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Variation in *Elymus repens* susceptibility to glyphosate

Liv Å. Espeby<sup>a</sup>, Håkan Fogelfors<sup>a</sup>, Sara Sjödal<sup>a</sup> & Per Milberg<sup>a,b</sup>

<sup>a</sup>Department of Crop Production Ecology, Swedish University of Agricultural Sciences, Box 7043, SE-750 07 Uppsala, Sweden

<sup>b</sup>IFM Biology, Conservation Ecology Group, Linköping University, SE-581 83 Linköping, Sweden

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The authors have no commercial interest in the findings presented.

*Correspondence to: Per Milberg, IFM Biology, Linköping University, SE-581 83 Linköping, Sweden*

Email: [permi@ifm.liu.se](mailto:permi@ifm.liu.se)

2 Figure

2 Tables

Approximately 5 600 words

## Abstract

Continuous increase in glyphosate use in Sweden has caused concern about resistance development, not least in connection with the possible introduction of crops resistant to glyphosate. In Sweden, the main weed targeted by glyphosate is *Elymus repens* (L.) Gould. We sampled 69 clones of *Elymus repens* to assess the magnitude and geographical distribution of variation in susceptibility to glyphosate. Clones originated from four habitat types: arable land intensively and extensively used, field vicinities and other habitats including natural vegetation. Susceptibility varied greatly between clones with GR50 (growth reduction to 50% of untreated) spanning over at least one order of magnitude, 17 to 278 ai ha<sup>-1</sup> in a pot experiment setting. There was a strong covariance between geographic and genetic distance, but no evidence of geographic or genetic differentiation in GR50. Nor did GR50 vary consistently between habitat types. We conclude that we found no indication of past selection towards resistance to glyphosate in *Elymus repens* clones in Sweden. The great variability in susceptibility suggests that there might be a potential for such selection.

**Key words:** *Agropyron repens* (L.) P. Beauv.; *Elytrigia repens* (L.) Desv.; herbicide tolerance; herbicide resistance, Sweden

## Introduction

*Elymus repens* (L.) Gould is a perennial, rhizomatous grass native to Eurasia but now distributed throughout the northern temperate zone (Hultén and Fries 1986). It spreads locally mainly by vegetative reproduction (Håkansson 1967; Tardif and Leroux 1991b), but can form a relatively short-lived soil seedbank of importance not least for re-colonization after effective control of vegetative plants (Håkansson 2003; Thompson et al. 1997; Williams 2006). As an almost obligate outcrosser (Beddows 1931), its production of viable seeds depends on the existence of more than one clone in the surroundings. The relative importance of asexual versus sexual reproduction therefore differs between populations (Szczepaniak et al. 2009). *Elymus repens* is an important weed in both rotational and perennial crops, and on waste-land and grassland (Häfliger and Scholz 1980). In the Nordic countries, including Sweden, it is the most widespread and abundant rhizomatous weed (Håkansson 2003).

The main herbicide used for control of *Elymus repens* in Sweden today is glyphosate (Swedish Board of Agriculture 2008), introduced on the Swedish market in 1978 (Olofsson and Nilsson 1999). Glyphosate makes up one third of the herbicides sold in Sweden (ton ai) (Swedish Chemicals Agency 2013). According to an interview study with farmers and other end users, 55 % of the glyphosate use in 1998 was for control of *Elymus repens* and other perennial weeds in cereal stubble or oil seed rape stubble while the remainder was used for termination of leys, burn-down of oil seed crops and catch crops (Swedish Board of Agriculture 2008). Among important factors behind the increase and change in the Swedish glyphosate use pattern since the mid-1990s are government and EU regulations and policies aimed at reducing nitrogen leakage (Olofsson and Nilsson 1999; Swedish Board of Agriculture 2008). Restrictions on autumn tillage and demands for “green cover” over the winter months have contributed to the shift from mechanical to chemical weed control, especially targeting perennials, a practice which has led to the decrease of *Elymus repens*, . in Denmark (Andreasen & Stryhn 2008) and Finland (Salonen et al. 2013). The range of situations where glyphosate is used for *Elymus repens* control has thus broadened, and the risk of sub-optimal treatments may have increased. Treatment in late autumn, or in conditions such as high soil water content, high amounts of straw, or low amounts of *Elymus repens* biomass present above ground, are all examples of situations where *Elymus repens* control may fail.

Field-evolved resistance to glyphosate in weed species is now well-documented, and has been reviewed by Preston and Powles (2006), Preston and Wakelin (2008), Tranel and Trucco (2009), Powles and Yu (2010), and Shaner et al. (2012). These reviews show that the level of such resistance is usually low to moderate, with survival at dose rates of 10 to 20-fold the level that kills a susceptible genotype. In cases where the genetic background has been elucidated, resistance is usually governed by single, nuclear genes with varying degrees of dominance.

There can be considerable intraspecific variation in the response of a weed to a herbicide (Gillespie and Vitolo 1993, Espeby et al. 2011) and this is also the case with glyphosate (Cerqueira and Duke 2006; Tranel and Trucco 2009). In fact, if such variation is large, the detection of glyphosate resistance may be difficult (Tranel and Trucco 2009). Examples of species with a documented natural variation to glyphosate include *Convolvulus arvensis* L. (Degennaro and Weller 1984) and *Amaranthus rudis* Sauer, and *A. tuberculatus* Moq. (Patzoldt et al. 2002). Variation in *Elymus repens* susceptibility to glyphosate has previously been shown both in field studies (Tardif and Leroux 1991a; Westra and Wyse 1978) and under controlled conditions (Ulf-Hansen 1989).

The aim of this study was to estimate the magnitude of variation in glyphosate susceptibility *Elymus repens* in Sweden, how this varies geographically and by habitat type, whether susceptibility varies with genetic distance, and to discuss the results in relation to risk of resistance evolution. With the design, we were able to address three questions: (i) how large is the variability in glyphosate susceptibility among clones? If substantial, that would indicate the potential for resistance selection by sorting among clones. (ii) Are susceptible clones more prevalent in habitats unexposed to glyphosate than in intensively cropped arable land? If so, that would indicate that selection has occurred. (iii) Is there is a correlation between genetic distance and geographic distance in the susceptibility among clones? If so, that would indicate that resistant genotypes have been spreading geographically (e.g. Andrews et al. 1998, Osuna et al. 2011, Aper et al. 2012, Yamada et al. 2013).

## **Materials and Methods**

### **Origin of *Elymus repens* Clones in the Glyphosate Dose-Response Study**

The glyphosate dose-response study included 69 clones of *Elymus repens*. They were collected in 2002-2003 from 63 locations in Sweden (Fig. 1), with emphasis on the agriculturally important provinces in southern Sweden, i.e. in Götaland (49 samples) and in Svealand (14 samples), but also northern Sweden (Norrland, 6 samples). One sample (rhizome) per location was taken, except at three locations in Svealand and two locations in northern Götaland where morphologically distinct co-occurring clones were sampled. The locations were always more than 150 meters apart.

Most samples were collected on arable land (Table I). In the fields situated on intensively cultivated land (32 samples), the land-use in the year of sampling was cereals, sugar beet, oil-seed rape, or fallow. On land that was more extensively and/or organically managed (16 samples), the crops were cereals, pulses, or leys. Collection was also done in field vicinities (7 samples) and locations such as roadsides and other ruderal sites, gardens, beaches, forests, meadows or permanent pastures (14 samples). In a few cases poor effect of glyphosate on *Elymus repens* had been noted at the site by the farmer, but in these cases it could not be ruled out that this was the result of inadequate conditions for treatment.

### **Rhizome Production and Planting**

Directly after collection, the rhizome samples were maintained by growing them in pots outdoors and, during winter, in a greenhouse.

In order to produce sufficient rhizome quantities and to minimize effects of growing conditions in the collection locations (such as differences in temperature, or in water and nutrient status) the clones were re-planted into 12-liter buckets in the first half of February the year after collection. One single piece of rhizome per clone was planted into potting soil. Liquid fertilizer (NPK 51-10-43 + micronutrients, 2 mL L<sup>-1</sup>) was added by watering each week to avoid growth being limited by nutrient shortage. Five to ten weeks after the re-planting (the time depended on growth rate), the rhizomes were harvested. For capacity reasons (space and manpower), the 69 clones were divided into three trial batches for the herbicide experiment. Batch 1 (25 clones) was planted on March 30 to April 2, batch 2 (26 clones) on April 20-22, and batch 3 (18 clones) on May 12-14. For each clone, 4 three-liter pots were planted with 12 pieces of rhizome each. Each single node rhizome piece was 4 cm

long of medium thickness, rendering each piece about the same weight. The oldest and youngest parts of rhizomes were avoided.

The soil used was a loamy sand to which *Sphagnum* peat had been added up to 25% w/w, and lime, resulting in a pH of 7.2. After planting, the pots were placed in a greenhouse with a light/darkness cycle of 16 h/8 h at 18-20°C/14°C for 10 weeks (batch 1 and 2), and then transferred outdoors. Batch 3 was already transferred outdoors after 4 weeks, as greenhouse conditions were considered too hot during the late summer season. Nutrients were added as granules (NPK 12-5-14) after 3 ½ weeks, corresponding to 17 kg N ha<sup>-1</sup>, and after 9 weeks, corresponding to 43 kg N ha<sup>-1</sup>. The growing period in the 3-liter pots prior to herbicide treatment was 13 weeks for all clones.

## **Glyphosate Treatment**

Glyphosate (Roundup Bio, 486 g L<sup>-1</sup> isopropylamine salt of glyphosate, SL, Monsanto Crop Science) was applied on June 29 (batch 1), July 20 (batch 2) and on August 10 (batch 3). One control clone was included in all three batches for comparison of possible batch effect. The dose rates of glyphosate were equivalent to 0, 146, 437, 875, 1458 and 2552 g of active ingredient per hectare for batch 1, and changed to 0, 146, 292, 437, 875 and 1458 g ai ha<sup>-1</sup> for batch 2 and 3, as the highest dose used in batch 1 was considered too high. A non-ionic wetting agent (Biowet® non-ionic wetting agent, alkyl alcohol 30-50 %, Tergent AB, Helsingborg, Sweden) was added at a rate of 0.3 mL per liter spray liquid. Distilled water was used. Four pots were treated for each clone and dose. The glyphosate treatments were done in a closed spray chamber (Experimental Pot Sprayer, 1992, Jens Kristensen, Ringsted, Denmark) with the equivalent of 130 L ha<sup>-1</sup> of spray liquid, using flat fan nozzles, a spray pressure of 300 kPa, and a spray boom speed of 5.5 km h<sup>-1</sup>. For one day prior and two days post glyphosate treatment, the pots were kept in a climate chamber with a daily cycle of 16 h/8 h at 18-20°C/14°C. Light was provided by fluorescent tubes supplying a minimum of 2-4 μmol m<sup>-2</sup> s<sup>-1</sup> of photosynthetically active light at pot level.

Three days post glyphosate treatment, the above-ground biomass was cut in all treatments. The fresh weight of this leaf material was recorded per pot for the 0 dose, to be used as a measure of plant size around the time of spraying.

After 6 weeks, the re-growth of above-ground biomass of the clones in batch 1 and batch 2 was harvested, and fresh weights recorded per pot for all treatments. Growth outdoors of the clones in batch 3 was reduced due to cool weather in the autumn. This material was therefore grown in the greenhouse with a daily cycle of 16 h/8 h at 18°C/14°C for 2 weeks before harvest, which gave a total period of post-treatment re-growth time of 9 weeks.

## **Genetic Analysis**

Forty-six *Elymus repens* clones in the dose-response study had earlier been assessed using AFLP-analysis with emphasis on genetic variation, a study that found moderate and no differentiation among regions and landscape type, respectively (Fahleson et al. 2008). In total, 126 AFLP loci for each clone were included in the present work in order to determine any linkage between glyphosate response and geographic distribution.

## Data Analysis

The software GraphPad Prism 5.02 (GraphPad 2008) was used to estimate GR50 (Growth Reduction to 50% of biomass of control plants). The logistic function (1) fitted to data assumed that biomass dropped from 100% to zero, with a fixed Hillslope of -1:

$$Y=100/(1+10^{(X-\text{LogGR50})}) \quad [1]$$

where X represents the log<sub>10</sub>-transformed concentration of ai ha<sup>-1</sup>. The data, which were analyzed per clone, were expressed as percentages of the mean values of unsprayed control plants. Confidence intervals (CI<sub>95%</sub>) for logGR50 were calculated, and back-transformed for presentation. The GR50 estimates were also averaged for each region, test batch and original habitat using meta-analysis methodology and the software Comprehensive Metaanalysis 2.0 (Borenstein et al. 2005). Meta analysis provides a flexible way of summarising data on estimates and incorporating the uncertainty of those estimates, in our case clone-wise GR50 and their CI<sub>95%</sub>., respectively. The mean values of GR50 per region, test batch and original habitat were calculated using a random model (Mengersen et al. 2013).

As a test for possible spatial and genetic differentiation in data, Mantel tests were conducted, using Brodgar 2.5.7 (Brodgar 2008) and 9999 permutations. Three pairwise distance matrices, based on 46 clones, were compared: geographical distance between clones, corresponding genetic distances (as judged by AFLP), and pairwise differences in logGR50. In addition, we also conducted partial Mantel tests using one of the three matrices as a covariable to adjust for possible confounding factors.

## Results

### How large is the variability in glyphosate susceptibility among clones?

The estimated GR50 values of the *Elymus repens* clones in our experiment spanned two orders of magnitude (4.3 to 395 ai ha<sup>-1</sup>; Fig. 2a), but there is a risk that the lowest values might be underestimates especially as GR50 is poorly validated when plants only survive at the lowest dose tested. Thus, in order to use a more conservative estimate of the range of GR50, we eliminated the two highest GR50 values (367 and 395 ai ha<sup>-1</sup>), that had the largest CI, and the two lowest GR50 values (4.3 and 7.9 ai ha<sup>-1</sup>), that were unexpectedly small (if we assume that logGR50 is normally distributed). Hence, our conservative estimate of the range of GR50 in *Elymus repens* populations in Sweden spans over a little more than one order of magnitude (17.2 to 277.7 ai ha<sup>-1</sup>).

### Are susceptible clones more prevalent in unexposed habitats?

GR50 did not vary consistently between the types of habitats where clones had been collected (Fig. 2b). A possible exception was the somewhat lower GR50 in clones from the intensively used arable land (Fig. 2b).

There was a clear batch effect (Fig. 2b) which is difficult to interpret because clones had not been randomly allocated to batches. Instead they had been grouped according to how soon after planting into the 12 L vessels each clone had accumulated sufficient biomass for the further experimentation, and it seems that the onset of growth depended on the geographic origin of a clone. The batch effect was also obvious in the results for our control clone: the largest GR50 for this clone was generated when it was included in batch 1. Removing the

batch effect (through normalization of GR50 estimates per batch), did not enhance any differences among types of location (data not shown).

There was a tendency for GR50 values to be higher in southern Götaland (Fig. 2b), but this effect cannot be distinguished from the batch effect mentioned above. Removing the batch effect through normalization (clone-wise GR50 estimates adjusted by batch mean) brought GR50 in southern Götaland on par with the other regions (data not shown).

There was a tendency for clones with smaller plants at the time of spraying to yield higher GR50 values: the regression equation for batch 1 was  $\log(\text{GR50})=2.597-0.0229*(\text{g biomass [3 days after spraying]})$ ,  $P=0.051$ ; for batch 2 and 3, considered together because they did not differ,  $(\log(\text{GR50})=2.631-0.0914*(\text{g biomass})$ ,  $P=0.00036$ ). Hence, plant size and, to some degree, possible differences in developmental stage at spraying have, either directly through larger leaf area, or indirectly through higher growth rate and more efficient translocation to apical parts, had some influence on our data. Our estimate of the extent of the variation in susceptibility, however, remains unaffected when we eliminate these trends from the two data sets (data not shown).

### **Is there a relationship between susceptibility, genetic and geographic distance?**

The Mantel test scored a significant covariance between geographic and genetic distance (Table II). In contrast, the other Mantel tests showed no evidence of geographic differentiation in susceptibility to glyphosate, as measured by GR50 (Table II), which would have been expected if there had been a strong selection pressure in arable land. Nor was there any correlation between the response to glyphosate in the clones and the genetic differences as measured by AFLP, which could have been the case if individual resistance mutation events were spreading, either by vegetative propagation or by sexual recombination. The correlations were only marginally affected by the inclusion of a covariable in the Mantel tests (Table II).

## **Discussion**

In this observational study we set out to study the potential for, and possible manifestations of, selection towards glyphosate resistance in the perennial grass weed *Elymus repens*, a main target for this herbicide during 25 years of usage in Sweden.

### **Variability in glyphosate susceptibility**

Our first aim was to establish the magnitude of variability in glyphosate susceptibility among clones. GR50 proved to span a bit more than one order of magnitude (17- 278 ai ha<sup>-1</sup>) in the 69 clones studied, and after eliminating the two largest and the two smallest values. This magnitude of differences is relatively large, at least compared with variability in growth reduction in annual grasses (Espeby et al. 2011). It is not possible to directly translate the effects of the dose rates employed in our pot study to the efficacy of a certain dose rate in the field. First, GR50 is not a relevant target for a farmer and second, the efficacy of a given dose is most likely higher in a pot-based system than in a field situation.

There was a tendency for the less vigorously growing clones to yield higher GR50 values, which is not unexpected (e.g. Reade & Cobb 2002, Espeby et al. 2011). If higher growth rate, through-more efficient translocation to apical parts, is one of the means by which clones can

exert differences in susceptibility, this means selection would favour the less vigorously growing clones. Biomass production can differ substantially between *Elymus repens* clones (Tardif and Leroux 1991b; Westra and Wyse 1981; Mercer et al. 2002), as can the production of spikes and rhizome buds (Westra and Wyse 1981) and the ratio of shoot growth to rhizome weight (Williams 1973). Varying ability of clones to utilize nitrogen is one additional factor behind differences in biomass production (Tardif and Leroux 1992). *Elymus repens* is generally favored by high nitrogen supply (Håkansson 2003) and increased availability of nitrogen has, in turn, been put forward as a factor reducing the inhibitory effect of glyphosate (Hunter et al. 1993; McIntyre and Hsiao 1982), perhaps due to its positive effect on growth. Tardif and Leroux (1993) could link reduced susceptibility to lack of accumulation of glyphosate in the apical parts of rhizomes in one particular biotype. This clone also had longer rhizomes, due to relatively rapid rhizome growth. For an equally tolerant clone in the same experiment, no explanatory causal factor was identified.

### **No Evidence of Selection Towards Resistance in Swedish *Elymus repens***

Our second aim was to search for manifestations of possible resistance selection 25 years after the introduction of the herbicide in Swedish agriculture. A development towards resistance leads to three predictions: (i) more susceptible clones in habitats unexposed to glyphosate (i.e. non-arable vs arable); (ii) more susceptible clones in regions where agriculture is less important and extensively conducted (i.e. northern vs. southern Sweden); (iii) resistant clones would be spreading, leading to geographical patterns in susceptibility. Our data could not support any of these predictions. First, there was no indication in our data for clones from non-arable situations to be, on average, more susceptible than those from intensively used arable land. If anything, there was a tendency for an opposite pattern: clones from the intensively used arable land were more susceptible. We hypothesize that such a pattern might reflect previous selection toward vigorous spring growth and the ability to more efficiently exploit nutrients in rich arable soil. Second, although there was a tendency of clones from southern Götaland to be less susceptible to glyphosate, this can be attributed to a batch effect, i.e. that clones that started to grow early in the spring, or were quick to accumulate biomass, were included in the first experimental batch. As the growth in clones from southern Sweden are likely to start first in a common garden experiment (e.g. Boström et al. 2013), we were unable to separate the clone effect from that of onset of growth and growth rate at time of herbicide treatment. Third, there were no genetic patterns to suggest that resistant clones would have spread in Sweden.

To conclude, 25 years after glyphosate was introduced in Sweden there was no clear evidence for a selection towards glyphosate resistance. The greater GR50 in southern Götaland (Fig. 2b) was probably due to those clones commencing growth earlier (and thereby being included in batch 1). The fact that clones from intensively cropped arable land had somewhat lower GR50 (Fig 2b) also points to a slow or non-existent resistance development in this combination of weed species and herbicide.

### **Potential Risk of Evolution of Glyphosate Resistance in Swedish *Elymus repens***

Two scenarios have been pinpointed as especially risky for development of glyphosate resistance: (i) continuous cultivation of glyphosate-resistant crops (Owen and Zelaya 2005), i.e. recurrent use as a selective herbicide, and (ii) intense use as weed burn-down before seeding in minimum or zero tillage (Neve et al. 2003). In this context, Tranel and Trucco (2009) expect that “natural tolerance” is likely to be due to the combined effects of multiple

genes, and thus prone to the evolution of quantitative resistance, if subjected to recurrent selection. On many Swedish farms, glyphosate is applied frequently also in situations that are not optimal for good efficacy. With the relatively large variation in susceptibility to glyphosate documented in our study, such practices could contribute to a risk scenario for development of resistance (Neve 2007), not least because application under sub-optimal conditions could favor resistance build-up especially of quantitatively inherited resistance traits (Neve and Powles 2005). On individual Swedish farms with no- or low-till practices, certain fields may be sprayed with glyphosate every year at reduced dose rates before drilling for a new crop. In such cases, the weeds mainly targeted will often be volunteers and annuals rather than *Elymus repens*, although this species may also be present. The selection pressure imposed on *Elymus repens* in Sweden is, however, far from the levels that have caused resistance in other weedy grass species such as *Sorghum halepense* (L.) Pers. (Gressel and Valverde 2006), *Lolium rigidum* Gaud (Powles et al. 1998; Pratley et al. 1999) or *Eleusine indica* (L.) Gaertn (Lee and Ngim 2000). Given the reproductive pattern of *Elymus repens*, the risk of glyphosate resistance evolution and its spread in this species in Sweden is not alarming. However, since no reduction of glyphosate usage is expected in the near-future, continued attention is called for and especially so for annual weeds.

The importance of seed, either freshly shed or from the seed bank, for *Elymus repens* reproduction is considered to be relatively limited at least in the shorter time perspective (Håkansson 2003). This could aid in restricting further spread of glyphosate resistance in this species. Seed set varies both among biotypes and years (Tardif and Leroux 1991b), and is reported to often be low to moderate. Theoretically, production of viable seed should also be limited by the availability of mating partners. Szczepaniak et al. (2009) found, however, that most genetic diversity between clones resided within populations, indicating that reproduction by seed can be important in the agricultural setting. Whether resources are allocated to seed setting or to rhizome production is also determined by the resource availability and the relative cost to the plant in a specific environment (Reekie 1991).

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Figure 1. Geographic locations in Sweden where the 69 *Elymus repens* clones were collected for the glyphosate susceptibility experiment. Open triangles show 23 clones lacking AFLP (Amplified Fragment Length Polymorphisms) data. Sweden stretches from 55°20' in the south to 69°3' in the north.

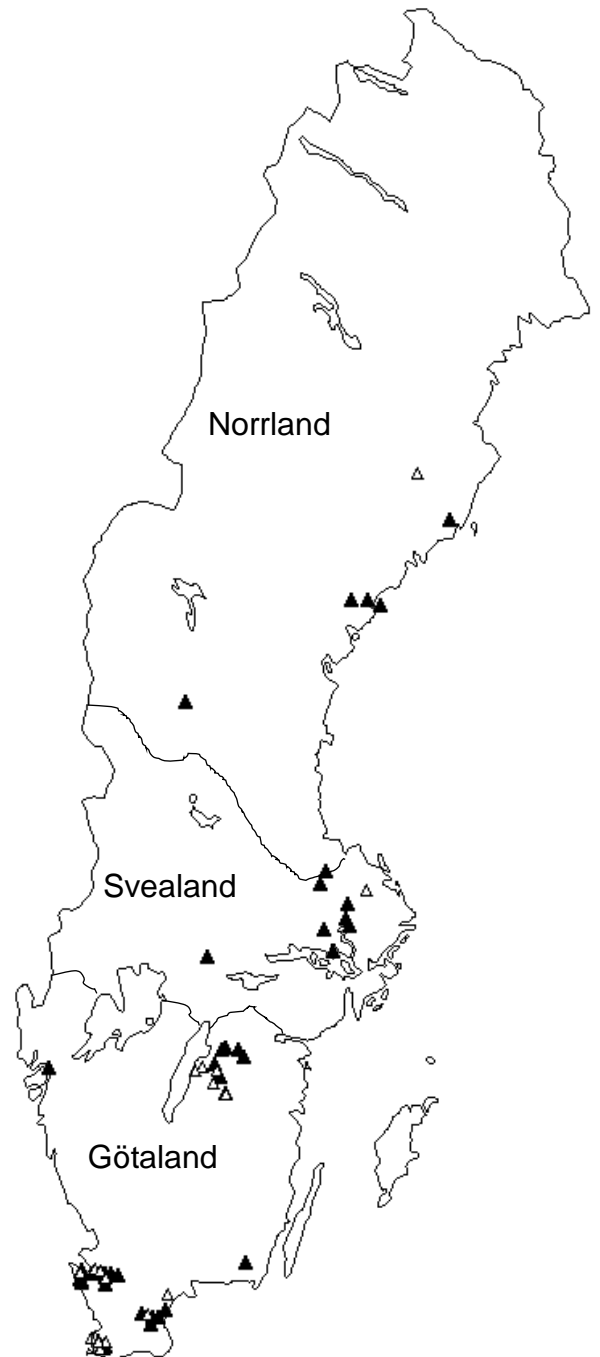


Figure 2. 2a) Estimated GR50 (effective dose, 50 % of biomass of control plants) values per clone, arranged by increasing latitude of collection site. 2b) Average GR50 values per region, test batch, and original habitat (see Methods for details). The three test batches differed mainly in spraying date, to some degree also in post-spraying growth conditions.

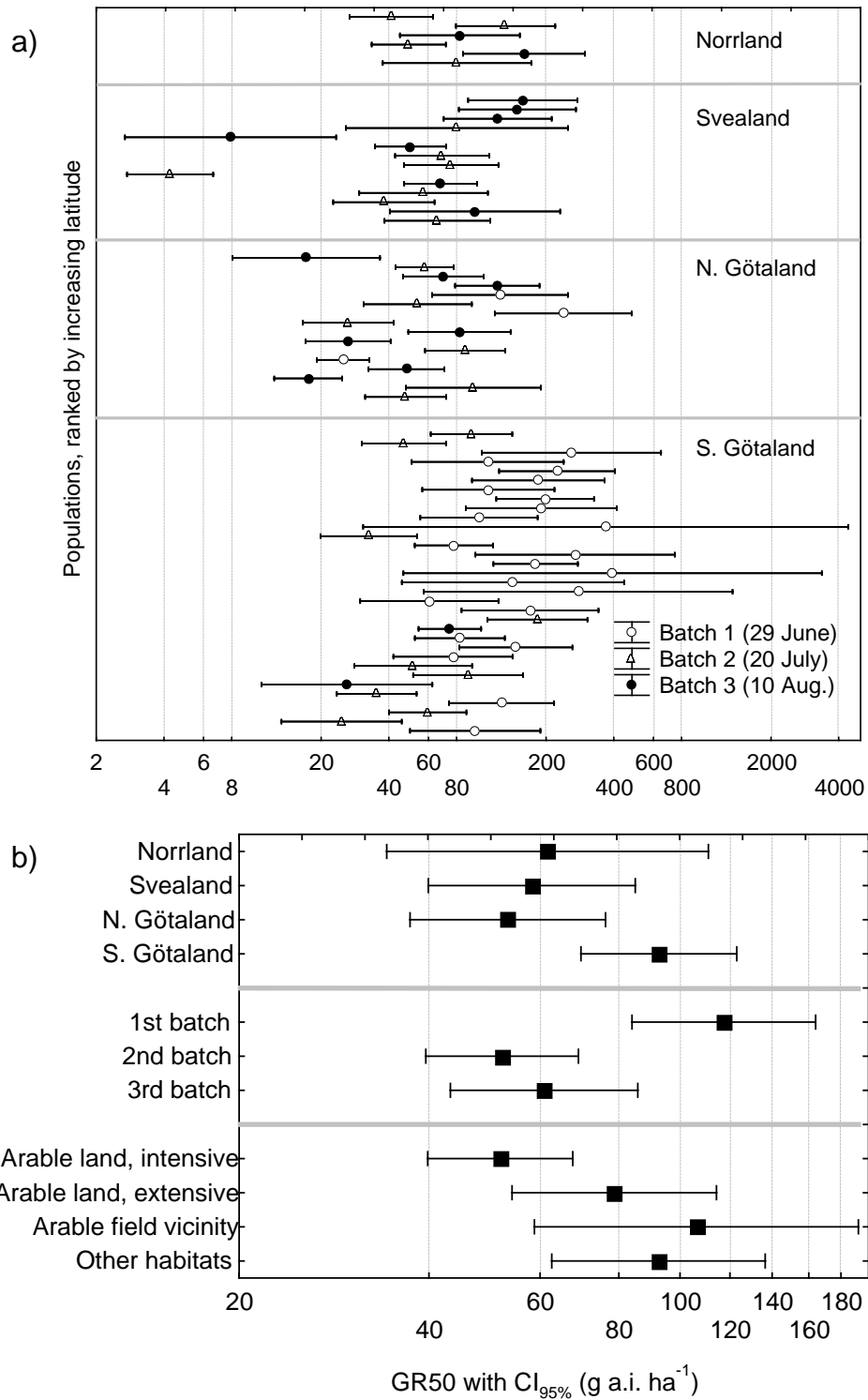


Table I. Number of clones of *Elymus repens* included in the study according to habitat and sampling latitude.

Region	Arable land, intensive	Arable land, extensive	Field vicinity	Other	Total	Latitude
Norrland	0	1	0	5	6	62.0-64.3°
Svealand	8	5	0	1	14	59.5-60.3°
N Götaland	11	4	1	0	16	58.1-58.5°
S Götaland	13	6	6	8	33	55.5-56.3°
Total	32	16	7	14	69	

Table II. Results from Mantel tests of matrices, based on 46 clones, on pairwise geographical distance, genetic distance as measured by AFLP (Amplified Fragment Length Polymorphisms) data, and difference in logGR50 (effective dose, 50 % of biomass of control plants). Top right contains Mantel tests, and bottom left partial Mantel tests. \*\*\* = P<0.001

	Geography	AFLP	LogGR50
Geography	-	0.272***	-0.0102
AFLP	0.273***	-	0.0632
LogGR50	-0.0286	0.0686	-