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Investigations on herbicide resistance in *Apera spica-venti* populations

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Chapter I

General Introduction

1. General Introduction

According to common definitions (Blatchley, 1912; Rademacher, 1948), weeds are ‘plants out of place’ or ‘growing where it is not wanted’. Other definitions describe weeds as ‘domesticated wild plants’ or ‘plants whose virtue has yet to be discovered’ (Emerson, 1878).

Assuming the correctness of all these different definitions, it follows that many plant species can become weeds under certain circumstances. In a more holistic view, every plant that ‘interferes with the intended use of land and water resources’ can be considered a weed (Molinar, 2002).

However, weeds are also identifiable by specific characteristics that differentiate them from other plants (Molinar, 2002). In fact, peculiar biological traits such as seed dormancy, germination and emergence over long time periods, long-term survival of buried seeds, abundant seed production, rapid population establishment, capacity to colonize new sites (including anthropized ecosystems), and multiple adaptations for spread, can confer weeds evolutionary advantages over cultural plants (Molinar, 2002).

Weeds are unanimously considered one of the most costly and limiting factors in crop production. Losses caused by weeds primarily derive from direct competition with crop plants for water, nutrients, light and space, which results in significant yield reductions (Roszkopf *et al.*, 1999). Further problems associated with weeds are the reduction of food, feed and fiber quality as well as the high costs associated with harvesting operations and soil preparation (Roszkopf *et al.*, 1999). In addition, some weed species are prone to cause strong allergies (e.g. hay allergies) and skin dermatitis in sensitive individuals (Molinar, 2002). Besides these direct implications, weeds can also serve as alternate hosts to insect pests and pathogens, often resulting in additional operating costs and increased risk of diseases (Wisler & Norris, 2005).

Intensive cropping systems are based on a strong reliance on herbicides, which belong to the most reliable and cost-effective tools for weed control (Powles & Shaner, 2001). Herbicides are compounds used to kill unwanted plants, mainly by inhibiting the activity of specific target enzymes associated with the catalysis of biosynthetic processes that are essential for plant growth (Powles & Yu, 2010). Over the past five decades, chemical weed control through the extensive use of herbicides has represented the main weed management option in developed countries (Wyse, 1992; Roszkopf *et al.*, 1999). However, repetitive long-term use of herbicides with the same mode of action, combined with the global changes in modern agricultural assets (e.g. intensive cropping systems, monoculture, short rotations etc.), has imposed selection for resistance in previously susceptible weed species and populations (Holt, 1992; De

Prado *et al.*, 1997). The application of herbicides to large weed communities characterized by a high rate of genetic diversity exerts a strong selection pressure that may promote the evolution of resistance (Maxwell & Mortimer, 1994; Powles & Shaner, 2001).

The evolution of resistance in weed populations had already been predicted by Blackman (1950) and Harper (1956) shortly after the appearance of herbicides in the global market (Grignac, 1978). In accordance with these correct predictions, the first case of resistance to herbicides inhibiting the electron transport in photosystem II (PSII-inhibitors) was reported in USA by Ryan (1970) and Radosevich & Appleby (1973) for some biotypes of *Senecio vulgaris* L. and *Amaranthus retroflexus* L..

Since then, further cases reporting resistance to PSII-inhibitors have been documented in several weed species [e.g. *Capsella bursa-pastoris* L., *Chenopodium album* L., *Setaria viridis* L., *Echinochloa crus-galli* L., *Alopecurus myosuroides* Huds. (Grignac, 1978)]. The quick spread of resistance to PSII-inhibitors occurred in consequence of repetitive treatments with this mode of action and the high residual activity of these compounds, which resulted in the selection for resistant biotypes. Nowadays, 68 weed species (49 dicotyles and 19 monocotyles) have evolved resistance to PSII-inhibitors, mostly in the USA, Canada and Europe (Heap, 2011).

Over the last three decades, increasing numbers of weed species with biotypes resistant to other herbicide modes of action have been reported (Holt, 1992). In 1978, herbicides inhibiting the activity of acetyl-CoA carboxylase (a key enzyme in the biosynthesis of fatty acids in monocotyle plants) made their first appearance in the market (Hock *et al.*, 1995). Only four years later, in 1982, resistance to acetyl-CoA carboxylase (ACCase)-inhibitors was reported in Australia for *Lolium rigidum* Gaud. biotypes (Heap & Knight, 1982; Powles & Howat, 1990) and in England for *Alopecurus myosuroides* Huds. biotypes (Moss & Clarke, 1994). Three years later, in 1985, resistance to ACCase-inhibitors was documented also in *Avena fatua* L. biotypes (Powles & Howat, 1990).

At the time of writing, 39 grass weed species worldwide have evolved resistance to this group of herbicides, with *Lolium* spp. and *Avena* spp. being mostly spread in Australia, USA and Canada and *Alopecurus* spp. in Europe and China (Heap, 2011).

At the beginning of the 1980's, a new group of herbicides inhibiting the activity of the acetolactate synthase (ALS) [the first enzyme involved in the biosynthetic pathway of branched-chain amino acids (Durner *et al.*, 1990)] was launched into the market for the control of broad-leaved and grass weeds. These herbicides, known as ALS-inhibitors (or AHAS-inhibitors, from acetohydroxyacid synthase), have enjoyed increasing popularity among farmers

worldwide and have been extensively used due to their high efficacy at low rates, low impact on non-target organisms (including mammals), low residual activity and persistence and high selectivity in several crops (Moss & Cussans, 1991; Heap, 1997).

Despite this, extensive usage of ALS-inhibitors in arable lands on a global scale, together with the proneness of weeds to evolve resistance to this mode of action, has resulted in the selection for resistant biotypes in over 100 weed species (107: 66 dicotyles and 41 monocotyles) (Heap, 1997; Heap, 2011). This makes resistance to ALS-inhibitors the most relevant resistance phenomenon actually documented worldwide.

Shortly after the appearance of ALS-inhibitors in the global market, the first cases of resistance were reported in Australia for *Lolium rigidum* Gaud. biotypes, which were also cross-resistant to ACCase-inhibitors (Llewellyn & Powles, 2001). In the United Kingdom, the first case of resistance to ALS-inhibitors was reported for *Alopecurus myosuroides* Huds. biotypes already in the year 1982 (Brown & Cotterman, 1994).

In 1986, resistance to ALS-inhibitors was documented in South Africa in *Avena fatua* L. biotypes which were also cross-resistant to ACCase-inhibitors (Cairns & Hugo, 1986) and in 1987 cases were reported in USA for *Kochia scoparia* L. Schrad. and *Lactuca serriola* L. biotypes (Reed *et al.*, 1989; Primiani *et al.*, 1990; Mallory-Smith *et al.*, 1990). In 1988 and 1989, resistance to ALS-inhibitors was reported in Canada for *Stellaria media* L. Vill. (O'Donovan *et al.*, 1994) and *Salsola iberica* Sennen & Pau biotypes (Saari *et al.*, 1992; Morrison & Devine, 1994) and in USA for *Lolium perenne* L. biotypes (Saari *et al.*, 1992).

Since the beginning of the resistance selection process, the number of reports on herbicide-resistant weeds has constantly increased until today. Furthermore, the introduction of more selective herbicides (inhibiting specific target sites essential in plant metabolism) for targeted weed control and crop injury reduction increased the selective force in favour of resistant individuals (Heap, 1997). At present time, 197 weed species (115 dicotyles and 82 monocotyles) and 352 biotypes across 420,000 fields worldwide have evolved resistance to different herbicide modes of action (Heap, 2011) (Fig. 1.1).

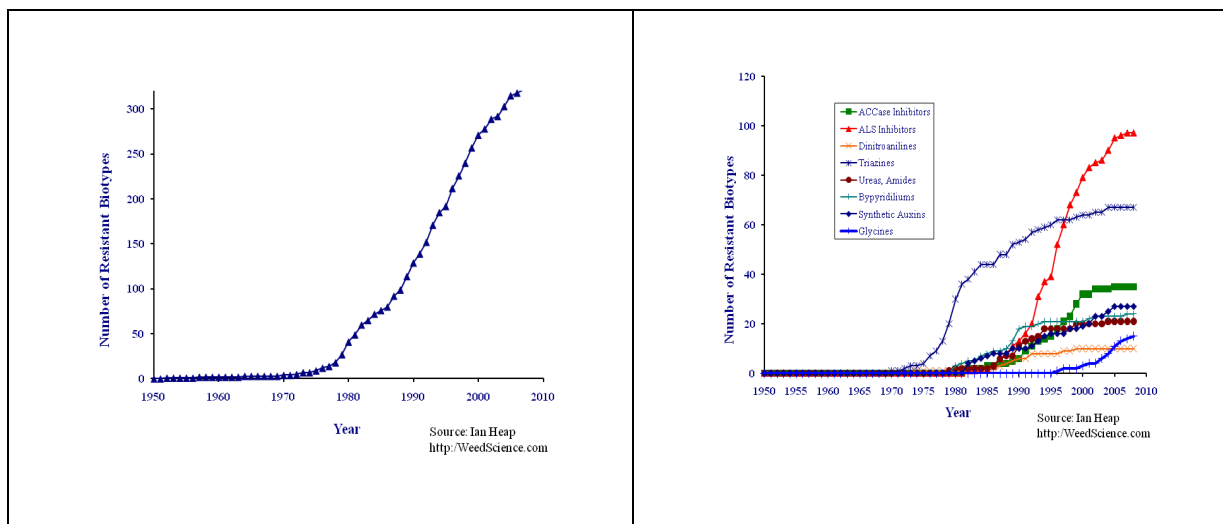


Fig. 1.1: Evolution of herbicide-resistant weed biotypes by year (left) and by mode of action (right), from 1950 until 2010. Since the beginning of the 1980's, a significant increase in herbicide-resistant weed biotypes has occurred (Source: Heap, 2011).

In Europe, at least 52 weed species (35 dicotyles and 17 monocotyles) from various botanical families have evolved resistance to different herbicide modes of action as well (De Prado *et al.*, 1997) (Fig. 1.2).

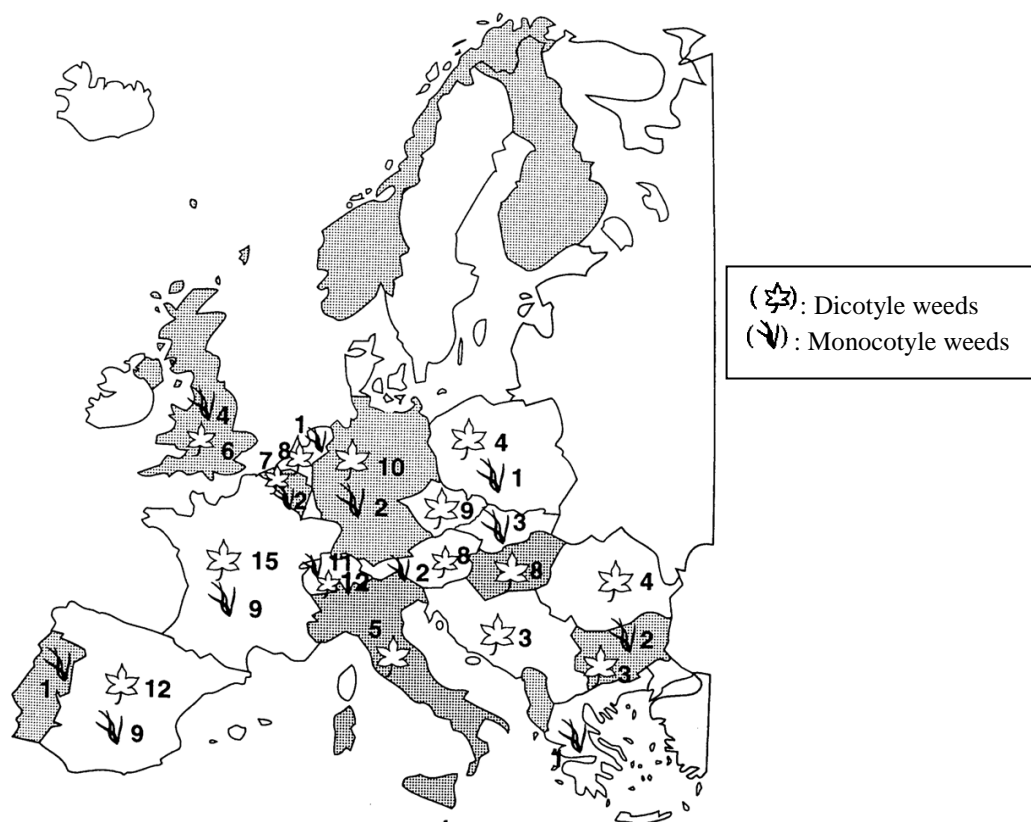


Fig. 1.2: Spatial distribution of herbicide-resistant weeds in Europe (Source: De Prado *et al.*, 1997).

1.1 Herbicide resistance

1.1.1 Definitions of herbicide resistance

According to the definition given by Heap and LeBaron (2001), herbicide resistance is ‘the evolved capacity for a previously herbicide-susceptible weed population to withstand a herbicide and complete its life cycle when the herbicide is used at its normal rate in an agricultural situation’. The Herbicide Resistance Action Committee (HRAC) defines herbicide resistance as ‘the naturally occurring inheritable ability of some weed biotypes within a given weed population to survive a herbicide treatment that would, under normal use conditions, effectively control that weed population. Selection of resistant biotypes may result in control failures’. A third definition, provided by the European and Mediterranean Plant Protection Organization (OEPP/EPPO), describes herbicide resistance as ‘the naturally occurring, inheritable adjustment in the ability of individuals in a population to survive a plant protection product treatment that would normally give effective control’.

More specifically, EPPO guidelines introduce an interesting distinction between resistance that can be verified at the laboratory level and resistance observed in the field situation, which is referred to as ‘practical resistance’ and defined as ‘the loss of field control due to a shift in sensitivity’. According to this distinction, the detection of herbicide resistance at the laboratory level does not necessarily imply that pest control in the field is reduced (EPPO, 1988). In fact, selectivity is the key requirement for actual resistance, in absence of which a population or an individual can be only defined as ‘tolerant’. Tolerance, unlike resistance, is defined as ‘the intrinsic, natural ability of a species or a plant to survive and reproduce after herbicide treatment, without any selection or genetic manipulation’ (Weed Science Society of America, WSSA).

1.1.2 Types of herbicide resistance

Herbicide resistance is an evolutionary process which strongly depends on genetic factors (frequency and number of resistant genes, mechanisms of inheritance, fitness costs associated with resistant alleles), weed species (self- or cross-pollination, pollen movement, seed production, dispersion and longevity), herbicide (chemistry, mode of action, residual activity) and operational factors (herbicide dose, environmental variables) (Powles & Yu, 2010). The different combinations of these factors can result in the selection of specific resistance genes within a given weed population (Powles & Yu, 2010).

In general terms, weed populations have the ability to evolve resistance to one or more herbicide active ingredients. One way of classifying herbicide resistance is based on mode of action, represented by the biochemical mechanism by which an active ingredient affects the target plant (Ross & Childs, 2011). When resistance to more than one class of active ingredients (e.g. sulfonylureas and sulfonylaminocarbonyltriazolinones) within the same herbicide group (e.g. ALS-inhibitors) is observed, this phenomenon is referred to as ‘cross resistance’ (Mallory-Smith & Namuth, 2011). On the other hand, resistance to more than one class of active ingredients (e.g. sulfonylureas and aryloxyphenoxypropionates) belonging to different herbicide groups (e.g. ALS-inhibitors and ACCase-inhibitors) is defined as ‘multiple resistance’ (Mallory-Smith & Namuth, 2011).

1.2 Mechanisms of herbicide resistance

1.2.1 Target site resistance

Another way of classifying resistance is based on intrinsic modifications occurring in resistant plants as compared with wild type plants. According to this classification, herbicide resistance can be generally divided into target site and non-target site resistance (Heap & LeBaron, 2001; Vila-Aiub *et al.*, 2009).

Target site resistance mechanisms consist of modifications occurring within the target site (e.g. a specific enzyme) that limits herbicide impact, albeit the target is reached at a lethal dose (Powles & Yu, 2010). Changes in target site sensitivity are conferred by point mutations (Single Nucleotide Polymorphisms, SNPs) at single major genes coding for the target sites; this results in altered proteins that prevent or reduce proper herbicide binding. Inheritance is determined by a single gene, is mainly a semi-dominant, nuclear trait and derives from nucleotide changes at specific amino acid positions within discrete gene domains (Balgheim *et al.*, 2007). This type of resistance mechanism can be also associated with negative pleiotropic effects on plant fitness in the original environment (Vila-Aiub *et al.*, 2009). This phenomenon, defined as ‘cost of adaptation’ (Purrington, 2000; Strauss *et al.*, 2002; Vila-Aiub *et al.*, 2009), can result in a fitness reduction that hampers the fixation of novel adaptive alleles (e.g. resistance-endowing alleles) and at the same time contributes to the conservation of genetic diversity in natural populations (Antonovics & Thrall, 1994; Tian *et al.*, 2003; Vila-Aiub *et al.*, 2009). Nevertheless, not all resistance-endowing alleles have a significant impact on plant fitness, as this seems to be also dependent on the position of the mutation within the gene and the substituted amino acid (Duggleby & Pang, 2000).

Target site resistance mechanisms account for the major part of documented field-evolved herbicide resistance (Jasieniuk *et al.*, 1996; Tranel & Wright, 2002). It has been demonstrated that mutant individuals carrying resistance-endowing alleles already exist within unselected sensitive weed populations before herbicides are applied. Spontaneous rates of ALS gene mutations in *Arabidopsis thaliana* Heynh. were estimated to be of the order of 10^{-9} (Haughn & Somerville, 1987). Studies conducted by Gressel & Segel (1978) and Diggle *et al.* (2003) on the evolution of herbicide resistance in the field estimated initial major resistance allele frequencies in herbicide-susceptible weed populations ranging between 10^{-7} and 10^{-5} . Preston & Powles (2002) reported initial major resistance allele frequencies ranging from 1×10^{-5} to 1.2×10^{-7} for target site ALS-resistant mutants in three unselected *Lolium rigidum* Gaud. populations. According to these estimations, a gene mutation rate of 10^{-7} in a population consisting of 10^6 individuals ha^{-1} would result in 10 mutant individuals 100 ha^{-1} carrying resistance-endowing alleles (Maxwell & Mortimer, 1994), which already represents a potential risk of resistance evolution under herbicide selection pressure (sensitive plants are easily removed by herbicide treatment while resistant individuals are steadily selected).

1.2.2 Non-target site resistance

Non-target site resistance is associated with physiological mechanisms aimed at reducing the amount of herbicide reaching the target site (Powles & Yu, 2010). These mechanisms mainly consist of decreased rate of herbicide penetration into the plant, decreased rate of herbicide translocation and increased rate of herbicide sequestration or metabolism (Hall *et al.*, 1997; Powles & Yu, 2010). Enhanced metabolization of herbicidal compounds accounts for the majority of non-target site resistance cases. Although several enzymatic complexes are known to be involved in non-target site resistance, cytochrome P450 monooxygenases, glutathione-S-transferases (GSTs) and aryl acylamidases have the highest relevance (Cole, 1994). In particular, cytochrome P450 monooxygenases are mostly responsible for the increased metabolism of xenobiotics (e.g. herbicides) in plants (Sandermann, 1992). Cytochrome P450 monooxygenases (P450s) are oxidoreducing enzymes that are bound to the endoplasmic reticulum or the plastid membranes (Powles & Yu, 2010). These enzymes are actively involved in several metabolic processes, such as the synthesis of hormones, sterols and fatty acid derivatives (e.g. suberine) (Hatzios, 1991; Bolwell *et al.*, 1994). Their active role in herbicide degradation mainly consists of hydroxylation or dealkylation reactions (Powles & Yu, 2010), by which an atom from molecular oxygen is incorporated into a substrate (with the formation of a more reactive compound) in presence of a cofactor (e.g. NADPH) and the second oxygen

atom is converted to water (Bolwell *et al.*, 1994; Siminszky *et al.*, 1999). The hydroxylation reaction of a given substrate (e.g. herbicide) is often followed by conjugation to glucose and sequestration into the vacuole (Kreuz *et al.*, 1996; Powles & Yu, 2010). The ability of crops such as wheat (*Triticum* spp.) and maize (*Zea mays* L.) to tolerate a broad spectrum of herbicide modes of action mostly derives from a P450-mediated herbicide metabolism (Siminszky, 2006; Powles & Yu, 2010).

Glutathione-S-transferases (GSTs) are enzymes that catalyze the conjugation of cysteine residues of the tripeptide glutathione to electrophilic and hydrophobic substances (Marrs, 1996). These enzymatic complexes are mostly involved in response to oxidative stress caused by active oxygen species (Dixon *et al.*, 1998; Powles & Yu, 2010). Detoxification of herbicidal compounds results from the conjugation of glutathione to herbicides and subsequent sequestration of the complex into the vacuole or exudation from the root system (Schröder *et al.*, 2007; Powles & Yu, 2010). GST-mediated herbicide metabolism is known in crops (e.g. *Zea mays* L. and *Sorghum* spp.) and weeds (e.g. *Alopecurus myosuroides* Huds. and *Abutilon theophrasti* Med.).

Aryl acylamidases play an active role in the catalysis of hydrolytic processes of amides (Frear & Still, 1968). These enzymes selectively act on carbon-nitrogen bonds, which are broken down into anilines and carboxylates (Mallory-Smith & Namuth, 2011), thus resulting in higher tolerance to amide herbicides. Increased aryl acylamidase activity is responsible for the tolerance to propanil (an amide herbicide, PSII-inhibitor) in rice (*Oryza sativa* L.). Resistance due to increased rates of propanil-metabolism has also been documented in weeds, such as *Echinochloa crus-galli* L. and *Echinochloa colona* L. (Yih *et al.*, 1968), with resistant populations showing higher levels of aryl acylamidase activity compared to the sensitive populations (Leah *et al.*, 1994). Treatments with carbamates and organophosphate insecticides, recommended for use in rice, can selectively inhibit the activity of this enzyme (Khodayari *et al.*, 1986; Wills & Street, 1988) and therefore represent a possible herbicide resistance management measure in rice fields (Mallory-Smith & Namuth, 2011).

Recent studies reported the correlation between the application of herbicides at low doses (which has gained an increasing popularity among farmers in Australia) and the evolution of high level, multiple non-target site resistance mechanisms in only three generations (Neve & Powles, 2005a; Neve & Powles, 2005b). This demonstrates the actual risk associated with the selection of this type of resistance in weed populations.

1.3 Resistance to the main herbicide modes of action

Resistance to ALS-, ACCase- and PSII-inhibitors has the highest relevance in weed populations worldwide, although other herbicide modes of action are increasingly involved as well (Heap, 2011). Herein, resistance to these three main modes of action is described.

1.3.1 Resistance to ALS-inhibiting herbicides

Acetolactate synthase or acetoxyacid synthase (ALS or AHAS, EC 2.2.1.6) is the first common enzyme in the biosynthetic pathway of branched-chain amino acids leucine and valine and the second enzyme in the biosynthesis of isoleucine (Singh & Shaner, 1995; McCourt *et al.*, 2006). This chloroplastic enzyme catalyzes the conversion of two molecules of pyruvate to 2-acetolactate or one molecule each of pyruvate and 2-ketobutyrate to 2-aceto-2-hydroxybutyrate (Mccourt *et al.*, 2006). ALS is the target site for the sulfonylurea (SU), imidazolinone (IMI), sulfonaminocarbonyltriazolinone (SCT), triazolopyrimidine (TP) and pyrimidylthiobenzoate (PTB) herbicides. These herbicides are potent inhibitors of the ALS enzyme and act selectively by stopping (or severely reducing) the biosynthesis of branched-chain amino acids, ultimately resulting in plant death (Powles & Yu, 2010). ALS belongs to a superfamily of thiamine diphosphate (ThDP)-dependent enzymes that have the ability to catalyze both the oxidative and non-oxidative decarboxylations of 2-ketoacids (Mccourt *et al.*, 2006). This enzyme is characterized by both a catalytic subunit (~65 kDa) and a regulatory subunit, whose size (ranging between 9 and 54 kDa) depends on the species of origin (Mccourt *et al.*, 2006). The regulatory subunit of plant ALS has no activity but can stimulate the activity of the catalytic subunit and confers sensitivity to feedback inhibition by branched-chain amino acids (Lee & Duggleby, 2001; McCourt *et al.*, 2006). Feedback inhibition is a regulatory mechanism by which high concentrations of certain effectors (in this case the branched-chain amino acids) inhibit the activity of the enzyme (in this case the ALS) catalyzing their biosynthesis. In this way, the enzymatic activity can be constantly adjusted depending on the needs of the plant (Wagner, 2004).

The gene encoding plant ALS protein is characterized by five discrete domains (A, B, C, D and E), each domain ranging from 4 to 19 amino acids in size (i.e. 12 to 57 bp) (Boutsalis *et al.*, 1999; Wagner, 2004). Domains C, A and D encompass residues 115 to 133, 191 to 203 and 205 to 210 of the ALS gene, respectively; domain B encompasses residues 573 to 576 and domain E encompasses residues 651 to 655 (Park & Mallory-Smith, 2004).

Mutations occurring at specific amino acid positions within each of these domains result in a reduction of sensitivity to certain classes of ALS-inhibitors. In particular, mutations at posi-

tions Ala₁₂₂ (Thr and Tyr), Ala₂₀₅ (Val), Ser₆₅₃ (Thr, Asn, Ile) and Gly₆₅₄ (Glu and Asp) (according to the numbering adopted for the ALS sequence of *Arabidopsis thaliana* Heynh.) result in resistance to IMI herbicides. Mutations at Pro₁₉₇ are associated with resistance to SU herbicides only, with the exception of Leu which also results in resistance to IMIs (Powles & Yu, 2010). Twelve amino acid changes at Pro₁₉₇ have been reported so far, including one novel substitution (Asn) that has been recently documented (Massa *et al.*, 2011). Mutations at position Asp₃₇₆ (Glu) and Trp₅₇₄ (Leu, Arg) confer resistance to all classes of ALS-inhibitors to different extents, whereas a His substitution at Arg₃₇₇ (not previously documented) confirmed to be involved in resistance to SUs, SCTs and TPs (Massa *et al.*, 2011). It has been recently found that resistance-endowing mutations act more by changing ALS sensitivity to branched-chain amino acid feedback inhibition (with subsequent accumulation of these amino acids) rather than by changing the ALS substrate affinity (Powles & Yu, 2010).

1.3.2 Resistance to ACCase-inhibiting herbicides

Acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) is a key enzyme involved in the biosynthesis of fatty acids. This enzyme acts by catalyzing the formation of malonyl-CoA from the ATP-dependent carboxylation of acetyl-CoA (Powles & Yu, 2010).

ACCase-inhibiting herbicides suppress the activity of the ACCase enzyme, therefore inhibiting the regular biosynthesis of fatty acids; this presumably blocks the production of phospholipids used in building new membranes required for cell growth (HRAC, 2011; WSSA, 2011). Two homomeric ACCase isoforms have been identified in grasses, which are encoded by two similar ACCase genes (Delye & Michel, 2005). One gene encodes a plastidic ACCase, which is located in the chloroplasts, and the other gene encodes a cytosolic ACCase, which is located in the cytosol. The plastidic ACCase isoform is highly sensitive to ACCase-inhibitors [i.e. aryloxyphenoxypropionates (APPs), cyclohexanediones (CHDs) and phenylpyrazolines (PPZs)] and is therefore the target site for this herbicide group, while the cytosolic isoform is tolerant to these herbicides (Price *et al.*, 2003; Delye & Michel, 2005). Both ACCase isoforms comprise three catalytic domains: 1) the biotin carboxyl carrier protein (BCCP), 2) the biotin carboxylase (BC) and 3) the carboxyl transferase (CT) (Thelen *et al.*, 2001).

The activity of the ACCase enzyme takes place in two different phases and catalytic sites. In the first phase, adenosine triphosphate (ATP) is used for the carboxylation of the biotin prosthetic group of the BCCP and in the second phase the carboxyl group is transferred to acetyl-CoA to give malonyl-CoA in the CT domain (Harwood, 2005). Since ACCase-inhibitors are known to interfere with the activity of the CT domain (Sasaki & Nagano, 2004), mutations

occurring within this region of the ACCase gene are likely to endow resistance to these herbicides (Balgheim, 2009).

Unlike in monocotyle plants, the plastidic ACCase of dicotyles is multimeric and is not sensitive to ACCase-inhibitors (Powles & Yu, 2010). In consequence of the selectivity toward monocotyle plants, these herbicides can be used to control grass weeds in dicotyle crops; for the control of grass weeds in monocotyle crops (e.g. cereals), a safener must be added to avoid herbicide injury. Safeners are compounds that minimize herbicide phytotoxicity in crops mainly by enhancing the activity of the enzymatic complexes that are involved in the deactivation of herbicides (Zwerger & Ammon, 2002).

So far, single mutations occurring at seven positions within the ACCase CT domain have been associated to a reduction of herbicide sensitivity of the ACCase enzyme (Powles & Yu, 2010). A Leu substitution at Ile₁₇₈₁, a Gly substitution at Asp₂₀₇₈ and an Arg substitution at Cys₂₀₈₈ are responsible for resistance to all classes of ACCase-inhibiting herbicides (APPs, CHDs and PPZs). A change of Trp₁₉₉₉ to Cys causes resistance to APPs only; Trp₂₀₂₇ to Cys endows resistance to APPs and PPZs as well as moderate resistance to CHDs; Ile₂₀₄₁ to Asn and Val results in resistance to APPs and eventually moderate resistance to CHDs and PPZs; finally, Gly₂₀₉₆ to Ala confers resistance to APPs and eventually moderate resistance to CHDs (Powles & Yu, 2010). According to studies conducted by Delye *et al.* (2005), mutations endowing resistance to ACCase-inhibitors occur within the catalytic cavity of the CT domain. In consequence of the change in shape of the cavity, herbicides are no longer able to access the binding site and act effectively.

1.3.3 Resistance to PSII-inhibitors

The light-dependent phase of the photosynthetic process implies the biophysical capture of sunlight by specialized pigments and its subsequent transduction to drive electron transport for the production of NAPH and ATP, which provide energy for the light-independent carbon reduction cycle (Powles & Yu, 2010). Photosystem II (PSII) is the first protein complex in the light-dependent phase of photosynthesis. It is located in the thylakoid membranes of plants, algae and cyanobacteria. PSII acts by using photons of light to excite electrons to a higher energy level. These electrons are used to reduce plastoquinone (PQ), an electron acceptor, which binds to the Q_b-binding niche within the D1 protein of the photosystem II and releases upon reduction and protonation by hydrogen atoms (formed through water photolysis). PSII-inhibiting herbicides act by binding to the same Q_b-binding niche on the D1 protein and therefore interfere with the transfer of electrons (Mallory-Smith & Namuth, 2011). As a con-

sequence, the formation of reduced plastoquinone and the transfer of excited electrons out of the system are impaired and the synthesis of NADPH and ATP in the chloroplast is compromised (Markwell & Namuth, 2011). Also, the inhibition of electron transfer results in the production of highly reactive triplet chlorophyll and ultimately in lipid peroxidation and membrane disruption (Mallory-Smith & Namuth, 2011). Specific mutations occurring within the chloroplastic *psbA* gene, encoding the D1 protein, confer resistance to PSII-inhibiting herbicides (Powles & Yu, 2010). The maternal inheritance of this gene decreases the movement of the resistance trait because it is not moved with the pollen (Mallory-Smith & Namuth, 2011). Furthermore, fitness costs are associated with mutations occurring within the D1 target site, as resistant plants show a reduced efficiency of photosynthesis compared to wild type plants (Mallory-Smith & Namuth, 2011).

So far, six mutations endowing resistance to different classes of PSII-inhibitors have been reported at five amino acid positions (Powles & Yu, 2010). In particular, a Gly substitution at Ser₂₆₄ is associated with resistance to triazine herbicides but not to non-triazines, whereas a Thr substitution confers resistance to both triazine and urea herbicides. An Ile mutation at Val₂₁₉, a Val mutation at Ala₂₅₁, an Ile mutation at Phe₂₅₅ and a Thr mutation at Asn₂₆₆ are known to be involved in resistance to specific non-triazine herbicides, to which changes at Ser₂₆₄ do not confer resistance (Powles & Yu, 2010).

1.4 Current situation of herbicide resistance in Germany

In Germany, as well as in many other European countries, resistance to ALS-, ACCase- and PSII-inhibitors is by far the most relevant phenomenon. With regard to ALS resistance, several cases have been documented in four weed species: *Alopecurus myosuroides* Huds., *Lolium perenne* L., *Matricaria chamomilla* L. and *Apera spica-venti* L. Beauv..

The first cases of ALS-resistant *Alopecurus myosuroides* Huds. populations in Germany were documented in 2001 by Niemann *et al.* (2002), who reported 6-10 sites across an area ranging from ~200 to ~400 ha. These populations were rated resistant to SUs and particularly to flupyrsulfuron-methyl. Six years later, in 2007, Petersen reported cases of multiple resistant *Alopecurus myosuroides* Huds. populations [ALS-, ACCase-, PSII- and very-long-chain fatty acids (VLCFA)-inhibitors] in 2-5 German sites across an area of 20-40 ha (Heap, 2011).

In 2008, the first ALS resistance cases (to iodosulfuron-methyl and pyroxsulam) were detected by Petersen in some German *Lolium perenne* L. populations (which were also cross-resistant to ACCase-inhibitors) and in some *Matricaria chamomilla* L. populations, which were rated resistant to the SU tribenuron-methyl (Heap, 2011). With regard to *Apera spica-*

venti L. Beauv., no ALS resistance cases were reported in Germany until 2005, when the first case of resistance to SUs (sulfosulfuron) was detected (Balgheim *et al.*, 2007). In 2009, Petersen reported the first case of multiple resistant (ALS-, ACCase- and PSII-inhibitors) *Apera spica-venti* L. Beauv. populations in 2-5 sites (Heap, 2011). Since then, several cases of resistance to ALS-inhibitors in *Apera spica-venti* L. Beauv. have been detected in Germany (Massa *et al.*, 2011; Massa & Gerhards, 2011).

Resistance to PSII-inhibiting herbicides has been documented in several weed species, probably due to the fact that PSII-inhibitors were the first herbicides used for weed control in agricultural fields. The first case of PSII resistance in Germany is dated 1978 and was reported for *Stellaria media* L. Vill. populations by Kees (1978). In 1980, further PSII resistance cases were reported for *Amaranthus retroflexus* L., *Atriplex patula* L., *Chenopodium album* L. and *Chenopodium ficifolium* Sm., *Galinsoga ciliata* Raf., *Poa annua* L., *Senecio vulgaris* L., and *Solanum nigrum* L. (Kees, 1988). In 1988, cases of PSII resistance in *Chenopodium polyspermum* L., *Epilobium tetragonum* L., *Fallopia convolvulus* L. Holub. and *Polygonum lapathifolium* L. were documented as well (Kees, 1988).

Finally, resistance to ACCase-inhibiting herbicides was reported for *Alopecurus myosuroides* Huds. (1983, 2003 and 2007), *Lolium perenne* L. (2008, cross-resistant to ALS-inhibitors), and *Apera spica-venti* L. Beauv. (2009, cross-resistant to ALS- and PSII-inhibitors) populations (Heap, 2011).

Alopecurus myosuroides Huds. is considered the most problematic grass weed to control in Germany, mainly due to the evolution of herbicide resistance. Petersen & Wagner (2009) recently estimated infestation rates ranging between 5 and 10% of German arable lands. Nevertheless, reports on herbicide-resistant *Apera spica-venti* L. Beauv. populations are accumulating (Niemann & Zwerger, 2006; Massa & Gerhards, 2011) and indicate that this weed will assume a primary role in the Central and Eastern European agricultural fields. Furthermore, the lack of new herbicide modes of action, combined with the inappropriateness of agricultural management measures in most intensive cropping systems, is likely to cause a shift in sensitivity that will eventually culminate in the selection for resistance to multiple herbicide modes of action.

1.5 Methods for the detection of herbicide resistance

The use of appropriate testing procedures for the verification of herbicide resistance in suspected weed populations is of primary importance for effective resistance management strategies. So far, several methods have been developed at both the greenhouse and laboratory lev-

el. The greenhouse-based tests normally include the conventional whole-plant pot assays, while laboratory-based tests ('rapid tests') include several approaches, such as Petri-dish germination, chlorophyll fluorescence, leaf disc flotation, pollen germination and enzyme sensitivity assays (Moss, 1995).

1.5.1 Greenhouse assays

The most widely used and reliable test for herbicide resistance is the whole-plant pot assay. This method implies growing plants in the greenhouse from seeds collected in the suspect field/s and spraying them with herbicides, applied either at a single dose or at a range of doses (HRAC, 2011). The final assessment of herbicide efficacy can be made through visual rating of whole-biomass reduction as compared with untreated controls or through measurement of fresh or dry weight of above-ground biomass (HRAC, 2011).

When testing a limited number of field populations, it is preferable to apply a wide range of herbicide doses (from below to above the recommended field rate) to obtain a dose-response curve. Once dose-response curves are generated, it is possible to estimate the ED_{50} (or LD_{50} , GR_{50}) of resistant and sensitive populations, which represents the dose at which 50% of the population is controlled. The ratio of ED_{50} (or any other herbicide dose) of a given population to that of a reference population (sensitive) will provide the Resistance Index (or Resistance Factor) (HRAC, 2011), which enables to estimate the resistance level at different herbicide doses.

On the other hand, dose-response assays are not feasible when dealing with large amounts of populations (mainly due to space and time reasons) and therefore not possible to carry out. In these cases, single-dose, double-dose or even fourfold-dose assays are preferable, as many more populations can be tested in different combinations and fewer pots per population are needed (HRAC, 2011).

Despite their main disadvantages (time-consuming, labour-intensive, at least five weeks to provide results), these methods are extensively used in weed science because they can realistically reproduce the situation occurring in the field (i.e. herbicide application) compared to other approaches (e.g. Petri-dish assays), and because resistance can be reliably detected on a phenotypic base regardless of mechanism (HRAC, 2011).

1.5.2 Laboratory assays

The main advantage of laboratory-based resistance tests is the possibility to obtain results even within days, thus enabling the screening of many more populations at the same time.

This has resulted in the development of several ‘rapid tests’, which are able to quickly provide information on eventual resistance evolution.

Despite the development of a broad range of resistance tests, the methods most commonly in use imply the simulation of the whole-plant pot assay procedure through the use of pre-germinated seeds (i.e. seedling bioassays), which are placed into Petri-dishes containing aqueous herbicide solutions (Kim *et al.*, 2000). Further variations in Petri-dishes include sowing pre-germinated seeds onto cooled agar (~1% wt/v) containing herbicides or placing young tillers in glass tubes containing ~0.2% (wt/v) agar incorporating herbicides (Kim *et al.*, 2000). Parameters to discriminate between resistant and sensitive biotypes mainly include the assessment of shoot and root length and fresh or dry weight (Burke *et al.*, 2006; Kim *et al.*, 2000; Kuk *et al.*, 2003). *In vitro* tests using non pre-germinated seeds are also available.

Enzyme assays are widely used and reliable tests for detecting resistance by measuring the activity of isolated target enzymes upon herbicide exposure. These assays have been described for both ALS- and ACCase-inhibitors (De Prado *et al.*, 2004). For PSII-inhibitors, resistance can be detected by measuring the oxygen production subsequent to the addition of herbicides to isolated chloroplasts (Hill’s reaction), by measuring chlorophyll fluorescence (Truelove & Hensley, 1982; Kim *et al.*, 2000) or by leaf disc flotation (Hensley, 1981; Kim *et al.*, 2000).

Although all these methods are able to provide results quickly and identify the mechanisms of resistance with higher precision compared to the whole-plant assays, their main disadvantage is that results can be difficult to interpret. This mainly derives from the fact that herbicides are applied in ways that are totally different from the field application (HRAC, 2011), and this could result in misleading conclusions.

Futhermore, none of the methods described so far is able to characterize the resistance profile at molecular level (e.g. gene mutations), which plays a primary role in studies aimed at clarifying the mechanisms underlying phenotypic resistance. Therefore, DNA-based marker technologies and other molecular methods have been developed during the past years to accomplish this task.

In particular, the Cleaved Amplified Polymorphic Sequence (CAPS) technology has gained increasing popularity among weed scientists and is therefore widely used for the detection of mutations occurring at specific amino acid positions within conserved gene domains. This method, rather inexpensive and time-saving, enables the detection of single nucleotide polymorphisms (SNPs) by the loss or gain of a recognition site of a restriction enzyme (Yu *et al.*, 2008). Unlike the CAPS technology, the dCAPS (derived Cleaved Amplified Polymorphic

Sequence) technique implies the introduction of a restriction enzyme recognition site, which includes the SNP of interest, into the PCR amplicon by a special primer (i.e. dCAPS primers) that contains one or more mismatches to the template DNA (Neff *et al.*, 1998). The PCR amplicon is subsequently digested by a restriction enzyme and the presence or absence of a given SNP is verified by the resulting restriction digestion pattern (Yu *et al.*, 2008).

Other methods include Polymerase Chain Reaction Amplification of Specific Alles (PASA), which is based on the detection of SNPs in DNA coding for a given gene (e.g. ALS) through the use of allele-specific primers (Kwok *et al.*, 1990; Sommer *et al.*, 1992). The 3' end of one primer matches for one allele and mismatches for the different allele at the mutation site (thus preventing primer elongation at the 3' end by *Taq* polymerase), which results in the amplification of a PCR fragment of expected size in the first case and in the absence of the corresponding DNA fragment in the second case (Wagner *et al.*, 2002). In this way, target alleles can be identified by the presence or absence of the expected amplicons after agarose gel electrophoresis (Wagner *et al.*, 2002). However, all these methods require at least partial information on the sequence of the gene/s of interest in order to amplify the desired DNA-fragments via PCR through the use of gene-specific primers (GSPs). Further methods, such as Pyrosequencing®, have been developed as well.

1.6 Loose silky bent grass (*Apera spica-venti* L. Beauv.)

Loose silky bent grass (*Apera spica-venti* L. Beauv.) is an annual grass weed in European winter grain fields (Northam & Callihan, 1992). It is mainly widespread in the Central and Eastern European countries, such as Germany, Switzerland, Austria, Poland, the Czech Republic, Slovakia and Hungary as well as in some Northern European countries, such as Denmark and Sweden (Melander *et al.*, 2008; Hamouzová *et al.*, 2011). The increase in recurrence of *A. spica-venti* infestations observed during the past 15 years can be mainly attributed to the plasticity of this species in adaptation to different agricultural environments (Soukup *et al.*, 2006; Melander *et al.*, 2008). As the common name under which *A. spica-venti* is known ('common windgrass') suggests, seed dispersal preferentially occurs via the wind due to the small size of the seeds and to the anatomy of the panicles, which are bent by the wind at maturity. *A. spica-venti* is a wind-pollinated and primarily self-incompatible species (Warwick *et al.*, 1987). Reproduction is exclusively generative and seed production can be extremely variable. Studies conducted by Warwick *et al.* (1985) report that individual plants produce up to 2000 seeds per plant. On the other hand, cases of single plants producing more than 20000 seeds were documented as well (Soukup *et al.*, 2006). Seeds exhibit a short primary dormancy

(Koch, 1968) and seedlings mainly emerge in autumn soon after harvest, overwinter in the two- to three-leaf stage and develop simultaneously with the crop in spring (Warwick *et al.*, 1985). Although *A. spica-venti* plants flower at the same time as the crop, most seeds ripen faster than those of the crop, which results in seeds falling from the panicles before harvest (Warwick *et al.*, 1985; Northam & Callihan, 1992). The longevity of seeds in soil can range between 1 and 4-7 years (Koch & Hurlle, 1978; Chomas & Kells, 2001), whereby ranges between 1 and 2 years are far more frequent (Zemanek, 1980; Chomas & Kells, 2001). Although seed germination mainly occurs in autumn, favourable pedoclimatic conditions such as light-textured soils, mild temperatures and high rainfall rates can promote germination in early-sown spring crops as well (Soukup *et al.*, 2006). The percentage increase in winter cereals and autumn-sown crops in the rotation systems, combined with the adoption of reduced and no soil tillage practices (which promote the permanence of seeds in the upper soil layers), resulted in an increased rate of *A. spica-venti* infestations in intensive cropping systems (Melander *et al.*, 1995; Pallutt & Moll, 2008). The economic threshold for this weed has been estimated at ~10-30 plants m⁻², with yield losses of the order of 30% at population densities of ~200 plants m⁻² (Melander *et al.*, 2008). During the last phases of the phenological cycle, *A. spica-venti* overgrows the crop (Szekeres, 1991), thus mainly competing for light (Gehring & Thyssen, 2011).

Repetitive long-term application of herbicides with the same mode of action (mostly ALS-inhibitors), together with the adoption of improper farm management strategies and measures, has resulted in the evolution of herbicide resistance in *A. spica-venti* as well as in many other weed species worldwide. Over the course of 15 years, an increasing number of herbicide-resistant *A. spica-venti* populations has been reported (Heap, 2011). Nowadays, populations resistant to photosystem II (PSII)-inhibitors, acetolactate synthase (ALS)-inhibitors and acetyl CoA carboxylase (ACCase)-inhibitors are mostly spread in Poland, the Czech Republic, Germany and Switzerland (Heap, 2011). In addition, several new cases have been documented during the course of the last three years (Massa *et al.*, 2011; Massa & Gerhards, 2011). Therefore, the further spread of herbicide resistance in *A. spica-venti* populations is likely to become a serious problem at European level in the near future.

1.7 Thesis objectives

The main objectives of the present work were:

- The screening of herbicide resistance in *Apera spica-venti* European populations at the greenhouse level using whole-plant bioassays (pot experiments) to verify actual resistance;
- The quantification of resistance to different herbicides in field-selected *A. spica-venti* populations using dose-response assays and digital image analysis;
- The elucidation of the ALS target site resistance mechanisms at the laboratory level by using previously and newly developed molecular markers, sequencing the whole ALS gene of resistant and wild type *A. spica-venti* populations and multiple sequence alignments;
- The development of a geo-referenced ‘weed database’ for documenting the spread of herbicide-resistant *A. spica-venti* populations across Europe over time and space;
- The evaluation of the influence of agricultural and biological factors on the probability of resistance occurrence in *A. spica-venti* populations using a risk assessment model;
- The verification of the results from the greenhouse assays under field conditions through studies conducted over the course of two years (2008/2010) and the detection of the introgression of herbicide resistance traits into the sensitive population after two generations.

The work is presented as a cumulative thesis. Four publications have resulted from this project during these three working years, three of which are in press and one of which is submitted and currently under review.

The first paper, published in the *Weed Research Journal*, deals with molecular genetic investigations of the ALS gene of resistant and wild type *A. spica-venti* populations. The paper presents the results from greenhouse and laboratory experiments conducted during two years on 72 European populations which were rated resistant to ALS-inhibiting herbicides. The mechanisms and the origin of resistance are explained and discussed.

The second paper, published in the *Journal of Plant Diseases and Protection*, presents the results of two-year investigations in the greenhouse (whole-plant pot assays) conducted on 265 *A. spica-venti* populations suspected to be resistant to ALS-inhibitors. Dose-response assays and digital image analysis were additionally carried out with the aim of quantifying resistance to different herbicides. The newly developed approach based on digital image analysis for the

quantification of herbicide resistance under greenhouse conditions is only at its initial stage. However, it has proven to represent a potential alternative to the more laborious dose-response assays.

The third paper, submitted to the Crop Protection Journal, presents a newly developed geo-referenced database for mapping the spread of herbicide-resistant *A. spica-venti* populations across Europe. This paper also evaluates the influence of agricultural and biological factors on the probability of resistance occurrence in *A. spica-venti* through the use of a risk assessment model, with the aim of preventing the further spread of this weed across European arable lands.

Finally, the fourth paper, published in the Gesunde Pflanzen Journal, presents the results of two-year field studies conducted at the Ihinger Hof research station, where herbicide-resistant and susceptible *A. spica-venti* populations were sown in winter wheat plots. The main aims of this paper were to verify the results obtained from the greenhouse under more realistic environmental conditions and to determine whether introgression of herbicide resistance traits into the sensitive population could be observed already in the second generation.

Overall, this work should provide weed scientists as well as consultants with useful tools for the detection of actual herbicide resistance in suspected weed populations, and most importantly, for the prevention of the spread of this problem across European arable lands.

Chapter II

Target-site resistance to ALS-inhibiting herbicides in *Apera spica-venti* populations is conferred by documented and previously unknown mutations

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2. Target-site resistance to ALS-inhibiting herbicides in *Apera spica-venti* populations is conferred by documented and previously unknown mutations

Summary

In this study, whole-plant bioassays were performed on 72 *Apera spica-venti* populations which have survived application of acetolactate synthase (ALS)-inhibiting herbicides in recent years. Molecular genetic analysis of the ALS gene revealed a Thr mutation at Pro₁₉₇ within 67 populations. Amplification and sequencing of the whole ALS gene from further resistant plants not carrying the above-mentioned mutation revealed the presence of a Leu mutation at Trp₅₇₄ within two populations and an Asn mutation at Pro₁₉₇ within two populations. Since the Pro₁₉₇-Asn substitution is reported for the first time in a field-selected weed population, a CAPS (Cleaved Amplified Polymorphic Sequences) marker was developed for its quick detection. In addition, one novel mutation was found within a population that coded for a His substitution at Arg₃₇₇. Enzyme assays confirmed a significantly reduced inhibition of ALS activity after herbicide exposure compared to the wild type. This population showed resistance to sulfonylureas (SUs) and cross-resistance to sulfonylaminocarbonyltriazolinones (SCTs) and triazolopyrimidines (TPs) within the whole-plant bioassays. ALS protein sequence alignments from weedy and cultural plants revealed that the Arg₃₇₇ is highly conserved among known wild type ALS enzymes. In agreement with existing literature concerning the structure and mechanisms of inhibition of plant ALS, this mutation is probably involved in target-site resistance to ALS-inhibitors. Our results suggest that further single nucleotide polymorphisms (SNPs) impairing proper herbicide performance might be selected within field populations in the near future, making the short and long range evolution of target-site resistance difficult to predict depending solely on herbicide use history.

Keywords: acetolactate synthase, ALS-inhibitors, loose silky-bent, target-site resistance, SNPs, sequencing

Chapter III

Investigations on herbicide resistance in European silky bent grass (*Apera spica-venti* L. Beauv.) populations

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3. Investigations on herbicide resistance in European silky bent grass (*Apera spica-venti*) populations

Abstract

In this study, the results of two-year investigations on herbicide resistance in silky bent grass (*Apera spica-venti*) populations are presented. Greenhouse assays were conducted with different herbicides on more than 250 silky bent grass populations from Central and Eastern European agricultural fields where herbicides had not performed satisfactorily. Results revealed that more than 60% of suspected populations were resistant to acetolactate synthase (ALS)-inhibitors. In contrast to this, resistance to acetyl-CoA carboxylase (ACCase)-inhibitors could be observed only sporadically and no resistance to photosystem II (PSII)-inhibitors could be detected. Dose-response assays conducted in the greenhouse on field-selected populations revealed resistance factors at the ED₅₀ and ED₉₀ ranging from 11 to 142 after treatment with flupyr-sulfuron-methyl, from 2 to 15 after treatment with mesosulfuron+iodosulfuron and from 4 to 6 after treatment with fenoxaprop-P-ethyl, thus confirming a prevalent resistance to ALS-inhibiting herbicides in silky bent grass. Within further greenhouse experiments, percent canopy cover after treatment with sulfosulfuron and fenoxaprop-P-ethyl was determined in sensitive and resistant populations by using digital image analysis. A significant effect of herbicide dose on canopy cover was observed in sensitive plants 7 and 15 days after treatment with sulfosulfuron and in all populations after treatment with fenoxaprop-P-ethyl. Statistical analysis revealed significant correlations between canopy cover and plant dry weight in all populations. This work, through the presentation of previously established and newly-developed methods, provides weed scientists with a broad range of practical tools for a reliable diagnosis of herbicide resistance in weed populations.

Key words: ALS-inhibitors, dose-response assays, digital image analysis, dry weight, plant cover, whole-plant bioassays

Chapter IV

Evaluation of agricultural and biological factors affecting the occurrence of herbicide resistance in silky bent grass (*Apera spica-venti*)

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4. Evaluation of agricultural and biological factors affecting the occurrence of herbicide resistance in silky bent grass (*Apera spica-venti*)

Abstract

During the last decades, the evolution of resistance to herbicides in weed populations has become a problem of increasing importance. In this work, we evaluate the role of agricultural and biological factors in the occurrence of herbicide resistance in silky bent grass (*Apera spica-venti*). During a period of three years, more than 250 populations were collected in several fields across Europe where herbicide treatment could not achieve satisfactory control and tested for resistance in the greenhouse. After recording the field history of locations, a geo-referenced database has been developed to map the distribution of herbicide-resistant silky bent grass populations in Europe. A Logistic Regression Model (LRM) has been fitted to describe and quantify the relationships between agrobiological factors (crop rotation, soil tillage, sowing date, soil texture and weed density) and the probability of resistance occurrence. Our results revealed that rotation management and sowing date of the winter crop are the factors that have the greatest influence on the model, with a risk of resistance emergence more than 90 times higher by high percentage of winter crops in the rotation (>75%) and more than three times higher by early sowing. Conservation tillage and high population density also significantly increase the risk of resistance in silky bent grass, whereas soil texture has no significant influence. The developed model can find practical application in risk assessment studies aimed at preventing the evolution and spread of herbicide resistance in weed populations.

Keywords: herbicide resistance, silky bent grass, geo-referenced weed database, Logistic Regression Model (LRM), risk assessment

4.1 Introduction

Since its appearance on the market more than five decades ago, chemical weed control through the use of herbicides has gained an increasing popularity among farmers worldwide. In fact, herbicides revolutionized the traditional concept of plant protection by removing weeds selectively while avoiding destructive soil cultivation practices, high work inputs and higher costs, ultimately resulting in safe and profitable global food production (Powles and Yu, 2010).

For this reason, herbicides represent the most important weed control option used, accounting for up to 50% of the whole plant protection market (Zwerger and Ammon, 2002). Despite this, at present time over 190 weed species (~60% dicotyles and ~40% monocotyles) across 400,000 fields worldwide have evolved resistance to several herbicide modes of action (Heap, 2011).

So far, recommendations for delaying the selection and spread of this phenomenon mostly focused on herbicide group rotation (Beckie et al., 2004). However, also agricultural and biological factors are known to play a role in the development of herbicide-resistant weed populations.

Increasing winter crops (e.g. winter cereals) in the rotation systems represents a major trend in modern agro-ecosystems, particularly those characterized by intensive cropping. The reason for this change in modern agriculture is merely economic as winter crops are, in most cases, far more profitable than spring-sown crops (Melander, 1995). However, such strategies were partly responsible for the higher weed infestation rates that have been reported during the past years; this ultimately resulted in income losses deriving from the increased weed control costs (Melander, 1995), whose effectiveness can be severely reduced by herbicide resistance.

Soil tillage is another management measure that is known to play an important role in the spread of weed populations, potentially including resistant ones. In fact, although reduced (e.g. tine cultivation prior to drilling) and no-till methods still have many benefits (conservation of soil structure and moisture, reduction of water and wind erosion, time optimization, lower work inputs), they can also promote the germination of certain weed species as a consequence of the permanence of their seeds in the upper soil layers (Murphy and Lemerle, 2006). This eventually results in higher rates of seedling emergence and population density, faster spread throughout the fields and higher competition ability, which may increase the risk of resistance evolution.

Finally, sowing date and weed density have a relevant impact on weed seed production, selection and survival (Murphy and Lemerle, 2006). In particular, early sowing and high weed density may enhance crop-weed competition, which may lead to short-term spread of weeds (Mohler, 1996) and therefore increased probability of resistance development.

Among the numerous weed species spread worldwide, silky bent grass (*Apera spica-venti* L. Beauv.) is one of the most abundant in the agricultural fields of Central and Eastern Europe. It is an annual grass weed that can be mostly found in winter crops, especially winter cereals and winter oilseed rape (*Brassica napus* L.) (Hurle, 1993; Melander et al., 2008). The

seeds exhibit short primary dormancy (Koch and Hurle, 1978; Wallgren and Avholm, 1978) and germinate in late summer and autumn, mostly on light-textured, humid soils (Rola, 1990; Northam and Callihan, 1992). Nevertheless, during mild winters the seeds may continue to germinate, eventually causing infestations also in spring-sown crops (Andreasen, 1990; Melander et al., 2008). Despite the presence of silky bent grass also in North America and Canada, its relevance is higher in the Central and Eastern part of Europe (Germany, Switzerland, Poland, the Czech Republic, Slovakia and Hungary), and more recently in Northern Europe (Denmark and Sweden) (Melander et al., 2008). The economic importance of this weed has been quantified in yield losses of up to 30% (Gehring and Thyssen, 2011) and the economic threshold has been estimated at ~10-30 plants m⁻² (Kötter, 1991). As the weed overgrows the crop during the last phenological development stages (July), silky bent grass strongly competes for light (Gehring and Thyssen, 2011).

During the last years, reports on herbicide-resistant silky bent grass populations have accumulated (Heap, 2011). In 1994, the first case of resistance to photosystem II (PSII)-inhibiting herbicides was reported in Switzerland (Mayor and Maillard, 1997). Since then, several cases have been documented. In 1997, the first case of PSII-resistance was reported in Germany (Niemann, 2000); in 2005, three cases of resistance to acetolactate synthase (ALS)-inhibiting herbicides were documented in Poland, the Czech Republic and Germany (Marczewska and Rola, 2005; Novakova et al., 2006; Balgheim et al., 2007); in 2006 the first case of resistance to ALS inhibitors was confirmed in Switzerland (Delabays et al., 2006) and in 2009 the first case of multiple resistance to PSII-, ALS-, and acetyl-CoA-carboxylase (ACCase)-inhibiting herbicides was reported in Germany (Heap, 2011).

The main objectives of this study were 1) the development of a geo-referenced database for mapping the spread of herbicide-resistant silky bent grass populations across Europe; 2) the evaluation of the influence of agricultural and biological factors (crop rotation, tillage, sowing date, soil texture and weed density) on the probability of resistance occurrence in silky bent grass.

4.2 Materials and Methods

4.2.1 Sampling strategy and field history

During a period of three years, seeds from 263 silky bent grass populations were collected in several agricultural fields of Germany, Poland and the Czech Republic where herbicide treatment could not achieve total control. Seed samples were collected at random, from different patches across each field and from different plants (>60 panicles) shortly before win-

ter crop harvest (July). Samples were collected at a distance of >4 m from the borders of fields. The harvested material from each field was pooled together (in mixed populations) and stored in paper bags for greenhouse screening. The field history of the samples was provided by the farmers, who were requested to complete questionnaires reporting the following information: 1) field location (address or GPS coordinates); 2) crop rotation of the last 5-7 years, 3) soil tillage, 4) soil texture, 5) sowing date of the winter crop and 6) weed density. Categories within each factor are shown in Table 4.1. The factors 2-6 functioned as explanatory variables for the developed model. Results from the greenhouse assays for verification of actual resistance after herbicide treatment functioned as categorical response for the model, using 'yes' (resistance confirmed) and 'no' (resistance not confirmed).

4.2.2 *Technical features of the geo-referenced database 'Weedscout'*

The information obtained from the questionnaires (field history) and the results of the greenhouse assays (resistant/non-resistant) were used to develop a database in which the distribution of herbicide-resistant silky bent grass populations is mapped. Weedscout 2.0, provided by Proplanta (www.proplanta.de), is a geo-referenced database and role-based web application for the storage, management and spatial representation of weed species and other location-related parameters. The multilingual administration interface of the software is based on the HTML PUBLIC "-//W3C//DTD HTML 4.01 Transitional//EN", which is CCS3-compliant and Crossbrowser compatible (IE 6.0+®, FF 2+®, Safari 3.0+®, Opera 9.0+®, Chrome®). The server-side scripting language is based on PHP® 5.2.10 and the web database on MySQL® 4.0.27-standard de-iso-8859-1. The cartographic representation of the data (available in aerial and terrain view) is run by Google maps 2.0. Different zoom levels allow detailed view of target areas. An online form was created for the upload of raw data (questionnaires), with the geocoding (i.e. the conversion of addresses into geographic coordinates) being automatically carried out by Google GClientGeocoder (Google maps-API). If no exact address can be given, the GPS coordinates (i.e. longitude and latitude) of locations are obtained through the marker function of Google Maps and automatically transferred into the form. After the greenhouse assays for verification of herbicide resistance, each population was identified by a colored flag, which is red in case of verified resistance, green in case of unverified resistance and yellow in case of suspected populations that could not be tested due to seed scarcity or inability to germinate (Fig. 4.1). The search results can be modulated by several filters (e.g. country, populations of interest, weed species) and specific geo-tools allow

the calculation of distances and surfaces. Finally, an export function enables the backup and processing of metadata in XLS format.



Fig. 4.1: Screenshot example (terrain view) showing the distribution of verified (red flags), unverified (green flags) and suspected (yellow) herbicide-resistant silky bent grass populations in Germany, Poland and the Czech Republic.

4.2.3 Presentation of the model and statistical analysis of the data

Statistical analysis of the collected data was performed with the software SPSS® (version 15.0). The relationships between variables were analyzed using Spearman correlations, multicollinearity detection and contingency tables. A Logistic Regression Model (LRM) was used to describe the relationships between the explanatory variables (crop rotation, soil tillage, soil texture, sowing date and weed density) and herbicide resistance. Logistic regression is a statistical method that uses one or more explanatory variables to predict the probability of a categorical response. The response variable in LRM is the log of the odds ratio (logit). The logit transforms a variable constrained between zero and one (p) into a continuous variable, which is linear with respect to the vector of the explanatory variables:

$$\text{Logit}(p) = \log \left[\frac{p}{1-p} \right] = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \dots + \beta_k X_k$$

where p is the probability of the occurrence of the event, β_0 is a scalar intercept parameter and $\beta_1 \dots \beta_k$ the slope coefficients for the explanatory variables $X_1 \dots X_k$.

The maximum likelihood method was carried out to estimate the parameters of the model. The criteria for the inclusion/exclusion of variables were: probability of inclusion of 0.05 and probability of exclusion of 0.10. The Wald statistic indicated the significance of the explanatory variables in herbicide resistance. The interpretation and assessment of significance for the explanatory variables were performed by using the p -value and the 95% confidence interval (CI) of the exponents of the coefficients β ($e^\beta = \text{OR}$). Explanatory variables were accepted with significance levels close to 10%. To assess the goodness of fit of the developed model, statistical measures were used (Log Likelihood, Cox-Snell R^2 , Nagelkerke R^2), a calibration test (Hosmer-Lemeshow test) and a measure of discrimination (percentage of correctly classified cases, area enclosed under the curve ROC: AUC).

In the ROC (receiver operating characteristic) analysis, sensitivity (true positive rate) is plotted against 1-specificity (false positive rate), to show the relationship between true positives (i.e. resistant cases correctly classified by the model) and false positives (i.e. non-resistant cases wrongly classified by the model). The area enclosed under the curve (AUC) is a measure of the overall performance of the predictive model and is expressed as the average value of sensitivity for all possible values of specificity. The perfect classification corresponds to 100% sensitivity (no false negatives) and 100% specificity (no false positives), with AUC=1.

The differences between the log likelihood of the full model and the log likelihood of a model without each predictor variable were calculated. The relative contribution of a predictor variable was defined as the ratio of its log likelihood difference to the sum of the all log likelihood differences multiplied by 100 (Escobar et al., 2008).

4.3 Results

The results of the greenhouse screening showed that most suspected populations (210) were resistant to herbicides (mainly ALS-inhibitors). In 53 cases (20.2%) resistance could not be confirmed. Herbicide-resistant populations accounted for 79.8% of observations. High proportion of winter crops in the rotation (>75%) accounted for 71.5% of cases, whereas the rest of the cases (<50% and 50-75%) had a smaller proportion. Early sowing was less represented than middle and late sowing (30% of observations). Finally, conventional/conservation soil tillage and low/high weed density had a similar proportion in their categories (Table 4.1).

Table 4.1: Frequency distribution of cases (in %) in each category of analyzed factors (resistance, crop rotation, soil tillage, sowing date, soil texture and weed density). Frequency distribution of herbicide resistance is shown in each factor category.

Factors	%	Herbicide Resistance		
		Yes (%)	No (%)	Total (%)
Resistant				
Yes (verified)	79.8			
No (not verified)	20.2			
Total	100			
Crop Rotation*				
<50%	11.4	16.7	83.3	100
50-75%	17.1	55.6	44.4	100
>75-100%	71.5	95.7	4.3	100
Total	100			
Soil Tillage				
Plough	57.0	74.0	26.0	100
Reduced/No-Till	43.0	87.6	12.4	100
Total	100			100
Sowing Date				
Middle/Late	70.0	76.1	23.9	100
Early	30.0	88.6	11.4	100
Total	100			100
Soil Texture				
Middle/Heavy	88.6	82.4	17.6	100
Light	11.4	60.0	40.0	100
Total	100			100
Weed Density**				
Low/Middle	43.3	73.7	26.3	100
High	56.7	84.6	15.4	100
Total	100			

* % of winter crops in the rotation; ** Plants m⁻² (Low: <10; Middle: 10-40; High: >40).

The developed LRM showed a good fitness, calibration and discrimination (Table 4.2). In accordance with the Nagelkerke R^2 , the model could explain the 58% of total variability in the occurrence of herbicide resistance. The discrimination capability of the fitted model resulted in 88.2% of correctly classified cases, with greater sensitivity (it correctly classified 92.4% of resistant cases) than specificity (it correctly classified 71.7% of non-resistant cases). ROC analysis showed a good discrimination, with an AUC of 0.89 (95% CI: 0.83 - 0.95) (Fig. 4.2).

Table 4.2: Fitness, calibration and discrimination of the LRM. The goodness of fit of the developed model was tested by Log Likelihood, Cox-Snell R^2 and Nagelkerke R^2 . Calibration was tested by Hosmer-Lemeshow statistic (χ^2 , degrees of freedom and p -value) and discrimination by percentage of correctly classified cases.

Statistical method	Value	Degrees of freedom	p -value
Goodness of fit			
Log Likelihood (LL)	-72.23		
Cox-Snell R^2	0.37		
Nagelkerke R^2	0.58		
Calibration			
Hosmer-Lemeshow χ^2	12.98	6	0.043
Discrimination			
Percentage of correct classification	88.20		
Valid cases	263		

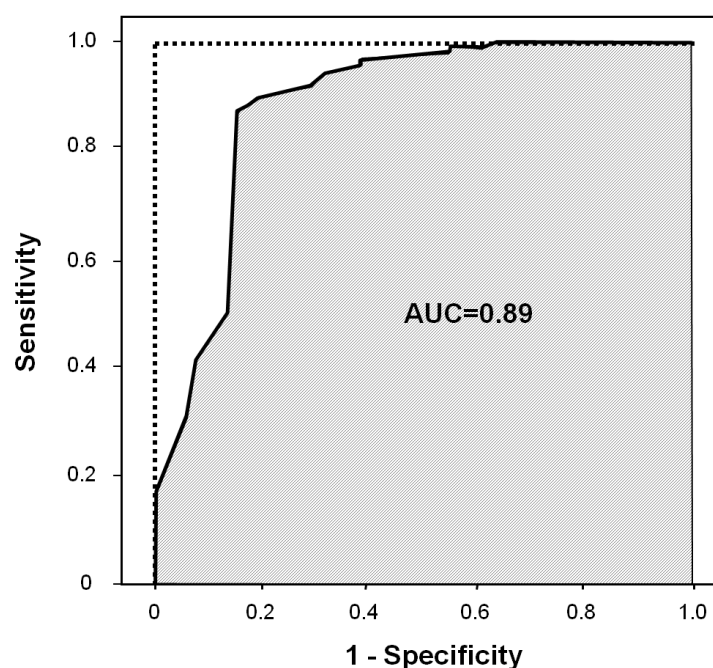


Fig. 4.2: ‘Sensitivity’ vs ‘1 – Specificity’ plot. Solid line represents the calculated ROC curve, which indicates the discrimination capability of the LRM. Dotted line represents the perfect ROC curve (AUC=1). Non-resistant cases wrongly classified by the model (false positives) are represented in the x-axis (1-Specificity). Resistant cases correctly classified by the model (true positives) are represented in the y-axis (Sensitivity).

LRM for the predictors resulted in two predictors with greater relative influence on the model. All factors, except for soil texture ($p=0.462$), were statistically significant at 95% (rotation and sowing date) and 90% confidence level (soil tillage and weed density). Therefore, rotation management and sowing date of the crop were the most important predictor variables. According to the analyzed data, the highest risk of herbicide resistance occurred in presence of low percentage of spring-sown crops in the rotation (<25%); in these cases, the probability of resistance occurrence showed to be 94.06 times higher compared to the reference category (up to 50% of winter crops). This probability decreased as the proportion of spring-sown crops in the rotation increased (50-75%), reaching the limit of 5.25 times compared to the reference category.

In the same way, the rest of the agronomic factors resulted in a potential risk of resistance emergence. Early sowing resulted in a 3.65 times higher probability of resistance occurrence compared to middle and late sowing. Conservation tillage also increased the chances of resistance occurrence (OR=2.18) compared to conventional tillage. Finally, high densities of silky bent grass (>40 plants m⁻²) resulted in a two times higher risk of resistance emergence compared to low and middle density (Table 4.3).

In conclusion, the predominant presence of winter crops in the rotation systems (>75%), together with early sowing, conservation tillage and high population density resulted in increased probability of resistance occurrence in silky bent grass (Fig. 4.3).

Table 4.3: Predictor variables of herbicide resistance for the LRM. The probability of resistance occurrence with respect to the reference category is shown for each statistically significant factor and is given by the crude (univariate model) and adjusted odds ratio (multivariate model with all factors considered as independent variables in the regression). The significance of the predictor variables is shown by Wald statistic and 95% CI of the adjusted OR. Percentage distribution of the factors influencing herbicide resistance is given in terms of relative contribution of the predictor variables.

Factors ^a	OR ^b	OR ^c	Wald	95% CI	% ^d
Crop Rotation (ref. <50%)			60.44		88.31
50-75%	6.25	5.25**	7.46	1.60-17.27	
>75-100%	112.50	94.06**	52.07	27.38-323.14	
Soil Tillage (ref. Plough)	2.49	2.18*	2.77	0.87-5.48	2.77
Sowing Date (ref. Middle/Late)	2.44	3.65**	6.07	1.30-10.22	6.50
Weed Density (ref. Low/Middle)	1.96	2.07*	2.48	0.84-5.09	2.42
Constant		0.00	0.084		

* Factor significant at 90% confidence level (p -value ≤ 0.100); ** Factor significant at 95% confidence level (p -value ≤ 0.050); ^a The displayed factors are maintained in the final model after the modeling process; ^b Crude Odds Ratio (OR); ^c Adjusted Odds Ratio (OR); ^d Relative contribution of predictors.

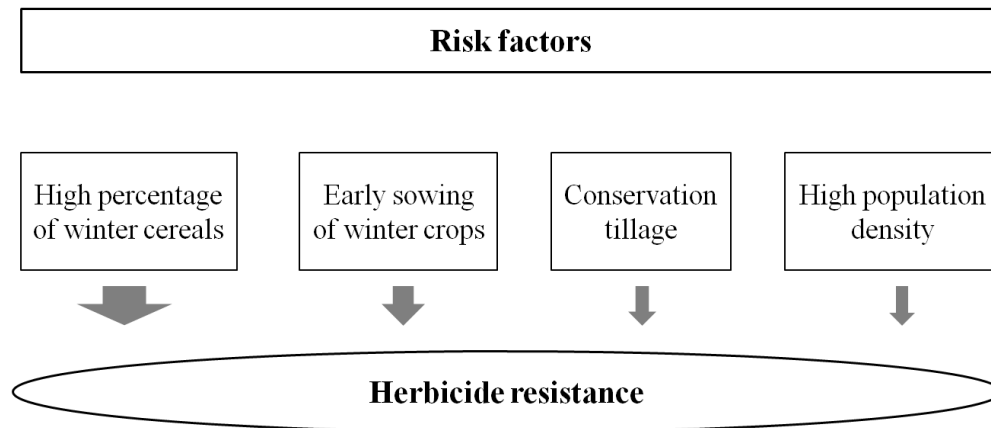


Fig. 4.3: Flow chart showing the risk factors involved in the development of herbicide resistance. All factors, with the exception of soil texture, significantly increased the probability of resistance occurrence in silky bent grass.

4.4 Discussion

Our results showed that herbicide resistance in silky bent grass is a phenomenon of growing importance. In fact, most populations were resistant to herbicides, particularly ALS-inhibitors. Since the collected populations were suspected of having evolved resistance (i.e. targeted survey), these results are not representative of the general situation of herbicide resistance in European winter crops. Nevertheless, the number of observations constituting the sample (263 populations) and the size of the sampling area (which covered agricultural fields across three Countries) confer robustness to the study, whose emphasis was placed on the relationships between farm management and resistance occurrence.

The advantages of developing a geo-referenced database of herbicide-resistant silky bent grass populations across Europe are manifold: 1) easy and fast acquisition of data for different purposes (not limited to herbicide resistance); 2) easy and fast acquisition of spatiotemporal features (e.g. evolution and spread of herbicide-resistant populations); 3) easy access to other geographic information system (GIS) sources, such as point locations, distances and further documentary data; 4) the creation of a specific weed-database with global coverage ability. Further benefits include: 1) online access, which enables users to work on their data safely and effectively (many users can work on their own data at the same time); 2) free scalability, which enables data analysis at different levels (global, national, regional, target-specific); 3) multi-filtering features, which enables a flexible access to the metadata through the selection of specific criteria (weed species, populations of interest, Country of provenance, field history etc.). Overall, the developed database represents a useful instrument for studies aimed at monitoring the evolution and spread of herbicide resistance in silky bent grass populations over

time and space, thus allowing precise identification of agricultural areas with higher risk of resistance emergence.

Statistical analysis of the collected data revealed that each factor, with the exception of soil texture, significantly increased the risk of resistance development. Rotation management proved the most significant factor influencing the probability of resistance occurrence in silky bent grass. The high proportion of winter crops in the rotations revealed to be decisive for the evolution of herbicide resistance as compared with situations in which winter and summer crops are more evenly distributed over the years. This is in accordance with most existing literature and can be attributed to two main reasons: 1) continuous cropping tends to favour and select the species within the weed flora that have phenological and physiological similarities to the crop (e.g. same class) (Bárberi, 2003). This eventually results in a more homogeneous weed community, with more dominant and troublesome species as it would be in a diverse cropping system (Cardina et al., 1998; Bárberi, 2003); 2) in consequence of repeated use of the same cultural practices over several years, the tolerance of weeds to the direct control methods (e.g. chemical weed control) is likely to develop and spread more quickly (Bárberi, 2003).

Sowing date confirmed as well its influence on the probability of resistance occurrence in silky bent grass populations. Our results revealed that early sowing of the winter crop increases the risk of herbicide resistance when compared to later sowing dates. Studies have shown that weed species characterized by early germination are favored by early sowing. Hartzler (2000), for example, documented the correlation between increase in the occurrence of giant ragweed (*Ambrosia trifida* L.) in the cropping regions of the U.S.A. and move to earlier sowing dates of maize and soybean; this was consequent to weed seedling emergence after planting, which results in reduced effectiveness of control measures in both pre- and post-emergence. As silky bent grass is a weed notoriously characterized by a short dormancy period and early emergence, the anticipation of winter crop sowing date can play a decisive role in promoting recurrence of infestations, therefore increasing the risk for selection of herbicide-resistant populations. This problem can be particularly relevant in years with favorable weather conditions which promote high seed germination rates, high population densities and ultimately higher weed competition ability.

Also conservation tillage practices resulted in a more than two-fold increased risk of resistance emergence in silky bent grass compared to conventional tillage situations (e.g. mouldboard ploughing). It has been widely documented that low soil disturbance systems can cause a shift of the weed flora towards a troublesome composition (Bárberi, 2003). In the case

of winter cereal cropping systems, for example, minimum tillage practices can promote the dominance of grasses with low-dormant seeds (e.g. *Alopecurus myosuroides*, *A. spica-venti*, *Bromus* spp.) within only few years (Bárberi, 2003). Consequently, annual grass weeds, especially those whose seed anatomy enables easy wind dispersion, are becoming more prevalent since the adoption of conservation tillage practices (Froud-Williams et al., 1981; Gill et al., 1987). Furthermore, inappropriate use of chemical inputs (i.e. herbicides) for the control of such weeds acted as an additional selection factor that speeded up the establishment and spread of herbicide-resistant populations.

Lastly, high silky bent grass densities (>40 plants m^{-2}) proved to increase the probability of resistance occurrence. In accordance with several sources, weed density can have a relevant influence on the rate of herbicide resistance development (Murphy and Lemerle, 2006). Beside the direct influence of this factor on resistance evolution (i.e. higher weed competition ability, higher seed production and faster spread, reduced herbicide penetration into the plant foliage), populations density also correlates with soil tillage, as fecundity and seed bank longevity are strictly dependent on the tillage method used. Studies have shown that weed density can be reduced by alternating tillage methods (e.g. mouldboard ploughing and tine harrowing) compared with situations in which the same tillage system is used year after year (Bárberi, 2003). Therefore, the high silky bent grass densities may be the consequence of the adoption of conservation tillage. Overall, this factor resulted in silky bent grass being twice as prone to evolve resistance in high density patches as in areas with low weed density.

The role of soil texture in the probability of resistance occurrence in silky bent grass could not be statistically confirmed. Although the preference of this weed for light-textured soils (i.e. sandy soils) has been widely documented, most infested fields were characterized by a middle soil texture. Light soils and heavy soils (e.g. clay soils) were under-represented. This underlines once more the role of conservation tillage in the spread of this weed as a consequence of the permanence of seeds in the upper soil layers. Conventional tillage systems, such as mouldboard ploughing, would be highly beneficial in containing silky bent grass infestations. In fact, the inversion of soil layers after the light induction of the seeds (which is necessary for germination) will result in impaired emergence of the deeply buried seedlings ('fatal germination') and therefore in reduced weed density and more effective control (Davis and Renner, 2007).

In conclusion, this study confirmed the significant role of rotation management, sowing date, soil tillage and weed density in the occurrence of herbicide resistance in silky bent grass. According to these results, growers should focus more effort on preventing the evolution and

spread of herbicide-resistant populations. The rotation of crops having different growing periods is a mandatory management measure for ‘breaking’ the regular life cycle of the weed and for delaying the selection of resistant plants. This should be corroborated by measures aimed at keeping the population density as low as possible, by applying conventional tillage methods (i.e. ploughing) to suppress seedling emergence from deep soil layers and by enhancing the competitive ability of crops (e.g. higher seed density). Finally, early sowing of winter crops should be avoided when dealing with silky bent grass infestations, as delaying the sowing date would facilitate weed suppression by seedbed preparation prior to planting. These measures, adequately combined together, will provide growers with useful tools for preventing and containing the spread of silky bent grass in arable lands and will result in decreased likelihood of resistance emergence. Strategies relying solely on rotation of herbicide modes of action, although undoubtedly effective, will not ultimately result in successful long-term management of silky bent grass in intensive cropping systems.

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Chapter V

Two-year investigations on herbicide-resistant silky bent grass (*Apera spica-venti* L. Beauv.) populations in winter wheat – population dynamics, yield losses, control efficacy and introgression into sensitive population

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5. Two-year investigations on herbicide-resistant silky bent grass (*Apera spica-venti* L. Beauv.) populations in winter wheat – population dynamics, yield losses, control efficacy and introgression into sensitive population

Abstract

In this study, the results of two-year investigations (2008/2010) on herbicide resistance in silky bent grass (*Apera spica-venti*) populations are presented. Silky bent grass populations A and B were sown in winter wheat plots at the ‘Ihinger Hof’ research station (Southern Germany) in October 2008. Whole-plant bioassays conducted in the greenhouse before setting up field experiments revealed that population A was strongly resistant to acetolactate synthase (ALS)-inhibitors, whereas population B was sensitive to these herbicides. Plots were treated with isoproturon, fenoxaprop-P-ethyl, sulfosulfuron and meso-iodosulfuron at the recommended field rate. One treatment remained unsprayed. It was found that the average number of silky bent grass panicles was significantly higher in the second year (2010), with 343 panicles m^{-2} in the untreated control compared to the 44 panicles m^{-2} in the first year (2009). Efficacy of both ALS-inhibitors was significantly reduced in the resistant population compared to the sensitive population. Silky bent grass plants surviving treatments with ALS-inhibitors produced the same number of seeds as untreated plants. It was found that germination rate of seeds from the resistant population was more than threefold higher than in the sensitive population. Grain yield was equal in all treatments and populations in 2009. In 2010, applications of isoproturon and fenoxaprop-P-ethyl resulted in higher grain yields (4.6 t ha^{-1}) when compared with the plots treated with sulfosulfuron and meso-iodosulfuron (3.9 t ha^{-1}) and the untreated plots (4.3 t ha^{-1}). However, these differences were not statistically significant. The sensitive population of the second generation (2009/2010) was approximately 20% more tolerant to ALS-inhibitors than the sensitive population of the first generation (2008/2009), which indicates introgression of herbicide resistance traits already after one year. These results clearly show that herbicide resistance in silky bent grass is likely to spread rapidly and can potentially result in significant economic losses. Therefore, management strategies need to be developed and tested to prevent and overcome the further spread of herbicide resistance in European cereal production systems.

Key words: herbicide resistance, ALS-inhibitors, yield losses, bioassays

Chapter VI

General discussion

6. General discussion

Resistance to herbicides in weed populations is an evolutionary process that affects agricultural fields worldwide. The relevant increase in reports on reduced herbicide performance observed since the massive adoption of chemical weed control demonstrates that the improper use of this powerful tool leads undeniably to negative consequences in the long term.

Special committees, working groups, and task forces were formed over the course of the years with the aim of facing agricultural problems associated with the rise of herbicide-resistant weeds on a global scale (Holt, 1990). Although much has been achieved in the understanding of whys and wherefores of herbicide resistance, the complexity of the evolutionary dynamics underlying this phenomenon still needs further attention.

In this work, investigations on herbicide resistance in *Apera spica-venti* populations were carried out at three main levels, namely greenhouse, laboratory and field. Greenhouse experiments (i.e. whole-plant pot assays) were conducted for the purpose of verifying actual herbicide resistance in hundreds of suspected *A. spica-venti* populations from different European countries under controlled conditions. Laboratory experiments (i.e. molecular genetic investigations) directly followed whole-plant assays to clarify the mechanisms underlying resistance in field-selected populations, particularly at the ALS target site level. Finally, field experiments were carried out over the course of two years by sowing herbicide-resistant and susceptible populations in winter wheat plots with the aim of comparing results with those obtained from the whole-plant assays and therefore ascertaining the reliability of greenhouse experiments for a safe diagnosis of herbicide resistance in suspected weed populations.

In this section, the main results of this work are summarized, reviewed and discussed, and the point of view of the author on the future development of herbicide resistance in European arable lands is given.

6.1 Greenhouse experiments

Greenhouse assays proved a powerful and practical tool in studies aimed at investigating the rate of sensitivity of weed populations to different herbicides (Streibig, 1988), thus providing an extensive amount of information that can lead to reliable results. However, the interpretation of the outcomes of experiments conducted under controlled conditions has to be taken ‘with a pinch of salt’ in consequence of some limitations that characterize greenhouse experiments compared to the field situation: 1) the controlled conditions (e.g. temperature, relative humidity, illumination etc.) under which greenhouse experiments are normally carried out are

impossible to obtain in the field situation, where countless variables can affect plant fitness, competition ability and herbicide performance; 2) greenhouse assays are carried out in absence of the crop, and this prevents the estimation of important parameters at the field level, such as interactions of weed populations with crop performance, eventual allelopathic effects etc.; 3) under greenhouse conditions, herbicides are normally applied when most of the seedlings are in the same growth stage (2-3 leaves). On the contrary, under field conditions weeds can emerge over long periods of time, even over more seasons (e.g. *A. spica-venti* can emerge from autumn until spring). This results in the seedlings being in different growth stages at the time of herbicide application; 4) the duration of greenhouse assays (~5-6 weeks) does not enable the estimation of parameters related to population dynamics, such as weed recovery after herbicide treatment, competition ability in different growth stages, seed production, germination capacity and so on.

Due to these main reasons, we conducted field experiments. In this way, we could test the results obtained from the greenhouse assays against more realistic and practical conditions. Our findings indicate that if all the above-mentioned limitations are carefully taken into account, it is still possible to rely on the outcomes of the greenhouse assays.

Results from the single- and fourfold-dose assays conducted on over 350 *A. spica-venti* populations collected across five European countries (Germany, Poland, the Czech Republic, Switzerland and Sweden), showed a prevalent resistance to ALS-inhibiting herbicides in more than 250 populations. All of them (~70% of the tested populations) were rated resistant to at least one ALS-inhibitor (i.e. flupyr-sulfuron-methyl or chlorsulfuron, SUs) applied at the recommended field rate. These results also comprise further tests carried out during the year 2010 [in which another 108 populations were screened and two more herbicides (florasulam-pyrox-sulam and propoxycarbazone-Na) were included] that are not mentioned in the Chapter III of this thesis.

In contrast to flupyr-sulfuron-methyl and chlorsulfuron, which performed very poor control of *A. spica-venti* at the recommended field rate, treatments with meso-iodosulfuron (SU), sulfometuron-methyl (SU) and florasulam-pyrox-sulam (TP) showed higher efficacy under greenhouse conditions. The performance of propoxycarbazone-Na (SCT) and sulfosulfuron (SU) could be classified somehow intermediate between the worst case (flupyr-sulfuron-methyl and chlorsulfuron) and the best case scenarios (meso-iodosulfuron, sulfometuron-methyl, florasulam-pyrox-sulam). Resistance to ACCase- and PSII-inhibitors did not have relevance with regard to the number of screened populations.

Dose-response studies substantially confirmed the outcome of the single- and fourfold-dose assays. In particular, flupyr-sulfuron-methyl seems to be not anymore a feasible herbicide for the control of *A. spica-venti*. Resistance factors of up to >140 were estimated at the ED₉₀ dose (ED₉₀ resistant / ED₉₀ sensitive), which would mean, in an agricultural situation, that 140-fold the field rate (1400 g.a.i./ha instead of 10 g.a.i./ha) of this herbicide would be necessary to control 90% of the resistant population. Similar results have been obtained after treatment with chlorsulfuron in several Czech populations, with resistance factors at ED₅₀ greater than 200 (Hamouzová *et al.*, 2011). Unlike for ALS-inhibitors, treatments with fenoxaprop-P-ethyl resulted in much better control, with resistance factors at ED₅₀ ranging between 4 and 6 in only two cases and below 1 in the other cases (at ED₉₀ differences were statistically significant in two cases as well, but the biological significance of these observations is negligible). Results from the digital image analysis for the quantification of biomass reduction after treatment with the herbicide sulfosulfuron showed the same tendency observed in the other greenhouse experiments. Treatments with sulfosulfuron, as expected, could significantly affect canopy cover and dry weight of the sensitive standard but not of the rest of the populations. On the other hand, treatments with fenoxaprop-P-ethyl again showed to be rather effective, with a significant reduction in canopy cover and dry weight observed in all tested populations. The strong correlation of dry weight with canopy cover demonstrated the appropriateness of this parameter for the estimation of biomass reduction following herbicide exposure. Therefore, canopy cover could represent a reliable and valid alternative to the dry weight assessment (which is notoriously a time-consuming and labour-intensive operation) for quantifying herbicide resistance in suspected weed populations. Nevertheless, this new approach is still at its initial developmental stage, and further efforts are needed in order to implement this promising method.

Overall, the outcomes of the greenhouse assays evidenced that ALS-inhibitors are resistance-prone herbicides, in line with the number of documented weed species worldwide (over 100) whose populations have evolved resistance to these compounds. In addition, ALS-inhibitors (especially SUs) have been and are still extensively used for the control of *A. spica-venti* in agricultural fields. This clearly explains the high number of resistant populations observed within the whole-plant assays. In fact, the strong selective force exerted by repetitive application of these herbicides can shift the selection in favour of resistant individuals within only few years (Heap, 1997).

On the other hand, applications of ACCase- and PSII-inhibitors are not so frequent for the control of *A. spica-venti*, which also explains the sporadic resistance observed to these modes

of action. Despite this, the first case of herbicide resistance in *A. spica-venti* was documented with regard to isoproturon (PSII-inhibitor) and cases of multiple resistance mechanisms including ACCase-inhibitors are being reported (Heap, 2011). This suggests that although rotation of herbicide modes of action is a highly recommended measure to adopt for delaying resistance evolution, it may also result in the selection for multiple resistance mechanisms, therefore representing more a palliative measure than a solution to the problem ('postponing the problem rather than solving it'). In this view, efforts should be focused on preventing the emergence of the problem rather than healing it, mainly by corroborating chemical weed control measures with proper farm management strategies.

6.2 Laboratory experiments

Laboratory work proved essential for the characterization of resistance at the genotypic level. In fact, it enables the identification of resistance mechanisms in a more precise and reliable way compared to the greenhouse assays (Kaundun & Windass, 2006). Although the present study was mainly focused on target site-based resistance (i.e. SNPs occurring within specific gene domains), several methods have been described for the detection of non-target site resistance as well. McIntyre, for example, described the use of radioactive-labelled (^{14}C) herbicides for translocation studies in *Pteridium aquilinum* L. Kuhn already at the beginning of the 60's (McIntyre, 1962). Since then, plenty of studies have followed, many of which were aimed at investigating the translocation of systemic herbicides upon uptake in both sensitive and resistant weed biotypes (Preston & Wakelin, 2008).

This method is able to provide accurate results and enables to 'trace' the route taken by herbicidal compounds with highest precision. However, the necessity to work with radioactive substances and therefore the high costs of these experiments represent two big disadvantages that significantly restrict the field of application of this technology to only few laboratories. Other methods, not as direct and informative as the above-mentioned one, are based on the detection of herbicide-metabolites (i.e. the products resulting from herbicide detoxification by enzymatic complexes) via High Performance Liquid Chromatography (HPLC) and combined Liquid Chromatography/Mass Spectrometry (LC/MS). Despite the evident disadvantages in terms of information that can be obtained compared to the previously described method (this technique does not allow to follow the dynamics of herbicide translocation upon uptake but relies on the detection of metabolites of known chemical structure in the plant), this approach does not involve health risks for the operators and can be carried out in almost every chemistry laboratory. In addition, the chemical structure of most herbicide-metabolites, as well as

the reactions by which they are produced, is known. In this way, it is possible to detect non-target site resistance mechanisms by the quantification of the amount of herbicides and their metabolites over time in both sensitive and resistant plants. This method has been developed and tested during the last three years in cooperation with the chemistry laboratory of our Institute. The first experiments, conducted on both sensitive and resistant *A. spica-venti* populations, produced encouraging results, which are indeed not presented in this work due to the lack of time necessary for the implementation of this approach.

With regard to the detection of target site resistance, a broad range of molecular methods have been established during the last years. In this work, CAPS markers were extensively used for the detection of SNPs at known amino acid positions within the five conserved ALS gene domains. Our results confirmed that target site resistance still plays a primary role in *A. spica-venti*. Besides the detection of previously documented mutations within the ALS gene (Pro₁₉₇ to Thr and Trp₅₇₄ to Leu), two novel amino acid changes were documented for the first time in three field-selected weed populations (Pro₁₉₇ to Asn and Arg₃₇₇ to His), one of which (Arg₃₇₇ to His) occurred at one position that had never been reported (Massa *et al.*, 2011). The role of this mutation in resistance to ALS-inhibiting herbicides is corroborated by the results of whole-plant and enzyme assays, but most importantly, is supported by recent specialized literature (See Chapter II). Delye *et al.* (2009), among others, indicate the Arg₃₇₇ residue as both constitutive of ALS active site and critical for herbicide binding. According to this and other sources (Duggleby *et al.*, 2008), positions Gly₁₂₁, Ala₁₂₂, Val₁₉₆, Pro₁₉₇, Met₂₀₀, Ala₂₀₅, Lys₂₅₆, Val₅₇₁, Ser₆₅₃ and Gly₆₅₄ are critical for herbicide binding but they do not constitute ALS active site, whereas positions Phe₂₀₆, Met₃₅₁, Asp₃₇₆, Arg₃₇₇, Met₅₇₀ and Trp₅₇₄ are both constitutive of ALS active site and critical for herbicide binding (Delye *et al.*, 2009). So far, mutations at positions Ala₁₂₂, Pro₁₉₇, Ala₂₀₅, Asp₃₇₆, Trp₅₇₄, Ser₆₅₃ and Gly₆₅₄ have been documented in several weed species, with the His mutation at Arg₃₇₇ reported only for *A. spica-venti* (Massa *et al.*, 2011). For all other above-mentioned positions involved in herbicide binding, no mutations have been documented in field-selected weed populations. This suggests that further mutations impairing or reducing herbicide performance are likely to be selected in the near future, potentially threatening the effectiveness of ALS-inhibitors for weed control. In addition, cases have been reported on weed populations carrying more than one resistance-endowing mutation within the gene coding for the target site. Warwick *et al.* (2008), for example, documented double ALS-mutations at both Pro₁₉₇ and Trp₅₇₄ in 23 Canadian *Kochia scoparia* L. Schrad. plants as well as mutations at both Pro₁₉₇ and Asp₃₇₆ in 7 plants. In consequence of the different geographic provenance of the plants carrying these mutations, these

authors suggest a multiple, independent origin of target site resistance traits. Therefore, it is reasonable to assume that multiple mutations occurring within single genes are likely to be selected as well, especially in weed species characterized by cross-pollination and high genetic diversity such as *Alopecurus myosuroides*, *Apera spica-venti*, *Lolium* spp. etc. (Warwick *et al.*, 1987). Further work should be carried out in the laboratory with the primary aim of testing, validating, and implementing existing methods for a quick, inexpensive, safe and reliable detection of herbicide resistance at the genotypic level. In this way, a remarkable amount of information can be obtained on the dynamics and evolution of resistance mechanisms (e.g. segregation of resistant and wild type alleles, introgression of resistance traits into sensitive populations etc.). In this view, CAPS technology as well as other tools (e.g. gene expression studies) described in the previous sections will still play an essential role in studies aimed at genotyping herbicide-resistant weed populations and evaluating their spreading potential over time and space.

The same assumptions apply to non-target site resistance mechanisms, whose potential danger is even greater if the role of the crop is taken into account. In fact, the use of herbicides in crops with the ability to metabolize a broad spectrum of modes of action (mainly through the action of P450s) also results in a strong selection pressure in favour of weed individuals having the same ability (Powles & Yu, 2010). In this respect, the evolution of weed populations that are able to metabolize different herbicide modes of action, potentially including herbicides that were never applied, represents a real threat to the future of resistance management (Powles & Yu, 2010). This, in the author's opinion, may be one of the reasons for the observed increase in reports on multiple resistance mechanisms during recent years and underlines once more the narrowness of measures aimed exclusively at delaying resistance evolution by rotating herbicide modes of action. As already mentioned before, it would be highly auspicious that preventive measures, rather than curative ones, could represent the 'cornerstone' of a successful weed management on a global scale.

6.3 The influence of agricultural and biological factors on the risk of herbicide resistance in Apera spica-venti

The role of repetitive long-term application of herbicides with the same mode of action in the evolution of resistant weed populations has been widely documented (Holt, 1992; Park & Mallory-Smith, 2004). However, also agricultural management measures and biological factors are known to play a decisive role in the risk for resistance selection and spread (Murphy & Lemerle, 2006). For this reason, part of our studies was focused on the evaluation of the

influence of specific agricultural and biological factors (crop rotation, soil tillage, soil texture, sowing date of the crop and weed density) on the probability of resistance occurrence in *A. spica-venti* (See Chapter IV). In order to accomplish this task, a risk assessment model and a geo-referenced database for mapping the distribution of herbicide-resistant *A. spica-venti* populations in Europe have been developed. The geo-referenced database can represent a powerful tool and a rich source of information for studies aimed at evaluating the relationships between field history and resistance spread over time and space. The flexibility deriving from the multiple features of the database also enables its application in other fields beyond weed science, such as phytopathology, plant ecology, plant breeding etc. The risk assessment model can be employed in studies aimed at assessing the extent to which different variables (namely biotic and abiotic factors) are related with the development of resistance and therefore the corresponding risk associated with each variable. The use of predictive models should be stressed in the years to come; in this way, it will be possible to elaborate preventive strategies for effective and reliable weed management measures.

Results from the statistical analysis conducted on a sample of 263 *A. spica-venti* populations across Europe are in line with most existing literature and confirmed the significant role of all considered factors (with the exception of soil texture) in the risk of resistance emergence. In particular, crop rotation and sowing date were the factors that had the greatest influence on the model, followed by soil tillage and weed density. The non-significant role of soil texture in the risk of resistance emergence was not expected and somehow surprising, as *A. spica-venti* is notoriously a species displaying a preference for light-textured soils (i.e. sandy soils). This could be attributed to the fact that light soils were represented by a negligible minority of locations. On the other hand, this also indicates that the majority of locations reporting reduced herbicide performance were characterized by finer-textured soils and therefore that other factors (e.g. soil tillage), rather than soil texture, seem to have a more prevalent influence on the risk of resistance emergence. In this view, the adaptability of this species to different environmental conditions (not restricted to areas characterized by light soils) and therefore its colonizing potential are once more demonstrated.

For future studies, it would be interesting to conduct random surveys carried out by collecting seeds across a pre-defined sampling area including fields that are not 'suspect'. In this way, it would be possible to further increase the representativeness of the sample and therefore the significance of results. This aspect has not been considered in our studies, in which seeds were collected from fields where herbicides had not performed satisfactorily and resistance evolution was therefore assumed (targeted surveys).

Overall, our results underline the importance of appropriate management measures for the prevention of herbicide resistance selection in *A. spica-venti* populations and demonstrate that resistance management strategies relying only on rotation of herbicide modes of action are not sufficient to overcome the spread of this increasing problem in European arable lands. In particular, higher percentage of spring-sown crops in the rotations, conventional tillage practices (i.e. plough), delay in sowing date and low weed density are the key points that must be considered for minimizing the risk of resistance evolution in *A. spica-venti*.

6.4 Field trials

Field experiments were conducted over the course of two years (2008/2010) with the aims of validating results obtained from the greenhouse assays and evaluating the influence of several factors that are not reproducible under controlled conditions. Competition ability of weeds with the crop, seedling emergence over long time periods and ability to recovery after herbicide application are the most important parameters that cannot be estimated in the greenhouse or in the laboratory. Although experiments were set up by sowing seeds from ALS-resistant and susceptible *A. spica-venti* populations in winter wheat plots and therefore not conducted in fields infested with naturally-occurring populations, obtained results substantially confirmed the outcomes of the greenhouse assays. The ALS-inhibitor-resistant population was not effectively controlled by sulfosulfuron and meso-iodosulfuron even in presence of the crop, but was well controlled by fenoxaprop-P-ethyl (ACCCase-inhibitor) and isoproturon (PSII-inhibitor). These two non-ALS herbicides were included in the experiments as ‘positive controls’ to be sure that the lack of herbicide performance after treatment with ALS-inhibitors was not due to application errors.

Our results revealed that the plants from the resistant population efficiently recovered after herbicide treatment and no effects on general fitness could be observed, whereas the few surviving plants from the sensitive population showed a general reduction in growth, panicle size, seed production and germination ability. In particular, the low size of the plants that were not killed by herbicide treatment suppressed their competition ability toward the crop. Despite this, no statistically significant differences in terms of crop yields could be observed between treated and untreated plots in both sensitive and resistant populations. According to these findings and considering that *A. spica-venti* densities in the year 2010 was up to >400 panicles m⁻², it seems that the economic threshold of this weed, previously estimated at 10-30 plants m⁻² (Kötter, 1991), is overrated. Also Melander *et al.* (2008) recently reported yield losses of the order of 30% at *A. spica-venti* densities of ~200 plants m⁻². This could not be

confirmed in our studies, in which maximum yield losses of ~10% could be quantified at *A. spica-venti* densities of >400 panicles m⁻².

This great difference observed in results, together with the lack of statistical significance in grain yields between herbicide-treated and untreated control plots, indicates variability in weed-crop competition under different pedoclimatic conditions and therefore demonstrates the ambiguity of concepts such as ‘economic threshold’ and ‘biological threshold’, which instead revealed to be strictly dependent on the local environmental conditions. In addition, *A. spica-venti* populations were sown in winter wheat plots, as this weed is not common in the agricultural fields of Baden-Württemberg. This may have had an influence on the competition ability of the plants in consequence of the non-optimal conditions (e.g. lower rainfall rates and lower temperatures in autumn) for the development of this weed. However, the relevant increase in weed density m⁻² observed in the second year (2010) clearly indicates that *A. spica-venti* possesses high colonization ability through the constitution of a dense seed bank already after one year (2009), in which a maximum of only 70 panicles m⁻² was counted. In fact, it seems unlikely that the high number of panicles counted in the second year could be only attributed to the germination of the seeds sown in that year. If it had been so, at least a similar number of panicles would have been counted also in 2009. In this respect, and in the author’s opinion, this study indicates that the real danger of *A. spica-venti* lies in its ability to establish, adapt and spread very quickly due to its peculiar anatomical-morphological and biological characteristics that have been discussed in the previous sections of this work.

With regard to the non-significant differences in terms of grain yield, it should be kept in mind that statistical significance is not always a reliable parameter for the interpretation of reality. In this view, any observed phenomenon can have a ‘biological significance’, or an ‘economic significance’, or still a ‘social significance’ even in the absence of statistical significance, and *vice versa*. In this particular case, for example, although a 10% grain yield loss resulted to be statistically not significant, it does not follow that farmers would consider this amount negligible. On the contrary, 10% yield losses should be already considered a significant economic loss.

The high fitness level observed in the resistant population after herbicide application proved once more that these plants were able not only to complete their life cycle, but also to set plenty of vital seeds from which an increasing amount of resistant plants will emerge year after year. It is also important to stress that the alleles regulating the phenotypic resistance response are dominant or semi-dominant in most cases, and this enables the rapid introgression of these traits into sensitive plants.

This seems to have occurred already after the first year (2009), in which plants from seeds harvested in the sensitive plots showed an increased tolerance to ALS-inhibitors (~20%) when compared with the sensitive plants sown in 2008. Of course, the closeness between the plots may have enhanced outcrossing between resistant and sensitive plants to a greater extent than in a situation in which populations are much more unevenly distributed over larger areas. Also, not all the increase in tolerance to ALS-inhibitors may derive from outcrossing. A certain amount of seed transfer from one plot to the other may also have occurred by wind and water or during seedbed preparation. Further studies should be conducted in order to verify these results and evaluate the goodness of the experimental approach (i.e. no naturally-occurring *A. spica-venti* populations).

6.5 Conclusions and future prospects

The present work was focused on investigations of herbicide resistance in *A. spica-venti* populations at the greenhouse, laboratory and field level. The outcomes of the present work will help to define clear guidelines for a safe and effective diagnosis of resistance in suspected weed populations at both the phenotypic and genotypic level. Furthermore, the screening of the ALS gene from target site resistant populations confirmed that amino acid substitutions occurring at positions that are critical for herbicide binding and for ALS activity still represent one of the main resistance mechanisms in *A. spica-venti*. Since inheritance is determined by single genes and is mostly a semi-dominant, nuclear trait, a rapid selection of resistant alleles is likely to occur under intense and prolonged selection pressure consequent to repetitive one-sided herbicide treatments. The selection for further mutations (and eventually multiple mutations) occurring at key positions of the genes coding for the herbicide target sites seems to be a highly probable scenario in the near future. New technologies based on the use of genetically modified crops (genetically modified organisms, GMOs) have been extensively employed in agriculture during the last decades. The principle of these technologies implies the introduction into the crops of specific transgenes associated with favourable characteristics, such as resistance to herbicides, pathogens, insects, low temperatures, as well as increase in crop performance (i.e. productivity). The system Roundup Ready® is the most representative example. Roundup Ready® crops (e.g. soybean, maize, alfalfa, rapeseed, sugar beet) are resistant to Roundup® (glyphosate), a non-selective, systemic herbicide used for the control of broad-leaved and grass weeds in intensive cropping systems. Resistance is induced through the insertion of resistance genes from different organisms (e.g. bacteria) with the purpose of reducing treatments with pre-emergence herbicides (with high residual activity and therefore

more dangerous for the environment) in favour of post-emergence herbicides (i.e. glyphosate, quickly inactivated by binding to soil particles or degraded by the telluric microflora) without risks for the crop plants. A more recent technology, mostly in use for winter wheat and rice (Clearfield®), implies the induction of resistance to ALS-inhibitors (IMIs) by traditional mutagenesis without the insertion of exogenous genetic material. The biggest advantage of this technology, besides not being based on the use of genetically modified organisms, lies in the selective control of weed species characterized by high genetic similarity to the crop (e.g. *Aegilops* spp. with wheat, *Oryza* spp. with rice etc.). In this way, herbicides such as imazamox (Beyond®), highly effective and extensively used in the USA for the control of *Aegilops cylindrica*, *Bromus tectorum*, *Avena* spp. and other grass weeds, can be applied in IMI-resistant winter wheat fields with no risk of crop injury (Ball *et al.*, 1999; Dahmer *et al.*, 2002; Rainbolt *et al.*, 2004).

The spread of technologies based on the use of genetically engineered crops (Roundup Ready®) or traditional mutagenesis (Clearfield®) seems to be one of the main trends of modern agricultural assets and is likely to become very popular in the years to come. However, several concerns are associated with this approach. The main one is the risk of a rapid resistance evolution in weed species that are effectively controlled by the herbicides to which also the crops are resistant. In the case of the Clearfield® technology, for example, an increase in the use of ALS-inhibiting herbicides in IMI-resistant winter wheat fields can be assumed within the next years, especially if a prevailing presence of this crop is foreseen in the rotation (Rainbolt *et al.*, 2004). The same situation occurred over two decades ago with regard to ALS-inhibitors used for the control of broad-leaved weed species (Mallory-Smith *et al.*, 1990). Therefore, in the author's opinion, the spread of this technology on a global scale will definitely not result in an improvement of herbicide performance and resistance management in the long-term perspective. As already mentioned several times throughout this work, *a priori* strategies (compatibly with the market trends) represent the only practicable way to contain the spread of herbicide resistance in *A. spica-venti* across European arable lands. Nevertheless, it is important to underline that chemical control options have played a decisive role in crop protection since their appearance in the market and will continue to serve as a powerful tool for affordable weed management if their usage will be corroborated by strategies aimed at preventing the establishment and spread of resistant populations. This is particularly important for weed species (e.g. *A. spica-venti*) whose pollen (and seeds) can be transported by wind over long distances. Furthermore, since the cultivated species of highest economic importance belong to the Poaceae family (i.e. maize, wheat and rice), the author assumes with

a fair degree of confidence that herbicide-resistant grass weeds will outnumber broad-leaved weeds in the next years. This especially applies to continuous cropping, which tends to favour and select the species within the weed flora that share phenological and physiological traits with the crop (Bárberi, 2003; Murphy & Lemerle, 2006).

In conclusion, the future of weed and resistance management will strongly depend on the future trends of the global agricultural assets. The actual situation, mainly consisting of intensive cropping systems heavily relying on chemical weed control and underestimating the importance of farm management strategies of proven efficacy, will not lead to a significant containment of this problem. Furthermore, the absence of new modes of action available in the market represents an obstacle that will limit the choice of the farmers relying exclusively on herbicides for weed control.

On the other hand, a certain degree of ‘extensivization’ of cropping systems through the partial restoration of diversity at the farm level (e.g. long rotations and well-balanced alternation of crop species, diversified production, grazing), together with the adoption of preventive and integrated weed management strategies, will presumably slow down and contain to a significant extent the spread of herbicide resistance across arable lands.

Summary

Zusammenfassung

7. Summary

Weeds belong to the main biotic yield-limiting factors in agricultural fields worldwide. Since the introduction of herbicides in the global market more than six decades ago, agroecosystems have been increasingly characterized by a strong reliance on herbicides for profitable weed management. However, the selection pressure exerted on weed populations by long-term application of herbicides with the same mode of action has imposed selection for resistance within several weed species.

The reduction in sensitivity of herbicide-target sites (target site resistance) is one of the most frequent mechanisms of resistance. It implies the alteration of the target enzymes that impair or reduce proper herbicide binding. These alterations result from point mutations (Single Nucleotide Polymorphisms; SNPs) occurring within conserved regions of the genes coding for the target enzymes. Nowadays, several weed species have evolved resistance to herbicides. *Apera-spica-venti* (L.) Beauv. represents one of the most abundant annual grass weeds in autumn-sown crops of Central and Eastern European arable lands. Since the first report on herbicide-resistant *A. spica-venti* populations in 1994, several new cases have been documented. Therefore, it is assumed that this species is rapidly evolving resistance to herbicides.

The main objectives of the present work were:

1. The development of reliable testing procedures under greenhouse conditions for the verification of herbicide resistance in suspected *A. spica-venti* populations;
2. The quantification of resistance in silky bent grass at different herbicide doses using dose-response assays and digital image analysis;
3. The elucidation of the mechanisms underlying resistance in field-selected *A. spica-venti* populations at the molecular genetic level through laboratory experiments;
4. The evaluation of the influence of agricultural and biological factors on the occurrence and spread of herbicide-resistance in *A. spica-venti* populations through the use of statistical modeling;
5. The development of a geo-referenced database for documenting the spatial and temporal distribution of herbicide-resistant *A. spica-venti* populations in Europe;
6. The verification of the results obtained from the greenhouse assays under realistic field conditions and the detection of the introgression of herbicide resistance traits into the sensitive population after two generations.

The main resistance testing procedure for *A. spica-venti* consisted of growing plants in the greenhouse (in potting compost) from seeds collected in the suspect fields and spraying them with herbicides. Applications were carried out with an automated precision sprayer either at a single dose or at a range of doses. Herbicide efficacy was then assessed at 15 and 30 days after treatment by direct comparison with untreated controls. Results from the greenhouse assays showed that the majority of suspected *A. spica-venti* populations (~70%) have indeed evolved resistance to herbicides, particularly acetolactate synthase (ALS)-inhibitors.

Dose-response assays conducted on field-selected populations revealed resistance factors at the ED₉₀ (ED₉₀ resistant / ED₉₀ sensitive) of up to 140 after treatment with flupyr-sulfuron-methyl, thus indicating that *A. spica-venti* is a resistance-prone grass weed. A novel method for quantifying resistance based on the assessment of percent canopy cover using digital image analysis has been developed in the greenhouse to provide a potential alternative to the labour-intensive and time-consuming dry weight assessments.

Laboratory experiments conducted on over 70 ALS-resistant *A. spica-venti* populations revealed that target site mutations were responsible for the observed resistance. Cleaved Amplified Polymorphic Sequences (CAPS) marker analysis and sequencing of the amplified 3' and 5' ends of the ALS gene by Rapid Amplification of cDNA Ends (RACE) revealed the presence of previously known as well as novel mutations endowing resistance to ALS-inhibitors.

The development of a risk assessment model allowed the evaluation of the influence of specific agricultural and biological factors on the probability of resistance occurrence in *A. spica-venti* populations. Results showed that a high percentage of winter crops in the rotation (>75%), together with reduced or no-till practices, early crop sowing and high population density significantly increase the risk of resistance emergence. An internet-based geo-referenced database was developed to document and analyze the spatial and temporal distribution of herbicide-resistant *A. spica-venti* populations in Europe.

Finally, field experiments were carried out over the course of two years (2008/2010) by sowing ALS-resistant and susceptible *A. spica-venti* populations in winter wheat plots. Results substantially reproduced the tendency observed in the greenhouse, with resistant plants surviving herbicide application, completing their life cycle and setting vital seeds. Resistant populations also showed a significantly higher seed germination rate when compared with the sensitive population. However, maximum yield losses of only ~10% could be observed at *A. spica-venti* panicle densities of >400 m⁻². Greenhouse bioassays conducted at the end of the first year with plants from seeds collected in the sensitive plots showed an increase in toler-

ance to ALS-inhibitors of ~20%, thus suggesting introgression of herbicide resistance traits into the sensitive population already after one year.

The outcomes of this work add knowledge to the understanding of the mechanisms underlying resistance to herbicides in *A. spica-venti* populations and provide weed scientists and consultants with useful tools for the reliable diagnosis and prevention of herbicide resistance in weed populations.

8. Zusammenfassung

Unkräuter gehören zu den wichtigsten Schadorganismen und gelten als ertragslimitierender Faktor im Ackerbau. Seit dem massiven weltweiten Einsatz von Herbiziden zur effektiven und leistungsfähigen Unkrautbekämpfung wurden Agrarökosysteme zunehmend von chemischen Maßnahmen abhängig. Der Einsatz von Herbiziden mit demselben Wirkungsmechanismus führt zu einem hohen Selektionsdruck auf verschiedene Populationen und damit zu herbizidresistenten Unkrautarten.

Die wirkortspezifische Resistenz zählt zu den am Häufigsten vorkommenden Resistenzmechanismen. Hierbei ist das Zielenzym des Herbizids so verändert, dass keine oder nur eine verringerte Bindung erfolgen kann. Diese Veränderungen im Zielenzym werden durch Punktmutationen (Single Nucleotide Polymorphisms; SNPs) innerhalb konservierter Domänen von Genen, die für den Zielort kodieren, hervorgerufen.

Heutzutage haben verschiedene Unkräuter sowie Ungräser Resistenzen gegenüber Herbiziden entwickelt. Der gemeine Windhalm (*Apera-spica-venti* L. Beauv.) gehört zu den wichtigsten Ungräsern im europäischen Getreideanbau. Seit dem ersten Resistenzfall in Windhalmpopulationen, dokumentiert im Jahr 1994, beobachten Landwirte eine zunehmend geringere Wirksamkeit von häufig verwendeten Herbiziden in Europa. Es wird angenommen, dass sich herbizidresistente Windhalmpopulationen mit der Zeit etabliert haben und sich somit das Problem der Herbizidresistenz in den nächsten Jahren verstärken wird.

Ziele dieser Arbeit waren:

1. Die Entwicklung geeigneter Testverfahren zum Nachweis der Herbizidresistenz unter Gewächshausbedingungen in verdächtigen *A. spica-venti* Populationen;
2. Die Quantifizierung der Resistenz bei verschiedenen Herbizidkonzentrationen mit Hilfe von Dosis-Wirkungsversuchen und digitalen Bildanalysen;
3. Die Aufklärung der Resistenzmechanismen in ausgewählten Windhalmpopulationen auf genetischer Ebene durch Laboruntersuchungen;
4. Die Evaluierung des Einflusses biologischer und Bewirtschaftungsfaktoren auf das Auftreten von Resistenzen in Windhalmpopulationen durch statistische Modellierung;
5. Die Entwicklung einer geo-referenzierten Datenbank zur Dokumentation der räumlichen Verteilung und der zeitlichen Verbreitung von herbizidresistenten Windhalmpopulationen in Europa;
6. Die Validierung der Ergebnisse aus den Gewächshausuntersuchungen unter Feldbedingungen und die Untersuchung zur Introgression von Resistenzmerkmalen in eine sensitive Population über zwei Generationen hinweg.

Bei dem Haupttestverfahren (Biotests) wurden *A. spica-venti* Samen von Resistenz verdächtigen Feldern gesammelt, die Pflanzen im Gewächshaus angezogen und im Anschluss mit Herbiziden gespritzt. Die Behandlung, bei der einzelne oder mehrere Herbizidkonzentrationen appliziert wurden, erfolgte in einer automatisierten Spritzanlage. Die Wirksamkeit der Herbizide wurde durch den direkten Vergleich mit den unbehandelten Kontrollen 15 und 30 Tage nach der Behandlung evaluiert.

Die Ergebnisse der Gewächshausversuche bestätigten eine Resistenz gegenüber ALS-Inhibitoren in ca. 70% der getesteten Populationen. Dosis-Wirkungsversuche, die mit ausgewählten Windhalmpopulationen durchgeführt wurden, zeigten Resistenzfaktoren auf Basis der ED₉₀ (ED₉₀ resistant / ED₉₀ sensitiv)-Werte von bis zu 140 nach einer Behandlung mit ALS-Inhibitoren (flupyrsulfuron-methyl), was auf eine starke Tendenz für eine Resistenzentwicklung gegenüber diesen Herbiziden hinweist. Ein neues Verfahren, welches auf der Bestimmung des Deckungsgrades mit Hilfe der Digital Image Analysis zur Quantifizierung der Resistenz basiert, wurde als Alternative zu der zeit- und arbeitsaufwändigen Trockengewichtbestimmung entwickelt.

Laboruntersuchungen von über 70 ALS-resistenten Windhalmpopulationen ergaben, dass wirkortspezifische Mutationen für die beobachteten Resistenzen verantwortlich sind. Cleaved Amplified Polymorphic Sequences (CAPS)-Markeranalysen und Sequenzierung der amplifizierten 3' und 5' Enden des ALS-Gens durch Rapid Amplification of cDNA Ends (RACE) bestätigten das Vorhandensein von bekannten sowie bisher noch nicht dokumentierten zur Resistenz führenden Mutationen.

Mit Hilfe der Entwicklung eines Risk Assessment Models konnten wir die biologischen und Bewirtschaftungsfaktoren, die zu einem Auftreten des Resistenzproblems in Windhalm führen, evaluieren. Die Resultate ergaben, dass ein hoher Prozentsatz von Winterungen in der Rotation (>75%), zusammen mit reduzierter Bodenbearbeitung, Frühsatterminen und hohe Populationsdichten das Auftreten von Resistenzen signifikant verstärken. Zur Dokumentation und Analyse der räumlichen und zeitlichen Verbreitung der ALS-Resistenz von *A. spica-venti* Populationen in Europa wurde eine internetbasierte georeferenzierte Datenbank entwickelt.

Schlussendlich wurden Feldversuche mit ALS-resistenten und sensitiven Windhalmpopulationen über einen Zeitraum von zwei Jahren (2008/2010) in Winterweizen-Parzellen durchgeführt. Die Resultate bestätigten die Ergebnisse der Gewächshausversuche, in denen resistente Pflanzen ihren Lebenszyklus vollendeten und lebensfähige Samen produzierten. Im Vergleich zu den sensitiven zeigten die resistenten Populationen eine signifikant höhere Keimrate. Es wurde jedoch nur ein maximaler Ertragsverlust von 10% bei einer Windhalmdichte von >400

m⁻² beobachtet. Gewächshausversuche mit Samen von sensitiven Pflanzen die am Ende des ersten Jahres gesammelt wurden, zeigten eine Zunahme der Toleranz zu ALS-Inhibitoren von ~20%. Dies weist auf eine Introgression von Resistenzmerkmalen in die sensitive Population nach schon einem Jahr hin und bestärkt weiterhin die Befürchtung für ein baldiges, verstärktes Auftreten weiterer Resistenzen.

Die Ergebnisse dieser Arbeit führen zu einem besseren Verständnis über die zugrundeliegenden Resistenzmechanismen gegenüber Herbiziden (meist ALS-Inhibitoren) in Windhalmpopulationen und bieten Herbologen sowie Beratern verschiedene Möglichkeiten für die Detektion und Vorbeugung der Herbizidresistenz in Unkrautpopulationen.

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9. General References

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Erklärung

Hiermit erkläre ich, dass ich diese Dissertation selbständig angefertigt habe, nur die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet habe.

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