Within-species Variation in Grass Weeds in Sweden

Dormancy, Herbicide Response, Genetic Relationships

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Abstract

Variation within a weed species enables it to persist through varying conditions and is thus an important component of weediness. In this thesis, intra-specific variation in two agronomically important attributes - herbicide susceptibility and seed dormancy - are studied in Swedish *Apera spica-venti* (L.) Beauv. and *Alopecurus myosuroides* Huds., both serious annual weeds in winter cereals, and with many cases of herbicide resistance. Swedish *Elymus repens* (L.) Gould, a perennial, rhizomatous grass, is investigated for its genetic variability and variation in glyphosate response.

The susceptibility to new and established herbicides in greenhouse studies in the two annual grasses ranged 0.5-4 orders of magnitude among populations, which mostly came from fields with no previous suspicion of resistance. The greatest variation was found in *A. myosuroides* in response to fenoxaprop-P-ethyl (an old herbicide in the sense that it had been used for a decade), with significant correlation with response to flupyrsulfuron-Na (a newly introduced herbicide). One fifth of the *A. spica-venti* populations were significantly less susceptible to isoproturon (old) than a susceptible reference population, but without correlation in response to sulfosulfuron (new). The findings indicate field evolved resistance at a scale of practical importance with possible cross-resistance to one new herbicide.

Both *A. spica-venti* and *A. myosuroides* exhibited considerable primary and seasonally variable seed dormancy, with variation between seed collects. Light requirement in *A. myosuroides* was affected by stratification, and soil disturbance did not greatly enhance the low spring emergence occurring in this winter annual.

Swedish *E. repens* seem to consist of one large, fairly homogeneous group, with a moderate geographic differentiation. Glyphosate dose-response patterns varied greatly between clones but were not linked to type of habitat or to genetic or geographic distance.

The results are discussed in relation to methods for assessing herbicide susceptibility, for early detection of resistance, and for weed management.

Keywords: Elymus repens, *Apera spica-venti*, *Alopecurus myosuroides*, herbicide resistance, dose-response, dormancy, genetic variation, intra-specific variation

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Fahleson, J., Okori, P., Åkerblom-Espeby, L., Dixelius, C. (2008). Genetic variability and genomic divergence of *Elymus repens* and related species. *Plant Systematics and Evolution* 271 (3-4):143-156.

II Espeby, L.Å., Fogelfors, H., Sjödal, S., Milberg, P. Variation in quackgrass susceptibility to glyphosate. (manuscript).

III Andersson, L., Åkerblom Espeby L. (2009). Variation in seed dormancy and light sensitivity in *Alopecurus myosuroides* and *Apera spica-venti*. *Weed Research* 49 (3): 261-270.

IV Espeby, L.Å., Fogelfors, H., Milberg, P. Isoproturon resistance in populations of *Apera spica-venti* (L.) Beauv. in Sweden. (manuscript).

V Espeby, L.Å., Fogelfors, H., Milberg, P . Susceptibility variation to new and established herbicides - examples of interpopulation sensitivity of grass weeds. (manuscript).

Papers I and III are reproduced with the permission of the publishers.

The contribution of Liv Åkerblom Espeby to the papers included in this thesis was as follows:

In paper I, II, IV and V, I have, together with co-authors, designed the field, greenhouse and common-garden work. I contributed to the design of the laboratory work for paper I and II, and to the design of one of the experiments in paper III (expt 4). I was responsible for all greenhouse and common-garden experiments in papers II, IV, and V, and participated in laboratory work that contributed to papers I, II, and III. I conducted or participated in the statistical analyses in papers II-V. As main author, I wrote most of papers II and IV. As co-author, I contributed substantially to paper V and to sections of paper III and to a lesser degree to paper I.

Abbreviations

a.i.	active ingredient
ED50	efficient dose 50 %
LD50	lethal dose 50 %
GR50	growth reduction 50 %
RF	resistance factor
ACCase	acetyl CoA carboxylase
ALS	acetolactate synthase
PCR	polymerase chain reaction
AFLP	amplified fragment polymorphisms
CI	confidence intervals
CV	coefficient of variance

1 Introduction

1.1 Variation as a component of weediness

In comparison to crops, the weed flora is diverse both in number of species and in the variation residing within the individual species. Whereas crops have been bred for many generations to become homogeneous, the success of weeds in cultivated systems depends on their ability to fill the varying niches that appear there. While natural habitats may also be variable over time, the cultivated habitats are highly disturbed systems which will offer different opportunities in different years and crops. Some weed species succeed by finding niches different to that occupied by the crop, others exploit the same niche as the crop, but with some competitive advantage that enables them to thrive in spite of the competition (Dekker, 1997). Weed species composition in arable fields changes over time (Hyvönen *et al*, 2003) as a consequence of changes in crop rotations and cultivation (Anderson & Milberg, 1998; Andreasen & Stryhn, 2007; Andreasen & Skovgard, 2009) and not least as a response to changing weed management practices (Cousens & Mortimer, 1995; Murphy & Demerle, 2006).

The ability to explore new niches requires a certain degree of flexibility in a species. This can be accomplished in two ways, which are not mutually exclusive; individual plants may have the ability to adjust to different environments by phenotypic plasticity, or the population may have the ability to adapt through variation in its off-spring.

1.2 Reproductive strategies

The ability to persist over time on a location is determined by the reproductive success. Dispersal pattern and the survival and germination characteristics of propagules are thus important attributes for persistence of weedy species. Many weed species exhibit seed dormancy, i.e. an ability to emerge at a time and in conditions that favour seedling growth and competitiveness with crops and other weeds, and remain un-germinated (but viable) when conditions are less favourable (Bewley & Black, 1994; Vleeshouwers *et al*, 1995). Dormancy contributes to a winter or summer annual germination behaviour in plants, with the main emergence periods in autumn and in spring, respectively (Froud-Williams *et al*, 1984).

The level of primary dormancy determines to what extent fresh seeds will germinate in the time period following upon dispersal. The primary dormancy may vary among populations (Frost & Cavers, 1975; Naylor & Abdalla, 1982; Andersson & Milberg, 1998) and such differences have been linked to weather effects during seed maturation (Strand, 1989; Fenner, 1991), the light environment of the mother plant (Fenner, 1991) and genetic differences are also implicated (Probert *et al*, 1984; Beckstead *et al.*, 1996).

Secondary, induced dormancy enables a seasonal variation in dormancy levels. The response among populations to dormancy-inducing factors is also subject to variation between populations (Probert *et al*, 1984).

1.3 Variation and cropping systems

Cropping is believed to typically reduce variation, and the more intense and one-sided, with continuous monoculture being one extreme, the less diversity will reside within the cropping system. All cropping systems and cultivation measures may exert directional selective pressure. Not least are pesticides powerful agents in reducing diversity, not only at the intended levels (Geiger et al, 2010).

The use patterns and attitudes towards pesticide use within agriculture has undergone several shifts since the advent of selective herbicides first drastically changed weed management strategies, and enabled shifts in crop rotations. Environmental concerns, legislation and levies on pesticides have all contributed to changes in pesticide use patterns in the last few decades. Reduced herbicide dose rates have become a recommended practice (Jordbruksverket, 2010b), but even so, in Sweden weed control chemicals remain the most applied type of pesticide in the majority of crops (with the most notable exceptions being spring sown Brassicas and potatoes, where insecticides and fungicides, respectively, dominate in acreage treated) (Statistiska centralbyrån, 2009).

1.4 Herbicide resistant weeds

Herbicides exert a very strong selective pressure on the weed flora in favour of species, and of genotypes within species, with low susceptibility to the used herbicide. The intra-specific variation in the response of a weed species to a herbicide can be considerable (Gillespie &Vitolo, 1993).

Most herbicides used today function by inhibiting a vital, biochemical process within the weed plant. Often the mode of action involves binding to a specific plant enzyme, blocking out the normal substrate(s). Crops that are able to survive herbicide treatments often do so by detoxifying and/or degrading the herbicide before serious injury occurs to the plant. Another way of surviving treatment, both in crops and in weed species, is to have an insensitive form of the enzyme that the herbicide targets. One such example is the enzyme acetyl CoA carboxylase, active in fatty acid synthesis, which is inhibited in monocotyledonous plants by certain herbicides, but is present in an insensitive form in dicotyledonous species (Cobb, 1992; Reade & Cobb, 2002)

Genotypes that carry one or several mutations rendering them less susceptible to a certain herbicide than what is normal in the species can be naturally present in a field. By repeated selection, the proportion of normally susceptible genotypes will be reduced in the population, which will gradually change into a resistant population, in reference to that particular herbicide, or to several (Moss, 2002).

There are many reports on cross-resistance due to an insensitive target site between herbicides sharing the same mode of action, but all members of a herbicide group need not necessarily be affected (the term "cross-resistance" is here used in reference to resistance affecting more than one herbicide, and which is caused by the same mutation(s)). Metabolic cross resistance, due to greater efficiency in the plant metabolism of herbicides, can affect groups of herbicides with different modes of action and is thus even more difficult to predict (Moss, 2002; De Prado & Franco, 2004). Resistance can also occur simultaneously in plants but be caused by different mutations and resistance mechanisms. For the majority of herbicide resistance cases (resistance to triazines excepted) a fitness cost of the resistance gene(s) has not been determined, or appears to be low (Moss, 2002).

The strong directional selection for resistance by herbicide application is thus not typically off-balanced by a strong force for reversal in the absence of the herbicide.

1.5 Scope of the thesis

In this work, the intra-specific variation in two attributes of weeds is studied: i) the susceptibility to herbicides normally used to control the species in question (Table 1), and ii), the ability to steer germination to a favourable time by seed dormancy. The plant material studied are Swedish populations of three agronomically important grass weeds; *Elymus repens* (couch or quackgrass, kvickrot), *Apera spica-venti* (silky bentgrass, åkerven), and *Alopecurus myosuroides* (blackgrass, slender foxtail, renkavle). While *E. repens* is the most wide-spread and abundant rhizomatous grass weed in Sweden, *A. spica-venti* and *A. myosuroides* are serious annual weeds in winter cereals, and recognised (especially the latter) as species with multiple cases of field evolved herbicide resistance. The genetic variation in Swedish *E. repens* is also studied.

Table 1. Herbicide susceptibility experiments

Species	Test type	Response parameter	Herbicide (active ingredient)
Elymus repens	Dose-response, pots outdoors	Re-growth	glyphosate
Apera spica-venti	Dose-response, greenhouse	Survival, biomass reduction	Isoproturon and sulfosulfuron
Alopecurus myosuroides	Dose-response, greenhouse	Survival, biomass reduction	Fenoxaprop and flupyrsulfuron

2 Aims

The aim of this project was to learn more about the intra-specific variation in the three grass weed species *E. repens*, *A. spica-venti*, and *A. myosuroides*, with the main objective of increasing the knowledge base for future management strategies for their control. The focus was on the Swedish populations of these three species in agricultural habitats, and on two attributes of importance for weed management; seed dormancy and herbicide susceptibility. For one of the species, *E. repens*, an additional aim was to investigate the genetic variability in the Swedish population.

The following questions related to the above objectives are addressed in the five papers on which this work is based:

Paper I and III:

- What are the patterns of genetic variability in the Swedish population of *E. repens*, with focus on three important agricultural regions in Sweden?
- What is the magnitude and pattern of variation in glyphosate susceptibility *in E. repens* in Sweden, in regards to genetic and geographic distance, and type of habitat?

Paper II:

- To what extent are *A. myosuroides* and *A. spica-venti* restricted to a winter annual germination behaviour?
- What is the magnitude of variation in dormancy among populations of the respective species? Is it of a size large enough to be of agronomical consequence?
- To what degree do populations vary in how emergence is affected by temperature, light and soil disturbance?

Paper IV:

- What is the size and geographic distribution of variation in susceptibility to isoproturon in Swedish *A. spica-venti*?
- How does the susceptibility in Swedish *A. spica-venti* to isoproturon, at the population level and at the level of individual plants, compare to that in European reference populations, previously characterised as susceptible or resistant?

Paper V:

- What is the intra-specific variation in herbicide susceptibility in Swedish field samples of *A. myosuroides* and *A. spica-venti* to the established herbicides fenoxaprop-P-ethyl and isoproturon, respectively?
- What range of LD50 and GR50 estimates for susceptibility to recently introduced sulfonylurea herbicides (here flupyrsulfuron and sulfosulfuron) can be expected in populations of annual grass weeds with little or no prior exposure to sulfonylurea graminicides?
- Is there a correlation in the response to the established and the recently introduced herbicides (isoproturon vs. sulfosulfuron and fenoxaprop vs. flupyrsulfuron)?

3 Species studied

3.1 *Elymus repens* (L.) Gould

Synonyms: *Elytrigia repens* (L.) Nevski, *Agropyron repens* (L.) PB., *Triticum repens* L.. English names: couch grass (Br), quackgrass (Am). Swedish name: kvickrot.

Elymus repens belongs to the Triticeae (wheat) tribe of Poaceae. It is a perennial grass native to Eurasia and introduced in North America, and a successful rhizomatous weed throughout the northern temperate regions (Hultén & Fries, 1986) in both rotation and perennial crops, in grassland and in waste places (Häfliger & Scholz, 1981). In Sweden, *E. repens* is present nationwide (Håkansson 2003).

E. repens was much favoured by changes in cropping systems from the 1950s and onwards, with cereals replacing leys, and with reduction of mechanical weed control measures, and its importance as a weed in Sweden increased during that time. *E. repens* is known to thrive on nitrogen-rich soils, but is very versatile, and can survive and spread in very differing habitats and environmental conditions. Mechanical control by repeated fragmentation of below-ground structures ("black fallow") was the prevailing method for control until the advent of chemical herbicides. (Håkansson 2003).

Today, the possibilities of control through mechanical means have been curtailed by both environmental regulation and by economics of different cropping systems (Jordbruksverket, 1999). In Sweden, *E. repens* is now mainly controlled by chemicals, in particular with glyphosate, and control of it accounts for a substantial share of glyphosate (and indeed of total herbicide) use (Jordbruksverket, 1999; Kemikalieinspektionen, 2009).

No resistance cases have been reported in *E. repens* to date in the web-based international survey on herbicide resistant weeds (Heap, 2010).

E. repens is an allogamous, wind-pollinated hexaploid, and an almost obligate out-crosser (Beddows, 1931; Redinbaugh et al., 2000). Vegetative growth in the form of rhizomes is considered to be the main way in which *E. repens* spreads and survives (Håkansson, 1967; Tardif & Leroux, 1991). The availability of suitable mating partner in the surroundings will affect the success of seed production, and the relative importance of vegetative reproduction versus propagation through seeds differs between populations (Szczepaniak et al., 2009). *E. repens* is often described to produce a low to moderate number of viable seeds, and to form a short-lived seed bank (Håkansson, 2003; Thompson et al., 1997; Williams and Attwood, 1971; Williams, 1973).

3.2 *Apera spica-venti* (L.) Beauv.

Synonym: *Agrostis spica-venti* L.; English name: silky bentgrass. Swedish name: åkerven.

Apera spica-venti is an annual grass, native to Eurasia, and introduced in North America. It is a weed in rotation crops and in waste places, and causes serious problems in winter cereals in a large part of Europe, not least in the eastern and northern parts (Weber & Gut, 2005; Warwick *et al.*, 1987) The spread of *A. spica-venti* in Sweden as a serious weed in agricultural fields coincides with the northern border of cultivation of winter cereals, not surprisingly, since it is known to benefit from frequent cultivation of winter cereals (Aamisepp & Avholm, 1970; Warwick *et al.*, 1985; Pallutt, 1999).

A. spica-venti exhibits a winter annual emergence behaviour with seasonal, secondary dormancy steering germination to the autumn period (Aamisepp & Avholm, 1970). The species does however also occur occasionally in spring crops (Soukup et al., 2006).

The range of herbicides and modes of action available for chemical control of *A. spica-venti* is fairly wide, in Sweden currently representing four different modes of action (Jordbruksverket, 2010b). Many resistance cases are however reported in European *Apera* populations, and against substances representing three of these modes of action; inhibitors of photosystem II (PSII), of acetyl CoA carboxylase (ACCase), and of acetolacate synthase (ALS). Both target site resistance (due to insensitive versions of the enzymes targeted by a herbicide) and enhanced detoxification/metabolism have been pinpointed as mechanisms causing reduction in susceptibility in this species. (Heap, 2010).

A. spica-venti is wind-pollinated, self-compatible and cross-pollinated, and a prolific seed producer. Studies (of allozyme characters) have revealed a high genetic variability within *A. spica-venti* populations, but very low variability between populations (Warwick et al., 1987).

3.3 *Alopecurus myosuroides* Huds.

English name: blackgrass, slender foxtail. Swedish name: renkavle

A. myosuroides is an annual grass native to Europe and the Mediterranean region. Today it is present in Europe, Asia, the Americas and Oceania (Häfliger & Scholz, 1981). *A. myosuroides* exhibits a predominantly winter annual germination pattern (Froud-Williams *et al.,* 1984). Like *A. spica-venti*, *A. myosuroides* is a grass weed in waste places and in rotation crops, and is especially favoured by cultivation of winter cereals (Naylor, 1972).

The distribution of *Alopecurus* as a serious agricultural weed in Sweden is mainly concentrated to the southwest part of Skåne. Field infestations have long been reported also on the island of Gotland, and recently, in the provinces of Västergötland and Östergötland. The species has increased in relative importance since the 1950s, when the introduction of selective chemical control of dicotyledonous weeds in cereals changed the weed flora composition in favour of grass weed species (Cousens & Mortimer, 1995). Its increase is also closely linked to the increase in winter cereal cultivation.

Resistance to herbicides constitutes a great problem in the control of *A. myosuroides*. Both targets site resistance and enhanced detoxification and metabolism are common, and the modes of action affected include inhibitors of photosystem II, ACCase, ALS and microtubule assembly (Heap, 2010).

A. myosuroides is allogamous and largely self-incompatible (Chauvel and Gasquez, 1994). The flowers are wind-pollinated and protogynous (Naylor, 1972). A similarity to *A. spica-venti* is the high level of genetic polymorphism present within populations, and the very low genetic differentiation among populations (Chauvel and Gasquez, 1994). This pattern has been explained as an effect of pollen flow typical of an out-crossing species (Hamrick & Godt, 1996). Propagation is solely by seeds and *A. myosuroides* has a moderate to high seed production, and forms a relatively short-lived seed bank (Moss 1983, 1985).

4 Materials and methods

4.1 Plant materials

4.1.1 Plant materials for investigations of genetic variation, phylogenetic relationships and variation in glyphosate susceptibility in *Elymus repens* (Papers I and II)

To study the genetic variation and phylogeny of *Elymus repens* in Sweden, 85 rhizome samples were collected throughout the country, from varying types of landscapes and habitats. The majority of samples came from cultivated land in three agriculturally important regions in southern Sweden; Skåne, Östergötland, and Uppland. Most samples were collected in 2003. Other plant materials included in the phylogenetic study were gene bank accessions of E. repens and of 18 related species, and samples of six commercial ley grass varieties for assessment of possible hybridisation events.

A subset of the Swedish *E. repens*-samples were used also in an experiment to estimate the range and distribution pattern of glyphosate susceptibility in *E. repens*, which is the main weed target in Swedish agriculture of this herbicide.

4.1.2 Sampling strategy for investigations of variation in dormancy and light sensitivity in *A. spica-venti* and *A. myosuroides* (Paper III)

The aim of the sampling strategy for seeds to use in studies of variation in dormancy and light sensitivity in *A. spica-venti* and *A. myosuroides* was to get seed collects from different field locations, but also seeds emanating from both autumn and spring emerged mother plants (by collection in crops sown in autumn and spring, respectively). For *A. myosuroides*, an additional intention was to collect seeds from the same site and crop, but that differed in maturation time, and thus had experienced different environmental conditions during ripening. In the case of *A. spica-venti,* seed maturation and shedding is typically very uniform for entire panicles, plants and indeed fields, so no such temporal separation was attempted. For each species, collections were made on six separate field sites, in total eight seed collects per species.

4.1.3 Sampling strategy for seed material to estimate range of variation in herbicide susceptibility in *A. spica-venti* and *A. myosuroides* (Papers IV and V)

For the two annual grasses studied, the focus was on populations in agricultural fields in southern Sweden.

To estimate the range of variation in *A. spica-venti* and *A. myosuroides* in susceptibility to one established and one newly introduced herbicide per species, seeds were collected in agricultural fields in Skåne, Östergötland and Öland (*Apera*), and in west Skåne (*Alopecurus*). Most samples were taken in 2002, and the objective was to obtain samples representative of the populations present on agricultural land in Sweden, rather than focusing on fields with hard-to-control populations with suspected resistance.

A true random choice of collection site was difficult to achieve in practice. Part of the *Apera*-collection sites in northeast Skåne was thus picked out from randomly assigned map points, but otherwise most of the sampled infestations were located by either contacting the local extension services and/or farmers directly, or by roadside surveys.

4.2 Molecular studies of genetic variation and phylogenetic relationships in *Elymus repens* (Paper I)

The methods used in the molecular studies of *E. repens* were amplified fragment length polymorphism (AFLP), and sequence-analysis. Differences in AFLP-results based on 126 loci for each of 85 *E. repens* samples were estimated by geographic location, landscape type, and between and within field samples.

To explore the genetic relationships, a comparison was done based on 139 AFLP-loci for a sub-set from the *E. repens* collection and for the related grass species. Sequence analysis was done for the nuclear gene phytochrome B, *phy*B, and for the chloroplast ribosomal protein encoding gene *rps*4, for a sub-set of the *E. repens* material and for the related grass species, and results compared to GenBank sequences.

4.3 Dormancy and light sensitivity experiments (Paper III)

The variation in seed dormancy and light sensitivity in Swedish populations of *A. spica-venti* and *A. myosuroides* was studied in three and four experiments respectively.

In experiment 1, variation in primary dormancy in the seed collects and the temperature effects of the process of after-ripening were studied. The primary dormancy was tested in vitro in light and in darkness.

In the second experiment, the focus was on seasonal variation in dormancy. Seeds were buried in soil in pots outdoors, exhumed on 16 occasions during 32 months, and germination tested in vitro in light, in darkness and after a 5-s exposure to light.

Experiment 3 was designed to study seasonal emergence and the effects of sol disturbance, which was recorded for seeds sown in pots outdoors.

In experiment 4, the light requirement of *A. myosuroides* seeds for germination was investigated by exposing temperature stratified seeds to photon irradiances ranging from 0.1 to 25 600 μmol m^{-2}, and recording the subsequent in vitro germination.

4.4 Glyphosate dose response experiment in *Elymus repens* (Paper II)

A total of 69 clones of Swedish *Elymus repens* were assessed in whole plant biosassays for their ability to regrow after glyphosate treatment. Of these clones, 46 had previously been examined for their genetic distance by AFLP (Paper I).

The magnitude and distribution of variation in susceptibility to glyphosate were estimated from a dose response study in pots. A single rhizome piece per clone was planted to produce large amounts of rhizome grown under similar conditions. In spring the year after collection, the rhizome was harvested, and one-node pieces of equal size were planted into 3-litre pots filled with potting soil (11 pieces per pot), and allowed to grow first in greenhouse, then outdoors. Herbicide treatments were done 13 weeks after replanting. Three days after treatment, the above-ground biomass was cut to stop further translocation, and the clones were allowed to regrow. The above-ground material was harvested per pot.

4.5 Herbicide dose response experiments in annual grass weeds (Papers IV and V)

The range of variation in susceptibility of Swedish populations of *A. spica-venti* and *A. myosuroides* to one established and one newly introduced herbicide per species was tested in dose-response experiments in a greenhouse setting.

Plant material for the herbicide dose-response experiments was generated by germination of seeds in vitro, planting of the resulting young seedlings to flat bowls with loamy sand low in organic matter, and allowing a growth period in greenhouse until plants were in the normal stage for each respective herbicide treatment. Visual assessment of each plant and individual harvest and determination of aboveground biomass were made when plants showed clear symptoms.

5 Results and Discussion

5.1 *Elymus repens* (Papers I and II)

- What are the patterns of genetic variability in the Swedish population of *E. repens*, with focus on three important agricultural regions in Sweden?
- What is the magnitude and pattern of variation in glyphosate susceptibility in *E. repens* in Sweden, in regards to genetic and geographic distance, and type of habitat?

5.1.1 Genetic variation (AFLP) in *Elymus repens*

The AFLP analysis of the genomes of 83 Swedish *E. repens* showed that the Swedish clone samples all belonged to one large and fairly homogenous group, without clear sub-groups. In the AMOVA analysis of the same set of 85 samples, a moderate genetic differentiation was found between clones originating from the three provinces Skåne, Östergötland and Uppland, but no differentiation due to landscape type was detected. There was also a moderate genetic differentiation when comparing 16 samples from different fields in Östergötland to 12 samples from one single field in the same province..

Similar results were found when analysing genetic and geographic distance based on the same 126 AFLP loci, but for the sub-set of 46 quackgrass clones that was also tested for glyphosate susceptibility. The covariance between geographic and genetic distance was apparent in the resulting cladogram and significant in a Mantel test comparing geographic and genetic dissimilarity.

From the combined data generated by the AFLP analysis and the sequence analysis of the phyB and rps4 genes, the Swedish population of *E.*

repens appeared to be slightly heterogeneous and to contain multiple origins of genome donors.

The genetic variation revealed by the AFLP-markers in Swedish *E. repens* was moderate, and related to its north-to southward distribution and to the regions in which the samples had been collected. The lack of distinct groups and the low diversity among populations is in line with expectations for out-crossing species in general (Hamrick & Godt, 1996). A degree of heterogeneity along a geographic gradient is rather to be expected, and can be due to mutations during a longer time period since introduction, or to multiple and also more recent introductions. In the case of *E. repens*, it cannot be ruled out that new genomic material may have been introduced by hybridisation events. *E. repens* is an allopolyploid species, with ability to exchange genomic material with wild relatives (Mahelka *et al* 2007). A likelier source of new introduction of variation is perhaps *E. repens* seeds as contaminants of cereal seeds lots (McCarthy & Culleton; 1987; Tonkin & Phillipson, 1973; Tonkin, 1987).

As for genetic variation within fields, this was not the main focus of the study but a limited comparison was made. The variation in AFLP-results on the within-field and among-field scales was compared for 12 samples from a single field and for 16 samples originating from different fields in the region in question. This comparison showed that more than one clone was present in the single field, that none of the clones had an identical marker composition in AFLP, and that the within-field variation differed from the among-field variation. Vegetative spread via rhizomes probably explains a large part of the homogeneity in the within-field population data.

In our study, we were not able to link differences in AFLP markers to landscape type or type of habitat. Possibly more narrowly defined habitat types and a more spatially structured sampling of habitats could have given a different result. In *Cirsium arvense,* also a clonal, perennial species, and with high morphological plasticity (Hettwer & Gerowitt, 2004), genetic differentiation in AFLP markers was found among habitat types, including different cropping systems, in a recent study (Bommarco *et al.*, 2010).

It has been argued that a wide ecological amplitude and indeed evolutionary success of allopolyploid species is increased by the introduction of genetic variability of multiple origins (Meimberg *et al.*, 2009) Thus the allopolyploid nature of *E. repens* may play an important role in a survival and persistence strategy based on adaptive traits, rather than on adaptation through variation and subsequent selection of the off-spring.

5.1.2 Variation in glyphosate response

The dose-response pot experiment with glyphosate revealed a large variation in susceptibility among clones, with ED50-values ranging from 17 to 278 g a.i./ha. Mantel tests showed however no covariance between ED50 and either of the factors genetic and geographic distance. Nor did ED50 vary consistently with the four categories of habitats (arable land in intensive or extensive use, field vicinities, and "other", including natural habitats and ruderal sites).

The range of glyphosate susceptibility in the 69 clones included in our dose-response experiment was more than tenfold, even after eliminating the two highest and two lowest values (which had the largest uncertainties in their estimates) from the datasets. It should be noted that the tested dose range was selected to give differential responses in the pot experiment setting, and that the resulting susceptibility range cannot be directly translated to treatment under field conditions.

It was not possible to detect any pattern indicating effects of a strong selection pressure, and/or of spreading resistance mutations in the variation in glyphosate susceptibility in our experiment. Neither geographic nor genetic distance (as measured by AFLP) could be linked to the variation in ED50-values in Mantel tests. There was no evident differentiation in ED50 due to habitat type. Had there been a strong selection pressure, it could have been visible as a link between glyphosate susceptibility and geographic distance, and especially to habitat types. In the case of individual resistance mutation events spreading by vegetative propagation or by sexual recombination, this could have been detectable as covariation with genetic distance (AFLP).

E. repens is the key weed target for glyphosate application, and considering the dominant role of glyphosate in share of herbicide sales in Sweden, it would not have been surprising to see some effects of selection pressure. As was pointed out in the discussion of the pattern of genetic variation in AFLP-markers, a more carefully structured design could possibly have given another results, but studying the results on individual clones, it was notable that among the most tolerant clones were several from sites without any recorded (or probable) history of glyphosate use. Although glyphosate has dominated the control of *E repens* in Sweden for a considerable time period, on the vast majority of agricultural locations, it has been used at 3-6 year intervals. This does not constitute a great selective pressure of a herbicide lacking persistent residual activity. In contrast, the glyphosate use patterns against species in which glyphosate resistance has evolved in the field has been intensive, sometimes with several application per year in plantations, or in some cases, used as a selective herbicide in glyphosate tolerant GM-crops. It is however noteworthy that glyphosate use patterns are changing in Swedish cropping systems and that more frequent applications in individual fields are now practiced at some farms.

5.2 *A. spica-venti* and *A. myosuroides*: Dormancy and light sensitivity (Paper III)

- To what extent are *A. myosuroides* and *A. spica-venti* restricted to a winter annual germination behaviour?
- What is the magnitude of variation in dormancy among populations of the respective species? Is it of a size large enough to be of agronomical consequence?
- To what degree do populations vary in how emergence is affected by temperature, light and soil disturbance?

5.2.1 *A. myosuroides* seed dormancy

The primary dormancy exhibited in *A. myosuroides* in our study (Expt 1) was fairly strong. There was no overall significant difference in initial germination between seed collects, but a significant interaction between light and seed collect. The initial germination range in the collects was 15-73% in light but less than 4% in darkness for all collects. Seed samples collected in the same site and crop, but at different times and from different parts of the panicle differed significantly in primary dormancy, with the early maturing seeds germinating to higher percentages.

As for seasonal dormancy changes, *Alopecurus* seeds germinated mainly in autumn, probably due to the greater light sensitivity at this time. Cold desensitized the seeds and rendered them more dependent on light for germination in spring. This greater light requirement in spring was seen in Expt 2, where exhumed seeds germinated to high percentages in full light all year, but a seasonal pattern was apparent when seeds were tested in germination conditions with short or no light exposures. A considerably higher light requirement in seeds that had experienced a cold versus a warm stratification before the germination test was also evident in Expt 4.

5.2.2 *A. spica-venti* seed dormancy

In *Apera*, the primary dormancy was stronger than in *Alopecurus*, and the seed collects differed significantly with initial germination ranging from 1-36%. Both temperature and light in the germination test interacted with seed collects.

The seasonal dormancy was also stronger in *Apera* than in *Alopecurus* in our study. In Expt 2, seeds had near zero germination if exhumed in winter or spring, regardless of light conditions. The strongest emergence peak in the experiment on seasonal emergence (Expt 3) took place in the first autumn. In spring, emergence was near zero, and a small emergence peak followed in the next autumn. This pattern was similar in all populations.

5.2.3 Phenotype or genotype?

Although our experimental design did not permit any definite conclusions to be drawn on whether the variation recorded was due to phenotypic effects or to different genotypes among and within the field populations, it seems most likely, based on the literature, that the apparent variation found between samples from different collection times on the same site in *Alopecurus* was due to environmental conditions affecting seed maturation. Weather conditions pre-and post-dispersal have been reported to be linked to dormancy characteristics (Strand, 1989; Fenner, 1991; Probert *et al*).

On the other hand, as seed dormancy is a life history trait that could potentially have a large influence on the persistence of a species in a particular field, it is not impossible that adaptation could have a role in the demonstrated differences among populations from different sites. Genetically determined components of seed germination and after-ripening patterns have been suggested in more extreme, but predictable environments, while results from more favourable environments suggested a greater role for phenotypic plasticity in the grass *Bromus tectorum* (Beckstead *et al*, 1996).

5.3 Resistance to isoproturon in *A. spica-venti* (Paper IV)

- What is the size and distribution of variation in susceptibility to isoproturon in Swedish *A. spica-venti*?
- How does the susceptibility to isoproturon vary in Swedish *A. spica-venti,* at the population level and at the level of individual plants, compared to that in European reference populations, previously characterised as susceptible or resistant?

5.3.1 Range of susceptibility among populations

With the analyses chosen, that strictly followed the design, GR50 turned out to be a very blunt instrument. Therefore, only LD50 will be considered here. The estimated LD50-values for isoproturon in the Swedish *A. spica-venti* populations varied from 234 to 1147 g a.i./ha, and the null hypothesis of a shared log LD50 among the Swedish populations was rejected.

5.3.2 Comparisons to reference populations

In this experiment, three reference samples were included: one with normal susceptibility, and two references with varying degrees of resistance. The dose range used, however, turned out to narrow for the most resistant one. Of 60 tested Swedish populations, 11 populations had LD50 estimates significantly higher (at the 5% level) than the normally susceptible reference sample. The most tolerant Swedish population was almost five times more tolerant to isoproturon than the susceptible reference as judged by the resistance factor (RF = 4.7), thus approaching the resistance level of the moderately resistant reference (RF=5.6).

An effect of describing resistance levels in the above way is that the choice of susceptible reference will greatly influence the conclusions drawn. A self-evident requirement is that the reference should have been shown to be well controlled by a normally recommended field rate of the herbicide. The question then arises of just how susceptible the reference is, in comparison to other populations that are also satisfactorily controlled by recommended dose rates. It has therefore been suggested (Heap, 2005) to base resistance factors instead on the average of a number of populations. In our study on isoproturon susceptibility, the most tolerant Swedish sample would then have an RF of 2.4.

The obvious difficulties in detecting early resistance evolution (especially when the resistance mechanisms present confer low level resistance) have been discussed by several authors. Not only is it difficult to note at the farmer level. Studying field trial results from the regions in the sampling year, the match between greenhouse results and field results for the same population varied considerably (data not shown), which is not uncommon especially for herbicides with soil activity (Moss, 1995).

5.3.3 Detection of within-population variation

Another difficulty in early detection is how to perform statistically valid analyses of field collected populations, where typically a mix of varying frequency of susceptible and less susceptible genotypes will be present. The Coefficient of Variance (CV) may be used as a measure of within-population variation in GR50 (growth reduction to 50% of untreated plants), and was generally large in our isoproturon study, indicating presence of individuals with highly variable degrees of susceptibility within the same samples. As a large CV-value will however represent many sources of uncontrolled variation in an experiment, individual plant observations are an advisable complement to population level studies.

Looking at the results at the individual plant level in our study of *Apera* and isoproturon, it is clear that occurrence also of plants with high tolerance to the tested herbicide may go undetected in analyses based on 50%-reduction values (GR50, LD50), A sample from Rystad in Östergötland contained plants which were not reduced in size by any of the tested doses, in which they responded in a manner more resembling the highly resistant reference, while such near unaffected plants did not occur in the moderately resistant reference. Nevertheless, the LD50-value of Rystad was not significantly different from the susceptible reference sample in the study (P=0.0916).

5.3.4 Isoproturon resistance levels

Although our conclusion is that isoproturon resistance is present in Swedish *Apera* populations, the levels are still moderate in comparison to results from other countries where this weed is abundant. One possible explanation is of course that selection pressure may not have been as intense as elsewhere. Pesticide statistics (where existing) are however typically based on sales rather than on usage, and reliable data for such comparisons are thus not readily available. It is also worth noting that we sampled more or less random populations, while other reports may be based on a sample biased towards cases with suspected resistance.

To some degree the moderate levels could be due to an untypically small sampling bias for resistance in our collection year. Field samples are commonly biased in herbicide susceptibility, as collection is often made after spraying, when the more sensitive part of the emerged populations that year have been killed, or at least hampered in their seed set. For early detection of field evolution of resistant populations, this can be an advantage as resistant individuals will have higher frequencies. In our study, however, there were no differences in logLD50 between samples taken in isoproturon-treated crops versus those from unsprayed fields. In fact, the crop year in which the samples were collected, conditions for autumn treatments had been difficult, and many of the populations were not spring treated. Hence, it can be assumed that the resulting samples included a larger part of susceptible plants and less bias towards high tolerance.

Taken together the results suggest that isoproturon resistance of a magnitude of practical importance is present in Swedish *Apera spica-venti*.

5.4 *A. spica-venti* and *A. myosuroides*: Herbicide susceptibility to new and established herbicides (Paper V)

- What is the intra-specific variation in herbicide susceptibility in Swedish field samples of *A. myosuroides* and *A. spica-venti* to the established herbicides fenoxaprop-P-ethyl and isoproturon, respectively?
- What range of LD50 and GR50 estimates for susceptibility to recently introduced sulfonylurea *herbicides (her*e fl*upyrsulfuron and* sulfosulfuron) can be expected in populations of annual grass weeds with little or no prior exposure to sulfonylurea graminicides?
- Is there a correlation in the response to the established and the recently introduced herbicides (isoproturon vs. sulfosulfuron and fenoxaprop vs. flupyrsulfuron)?

5.4.1 Range and shape of GR50 and LD50-estimates

The ranges of GR50 and LD50 estimates for the four herbicides included in this study are summarised in Table 2.

Table 2. Grenhouse dose-response experiments. Recommended and tested herbicide doses. Estimated GR50- and LD50-values for field collected populations of two grass weed species.

Species Herbicide active ingredient (time of introduction)	Recommended field rate (g a.i. ha^{-1})	Doses tested (g a.i. ha^{-1})	GR50-estimates (g a.i. ha^{-1})	LD50-estimates (g a.i. ha^{-1})
Apera spica-venti				
isoproturon (1970s)	1250-1500	28-1380	229-753	100-450
sulfosulfuron (1999)	20-25	2.2-30.0	1.8-8.9	1.6-12.2
Alopecurus myosuroides				
fenoxaprop-P-ethyl (1992)	56-70	22.4-364	7.8-10865	15-102031
flupyrsulfuron-methyl-Na (2000)	10	1.5-40.0	1.66-6.43	3.0-87

In three of the eight cases, there were indications that the sample of populations deviated from normality (logGR50 *Apera*/sulfosulfuron and both logGR50 and logLD50 for *Alopecurus*/fenoxaprop-P-ethyl). When considering skewness, i.e. degree of asymmetry (if clearly different from 0, then asymmetrical) and degree of kurtosis, i.e. peakiness of data (if clearly different than 0, then the distribution is either flatter or more peaked than expected from a normal distribution), these three cases also stand out as non-normal, together with logLD50 for *Apera*/sulfosulfuron. Hence, one of the old and one of the new herbicides generated non-normal distributions of logGR50 and logLD50. Non-normality is thus not necessarily an indicator of past selection pressure.

The possibilities of using ranges and shapes of distributions of herbicide susceptibility estimates in detecting early signs of resistance will perhaps be greater in future, when – hopefully – changes in ranges and patterns over time can be compared, rather than just studying the patterns at a given time. Nowadays, it is a requirement for new registration and re-registration of herbicides within the European Union to provide a resistance risk assessment (Kalamarakis & Markellou, 2007; EPPO, 2004), in which so called base line sensitivity data may constitute one component, especially for high-risk organisms (Heimbach et al, 2002). Guidelines for industry on how to obtain such data have been provided (EPPO 2004). An important aspect in this context is that in species with many resistance cases, cross resistance risks should be evaluated for the new compounds (Moss, 2001; EPPO 2004).

5.4.2 Cross-correlation

For *Alopecurus*, there was a positive correlation between the logGR50 and logLD50 for fenoxaprop-P-ethyl and flupyrsulfuron (P=0.00026 and 0.0664, respectively). No correlation was found in the *Apera* populations between susceptibility to isoproturon and sulfosulfuron.

These findings are in line with literature on cross-resistance patterns for the concerned herbicides. Enhanced metabolism as a probable cause of cross-resistance between several herbicides, including fenoxaprop and flupyrsulfuron, has been reported in *Alopecurus myosuroides* (reviewed by Marshal, 2008) while resistance cases in *Apera* to sulfosulfuron have instead been linked to target site resistance (Balgheim *et al*, 2007).

From literature, it is thus likely that the correlation of susceptibility between fenoxaprop and flupyrsulfuron in the Swedish *Alopecurus* populations could be due to the same metabolism-enhancing genes, though with less efficiency so far in detoxifying the flupyrsulfuron.

The hypothesis that the low susceptibility to fenoxaprop in Swedish populations is typically caused by metabolic resistance was further supported by the cross resistance patterns in an in vitro seed germination assay. In this assay, where herbicides are added in the germination water (Moss, 1999) the Swedish material was compared to a susceptible reference sample, and to a resistant reference, Peldon 2000. *A. myosuroides* populations originating from the UK site Peldon have to date been reported to have metabolic resistance affecting six different herbicidal modes of action (Marshal, 2008), and including resistance to fenoxaprop-P-ehtyl and pendimethalin (a microtubule assembly inhibitor) (Hall et al, 1997; Moss, 1990). Target site resistance to the ACCase-inhibitors fenoxaprop, sethoxidim, and cycloxidim was indicated only in one of the Swedish samples (data not shown). Analysis by selective PCR (polymerase chain reaction) (Delye *et al,* 2002) confirmed presence of the mutation Ile 1781 (chloroplastic ACCase, nucleotide position 5341), the most commonly reported point mutation (Délye *et al*, 2007) to confer the target site resistance pattern we found in the seed assay in this one Swedish population (data not shown).

For all other Swedish populations with high tolerance to fenoxaprop, there was no cross-resistance with the tested ACCase-inhibitors in the seed assay. Plants from three of the Swedish populations with low fenoxaprop-susceptibility were selected in vitro with fenoxaprop and found to lack the Ile 1781-mutation, as did plants from one fenoxaprop-susceptible Swedish sample (data not shown). Metabolic resistance thus remained a plausible mechanism for the majority of the Swedish *Alopecurus* populations with low fenoxaprop-susceptibility.

In addition, in the population that scored the highest for tolerance to flupyrsulfuron in our green-house dose-response study, a small proportion of survivors were found in the pendimethalin treatment in the seed assay (data not shown). This treatment was included as an indicator of one variant of enhanced metabolism resistance. This finding was not further investigated, but was in line with the hypothesis of enhanced metabolism causing cross-resistance in the Swedish populations. On the practical scale, a field survey conducted in west Skåne in 2009 (Jordbruksverket, 2010a) identified *Alopecurus* populations in which resistance to fenoxaprop co-occurred with reduced susceptibility to flupyrsulfuron. Also samples with reduced susceptibility to prosulfokarb and to fenoxaprop/cycloxidim were found. These results indicate that there is continuing field evolution of populations with enhanced metabolism in Swedish *Alopecurus myosuroides,* in parallel with new target site resistance cases being discovered.

6 Main Conclusions and Agronomical Implications

The variation in susceptibility to all tested herbicides was large, both between populations and within the field-collected seed samples. It was not possible to distinguish between pre-existing variation and evolving resistance based solely on the size of the range of variation and on whether susceptibility estimates were normally distributed.

There were strong indications of field evolved resistance at a scale of practical importance to the long-established grass herbicides isoproturon in *Apera spica-venti*, and fenoxaprop-P-ethyl in *Alopecurus myosuroides*. The significant correlation in the responses to fenoxaprop-P-ethyl and flupyrsulfuron-Na in *A. myosuroides*, together with cross resistance patterns in a seed assay (unpublished data), indicates that enhanced metabolism is the most common mechanism behind low herbicide susceptibility in Swedish *A. myosuroides*, and may affect different mode of action groups.

Taken together, there is an obvious and urgent need for good resistance strategies, not including just shifting between modes of action but also other measures. As the increase and success of these annual species is largely due to the increase in winter cereal cultivation and reduced soil tillage, obvious counter-strategies are the inclusion of other crops and timely soil tillage – preferably before and not after great resistance problems are encountered at the farm. The indication of enhanced metabolism being the most common resistance mechanism at least in *A. myosuroides* should be seen as a warning against a continuous practice of dose reduction.

Primary and seasonal seed dormancy varied significantly between seed collects of both *A. spica-venti* and *A. myosuroides*, but a winter annual germination pattern dominated in all samples. For *A. myosuroides,* variation in dormancy was linked to light sensitivity. In spring, the light requirement of this species for germination was increased by cold temperatures and emergence was low even after soil disturbance. Depletion of the soil seed bank by tillage in spring time will thus not be a successful strategy.

Glyphosate dose-response in *E. repens* varied greatly between clones, but was not linked to different types of habitat or to genetic or geographic distance. Thus it could not be shown that the variation was due to past selection pressure.

The genetic variation found in Swedish *E. repens* was low, but no samples had an identical result in the AFLP analysis. It can thus be assumed that even though a lot of the within-field spread is probably due to farm machinery moving rhizome fragments of the same clonal material across fields, there is genetic variation present within fields, and thus potential for selection by weed management strategies, including herbicide treatments.

The use patterns of glyphosate are changing towards more frequent applications on many farms, and as it can be very difficult to separate effects of sub-optimal treatment conditions from effects of low susceptibility in the clones, caution is called for.

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