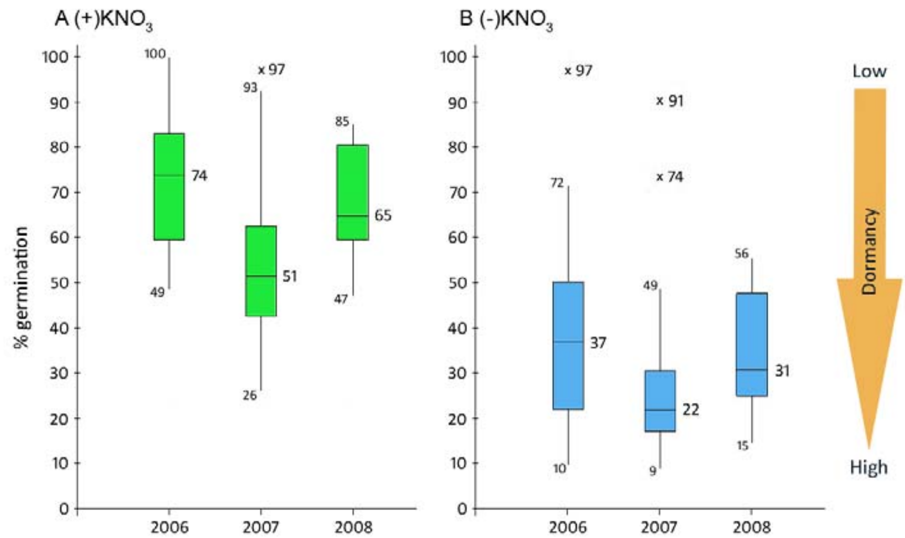

Figure 5. Box plot with the data obtained from the dormancy tests in Petri dishes with (A) and without (B) KNO_3 solution carried out within 7 days of collection of *L. multiflorum* seeds collected from winter wheat fields in England in 2006, 2007 and 2008. The central horizontal line represents the median, boxes encompass 50% of values and whiskers delineate the range. The symbol 'x' indicates extreme outliers.

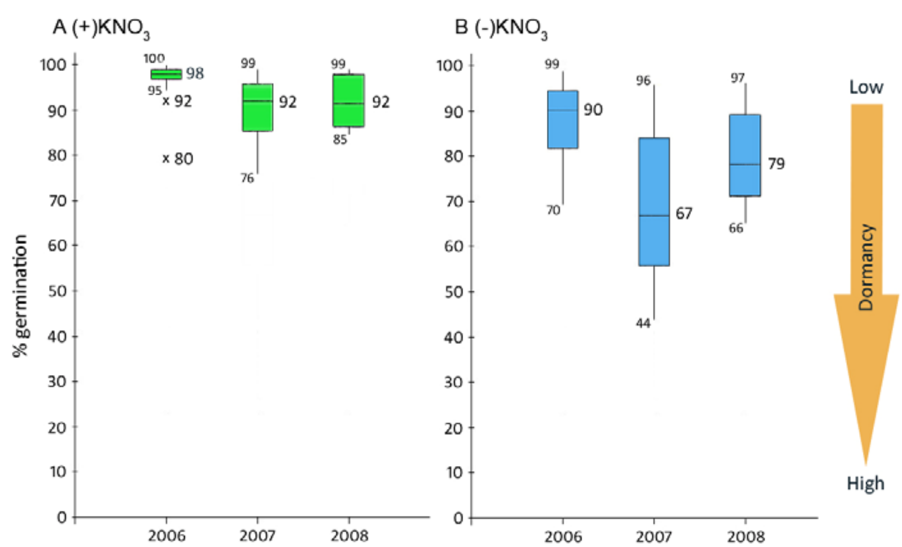

Figure 6. Box plot with the data obtained from the germination tests in Petri dishes with (A) and without (B) KNO_3 solution carried out 2 months after collection with *L. multiflorum* seeds collected from winter wheat fields in England in 2006, 2007 and 2008. The central horizontal line represents the median, boxes encompass 50% of values and whiskers delineate the range. The symbol 'x' indicates extreme outliers.

In years when there are cooler and wetter than average conditions in June and July and with more days with rain than average, a higher proportion of non-dormant seeds and a longer dormancy will be expected (
Figure 5, Figure 6). Thus, germination of *L. multiflorum* seeds would be expected to be delayed.

The great variability in the proportion of non-dormant seeds found between field samples grown under similar weather conditions might be explained by an intrinsic genetic dormancy.

Pot experiments on the effects of temperature and soil moisture on seed dormancy

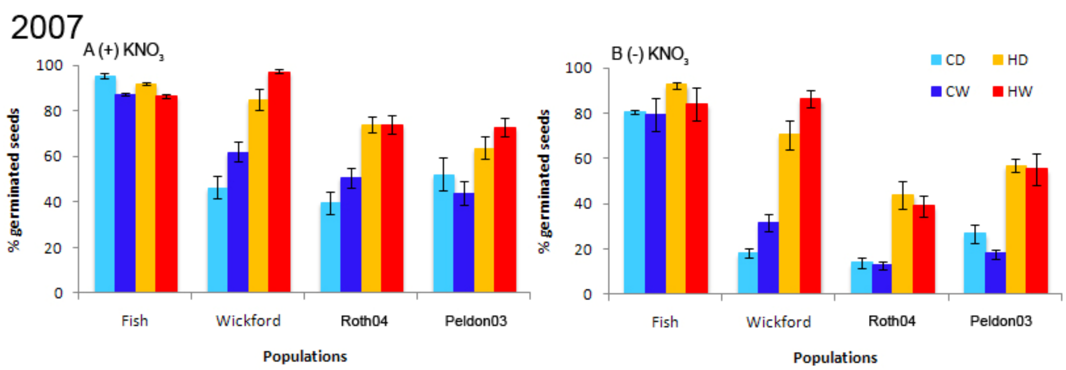

Figure 7. Effect of four temperature and soil moisture regimes applied during the period of spike emergence and seed maturation on the proportion of non-dormant seeds obtained on tests carried out on Petri dishes with (A) and without (B) a KNO_3 solution of two *L. multiflorum* populations (Fish and Wickford) and two *A. myosuroides* populations (Roth04 and Peldon03) in 2007.

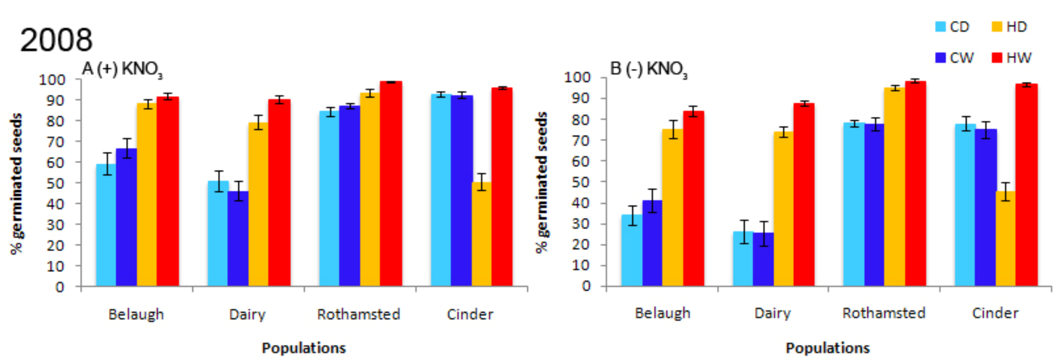

Figure 8. Effect of four temperature and soil moisture regimes applied during the period of spike emergence and seed maturation on the proportion of non-dormant seeds obtained on tests carried out on Petri dishes with (A) and without (B) a KNO_3 solution of four *L. multiflorum* populations, Belaugh, Dairy, Rothamsted and Cinder in 2008.

Two different kinds of *L. multiflorum* populations were found; those which are non-dormant and not affected by weather conditions during seed development and maturation and those which are dormant and affected by weather conditions during that period (Figure 7, Figure 8).

High temperature during seed development and maturation in dormant cultivars of *L. multiflorum* decreased initial seed dormancy and seemed to be the most important parameter responsible for changes in dormancy.
Soil moisture was not clearly correlated with dormancy.

Under similar weather conditions during the seed maturation period *A. myosuroides* seeds were more dormant than those of *L. multiflorum* seeds.

Effects of crop and weed densities on the interactions between winter wheat and Lolium multiflorum and Alopecurus myosuroides

Both *Lolium multiflorum* and *Alopecurus myosuroides* have a highly detrimental effect on winter wheat yield causing losses of up to 89%.

A yield loss of 5% can result from just two and four *A. myosuroides* and *L. multiflorum* plants m^{-2}, respectively at a crop density of 100 winter wheat plants m^{-2} (Table 4).

A. myosuroides and *L. multiflorum* are both highly competitive with *A. myosuroides* being slightly more competitive.

Increasing crop seeding rate has a beneficial effect on weed suppression with reductions of head numbers and seeds $plant^{-1}$ of up to 58 and 67% on *A. myosuroides* and *L. multiflorum* plants, respectively.

Increasing crop density can be used as a weed control measure as winter wheat densities of 300 plants m^{-2} have a significant effect on weed suppression, compared to that of 100 plants m^{-2}, while there are is no significant effect on crop yield (Figure 9).

The use of higher crop seeding rates as a weed control measure could complement the use of herbicides and thus decrease the selection pressure imposed on the weed by herbicides.

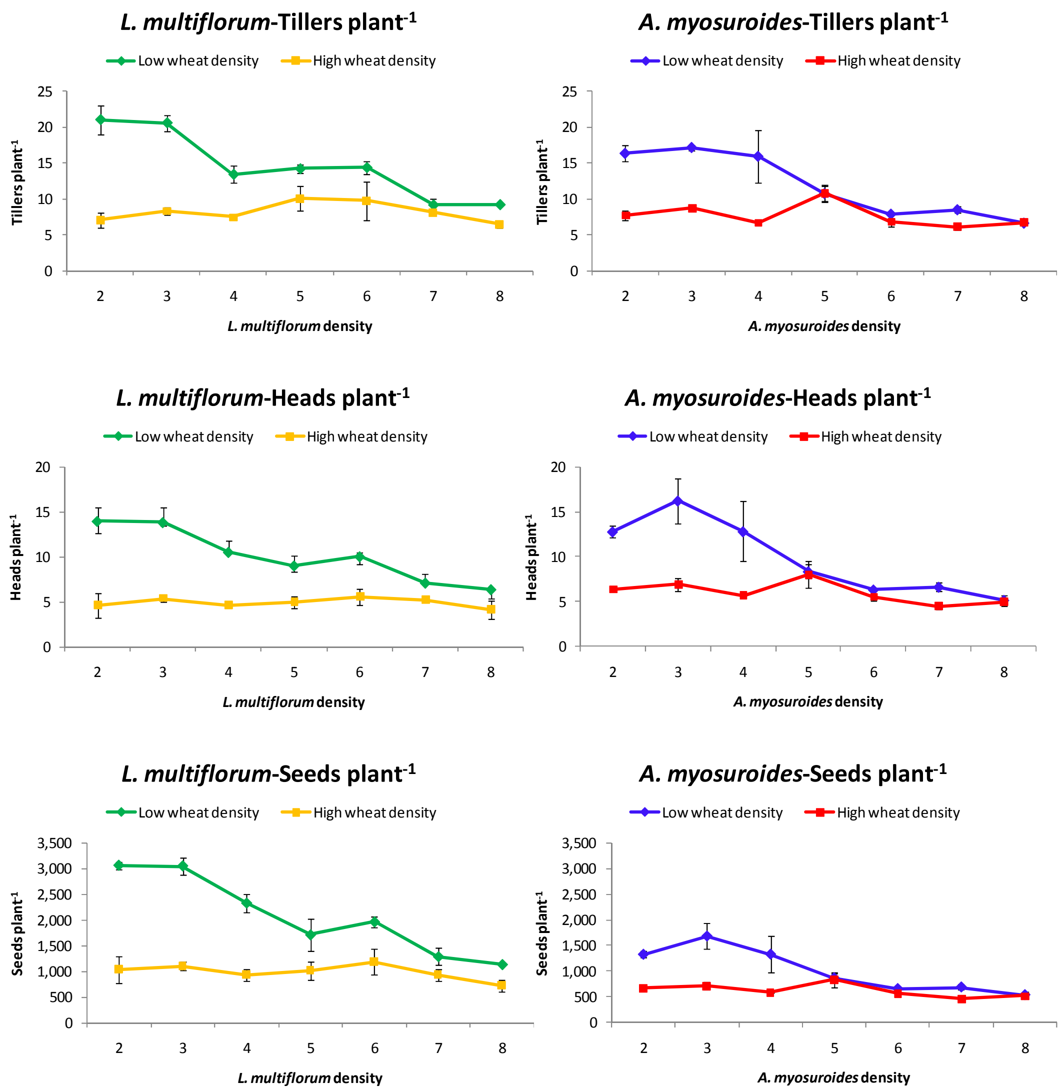

Figure 9. Effect of two different wheat densities on the number of tillers plant^{-1}, heads plant^{-1}, seeds plant^{-1}, in *L. multiflorum* and *A. myosuroides* plants.

Table 4. Relationship between *A. myosuroides* and *L. multiflorum* plant density and % yield loss of wheat, at crop densities of 100 and 300 plants m^{-2}.

Weed plants m^{-2}	*A. myosuroides*		*L. multiflorum*	
	100 wheat plants $^{-2}$	300 wheat plants m^{-2}	100 wheat plants $^{-2}$	300 wheat plants m^{-2}
1	2	1	1	1
5	9	3	7	3
10	17	7	12	6
15	23	10	16	8
20	28	12	20	10
25	32	15	24	13
50	48	26	35	22
75	56	33	42	29
100	62	40	46	35
200	73	55	55	49
300	78	63	58	57
400	81	68	60	61
500	82	71	62	65
600	83	73	62	67
700	84	75	63	69
800	85	77	64	70
900	85	78	64	71
1000	86	79	64	72

Resistance at the whole plant and molecular levels

Resistance in *Lolium multiflorum* to at least one ACCase inhibiting herbicide is widespread in England as it has been detected in 35 (70%) out of 50 individual semi-randomly sampled farms included in the survey (Figure 10).

Resistance to the herbicides diclofop-methyl and tralkoxydim is the most widespread as it was detected on 31 (62%) and 30 (60%) farms, respectively. Resistance to fluazifop-P-butyl was detected on 18 farms (36%) and resistance to cycloxydim and pinoxaden was less common and was detected only on 10 (20%) and 9 (18%) farms, respectively.

The ACCase-inhibiting herbicides diclofop-methyl, fluazifop-P-butyl and tralkoxydim seem to be affected by both non-target site (probably enhanced metabolism) and ACCase target site resistance mechanisms, and possibly additional mechanisms too. Cycloxydim seems to be affected only by ACCase target site resistance.

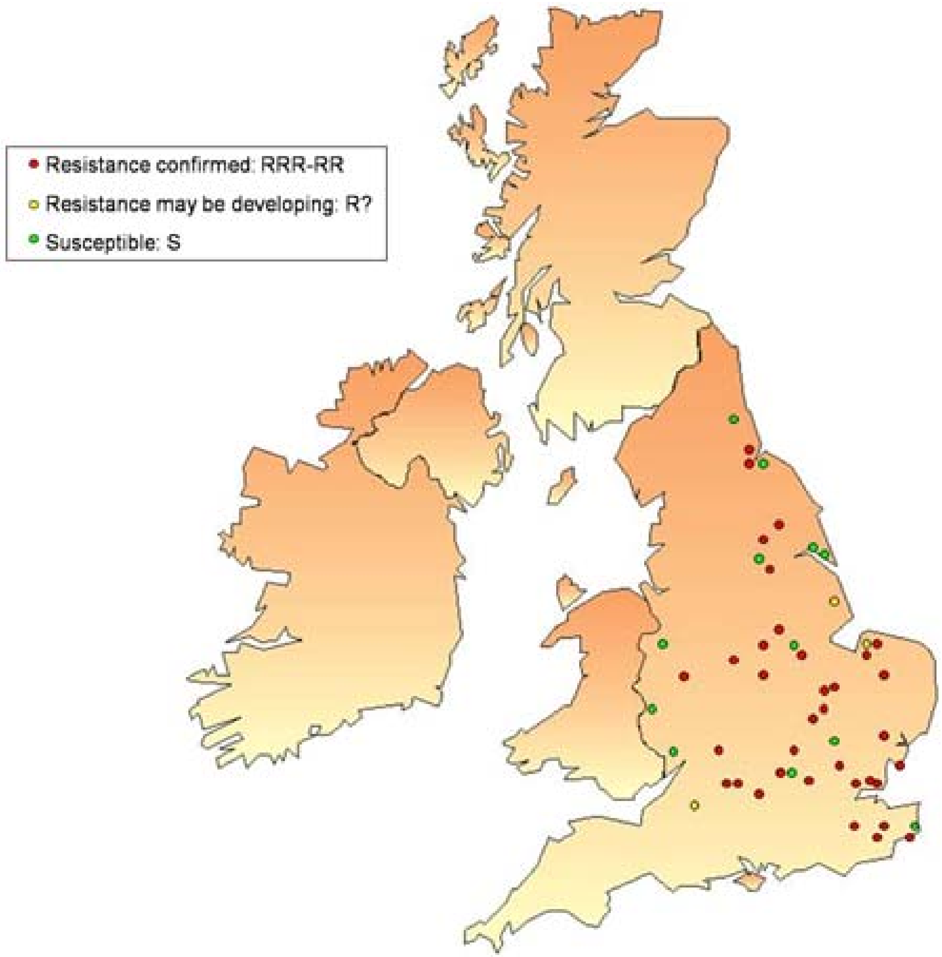

Figure 10. Frequency of resistance to at least one ACCase-inhibiting herbicide in *Lolium multiflorum* populations from 50 farms in England.

The ACCase-inhibiting herbicide pinoxaden seems to be affected mainly by ACCase target site resistance. It is possible that non-target site resistance mechanisms exist, but that they appear not to be very widespread in *L. multiflorum* in the UK at present.

24% of the populations tested were found to be sensitive to all the herbicides assayed, 22% were found to be resistant to all the herbicides and 55% were found to be resistant only to diclofop-methyl, fluazifop-P-butyl and tralkoxydim.

Differences in the degree of resistance to the herbicides cycloxydim and pinoxaden were found between populations known to possess ACCase target site resistance, thus indicating that not all the target site point mutations confer the same levels of resistance to the same herbicides.

Even though a point mutation was found in 40% of the 384 resistant plants from the 54 populations studied, the main mechanism conferring resistance in 26 out of the 38 populations (68%) with confirmed resistance to at least one of the herbicides assayed was a non target site resistance mechanism, most probably enhanced metabolism.

The most common mechanism of resistance to ACCase-inhibiting herbicides in UK *L. multiflorum* populations appears not to be target site resistance but, most probably, enhanced metabolic resistance.

In only 12 out of 38 populations (32%) from 35 farms with confirmed resistance to at least one of the five herbicides studied was the main mechanism conferring resistance target site resistance. However, 7 of these 12 populations also contained resistant individuals possessing no mutations, thus showing the importance of non-target site resistance mechanisms.

In total, six of the seven known ACCase mutations were detected in resistant plants. The most common mutations found conferring resistance to ACCase-inhibiting herbicides in UK *L. multiflorum* populations were Asp-2078-Gly and Ile-1781-Leu. The six mutations found, and their frequencies as a proportion of the total number of resistant plants assayed (384), were: Asp-2078-Gly (24.5%), Ile-1781-Leu (13.3%), Ile-2041-Asn (2.1%), Cys-2088-Arg (1.8%), Trp-2027-Cys (1.0%) and Trp-1999-Cys (0.3%). No plants with the Gly-2096-Ala mutation were found.

Resistance to pinoxaden was found in a few plants carrying one of the Asn-2041, Cys-1999 and Cys-2027 alleles or carrying none of the seven known mutations. This might imply that a non-target site resistance mechanism and/or the presence of one of these three alleles could be responsible for the resistance to pinoxaden in some *L. multiflorum* plants.
Resistance to ACCase-inhibiting herbicides in the UK *L. multiflorum* population Pyl is conferred by a change from Adenine (A) to Guanine (G) in

nucleotide position 6233 of the plastidic ACCase gene, corresponding to a change from aspartate (Asp) to glycine (Gly) in amino acid position 2078 of the CT domain of the ACCase protein.

A change from Thymine (T) to Adenine (A) at nucleotide position 6230 of the plastidic ACCase gene, corresponding to a change from isoleucine (Ile) to asparagine (Asn) in amino acid position 2077 of the CT domain of the ACCase, could be responsible for resistance to ACCase inhibitors in the *L. multiflorum* population Thornham (Figure 11). Further work should be carried out to confirm this.

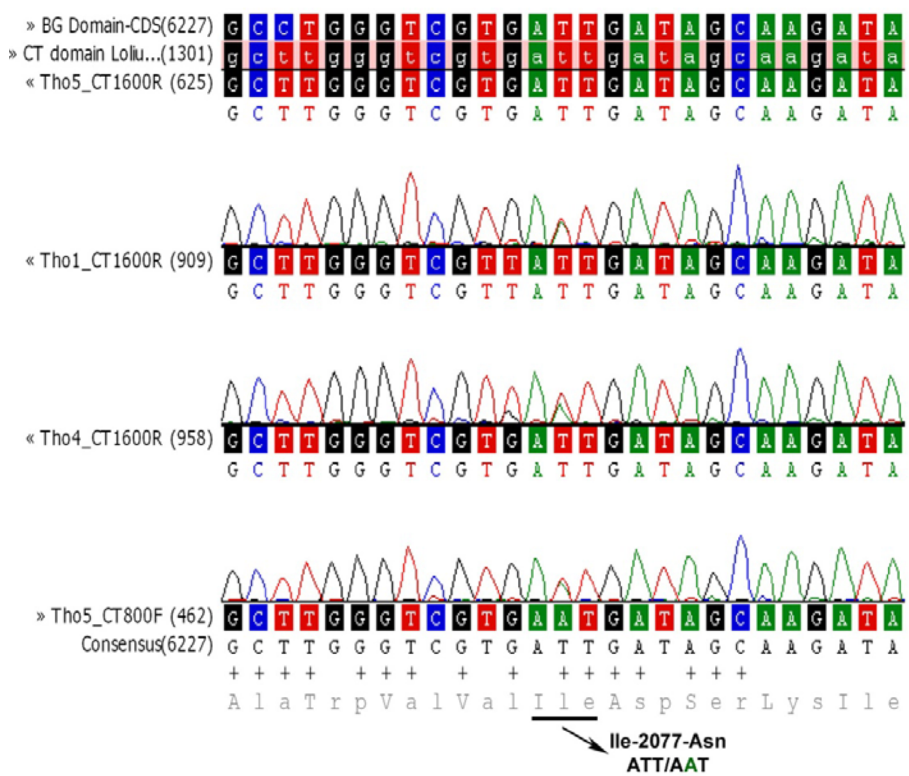

Figure 11. Alignment of acetyl CoA carboxylase partial nucleotide sequences centred around the 6230 nucleotide position, which corresponds with the amino acid position 2077, of a herbicide resistant *L. multiflorum* plant from the population Thornham and GeneBank sequences of *L. multiflorum* (AY710293) and *A. myosuroides* (AJ310767).

DISCUSSION

The series of experiments carried out to study and understand the agro-ecology of *L. multiflorum* in UK conditions described here produced interesting results that could prove useful when deciding which weed control strategy to implement. It was shown that both *L. multiflorum* and *A. myosuroides* have a highly detrimental effect on wheat yield. At a crop density of 100 winter wheat plants m^{-2}, just two and four *A. myosuroides* and *L. multiflorum* plants m^{-2}, respectively, caused a mean yield loss of 5%, a value often taken as an economic threshold at which the cost of weed control approximately equals the return from increased yields. Much higher infestation levels were recorded on many fields, demonstrating the potential for much higher yield losses. In addition, apart from short term effects on yield, the results show that weed seed return should also be taken into consideration. This is important for achieving longer term control. *L. multiflorum* plants are highly adaptable to different weed densities and very high seed production is possible from low density populations. In the studies described here, just three *L. multiflorum* plants m^{-2} produced an average of 6,816 seeds $plant^{-1}$ in a winter wheat crop. This means that, if even a small number of plants survive a weed control treatment, they could cause significant longer term damage because of their high potential seed return. Most seed shedding occurred in July or August, just prior to the harvest of winter wheat. When chemical control is inadequate, as may occur with increasing herbicide resistance, a more integrated approach should be considered, combining both chemical and non-chemical methods. Factors such as weed dormancy or emergence pattern should be taken into consideration in order to develop the optimum control strategies.

Results from dormancy studies with *L. multiflorum* showed that the most important factor affecting the initial dormancy in *L. multiflorum* was temperature during spike emergence and seed maturation. Higher temperatures during seed maturation were associated with lower dormancy. However, dormancy in *L. multiflorum* lasted for only a relatively short period in most cases, with most seeds germinating within two months of collection in laboratory assays. This agrees with the results of the emergence pattern studies conducted in the field. However, environmentally induced dormancy had much less of an impact on *L. multiflorum* than *A. myosuroides*.

These studies gave very consistent results and showed that, averaged over five field experiments, 88.1% of *L. multiflorum* seedlings emerge in autumn in winter wheat crops. This indicates that there is little or no innate dormancy in *L. multiflorum* seeds retained by the time winter cereal crops are sown, which is typically late September or October under UK conditions. All of this information has important implications for weed management.
A prediction of the dormancy status of *L. multiflorum* seeds as influenced by the weather conditions during seed development and maturation would help in the development of control strategies. Thus, in a year when it is hot during the time of seed maturation (June and July in the UK) *L. multiflorum* seeds would be expected to have a high degree of non-dormancy and germinate as soon as soil and weather conditions are conducive for germination. In such years there is the potential to reduce the seed burden by encouraging as much germination as possible in early autumn and destroying the seedlings prior to sowing the next crop. In contrast, in a cool year, germination will be expected to be delayed slightly as a higher proportion of seeds will be dormant. Delaying drilling may give more opportunity for emergence or, alternatively, the application of a pre-emergence residual herbicide would be advisable to improve control of later emerging plants after sowing. However, regardless of weather conditions during the seed development and maturation period, the majority of *L. multiflorum* plants emerge in autumn. Therefore, spraying herbicides in the autumn would be recommended in any year in order to remove weeds early, thereby reducing crop competition. By spring, weed plants would be bigger and more difficult to kill, crop yield might already be compromised by the competition with the weed and the more advanced crop growth stage in spring might shield weed plants, restricting spray coverage (Clarke, 2002). Thus, delaying the sowing date of autumn sown crops, and having an effective chemical control in autumn could potentially greatly reduce *L. multiflorum* infestations. However, in the UK, there is a widely-held perception by farmers that many *L. multiflorum* plants emerge in spring and

these could still pose a big threat if weed control was confined to the autumn in winter wheat crops. Farmers are particularly concerned about seed production in plants they perceive to have emerged in spring. Results on seed production potential do indeed indicate that *L. multiflorum* plants can produce a large number of seeds per unit area even at low plant densities. This could mean that even a small number of plants emerging in spring could potentially produce a considerable number of heads and consequently a high seed return. In these studies, seed return ranged from 546 to 6,816 seeds plants^{-1}, with an average value of 3,759 seeds plants^{-1}, which, in terms of unit area ranged from 18,262 to 262,131 seeds m^{-2} and had an average value of 143,137 seeds m^{-2}. However, studies of the comparison of seed production potential between autumn and spring cohorts showed that spring emerging plants are much less productive with 23 times less seeds plant^{-1} than autumn emerging plants. These plants were not fully developed due to the high competition exerted by other plants which had emerged previously and so produce very little seed return. Therefore, seed production from spring plants will not have a significant impact in terms of seed return since few plants emerge in spring and those that do produce very few seeds per plant. Many of the plants that farmers spot in spring are probably autumn emergers that were previously unnoticed because of the small size and are only seen in spring when they get bigger.

Small numbers of surviving plants should not be ignored, however, especially if they are potentially herbicide-resistant. In such cases, seed return from herbicide resistant *L. multiflorum* plants could be significant with just a few survivors. Additional non-chemical control measures should be taken to reduce seed return. In the studies described here it was shown that increasing crop seeding rate from 100 to 300 plants m^{-2} helped reduce weed seed return by up to 66% without significantly affecting crop yield.

In summary, more non-chemical, cultural control measures should be adopted to reduce dependence on herbicides. This is important both to reduce the risk of resistance developing and to manage it once it has evolved. These measures have been recommended elsewhere for other grasses and include those previously mentioned, such as increasing crop competition by the use of more competitive crop varieties and higher crop seed rates, delaying drilling date, crop rotation, cultivation and non-cropping (Moss *et al.*, 2007). However, more studies are still needed to determine the best management options for *L. multiflorum* in UK cropping systems. The agro-ecology results presented here provide a starting point for the development of better control strategies for *L. multiflorum* populations.

Resistance studies in *L. multiflorum* were focused on ACCase-inhibiting herbicides since this is the major class of herbicides used in the control of this weed in the UK and many other countries. Results from whole plant screening tests showed that resistance is widespread in England with resistant populations present on 70% of the 50 farms included in the survey. ACCase mutations did not fully account for the resistance detected at the whole plant level in all 38 populations from 35 farms that showed confirmed resistance to at least one herbicide. In only 12 populations was the main mechanism conferring resistance an insensitive ACCase as mutations were detected in almost every plant within these populations. Out of a total of 384 plants from 54 populations, 155 plants possessed at least one ACCase mutation. The most common mutations were the Asp-2078-Gly mutation that was found in 94 plants (24.5%) and nine populations, and the Ile-1781-Leu mutation that was found in 51 plants (13.3%) and seven populations. The Cys-2088-Arg mutation was found in seven plants (1.8%) all of which were within a single population. The Ile-2041-Asn mutation was found in eight plants (2.1%) that belonged to three populations. The Trp-2027-Cys mutation was found in four plants (1.0%) from three populations. The Trp-1999-Cys mutation was found in just one plant (0.3%) from one population. No plant was found possessing the Gly-2096-Ala mutation.

However, even though a point mutation was found in 40% of the resistant plants, the main mechanism conferring resistance in 26 out of the 38 populations (68%) with confirmed resistance to at least one of the herbicides assayed was a non target site resistance mechanism. This indicates that the most common resistance mechanism in UK *L. multiflorum* populations is not target site resistance (TSR) but a non-target site resistance (NTSR) mechanism. As other studies carried out previously in UK *L. multiflorum* populations show (Cocker *et al.*, 2001) this NTSR mechanism could be enhanced metabolic resistance. Knowing the proportion of resistant populations which have each of the different mechanisms responsible for resistance is important since this can help in the study of the evolution of resistance and in the development of future control strategies. To prevent the exertion of a high selection pressure for resistance, integrated weed management strategies should be adopted, which means the use of cultural and mechanical control methods combined with chemical control. The work in this research project provides useful information on which to develop further integrated control strategies that are relevant, not only in the UK, but also in many other countries where this weed is an increasing threat to arable cropping.

CONCLUSIONS

- Innate dormancy in *L. multiflorum* is relatively short and is determined by both genetics and weather conditions. Higher temperatures during seed maturation are associated with reduced seed dormancy, but this effect is relatively weak compared with some other grass species. However, genetically non-dormant populations seem to be less affected by weather conditions.
- The majority of *L. multiflorum* plants in winter wheat fields in the UK emerge in autumn. On average, 94% of plants emerge in October, November and December and only 6% in spring. Plants which emerged in autumn were much larger and produced on average 23 times more seeds per plant than spring emerging cohorts.
- *L. multiflorum* plants are highly adaptable to different weed densities and very high seed production is possible from low density populations. The success of *L. multiflorum* as a weed of winter cereals appears to be linked to its ability to produce a high number of heads and seeds, even at low weed densities.
- *L. multiflorum* plants are highly competitive and have a highly detrimental effect on winter wheat yield with losses of up to 89%.

Increasing crop seed rate can be used as a weed control measure with winter wheat crops. Winter wheat plant densities of 300 plants m^{-2} reduced the number of *L. multiflorum* heads and seeds $plant^{-1}$ by up to 67% compared to wheat densities of 100 plants m^{-2}.

- Resistance in *L. multiflorum* to at least one ACCase inhibiting herbicide is widespread in England and was detected on 35 out of 50 (70%) semi-randomly sampled farms included in a survey. Resistance to the herbicides diclofop-methyl and tralkoxydim was the most widespread as it was detected on 31 (62%) and 30 (60%) of sampled farms, respectively. Resistance to fluazifop-P-butyl was detected on 18 farms (36%) and resistance to cycloxydim and pinoxaden was less common, being detected on only 10 (20%) and 9 (18%) farms, respectively.
- The most common mutations found conferring resistance to ACCase-inhibiting herbicides in UK *L. multiflorum* populations were Asp-2078-Gly and Ile-1781-Leu. The six mutations found, and their frequencies as a proportion of the total number of resistant plants assayed (384), were: Asp-2078-Gly (24.5%), Ile-1781-Leu (13.3%), Ile-2041-Asn (2.1%), Cys-2088-Arg (1.8%), Trp-2027-Cys (1.0%) and Trp-1999-Cys (0.3%). No plants with the Gly-2096-Ala mutation were found.
- ACCase target site resistance was the main mechanism conferring resistance in only 12 populations (32%) out of the 38 populations (from 35 farms) with confirmed resistance detected in whole plant assays. These involved five herbicides, diclofop-methyl, tralkoxydim, fluazifop-P-butyl, cycloxydim and pinoxaden. Thus, the most common mechanism of resistance to ACCase-inhibiting herbicides in UK *L. multiflorum* populations appears, at present, to be a non-target site resistance mechanism, most likely, enhanced metabolic resistance.

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