On the optimization of low dosage application systems: Improvement of dose advice and early detection of herbicidal effects

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Propositions

1. Pre-spraying weather conditions do have a strong effect on herbicide efficacy, mainly through their effect on herbicide uptake.
   This thesis

2. Acetolactate synthase inhibiting herbicides produce a rapid inhibition of photosynthesis.
   This thesis

3. Widespread use of transgenic herbicide resistant crops will result in an increased herbicide use in the long term.

4. Even minor tampering with nature can have serious consequences.

5. Careful nutrition is a key factor in preventive healthcare.

6. Researchers are not pragmatic enough.

7. If you cannot control the weeds, eat them!
   Prof. Bianco Vito Vincenzo, University of Bari

Propositions belonging to the PhD thesis of Ingrid Riethmuller-Haage:

On the optimization of low dosage application systems: Improvement of dose advice and early detection of herbicidal effects

Wageningen, 12 May 2006

Abstract

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Application of herbicides at rates below the recommended label dose has received considerable attention in recent years as it is a means of reducing overall herbicide use. To minimize the risk of inadequate weed control in these situations, the Minimum Lethal Herbicide Dose (MLHD) technology, which was specifically developed for the use of low rates of photosynthesis inhibiting herbicides, relies on an appropriate calculation of the optimum herbicide dose and on an early detection method of herbicidal effects to evaluate the efficacy of the treatment shortly after application. The study described in this thesis was undertaken to further improve the prediction of the optimum dose of photosynthesis-inhibiting herbicides and to explore the opportunities of expanding the MLHD-technology to acetolactate synthase (ALS) inhibiting herbicides.

The first part of the study examined the influence of pre-spraying weather factors on the efficacy of photosynthesis-inhibiting herbicides. The results put forward that pre-spraying weather conditions have a considerable effect on herbicide efficacy and that this effect is mainly through an effect on herbicide uptake. Herbicide-specific correlations between uptake and individual weather parameters were found. These correlations were non-species specific and indicate that inclusion of pre-spraying weather conditions in advisory systems that recommend on the use of reduced herbicide dose rates is recommendable.

In the second part of the study the extension of the MLHD technology to acetolactate synthase (ALS) inhibiting herbicides, particularly the development of an early detection method of herbicidal efficacy, was explored. Application of the ALS inhibiting herbicides metsulfuron-methyl on *Solanum nigrum* and *Polygonum persicaria* plants resulted in a progressive reduction in CO_2 fixation and the relative quantum efficiency of photosystem I (Φ_{PSI}) and photosystem II (Φ_{PSII}) electron transport, emerging a couple of days after herbicide application. Φ_{PSII} turned up to be one of the most promising and practical parameters to use when designing an early detection method for sensing the toxicity of metsulfuron-methyl. The loss of Φ_{PSII} was light-dependent and could be earliest detected at the base of the youngest leaf of treated plants.

Keywords: Acetolactate inhibiting herbicides, dose, herbicide efficacy, fluorescence, photosynthesis, photosynthesis inhibiting herbicides, uptake, weather, weed.

Contents

Abbreviations

ΔA_{820nm}	Parameter corresponding to complete oxidation of P700
Φ	Maximal apparent quantum yield (non-rectangular hyperbola)
Φ_{CO2}	Quantum efficiency of gross CO_2 fixation
Φ_{PSI}	Quantum efficiency of photosystem I electron transport
Φ_{PSII}	Quantum efficiency of photosystem II electron transport
$\Phi_{PSII560nm}$	Quantum efficiency of photosystem II electron transport measured at 560 nm
$\Phi_{PSII660nm}$	Quantum efficiency of photosystem II electron transport measured at 660 nm
θ	Curvature term of light response curve
+	Little sensitive herbicide × species combination
++	Normal sensitive herbicide × species combination
+++	Very sensitive herbicide × species combination
A	CO_2 fixation rate (μmol m^{-2} s^{-1})
A_{max}	Light saturated rate of CO_2 fixation (μmol m^{-2} s^{-1})
a.i.	active ingredient
ANOVA	Analysis of variance
b	Slope of the logistic curve at dose ED_{50}
c	Upper asymptote of the logistic curve (g)
CHEAL	*Chenopodium album*
[Chl]	Chlorophyll content
CV	Coefficient of variation
DAS	Days after Sowing
DAT	Days after Treatment
DNA	Deoxyribonucleic acid
DSS	Decision support system
DW	Dry Weight (g)
ED_{50}	Herbicide dose giving 50% response (e.g. 50% reduction of plant DW)
ED_{90}	Herbicide dose giving 90% response (e.g. 90% reduction of plant DW)
FL	Full Light
F_m	Dark adapted maximum fluorescence
F_{ss}	Relative fluorescence yield obtained under steady-state irradiance
F_v	Variable fluorescence
GR	Growth Rate (g day^{-1})
G. Rad.	Global radiation (KJ m^{-2} h^{-1})
I	Irradiance (μmol m^{-2} s^{-1})
J_{PSI}	Index of linear photosynthetic electron transport through PSI

J_{PSII}	Index of linear photosynthetic electron transport through PSII
k_e	Rate constant for $P700^+$ reduction by electrons coming from PQH_2 pool
L	Light conditions
LA	Leaf Area (cm^2)
LAR	Leaf Area Ratio (m^2 leaf kg^{-1} plant)
LSD	Least Significant Difference
LWR	Leaf Weight Ratio (kg leaf kg^{-1} plant)
MLHD	Minimum Lethal Herbicide Dose
OL	Oldest leaves
PAR	Photosynthetically active radiation
POLPE	*Polygonum persicaria*
PSI	Photosystem I
PSII	Photosystem II
PUL	Penultimate leaf
Q1	Quartile 1
Q2	Quartile 2
Q3	Quartile 3
RGR	Relative Growth Rate (d^{-1})
RH	Relative humidity (%)
RNA	Ribonucleic acid
SENVU	*Senecio vulgaris*
SLA	Specific Leaf Area (m^2 leaf kg^{-1} leaf)
SOLNI	*Solanum nigrum*
YL	Youngest leaf

Chapter 1

General introduction

Changes in crop production after 1960

In the second half of the last century, developments in cultivation techniques, mechanization and plant breeding have resulted in drastic improvements in crop productivity in Europe. Between 1960 and 2000, cereal yields have increased from 2.1 to 6.0 t ha^{-1}, maize yields from 2.5 to 6.0 t ha^{-1} and sugar beet yields from 33.6 to 63.3 t ha^{-1} (FAOSTAT data, 2005). These yield increases have also been due to higher levels of external inputs, particularly fertilisers and chemical crop protection agents.

Historians have traced the use of plant protection products to the time of Homer around 1000 B.C., but humans began to seriously develop and use modern plant protection products extensively during the second half of the 20th century. From the 2.27 million tons of plant protection products used in 2001 worldwide, herbicides accounted for the largest proportion of total use (40%), followed by insecticide use (25%) and fungicide use (10%) (EPA, 2004). That same year, about 0.33 million tons of these pesticides were used in Europe (Eurostat, 2005). Fungicides made up the largest pesticide group accounting for 41% of the total weight of active ingredients, followed by herbicides (39%) and insecticides (12%). However, the situation varies from one country to another due to differences in climatic conditions and type of crops grown. In southern European countries (France, Italy, Portugal, Spain, and Greece), where fungal diseases are the main problem, fungicides dominate. In France, Italy and Portugal fungicides accounted for more than 50% of the sales. Herbicides made up the largest group of pesticide sales for central and northern European countries. In the United Kingdom, herbicides and desiccants accounted for 69% of the total, whereas fungicides accounted for 18% and insecticides for 2%. In Denmark, Sweden and Finland, herbicides and desiccants accounted for more than 80% of the sales (Figure1).

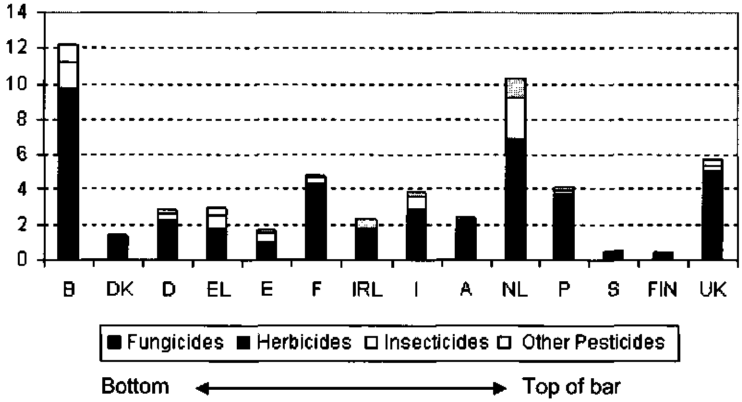

Figure 1: Pesticide use (kg) per hectare in 1996 (Eurostat, ECPA).

It is also noteworthy that The Netherlands, with its very intensive agriculture, was one of the countries with the highest pesticide use in kg ha^{-1}.

Strong drive to reduce pesticide use in Europe

When DDT became available for civilian use in 1945, there were only a few people who expressed second thoughts about this new miracle compound. However, by 1962, Rachel Carson exposed the hazards of the pesticide DDT in her book Silent Spring. Her research revealed the harmful side-effects of DDT: contamination of the food chain, risks of cancer and genetic damage and the extinction of species (Lear, 2005). Since then, public concerns about the side-effects of pesticides on the environment and on human health have increased (DeFelice et al., 1989; Kudsk, 1989). The rapid progress in analytical chemistry has made it possible to detect pesticides in very small concentrations, and as a consequence the public has been concerned about the presence of pesticides in the environment and in agriculture produce. Additional drivers to reduce the use of pesticides were the need to reduce the cost of the crop production and the rapid progress in the development of pesticide resistance.

The EU Fifth Environmental Action Programme (5EAP) set out a series of targets for the year 2000, including 'the significant reduction in pesticide use per unit of land under production, and conversion to methods of integrated pest control, at least in areas of importance for nature conservation'. In Denmark, Sweden and later The Netherlands, pesticide action plans, demanding reduced use and emission of pesticides, were passed through national Parliaments.

In October 2000, the 'Directive 2000/60/EC of the European Parliament and of the Council establishing a framework for the Community action in the field of water policy' (EU Water Framework Directive) was adopted. The purpose of this Directive is to establish a framework for the protection of inland surface waters (rivers and lakes), transitional waters (estuaries), coastal waters and groundwater. It is hoped that this Directive will encourage the European community to meet specific standards and that all aquatic ecosystems and, with regard to their water needs, terrestrial ecosystems and wetlands, meet 'good status' by 2015.

Dose optimization of herbicides in Europe

Herbicide labels contain the dose recommendations of the manufacturer and as agrochemical companies can be held liable by farmers in case control is not satisfactory, label recommendations tend to reflect worst-case conditions. Hence, it is not surprising that under optimum conditions, herbicide doses can often be reduced below label recommendations and still provide adequate control (Kudsk & Streibig, 2003). The use of optimized herbicide doses has been widely adopted by farmers in European countries such as Denmark, Sweden, Finland and The Netherlands.

Adjusting herbicide doses to the prevailing conditions in the field by taking into account, for example, both abiotic and physiochemical factors, is an overwhelming task to most end-users. Development of decision support systems (DDS) is one way to motivate farmers and agricultural contractors to adopt knowledge on herbicide dose optimization.

In Denmark a computer-based weed DSS 'PC Plant Protection' has been developed to achieve this task (Kudsk, 1999). The system provides farmers with herbicide solutions for specific fields, not only by specifying the most suitable herbicides, but also by suggesting specific doses on the basis of the weed flora and the spraying conditions. This DSS is currently under evaluation in Norway (Netland, 2005). In Sweden, decision support guidelines have been developed for spring cereals incorporating information on total weed density, competitive ability of the weed species, climatic conditions and crop vigour (Bostrom & Fogelfors, 2001). In Sweden the 1995/96 survey (Eurostat ECPA, 1996) showed a reduction in overall pesticide use of 46% in tonnes of active ingredient applied compared to 1990. The largest reduction was for fungicides and insecticides followed by herbicides (–40%). In 1995/96, low herbicide doses were used on more than 50% of the cereal area cultivated in Sweden.

The Minimum Lethal Herbicide Dose methodology

The Minimum Lethal Herbicide Dose (MLHD) methodology (Kempenaar et al., 2002; Ketel et al., 1996), specifically developed for photosynthesis inhibiting herbicides, has shown to be a decision support system leading to the use of herbicides at lower rates. The methodology allows the calculation of the minimum dose of a photosynthesis inhibiting herbicide that is just appropriate for killing the weeds in the field. A couple of days after application, photosynthesis related techniques based on fluorescence signals emitted by treated plants are used to verify whether or not the herbicide application will, ultimately, result in the death of the weeds. Under field conditions, particularly under low temperatures in spring, the visible effects of herbicide action are often only apparent one or two weeks after spraying, which is too long a delay in the event that a second application of herbicide is necessary. An early detection method permits a prompt second herbicide application in case of failure. This last element is of particular importance for ensuring that even though minimal doses of herbicides have been employed, there is a guarantee that the treatment will be successful in eliminating the weeds. Such a guarantee contributes to the adoption of this methodology by farmers, agricultural contractors and others (Kempenaar et al., 2004).

Outline of the thesis

The general objective of this study was to investigate whether or not there was a possibility for possible improvements of the Minimum Lethal Herbicide Dose (MLHD) methodology. The following sub-questions were asked:

- Would an extension of the MLHD, in which the advised dose would also be based on weather conditions, result in a further optimization of herbicide use, without lowering herbicide efficacy?
- Would it be possible to develop an early assessment tool to reliably predict, in the field, whether or not the application of acetolactate synthase inhibiting herbicides (ALS inhibitors), an increasingly important group of herbicides, is affecting weed plants?

Taking into account pre-spraying weather conditions

Several authors suggested that environmental conditions before, during and after herbicide application might be an important factor determining the efficacy of herbicides (Caseley, 1987, 1989; Kudsk, 1989; Kudsk & Kristensen, 1992; Lundkvist, 1997a). For that reason, weather factors appear to be a good basis for determining the appropriate reduced dose rate. Taking into account the weather component could be a useful addition in improving the prediction of the MLHD. Given the risk-avoiding character of the MLHD-methodology, and the uncertainty of weather predictions, pre-spraying weather conditions seems the most logical basis for helping improve the advised dose rate.

In the current research, presented in **Chapter 2**, special attention was paid to the effect of pre-spraying weather conditions on the weed plant growth rate and final herbicide efficacy. To this purpose, a number of pot experiments were conducted in which the foliar-applied herbicide metribuzin (photosystem II inhibitor, HRAC group C1) was sprayed on *Polygonum persicaria* plants grown under greenhouse conditions (2002) and field conditions (2003). In 2004, research was undertaken to determine the influence of pre-spraying weather conditions on herbicide uptake and efficacy for a combination of four weed species and two herbicides.

Develop an early assessment tool to predict the effects of ALS inhibitors on weeds

The acetolactate synthase inhibitors herbicides (ALS inhibitors, HRAC group B), were discovered in the mid-1970's and are an increasingly important group of herbicides. ALS inhibitors provide a broad-spectrum weed control at doses of 2-75 g ha^{-1}. Such low use rates have reduced the amount of chemicals applied to the field by a factor 100-1000 over conventional herbicides and it helped solving handling of the herbicide as well as application and container-disposal issues. These herbicides are widely used around the world because in addition to their relatively low use rates they have a limited environmental impact, a low mammalian toxicity, a high margin for crop safety, a large application window and a high efficacy (Green & Strek, 2001; Peterson, 2001). ALS inhibitors selectively inhibit acetolactate synthase (EC 4.1.3.18), which is the first common enzyme involved in chloroplastidic biosynthesis of essential branched chain amino acids (valine, leucine and isoleucine).

There are no published methods for predicting the effectiveness of the application of an ALS inhibitor, but changes in photosynthesis related parameters of treated plants have been observed. Photosynthesis related parameters are commonly used parameters and are used in areas like screening for environmental stress tolerance in plant breeding and production programs, in air pollution studies (Lichtenthaler & Rinderle, 1988; Odasz-Albrigtsen et al., 2000; Popovic et al., 2003), in studies on the toxicity induced by herbicides (Judy et al., 1991; Percival & Baker, 1991) and in studies on environmental stress such as chilling, freezing, heat stress and nutrient deficiency (De Groot et al., 2003; DeEll & Toivonen, 1999; Kingston-Smith et al., 1997, 1999).

The purpose of the first investigations, presented in **Chapter 3**, was to determine if and how long after application the photosynthetic apparatus of two common weed species were affected by an ALS inhibiting herbicide. Once it was clearly established that the photosynthetic apparatus of both weed plants was affected by an ALS inhibiting herbicide, a more detailed study, presented in **Chapter 4**, on the influence of the ALS inhibitor metsulfuron-methyl on the operation, regulation and organization of photosynthesis in *Solanum nigrum* was conducted. The potential use of the different photosynthesis related parameters for assessing the effects of metsulfuron-methyl on weed plants was investigated and the relevance of the different parameters for field use was discussed in **Chapter 5**. The effects of light intensity after application of the ALS inhibitors on the detection of Φ_{PSII} efficiency were discussed in **Chapter 6**. In **Chapter 7**, the results of four years of experimentation were combined and placed into perspective.

Chapter 2

Are pre-spraying weather conditions a major determinant of herbicide efficacy?

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Submitted

Abstract

To evaluate whether the effect of pre-spraying weather conditions on herbicide efficacy was mainly through an effect on plant growth rate, *P. persicaria* plants were exposed to different light intensities for one to four days before application of metribuzin. Specific leaf area, rather than plant growth rate or plant size, was the only parameter that correlated well with herbicide efficacy in both years of experimentation. The negative relationship between the ED_{50} and the specific leaf area puts forward that leaf characteristics might be an important determinant of herbicide efficacy, for instance through an effect on herbicide uptake. In the third year of experimentation, this hypothesis was confirmed as clear relations between uptake and herbicide efficacy were found for a combination of four plant species (*Solanum nigrum*, *Senecio vulgaris*, *Chenopodium album*, *Brassica napus*) and two herbicides (phenmedipham and bentazon). For phenmedipham, an apolar herbicide, uptake was negatively correlated with global radiation and positively correlated with relative humidity. For the polar herbicide bentazon exactly the opposite was found. These results were non-species specific. This research shows the importance of the sensitivity of herbicide × species combinations and indicates that including pre-spraying weather conditions in the establishment of the reduced dose rate advice is relevant.

Introduction

In 2000, herbicides accounted for 40% of pesticide use worldwide in terms of the volume of active ingredient (EPA, 2004). Increased concerns about environmental side effects of herbicides, development of herbicide resistance in weeds and the economic drive to reduce the cost of inputs have resulted in an increasing pressure on farmers to reduce the use of herbicides. The quantity of herbicides applied to crops can be reduced by cutting down the number of applications, by applying spot spraying rather than full-field applications or by using reduced dose rates. However, the reverse side of all of these tactics is that they increase the risk of inadequate control.

The Minimum Lethal Herbicide Dose (MLHD) technology (Kempenaar et al., 2002a; Ketel, 1996) is a decision support system developed in the Netherlands for photosynthesis-inhibiting herbicides which advices on the application of low dosages while minimizing the risk of inadequate control. Based on weed species and weed size it provides an estimate of the lowest dose of the herbicide needed to control the weed population. Photosynthesis measurements, conducted a few days after application, point out whether the herbicide application will ultimately result in the death of the weeds. This is of specific importance, as under field conditions in spring the visible effects of herbicide action are often only apparent several days after spraying, which is too long a delay in the event that a second application of herbicide is necessary. Early detection is an essential element of the MLHD-technology, as it provides a guarantee that even though minimal doses have been employed, the treatment will be successful in killing the weeds. Several authors suggested that environmental conditions before, during and after herbicide application might be an important basis for determining the reduced dose rate (Caseley, 1987, 1989; Kudsk, 1989; Kudsk & Kristensen, 1992; Lundkvist, 1997a). For that reason it seems useful to explore whether inclusion of a weather component could help improve the prediction accuracy of the MLHD methodology. Given the risk-avoiding character of the MLHD-methodology, and the uncertainty of weather predictions, pre-spraying weather conditions seem the most logical basis for helping to improve the advised dose rate. The current research explored whether pre-spraying weather conditions do have a clear and consistent effect on the efficacy of herbicides

Studies in controlled environments have shown that conditions that promote growth rate, such as temperature (Devine, 1989; Wanamarta & Penner, 1989), high relative humidity (Caseley, 1989; Kudsk et al., 1990) and water supply, improve the activity of many foliar-applied herbicides. The importance of weed growth rate for herbicide efficacy has been stressed by several authors, among them are Hammerton (1967), Coupland (1989) and De Ruiter (1999). Lundkvist (1997b) quantitatively demonstrated that, under Swedish field conditions, performance of herbicides applied under conditions that facilitated higher growth rates gave better results for the two tested herbicides [mixture of dichlorprop-P/MCPA (synthetic auxins, HRAC group O) and tribenuron-methyl (ALS inhibitor, HRAC group B) mixed with a wetting agent] when applied on a natural weed population. Lundkvist (1997a) also found that the most pronounced effects of weather could be ascribed to the day of application and the day before.

In this study, special attention was paid to the effect of pre-spraying weather conditions on herbicide efficacy. For this purpose, a number of pot experiments were conducted in three consecutive years. In 2002 and 2003, the combination of metribuzin and *Polygonum persicaria* was used to evaluate whether the effect of pre-spraying conditions on herbicide efficacy was mainly through an effect on plant growth rate. In 2004, combinations of four weed species (*Solanum nigrum, Senecio vulgaris, Chenopodium album, Brassica napus*) and two herbicides (bentazon and phenmedipham), which differed in polarity, were used to verify whether the effect of pre-spraying weather conditions was brought about through an effect on herbicide uptake.

Materials and methods

2002 Experiment

In 2002, a greenhouse pot experiment was conducted to assess the influence of plant growth rate on the activity of the foliar-applied herbicide metribuzin. Growth rates were manipulated by exposing the plants to two levels of light intensity. Seeds of *Polygonum persicaria* (ladysthumb) were germinated in a greenhouse (22°C / 18°C for day / night temperatures, RH 70% and a photoperiod of 12 hours) on a moistened potting soil and sand mixture (2:1). Eleven days after sowing (11 DAS) plants were transferred into 1 dm^3 pots, with one plant per pot. The transplants were grown in a greenhouse from 11 to 17 DAS with day / night temperatures of 18°C / 14°C, RH 70% and a photoperiod of 12 h provided by natural light supplemented with high-pressure mercury lamps. At 17 DAS, half of the *P. persicaria* plants remained under full light conditions (FL; treatments A and B), whereas the other half of the plants were put under reduced light conditions (RL; treatments C and D). The light intensity was reduced by 65 to 70% by placing two layers of cheese cloth 1 m above the top of the plants. The presence of cheese cloth did not significantly affect the light spectrum (data not shown). The experiment consisted of four blocks and within each block a full light and a shaded area were randomly assigned. Within each block, each treatment was represented by 88 plants. The plants remained in those conditions for 6 days. At

23 DAS, 8 plants of treatments A and B referred to as AB (23 DAS) and 8 plants from treatments C and D referred to as CD (23 DAS) were sprayed. Right after that, treatment B was transferred to reduced light conditions, whereas treatment C was transferred to full light conditions. Treatment A and treatment D remained under the same light conditions as before. From 24-27 DAS, 8 plants per treatment were selected daily and sprayed with an air-pressurized laboratory track sprayer delivering 400 litres ha^{-1} at 3.0 bar (1.2 mm Birchmeier nozzles, boom rate 3.5 km h^{-1}). Plants were sprayed with metribuzin (Sencor, Bayer CropScience), at a range of 8-herbicide doses (from untreated to recommended field dose; logarithmic scale). After the herbicide treatment all plants were put under full light conditions. Two weeks after spraying, plant dry weight (after at least 48 h at 70°C) was assessed.

Throughout the experiment, regular observations were made to determine leaf area (LI-3100, LI-COR LAMBDA Instruments Corporation, Lincoln, Nebraska, USA) and leaf and stem dry weight (after at least 48 h at 70°C). From 11 DAS to 23 DAS these observations were made every two days, whereas daily observations were made from 23 DAS to 27 DAS. Sample size on each observation day was 4×4 plants per treatment (A to D). Observations were used to estimate the relative growth rate (RGR) for plants growing in the different light environments. Additionally, leaf area ratio (LAR), leaf weight ratio (LWR) and specific leaf area (SLA) were calculated.

2003 Experiment

In 2003, a pot experiment was conducted to assess the effect of plant growth rate and plant size on the activity of the foliar-applied herbicide metribuzin. The trials were conducted in April / mid-May 2003 (trial 1), mid-May / June 2003 (trial 2) and mid-June / July (trial 3). For each trial, 3 batches of *P. persicaria* plants were sown at a-5-day time interval (sowing time 1, 2 and 3), to create plants differing in size. For trial III, only data of sowing time 1 and 3 were obtained due to poor establishment of the plants of the second sowing. Sowing procedure, initial growing conditions and greenhouse settings were similar to those in 2002. Eleven days after sowing, seedlings were transplanted and kept in the greenhouse for about 5 days to get an optimal establishment and a gradual adaptation to outside conditions (greenhouse settings: 18°C / 12°C for day / night temperatures, RH 70% and a photoperiod of 10 h). All plants of one trial were sprayed at the same time.

From transplanting to the beginning of the light treatment, pots were randomly put in 100 pots-trays and put outside on trolleys. Four days before application of the herbicide (beginning of the light treatment), half of the plants were put under full light (FL) and the other half under reduced light (RL) conditions. The light intensity was reduced by 65 to 70% by placing two layers of cheesecloth 1 meter above the top of the plants. During the light treatment the trays were put in a split plot design with four blocks (first split: light regime, second split: sowing time). Within each light regime and each sowing time, 8 weed plants were distributed randomly. *P. persicaria* plants were sprayed with metribuzin (Sencor, Bayer CropScience) with a range of 8-herbicide doses (from untreated to recommended field dose; logarithmic scale) and were placed under full light conditions after spraying. At application time, plants sown at sowing time 1, 2 and 3 were respectively 38, 33 and 28 days old. Spraying equipment was comparable to 2002. Two weeks after spraying, plant dry weight (after at least 48 h at 70°C) was assessed.

Plant dry weight was assessed 4 days before application of the herbicide and at spraying time, using 4×4 plants for each combination of sowing time and light condition. Leaf area, and leaf and stem dry weight (after at least 48 h at 70°C) were determined.

2004 Experiment

In 2004, an outside trial was conducted to sort out whether the main effect of pre-spraying weather conditions on herbicide efficacy was through an effect on herbicide uptake. Plastic trays of 0.25 m^2 were filled with a mixture of pot ground and sand (3:1). After flattening of the soil surface, seeds of four species, particularly *Solanum nigrum* (black nightshade), *Senecio vulgaris* (groundsel), *Chenopodium album* (fat hen) and *Brassica napus* (oilseed rape) were sown in trays at ca. one millimetre below soil surface. All trays were covered with a plastic sheet and put in the greenhouse with day / night temperatures of 22°C / 18°C, RH 70% and a photoperiod of 12 h to get an optimal emergence. From 14 to 20 DAS, plants were put outside the greenhouse. Few days before application of the herbicide, the number of weed plants per tray was thinned to 35 plants for *S. nigrum* and *S. vulgaris*, 20 plants for *C. album* and 15 plants for *B. napus*.

In order to assess whether pre-spraying weather conditions had an influence on uptake and herbicide efficacy, 6 groups of plants were raised with a one-week interval. Each group was sprayed at 3 weeks after sowing, from July 22 till August 26. Each group was sub-divided into three sub-groups and sprayed with (1) bentazon (Basagran, 480 g L^{-1}, BASF) a polar herbicide, (2) bentazon + 1% v/v of oilseed rape oil or (3) phenmedipham (Herbasan, 160 g L^{-1}, Bayer Cropscience) an apolar herbicide. Plants were sprayed with a range of 5-herbicide doses (from untreated to half recommended field dose; logarithmic scale), using a hand carried 2-m wide boom sprayer delivering either 400 litres ha^{-1} (bentazon) or 300 litres ha^{-1} (phenmedipham) at 2.0 bar (Teejet XR11004VS nozzles, 4 km h^{-1}). The experiment consisted of a completely randomized design with 240 trays (4 block × 4 species × 3 herbicides × 5 doses) per group.

After spraying, trays were randomly put into a greenhouse to standardize as much as possible the post-spraying weather conditions (day / night temperatures of 18°C / 14°C and 70% relative humidity). Two weeks after spraying, plant dry weight (after at least 48 h at 70°C) and plant height (from soil surface to plant tip) were assessed.

Simultaneous with each of the regular herbicide applications, application of [^{14}C] bentazon, [^{14}C] phenmedipham and [^{14}C] bentazon + oil was conducted in an isotop-laboratory on a separate set of plants grown under the same conditions. [^{14}C] herbicides were applied with a Burkard Microapplicator PAX 100 fitted with a 50 μL syringe to the youngest fully expanded leaves, e.g. the second leaf of *S. nigrum*, *S. vulgaris* and *B. napus* plants, and the first pair of leaves of *C. album* plants. The experiment consisted of a complete randomized design with 48 plants (4 blocks × 4 species × 3 herbicides) per group. The [^{14}C] herbicides solutions were applied as four 1 μL drops (0.21 kBq μl^{-1}) to a discrete area (diameter < 1 cm) outlined with a water-proof pen on the adaxial surface in the median part of the leaf. The concentration of the herbicides was equivalent to the molarity of the herbicides when these compounds were applied at a rate of 315 g ha^{-1} at a spray volume of 400 (bentazon) and 300 (phenmedipham) L ha^{-1}. To find out how much [^{14}C] herbicide was applied to the leaves, a series of four 1 μL drops were dispensed into a scintillation vial prior to each treatment. After 24 h, the treated leaf was cut and washed for 40 s with 5 ml demineralized water (bentazon and bentazon + oil) or aqueous alcohol at 1:1 v/v (phenmedipham) to remove residual chemical deposits. The amount of [^{14}C] herbicides was counted in a sample of 0.5 mL of the washing solution dissolved in scintillation liquid (4.5 mL; Packard Ultima Gold, Packard Instruments BV, The Netherlands). The efficiency of the washing procedure was tested in two ways as described by De Ruiter and Meinen (1998). When the leaf surface was washed directly after application of the [^{14}C] herbicides, a recovery of 97% was measured for bentazon and bentazon + oilseed rape oil and 93% for phenmedipham. To determine the efficiency of the washing after the drops had dried, four 1-μL drops were applied on glass slides and put in climate chamber for 24 h. The glass slides were washed for 40 s with 5 ml demineralized water (bentazon and bentazon + oil) or aqueous alcohol at 1:1 v/v (phenmedipham). A recovery of 99% was measured for bentazon and bentazon + oilseed rape oil and 94% for phenmedipham.

Data analysis The response of above ground plant dry matter (y) to increasing herbicide dose (x) was analysed using a log-logistic curve (Christensen et al., 1990; Seefeldt et al., 1995; Streibig, 1992; Streibig et al., 1993):

$$y = a + \frac{c}{1 + e^{(-b(Log(x) - Log(ED50)))}} \qquad \text{Equation 1}$$

where *c* is the upper asymptote of the curve and the mean response of the control plants, *a* is the lower asymptote of the curve and the mean response at very high doses (usually set to zero), ED_{50} is the dose giving 50% response (e.g. 50% reduction of plant DW) and *b* is the slope of the curve around ED_{50}. Logistic regressions were performed using Genstat 8 (Lawes Agricultural Trust, IACR-Rothamsted, UK). The same software was used for simple linear regressions, correlations analysis and analysis of variance. Simple linear regressions and correlation analysis were used to explore possible relationships between ED_{50}, GR, RGR, plant size and SLA (2002, 2003) and herbicide uptake and individual weather parameters (2004). Analysis of variance was performed on growth rate, plant dry weight and other plant characteristics, as well as on herbicide efficacy and uptake.

Results

2002 Experiment

Plant growth At 17 DAS, plants of *P. persicaria* had an average dry weight of 9.75 mg (Table 1), a leaf area of 2.66 cm^2 per plant, and values of LAR, SLA and LWR which are typical of herbaceous species (Poorter & Van der Werf, 1998). Plants exposed to full light conditions from 17 to 23 DAS (treatments A and B) grew significantly faster than plants exposed to reduced light conditions (treatments C and D) with relative growth rates of 1.18 and 1.13 d^{-1}, respectively. Also from 23 to 27 DAS *P. persicaria* plants exposed to full light conditions (treatment A and C) grew significantly faster than plants exposed to reduced light conditions (treatment B and D) with relative growth rates of 1.26 and 1.17 d^{-1}, respectively. The use of cheese cloth thus generated significant differences in relative growth rate between covered and uncovered plants.

At both 23 and 27 DAS this resulted in significant differences in plant dry weight between plants that remained unshaded (treatment A) and plants that were consistently shaded from 17-27 DAS (treatment D). Apart from differences in growth rate and plant dry weight, differences in plant characteristics were obtained. In response to the reduced light level, the LAR increased. This increase in LAR was almost fully due to an increase in SLA as the LWR remained constant at around 0.72 kg leaf kg^{-1} plant. No significant effect on leaf area (LA) was observed, as under reduced light conditions the reduction in plant dry weight was compensated by an increased LAR.

Table 1: Dry weight (DW, g plant^{-1}), leaf area (LA, cm^2), leaf area ratio (LAR, m^2 leaf kg^{-1} plant), specific leaf area (SLA, m^2 leaf kg^{-1} leaf) and leaf weight ratio (LWR, kg leaf kg^{-1} plant) and the RGR from the exponential curve fitting for treatment A to D measured at 17, 23 and 27 DAS. Figures followed by different letters are significantly different.

DAS	Treatment	DW	LA	LAR	SLA	LWR	RGR
17	A B C D	9.75×10^{-3}	2.66	27.28	41.68	0.66	
23	A B	26.73×10^{-3} ^{b}	8.76	32.76^{a}	45.74^{a}	0.72	1.18^{b*}
	C D	20.23×10^{-3} ^{a}	8.45	41.75^{b}	58.34^{b}	0.72	1.13^{a*}
	s.e.d	2.33×10^{-3}	n.s	1.38	2.35	n.s	
27	A	63.25×10^{-3} ^{b}	22.62	35.76^{a}	48.80	0.73	1.26^{b**}
	B	50.61×10^{-3} ^{ab}	20.22	39.96^{bc}	54.93	0.73	1.17^{a**}
	C	45.69×10^{-3} ^{ab}	18.14	39.70^{ab}	55.78	0.72	1.26^{b**}
	D	36.56×10^{-3} ^{a}	17.62	48.19^{c}	67.24	0.72	1.17^{a**}
	s.e.d	19.59×10^{-3}	n.s	3.36	n.s	n.s	

* RGR for the period 17-23 DAS; ** RGR for the period 23-27 DAS.

Table 2: Dry weight at spraying (DW, g plant^{-1}), growth rate (GR, g day^{-1}), leaf area (LA, cm^2) and parameters b, c (g plant^{-1}) and ED$_{50}$ (g ha^{-1}) of the logistic regression for treatments A, B, C and D sprayed at 23 to 27 DAS. Final harvest at 14 days after application of the herbicide. Figures followed by different letters are significantly different.

Spraying	Treatment	DW	GR	LA	b	c	ED$_{50}$
23 DAS	A B	26.73×10^{-3}		8.76	−1.712	0.316	12.64
24 DAS	A	31.62×10^{-3}	4.89×10^{-3}	11.16	−1.275	0.376	9.55
	B	30.50×10^{-3}	3.77×10^{-3}	11.37	−1.275	0.308	14.49
25 DAS	A	39.52×10^{-3}	8.22×10^{-3}	14.57	−1.382	0.322	13.84
	B	36.99×10^{-3}	6.49×10^{-3}	13.40	−1.382	0.303	18.37
26 DAS	A	50.20×10^{-3}	10.36×10^{-3}	17.32	−1.518	0.402^{b}	20.32
	B	43.27×10^{-3}	6.28×10^{-3}	16.55	−1.518	0.317^{a}	10.72
27 DAS	A	63.25×10^{-3}	13.05×10^{-3}	22.62	−2.252	0.436	35.89
	B	50.61×10^{-3}	7.35×10^{-3}	20.22	−2.252	0.413	20.46
23 DAS	C D	20.23×10^{-3}		8.45	−1.712	0.251	8.11
24 DAS	C	22.84×10^{-3}	2.62×10^{-3}	10.15	−1.486	0.231	11.07
	D	22.03×10^{-3}	1.81×10^{-3}	9.40	−1.486	0.233	9.51
25 DAS	C	28.78×10^{-3}	5.94×10^{-3}	12.39	−1.182	0.245	9.57
	D	26.72×10^{-3}	4.68×10^{-3}	12.01	−1.182	0.245	4.19
26 DAS	C	36.26×10^{-3}	7.48×10^{-3}	13.53	−1.661	0.329^{b}	13.27
	D	31.26×10^{-3}	4.54×10^{-3}	15.38	−1.661	0.256^{a}	14.55
27 DAS	C	45.69×10^{-3}	9.43×10^{-3}	18.14	−1.243	0.463^{b}	4.02
	D	36.56×10^{-3}	5.31×10^{-3}	17.62	−1.243	0.332^{a}	6.71

Herbicide efficacy For each herbicide application date, data on plant dry weight obtained at 14 days after application were fitted pair wise (treatment A and B; treatment C and D) to the logarithm of the herbicide dose. The log-logistic dose response curve gave an accurate description of plant dry weight ($R^2 > 0.85$). Only in one situation (treatment C and D at 25 DAS) the residuals seemed non-randomly distributed. The outcomes of the logistic regressions are summarized in Table 2.

For each pair the shape factor (*b*) of the log-logistic dose response curve did not differ significantly and for that reason the value was kept identical. Parameter *c*, which describes the upper asymptote of the response curve, representing the DW of the control plants, increased with herbicide application time, as plants were allowed to grow until exactly two weeks after application.

Significant differences in parameter *c* were only observed when plants had been exposed to different light conditions for either 3 (26 DAS) or 4 (27 DAS) days. Significant differences in ED_{50} between plants that were exposed to different light conditions for one to four days prior to herbicide application were not observed (Table 2).

As pair wise comparisons did not reveal significant differences in ED_{50}, simple linear regression was used to explore possible relationships between ED_{50} and GR, RGR, plant size (DW and LA) and SLA. These simple regression analyses demonstrated positive linear relationships between the ED_{50} and GR ($r = 0.62$; F pr = 0.011; Figure 1a), the ED_{50} and DW ($r = 0.69$; F pr < 0.001; Figure 1b) and the ED_{50} and LA ($r = 0.54$; F pr = 0.014; Figure 1c). The analysis also showed a nearly significant negative linear relationship between the ED_{50} and SLA ($r = -0.42$; F pr = 0.065; Figure 1e). No significant linear relationship was found between the ED_{50} and RGR ($r = 0.13$; F pr = 0.574; Figure 1d).

Correlation analysis demonstrated strong correlations between GR and DW ($r = 0.62$; F pr = 0.014), GR and LA ($r = 0.67$; F pr = 0.007) and DW and LA ($r = 0.92$; F pr < 0.001) indicating that GR and plant size (DW and LA) were confounded. No correlation was recorded between SLA and GR, DW or LA.

2003 Experiment

Plant growth In all three trials, the differences in sowing time resulted in significant differences in plant size at spraying (Table 3). Apart from plant dry weight (F pr < 0.001) and leaf area (F pr < 0.001) this difference was present in the number of leaves per plant. The largest difference in plant size between plants of sowing time 1 and plants of sowing time 3 was obtained in trial I, when *P. persicaria* plants of sowing time 3 were still extremely small and had just reached the 2-leaf-stage.

For all three trials, it appeared that only for the largest plants (trial I sowing time 1, trial II sowing times 1 and 2, trial III sowing time 1) a 4-day difference in light condition resulted in significant differences in plant DW at spraying. *P. persicaria* plants exposed to full light were heavier than plants exposed to reduced light conditions. The LA, however, remained mostly unaffected by light condition, as only in one situation (trial 3; sowing time 1) a significant difference was recorded (Table 3). Significant changes in plant characteristics, consistent with those observed in 2002, were obtained. In response to the reduction of the light level, the LAR increased significantly by 24 to 49%. The increased LAR was completely due to an increase in SLA, whereas LWR remained unaffected.

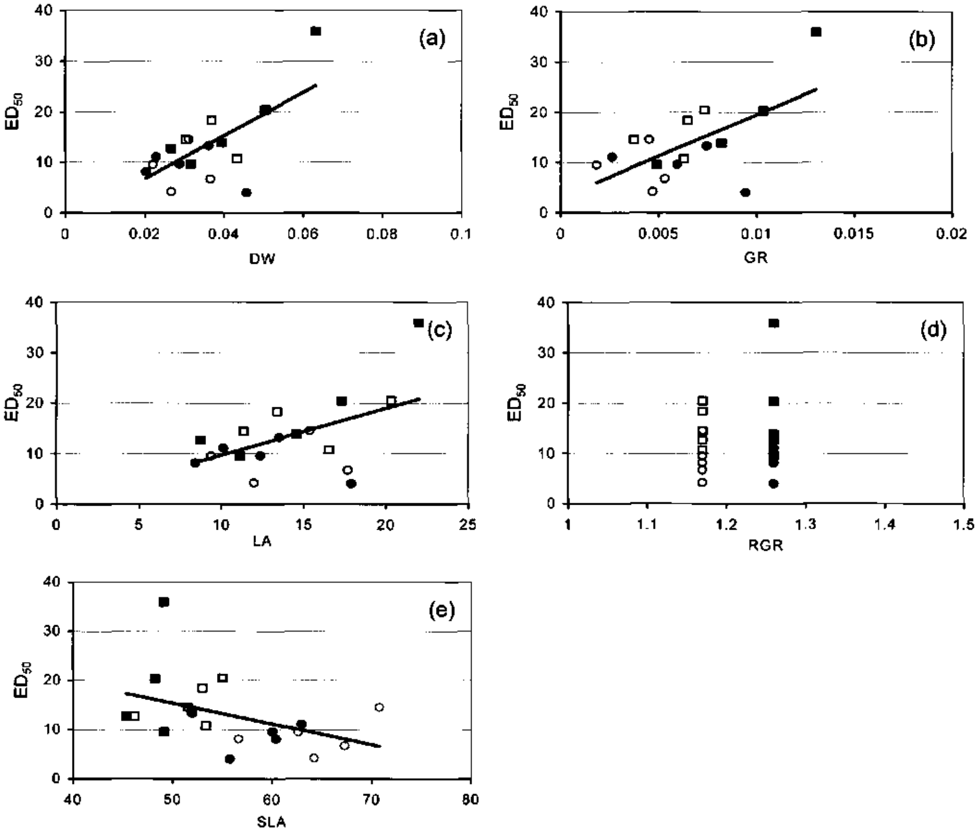

Figure 1: Relationships between the ED_{50} (g ha^{-1}) and dry weight in g $plant^{-1}$ (a), growth rate in g day^{-1} (b), leaf area in cm^2 (c), relative growth rate in d^{-1} (d) and specific leaf area in m^2 leaf kg $leaf^{-1}$ (e) for *P. persicaria* plant treated with metribuzin (2002 experiment) and exposed to either full light (closed symbols) or reduced light (open symbols). Linear regressions are included.

Table 3: Number of leaves, dry weight at spraying (DW, g plant^{-1}), leaf area (LA, cm^{2}), leaf area ratio (LAR, m^{2} leaf kg^{-1} plant), specific leaf area (SLA, m^{2} leaf kg^{-1} leaf), leaf weight ratio (LWR, kg leaf kg^{-1} plant) and parameters b, C (in g plant^{-1}) and ED$_{50}$ (g ha^{-1}) of the logistic regression for plants of sowing times 1 to 3 and replicate I to III exposed to 4 days full light (Full) or reduce light (Reduce) conditions. Figures followed by different letters are significantly different. Statistical analysis done per trial (2003 experiment).

Trial	Sowing	Light	Nb. of leaves	DW	LA	LAR	SLA	LWR	b	C	ED$_{50}$
I	1	Full	4	0.304^{a}	37.30^{a}	12.88^{e}	16.80^{d}	0.762^{b}	-1.78^{a}	0.669^{b}	206.06^{a}
I	1	Reduced		0.198^{b}	34.40^{a}	17.43^{d}	22.75^{c}	0.767^{b}	-1.78^{a}	0.757^{cd}	44.57^{b}
I	2	Full	2-3	0.085^{c}	15.30^{b}	18.01^{d}	21.58^{c}	0.835^{a}	-1.78^{a}	0.844^{d}	45.19^{b}
I	2	Reduced		0.076^{c}	18.30^{b}	24.00^{b}	29.01^{b}	0.827^{a}	-1.78^{a}	0.751^{bc}	59.43^{b}
I	3	Full	2	0.016^{c}	3.42^{c}	21.37^{c}	28.09^{b}	0.761^{b}	-1.78^{a}	0.196^{a}	56.62^{b}
I	3	Reduced		0.021^{c}	5.65^{c}	26.94^{a}	34.00^{a}	0.792^{b}	-1.78^{a}	0.236^{a}	54.95^{b}
			s.e.d.	0.0326	2.726	1.033	1.065	0.0182			
II	1	Full	6-7	0.541^{a}	70.10^{b}	13.03^{e}	18.98^{d}	0.687^{b}	-1.21^{a}	1.507^{a}	44.46^{a}
II	1	Reduced		0.430^{bc}	76.70^{b}	18.02^{d}	26.51^{c}	0.679^{b}	-1.21^{a}	1.426^{a}	30.62^{a}
II	2	Full	4-5	0.443^{b}	93.00^{a}	21.04^{c}	28.19^{c}	0.747^{a}	-1.21^{a}	2.042^{bc}	52.60^{a}
II	2	Reduced		0.356^{c}	101.80^{a}	28.33^{b}	37.89^{b}	0.745^{a}	-1.21^{a}	2.361^{c}	20.51^{a}
II	3	Full	3-5	0.192^{d}	40.70^{c}	21.25^{c}	27.78^{c}	0.765^{a}	-1.21^{a}	1.640^{ab}	65.16^{a}
II	3	Reduced		0.135^{d}	42.90^{c}	31.76^{a}	41.53^{a}	0.765^{a}	-1.21^{a}	1.542^{a}	25.18^{a}
			s.e.d.	0.0405	7.10	0.998	1.157	0.0107			
III	1	Full	6-7	0.712^{a}	103.10^{b}	14.81^{d}	23.15^{d}	0.639^{b}	-1.20^{a}	1.909^{b}	53.70^{b}
III	1	Reduced		0.595^{b}	121.20^{a}	20.46^{c}	30.11^{c}	0.679^{b}	-1.20^{a}	2.319^{c}	18.97^{a}
III	3	Full	3	0.129^{c}	36.50^{c}	28.45^{b}	34.45^{b}	0.825^{a}	-1.20^{a}	1.193^{a}	25.29^{ab}
III	3	Reduced		0.102^{c}	36.00^{c}	35.39^{a}	43.22^{a}	0.819^{a}	-1.20^{a}	1.179^{a}	10.28^{a}
			s.e.d.	0.0436	4.40	0.943	1.444	0.0178			

Herbicide efficacy Also in this case the log-logistic dose response curve gave an accurate description of plant dry weight against herbicide dose ($R^2 > 0.86$). The results of the logistic regressions are summarized in Table 3. Within a trial, no significant differences in shape factor (*b*) were recorded. Significant differences in the upper asymptote of the dose response curve (parameter *c*) were mainly present between early and late sown plants, except for trial II, where *c*-values for early and late sown plants were comparable and, surprisingly, plants of sowing time 2 had the highest *c*-value.

ED_{50} varied from 10 to 65 g ha^{-1}, except for the earliest sowing in trial 1, where an ED_{50} of 206 g ha^{-1} was obtained with plants that were exposed to full light conditions. The average ED_{50} (around 40 g ha^{-1}) dose was larger than the average ED_{50} recorded in 2002 (around 14 g ha^{-1}) when plants were grown in the greenhouse. Linear regression was used to investigate which of the parameters tested in 2002 (GR, DW, LA and SLA) was the best determinant of herbicide efficacy. Correlation analysis showed that, despite changes in the experimental set up, the positive correlation between GR and DW (r = 0.62; F pr = 0.014), GR and LA (r = 0.67; F pr = 0.007) and DW and LA (r = 0.67; F pr = 0.007) remained. No correlation was recorded between SLA and GR, DW or LA.

Contrary to what was found in 2002 for greenhouse grown weeds, statistical analyses demonstrated the absence of linear relationships between the ED_{50} and GR (r = 0.11; F pr = 0.686; Figure 2b), the ED_{50} and DW (r = –0.12; F pr = 0.674; Figure 2a) and the ED_{50} and LA (r = –0.35; F pr = 0.199; Figure 2c). Figure 2a-c clearly show that the difference between plants put under light and shaded conditions (the lines that connect the two treatments for each trial × sowing time combination) was much larger than the average trend (bold regression line), indicating that the investigated characteristics were not the main determinants for herbicide efficacy. Regression analysis confirmed the negative linear relationship between the ED_{50} and SLA (r = –0.60; F pr = 0.018; Figure 2d). For this parameter the lines that connect the light and shaded treatment within each sowing time × trial combination and the average trend were for most cases largely identical. This negative relationship between ED_{50} and SLA puts forward that leaf characteristics might be an important determinant of herbicide efficacy.

2004 Experiment

Pre-spraying weather conditions Both the highest mean air temperatures and the highest global radiation during the last 48 hours before application of the herbicides were recorded for sprayings 2, 3, 4 and 5 (Table 4). However, sprayings 2 and 3 were considerably dry with no precipitation, a mean RH around 65 to 70% and only 10 h

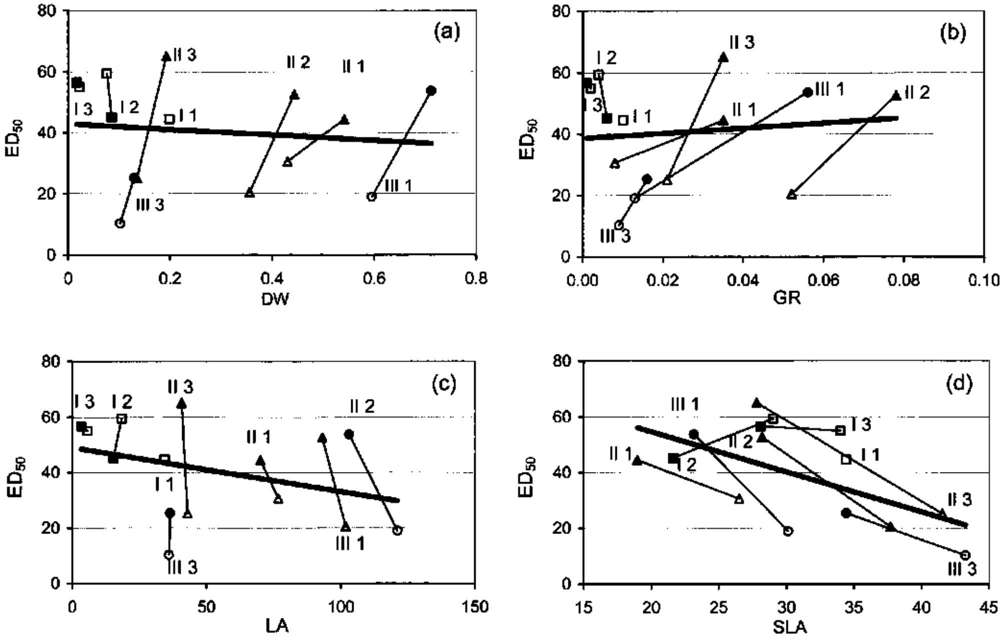

Figure 2: Relationships between the ED_{50} (g ha^{-1}) and dry weight in g $plant^{-1}$ (a), growth rate in g day^{-1} (b), leaf area in cm^2 (c) and specific leaf area in m^2 leaf kg $plant^{-1}$ (d) for *P. persicaria* plants treated with metribuzin (2003 experiment) and exposed to either full light conditions (full symbols) or reduced light conditions (empty symbols). I, II and III for trials 1, 2 and 3, respectively; 1, 2 and 3 for sowing time 1, 2 and 3, respectively. Linear regression for all trials × sowing times is included.

with more than 90% RH. Sprayings 4 and 5 had some precipitation (2.7 and 7.3 mm, respectively) and a mean RH around 77%. The minimum average air temperature and global radiation during the last 48 hours before application of the herbicides were recorded for sprayings 6 and 1, for which the average air temperature was 5 to 6 °C lower than the maximum recorded at spraying 3 and the global radiation was almost half of what was measured for sprayings 2 or 3. Sprayings 6 and 1 had an amount of precipitation of more than 5 mm in the 48 hours prior to application of the herbicides. Tendencies at 48 hours before application were comparable to those at 24 and 72 hours (data not shown). The last 12 hours before application, corresponding mainly to the night prior to spraying (8 pm to 8 am), were relatively dry with no rain at all for sprayings 2, 3, 4 and 5. Sprayings 1 and 6 had some rain so that RH was higher than 90% during the 12-hours period. For both 48 and 12 hours before application of the

Table 4: Mean air temperature (°C), global radiation (KJ m^{-2} h^{-1}), precipitation (mm) and mean relative humidity (%) during the last 12 and 48 hours before application of the herbicides for spray 1 to 6 (2004 experiment).

Parameters	Spray 1	Spray 2	Spray 3	Spray 4	Spray 5	Spray 6
12 hours before application						
Mean air temperature	17.4	16.4	19.6	19.5	17.9	15.2
Global radiation	297.3	420.5	175.6	240.3	152.4	112.6
Precipitation	2.0	0.0	0.0	0.0	0.0	5.2
Mean RH	95.0	77.1	89.8	84.8	90.2	95.1
48 hours before application						
Mean air temperature	17.8	17.9	22.7	21.4	20.6	16.4
Global radiation	534.7	872.8	816.9	589.0	692.4	409.2
Precipitation	5.2	0.0	0.0	2.7	7.3	14.1
Mean RH	92.0	70.6	65.8	77.0	78.4	90.0

Table 5: Linear regression coefficients (r) between mean air temperature (T°C), global radiation (G.Rad. in KJ m^{-2} h^{-1}), precipitation (P in mm) and mean relative humidity (RH in %) during the last 12 and 48 hours before application of the herbicides (2004 experiment).

		12 h			48 h		
		T°C	G. Rad.	P	T°C	G. Rad.	P
12 h	G. Rad.	– 0.07					
	P	– 0.73 [#]	– 0.42				
	RH	– 0.12	– 0.71	+ 0.64 [§]			
48 h	G. Rad.				+ 0.48		
	P				– 0.58	– 0.83 [*]	
	RH				– 0.70 [§]	– 0.89 [*]	+ 0.77 [*]

([*]) *P < 0.05*, ([#]) *P < 0.10*, ([§]) *P < 0.20.*

Table 6: Number of fully expanded leaves for *B. napus*, *C. album*, *S. nigrum* and *S. vulgaris* at spraying 1 to 6 (2004 experiment).

	Spray 1	Spray 2	Spray 3	Spray 4	Spray 5	Spray 6	Average (± stdev)
B. napus	1.9	2.0	2.3	3.0	2.2	2.0	2.2 (± 0.38)
C. album	2.3	2.3	3.3	4.0	3.8	3.0	3.1 (± 0.75)
S. nigrum	1.0	1.9	2.1	3.2	2.0	1.6	2.0 (± 0.73)
S. vulgaris	1.9	2.0	2.2	3.1	2.2	2.0	2.2 (± 0.44)

herbicides, significant negative correlations were found between the global radiation and precipitation and the global radiation and the mean relative humidity. Precipitation was positively correlated with the mean relative humidity (Table 5).

Plant size at spraying At the time of spraying, *B. napus* had on average 2.2 leaves, *C. album* 3.1 leaves (equivalent to 1.6 pairs), *S. nigrum* 2.0 leaves and *S. vulgaris* 2.2 leaves per plant. For the four species considered, plants were smallest at spraying 1 and largest at spraying 4 (Table 6). The coefficient of variation was largest for *S. nigrum* plants (36% compared to 17%, 24% and 20% for *B. napus*, *C. album* and *S. vulgaris*, respectively) indicating that the growth of *S. nigrum* plants was more influenced by pre-spraying weather conditions than that of the other three species.

Herbicide uptake Analysis of variance clearly showed that both the herbicide and the species had a significant effect on uptake. On average, the lowest uptake was recorded for bentazon used alone (34%), whereas the highest uptake was obtained when oil was added to bentazon (88%). Phenmedipham (63%) held an intermediate position (Table 7). The average uptake of *B. napus* (70%) and *C. album* (69%) was significantly higher than that of *S. nigrum* (57%) and *S. vulgaris* (51%).

No herbicide × species interaction was observed. Between the different sprayings, the uptake of bentazon was highly variable with coefficients of variation (CV) varying from 23% (*S. nigrum*) to 50% (*C. album*). Adding oil to bentazon clearly improved herbicide uptake and reduced its variability. The CV was still 16% for *S. vulgaris*, but lower than 6% for the three other species. Variability in uptake was intermediate with phenmedipham. For this herbicide the CV was 8% for *B. napus*, whereas it ranged between 17% (*C. album*) and 26% (*S. nigrum*) for the other species (Table 7).

Table 7: Uptake (%) of phenmedipham, bentazon and bentazon + oil for *B. napus*, *C. album*, *S. nigrum* and *S. vulgaris*. Figures followed by the same letter are not significantly different. Standard error of the uptake are between parentheses (2004 experiment).

	Herbicides						
Plant species	Bentazon		Bentazon + oil		Phenmedipham		Average
B. napus	41.2	(± 13.29)	96.9	(± 1.31)	71.6	(± 6.03)	69.9 ^{a}
C. album	42.7	(± 21.50)	93.0	(± 3.48)	71.4	(± 11.94)	68.9 ^{a}
S. nigrum	27.1	(± 6.14)	87.0	(± 5.01)	57.1	(± 15.28)	56.9 ^{b}
S. vulgaris	22.6	(± 8.43)	78.9	(± 12.95)	53.2	(± 11.83)	51.2 ^{b}
Average	33.8 ^{c}		88.4 ^{a}		62.9 ^{b}		

Herbicide efficacy For each spraying time, the data on plant dry weight at two weeks after spraying were fitted to herbicide dose for all plant species and herbicide combinations, using Equation 1. R^2 values were always larger than 0.90, whereas residuals were homogeneously distributed around the fitted curves, indicating a good fit of the experimental data to the model. For the shape factor (*b*) an average value of -3.80 (± 1.28) was obtained. No significant differences for this parameter were found among the combinations of spraying time × plant species × herbicide. For all four species, *c*-values were different from spraying time to spraying time (data not shown) as growth conditions after application of the herbicides were not completely identical.

As expected from the product recommendations, use of bentazon on *S. nigrum* and *S. vulgaris* resulted in a low average ED_{50} (Figures 3b, 3d). *C. album*, which was not supposed to be very well controlled by bentazon, had a larger average ED_{50} (388 g ha^{-1}) (Figure 3f). Results indicated that bentazon had an intermediate effect on *B. napus* plants with an average ED_{50} of 166 g ha^{-1} which is consistent with the fact that *Sinapis arvensis*, a closely related species, is rather sensitive to bentazon.

The dose required to obtain 50% control was however quite variable (Figure 3h). Adding oil to bentazon reduced the amount of herbicide required to obtain a sufficient level of control, with ED_{50} between 33 and 48 g ha^{-1} (for *B. napus* and *S. vulgaris*, respectively). Moreover, the variability in ED_{50}-doses was considerably reduced. Only *S. nigrum* which was already controlled very well by bentazon alone did not greatly benefit from the addition of oil. Phenmedipham provided a reasonable control of *C. album* with an ED_{50} of 241 g ha^{-1} (Figure 3e), while *S. vulgaris* was also quite responsive. The herbicide provided a poor control of *S. nigrum* plants (Figure 3a). Phenmedipham had only marginal effects on *B. napus*, with an average ED_{50} larger than the recommended herbicide dose (Figure 3g). This is not unexpected, as this herbicide is not recommended for *S. arvensis*.

Relationship between herbicide uptake and herbicide efficacy For all weed species × herbicide combinations isolines were included in Figure 3. These isolines consist of all combinations of ED_{50} and percentage uptake that result in an identical herbicide uptake as to the average uptake of herbicide at the ED_{50} point over the six sprayings. The figures show that in quite a number of cases the individual data points are reasonably well represented by the isolines, indicating that uptake is a major determinant of differences in ED_{50} between spraying times. A poor relationship was obtained for phenmedipham applied on *B. napus* (Figure 3g), for which the use of phenmedipham is not recommended. Large deviations from the isolines were also observed in a number of other situations. For example, a 40% uptake of bentazon by *C. album* (sprayings 1, 3 and 5) resulted in ED_{50} varying from 40 g ha^{-1} at spraying 1

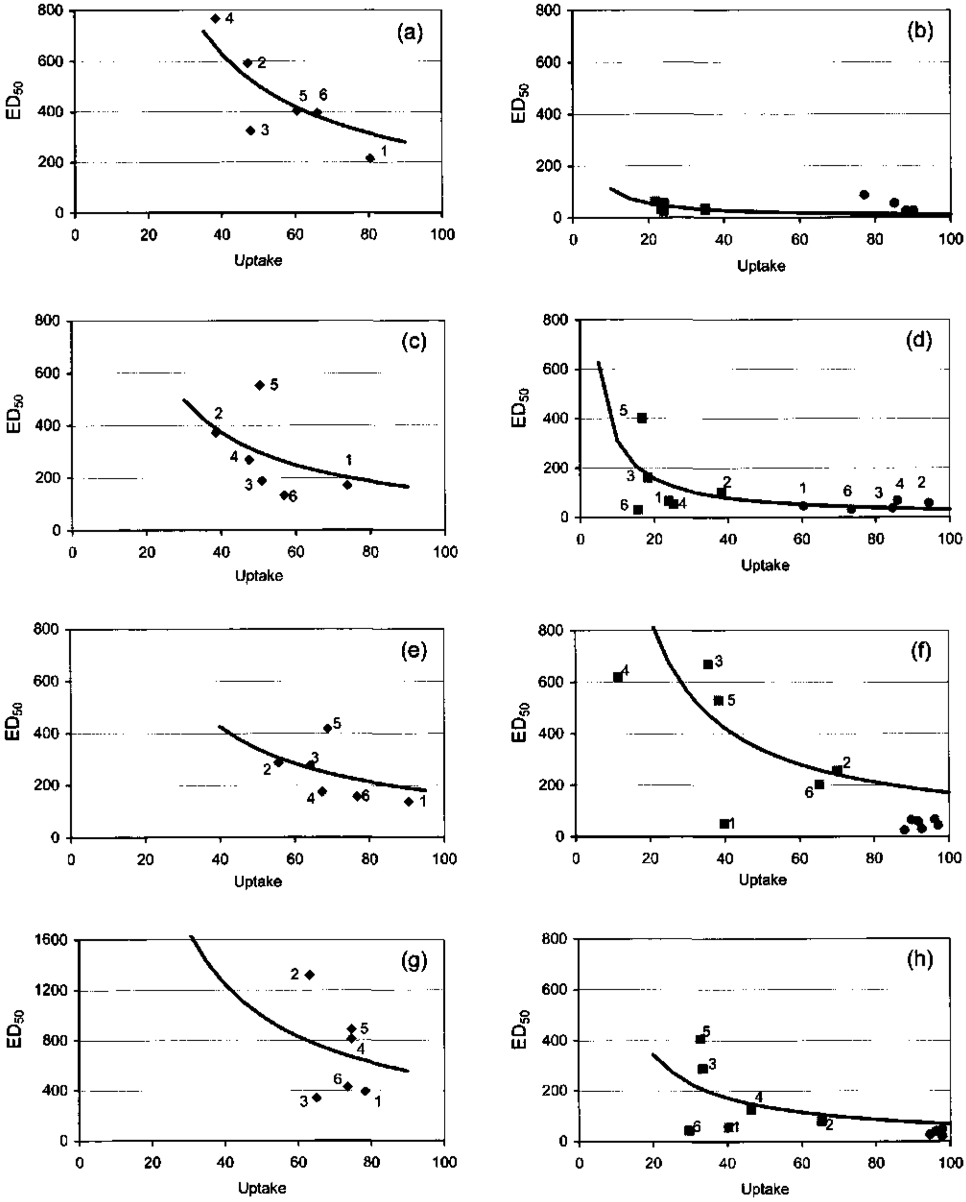

Figure 3: Relationship between ED$_{50}$ (g ha^{-1}) and uptake for *S. nigrum* (a), *S. vulgaris* (c), *C. album* (e) and *B. napus* (g) sprayed with phenmedipham and *S. nigrum* (b), *S. vulgaris* (d), *C. album* (f) and *B. napus* (h) sprayed with bentazon (squares) or bentazon + oil (circles). The average uptake / herbicide efficacy isolines are included for each herbicide (2004 experiment).

to 670 g ha^{-1} at spraying 3. Similar observations were recorded for phenmedipham where a 65% uptake by *C. album* (sprayings 3, 4 and 5) resulted in ED_{50} varying from 174 g ha^{-1} at spraying 4 to 418 g ha^{-1} at spraying 5. These deviations from the isolines indicate that other factors than uptake affected the outcome of the herbicide treatment as well. Results obtained for bentazon with oil were quite well described by the isolines derived for bentazon, indicating that the reduction in ED_{50} that followed from the addition of oil resulted completely from an increased uptake of herbicide. For *C. album*, however, the reduction in ED_{50} was greater than expected, suggesting an additional phytotoxic effect of the oil (Figure 3f).

Influence of pre-spraying weather parameters on uptake Correlation analysis revealed significant correlations between herbicide uptake and individual weather parameters. Correlations were tested for the last 12, 24, 48 and 72 hours before herbicide application but, depending on the plant species × herbicide combination, the strongest effects were found for either the last 12 or 48 hours before herbicide application. Regression analysis demonstrated that of the tested weather parameters, only global radiation and mean relative humidity were significantly correlated with herbicide uptake (Table 8).

Uptake of phenmedipham, by all four species, was significantly positively correlated with the mean relative humidity during the last 12 or 48 hours before application of the herbicide. A significant negative correlation between the global radiation during the last 48 hours before application and uptake of phenmedipham was found for *B. napus*. For the other three species also negative, but non-significant, correlations were found between global radiation and uptake of phenmedipham.

Uptake of bentazon for *B. napus*, *S. nigrum* and *S. vulgaris* was positively correlated with the global radiation during the last 12 hours before herbicide application. Mean RH in this period was negatively correlated with herbicide uptake for *B. napus* and *S. vulgaris*. Analysis using weather data during the 48 h before application confirmed both trends, though no significant correlations were obtained. Adding oil to bentazon did not affect the nature of the correlations but the different weed species became much less responsive to individual pre-spraying weather parameters. In this case the uptake of bentazon for *C. album* and *S. vulgaris* was significantly negatively correlated to the mean relative humidity during the last 12 or 48 hours before application.

Table 8: Linear regression coefficients (r) between herbicide uptake and global radiation (GR) or mean relative humidity (mean RH) for combination where $P < 0.10$. For combinations where $P > 0.20$ only (+) or (–) are indicated. (+) = an increase in the value of the weather variable resulted in an increased uptake. (–) = an increase in the value of the weather variable resulted in a reduced uptake (2004 experiment).

		Bentazon				Bentazon + oil				Phenmedipham			
Weather parameters	Time	BN	CA	SN	SV	BN	CA	SN	SV	BN	CA	SN	SV
Global radiation	12 h	+0.94 *	+	+0.85 *	+0.97 *	-	+	+	+	–	–	–	–
Global radiation	48 h	+	+	+	+	+	+	+	+	–0.81 *	–0.77 #	-	–
Mean RH	12 h	–0.89 *	–	–	–0.84 *	–	–0.64 #	(-)	–0.84 *	(+)	+0.83 *	+0.74 *	+0.84 *
Mean RH	48 h	–	–	+	–	–	–0.77 *	+	–0.76 *	+0.83 *	+0.88 *	+0.81 *	+0.77 *

(*) $P < 0.05$, (#) $P < 0.10$. BN = *B. napus*, CA = *C. album*, SN = *S. nigrum*, SV = *S. vulgaris*.

Discussion

Herbicide labels contain the dose recommendations of the manufacturer and are chosen such that even under non-favourable conditions the application still results in a sufficient level of weed control. For this reason it is not surprising that under optimum conditions herbicides still provide adequate control at doses well below these label recommendations (Kudsk & Streibig, 2003). Reduced dose strategies try to exploit this gap in required herbicide dose. Tailor-made dosages are recommended based on factors that influence the efficacy of herbicide applications. Among these factors are the sensitivity of the weed species to the specific herbicide, the size of the weed plants, the precision of the spraying equipment and the environmental conditions before, during and after herbicide application. The MLHD-technology, developed in The Netherlands, aims at recommending the lowest dose of a herbicide which is just sufficient to kill the weed plants and bases this reduced dose rate advice on plant species and plant size. If, for what ever reason, a farmer considers the spraying conditions non-optimal the system recommends to increase this advised dose rate with 50%. The MLHD-technology is restricted to photosynthesis-inhibiting herbicides.

The results of the experiment conducted in 2004 offer an opportunity to illustrate the gains and risks associated to the use of reduced dose rates. In 2004, three out of the nine herbicide × weed species combinations studied were classified as highly sensitive (+++). This involves the use of phenmedipham for the control of *C. album* and the use of bentazon and bentazon + oil used against *S. nigrum*. All of these combinations were used on all 6 spraying dates, resulting in 18 events. In addition, two normal sensitive (++) combinations (bentazon and bentazon + oil used against *S. vulgaris*, n = 12) and four less sensitive (+) combinations (phenmedipham for the control of *S. vulgaris* and *S. nigrum*, bentazon and bentazon + oil used against *C. album*, n = 24) were studied. For each event the ED_{90} dose derived from the fitted log-logistic curve was compared with the advised MLHD-dose, the 1.5 × MLHD-dose and the recommended (or label) dose. In Figure 4, the percentage of events in which the ED_{90} dose was equal to or below the specified doses, and consequently a sufficient level of weed control would have been obtained, is presented. This figure shows that for the highly sensitive (+++) herbicide × weed species combinations the recommended label dose gave an appropriate control for more than 95% of the applications. Use of the MLHD advice resulted in an appropriate weed control for 70% of the applications. Introduction of a safety margin increased this figure to 80%. These figures are lower than what is experienced by farmers in the Netherlands who are using the MLHD-technology. Under practical conditions the MLHD advice is reported to result in appropriate weed control in about 90% of the situations (Kempenaar & Uffing, 2002). One major reason for the lower success rate might be that in the experiment, because of a preset spraying schedule, some of the sprayings were conducted under more extreme conditions, with hardened weed plants, than what would have been considered acceptable by farmers. In practice, under such conditions, farmers would most likely have chosen not to use the MLHD advice, or they would have postponed the application time of the herbicides. In the applications of the experiment this kind of expert knowledge was not included.

For the normal sensitive (++) herbicide × weed species combinations the recommended label dose gave an appropriate control for around 70% of the combinations. The use of the MLHD advice resulted in an unacceptable decrease in the number of successful applications (35%), whereas the introduction of a safety margin of 50% provided a marginal increase in the number of appropriate applications (45%). For little sensitive (+) herbicide × weed species combinations the recommended dose gave an appropriate control for an even smaller proportion of the combinations (60%), whereas the number of successful applications with the MLHD advice was stable at around 35 to 45% of the combinations. It is obvious that in many cases a successful weed control can be realized with herbicide rates well below the recommended dose. However, especially for normal sensitive and little sensitive herbicide × weed species combinations, an advice just based on plant species and plant

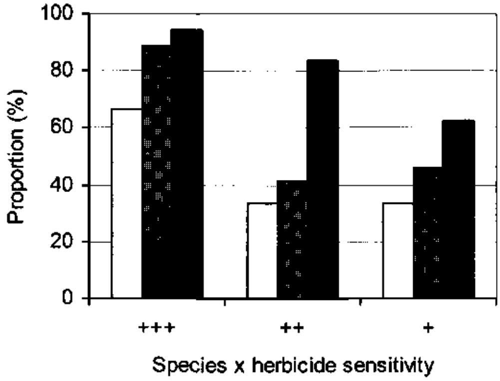

Figure 4: Fraction of events in which ED_{90} dose was smaller than the advised MLHD-dose (white bar), the 1.5 × MLHD-dose (middle bar) and the recommended dose (black bar) for the three highly sensitive (+++) herbicide × weed species combinations (n = 18), two normal sensitive (++) herbicide × weed species combinations (n = 12) and four little sensitive (+) herbicide × weed species combinations (n = 24). Only combinations for which official recommendations are provided were included in this analysis (2004 experiment).

size alone seems not sufficient, as only in about 35% of the situations this resulted in an adequate control. As plant size was identical, environmental conditions seem to have a significant influence on the efficacy of the herbicides and inclusion of this factor as one of the determinants of the reduced dose rate advice seems a prerequisite for a secured use of a reduced dose strategy. In this context, the current research tried to reveal the importance of pre-spraying weather conditions.

In the first two years of experimentation, the hypothesis that pre-spraying weather conditions have an effect on herbicide efficacy mainly through plant growth rate was verified using the combination of metribuzin with *P. persicaria*. Several authors (Hammerton, 1967; Peterson, 2001) reported that an increased growth rate resulted in a higher efficacy. In this research, plant growth rate (both relative growth rate (RGR) and absolute growth rate (GR)) prior to herbicide application was successfully manipulated by covering half of the plants with cheese cloth. Using two layers of cheese cloth resulted in a reduction of light intensity by 65 to 70%. In 2002, plants were exposed to these conditions for a different period of time, varying from one to four days, resulting in weed plants of different size. Furthermore, shading increased LAR through an increase in SLA which was consistent with previous findings from Hughes (1973), Hunt and Halligan (1981) and Powels and Critchley (1980). Regression analysis demonstrated a significant positive linear relationship between the ED_{50} and GR, suggesting that for this specific herbicide × weed species combination an increased weed growth rate resulted in a poorer performance of the herbicide (Kudsk & Kristensen, 1992). However, plant growth rate was confounded with plant size (expressed in dry weight or leaf area), making it impossible to attribute the differences in herbicide efficacy to either one of these factors. On top of that, a nearly significant ($p = 0.065$) negative relationship between ED_{50} and SLA was found, indicating that also changes in leaf characteristics might have contributed to the differences in herbicide efficacy.

The use of differently sized plants of *P. persicaria* in the 2003 experiment was not sufficient to remove the correlation between plant size and plant growth rate. However, through the use of differently sized plants it was possible to demonstrate that the differences in plant growth rate and plant size initiated by the light treatment were by far not sufficient to explain the differences in ED_{50}. At the same time, the results confirmed the negative relation between SLA and ED_{50}, which was truly significant this time ($p = 0.018$). This significant relationship does not necessarily mean that the higher SLA is causally related to a lower ED_{50}, though the increased area-weight ratio of the leaf might contribute to a better translocation of herbicide to the side of action. It is more likely that the increased SLA, a typical shade response, is indicative for other changes in leaf characteristics, like a thinner cuticle (Maier & Post-Beittenmiller, 1998; Mummigatti et al., 1995; Nobel, 1976), which in turn results in a better herbicide uptake (Mummigatti et al., 1995).

The 2004 experiment was based on the outcomes of the 2003 experiment and was designed to verify whether pre-spraying weather conditions are mainly having an effect on herbicide efficacy through their effect on herbicide uptake. For this reason, a direct measurement of herbicide uptake, using radio-active labelled herbicides, was added. Apart from that two herbicides which differed in polarity were used and the experiments were conducted with four different weed species, as also leaf characteristics between weed species will differ. Finally, the artificially created differences in light intensity were replaced. Instead plants were raised in consecutive batches with intervals of one week, to obtain more natural pre-spraying weather conditions that varied in more than just light condition. This widened the research as it allowed us to study not only whether herbicide uptake was an important cause of differences in herbicide efficacy, but also which weather parameters and which conditions promoted the uptake of herbicides. The close relationship between the experimental data and the average uptake / herbicide efficacy isolines showed that herbicide uptake was a major determinant for herbicide efficacy under field conditions, as was previously demonstrated by Merritt (1984). Two pre-spraying weather parameters, the global radiation and the mean relative humidity during the last 12 or 48 hours before application of the herbicides were strongly correlated to herbicide uptake. The nature of these correlations (positive / negative) was herbicide specific rather than species specific. For phenmedipham, representing apolar herbicides, herbicide uptake was negatively correlated with global radiation and positively correlated with mean relative humidity. Several authors showed that high global radiation and / or low mean relative humidity induced lower herbicide uptake (Baker, 1980; De Ruiter & Meinen, 1998; Merritt, 1984) and herbicide efficacy (Merritt, 1984). Devine (1989), Green and Strek (2001) and Kudsk and Kristensen (1992) also pointed out that high global radiation or low mean relative humidity usually have a negative influence on herbicide efficacy. Baker (1980) and Merritt (1984) demonstrated that herbicide uptake was affected through changes in cuticle characteristics. As both weather parameters were negatively correlated to each other at both 12 hours ($r = -0.71$) and 48 hours ($r = -0.89$) before application of the herbicides, it was not possible to identify which of the two parameters was the best determinant of herbicide uptake.

For bentazon, representing polar herbicides, herbicide uptake was positively correlated with global radiation and negatively correlated with mean relative humidity. These correlations were not expected. Adding oil to the polar herbicide bentazon did not alter the nature of the correlations between herbicide uptake and global radiation or mean relative humidity, but made the different weed species much less responsive and thus less dependent on pre-spraying weather parameters because of increased herbicide uptake. Irrespective of pre-spraying weather conditions herbicide uptake was always larger than 80%.

The number of hours before application to take into account for the correlations between pre-spraying weather parameters and herbicide uptake was also herbicide specific rather than species specific. The uptake of the apolar herbicide phenmedipham was best correlated when pre-spraying weather parameters of the last 48 hours before herbicide application were used. For bentazon, with or without oil, 12 hours seemed sufficient. These timing were in line with findings from Lundkvist (1997a).

This research demonstrates that pre-spraying weather parameters do have a clear effect on herbicide efficacy, mainly through their effect on herbicide uptake. The correlations between pre-spraying weather parameters and herbicide uptake were herbicide specific and not species specific, which has clear benefits for transfer into practice. This research also shows the importance of the sensitivity of herbicide × weed species combinations in the calculation of optimized herbicide doses. In many situations, considerable reductions in herbicide rate can still result in appropriate weed control. However, safe utilization of this knowledge is only possible if the parameters determining the required herbicide dose are identified. Although pre-spraying weather conditions are definitely not the only factor influencing herbicide efficacy, the current results demonstrate the relevance of including this factor in the establishment of the reduced dose rate advice.

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Chapter 3

Early assessment of herbicide efficacy after application with ALS inhibitors – a first exploration

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Abstract

Plants of *Solanum nigrum* (black nightshade) and *Polygonum persicaria* (ladysthumb) were grown in a greenhouse until the fourth leaf stage. These plants were sprayed with a mixture of metsulfuron-methyl (40 and 30 g a.i. ha^{-1}, respectively) and isodecyl alcohol ethoxylate (0.75% v/v) using an air-pressured laboratory track sprayer. The level of carbon dioxide (CO_2) fixation as well as the relative quantum efficiency of photosystem II electron transport (Φ_{PSII}) and the relative quantum efficiency of photosystem I electron transport (Φ_{PSI}) were simultaneously assessed between 2 and 4 days after treatment. Measurements showed that CO_2 fixation, Φ_{PSI} and Φ_{PSII} for treated plants were much lower than for unsprayed control plants. These results suggest that already a few days after application the photosynthetic apparatus of *S. nigrum* and *P. persicaria* were affected by ALS inhibiting herbicides. An extensive set of experiments is being prepared to study these effects in more detail and to explore whether photosynthesis parameters can be used for early assessment of herbicide efficacy under field conditions.

Introduction

In 1991, herbicides accounted for almost half of the pesticide used worldwide in terms of the volume of active ingredient. Increased concerns about environmental side effects of herbicides, development of herbicide resistance in weeds and the necessity to reduce the cost of the inputs have resulted in increasing pressure on farmers to reduce the use of herbicides. The quantity of herbicides applied to crops can be reduced by cutting down the number of applications, by applying spot spraying rather than full-field applications or by using reduced dose rates. However, these methods increase the risk of inadequate control.

Therefore, weed management strategies aiming at using low herbicide doses ideally consist of a combination of two components. First, a method should be available to reliably predict the dose rate that is just appropriate for killing the weeds in the field. Second, a means should be available to rapidly verify whether or not the herbicide application will, in fact, result in the death of the weeds. Under field conditions, the visible effects of herbicide action are only apparent 10 to 20 days after spraying, which is too long a delay in the event that a second application of herbicide is necessary. An early detection method would permit a prompt second herbicide application in case of failure. This last element is of particular importance for ensuring that even though minimal doses of herbicides have been employed, there is a guarantee that the treatment will be successful in eliminating the weeds. Such a guarantee contributes to the adoption of this methodology by farmers, agricultural contractors and others.

The Minimum Lethal Herbicide Dose (MLHD) technology, developed by Plant Research International in The Netherlands (Kempenaar et al., 2002a; Ketel, 1996), has shown itself to be a promising decision support system leading to the use of lower rates of photosynthesis-inhibiting herbicides. This method allows the calculation of the minimum dose of a photosynthesis inhibiting herbicide needed to control a weed population. Simple and rapid measurements of photosynthetic activity are used to evaluate the efficacy of the treatment shortly after application. Only a minority of herbicides, however, act directly to inhibit photosynthesis, and photosynthesis is the only biological process in the plant whose activity can be rapidly and simply measured in the field. An obvious question is "How effectively can measuring instruments and methods that work so well to measure the effect of photosynthetic herbicides work in detecting the effect of non-photosynthetic herbicides by their indirect effect on photosynthesis?".

Acetolactate synthase (ALS) inhibitors are an important group of herbicides. They selectively inhibit acetolactate synthase, which is the first common enzyme involved in chloroplastidic biosynthesis of essential branched chain amino acids (valine, leucine and isoleucine). Photosynthesis is not regarded to be a primary target of ALS inhibiting herbicides, but changes in chlorophyll fluorescence responses have been observed in treated plants. Judy et al. (1990) found effects on the fluorescence from barley 2 h after treatment with imazaquin and Percival and Baker (1991) found effects on the fluorescence from wheat leaves 24 h after treatment with the ALS-inhibitor imazamethabenz methyl at the recommended field rates. Van den Boogaard and Harbinson (unpublished data) worked with photosystem I, which contained P700, the chlorophyll *a* dimer that functions as a primary electron donor. They observed that two days after sunflower plants (*Helianthus annuus* L.) had been treated with the ALS inhibitor amidosulfuron, the rate-constant for the reduction of the $P700^+$ pool decreased significantly. For rape (*Brassica napus*), though treatment with amidosulfuron had no effect on the rate-constant for $P700^+$ reduction, there was an easily detectable effect on the pattern of P700 oxidation following the start of the illumination of dark-adapted leaves. How exactly these events are related to the primary events caused by ALS-inhibiting herbicides remains unknown. Madsen et al. (1995) found that photosynthetic parameters such as fluorescence and carbon dioxide exchange rate were significantly affected after treatment with glyphosate, another well-known herbicide which interferes with the essential aromatic amino acid synthesis.

There are no published methods for predicting the effectiveness of an ALS treatment after a relatively short period of time. However, these previous studies have demonstrated the effects of various ALS inhibitors on photosynthesis within a few hours or days of application suggesting that photosynthetic parameters might be useful indicators of herbicidal efficacy. The purpose of this study was to determine if, how and when the photosynthetic apparatus of *Solanum nigrum* (black nightshade) and *Polygonum persicaria* (ladysthumb) were affected by ALS inhibiting herbicides.

Materials and methods

Plant material

S. nigrum and *P. persicaria* were raised from seeds (Herbiseed, UK) and grown in a greenhouse between January and March 2003 at Plant Research International (Wageningen, The Netherlands). The plants were grown with day / night temperatures of 18°C / 14°C, 70% relative humidity and a 12 h photoperiod provided by natural light supplemented with high-pressure mercury lamps. Water and soil nutrients were kept at a level so that they were not limiting for growth.

Spraying procedure

Five week old *S. nigrum* and *P. persicaria* plants (both at the 3-leaf stage) were sprayed with an air-pressurized laboratory track sprayer delivering 400 litres ha^{-1} at 303 kPa. *S. nigrum* plants were treated with 40 g a.i. ha^{-1} of metsulfuron-methyl (Ally, DuPont) and 0.75% v/v isodecyl alcohol ethoxylate (Trend 90, DuPont). *P. persicaria* plants were sprayed with 30 g a.i. ha^{-1} of metsulfuron-methyl and 0.75% v/v isodecyl alcohol ethoxylate. Isodecyl alcohol ethoxylate is used as a surfactant to improve the uptake of metsulfuron-methyl by the leaves.

Photosynthesis measurements

To have an indication on the relative performance of the photosynthetic apparatus of ALS-treated plants the level of carbon dioxide (CO_2) fixation, the relative quantum efficiency of photosystem II electron transport (Φ_{PSII}) and the relative quantum efficiency of photosystem I electron transport (Φ_{PSI}) were assessed for both control and treated plants. Equipment similar to that described by Kingston-Smith et al. (1999, 1997) was used.

CO_2 fixation was measured using an infra-red gas analyser (Mark 3, Analytical Development Company, Hoddesdon UK). Actinic light was provided by a quartz halogen lamp filtered by NIR and Calfex dichroic mirrors (Balzers, Liechtenstein), and light-intensity was adjusted using metal film neutral density filters (Balzers, Liechtenstein) (Kingston-Smith et al., 1997). Two wavelengths (560 and 660 nm) were used to excite the chlorophyll fluorescence in order to measure Φ_{PSII}. These two excitation wavelengths penetrate the leaf differently and the fluorescence they each produce is electronically recovered and displayed separately. Φ_{PSI} was measured using the irradiance-induced absorbance change around 820 nm. The CO_2 fixation and efficiency measurements were made in air consisting of 21% (v/v) oxygen (O_2), 370 μmol mol^{-1} CO_2 with the remainder nitrogen (N_2), at a temperature of 21 to 23°C. Photosynthesis parameters were measured when photosynthesis was in a steady state after acclimatization to the irradiance level, which typically occurred after 45 to 60 minutes.

The light response curves of CO_2 fixation (μmol m^{-2} s^{-1}), Φ_{PSI} and Φ_{PSII} to increasing irradiance were measured for both control and treated plants of *S. nigrum* and *P. persicaria*. The actinic light source was used to provide the step increase in irradiance from 0 to 7500 μmol m^{-2} s^{-1} (light level $step_n \approx 1.5 \times$ light level $step_{(n-1)}$). Based on these observed light response curves, a single light-intensity was selected for each species that was sufficient to saturate CO_2 fixation and which would serve as a standard irradiance for the comparison of Φ_{PSI} and Φ_{PSII} in the presence or absence of herbicide. Once this standard had been determined the same parameters were measured at this light intensity on additional plants. For *S. nigrum* the measurements were made 2 and 3 DAT on the second or third leaf. For *P. persicaria* the measurements were made 3 and 4 DAT on the third leaf.

Results

At 2 to 4 DAT CO_2 fixation, Φ_{PSII} and Φ_{PSI} were always higher for control plants than for treated plants for both *S. nigrum* (Figure 1) and *P. persicaria* (data not shown). The rate of CO_2 fixation was most strongly reduced especially at high irradiance (irradiance from 500 to 1000 μmol m^{-2} s^{-1}). These results clearly demonstrate that the ALS inhibiting herbicide metsulfuron-methyl has major effects on photosynthesis. Whether these effects are primary or secondary effects remains unknown. Based on the light response curves for *S. nigrum* it appeared that the most appropriate irradiance for later measurements was 500 μmol m^{-2} s^{-1}. For *P. persicaria* the irradiance selected was 700 μmol m^{-2} s^{-1}.

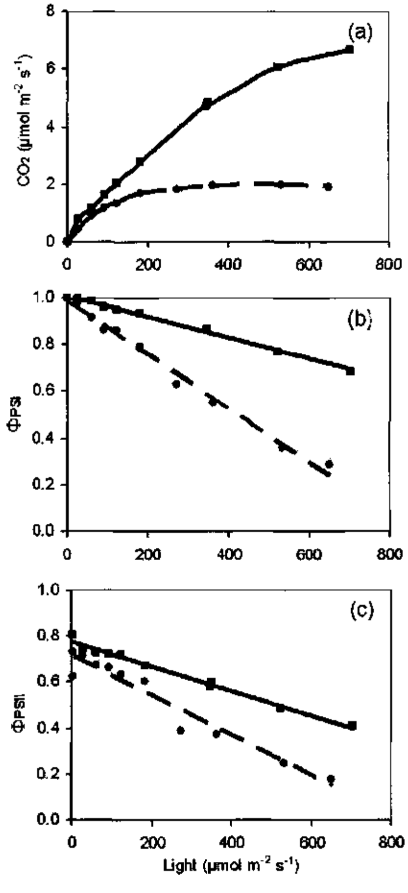

Figure 1: The effect of irradiance (μmol m^{-2} s^{-1}) on (a) CO_2 fixation (μmol m^{-2} s^{-1}); (b) Φ_{PSI}; (c) Φ_{PSII} for one *S. nigrum* control plant (squares) and one *S. nigrum* treated plant (3 DAT, circles). Six week old plants (5 leaves, measurement on the fourth leaf) treated with 40 g ha^{-1} metsulfuron-methyl + 0.75% v/v isodecyl alcohol ethoxylate.

CO_2 fixation, Φ_{PSI} and Φ_{PSII} were assessed at 2 and 3 DAT for both control and treated *S. nigrum* plants. For *P. persicaria* plants the measurements were performed at 3 and 4 DAT. This data clearly demonstrated that for *S. nigrum* at both 2 and 3 DAT the 3 parameters presented here were strongly reduced by the ALS inhibiting herbicide (78 to 82% reduction for the CO_2 fixation, 28 to 34% reduction for Φ_{PSI} and 40 to 45% reduction for Φ_{PSII}). Already 2 DAT, the differences between control and treated *S. nigrum* plants were highly significant, as given in Table 1.

For *P. persicaria* the results show that 3 or 4 DAT CO_2 fixation, Φ_{PSI} and Φ_{PSII} were reduced by the herbicide. These reductions were only statistically significant for CO_2 fixation and Φ_{PSII} (Table 2).

Table 1: Mean values for CO_2 fixation (μmol m^{-2} s^{-1}), Φ_{PSI} and Φ_{PSII} measured on control and treated *S. nigrum* plants sprayed with 40 g a.i. ha^{-1} metsulfuron-methyl + 0.75% v/v isodecyl alcohol ethoxylate at 2 DAT and 3 DAT. Figures followed by similar letters are not significantly different from each other.

Species	Treatment	DAT	CO_2 fixation	Φ_{PSI}	Φ_{PSII}
S. nigrum	Control	2	5.66 ^{a}	0.759 ^{a}	0.507 ^{a}
S. nigrum	Treated	2	1.26 ^{b}	0.543 ^{b}	0.304 ^{b}
	$LSD_{0.05}$		1.15	0.073	0.141
S. nigrum	Control	3	5.76 ^{a}	0.786 ^{a}	0.512 ^{a}
S. nigrum	Treated	3	1.05 ^{b}	0.522 ^{b}	0.282 ^{b}
	$LSD_{0.05}$		0.857	0.122	0.110

Table 2: Mean values for CO_2 fixation (μmol m^{-2} s^{-1}), Φ_{PSI} and Φ_{PSII} measured on control and treated *P. persicaria* plants sprayed with 30 g a.i. ha^{-1} metsulfuron-methyl + 0.75% v/v isodecyl alcohol ethoxylate at 3 DAT and 4 DAT. Figures followed by similar letters are not significantly different from each other.

Species	Treatment	DAT	CO_2 fixation	Φ_{PSI}	Φ_{PSII}
P. persicaria	Control	3	6.33 ^{a}	0.669 ^{a}	0.443 ^{a}
P. persicaria	Treated	3	4.60 ^{b}	0.545 ^{a}	0.348 ^{b}
	$LSD_{0.05}$		1.73	NS	0.048
P. persicaria	Control	4	6.45 ^{a}	0.645 ^{a}	0.426 ^{a}
P. persicaria	Treated	4	4.60 ^{b}	0.536 ^{a}	0.350 ^{b}
	$LSD_{0.05}$		0.790	NS	0.045

Discussion

This exploratory study showed that the photosynthetic apparatus of both *S. nigrum* and *P. persicaria* plants were affected by an ALS-inhibiting herbicide. The effects were detectable shortly after application (2 to 4 days). This indicates that there might be scope for utilizing easily measured photosynthetic characteristics, such as Φ_{PSII}, as practical early indicators for the success of an ALS-herbicide application. Still many scientific and practical questions arose. More experiments are needed to answer questions like: "How soon after application are photosynthetic parameters affected?" or "Are photosynthetic parameters predictive enough to be used as early indicators of plants death?". An extensive set of experiments is being prepared to study these effects in more detail. Lastly, the question as to how these non-photosynthetic herbicides affect the operation of photosynthesis only a few days after application, and how different species (e.g., *Brassica* and *Helianthus*) can respond so differently, still needs to be answered.

Acknowledgements

André Uffing is greatly acknowledged for assistance with the plants, Manon Mensink and Joost Ruijsch for technical assistance with the photosynthetic measurements. This research was supported by the Technology Foundation STW, applied science division of NWO and the technology programme of the Ministry of Economic Affairs.

Chapter 4

Influence of the ALS inhibitor metsulfuron-methyl on the operation, regulation and organization of photosynthesis in *Solanum nigrum*

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Photosynthesis Research (in press)

Abstract

The influence of the ALS inhibitor metsulfuron-methyl on the operation of the photosynthetic apparatus was examined for four weeks old climate chamber grown *Solanum nigrum* (black nightshade) plants. To have an indication on the relative performance of the photosynthetic apparatus of ALS-treated plants the level of carbon dioxide (CO_2) fixation, the relative quantum efficiency of photosystem I (Φ_{PSI}) or photosystem II (Φ_{PSII}) electron transport and leaf chlorophyll content were assessed for both control and treated plants at 2, 4 and 7 days after application of the herbicide. Results indicated a progressive inhibition of the level of carbon dioxide (CO_2) fixation, the relative quantum efficiency of photosystem I (Φ_{PSI}) and II (Φ_{PSII}) electron transport and the leaf chlorophyll content already two days after application of the herbicide. The linear relationship between the photosystem I and II was unaltered by herbicidal treatment and was sustained under conditions where large changes in pigment composition of the leaves occurred. It appears that the stress-induced loss of leaf chlorophyll is not a catastrophic process but rather is the consequence of a well organized breakdown of components. Under photorespiratory and non-photorespiratory conditions, the relationship between the index of electron transport flow through photosystem I and II and the rate of CO_2 fixation is altered so that electron transport becomes less efficient at driving CO_2 fixation.

Introduction

The acetolactate synthase inhibitors herbicides (ALS inhibitors, Herbicide Resistance Action Committee group B), from which the sulfonylureas are a particular sub-group, selectively inhibit acetolactate synthase (EC 4.1.3.18), which is the first enzyme involved in chloroplastidic biosynthesis of essential branched-chain amino acids. For the biosynthesis of valine and leucine ALS catalyses the condensation reaction between two molecules of pyruvate to form 2-acetolactate with elimination of CO_2, while a molecule of pyruvate is condensed with 2-ketobutyrate in a similar reaction leading to the biosynthesis of isoleucine. Shortly after application of ALS herbicides profound changes take place within the plants. The first physiological response of plants treated with ALS herbicides is the cessation of growth within hours after the application (Rhodes et al., 1987; Shaner & Singh, 1991). Several authors suggested that this inhibitory effect was not due to an inhibition of photosynthesis, respiration, hormone-regulated cell elongation, protein synthesis or RNA synthesis (Pillmoor & Caseley, 1987; Ray, 1982; Shaner & Reider, 1986). Visual symptoms, including anthocyanin accumulation (Suttle & Schreiner, 1982), leaf chlorosis, terminal bud death and necrosis, develop slowly and may not appear until several days to weeks after herbicide application (Peterson, 2001). ALS inhibiting herbicides rapidly disrupt protein synthesis and cell division (Ray, 1982; Rost & Reynolds, 1985). Rhodes et al. (1987) showed that the increase in amino acids following chlorsulfuron treatment is caused by protein hydrolysis. These inhibitors also cause rapid decrease in the level of soluble proteins (Shaner, 1989). Mitosis and DNA synthesis decrease within a few hours after the application of ALS-inhibiting herbicides (Ray, 1982; Rost et al., 1990). These herbicides also cause a rapid decrease in the translocation of photosynthate to the growing points of the plant. Thus, the meristematic tissue is not only deprived of the branched-chain amino acids, but is also starved for carbon. It has been suggested that this combination of stresses kills the plants (Shaner & Singh, 1992).

Photosynthesis is not regarded to be a primary target of ALS inhibiting herbicides, but changes in chlorophyll fluorescence responses have already been observed in treated plants. Judy et al. (1990) found effects on the fluorescence from barley 2 h after treatment with imazaquin and Percival and Baker (1991) found effects on the fluorescence from wheat leaves 24 h after treatment with the ALS-inhibitor imazamethabenz-methyl at the recommended field rates.

Being able to detect early effects on photosynthesis of herbicides whose mode of action does not directly affect photosynthesis also has practical implications. Increased concerns about environmental side effects of herbicides, development of herbicide resistance in weeds and the economic drive to reduce the cost of the inputs have resulted in increasing pressure on farmers to optimize the use of herbicides. The Minimum Lethal Herbicide Dose (MLHD) technology (Kempenaar et al., 2002a; Ketel, 1996) is a decision support system leading to the use of lower rates of photosynthesis inhibiting herbicides. The method allows the calculation of the minimum dose of a photosynthesis inhibiting herbicide that will be needed to control a weed population. A method for the early detection of herbicidal effects, based on simple and rapid measurements of photosystem II activity, is then used to evaluate the efficacy of the treatment shortly after application. Extension of the MLHD technology to the acetolactate synthase inhibitors herbicides requires the identification of suitable parameters for evaluation of the activity of the herbicides shortly after application. Under field conditions, the visible effects of herbicide action are only apparent 10 to 20 days after spraying, which is too long a delay in the event that a second application of herbicide is necessary. An early detection method would permit a prompt second ALS inhibiting herbicide application in case of failure of the first low rate application. This last element is of particular importance for ensuring that even though minimal doses of herbicides have been employed, there is a guarantee that the treatment will be successful in eliminating the weeds. Such a guarantee has contributed to the adoption of the MLHD methodology (Kempenaar et al., 2004).

The response of the photosynthetic system to stress involves changes at the metabolic and electron transport level; stress results in decreases in the rate of CO_2 fixation, and chloroplast electron transport monitored by means of measurements of the quantum efficiencies for electron transport by photosystems I and II (De Groot et al., 2003; Kingston-Smith et al., 1997, 1999). The individual responses of these processes are interesting in their own right, but in addition by comparing these processes with each other it is possible to understand more of how they regulate in response to stress and what the limits of this response are (De Groot et al., 2003; Harbinson et al., 1990a; Kingston-Smith et al., 1997, 1999; Laisk & Oja, 1994). This work has so far shown that in the short-term regulation of electron transport is achieved by changing the capacity of the electron transport chain between photosystems I and II probably by adjusting intrathylakoid pH (Genty & Harbinson, 1996; Laisk & Oja, 1994); it is not known to what extent this mechanism operates during long-term stress. It is clear, however, that in long-term stress the overall co-ordination of the electron transport chain, and its relationship with photosynthetic metabolism is remarkably stable (De Groot et al., 2003; Kingston-Smith et al., 1997, 1999).

The aim of the study was to see whether sulfonylurea herbicides had an effect, even though indirect, on the operation of the photosynthetic apparatus of *S. nigrum* plants. Special emphasis was put on how these effects on photosynthesis developed in time. Another objective of the work was to determine how the regulation and protection of the photosynthetic apparatus operates under conditions of prolonged and severe metabolic dysfunction provoked by the hydrolysis of soluble proteins. Finally, this work should give more understanding on the possible processes of morbidity related to the type of herbicide used and the cessation of growth.

Materials and methods

Plant material and spraying procedure

Seeds of *Solanum nigrum* (black nightshade) were germinated on a moist mixture of potting soil and sand (2:1). Fifteen days after sowing individual plants were transferred into 1 dm^3 pots and placed in a growth chamber with a photosynthetically active radiation (PAR) of 220 μmol m^{-2} s^{-1} for 12 hours (lamps: TL-D-HF, Philips, Eindhoven, The Netherlands). The plants were grown at day / night temperatures of 22°C / 18°C and 70% relative humidity. Water and soil nutrients were supplied so as not to be limiting for growth.

Four-week-old (3 leaf stage) *S. nigrum* plants were sprayed with an air-pressurized laboratory track sprayer delivering 400 litres ha^{-1} herbicide solution at 303 kPa. *S. nigrum* plants were treated with 16 g a.i. ha^{-1} of metsulfuron-methyl (Ally, 40 g L^{-1}, DuPont) and 0.75% v/v isodecyl ethoxylate (Trend 90, DuPont). Isodecyl ethoxylate was used as a surfactant to improve the penetration and uptake of metsulfuron-methyl by the leaves.

Photosynthesis measurements

To have an indication on the relative performance of the photosynthetic apparatus of ALS-treated plants the level of carbon dioxide (CO_2) fixation, the relative quantum efficiency of photosystem II electron transport (Φ_{PSII}) and the relative quantum efficiency of photosystem I electron transport (Φ_{PSI}) were assessed for both control and treated plants. Equipment similar to that described by De Groot et al. (2003) was used. CO_2 fixation was measured using an infra-red gas analyser (Mark 3, Analytical Development Company, Hoddesdon UK). Actinic light was provided by a quartz halogen lamp filtered by Near Infra Red (NIR) and Calflex dichroic mirrors (Balzers, Liechtenstein), and light-intensity was adjusted using metal-film neutral density filters (Balzers, Liechtenstein) (De Groot et al., 2003). Two excitation wavelengths, 560 and 660 nm, were used to excite the chlorophyll fluorescence in order to measure Φ_{PSII}; the fluorescence they each produce was electronically recovered and displayed separately. The efficiencies derived from the two excitation wavelengths will be referred to as

$\Phi_{PSII560nm}$ and $\Phi_{PSII660nm}$, respectively. Φ_{PSI} was measured using the irradiance-induced absorbance change around 820 nm (Harbinson & Woodward, 1987). The reason for using two different excitation wavelengths for the measurement of Φ_{PSII} was to sample the same chloroplast populations within the leaves as were being sampled by the CO_2 fixation measurements and PSI measurement system. A problem with light-based measurement systems is that at different wavelengths leaves will have different effective extinction coefficients for the measuring beams, thus producing a wavelength dependent gradient of measurement light through the leaf. As a result of this, different measurement wavelengths will sample different populations of chloroplasts within the leaf. So, a strongly absorbed measuring beam (e.g., in the red or blue part of the spectrum) will tend to report upon the operation of chloroplast near to the surface of the leaf at which the measuring beam arrives. A weakly absorbed measuring beam (e.g., green, near infra-red) will penetrate the leaf further and thus report on the operation a population of chloroplasts distributed, on average, more deeply in the leaf. Broad-band 'white' light, the actinic source for CO_2 fixation, is absorbed strongly in the upper layers of the leaf, similar to the 660 nm excitation wavelength for fluorescence, whereas the 820 nm measuring wavelength used for photosystem I measurements is not strongly absorbed by the leaf, similar to the 560 nm excitation wavelength. Thus the $\Phi_{PSII560nm}$ is used in correlations with Φ_{PSI} and the $\Phi_{PSII660nm}$ is used in correlations with CO_2 fixation (Kingston-Smith et al., 1997).

The 820 nm absorbance changes used to measure Φ_{PSI} efficiency also permitted the measurement of the kinetics of electron transport between the plastoquinol pool and P-700^+ by measuring the reduction of the photochemically formed P-700^+ pool following a light-on light-off transition (Harbinson & Hedley, 1989; Laisk & Oja, 1994). The kinetics of the relaxation of the 820 nm absorbance change due to P-700^+ reduction is pseudo-first order. The recording of the absorbance change and its analysis to obtain the first-order rate constant for electron transport from the plastoquinol pool and P-700^+ were carried out as described in Kingston-Smith et al. (1999).

The CO_2 fixation and efficiency measurements were made in air consisting of 20% (v/v) oxygen (O_2), 350 ppm CO_2 with the remainder as nitrogen (N_2), at a temperature of 20 to 23°C. During experiments dark-adapted leaves were initially exposed to the lowest excitation irradiance to be used (50 μmol m^{-2} s^{-1}), and then to increasing levels of irradiance. An actinic light source was used to provide the step increase in irradiance from 0 to 750 μmol m^{-2} s^{-1} (light level $step_n \approx 2 \times$ light level $step_{(n-1)}$). Leaves were allowed to establish steady state photosynthesis, which took between 20-40 min, before photosynthetic measurements were made. A dark respiration measurement was made at the end of each irradiance step and all CO_2 fixation rates were calculated as gross rates. To investigate the possible development of sink-limitation of photosynthesis following herbicidal application, the photosynthetic measurements made at 20% (v/v) oxygen (irradiance from 0 to 750 μmol m^{-2} s^{-1}) were followed by measurements in 2% (v/v) oxygen (Foyer & Galtier, 1996). Forty-five minutes after changing the oxygen concentration from 20 to 2%, during which time the leaf was in darkness, the photosynthetic parameters were measured again from lower to higher irradiance levels. Unsprayed four-week-old (3-leaf stage) *S. nigrum* were measured at 1 and 3 DAT (no significant differences between 1 and 3 DAT, while treated *S. nigrum* plants were measured at 2 and 4 DAT. The quantum efficiency of gross CO_2 fixation (Φ_{CO2}) at each irradiance level was calculated as the ratio of CO_2 fixation to incident irradiance.

The light-saturated rate of CO_2 fixation and the curvature factor of the irradiance response curves were calculated by fitting a non-rectangular hyperbola (Thornley & Johnson, 1990):

$$A = \frac{\Phi \times I + A_{\max} - [(\Phi \times I + A_{\max})^2 - 4 \times \Phi \times I \times A_{\max} \times \theta]^{1/2}}{2 \times \theta}$$

where A (μmol m^{-2} s^{-1}) is the CO_2 fixation rate, I (μmol m^{-2} s^{-1}) is the irradiance, Φ is an estimate of the maximal apparent quantum yield, $A_{\max}$ (μmol m^{-2} s^{-1}) is the light-saturated rate of photosynthesis at infinitely high irradiances and θ is the curvature term that describes the transition between the light-limited and light-saturated regions of the CO_2 fixation – irradiance curve. The non-rectangular hyperbolic model assumes that there is a completely non-linear relationship between CO_2 fixation and irradiance, an assumption that sometimes leads to an erroneous estimation of Φ. For that reason, the light-limited quantum yield of CO_2 fixation was more directly estimated as the difference between CO_2 fixation at zero irradiance and 50 μmol m^{-2} s^{-1} divided by the difference in irradiance (50 μmol m^{-2} s^{-1}).

Plant growth, leaf absorptance and chemical analysis

The growth of another set of identically grown plants (10 plants per treatment) was followed from 2 to 11 DAT. Leaves were separated into three classes: fully expanded (larger than 3 cm), partially expanded (1 to 3 cm) and very small (smaller than 1 cm). Leaf development was assessed every two to four days. Plant height (from soil surface to plant tip), plant fresh weight and plant dry weight (after at least 48 h at 70°C) were determined at 11 DAT.

Another set of plants (4 plants per treatment) was used for the determination of the leaf absorptance and leaf chlorophyll content. The absorptance of the third leaf (counted from bottom to top) in the spectral range 400-800 nm was measured at 2 nm intervals using a Taylor Sphere (for a non-diffuse incident irradiance) (LI-COR, Lincoln, Nebraska, USA) and an Instaspec CCD spectrometer (Oriel Scientific, Stratford, CT, USA). A weighed sample (0.1 to 0.2 g) from the third leaf was taken and the chlorophyll extracted with dimethylformamide. The extraction took 3 to 6 days in darkness at 4°C. Subsequently, the absorbance of the extract was measured with a spectrophotometer (Shimadzu UV 160-A; Shimadzu Scientific Instrument Corp., Columbia, Md., USA) at 647.0 and 664.5 nm. Chlorophyll concentration (Chl $a+b$ in mg g^{-1} FW) and chlorophyll a/b ratio were calculated (Inskeep & Bloom, 1985).

Statistics

All measurements were conducted three times. Data were analysed at a significance level of $\alpha = 0.05$ with one-way ANOVA using Genstat 7.2 (Lawes Agricultural Trust, IACR-Rothamsted, UK).

Results

Plant growth

Plant growth was assessed from 0 to 11 DAT by counting the number of fully expanded leaves at 2 to 4 day intervals. Control and treated plants had a comparable number of fully expanded leaves until 2 DAT (Figure 1). From 4 DAT onwards, control plants had significantly more fully expanded leaves than treated plants. The growth of treated plants was almost completely inhibited (3.1, 3.3, 3.3 leaves at 4, 8 and 11 DAT, respectively). At 11 DAT (final assessment) control plants had 6.5 leaves larger than 3 cm whereas treated plants had only 3.3. The dry weight and plant height of treated plant were also significantly reduced (Table 1). From these data it is evident that the ALS-inhibiting herbicide treatment had strong and rapid effects on the growth of *S. nigrum* plants.

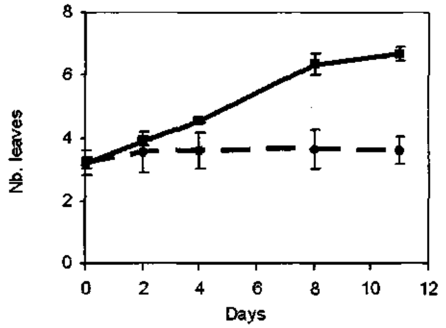

Figure 1: Number of fully expanded leaves (per plant) plotted against days after treatment (DAT). Solid line for control plants and dotted line for treated plants. Vertical bars indicate standard error of the mean.

Table 1: Number of fully expanded leaves (per plant), dry weight (g per plant) and plant height (cm) for control plants and treated plant at final harvest (11 DAT). For each measured parameter, numbers followed by different letters are significantly different ($P<0.05$).

	Number of fully expanded leaves	Dry weight	Plant height
Control	6.5 ^{a}	0.714 ^{a}	6.2 ^{a}
Treated	3.3 ^{b}	0.206 ^{b}	3.5 ^{b}
$LSD_{0.05}$	0.33	0.0594	0.28

Photosynthesis light response curves

At 20% O_2 concentration, the fixation of CO_2 by control plants increased with increasing irradiance up to the maximum irradiance employed (750 μmol m^{-2} s^{-1}). For treated plants (at both 2 and 4 DAT) CO_2 fixation approached light saturation at much lower irradiance (400 μmol m^{-2} s^{-1}, Figure 2). At 20% O_2 concentration the maximum rate of CO_2 fixation, estimated by the non-rectangular hyperbola regression, was significantly higher for control plants (11.9 μmol m^{-2} s^{-1}) than for treated plants (5.0 and 2.8 μmol m^{-2} s^{-1} at 2 and 4 DAT, respectively). At both 2 and 4 DAT the difference in CO_2 fixation between control and treated plants was significant from 200 μmol m^{-2} s^{-1} onwards ($P<0.05$). At ambient atmospheric CO_2 concentration reduction in atmospheric O_2 concentration from 20% to 2% produces an almost complete suppression of photorespiratory activity in leaves, and, consequently an increase in the rate of CO_2 fixation occurred (Figure 2). At 2% O_2 a significant reduction in CO_2

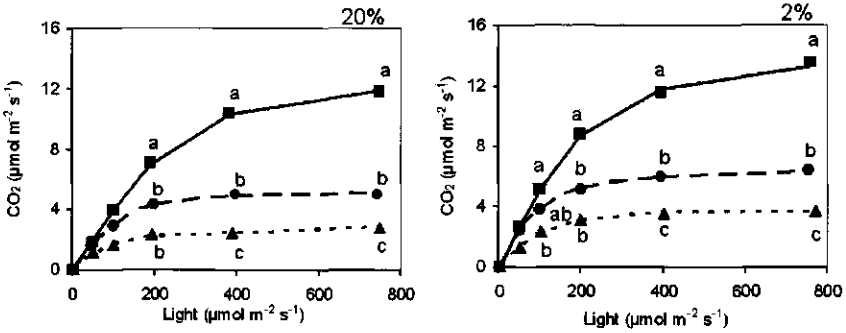

Figure 2: Relationship between CO_2 fixation (μmol m^{-2} s^{-1}) and irradiance (μmol m^{-2} s^{-1}) at 20% and 2% oxygen for control plants (squares) and treated plants at 2 DAT (circles) and 4 DAT (triangles). Non-rectangular hyperbola fit for control (solid line), treated plants at 2 DAT (dashed line) and 4 DAT (dotted line).

fixation rate of treated plants was observed from 200 μmol m^{-2} s^{-1} onwards ($P<0.05$) at 2 DAT but observed from 100 μmol m^{-2} s^{-1} onwards ($P<0.05$) at 4 DAT. When O_2 was depleted from 20 to 2%, CO_2 fixation was enhanced over a wide range of irradiances by an average of 20%, 24% and 37% for control *S. nigrum*, treated *S. nigrum* 2 DAT and treated *S. nigrum* 4 DAT, respectively. The light-limited quantum yield of CO_2 fixation, estimated as the difference between CO_2 fixation at zero irradiance and 50 μmol m^{-2} s^{-1} divided by the difference in irradiance (50 μmol m^{-2} s^{-1}), was equal to 0.043 and 0.034 for O_2 concentrations of 20% and 2% with no significant differences between control and treated plants. The curvature factor (θ) was equal to 0.87 and 0.82 for O_2 concentrations of 20% to 2%, respectively with no significant differences between control and treated plants.

Quantum efficiency for electron transport by PSII and PSI

The quantum efficiency for electron transport by PSII at 660 nm wavelength ($\Phi_{PSII660nm}$) decreased with increasing irradiance at both atmospheric (20%) and reduced (2%) oxygen concentrations (Figure 3). As the relationship between $\Phi_{PSII660nm}$ and irradiance was not linear, the $\Phi_{PSII660nm}$ measurements of the different treatments were compared separately for each irradiance level. For both oxygen concentrations, $\Phi_{PSII660nm}$ decreased more with increasing irradiance in treated plants (at both 2 and 4 DAT) than in the control plants. The measurements at zero irradiance, equivalent to the dark-adapted ratio of variable / maximum fluorescence (F_v/F_m), were similar for both control and treated plants (at both 2 and 4 DAT) indicating the absence of photoinhibition at atmospheric oxygen concentration (Kingston-Smith et al., 1999). At

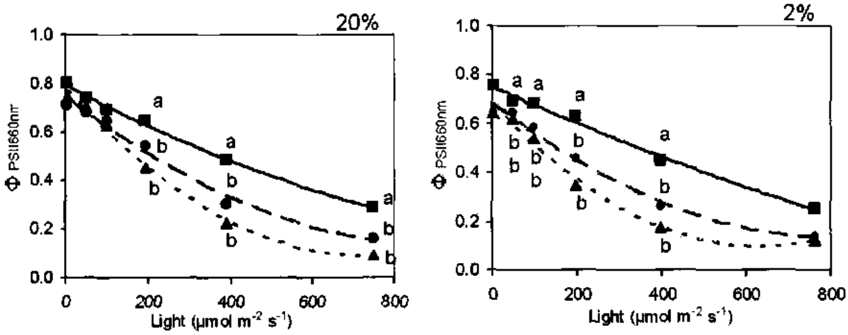

Figure 3: Relationship between $\Phi_{PSII660nm}$ and irradiance (μmol m^{-2} s^{-1}) at 20% oxygen and 2% oxygen for control (squares), treated plants at 2 DAT (circles) and 4 DAT (triangles). For each irradiance level, letters indicate whether figures are significantly different from each other ($P<0.05$).

atmospheric oxygen concentration, the difference in $\Phi_{PSII660nm}$ between the control and treated plants (at both 2 and 4 DAT) was significant at 200, 400 and 750 μmol m^{-2} s^{-1} ($P<0.05$). At 2% oxygen concentration differences between control and treated plants (at both 2 and 4 DAT) were observed at 100, 200 and 400 μmol m^{-2} s^{-1} ($P<0.05$). No significant differences were recorded between the measurements of $\Phi_{PSII660nm}$ done at 2 DAT and at 4 DAT. At high irradiance (750 μmol m^{-2} s^{-1}) the $\Phi_{PSII660nm}$ of both control and treated plants were low indicating progressive limitation of PSII efficiency due to a combination of increasing irradiance and a finite capacity for thylakoid electron transport. At high irradiance the photochemical efficiencies were so low in both the control and treated leaves that it becomes difficult, at least at 2% O_2, to see any difference that could be attributed to the herbicide treatment. In the range of irradiances used the difference between $\Phi_{PSII660nm}$ values measured in 20% and 2% oxygen was small (Figure 4) suggesting only a small down-regulation of electron transport in response to the inhibition of photorespiration.

Measurements of Φ_{PSI} provide similar information as for Φ_{PSII}, but with additional information about the redox state of the PSI acceptor pool and the kinetics of the electron transfer between the photosystems (Harbinson & Hedley, 1993). Similar to what was observed for $\Phi_{PSII660nm}$, Φ_{PSI} decreased with increasing irradiance (Figure 5). This finding was previously reported (Genty et al., 1990; Harbinson et al., 1990b). In treated plants the decrease in Φ_{PSI} at both 2 and 4 DAT was stronger than in the control plants under both atmospheric and reduced oxygen concentrations. At atmospheric oxygen concentration, the difference in Φ_{PSI} between control and treated plants increased with the number of days after application of the herbicide.

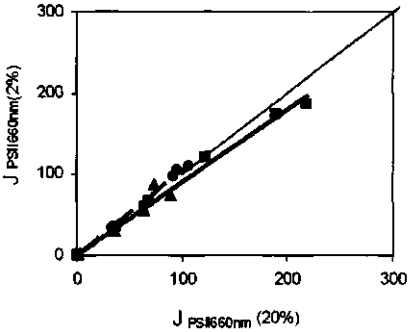

Figure 4: Relationship between $\Phi_{PSII660nm}$ at 2% oxygen and $\Phi_{PSII660nm}$ at 20% oxygen for control (squares), treated plants at 2 DAT (circles) and treated plants at 4 DAT (triangles). The trend lines are linear regressions constrained to pass through the origin. A 1:1 trend line has also been drawn.

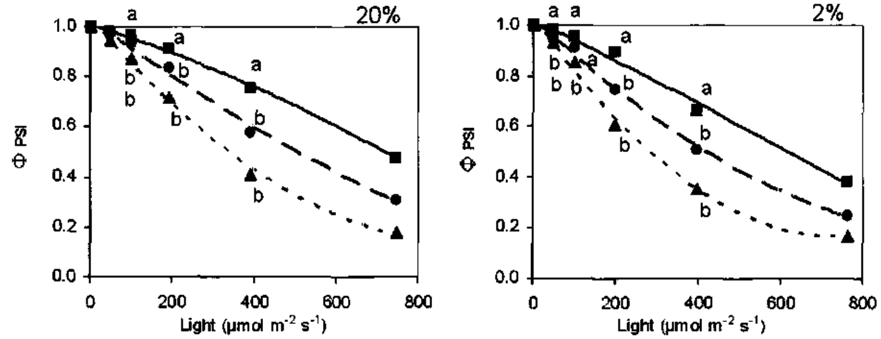

Figure 5: Relationship between Φ_{PSI} and irradiance (μmol m^{-2} s^{-1}) at 20% oxygen and 2% oxygen for control (squares), treated plants at 2 DAT (circles) and 4 DAT (triangles). For each irradiance level, letters indicate whether figures are significantly different from each other (*P*<0.05).

At both 2 and 4 DAT, the difference in Φ_{PSI} between control and treated plants was significant at 100, 200 and 400 μmol m^{-2} s^{-1} (*P*<0.05) but not at 750 μmol m^{-2} s^{-1} (*P*<0.05). Under reduced oxygen concentration, the difference in Φ_{PSI} between control and treated plants (at either 2 or 4 DAT) was significant from 50 to 400 μmol m^{-2} s^{-1} (*P*<0.05) but not at 750 μmol m^{-2} s^{-1}. Hardly no significant differences were recorded between treated plants at 2 and 4 DAT. Measurements of Φ_{PSI} done at 20% oxygen were 10 to 15% higher than measurements obtained at 2% oxygen concentrations.

The predominantly linear relationship between $\Phi_{PSII560nm}$ and Φ_{PSI} (Figure 6), passing close by the origin, demonstrates that over a wide range of irradiance (from 100 to 750 μmol m^{-2} s^{-1}), oxygen concentrations (20 or 2%) and plant status (control or treated with an ALS inhibiting herbicide), the plants maintain a close co-ordination between the quantum efficiencies of PSI and PSII in relation to changing capacities for CO_2 fixation. Figure 7 shows the relationship between the values of Φ_{CO2} obtained from treated (2 and 4 DAT) and untreated *S. nigrum* leaves under non-photorespiratory and photorespiratory conditions. Clearly the quantum yield of CO_2 fixation under photorespiratory condition is linearly related to the yield under non-photorespiratory conditions over the greater part of a range of values obtained, and there appears to be no significant difference between treated and untreated leaves in respect of the response of their CO_2 fixation efficiency to the elimination of photorespiration.

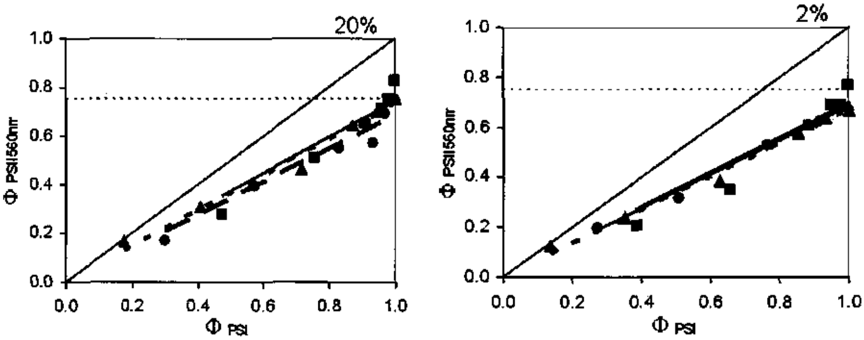

Figure 6: Relationship between $\Phi_{PSII560nm}$ and Φ_{PSI} at 20% oxygen and 2% oxygen for control (squares), treated plants at 2 DAT (circles) and 4 DAT (triangles). The trend lines are linear regressions constrained to pass through the origin. A 1:1 trend line has also been drawn. Dotted line at $\Phi_{PSII560nm}$ equivalent to 0.78 (maximal theoretical value for $\Phi_{PSII560nm}$).

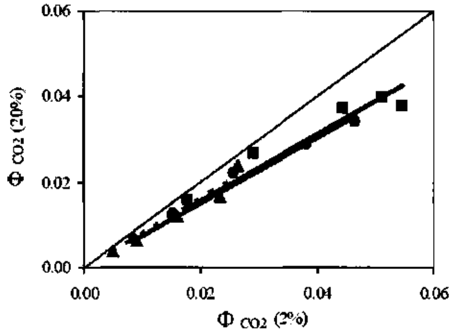

Figure 7: Relationship between the quantum yield of CO_2 fixation (Φ_{CO2}) at 20% oxygen and quantum yield of CO_2 fixation (Φ_{CO2}) at 2% oxygen for control (squares), treated plants at 2 DAT (circles) and treated plants at 4 DAT (triangles). The trend lines are linear regressions constrained to pass through the origin. A 1:1 trend line has also been drawn.

The relationship between CO_2 fixation and the index of linear photosynthetic electron transport through PSII, J_{PSII} (which is the product of Φ and irradiance) is presented in Figure 8. J_{PSII} and J_{PSI} (index of linear photosynthetic electron transport through PSI which is the product of Φ_{PSI} and irradiance, data not shown) give a 'rate' of electron transport which is proportional to the rate of photosynthetic electron transport through PSII or PSI (Kingston-Smith et al., 1999, 1997). In the range of irradiance tested the relationships between $J_{PSII660nm}$, J_{PSI} (data not shown), and CO_2

fixation were linear under both atmospheric and reduced oxygen concentrations. Surprisingly, the slopes of the relationship between $J_{PSII660nm}$, J_{PSI} (data not shown) and CO_2 fixation were different for control and treated plants at 2 and 4 DAT irrespective of the oxygen concentration. The apparent rate constant for photosynthetic electron transport was independent of irradiance in the range 200 to 750 μmol m^{-2} s^{-1}. No significant differences were recorded between control and treated plants exposed to 20% oxygen concentrations. Comparable values were obtained for plants exposed to 2% oxygen. The ΔA_{820nm} parameter corresponding to complete oxidation of P700 ($\Delta A_{820nmmax}$) was higher for control plants (around 84 arbitrary units) than for treated plants (43.5 and 41 arbitrary units at 2 and 4 DAT, respectively) and rather stable over the range of irradiance and oxygen concentrations tested. The maximum rate of CO_2 fixation appeared to be substantially independent of the apparent rate constant for P700 reduction following a light / dark transition at both 20 and 2% oxygen concentrations (Figure 9). At 20% O_2, rate constant measurements were 10 to 27% larger than measurements done at 2% O_2.

Chlorophyll content

Compared to control plants the level of chlorophyll *a+b* was significantly lower in treated plants (Table 2). Already at 2 DAT the total chlorophyll concentration of treated plants (Treated, 2 DAT) was reduced by 25%. At 4 DAT the chlorophyll content of treated plants (Treated, 4 DAT) was 45% lower than of control plants (Control, 4 DAT). No significant differences were observed between control plants at 2 and 4 DAT. After herbicide treatment a slight change in chlorophyll *a/b* ratio was observed (Table 2).

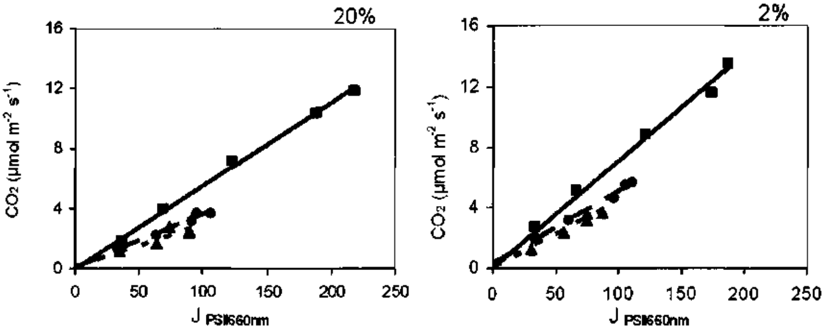

Figure 8: Relationship between CO_2 fixation (μmol m^{-2} s^{-1}) and $J_{PSII660nm}$ at 20% oxygen and 2% oxygen for control plants (squares), treated plants at 2 DAT (circles) and 4 DAT (triangles). The trend lines are linear regressions constrained to pass through the origin.

Table 2: Chlorophyll concentration (Chl *a*+*b* in mg g^{-1} fresh weight) and chlorophyll *a*/*b* ratio for control plants and treated plants at 2 and 4 days after treatment. Figures followed by different letters are significantly different ($P<0.05$).

Treatments	DAT	Chl *a*+*b*	Chl *a*/*b*
Control	2	1.705 ^{a}	3.600 ^{a}
Control	4	1.760 ^{a}	3.526 ^{a}
Treated	2	1.272 ^{b}	3.376 ^{b}
Treated	4	0.992 ^{c}	3.364 ^{b}
$LSD_{0.05}$		0.0896	0.0877

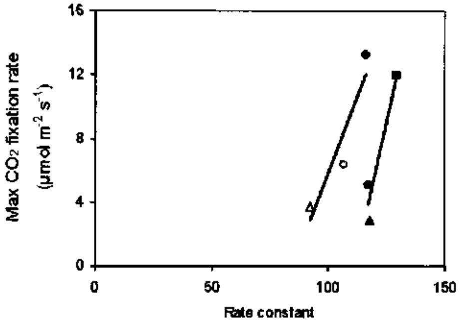

Figure 9: Maximum CO_2 fixation (μmol m^{-2} s^{-1}) and the apparent rate constant for P700 reduction following a light/dark transition at 20% (closed symbols) and 2% oxygen (open symbols) for control plants (squares), treated plants at 2 DAT (circles) and 4 DAT (triangles). The trend lines are linear regressions.

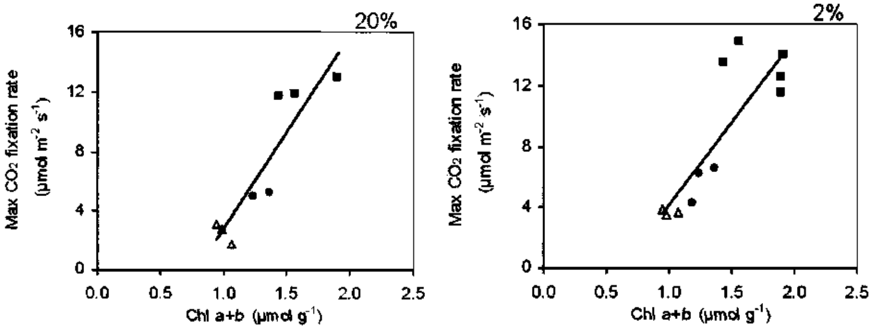

Figure 10: The relationship between maximum CO_2 fixation (μmol m^{-2} s^{-1}) and chlorophyll *a*+*b* concentration for leaves exposed to 20% ($r^2 = 0.82$) and 2% ($r^2 = 0.74$) oxygen. Control plants (squares), treated plants at 2 DAT (circles) and treated plants at 4 DAT (triangles). Linear regression included.

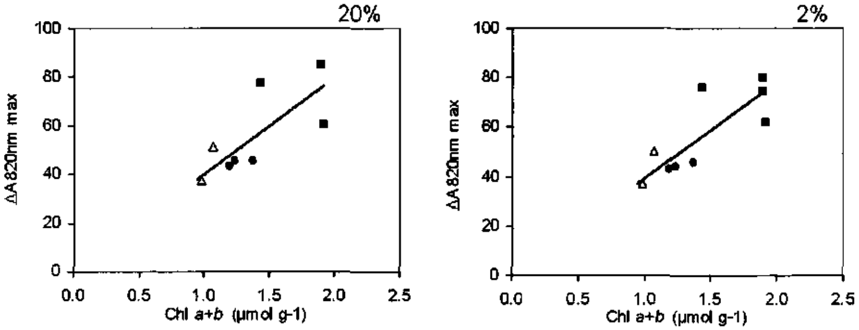

Figure 11: The relationship between ΔA_{820nm} corresponding to complete oxidation of P700 and chlorophyll *a+b* concentration for leaves exposed to 20% (r^2 = 0.54) and 2% (r^2 = 0.65) oxygen. Control plants (squares), treated plants at 2 DAT (circles) and treated plants at 4 DAT (triangles).

The chlorophyll *a/b* ratio of treated plants (3.376 and 3.364 at 2 and 4 DAT, respectively) was lower than that of control plants (3.600 and 3.526 at 2 and 4 DAT, respectively). This change indicates that in the treated plants the relative loss of chlorophyll *a* was only slightly greater than the loss of chlorophyll *b*. Positive linear relationships were observed between both the maximum rate of CO_2 fixation (Figure 10) or the $\Delta A_{820nmmax}$ parameter (Figure 11) and the concentration of chlorophyll *a+b* present in the plants.

Leaf absorptance versus chlorophyll content

The herbicide treatment had little effects on leaf absorptance. The reduction in leaf absorptance was in average 5% for treated plants at 2 DAT and 10% for treated plants at 4 DAT. A linear relationship was found between absorptance and the total chlorophyll concentration (data not shown).

Discussion

Herbicide effects on plant growth

The application of the herbicide metsulfuron-methyl produced a strong and rapid reduction in the growth of *S. nigrum* plants. Data presented suggest a very short time lag before an effect on growth was apparent. This is consistent with results from Gaston et al. (2003) and earlier observations from Shaner and Singh (1992) that the growth of plants treated with ALS inhibiting herbicides such as sulfonylurea, slows within hours and ceases within a few days. At final harvest, treated plants exhibited stunting which is known to be one possible ALS-inhibiting-herbicide symptom (Peterson, 2001). As expected, treated plants also exhibited interveinal chlorosis, red leaf venation, purpling and gradual death. Visual symptoms were first noticeable 4 to 7 DAT while death of treated plants only happened at 15 to 20 days after treatment. The question arises as to how this cessation of growth relates to changes in the operation of photosynthesis, and especially whether growth limits photosynthesis or *vice versa.*

The relationship between the herbicide, photosynthesis and growth

It is evident that even though methsulfuron-methyl is not a herbicide which targets the photosynthetic machinery, its application produces a rapid progressive reduction in photosynthesis, with both CO_2 fixation (Figure 2) and electron transport showing a reduction in activity following application of the herbicide. Given the rapid cessation of growth this might not appear surprising. However the absence of any effect of herbicide application on the relative efficiencies of CO_2 fixation at 20% and 2% O_2 suggests that the down-regulation of CO_2 fixation was not due to sink-limitation. If sink limitation of photosynthesis would have developed, it would be expected to produce some degree of oxygen insensitivity in the operation of CO_2 fixation. This would have revealed itself as a reduced increase in the rate of CO_2 fixation following the elimination of photorespiration by using an atmosphere containing 2% O_2 (Foyer & Galtier, 1996). The absence of sink limitation is supported by the lack of any effect of herbicide application on the Φ_{PSII} or J_{PSII} irradiance response measured on leaves in either 2% or 20% O_2 (Figure 3). Regardless of whether or not the plants had been treated with herbicide, the values of Φ_{PSII} or J_{PSII} obtained over a range of irradiances were only slightly lower in 2% O_2 compared to 20% (Figure 4). This means that photosynthetic electron transport was only slightly down-regulated by the elimination of photorespiration, a response that implies only minor sink-limitation in either the control or treated plants. Though it appears that the reduction in photosynthesis was not due to the restriction of growth, it cannot, however, be inferred from these data that the reverse was true. The reduction of growth is rapid (within 2 days) but the reduction in photosynthetic capacity was more gradual, so it may be that the inhibition of photosynthesis and growth were not directly dependent on each other, though sharing a common cause.

Herbicidal treatment and the internal co-ordination of PSI and PSII activity

The irradiance dependencies of both Φ_{PSII} and Φ_{PSI} are strongly affected by herbicidal treatment (Figures 3, 5). However the relationship between the efficiencies of both photosystems, whether revealed by comparing the changing efficiencies or electron fluxes, is unaltered by herbicidal treatment (Figure 6). The efficiencies of both photosystems decline in parallel, suggesting a predominant role for linear electron transport – a phenomenon that has been widely reported (Harbinson & Foyer, 1991; Harbinson et al., 1990a; Kingston-Smith et al., 1999). It is noteworthy that the consistent, linear relationship between the efficiencies of both photosystems is sustained under conditions where large changes are occurring in the pigment composition of the leaves (Table 2). This loss of pigments is not accompanied by any photoinhibition of PSII, and the value of $\Delta A_{820nnmax}$ value decreases in proportion to the decreasing leaf chlorophyll content (Figure 11). The chlorophyll *a/b* ratio is also largely unchanged by the effects of the herbicide (Table 2). Taken together these data imply that the loss of chlorophyll is sufficiently organized so as to maintain the balance of pigment-protein complexes and function between the photosystems close to that found in control leaves. This situation is similar to that found in cold-grown maize leaves, where in spite of large reductions in leaf chlorophyll provoked by prolonged exposure to low temperatures, the balance between photosystem I and photosystem II function was maintained (Kingston-Smith et al., 1999). It appears that the stress-induced loss of leaf chlorophyll is not a catastrophic, uncontrolled process, but rather the consequence of a well organized breakdown of components.

The rate constant data are especially interesting with regards to the loss of photosynthetic activity of the leaves. Though light saturated CO_2 fixation rates are progressively decreased by herbicidal activity, the values of the rate constant for P700 reduction by electrons coming from PQH_2 pool (k_e) remain relatively unchanged. The rate constant for electron transport is, as the name suggests a measure of the capacity for electron transport through the rate-limiting step of linear electron transport. In measurements made upon individual leaves the value of k_e is closely correlated with the maximum (light-saturated) rate of CO_2 fixation. The apparent contradiction between these observations of the relationship observed here and those reported previously can be partly explained by the changing leaf chlorophyll contents. The actual rate of electron transport associated with a particular rate constant will depend on the quantity of P700 present in the leaf. If the P700 pool decreases then an unchanging rate constant will create a decreasing rate of electron transport. The decrease in chlorophyll content is associated with a decrease in the ΔA_{820max}; the latter reflects the amount of P700 present in the leaf. The decrease in leaf chlorophyll observed during experiments, though large, was insufficient to produce any significant change in leaf absorbance. This implies that the changes in electron transport that occur while k_e remains constant cannot be attributed to a loss of overall light-trapping by the leaves. It may even be that the stability of the rate-constant is the goal of the control process that modifies leaf chlorophyll during the stress created by the herbicide application.

The relationship between electron transport and CO_2 fixation

The relationship between the gross rate of CO_2 fixation and the index of linear photosynthetic electron transport through PSI (J_{PSI}) or PSII (J_{PSII}) were clearly linear under both atmospheric and reduced oxygen concentrations (Figure 8) for the different treatments considered (untreated plants as well as treated plants 2 and 4 DAT). The linear relationship between gross CO_2 fixation and J_{PSII} observed for plants exposed to non-photorespiratory conditions is in agreements with the findings of Harbinson et al. (1990a). Herbicidal treatment, however, affects the quantitative relationship between electron transport and gross CO_2 fixation. In both photorespiratory and non-photorespiratory conditions, the relationship between the index of electron flow through photosystem I or II, and the rate of CO_2 fixation is altered so that electron transport becomes less efficient at driving CO_2 fixation (Figure 8). The effect occurs even under non-photorespiratory conditions so it is not due to an increase in photorespiration provoked by the herbicidal treatment. It also cannot be due to a change in leaf absorbance, as this is scarcely changed by the herbicidal treatment. It cannot likewise be due to the loss of chlorophyll acting in some other unexpected way as the effects were the same at 2 and at 4 DAT, whereas a large drop in chlorophyll content was observed between 2 and 4 DAT. One simple explanation is that the loss of efficiency of electron transport with respect to CO_2 fixation is due to the development of another sink for electron transport. It cannot be stated what this sink is, though speculation that it is O_2 is inevitable. If O_2 were the acceptor this could be a factor in the herbicidal effect produced by metsulfuron-methyl. Considering the response in more detail, a change in atmospheric O_2 concentration from 2% to 20% produces a 22% drop in the rate of CO_2 fixation of control leaves measured when J_{PSII} is 100. In treated leaves under the same circumstances the drop was 28% (Figure 8). So though there appears to be some alternate acceptor activity in the treated leaves, the electron transport-CO_2 relationship is still responding to changes in O_2 concentration in a way that is broadly consistent with the existence of photorespiration.

Early detection of the activity of ALS inhibiting herbicides on weeds

As results clearly demonstrated difference in carbon dioxide fixation, the quantum efficiency for electron transport by PSII (Φ_{PSII}) or PSI (Φ_{PSI}) and the total chlorophyll content between control and treated *S. nigrum* plants, early detection of the activity of ALS inhibiting herbicides on weeds seemed practical. Further experiments confirmed the present results for both *S. nigrum* and *Polygonum persicaria* plants grown under greenhouse and *S. nigrum* plants grown under field conditions. Possibilities of using the different photosynthesis related parameter to assess the effect of an ALS inhibiting herbicide are discussed elsewhere (Chapter 5).

Acknowledgements

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Chapter 5

Can photosynthesis related parameters be used to establish the activity of acetolactate synthase inhibiting herbicides on weeds?

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Submitted

Abstract

The application of the ALS inhibiting herbicide metsulfuron-methyl on greenhouse and field grown *S. nigrum* (black nightshade) and greenhouse grown *P. persicaria* (ladysthumb) resulted in a progressive inhibition of the level of carbon dioxide (CO_2) fixation, the relative quantum efficiency of electron transport through photosystem I (Φ_{PSI}) and II (Φ_{PSII}) and the leaf chlorophyll content. Photosynthetic related measurements, measured 2 to 4 days after treatment at irradiance of 400-500 μmol m^{-2} s^{-1} provided valuable information that was strengthening the visual symptoms that first appeared at 7 to 10 days after application of the herbicide. Measurement of the quantum efficiency for electron transport by photosystem II and the loss in leaf chlorophyll content appeared to be two of the most practical parameters to use when designing an early detection method to assess the toxicity of metsulfuron-methyl. The use of chlorophyll fluorescence would require a comparison of steady-state Φ_{PSII} measurements for control and treated plants which could be realized by either measuring in time (before / after application) or space (treated / untreated patch).

Introduction

In 2000/2001, herbicides accounted for almost 40% of the pesticide use worldwide in terms of the volume of active ingredient (EPA, 2004). Increased concerns about environmental side effects of herbicides, development of herbicide resistance in weeds and the economic drive to reduce the cost of the inputs, have resulted in increasing pressure on farmers to reduce the use of herbicides. The Minimum Lethal Herbicide Dose (MLHD) technology (Kempenaar et al., 2002a; Ketel, 1996) has shown itself to be a promising decision support system leading to the use of lower rates of photosynthesis inhibiting herbicides. This method allows the calculation of the minimum dose of a photosynthesis inhibiting herbicide that will be needed to control a weed population. A method for the early detection of herbicidal effect, based on simple and rapid measurements of photosystem II activity, is then used to evaluate the efficacy of the treatment shortly after application. Extension of the MLHD technology to other common groups of herbicides requires the identification of suitable parameters for evaluation of the activity of the herbicides shortly after application.

Developed over the past 20 years, the acetolactate synthase (ALS) inhibitors are an increasingly important group of herbicides. ALS inhibiting herbicides are widely used around the world due to the relatively low rates of application that are required and their limited environmental impact, low mammalian toxicity, wide crop selectivity and high efficacy (Peterson, 2001). The ALS inhibiting herbicides selectively inhibit acetolactate synthase (EC 4.1.3.18), which is the first enzyme involved in chloroplastidic biosynthesis of essential branched-chain amino acids. ALS inhibiting herbicides rapidly disrupt protein synthesis and cell division. The rapid inhibition of cell division is a specific effect of ALS inhibiting herbicides, since cell division is not similarly affected by herbicides with a different mode of action. Rhodes et al. (1987) showed that the increase in amino acids following application of the ALS-inhibiting herbicide chlorsulfuron was due to protein hydrolysis. ALS inhibiting herbicides also cause rapid decrease in the level of soluble proteins (Shaner, 1989). Mitosis and DNA synthesis decrease within a few hours after the application of ALS-inhibiting herbicides (Rost et al., 1990). The herbicides also cause a rapid decrease in the translocation of photosynthate to the growing points of the plant (Devine, 1989). Thus, the meristematic tissue is not only deprived of the branched-chain amino acids, but is also starved of carbohydrate. It has been suggested that this combination of stresses ultimately kills the plants (Shaner & Singh, 1992). The growth of plants treated with an ALS inhibiting herbicide slows within hours and ceases within a few days (Gaston et al., 2003; Shaner & Singh, 1992). Visual symptoms including anthocyanin accumulation, leaf chlorosis, terminal bud death and necrosis develop slowly and may not appear until several days to weeks after herbicide application (Peterson, 2001).

Photosynthesis is not regarded to be a primary target of ALS inhibiting herbicides, but changes in chlorophyll fluorescence responses have been observed in treated plants. Judy et al. (1990) found effects on fluorescence of barley 2 h after treatment with imazaquin (ALS inhibitor, imidozolinones sub-group). Percival and Baker (1991) found effects on the fluorescence of wheat leaves 24 h after application of imazamethabenz-methyl (ALS inhibitor, imidozolinones sub-group) at its recommended rate. Barbagallo et al. (2003) also demonstrated that many inhibitors of metabolic processes that are not directly involved in photosynthetic metabolism can produce modifications to fluorescence kinetics. In an extensive study, Riethmuller-Haage et al. (Chapter 4) showed that the rate of carbon dioxide (CO_2) fixation, the relative quantum efficiency of photosystem II electron transport (Φ_{PSII}), the relative quantum efficiency of photosystem I electron transport (Φ_{PSI}) and total chlorophyll content of *S. nigrum* plants were all significantly reduced after application of the ALS inhibiting herbicide metsulfuron-methyl. These last observations were made on climate chamber grown plants.

The purpose of the present experiments were to determine whether or not rapid changes in the operation of photosynthesis after application of metsulfuron-methyl as observed on climate chamber grown *S. nigrum* plants can also be detected on *S. nigrum* plants grown under more natural conditions. Observations on *P. persicaria*, a second weed species, were made to check the general validity of the results. Possibilities of using a photosynthesis related parameter to assess the effect of an ALS inhibiting herbicide are discussed.

Materials and methods

Plant material and spraying procedure

Seeds of *S. nigrum* or *P. persicaria* were put on a tray containing a moistened mixture of potting soil and sand (2:1), which was placed in a climate chamber at 22°C / 18°C (day / night) and 70% relative humidity for fifteen days. After germination, individual weed seedlings were transferred into 1 dm^3 pots and, depending on the trial, placed in a climate chamber, in a greenhouse or outside. For the first trial (S-1), which took place in October 2003, *S. nigrum* plants were grown in a climate chamber at 22°C / 18°C (day / night) and 70% relative humidity. The photon flux density was 220 μmol m^{-2} s^{-1} for 12 hours (TL-D-HF, Philips, Eindhoven, The Netherlands). Two trials were conducted in February 2004. *S. nigrum* (trial S-2) and *P. persicaria* plants (trial P-2) were grown in a greenhouse at 18°C / 14°C (day / night), 70% relative humidity and a

12 h photoperiod provided by natural light supplemented with high-pressure mercury lamps. For the last trial (trial S-3), *S. nigrum* plants were grown in the field between April 29 and June 18, 2004. The average temperature was 15.5°C (maximum 30.6°C, minimum 4.9°C). Weed plants were irrigated daily.

Plants of *S. nigrum* and *P. persicaria* were always sprayed in their 3-leaf stage, with an air-pressurized laboratory track sprayer delivering 400 litres ha^{-1} herbicide solution at 303 kPa. *S. nigrum* plants were treated with 16 g a.i. ha^{-1} of metsulfuron-methyl and 0.75% v/v isodecyl ethoxylate. A preliminary test showed that the recommended field rate for metsulfuron-methyl (8 g a.i. ha^{-1}) was insufficient to kill growth chamber raised *S. nigrum* plants. For that reason an application rate of 16 g a.i. ha^{-1} was used as standard in the various trials (S-1, S-2, P-2 and S-3). For *S. nigrum* plants grown under field conditions (trial S-3) both 16 and 8 g a.i. ha^{-1} were tested and no significant differences were observed (data not shown).

Photosynthesis measurements

To have an indication of the relative performance of the photosynthetic apparatus of ALS-treated plants the level of carbon dioxide (CO_2) fixation, the relative quantum efficiency of photosystem II electron transport (Φ_{PSII}) and the relative quantum efficiency of photosystem I electron transport (Φ_{PSI}) were assessed for both control and treated plants. Equipment essentially identical to that described by De Groot et al. (2003) was used. CO_2 fixation was measured using an infrared gas analyser (Mark 3, Analytical Development Company, Hoddesdon UK). Actinic light was provided by a quartz halogen lamp filtered by Near Infra Red (NIR) and Calfex dichroic mirrors (Balzers, Liechtenstein), and light-intensity was adjusted using metal film neutral density filters (Balzers, Liechtenstein) (De Groot et al., 2003). A wavelength of 660 nm was used to excite the chlorophyll fluorescence in order to measure the relative quantum efficiency of photosystem II electron transport. The relative quantum efficiency of PSI electron transport was measured using the irradiance-induced absorbance change around 820 nm (Harbinson &Woodward, 1987).

The CO_2 fixation and efficiency measurements were made in air consisting of 20% (v/v) oxygen (O_2), 350 ppm CO_2 with the remainder nitrogen (N_2), at a temperature of 20 to 23°C. During experiments dark-adapted leaves were initially exposed to the lowest excitation irradiance level (50 μmol m^{-2} s^{-1}), and then to increasing levels of irradiance. The actinic light source was controlled to provide a step-wise increase in photon flux density from 0 to 800 μmol m^{-2} s^{-1}. At each irradiance level, leaves were allowed to establish steady state photosynthesis, which took between 20 to 40 min, before the measurements of CO_2 fixation, Φ_{PSII} and Φ_{PSI} were made. Unsprayed and treated 3-leaf stage *S. nigrum* plants were measured at 2 and 4 days after treatment

(DAT) (trials S-1 and S-2), *S. nigrum* plants grown in the field (trial S-3) were measured at 2, 4 and 7 DAT and *P. persicaria* plants (trial P-2) were measured at 2 DAT.

Plant growth and chemical analysis

Plant height (from soil surface to plant tip) and plant dry weight (after at least 48 h at 70°C) were determined at 11 (trial S-1), 14 (trials S-2 and P-2) or 15 days after treatment (DAT) (trial S-3). Additionally, in trials S-1 and S-3, the number of fully expanded leaves (larger than 3 cm) was assessed every two to four days from application day to final harvest. Four plants per treatment were used for chlorophyll extraction at 2 and 4 DAT (trial S-1) or 2, 4 and 7 DAT (trial S-3). A sample (0.1 to 0.2 g fresh weight) from the third leaf was taken and chlorophyll content was extracted in 3 ml dimethylformamide. The extraction, carried out in darkness, took 3 to 6 days at 4°C. Subsequently the absorbance of the chlorophyll solution was measured with a spectrophotometer (Shimadzu UV 160-A; Shimadzu Scientific Instrument Corp., Columbia, Md., USA) at 647.0 and 664.5 nm. The chlorophyll concentration (Chl $a+b$ in mg g^{-1} FW) and chlorophyll a/b ratio were calculated according to Inskeep and Bloom (1985).

Statistics

Each trial was conducted in three replications. Data were analysed with one-way ANOVA using Genstat 7.2 (Lawes Agricultural Trust, IACR-Rothamsted, UK). Differences between means were evaluated at a significance level of $\alpha=0.05$.

Results

Plant growth

In all trials, application of metsulfuron-methyl resulted in significant reductions in plant dry weight and plant height. The dry weight of treated *S. nigrum* plants was significantly reduced from 67% (trial S-2) to 90% (trial S-3). Plant height was also significantly reduced as treated *S. nigrum* plants were on average 35% (trial S-2) to 72% (trial S-3) smaller than control plants. Treated *P. persicaria* plants grown in the greenhouse (trial P-2) had their dry weight reduced by 40% and their plant height reduced by 30% (Table 1). Very soon after application of the herbicide the number of fully expanded leaves on treated plants stayed behind the number observed on control plants (Figure 1). The increase in number of fully expanded leaves on treated plants was even completely inhibited from 4 (trial S-1) and 7 DAT (trial S-3) onwards.

Table 1: Dry weight and plant height of plants of *S. nigrum* (SOLNI) or *P. persicaria* (POLPE) treated with the ALS inhibiting herbicide metsulfuron-methyl. Plants were grown in climate chamber (CC), greenhouse (GH) or under field conditions (Field). Measurements done at 11 DAT (S-1), 14 DAT (S-2 and P-2) and 15 DAT (S-3). Figures followed by different letters are significantly different ($P<0.05$).

Trial	Species	Growth	Dry weight (g)			Plant height (cm)		
			Control	Treated	% diff.	Control	Treated	% diff.
S-1	SOLNI	CC	0.714^{a}	0.206^{b}	71.1	6.2^{a}	3.5^{b}	43.5
S-2	SOLNI	GH	0.625^{a}	0.209^{b}	66.6	5.7^{a}	3.7^{b}	35.0
S-3	SOLNI	Field	0.382^{a}	0.040^{b}	89.5	4.75^{a}	1.35^{b}	71.6
P-2	POLPE	GH	1.160^{a}	0.696^{b}	40.2	12.1^{a}	8.5^{b}	30.0

As expected, both treated *S. nigrum* and *P. persicaria* plants exhibited chlorosis, red leaf venation, purpling and gradual death (Peterson, 2001). For climate chamber and greenhouse grown *S. nigrum* (trials S-1 and S-2) and *P. persicaria* plants (trial P-2), visual symptoms were first noticeable 4 to 7 DAT while death of treated plants occurred at 2 to 3 weeks after application of the herbicide. For *S. nigrum* plants grown under field conditions (trial S-3) the time lag before visual symptoms appeared was slightly longer as symptoms were first noticeable 10 DAT. Death of treated plants occurred 3 to 4 weeks after application of the herbicide.

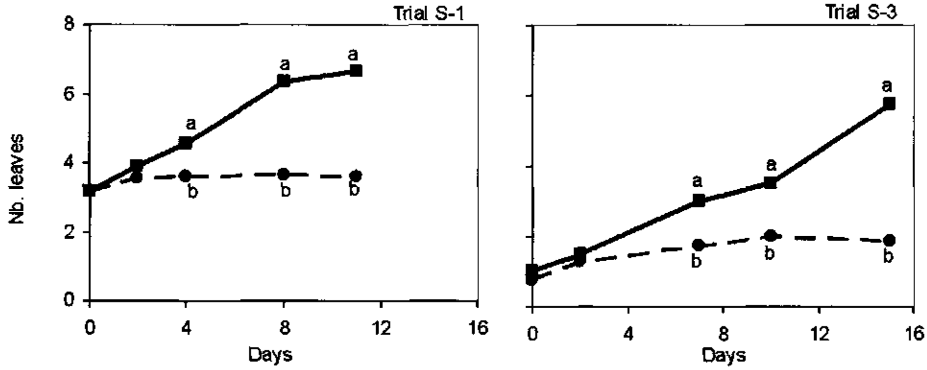

Figure 1: Number of fully expanded leaves (per plant) plotted against days after treatment (days) for *S. nigrum* plants grown under climate chamber conditions (S-1) or under field conditions (S-3). Control plants (squares) and treated plants (circles) with the ALS inhibiting herbicide metsulfuron-methyl. Difference between control and treated plants not significant when not stated otherwise.

Photosynthesis light response curves

The fixation of CO_2 by control plants increased with increasing irradiance (Figure 2). For control plants grown in the climate chamber (trial S-1) or in the greenhouse (trial S-2 and P-2) CO_2 fixation approached light saturation at around 750 μmol m^{-2} s^{-1}, with maximum rates of CO_2 fixation of around 13 and 5 μmol m^{-2} s^{-1}, respectively. Untreated *S. nigrum* plants grown in the field (trial S-3), which had acclimated to higher intensities, were clearly not close to light saturation at the highest irradiance used (750 μmol m^{-2} s^{-1}). At this irradiance a CO_2 fixation rate of around 20 μmol m^{-2} s^{-1} was obtained.

The maximum rate of CO_2 fixation of treated *S. nigrum* and *P. persicaria* plants decreased as the number of days after treatment increased. Treated plants were

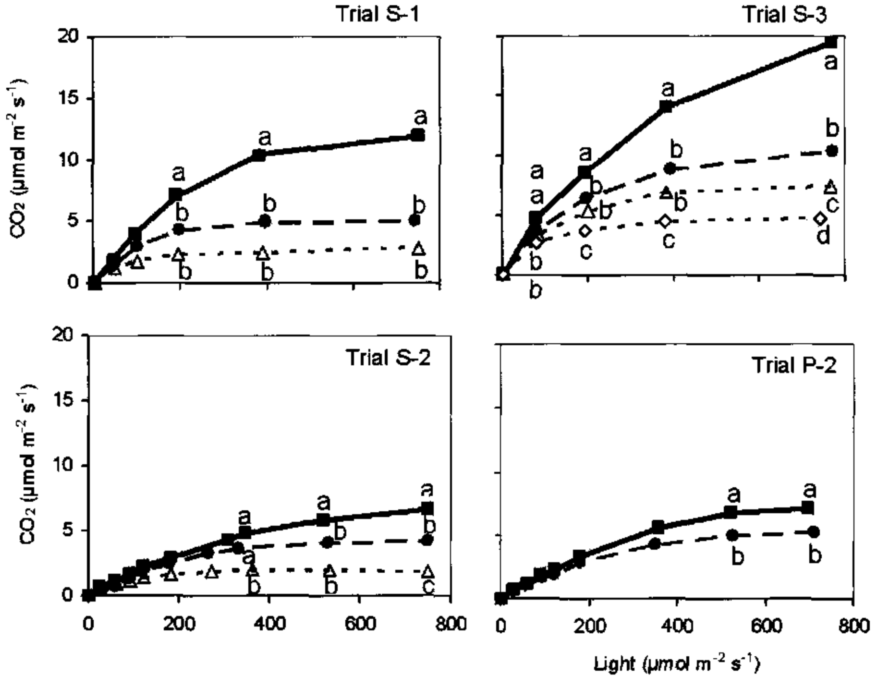

Figure 2: Relationship between CO_2 fixation (μmol m^{-2} s^{-1}) and irradiance (μmol m^{-2} s^{-1}) for *S. nigrum* plants grown in climate chamber (S-1), greenhouse (S-2) and under field conditions (S-3) and *P. persicaria* plants grown in greenhouse (P-2). Data for control plants (square) and metsulfuron-methyl treated plants at 2 DAT (circle), 4 DAT (triangle) and 7 DAT (diamond). Difference between control and treated plants not significant when not stated otherwise.

more affected at 7 DAT or 4 DAT than at 2 DAT (Figure 2). Treated plants also approached light saturation at a much lower irradiance level (400 μmol m^{-2} s^{-1}). For *S. nigrum* plants grown in a climate chamber (trial S-1), the differences in CO_2 fixation between control and treated *S. nigrum* plants were significant from a light intensity of 200 μmol m^{-2} s^{-1} onward. The maximum rate of CO_2 fixation of treated plants at 750 μmol m^{-2} s^{-1} was reduced with 58% at 2 DAT and 76% at 4 DAT. (Figure 2, S-1). For *S. nigrum* plants grown in the greenhouse (trial S-2), a nearly similar reduction of the maximum rate of CO_2 fixation (70%) was recorded at 4 DAT (Figure 2, S-2). The lowest light intensity at which control and treated plants could be significantly differentiated was around 350 μmol m^{-2} s^{-1}, which was close to the light intensity needed in the case of climate chamber grown plants (trial S-1). When *S. nigrum* plants were grown outside (trial S-3), the time required to produce a 70% reduction of the maximum rate of CO_2 fixation was slightly longer (7 days). However by this time, significant differences between control and treated plants could already be observed from a light intensity of 80 μmol m^{-2} s^{-1} onward (Figure 2, S-3). For *P. persicaria* plants grown in the greenhouse (trial P-2), the maximum rate of CO_2 fixation was reduced by 40% at 2 DAT, which was in line with observations done for greenhouse grown *S. nigrum* plants. The lowest light intensity at which control and treated plants could be differentiated was around 500 μmol m^{-2} s^{-1}, which was slightly higher than for *S. nigrum* plants (Figure 2, P-2).

Quantum efficiency for electron transport by PSII and PSI

The quantum efficiency for electron transport by PSII (Φ_{PSII}) measured at 660 nm wavelength at a photon flux density of 0 μmol m^{-2} s^{-1}, equivalent to a dark adapted F_v/F_m, were around 0.8 for both control and treated *S. nigrum* and *P. persicaria* plants, at all observation dates (Figure 3). Φ_{PSII} decreased with increasing irradiance and this decrease was stronger in treated plants than in control plants. The difference in Φ_{PSII} between control and treated plants also increased with time. For *S. nigrum* plants grown in a climate chamber (trial S-1), the difference in Φ_{PSII} between control and treated plants at 4 DAT was significant at irradiance levels above 200 μmol m^{-2} s^{-1} with reductions of 29%, 53% and 66% at 200, 400 and 750 μmol m^{-2} s^{-1}, respectively (Figure 3, S-1). For greenhouse grown *S. nigrum* plants (trial S-2), reductions in Φ_{PSII} at 4 DAT were largely similar to those found in trial S-1 (15%, 48% and 57% at 200, 500 and 700 μmol m^{-2} s^{-1}, respectively) (Figure 3, S-2).

For *S. nigrum* plants grown outside (trial S-3), the differences in Φ_{PSII} between treated and control pants were only 8% (200 μmol m^{-2} s^{-1}) and 32% (750 μmol m^{-2} s^{-1}) at 2 DAT.

These differences increased to 16 and 52% (at 200 and 750 μmol m^{-2} s^{-1}, respectively) at 4 DAT and did not increase any further, as comparable differences were recorded at 7 DAT (data not shown). Differences in Φ_{PSII} between control and treated plants (at 2, 4 and 7 DAT) measured at light irradiance of 200, 400 and 750 μmol m^{-2} s^{-1} were significant (Figure 3, S-3). For *P. persicaria* plants grown in a greenhouse (trial P-2), the difference in Φ_{PSII} between treated and control plants was 37 and 42% (at 500 and 700 μmol m^{-2} s^{-1}, respectively) at 2 DAT. This was close to the observations made for *S. nigrum* plants grown under similar circumstances (trial S-2). Significant differences between control and treated plants were observed at light intensities around 500 μmol m^{-2} s^{-1} (Figure 3, P-2).

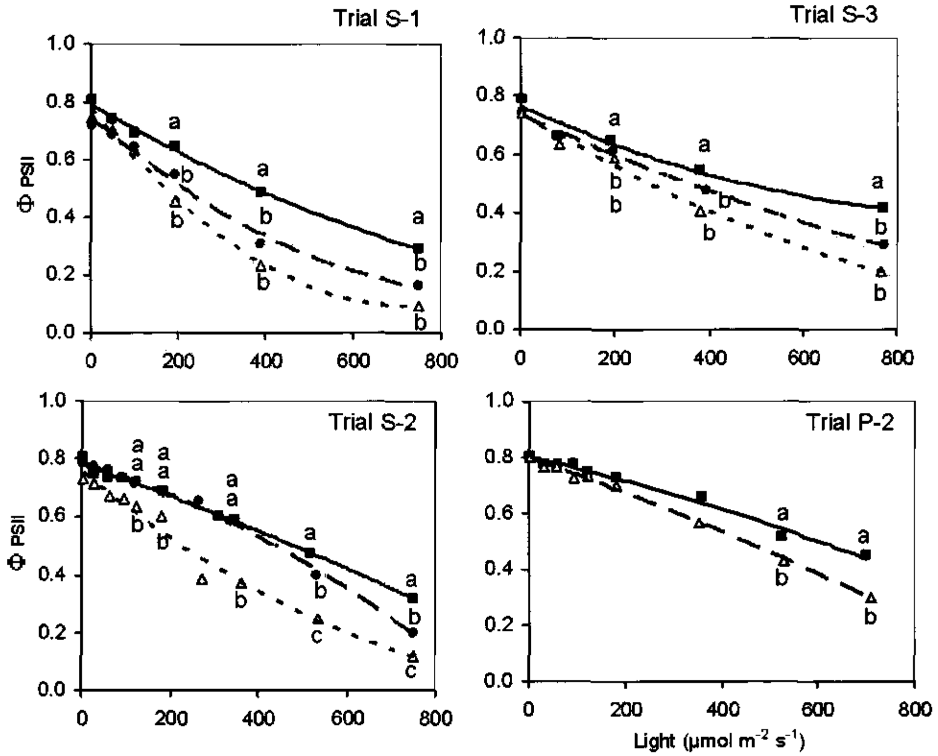

Figure 3: Relationship between Φ_{PSII} and irradiance (μmol m^{-2} s^{-1}) for *S. nigrum* plants grown in climate chamber (S-1), greenhouse (S-2) and under field conditions (S-3) and *P. persicaria* plants grown in greenhouse (P-2). Data for control plants (square) and metsulfuron-methyl treated plants at 2 DAT (circle), 4 DAT (triangle) and 7 DAT (diamond). Difference between control and treated plants not significant when not stated otherwise.

At a photon flux density of 0 μmol m^{-2} s^{-1}, Φ_{PSI} was equal to 1.0 for both control and treated plants of *S. nigrum* and *P. persicaria*, at all observation dates. Φ_{PSI} decreased with increasing irradiance. Similar to what was observed for Φ_{PSII} this reduction was stronger in treated plants than in the control plants. In all four trials the trends for the irradiance dependencies of Φ_{PSI} were mostly comparable to those obtained for Φ_{PSII} (Figure 4). For *S. nigrum* plants grown in a climate chamber (trial S-1), the percentage difference between control and treated plants were comparable to the differences recorded for Φ_{PSII}. Significant differences between control and treated plants were recorded 2 DAT at a light intensity of 400 μmol m^{-2} s^{-1} onward and 4 DAT at a light intensity of 80 μmol m^{-2} s^{-1} onward (Figure 4, S-1). For *S. nigrum* plants grown in greenhouse (trial S-2) or under field conditions (trial S-3), the extent to which the decrease of Φ_{PSI} with increasing irradiance was exacerbated by herbicide

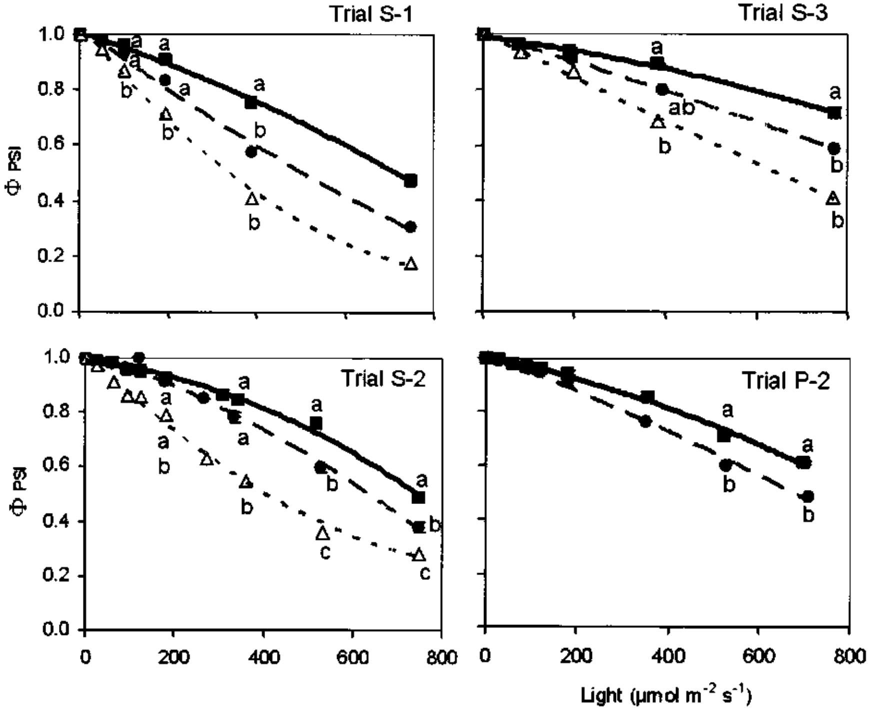

Figure 4: Relationship between Φ_{PSI} and irradiance (μmol m^{-2} s^{-1}) for *S. nigrum* plants grown in climate chamber (S-1), greenhouse (S-2) and under field conditions (S-3) and *P. persicaria* plants grown in greenhouse (P-2). Data for control plants (square) and metsulfuron-methyl treated plants at 2 DAT (circle), 4 DAT (triangle) and 7 DAT (diamond). Difference between control and treated plants not significant when not stated otherwise.

treatment was comparable to the responses described for Φ_{PSII}. In trial S-3, these differences went up to 15% and 43% (at 200 and 750 μmol m^{-2} s^{-1}, respectively) at 4 DAT (Figure 3, S-3) and did increase only slightly until 7 DAT (data not shown). For *P. persicaria* plants grown in greenhouse (trial P-2), the percentage difference between control and treated plants were comparable to the differences recorded for Φ_{PSII}.

Chlorophyll content

In treated plants the level of chlorophyll *a*+*b* was significantly lower than that of control plants (Table 2). Treated *S. nigrum* plants grown in climate chamber (trial S-1) had their chlorophyll *a*+*b* concentration reduced by approximately 25 and 44% at 2 and 4 DAT, respectively. When grown outside (trial S-3) the reduction in chlorophyll *a*+*b* was comparable (34, 45 and 39% at 2, 4 and 7 DAT, respectively). After herbicide treatment a slight change in chlorophyll *a/b* ratio was observed for *S. nigrum* plants grown in climate chamber (trial S-1) but the shift was much more dramatic for plants grown in the field (trial S-3). The chlorophyll *a/b* ratio of treated *S. nigrum* plants (trial S-3) was 19% (2 DAT) and 30% (7 DAT) lower than that of control plants (Table 2). This ratio indicates that in the treated plants the relative loss of chlorophyll *a* was greater than the relative loss in chlorophyll *b*.

Discussion

Herbicide symptoms and plant growth

In the current experiments the development of clear visual symptoms of metsulfuron-methyl toxicity took at least 7 days. A delay of at least 7 days or longer is too long in

Table 2: Chlorophyll concentration (Chl *a*+*b* in mg g^{-1} fresh weight) and chlorophyll *a/b* ratio for treated and control plants of *S. nigrum* (SOLNI) grown in a climate chamber (CC) or under field conditions (Field). Measurements done at 2, 4 and 7 days after treatment (DAT). Numbers followed by different letters are significantly different (P<0.05).

Trial	Species	Growth	DAT	Chl *a*+*b*			Chl *a/b*		
				Control	Treated	% diff.	Control	Treated	% diff.
S-1	SOLNI	CC	2	1.705^{a}	1.272^{b}	25.4	3.600^{a}	3.376^{a}	6.2
S-1	SOLNI	CC	4	1.760^{a}	0.992^{c}	43.6	3.526^{a}	3.364^{a}	4.6
S-3	SOLNI	Field	2	1.096^{a}	0.722^{b}	34.1	3.980^{a}	3.234^{a}	18.7
S-3	SOLNI	Field	4	1.069^{a}	0.586^{c}	45.2	3.915^{a}	2.728^{b}	30.3
S-3	SOLNI	Field	7	0.930^{a}	0.566^{c}	39.1	3.966^{a}	2.822^{b}	28.8

the event that a second application of herbicide would be necessary. From our experience with the development of the MLHD technology for photosynthesis-inhibiting herbicides it is clear that farmers and agricultural contractors demand an early detection method to assess within a few days following the application of the herbicides, whether the treatment will be successful in eliminating the weeds (Kempenaar et al., 2004). Such a guarantee has contributed to the adoption of the MLHD methodology.

In accordance with what was observed for climate chamber grown *S. nigrum* plants (trial S-1), the application of the herbicide metsulfuron-methyl produced a strong and rapid reduction of growth in field grown *S. nigrum* plants (trial S-3). The growth of the treated plants was almost completely inhibited from 2 to 4 DAT on and resulted in significant differences in plant dry weight between treated and control plants at final harvest. This is consistent with the observations of Shaner and Singh (1992) that the growth of plants treated with ALS inhibiting herbicides slows within hours and ceases within a few days and findings from Gaston et al. (2003) who observed a clear reduction of plant dry weights at only 7 days after application of the ALS inhibiting herbicides imazethapyr (ALS inhibitor, imidozolinones sub-group) and chlorsulfuron (ALS inhibitor, sulfonylureas sub-group). Though the assessment of growth by visual or other means is feasible under laboratory conditions, its use under field conditions, possibly on a large scale, would be at best inconvenient and more generally unsuitable as a means for the routine assessment of herbicidal toxicity.

Controlled breakdown or catastrophic loss of photosystem I and II?

The linear relationship between the efficiencies of photosystems I and II suggested a predominant role for linear electron transport – a phenomenon that has been widely reported (Harbinson & Foyer, 1991; Harbinson et al., 1990a, b; Kingston-Smith et al., 1999). It is noteworthy that the consistent, linear relationship between the efficiencies of both photosystems was mostly sustained under contrasting growing environments and plant species. The linear relationship between the efficiencies of photosystems I and II was unaltered by herbicidal treatment (Figures 3 and 4) even though large changes occurred in the pigment concentration of the leaves (Table 2). For field grown *S. nigrum* plant (trial S-3), however, data suggests that in treated plants the relative loss in Φ_{PSII} was slightly larger than the loss in Φ_{PSI} and that the relative loss of chlorophyll *a* was greater than the loss of chlorophyll *b*. This could be due to the greater percentage of chlorophyll *b* that is associated with photosystem II compared to photosystem I. Taken together this data implies that the stress-induced loss of chlorophyll is sufficiently organized in the climate chamber plants (trial S-1) so as to maintain the balance of pigment-protein complexes and function between the photosystems close to that found in control leaves. This loss of leaf chlorophyll in treated plants was not a catastrophic, uncontrolled process, but rather the consequence of a well-organized breakdown of components. This situation is similar to that found in cold-grown maize leaves, where in spite of large reductions in leaf chlorophyll provoked by prolonged exposure to low temperatures, the balance between photosystem I and photosystem II function was maintained (Kingston-Smith et al., 1999). Under field conditions (trial S-3) this pattern of controlled breakdown disappears. The greater loss of chlorophyll *a* compared to chlorophyll *b* implies a relatively greater loss of solely chlorophyll *a* containing chlorophyll-protein complexes, such as the bulk of those associated with photosystem I and the antenna and reaction complexes of photosystem II. The high Φ_{PSII} following dark-adaptation (the measurement at 0 μmol m^{-2} s^{-1}) in herbicide treated leaves implies an absence of photoinhibition of photosystem II, which rules out a loss of photosystem II activity by that route.

Early assessment of herbicidal effects under field conditions

CO_2 fixation of *S. nigrum* plants was more quickly affected by metsulfuron-methyl under climate chamber and greenhouse conditions (trial S-1 and S-2) than it was for early spring plants grown in the field (trial S-3). For *S. nigrum* plants grown in the field, a 75% reduction of the CO_2 fixation was observed at 7 DAT which was 3 days before the first symptoms became noticeable and 2-3 weeks before plant death. Treated *P. persicaria* plants (trial P-2) also had their CO_2 fixation largely reduced soon after application. Several portable gas analyzers are available for field measurement of gas exchange at leaf level and have been used in a number of field studies (Bernacchi et al., 2002; Myers et al., 1999; Rascher et al., 2000; Tissue et al., 1997). However portable systems for the measurement of CO_2 fixation are expensive, complicated and sometimes both, and the time required to make an individual measurement is normally several minutes, or longer, depending on the protocol employed. For these practical reasons, we do not believe that measurements of CO_2 fixation are a practical option for field assessment of the activity of metsulfuron-methyl on weeds.

Although when measured at low irradiance (from 0 to 50 μmol m^{-2} s^{-1}), no difference was observed between control and treated *S. nigrum* or *P. persicaria* plants, Φ_{PSII} measurements made at higher irradiance (400 and 500 μmol m^{-2} s^{-1} for *S. nigrum* and *P. persicaria*, respectively) revealed differences in the steady-state Φ_{PSII} within several days after the application of the ALS inhibiting herbicide metsulfuron-methyl (Figure 3). The inhibition of Φ_{PSII} that could be detected 2 to 4 days after application of the herbicide proved that Φ_{PSII} in illuminated leaves is a parameter to consider when looking for early assessment tools. Measurements of photosystem II intactness (estimated by means of a dark-adapted F_v/F_m measurement) or steady-state quantum efficiency of photosystem II in illuminated leaves are used to screen for environmental stress tolerance in plant breeding, in air pollution studies (Lichtenthaler & Rinderle, 1988; Odasz-Albrigtsen et al., 2000; Popovic et al., 2003), in herbicides toxicity studies (Barbagallo et al., 2003; Judy et al., 1991; Percival & Baker, 1991) or in environmental stress studies such as chilling, freezing, heat stress, nutrient deficiency (De Groot et al., 2003; DeEll & Toivonen, 1999; Kingston-Smith et al., 1999; Kingston-Smith et al., 1997). A recent review from Baker and Rosenqvist (2004) exposed the possible applications of chlorophyll fluorescence in crop production. With the development of smaller electronic components and optical systems, instruments for the measurement of dark-adapted F_v/F_m or Φ_{PSII} have become compact and more readily usable outside the laboratory. Moreover, measurements can be made within a few seconds. Instruments for measuring in vivo fluorescence kinetics include: the Multi-Flash Kinetic Fluorometer, the Fast-Repetition-Rate Fluorometer or the Double-Modulation Fluorometer. Imaging instruments based on Charge-Coupled Device (CCD) cameras have been successful in mapping the photosynthetic activity of a leaf (Bartak et al., 2005; Nedbal et al., 2000; Schreiber et al., 2003).

The current results demonstrate that the use of chlorophyll fluorescence to detect the toxicity of an ALS inhibiting herbicide would require the comparison of steady-state Φ_{PSII} for control and treated plants at irradiance around 400 or 500 μmol m^{-2} s^{-1}. Making observations before and after the herbicide treatment would be a more difficult option, as the steady-state Φ_{PSII} is affected by any environmental factor that affects CO_2 fixation, such as temperature, irradiance or drought. This implies that the measurements would need to be made under conditions of controlled irradiance and temperature, and drought or any other factor that could result in damage to the plant (e.g., frost) would need to be avoided. Producing such a controlled leaf environment, and allowing the leaf sufficient time to allow Φ_{PSII} to reach a steady-state in response to it would increase the complexity of the equipment and the time required to make the measurement. However, it would still be a simpler, cheaper measurement than a measurement of CO_2 fixation. Another way to deal with the comparison of measurements of steady-state Φ_{PSII} for control and treated plants would be to perform the measurements at different locations within the same field. Steady-state Φ_{PSII} of control plants should then be measured in a small patch of the field that is left untreated. The position of such a patch could be randomly chosen or put at a specific spot in the field (e.g., close to the field edge, start / end of a row).

Measurements of Φ_{PSI} could also detect herbicidal effects. The ΔA_{820nm} light-induced absorbance change which is used to estimate Φ_{PSI} (Harbinson & Hedley, 1993) can also be used to measure the rate constant for electron transport from plastoquinol through the cytochrome b6/f complex to P-700$^+$, which in turn can be used to provide an estimate of changes in the light-saturated rate of CO_2 fixation. As light-saturated rates of CO_2 fixation decrease following treatment with ALS inhibiting herbicides, the application of the ΔA_{820nm} measurement could be used as an indicator for herbicide activity. However, for this application to work the chlorophyll content of the leaf must not change, and here as in previous studies (Chapter 4) changes of chlorophyll content have been observed. As the maximum ΔA_{820nm} was also shown to decrease following the application of metsulfuron-methyl, measuring the maximum ΔA_{820nm} (i.e., that obtained under far-red illumination) which is proportional to the amount of P700 would be an alternative approach. Changes in ΔA_{820nm} are easy and rapid to assess (typically only requiring less than 10 s). However, as changes in P700 were broadly parallel to those of chlorophyll content, it might be even easier to evaluate the loss in leaf chlorophyll content using readily available portable chlorophyll meters. These can produce a measurement of leaf chlorophyll content within 5 to 10 seconds, and based upon the measurements of chlorophyll presented here would likely be a useful method for identifying the effectiveness of a metsulfuron-methyl treatment.

Although results demonstrated that the different parameters measured showed large differences between control and treated weed plants soon after application of the ALS inhibiting herbicide metsulfuron-methyl and that in principle all of them could be used as an early indication for a successful application, the practical applicability determines which parameter is really suitable for field assessment of herbicide efficacy. Measurement of the quantum efficiency for electron transport by photosystem II (Φ_{PSII}) and the loss in leaf chlorophyll content appeared to be two of the most practical parameters to use when designing an early detection method to assess the toxicity of metsulfuron-methyl. The use of chlorophyll fluorescence would require a comparison of steady-state Φ_{PSII} measurements for control and treated plants which could be realized by either measuring in time (before / after application) or space (treated / untreated patch).

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Chapter 6

The use of chlorophyll fluorescence imaging to detect the influence of light conditions on herbicidal effects and identify the most appropriate position for single point measurement of Φ_{PSII}

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To be submitted

Abstract

Images of the quantum efficiency for photosystem II electron transport derived from images of chlorophyll fluorescence (Φ_{PSII}) were captured for control and herbicide treated *S. nigrum* plants exposed to either high-light or low-light conditions after the application of the acetolactate synthase (ALS) inhibiting herbicide metsulfuron-methyl at the commercially recommended dose. The Φ_{PSII} images were recorded at 0, 2, 4 and 7 days after treatment (DAT) using a FluorCam kinetic imaging fluorometer. Metsulfuron-methyl produced a strong and rapid reduction in treated *S. nigrum* plants growth but only plants exposed to high-light conditions exhibited severe symptoms and died. It appears that the killing of the weed plants may require some photodynamic effect in addition to the metabolic stress created by the herbicide. In addition to the mean Φ_{PSII}, chlorophyll fluorescence images have their own statistical properties (variance and distribution of Φ_{PSII} values) which are useful as a means of detecting herbicidal effects. The mapping of the Φ_{PSII} distributions of individual leaves indicates that the youngest growing leaves are the most affected by the herbicide application; Φ_{PSII} of older leaves is relatively insensitive to the effects of herbicide. The basal part of the leaf is also relatively more sensitive than the apical region of the leaf. Implications for measurements of Φ_{PSII} as a means of quantifying herbicidal damage are discussed.

Introduction

Developed over the past 20 years, the acetolactate synthase (ALS) inhibitors are an increasingly important group of herbicides. The acetolactate synthase inhibiting herbicides (ALS inhibitors, Herbicide Resistance Action Committee group B), of which the sulfonylureas are a specific sub-group, selectively inhibit acetolactate synthase (EC 4.1.3.18), which is the first enzyme involved in chloroplastidic biosynthesis of essential branched-chain amino acids. For the biosynthesis of valine and leucine ALS catalyses the condensation reaction between two molecules of pyruvate to form 2-acetolactate with elimination of CO_2, while a molecule of pyruvate is condensed with 2-ketobutyrate in a similar reaction leading to the biosynthesis of isoleucine.

ALS inhibiting herbicides are widely used around the world due to the relatively low rates of application that are required coupled with their limited environmental impact, low mammalian toxicity, wide crop selectivity and high efficacy (Peterson, 2001). Although visual symptoms, including anthocyanin accumulation (Suttle & Schreiner, 1982), leaf chlorosis and necrosis of the growing points (Blair & Martin, 1988) may not appear until several days to weeks after herbicide application (Peterson, 2001), profound changes take place shortly after application of ALS herbicides within treated plants. The first physiological response of plants treated with ALS inhibiting herbicides is a cessation of growth which occurs within hours after the application (Gaston et al., 2003; Rhodes et al., 1987; Shaner & Singh, 1991). This inhibitory effect on plant growth was found not to be due to an inhibition of photosynthesis, respiration, hormone-regulated cell elongation, protein synthesis or RNA synthesis (Pillmoor & Caseley, 1987; Ray, 1982; Shaner & Reider, 1986). Further work from Ray (1982) and Rost and Reynolds (1985) showed that DNA synthesis was severely inhibited by the ALS inhibiting herbicide chlorsulfuron and that cell division was stopped soon after application of this herbicides, but the authors speculated that the effects of chlorsulfuron on DNA synthesis was a secondary response to its herbicidal action. Additional metabolic responses to ALS inhibiting herbicides include an increase in free amino acids (Rhodes et al., 1987), a rapid decrease in the level of soluble proteins (Shaner, 1989), and a decrease in the translocation of photosynthate to the growing points of the plant thus depriving the meristematic tissue not only of branched-chain amino acids but also of carbon (Devine, 1989 cited in Shaner & Singh, 1992). It has been suggested by Shaner and Singh (1992) that all these factors probably interact to kill the plants.

Van der Weide (personal communication) observed inconsistent results of weed control using acetolactate synthase inhibiting herbicides from the sulfonylurea subgroup such as rimsulfuron in maize or potato crops, and methsulfuron-methyl in winter cereals, for applications late in the season. Low herbicide efficacy is usually suggested to result from an inappropriate use of the herbicide (active ingredient or dose), the presence of weed plants bigger than the optimal size for treatment, or the use of inadequate spraying techniques. One additional factor are the weather conditions around the time of application of the herbicides (Kudsk & Kristensen, 1992; Chapter 2). Van der Weide (personal communication) suggested two additional reasons to explain the fore mentioned escapes in weed control: the crop plants might have shaded the weeds resulting in a poor and uneven spray deposition, or they might have reduced the light intensity experienced by the weeds because of a dense crop canopy. It is known that apart from a marked influence on the weed plants' anatomy and morphology (Brown, 2001; Mummigatti et al., 1995; Nobel, 1976), light conditions also can have direct effects on herbicide uptake (Chapter 2), herbicide translocation (Brown, 2001; Goodwin, 2002) and herbicide efficacy (Brown, 2001; Kudsk & Kristensen, 1992; Mummigatti et al., 1995).

The possible impact of light conditions on toxicity of ALS inhibiting herbicides is intriguing as photosynthesis is not regarded to be a primary target of ALS inhibiting herbicides. However changes in chlorophyll fluorescence responses have been observed in treated plants. Judy et al. (1990) found effects on the fluorescence of barley 2 h after treatment with imazaquin and Percival and Baker (1991) found effects on the fluorescence of wheat leaves 24 h after treatment with the ALS-inhibitor imazamethabenz-methyl at the recommended field rates, which suggests that photosynthesis is influenced by these herbicides, even though the effect may be indirect. Barbagallo et al. (2003) also demonstrated that many inhibitors of metabolic processes that are not directly involved in photosynthetic metabolism can produce modifications to fluorescence kinetics. An extensive study of Riethmuller-Haage et al. (Chapter 4) showed that the rate of carbon dioxide (CO_2) fixation, the relative quantum efficiency of photosystem II electron transport (Φ_{PSII}), the relative quantum efficiency of photosystem I electron transport (Φ_{PSI}) and total chlorophyll content of climate chamber grown *Solanum nigrum* plants were all significantly reduced after application of the ALS inhibiting herbicide metsulfuron-methyl. Another study by Riethmuller-Haage et al. (Chapter 5) confirmed these findings for greenhouse and field grown *S. nigrum* and greenhouse grown *Polygonum persicaria* plants. They came to the conclusion that the quantum efficiency for electron transport by photosystem II (Φ_{PSII}) was one of the most practical parameters to use when designing an early detection method to assess the toxicity of metsulfuron-methyl under field conditions.

One aim of this study was to investigate whether inconsistencies in weed control by sulfonylureas might be explained by the reduced light conditions experienced by weed plant under a dense crop canopy. For this reasons, control and treated *S. nigrum* plants were exposed to high-light and low-light conditions for 0, 2, 4 and 7 days after application of the ALS inhibiting herbicide methsulfuron-methyl. A FluorCam kinetic fluorimeter was used to visualise the change of Φ_{PSII} distribution at plant and leaf level. Consequently, another objective of this study became to identify which leaves and at what position within leaf herbicide effects become first noticeable.

Materials and methods

Plant material and spraying procedure

Seeds of *Solanum nigrum* (black nightshade) were germinated on a moist mixture of potting soil and sand (2:1). Fifteen days after sowing individual plants were transferred into 1 dm^3 pots and placed in a growth chamber with a photosynthetically active radiation (PAR) of 220 μmol m^{-2} s^{-1} for 12 hours (lamps: TL-D-HF, Philips, Eindhoven, The Netherlands). The plants were grown in a climate chamber at day/night temperatures of 22°C / 18°C and 70% relative humidity. Water was supplied on a daily basis. Four-week-old (3 leaf stage) *S. nigrum* plants were sprayed with an air-pressurized laboratory track sprayer delivering 400 litres ha^{-1} herbicide solution at 303 kPa. *S. nigrum* plants were treated with 12 g a.i. ha^{-1} of metsulfuron-methyl (Ally, 30 g L^{-1}, DuPont) and 0.75% v/v isodecyl ethoxylate (Trend 90, DuPont). Isodecyl ethoxylate was used as a surfactant to improve the penetration and uptake of metsulfuron-methyl by the leaves.

One hour after application of the herbicide, half of the *S. nigrum* plants were put under the standard radiation level of 220 μmol m^{-2} $s^{-}1$, further referred to as high-light conditions, whereas the other half of the plants was put under low-light conditions. The light intensity was reduced by 70 to 75% by placing two layers of cheese cloth 1 m above the plant canopy. The presence of cheese cloth did not significantly affect the light spectrum (data not shown). The experiment, therefore, consisted of 2 different light regimes (high-light and low-light), 2 different herbicide treatments (treated and control). For each light regime, the experiment consisted of 48 *S. nigrum* plants with 18 plants reserved for the photosynthetic measurements and 30 plants for assessing plant growth.

Measurements of chlorophyll fluorescence

Images of fluorescent emission were captured using a FluorCam kinetic imaging fluorometer (Photon Systems Instruments, Ltd.). The CCD camera of this system, combined with the pulsed measuring light, allows the imaging of relative fluorescence yields from objects such as leaves. These images of fluorescence yield obtained under various conditions (e.g., saturating light) can be used to produce images of physiologically more useful parameters, such as the relative quantum efficiency for PSII electron transport. The typical size of the object being imaged in this study was smaller than 6 × 6 cm, which is within the equipment capabilities (Photon System Instruments, 2002). The objective lens of the CCD camera was located approximately 7 cm perpendicular from the leaf surface. The continuous actinic irradiance was provided by two sets of 325 super-bright orange light emitting diodes (HLMP-EH08, Agilent Technologies, Palo Alto, CA) that are a standard component of the FluorCam system. The maximum intensity of the continuous actinic light was 180 μmol m^{-2} s^{-1}. The saturating light pulse was applied using a halogen lamp equipped with a shutter. The intensity of the saturating light pulse was 2500 μmol m^{-2} s^{-1}.

S. nigrum plants were light-adapted for 15 minutes with a continuous actinic irradiance at 180 μmol m^{-2} s^{-1}. During this period of illumination, a proportion of photosystem II reaction centers will, on average, be closed and non-photochemical quenching will also have become established. Both of these processes will reduce the quantum efficiency of photosystem II photochemistry, and thus the efficiency of linear electron transport. Using images of the relative fluorescence yield of chlorophyll fluorescence obtained under steady-state irradiance (F_{ss}) and during a saturating light-pulse (F_m), it is possible to obtain an image of the relative quantum yield for photosystem II electron transport (Φ_{PSII}) by combining the images pixel by pixel according to the following equation (Genty et al., 1989):

$$\text{Image } (\Phi_{PSII}) = (\text{Image } (F_m) - \text{Image } (F_{ss})) \,/\, \text{Image } (F_m)$$

The fluorescence measurements started with a 12.4 second period of continuous actinic light at 180 μmol m^{-2} s^{-1} that succeeded the light-adaptation period without interruption and during which the F_{ss} images were acquired. The saturating pulse (1.2 seconds, intensity of 2500 μmol m^{-2} s^{-1}) was applied at the end of the 12.4 seconds period and closed all the photosystems II centers bringing the photochemical yield to its minimum and fluorescence emission to its maximum (F_m); during this pulse the F_m images were acquired. Measurements of Φ_{PSII} done in this way were made at 0, 2, 4 and 7 days after treatment (DAT).

Plant growth

Another set of plants grown and treated identically was followed from 0 to 23 DAT. Leaves were separated into three classes: fully expanded (wider than 3 cm), partially expanded (1 to 3 cm wide) and small (less than 1 cm). Leaf development was assessed at 0, 2, 4, 7, 11 and 23 DAT. Plant height (from soil surface to plant tip) and plant dry weight (after at least 48 h at 70°C) were determined at 23 DAT.

Statistics

FluorCam images of the quantum efficiency for electron transport by PSII (Φ_{PSII}) were exported as histograms and descriptive statistics (average, standard deviation) were calculated. Both Φ_{PSII} data and information on plant growth were analysed at a significance level of $\alpha=0.05$ with ANOVA tests using Genstat 8 (Lawes Agricultural Trust, IACR-Rothamsted, UK). To evaluate the statistical dispersion of the data sets the first quartile (Q1 – cutting off the lowest 25% of the data set), the second quartile (Q2 or the median – cutting data set in half) and the third quartile (Q3 – cutting off the highest 25% of the data set) were calculated. The difference between the third quartile and the first quartile, the interquartile range, was computed as well.

Results

Plant growth and symptoms development

Plant growth was assessed from 0 to 23 DAT by counting the number of fully expanded leaves on a regular basis. From 2 DAT onwards, control plants had significantly more fully expanded leaves than the treated plants, but no significant difference was recorded between plants grown under high-light or low-light conditions until 12 DAT (Figure 1). At 23 DAT (final assessment), significant effects of both herbicide and light condition were recorded. Treated plants resulted in significantly smaller plants (lower dry weight and reduced plant height) with fewer leaves. Plants grown under low-light conditions also had lower dry weight but plants were taller (increased plant height) than the plants grown under high-light conditions (Table 1).

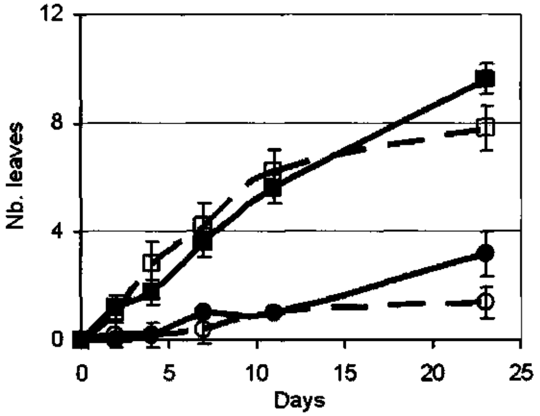

Figure 1: Number of fully expanded leaves (per plant) for control (square) and treated (circles) *S. nigrum* plants plotted against days after treatment (DAT). Solid line for plants exposed to high-light conditions and dotted line for plants exposed to low-light conditions. Vertical bars indicate standard error of the mean.

Table 1: Number of fully expanded leaves (per plant), dry weight (g per plant) and plant height (cm) for control plants and treated plant at final assessment (23 DAT) exposed to either high-light or low-light conditions. For each measured parameter, numbers followed by different letters are significantly different ($P < 0.05$).

		Number of fully expanded leaves	Dry weight	Plant height
Control	High-light	9.6^{a}	1.794^{a}	14.6^{b}
Control	Low-light	7.8^{b}	0.679^{b}	23.0^{a}
Treated	High-light	3.2^{c}	0.333^{c}	5.3^{c}
Treated	Low-light	1.4^{d}	0.107^{d}	7.1^{c}
	s.e.d.	0.50	0.083	1.32

Light conditions had a strong effect on the effect of herbicide treatment. When grown under high-light conditions *S. nigrum* plants exhibited a gradual deterioration of the plants which resulted in death 2 to 3 weeks after the application of the herbicide. Chlorosis also developed; four to seven days after application of the herbicide treated high-light plants had turned yellow, and developed a conspicuous purple colouration around the veins. The leaves also rolled in on themselves. Under low-light conditions, symptoms were far less pronounced and appeared later at around 7 to 10 DAT. Symptoms included a temporary stunting of the plants by a shortening of the internodes that were developing at the time of application. This later phenomenon was earlier observed after application of sub-lethal ALS inhibiting herbicide doses (Shaner, 1989). Only the youngest growing leaves turned yellow but displayed no clear purple colouration around the veins. Also the herbicide treatment was not fatal for the plants.

Loss of Φ_{PSII} for treated S. nigrum *plants exposed to high-light versus low-light conditions: plant level*

Figure 2 gives the absolute number of pixels in FluorCam images of the quantum efficiency for electron transport by PSII (Φ_{PSII}); this Φ_{PSII} distribution histogram was calculated by the FluorCam software. In this example (control *S. nigrum* plants exposed to high-light conditions for 2 days) the averages of Φ_{PSII} were 0.69, 0.70 and 0.69 for replicates 1, 2 and 3, respectively, and the pixel distribution was normal with 50% of the Φ_{PSII} values between 0.64 (the median of the lower half of the data, Q1) and 0.76 (the median of the upper half of the data, Q3). The average Φ_{PSII} for treated and control *S. nigrum* plants exposed to either high-light and low-light conditions after application of the ALS inhibiting herbicide metsulfuron-methyl are presented in Table 2.

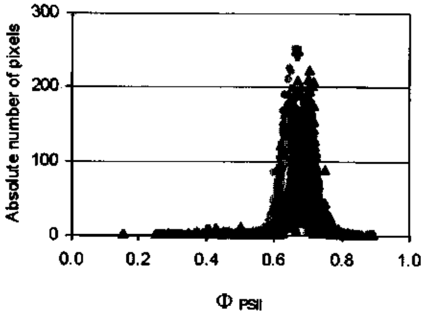

Figure 2: Absolute number of pixels for each Φ_{PSII} level for control *S. nigrum* plants exposed to high-light conditions for 2 DAT. Replicate 1 (black), replicate 2 (grey) and replicate 3 (light grey).

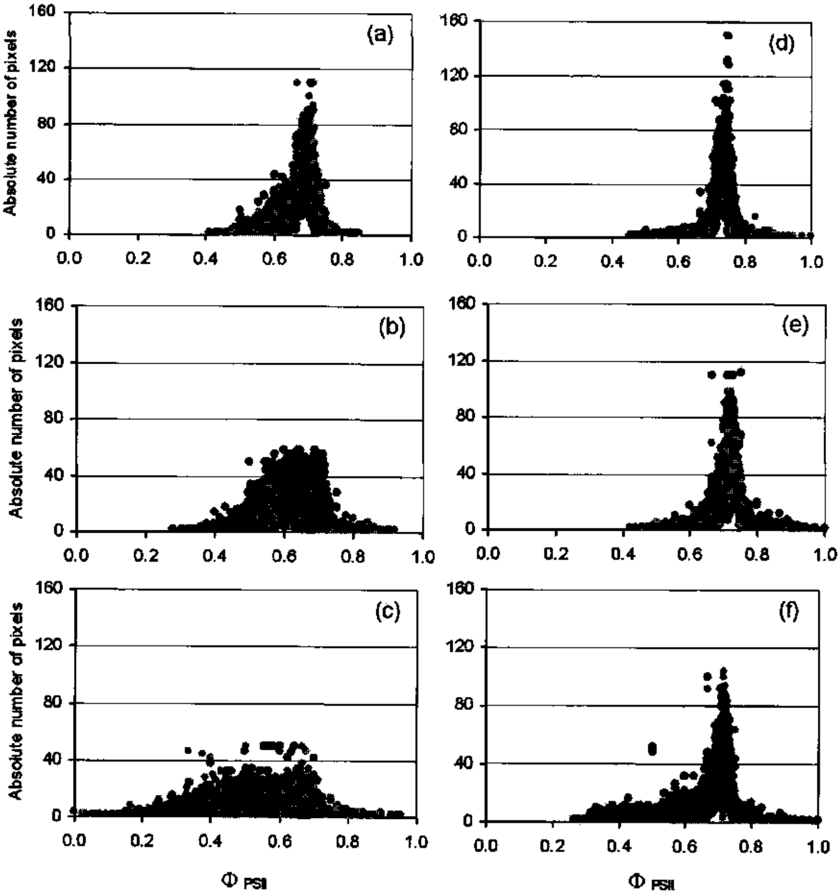

Figure 3: Absolute number of pixels for each Φ_{PSII} level for treated *S. nigrum* plants exposed to high-light conditions for 2 days (a), 4 days (b) or 7 days (c) or low-light conditions for 2 days (d), 4 days (e) or 7 days (f) after application of the ALS inhibiting herbicide methsulfuron-methyl.

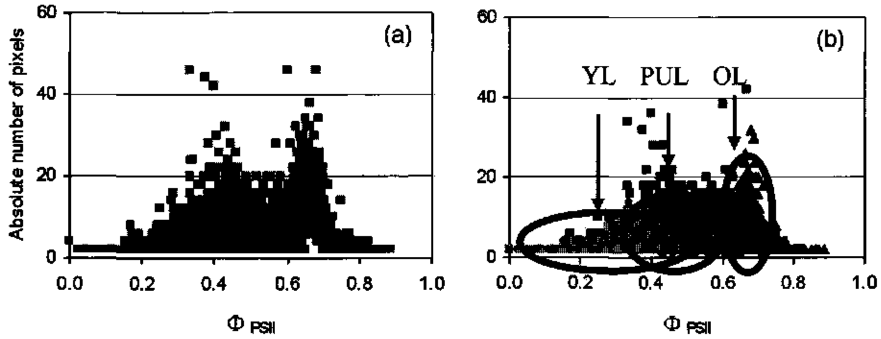

Figure 4: Absolute number of pixels for each Φ_{PSII} level for treated *S. nigrum* plants exposed to light conditions for 7 days. (a) Analysis done at plant level or (b) leaf level for the oldest leaf (OL), the penultimate leaf (PUL) and the youngest leaf (YL).

The average of the mean Φ_{PSII} measurements were similar (around 0.68) at all measurement dates for control *S. nigrum* plants exposed to either high-light or low-light conditions. The herbicide treated plants showed time dependent losses of Φ_{PSII} after herbicide application (F pr < 0.001), and these losses were greater for the herbicide treated plants exposed to high-light compared to the herbicide treated plants grown in low-light (F pr < 0.001). For the herbicide treated high-light plants Φ_{PSII} became significantly lower than that of the controls at 2 DAT, whereas for the herbicide treated low-light plants this difference was only significant at 7 DAT (Table 2).

In addition to changes in the mean Φ_{PSII} value, treatment with herbicide also affected the distribution of pixel values (Figure 3). In comparison to the control plants, the greatest changes in pixel distribution were recorded from treated *S. nigrum* plants exposed to high-light conditions (Figures 3a, 3b, 3c). The interquartile range was much larger for treated plants exposed to high-light conditions (0.03, 0.12, 0.15 and

Table 2: Average Φ_{PSII} for treated and control *S. nigrum* plants at 0, 2, 4 and 7 DAT exposed to high-light and low-light conditions. Analysis of variance done per herbicide treatment. Numbers followed by different letters are significantly different ($P < 0.05$).

		0 DAT	2 DAT	4 DAT	7 DAT	s.e.d.
Treated	High-light	0.69^{a}	0.64^{bc}	0.60^{c}	0.52^{d}	0.022
Treated	Low-light	0.70^{a}	0.72^{a}	0.69^{ab}	0.63^{c}	
Control	High-light	0.68^{a}	0.67^{a}	0.68^{a}	0.67^{a}	0.012
Control	Low-light	0.69^{a}	0.70^{a}	0.69^{a}	0.67^{a}	

Table 3: Q1-Q3 quartiles for treated and control *S. nigrum* plants at 0, 2, 4 and 7 DAT exposed to high-light and low-light conditions.

		0 DAT	2 DAT	4 DAT	7 DAT
Treated	High-light	0.67 - 0.70	0.58 - 0.70	0.53 - 0.68	0.41 - 0.63
Treated	Low-light	0.68 - 0.71	0.68 - 0.76	0.64 - 0.74	0.56 - 0.70
Control	High-light	0.66 - 0.70	0.65 - 0.69	0.66 - 0.70	0.64 - 0.69
Control	Low-light	0.67 - 0.71	0.68 - 0.72	0.67 -0.71	0.65 - 0.68

0.22 at 0, 2, 4 and 7 DAT) than for treated plants exposed to low-light conditions (0.03, 0.08, 0.10 and 0.14 at 0, 2, 4 and 7 DAT). The interquartile ranges also increased with the number of days after treatment (Table 3). The normal distribution observed for control *S. nigrum* plants grown under either high-light or low-light conditions (Figure 2) changed to a non-normal bimodal distribution for treated plants exposed to high-light conditions for 4 or 7 days following treatment (Figures 3b, 3c) and, to a lesser extent, for treated plants exposed to low-light conditions for 7 days following treatment (Figure 3f).

An example of this non-normal bimodal distribution of pixel values obtained from a treated *S. nigrum* plant exposed to high-light conditions for 7 days after treatment is analysed in more detail in Figure 4. The mean Φ_{PSII} for the 3 leaves of this plant taken as a whole was around 0.48. However when the image is analysed on a leaf-by-leaf basis clear differences emerge between the average Φ_{PSII} of the oldest leaf (average Φ_{PSII} = 0.67, the same as for the control plants), the penultimate leaf (average Φ_{PSII} = 0.49) and the youngest leaf (average Φ_{PSII} = 0.38) (Figure 4b). The interquartile range of the distribution of pixel values was also different between leaves, with the values of penultimate leaf and the youngest leaf (0.20 and 0.14, respectively) being larger than that of the oldest leaf (0.07). The loss of Φ_{PSII} seemed inversely related to the degree of maturity of the leaves.

Loss of Φ_{PSII} for treated S. nigrum plants exposed to high-light versus low-light conditions: leaf level

When analysed at the leaf level, the results clearly revealed differences in the mean Φ_{PSII} of the oldest leaves (OL), the penultimate leaf (PUL) and the youngest leaf (YL) of treated *S. nigrum* plants (Table 4). Analysis of variance showed clear differences in mean Φ_{PSII} between these leaves (leaf effect, $F\ pr < 0.001$) and with increasing DAT ($F\ pr < 0.001$ and $F\ pr = 0.006$ for plants exposed to high-light and low-light conditions, respectively). In control *S. nigrum* plants, no differences were identified between leaves or the time after treatment (Table 5).

Whether maintained in high-light or low-light, the oldest leaves (OL) were not affected by the ALS inhibiting herbicide metsulfuron-methyl. The Φ_{PSII} properties of the youngest leaves (YL) were the most dramatically affected by the herbicide with the first significant difference between the mean Φ_{PSII} of YL leaves and the mean Φ_{PSII} of the OL or the PUL leaves resolvable at 2 DAT when treated plants were exposed to high-light conditions and at 4 DAT when treated plants were exposed to low-light conditions (Table 4). The use of low-light conditions after application of the ALS inhibiting herbicide clearly delayed the effect of the treatment on Φ_{PSII}. An 18% decrease in the average mean Φ_{PSII} was observed at 4 DAT between the OL and the YL leaves of plants exposed to high-light conditions.

Table 4: Average Φ_{PSII} for the oldest leaf (OL), the penultimate leaf (PUL) and the youngest leaf (YL) of treated *S. nigrum* plants at 2, 4 and 7 DAT exposed to either high-light or low-light conditions. Numbers followed by different letters are significantly different ($P < 0.05$).

		2 DAT	4 DAT	7 DAT	s.e.d.
High-light	OL*	0.70^{a}	0.68^{a}	0.67^{ab}	0.022
High-light	PUL	0.67^{a}	0.62^{c}	0.53^{d}	
High-light	YL	0.63^{bc}	0.56^{d}	0.42^{e}	
Low-light	OL*	0.74^{a}	0.72^{a}	0.69^{ab}	0.027
Low-light	PUL	0.73^{a}	0.72^{a}	0.70^{ab}	
Low-light	YL	0.69^{ab}	0.66^{b}	0.58^{c}	

OL* - oldest leaves equivalent to cotyledons at 2, 4 and 7 DAT.

Table 5: Average Φ_{PSII} for the oldest leaf (OL), the penultimate leaf (PUL) and the youngest leaf (YL) of control *S. nigrum* plants at 2, 4 and 7 DAT exposed to either high-light or low-light conditions. Numbers followed by different letters are significantly different ($P < 0.05$).

		2 DAT	4 DAT	7 DAT	s.e.d.
High-light	OL*	0.67^{a}	0.69^{a}	0.65^{a}	0.020
High-light	PUL	0.67^{a}	0.66^{a}	0.66^{a}	
High-light	YL	0.65^{a}	0.68^{a}	0.66^{a}	
Low-light	OL*	0.70^{a}	0.69^{a}	0.67^{a}	0.021
Low-light	PUL	0.71^{a}	0.68^{a}	0.66^{a}	
Low-light	YL	0.68^{a}	0.68^{a}	0.66^{a}	

OL* - oldest leaves equivalent to cotyledons + 1st leaf at 2 DAT or cotyledons + 1st and 2nd leaf at 4 and 7 DAT.

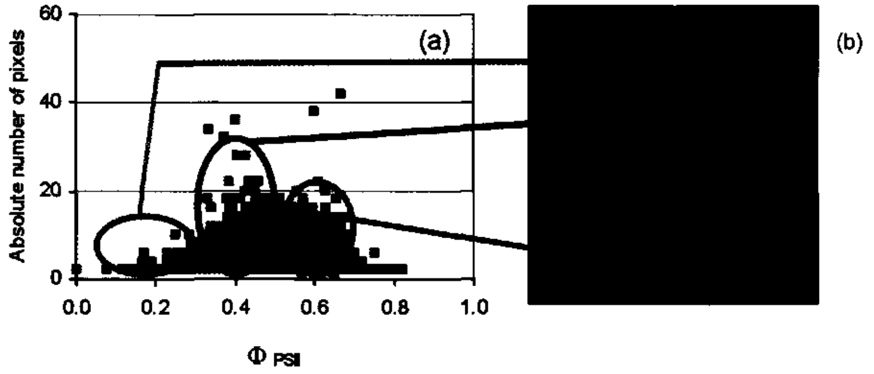

Figure 5: Absolute number of pixels for each Φ_{PSII} level for the leaf before the last growing leaf (PUL) of treated *S. nigrum* plants exposed to high-light conditions for 7 days (a) and its corresponding FluorCam image (b). Colour legend presented in Figure 6.

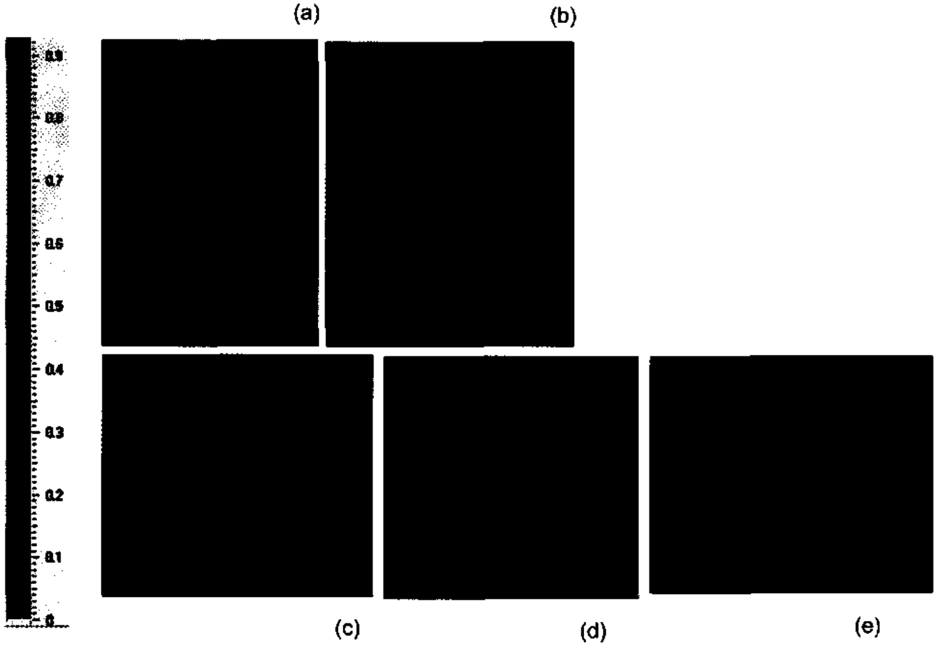

Figure 6: FluorCam images of treated *S. nigrum* plants exposed to high-light conditions for 4 (a, b) and 7 days (c, d, e) after treatment with methsulfuron-methyl.

However, a 7 day period was required to produce a similar difference between the OL and the YL leaves when plants were kept in low-light conditions (Table 4).

Finally, the variation of Φ_{PSII} within individual leaves was analysed. In Figure 5, the absolute number of pixels for each resolved Φ_{PSII} level for the penultimate leaf (PUL) of treated *S. nigrum* plants after a 7 days exposure to high-light conditions for 7 days and its corresponding FluorCam image are presented. The average Φ_{PSII} for this leaf was 0.49 with a large interquartile range (0.20), a low absolute minimum Φ_{PSII} value (0.08) and a high absolute maximum Φ_{PSII} value (0.83). The corresponding FluorCam image shows areas with low (0-0.3), intermediate (0.3-0.6) and high (0.6-0.83) Φ_{PSII} values. The lowest and highest efficiencies were recorded at the leaf base and close to the tip of the leaf, respectively. Figure 6 illustrates the dynamics of the loss of Φ_{PSII} in treated *S. nigrum* plants exposed to high-light conditions after application of the herbicide. At 2 DAT, the youngest leaf (YL) was affected with a greater loss of Φ_{PSII} around the leaf base (0.3-0.6). The penultimate leaf (PUL) was slightly affected with no specific pattern (Figures 6a, 6b). At 7 DAT, the youngest leaf (YL) was also the most affected with low Φ_{PSII} values (0-0.3) over a large part of the leaf. Only the tips of the leaves had intermediate efficiencies (Figures 6c, 6d, 6e). The penultimate leaf (PUL) was also affected with the greatest loss of Φ_{PSII} occurring around the leaf base.

Discussion

Herbicide-induced changes in plant growth and symptoms development

The application of the herbicide metsulfuron-methyl (ALS inhibitor, sulfonylurea subgroup) produced a strong and rapid reduction in the growth of treated *S. nigrum* plants exposed to both high-light and low-light conditions. Data presented suggest a very short time lag before an effect on growth was apparent. This is consistent with results from Gaston et al. (2003) and earlier observations from Shaner and Singh (1992) that the growth of plants treated with ALS inhibiting herbicides slows within hours and ceases within a few days. Treated plants exposed to high-light conditions exhibited chlorosis, red leaf venation, purpling and gradual death (Peterson, 2001). Treated plants also exhibited stunting, which is known to be one possible ALS-inhibiting-herbicide symptom (Peterson, 2001).

In treated plants exposed to high-light conditions, visual symptoms were first noticeable 4 to 7 DAT, with death of treated plants occurring 2 to 3 weeks after treatment (Chapter 5). However it is notable that under low-light conditions the severity of the symptoms was diminished. Symptoms required 7 to 10 days to become apparent, only the youngest growing leaves became chlorotic and there was no significant purple colouration on the leaves. More importantly the plants did not die during the course of the experiment. This suggests that under climate chamber conditions, the symptoms that develop in response to treatment with metsulfuron-methyl are secondary light-induced effects. Even the death observed under high-light conditions might not be a primary effect of the herbicide, but may be due to the effect of high-light conditions on the herbicide treated leaves. Metsulfuron-methyl still has a toxic effect on the low-light plants as growth is arrested (Table 1) and it is only the killing of the plants that may require some additional photodynamic effect. In measurements of the regulation of photosynthetic electron activity in metsulfuron-methyl treated plants, Riethmuller-Haage et al. (Chapter 4) noted some diversion of electron transport away from carbon dioxide fixation (i.e., a measurable alternative sink for electron transport developed in treated *S. nigrum* leaves). A working hypothesis is that treatment with metsulfuron-methyl produces metabolic imbalances which result in the use of molecular O_2 as an alternative electron acceptor for the photosynthetic electron transport chain (or possibly the mitochondrial chain acting in concert with the photosynthetic chain). The reactive oxygen species (ROS) formed by this route could be the source of the photodynamic damage that results in mortal damage under high-light conditions. Under low-light the ROS would still be formed, but at a rate low enough to be dealt with by the anti-oxidative mechanisms of the leaf mesophyll cells (Asada, 1996).

Light-induced changes in quantum efficiency for electron transport by photosystem II

The use of chlorophyll fluorescence as an instrument with which to detect herbicidal effect on plants has been studied in several occasions. However, it requires understanding on how the selected fluorescence-derived parameter (Barbagallo et al., 2003; Habash et al., 1985), in our case the steady state Φ_{PSII} (as could be measured under field irradiance conditions), was affected by the herbicide methsulfuron-methyl. This effect was time-dependent and also depended on the leaf measured. Under high-light conditions, results showed that there was a significant loss of steady-state Φ_{PSII} by 2 days after the application of the herbicide metsulfuron-methyl which is consistent with findings from Barbagallo et al. (2003) who found a significant decrease in Φ_{PSII} in the 48 hours following treatment of Arabidopsis seedlings with 7 different herbicides (among them were ALS inhibitors). Within 7 days, the difference between the high-light treated plants and the control plants was 0.15, a numerically large value. When grown under low-light conditions the loss in mean Φ_{PSII} was smaller, was not observed before 7 DAT (Table 2) and weed plants did not die. As the possible sources of variation were controlled as much as possible before and during application of the herbicide (e.g., homogeneous growth, minimization of potential differences in spray deposition and uptake), differences in mean Φ_{PSII} observed between the high-light and low-light conditions were most certainly due to the differential exposure to light conditions after application of the herbicide. It is interesting to note that early changes in mean Φ_{PSII} were not always indicative of plant death. Only the changes in mean Φ_{PSII} observed at 2 and 4 days after treatment for *S. nigrum* plants exposed to high-light conditions resulted in plant death. The small changes observed at 7 days after treatment for plants exposed to low-light conditions had no effect on plant viability. Consequently, other parameters than the mean Φ_{PSII} were considered.

Apart from providing information on changes in mean Φ_{PSII}, the examination of the distribution of pixels of leaves or whole-plant images of Φ_{PSII} gives a detailed description of the photosynthetic properties of the tissue being examined (Genty & Meyer, 1994; Schreiber et al., 2003) and provides a powerful tool for summarizing how a treatment is affecting photosynthesis (Genty & Meyer, 1994). These chlorophyll fluorescence images have their own statistical properties, such as the variance and peculiarities of the distribution of Φ_{PSII} values, which themselves may be useful as indicators of damage (Baker et al., 2001; Meyer & Genty, 1999). The images of Φ_{PSII} obtained in the present investigation makes it possible to recommend when, under which circumstances, and upon which leaves measurements of Φ_{PSII} should be made with non-imaging instruments. These images may also be used as a means of quantifying herbicidal damage, but they also suggest that the analysis of Φ_{PSII} images would be a means of detecting herbicidal injury. The Φ_{PSII} values of individual pixels in a healthy leaf is narrowly distributed around a mean value with only a small tail extending to lower values of Φ_{PSII} (Figure 2 and Table 3). This unimodal and almost symmetrical frequency distribution was also observed for unstressed, perfectly healthy *Rosa rubiginosa* L. plants (Meyer & Genty, 1999). In response to herbicide treatment and high-light conditions the narrow distribution, observed for control *S. nigrum* plants, becomes increasingly skewed to lower Φ_{PSII} values (Figure 3). Even by 2 DAT the pixel distribution is noticeably different from the control. This loss in Φ_{PSII} and the change from a symmetrical to a left-skewed distribution was already observed for water stressed dehydrated *Rosa rubiginosa* L. leaves (Meyer & Genty, 1999). The change in Φ_{PSII} distribution following herbicide treatment in *S. nigrum* plants is sufficiently conspicuous that it could be used as an indicator of herbicide treatment possibly within 2 DAT and certainly by 4 DAT. The large change in Φ_{PSII} distribution observed at 2 and 4 days after treatment for *S. nigrum* plants exposed to high-light conditions were, in fact, indicative of plant death. For S. nigrum plants exposed to low-light conditions changes in Φ_{PSII} distribution were not large enough to be indicative of plant death.

Φ_{PSII} value, which is what is measured by single point fluorimeters, are susceptible to changes in light intensity, CO_2 availability (e.g. as a result of stomatal closure) or other normal environmental factors that depress photosynthesis in the absence of damaging stress. For this reason a simple measure of Φ_{PSII} cannot be used as an unambiguous measure of herbicidal effect, and as a result the use of other chlorophyll fluorescence parameters as indicators of herbicidal effectiveness has been explored (Barbagallo et al., 2003). The change of Φ_{PSII} distribution shown in this work offers a less ambiguous means with which to determine herbicidal effectiveness. However, distribution of Φ_{PSII} is affected by water stress (Meyer & Genty, 1999) and possibly by other stresses (unpublished observations), and the vulnerability of the distribution properties of Φ_{PSII} in young weed plants to environmental stress is not known. In spite of these reservations we feel that the use of Φ_{PSII} distribution as a means with which to detect herbicidal effectiveness is promising. Though other damaging stresses may change the distribution of Φ_{PSII} we expect that these will be easier to account for. The speed of development and degree of skewness produced by herbicidal treatment is so great that we expect that it will be possible to distinguish it from changes in distribution produced by other stresses. Ultimately, however, this last point will have to be tested experimentally.

Most appropriate position for Φ_{PSII} measurements

Another goal of this investigation was to use Φ_{PSII} imaging to determine which leaves or tissues showed the greatest decrease of Φ_{PSII} following the application of herbicide. This knowledge will help to develop a more reliable application of chlorophyll fluorimetry in detecting herbicidal effectiveness. The mapping of the Φ_{PSII} distributions of individual leaves (Figures 4 and 6) shows clearly that the oldest leaf of the plants have a Φ_{PSII} distribution that is similar to that of control leaves, both in terms of mean value and distribution. Moving to increasingly younger tissue there is an increase in the effect of the herbicidal treatment, with the mean Φ_{PSII} decreasing and the variance in Φ_{PSII} increasing. This indicates that if single point measurements of Φ_{PSII} are to be used to determine herbicidal effectiveness then they should be made on the youngest leaves shortly after application of the herbicide (2 or 4 days after treatment). Even within individual leaves there is considerable variation in the effect of metsulfuron-methyl on Φ_{PSII} (Figures 5 and 6). The basal part of the leaf is more vulnerable than the apical region of the leaf, so again measurements of fluorescence should be made soon after application of the herbicide in the basal region where the effect on Φ_{PSII} is greater.

Metsulfuron-methyl produced a strong and rapid reduction in treated *S. nigrum* plant growth but only plants exposed to high-light conditions exhibited severe symptoms and died. The killing of the weed plants may require some photodynamic effect in addition to the metabolic stress created by the herbicide. In addition to the mean Φ_{PSII}, chlorophyll fluorescence images provide valuable information on the variance and the distribution of the Φ_{PSII} values. These last parameters seem more appropriate and less ambiguous than the mean Φ_{PSII} in quantifying herbicidal effects. The mapping of the Φ_{PSII} distributions of individual leaves indicates that the youngest growing leaves are the most affected by the herbicide application. The basal part of the leaf is also relatively more sensitive than the apical region of the leaf.

Acknowledgements

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Chapter 7

General discussion

Weed management: a combination of non-chemical and chemical options

For the last 40 years, weed management strategies in industrialized countries have become heavily dependent upon chemical weed control. Herbicides are, along with other pesticides, fertilizers, improved varieties and mechanized traction, one of the cornerstones of a high-yielding, labour-efficient agriculture. From the 2.27 million tons of plant protection products used in 2001 worldwide, herbicides accounted for the largest proportion of total use (40%), followed by insecticides (25%) and fungicides (10%) (EPA, 2004). There has, however, evolved a common concern toward the extensive use of pesticides and regulations to enforce a restricted use have already been introduced in some countries (Kudsk, 1989; Pedersen et al., 1993). Concerns include the problems of pesticide residues in food products and surface water and the possible threats of pesticides to human health, the growing problem of resistant pest populations and the economic drive to reduce the cost of crop production.

Options for non-chemical weed control

Effective weed control has always been an important part of crop husbandry and has traditionally been a labour intensive operation. In less developed countries this situation still exists and hand weeding often represents the peak labour requirement in agricultural systems (Parish, 1990). When reliance on herbicides is reduced or eliminated, the approach to weed management should encompass the whole cropping system (Liebman & Davis, 2000). A large range of possible alternative weed control measures are available to growers (Bond & Grundy, 2001; Parish, 1990), but usually none of these is sufficiently effective on its own. Cultural weed control methods include measures such as the selection of proper crop rotation and optimal soil cultivation methods and their appropriate timing, as well as manipulation of row spacing and crop density. Mechanical weeding includes the utilization of tools such as hoes, harrows, tines and brush weeders and cutting tools such as mowers and strimmers (Bond & Grundy, 2001). Also flaming equipment to burn off weeds has been developed in several countries.

A recent paper from Beckie and Kirkland (2003) concluded that the sole use of cultural weed control measures was not sufficient to provide an acceptable long-term control of wild oat populations in cereals. The authors proposed to combine cultural weed control methods with a reduced herbicide use for optimal weed management. Yarborough (1996) also put forward that successful weed management in wild blueberry fields required the integrated use of chemical and cultural methods. For control of the difficult noxious weed *Euphorbia esula* L. (leafy spurge), the North Dakota State University Extension Service found that the use of non-herbicide control options such as biological and cultural methods could provide good but not complete control. An integrated programme combining two or more control methods is likely to provide a more successful and cost-effective long-term solution to the leafy spurge problem than a single method used alone (Anon., 2005). Studies conducted in several European countries on weed management on hard surfaces demonstrated the apparent potential of mechanical weed control methods, such as brushing and mowing, and thermal weed control methods such as burning, using hot water or steam (Hansen et al., 2004). At the same time, these studies pointed out that generally non-chemical techniques were less effective and more expensive than chemical methods using glyphosate (Aeschimann & Gut, 1995; Hansen et al., 2004; Vermeulen et al., 2002). Also studies from Kempenaar et al. (2002b) clearly demonstrated that non-chemical methods for weed control were more expensive than chemical weed control because of higher treatment frequency and larger energy consumption, resulting in a reduced cost / benefit ratio. As a result, several Dutch municipalities decided to reintroduce the use of chemicals (Kempenaar & Spijker, 2004). An integrated weed control system combining non-chemical weed control methods with the use of glyphosate was tested in 2002 and 2003 in The Netherlands. Results showed clear reductions in herbicide use (−11% to −66%) and a reasonable to good weed control throughout the season. Costs related to this particular integrated weed control system were 10 to 25% higher than the use of chemical control alone (Kempenaar et al., 2006). Still this is two to ten times less expensive than the use of completely non-chemical weed control systems (Saft & Staats, 2002).

Integrated weed control programs, combining cultural and mechanical weed control methods together with a more efficient use of herbicides, will provide a more successful and cost-effective long-term solution for weed problems in conventional agriculture. Such integrated approaches are regarded as an important part of the strategy for meeting pesticide reduction and nutrient emission goals in The Netherlands (Proost & Matteson, 1997).

Options for a more efficient use of herbicides

A more efficient use of herbicides may be achieved through site-specific herbicide application, the use of advanced product formulation or an improved application technology or dose optimization (Waldron, 1989).

Site-specific weed management

Traditionally, much focus has been devoted to applying herbicides uniformly over the entire field, using a prescribed dose recommended by the manufacturer. With a large within-field variation in weed occurrence (spatially and temporally heterogeneous weed distribution) patch spraying, based on the site-specific need for weed control, may reduce treatment costs as well as the pesticide load to the environment (Gerhards & Christensen, 2003). In a 4 year study, Timmermann et al. (2003) demonstrated that application of post-emergence herbicides based on georeferenced maps of weed seedling distribution were an economically attractive option with positive effects for the environment. Herbicide use in winter wheat and winter barley was reduced by 60% when it concerned herbicides against broad-leaved weeds and by 90% for herbicides against grassy weeds. Average savings for herbicides against broad-leaved weeds were 11% in maize and 41% in sugarbeets, whereas for herbicides against grassy weeds, savings were 78% and 36% in maize and sugarbeets, respectively (Timmermann et al., 2003). Although site-specific weed management has a large potential to reduce herbicide use in agricultural crops, its financial requirements and need of advanced technical expertise has delayed its implementation. In recent years, a lot of effort has been put in obtaining a better understanding of the behaviour and the mechanisms underlying the spatial patterns of weeds. Such studies have focussed on the stability of weed patches (Dieleman & Mortensen, 1999; Gerhards et al., 1997; Heijting et al., submitted), on possible relations with the soil (Heijting et al., 2005; Walter et al., 2002) and on the contribution of harvesting equipment and other machinery to the dispersal of weed material (Blanco-Moreno et al., 2004; Heijting & Van der Werf, 2005).

Advanced product formulation

The presence of an adjuvant, usually biologically inactive, in the spray solution generally improves the effectiveness of post emergence herbicides by influencing retention (De Ruiter et al., 1990) and / or uptake of the herbicide (Chapter 2, 2004 results). Results obtained in 2004 (Chapter 2, 2004 results), with oil added to Bentazon, clearly demonstrated that application of an optimized herbicide formulation is an effective means to reduce the ED_{50} and minimize the variations in herbicide performance. Increasing the amount of adjuvant in the spray mixture has also shown to be beneficial for herbicide efficacy at low spray volumes (Ramsdale, 2000).

Improved application technology

Application technology is a key factor in the optimization of herbicide performance as well as in minimization of off-target movements of herbicides (Kudsk & Streibig, 2003). The efficiency of pesticide applications is generally very low with only a minute fraction of the applied amount reaching the site of action. This has led to pesticide application being described as the 'least effective industrial process on earth' (Rutheford (1985) cited by Kudsk and Streibig (2003)). Today, requirements for a sprayer are to apply an exact, low dose uniformly to obtain high target, low ground deposition and a minimization of drift. Recent developments in application technology include downwards air-assisted sprayers (small droplets size with a significant reduction in drift) and air-induction nozzles (very coarse spray at low volume rates and very effective in reducing pesticide drift) (Miller & Lane, 1999).

Dose optimization

Herbicide labels contain the dose recommendations of the manufacturer and tend to reflect worst-case conditions. Under optimum conditions, herbicide doses can often be reduced below label recommendations and still provide adequate weed control (Kudsk & Streibig, 2003). Adjusting herbicide doses to the prevailing conditions in the field, taking into account both abiotic and biotic factors, is an overwhelming task to most end-users. Development of decision support systems (DDS) is one way of motivating farmers and agricultural contractors to adopt knowledge on herbicide dose optimization. In Denmark a computer-based weed DSS 'PC Plant Protection' has been developed to achieve this task (Jensen, 2004; Kudsk, 1999). This DSS is currently under evaluation in Norway (Netland, 2005). In Sweden, decision support guidelines have been developed for spring cereals incorporating information on total weed density, competitive ability of the weed species, climatic conditions and crop vigour (Bostrom, 1999; Bostrom & Fogelfors, 2001). Reduced dose applications have also been proposed in Spain for sunflower cultivation (GarciaTorres et al., 1995), in the US for soybean cultivation (DeFelice & Kendig, 1994) and in Canada for wheat and oil seed rape cultivation (Blackshaw et al., 2005). In The Netherlands, several DSS have been developed to adjust herbicide use. The current MLHD-technology (Kempenaar et al., 2004; Ketel et al., 1996), specifically developed for photosynthesis inhibiting herbicides, has shown to be a DSS leading to the use of herbicides at lower rates. The present research project focused on exploring options to further develop this methodology.

Dose optimization and the MLHD technology

The MLHD technology focuses on the calculation of the minimum dose of a photosynthesis inhibiting herbicide that is appropriate for killing the weeds in the field. A couple of days after application, photosynthesis related techniques based on fluorescence signals emitted by treated plants are used to verify whether or not the herbicide application will, ultimately, result in the termination of the weeds. Under field conditions, particularly under low temperatures in spring, the visible effects of herbicide action are often only apparent one or two weeks after spraying, which is too long a delay in the event that a second application of herbicide is necessary. An early detection method permits a prompt second herbicide application in case of failure. This last element is of particular importance for ensuring that even though minimal doses of herbicides have been employed, the treatment will be eventually successful in killing the weeds. Such a tool contributes to the adoption of this methodology by farmers, agricultural contractors and others (Kempenaar et al., 2004). At this point, dose advices are weed species and size specific. The present research pointed out that particularly for less sensitive herbicide × weed species combinations, additional factors have to be taken into account to obtain adequate advice regarding the use of reduced herbicide doses.

Weed flora composition and plant size

Results presented in this thesis demonstrated clear differences in susceptibility between plant species. Results showed that seedlings of *C. album* and *S. vulgaris* were properly controlled with one-fourth of the recommended dose of phenmedipham, whereas half of the recommended dose was required to control *S. nigrum* plants. *S. vulgaris* and *S. nigrum* plants were much more easily controlled by bentazon than *C. album* plants (Chapter 2, 2004 results). The significance of the weed flora (Lundkvist, 1997a) and the influence of growth stage were highlighted by several authors (Ahmadi et al., 1980; Caseley, 1990; De Ruiter et al., 1999; Ivany, 1975; Kudsk, 1989; Whitehouse & Harries, 1988). Petersen and Jensen (1987, cited by Lundkvist (1997a)) showed, for example, that *Stellaria media* (L.) Vill was controlled with one-eighth of the recommended dose of chlorsulfuron, whereas the recommended dose was required to control *Veronica* spp. Lundkvist (1997a) confirmed the large differences in susceptibility to the herbicides dichlorprop-P/MCPA or tribenuron-methyl between *B. napus*, *C. album* and *S. media*. This difference in susceptibility between weed species has to be taken into consideration when heterogeneous weed populations are present in the field.

In addition, it was established that weed species susceptibility to herbicides is also dependent on their growing environment. In the 2003 experiment, the average ED_{50} of *P. persicaria* plants treated with the photosynthesis inhibiting herbicide metribuzin was around 40 g ha^{-1}. This average was much larger than the average ED_{50} recorded in 2002 (around 14 g ha^{-1}) when plants were grown in the greenhouse (Chapter 2). Clear positive linear relationships between the ED_{50} and plant growth rate and plant size were found in 2002 when *P. persicaria* plants were grown in the greenhouse. These relationships between herbicide efficacy and plant size are well documented for plants grown under controlled conditions. Kudsk (1989) found that the best effect of the herbicide mixture bentazon / dichlorprop was obtained when both *S. media* or *C. album* were between 0 and 2 leaves old. Larger plants were less efficiently controlled by the herbicide mixture. De Ruiter et al. (1999) also clearly demonstrated that *S. nigrum* plants became less susceptible at later stages of growth and almost completely lost their susceptibility at the flowering stage and the seed filling stage. Caseley (1990) proposed three reasons why seedlings are easier to control: there is less plant tissue to kill, herbicide movements are facilitated by active plant growth and seedlings have a reduced potential for regeneration. However, these relationships between herbicide efficacy and plant size were not observed in the 2003 experiment when the same species, *P. persicaria*, was grown under field conditions (Chapter 2). The differences between the results obtained in 2002 and 2003 illustrate how complex it is to transfer results from controlled environments into recommendations to farmers. The inconsistency in the results could be explained by the inherent methodological problems associated with the use of controlled environments: low light intensity, long photoperiod and the lack of natural variation in photoperiod, temperature and humidity (Kudsk & Kristensen, 1997). Based on this, it is concluded that validation of results obtained under controlled environments is not only useful but strictly necessary.

Weather related parameters

Apart from advice being crop and weed species specific, chemical companies recently also started to indicate to farmers the most suitable conditions for application of their herbicides. Particularly if herbicide doses are reduced, the weed × herbicide system becomes even more sensitive to the influence of weather (Caseley, 1987, 1989; Kudsk, 1989; Kudsk & Kristensen, 1992; Lundkvist, 1997a). The current research demonstrated that two pre-spraying weather parameters, global radiation and mean relative humidity during the last 12 or 48 hours before application of the herbicides, were strongly correlated to herbicide uptake. The nature of these correlations (positive/negative) was herbicide specific, whereas the responses were non-species specific.

For phenmedipham, representing apolar herbicides, herbicide uptake was negatively correlated with global radiation and positively correlated with mean relative humidity. Several authors showed that high global radiation and / or low mean relative humidity induced lower herbicide uptake (Baker, 1980; De Ruiter & Meinen, 1998; Merritt, 1984) and herbicide efficacy (Merritt, 1984). Devine (1989), Green and Strek (2001) and Kudsk and Kristensen (1992) also pointed out that high global radiation or low mean relative humidity usually have a negative influence on herbicide efficacy.

Baker (1980) and Merritt (1984) demonstrated that herbicide uptake was affected through changes in cuticle characteristics. As both weather parameters were negatively correlated to each other at both 12 hours and 48 hours before application of the herbicides, it was not possible to identify which of the two parameters was the best determinant of herbicide uptake. For bentazon, representing polar herbicides, herbicide uptake was positively correlated with global radiation and negatively correlated with mean relative humidity. Adding oil to the polar herbicide bentazon did not alter the nature of the correlations between herbicide uptake and global radiation or mean relative humidity.

The number of hours before application to take into account for the correlations between pre-spraying weather parameters and herbicide uptake was also herbicide specific rather than species specific. The uptake of the apolar herbicide phenmedipham was best correlated when average pre-spraying weather parameters were based on the last 48 hours before herbicide application. For bentazon, with or without oil, weather parameters based on the last 12 hours seemed sufficient. These timings are in line with findings from Lundkvist (1997a).

Farmer's acceptance of optimized dose technology

Development of decision support systems (DDS) is one way of motivating farmers and agricultural contractors to adopt knowledge on herbicide dose optimization. However, in order to be more readily adopted by farmers and agricultural contractors new concepts in weed management should address possible risks of inadequate weed control, should state benefits for the overall profitability and should not have undesired long-term consequences on the weed populations.

Risk of inadequate weed control

Compared to conventional farming, integrated systems are characteristically more complex and risky (Park et al., 1997; Wibberley, 1995). When reliance on herbicide is reduced or eliminated, the risk of inadequate weed control increases and certain techniques, such as threshold spraying, can expose the enterprise to greater risk (Park et al., 1997; Vereijken & Royle, 1989).

Weed management strategies aimed at using optimized herbicide doses ideally consist of a combination of two components. First, a method should be made available to reliably predict the dose rate that is appropriate for killing the weeds in the field. Second, a means should be available to rapidly verify whether or not the herbicide application will, in fact, result in the termination of the weeds. From the experience with the development of the MLHD technology for photosynthesis-inhibiting herbicides, it is clear that farmers and agricultural contractors demand an early detection method in order to assess within a few days following the application of the herbicide, whether or not the treatment with optimized herbicide doses will be successful in controlling the weeds (Kempenaar et al., 2004).

Two types of MLHD meters are available for an early assessment of herbicide efficacy shortly after application of photosynthesis inhibiting herbicides. The Plant Photosynthesis Meter (manufacturer and distribution: EARS, www.ears.nl) has been used in relation with MLHD since 1995. The PPM-meter measures the fluorescence of the plant tissue under investigation and gives a reading on a scale from 0 (no photosynthesis at all) to 80 (completely healthy). As threshold value, a reading lower than 15 predicts that 99% of the weeds will die if treated with a photosynthesis inhibiting herbicide (Kempenaar, 2004). The PS1-meter (manufacturer: Rometron; distribution: Agrifirm) has been used in relation with MLHD since 2002. This equipment measures the absorption of light by the plant tissue and gives a reading on a scale from 0 (no damage to photosystem I, completely healthy) to 100 (photosystem I is completely blocked, no photosynthesis at all). As threshold value, a reading higher than 80 predicts that 99% of the weed will die if the plant was treated with a photosynthesis inhibiting herbicide (Kempenaar, 2004).

Developed over the past 20 years, the acetolactate synthase (ALS) inhibitors are an increasingly important group of herbicides. ALS inhibiting herbicides are widely used around the world due to the relatively low rates of application that are required and their limited environmental impact, low mammalian toxicity, wide crop selectivity and high efficacy (Peterson, 2001). Extension of the MLHD technology to the acetolactate synthase inhibiting herbicides requires the identification of suitable parameters for field evaluation of the activity of the herbicides shortly after application. The application of the ALS inhibiting herbicide metsulfuron-methyl on climate chamber grown *S. nigrum* plants (Chapters 3 and 4), greenhouse and field grown *S. nigrum* plants (Chapter 5) and greenhouse grown *P. persicaria* plants (Chapter 5) resulted in a progressive inhibition of the level of carbon dioxide (CO_2) fixation, the relative quantum efficiency of electron transport through photosystem I (Φ_{PSI}) and II (Φ_{PSII}) and a gradual decline in leaf chlorophyll content. Photosynthetic measurements, measured 2 to 4 days after treatment at photon flux densities of 400-500 μmol m^{-2} s^{-1} provided valuable information that strengthened the visual symptoms that first appeared 7 to 10 days after application of the herbicide (Chapters 4 and 5). A delay of at least 7 days is too long in the event that a second application of herbicide would be necessary. Measurement of the quantum efficiency for electron transport by photosystem II and the loss in leaf chlorophyll content appeared to be two of the most practical parameters for assessing the actual toxicity of metsulfuron-methyl. The use of chlorophyll fluorescence would require a comparison of steady-state Φ_{PSII} measurements for control and treated plants which could be realized by either measuring in time (before / after application) or space (treated / untreated patch) (Chapter 5).

Overall profitability

Inputs Experiments conducted at the Dutch Institute of Sugarbeet Research (IRS) in 2000 demonstrated that the MLHD methodology led to an even higher dose reduction than the traditional low dosage system used in sugarbeets in The Netherlands (Betanal Trio OF, 2 to 4 times applied at 2 l per ha). Adequate control was achieved with an additional reduction of 30% when the MLHD system was used. Various on farm experiments conducted by Plant Research International (PRI) and Applied Plant Research (PPO) between 1998 and 2000 showed similar cuts in herbicide use: 35% in onions, 30% in potatoes, 25% in sugarbeet and 37% in maize (Kempenaar et al., 2002a). Comparable cuts in herbicide use were obtained in the UK for several long-term research projects on Integrated Farming Systems (Morris et al., 2001). In addition to cuts in herbicide use, a number of long-term research programmes on Integrated Farming Systems conducted in Western Europe demonstrated that also cuts in fungicides (52-79%), insecticides (40%), and fertilizers (17%) are feasible (Morris et al., 2001). El Titi (1992) also reported moderate to high reductions in pesticide use in projects conducted in Austria, Switzerland, Germany and The Netherlands. The MLHD technology appeared to be an adequate tool leading to optimization of herbicide use but integration in a wider Integrated Crop Management program should be pursued in order to be able to achieve additional cuts in pesticides and in fertilizer use.

Outputs and costs Use of optimized herbicide doses also resulted in slight increases of crop yield: 2% in onions, 6% in potatoes, 4% in sugarbeets and 6-8% in maize (Kempenaar & Uffing, 2002). Unlike organic farming, the produce of integrated systems is very often not rewarded by premium prices (Morris et al., 2001). Like any other IPM measure, the MLHD technology relies on more technical advice and is more knowledge-intensive, requiring time and effort from the growers as they have to develop new skills – observation, interpretation and anticipation (Proost & Matteson, 1997). Additional costs include the subscription to the technology, the measuring equipment and staff costs which are likely to increase due to training requirements and extra observations taken in the field (1 h ha^{-1}) (Kempenaar, personal communication).

Profitability No data have been published on the overall profitability of the MLHD methodology but a number of long-term research programmes on Integrated Farming Systems conducted in Western Europe have demonstrated that integrated approaches can potentially be at least equally profitable to conventional farming. Lower inputs of integrated systems may result in identical or slightly lower yields, but this is compensated for by lower variable and operating costs (Morris et al., 2001). Profitability was at least equal to conventional farming in 17 out of 25 case studies by the TALISMAN project conducted in the UK (Holland et al., 1994) or equal to conventional farming in an experiment conducted in Austria (El Titi, 1992). Profitability was higher in the Lautenbach project conducted in Germany (El Titi, 1992), the LIFE project conducted in the UK (Jordan et al. (1993) cited by Holland et al. (1994)) and in experiments conducted in Switzerland and The Netherlands (El Titi, 1992).

Undesired long-term consequences

Long-term consequences on weed densities Bostrom and Fogelfors (2001) found a rather good connection between the sum of the amount of herbicide applied during the experimental period and the density of weeds one year after the last application. A dose reduction from a full dose to a 75%-dose did, however, not significantly influence weed density in the long run and, based on this it was concluded that every now and then herbicides may be used at reduced doses without accumulation of weed problems. Salonen (1992) and Richards et al. (1997) came to a similar conclusion. Experiments in which the herbicide reduction exceeded 25% showed that weed problems were likely to increase. In the majority of the trials reported by Bostrom and Fogelfors (2001), a dose-reduction from 75% to 25% of a full dose caused both an increase in the weed density (from 48% to 137%) as well as an increase in the proportion of difficult-to-control weed species. As the MLHD-technology aims at optimizing herbicide use without affecting herbicide efficacy, the likelihood of getting an increase in weed density is minimal.

Long-term consequences on weed resistant weeds Since the first observation of resistance in *Senecio vulgaris* (L.) to triazine in the US in the 1970s (Ryan, 1970), herbicide-resistant biotypes have been found worldwide in 182 different weed species and have been found to be resistant to nearly all of the herbicide chemistries (Heap, 2005). In The Netherlands, nine cases of herbicide-resistant weeds have been reported in the last 20 years (Heap, 2005). Resistance is 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 (Powles et al., 1998). The dynamics of herbicide resistance evolution are governed by the biology of weedy plant species, by the genetic determination of the resistance trait and by herbicide characteristics and use patterns. Genetic factors include the frequency, dominance and inheritance of major (large phenotypic effect) and minor (small phenotypic effect) resistance-endowing alleles and the fitness of resistant genotypes in the presence and absence of the selecting agent (Georghiou & Taylor, 1986; Maxwell & Mortimer, 1994). Important herbicide-related factors include herbicidal mode of action, environmental persistence and frequency of use. The role of herbicide dose in determining the evolutionary dynamics of herbicide resistance has often not been fully considered.

Most of the documented cases of field-evolved resistance to pesticides resulted from the selection of single major genes (Darmency, 1994; Macnair, 1991; Roush & McKenzie, 1987; Shaw, 2000) and it was the prevalent cause of pesticide resistance until farmers started using reduced doses (Gressel, 1995). Where high herbicide doses are applied, only the small fraction of the population possessing resistance alleles conferring a high level of resistance (major alleles) are likely to survive. It can be argued that for this type of resistance the use of low doses will put a lower selection pressure on the weed population, as with a decreasing dose, apart from the survival of the highly resistant individuals, also an increasing number of non-resistant individuals will escape, decreasing the overall level of resistance in the population. Whether under practical circumstances the use of minimum lethal herbicide doses also contain this advantage is questionable, as this strategy aims to obtain a herbicide efficacy comparable to that of the recommended label dose. Consequently, minimum lethal herbicide doses will ultimately have the same effects on the development of monogenic herbicide resistance as the recommended label dose.

The concept that reduced dose rates increase the threat of resistance in weed populations has been brought up by Gressel and Gardner (Gardner et al., 1999; Gardner et al., 1998; Gressel, 1995). Gressel (1995) suggested that lowering herbicide rates to the minimum effective control level enhances the rapidity of evolution of polygenic resistance in weeds. McKenzie (2000) also argued that where high insecticide rates will select for monogenic resistance with large phenotypic effects, low insecticide rates will select for polygenic resistance. There is very little empirical evidence for the existence of polygenic resistance, but many studies have found a high degree of continuous variation for tolerance to low herbicide rates within and between weed populations (Neve & Powles, 2005a; Patzoldt et al., 2002; Prince et al., 1983). Neve and Powles (2005b) recently demonstrated that under recurrent selection at low herbicide doses, *Lolium rigidum* was able to rapidly evolve high levels of resistance as multiple weaker mechanisms were selected and gradually accumulated from one generation to another. In practice, application of minimum lethal herbicide doses is likely to be a driving force for selection of the polygenic type of resistance. However, in the European context, with a diverse crop rotation, several options like pesticide and dose rotations can be utilized in order to delay and weaken herbicide resistance development (Gardner et al., 1999). Apart from considering the benefits related to low dosage systems, farmers and agricultural contractors should be aware of or informed about the potential long-term effect of low herbicide dose applications on the development of herbicide resistant weeds. Also legislative bodies should consider such risks when designing new policies on herbicide use.

Main conclusions

In many situations, it has been shown that considerable reductions in herbicide rate can still result in appropriate weed control. However, safe utilization of this strategy is greatly enhanced if the parameters determining the required herbicide dose are identified. This research demonstrated that pre-spraying weather conditions have a clear impact on herbicide efficacy, mainly through their effect on herbicide uptake, though this factor is definitely not the only relevant factor influencing herbicide efficacy. The correlations between pre-spraying weather parameters and herbicide uptake were herbicide specific rather than weed species specific, which has clear benefits for transfer of this knowledge into practice.

The research presented in this thesis also demonstrated that there is a good scope for extending the principle of the MLHD-technology to ALS-inhibiting herbicides. It was demonstrated that soon after application of the ALS inhibiting herbicide metsulfuron-methyl various photosynthesis related parameters, such as the carbon dioxide (CO_2) fixation, the relative quantum efficiency of photosystem I (Φ_{PSI}) and II (Φ_{PSII}) electron transport and the leaf chlorophyll content, were clearly affected. This indicates that in principle all of these could be used as an early indication for a successful herbicide application. Measurement of the quantum efficiency for electron transport by photosystem II (Φ_{PSII}) and the loss in leaf chlorophyll content appeared to be two of the most practical parameters to use when designing an early detection method to assess the toxicity of metsulfuron-methyl. Finally, it was argued that farmers' acceptance of optimized dose technologies greatly depends on risks of inadequate control in both the short and the long term and overall profitability.

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Summary

In 2000, herbicides accounted for 40% of pesticide use worldwide in terms of the volume of active ingredient. Increased concerns about environmental side effects of herbicides, development of herbicide resistance in weeds and the economic drive to reduce the cost of the inputs have resulted in increasing pressure on farmers to reduce the use of herbicides. The quantity of herbicides applied to crops can be reduced by cutting down the number of applications, by applying spot spraying rather than full-field applications or by using reduced dose rates. However, the reverse side of all of these methods is that they increase the risk of inadequate weed control.

The Minimum Lethal Herbicide Dose (MLHD) technology has demonstrated to be a decision support system that leads to the use of herbicides at a lower rate. The method is specifically developed for photosynthesis inhibiting herbicides, and allows the calculation of the minimum dose of a herbicide needed to control a weed population. To evaluate the efficacy of the treatment soon after application fluorescence techniques are used. Early detection is an essential element of the MLHD-technology, as it provides an early indication that even though minimal doses have been employed, the treatment will be successful in killing the weeds. During the last four years, investigations were undertaken to further improve the MLHD technology and to explore the opportunities of expanding it to ALS-inhibiting herbicides.

In the first part of this thesis (**Chapter 2**) the role of pre-spraying weather conditions as major determinant of herbicide efficacy was investigated. To evaluate whether differences in pre-spraying weather conditions on herbicide efficacy was mainly through an effect on plant growth rate, *Polygonum persicaria* plants were exposed to different light intensities for one to four days before application of the photosynthesis inhibiting herbicide metribuzin. During two years of experimentation, specific leaf area, rather than plant growth rate or plant size, was the only parameter that correlated well with herbicide efficacy in both years. The negative relationship between the ED_{50} and the specific leaf area put forward that leaf characteristics might be an important determinant of herbicide efficacy, most likely through an effect on herbicide uptake. In the third year of experimentation (2004), this hypothesis was confirmed as clear relations between uptake and herbicide efficacy were found for a combination of four plant species (*Solanum nigrum*, *Senecio vulgaris*, *Chenopodium album*, *Brassica napus*) and two herbicides (phenmedipham and bentazon). For phenmedipham, an apolar herbicide, uptake was negatively correlated with global radiation and positively correlated with relative humidity. For the polar herbicide

bentazon exactly the opposite was found. These results were non-species specific and indicate that inclusion of pre-spraying weather conditions in advisory systems that recommend on the use of reduced herbicide dose rates is highly recommendable.

In the second part of the thesis, the extension of the MLHD technology to acetolactate synthase (ALS) inhibiting herbicides was investigated. Photosynthetic measurements, presented in **Chapter 3**, showed that for *S. nigrum* and *Polygonum persicaria* plants treated with metsulfuron-methyl, CO_2 fixation, the relative quantum efficiency of photosystem I electron transport (Φ_{PSI}) and the relative quantum efficiency of photosystem II electron transport (Φ_{PSII}) were much lower than for unsprayed control plants. These results demonstrate that already few days after application the photosynthetic apparatus of both *S. nigrum* and *P. persicaria* plants was affected by the ALS inhibiting herbicide.

The influence of the ALS inhibitor metsulfuron-methyl on the operation, regulation and organisation of photosynthesis in *S. nigrum* was studied in more detail in **Chapter 4**. It became evident that even though the acetolactate synthase inhibiting herbicide methsulfuron-methyl is not a herbicide that targets the photosynthetic apparatus, its application on four weeks old climate chamber grown *S. nigrum* plants resulted in a progressive reduction in photosynthesis. Both CO_2 fixation and electron transport showed a reduced activity at 2 and 4 days after herbicide application. The linear relationship between photosystem I and II was unaltered by herbicidal treatment and was sustained under conditions where large changes in pigment composition of the leaves occurred. From these observations it was concluded that the stress-induced loss of leaf chlorophyll was not a catastrophic process but rather the consequence of a well organised breakdown of components. Under both photorespiratory and non-photorespiratory conditions, the relationship between the index of electron transport flow through photosystem I and II and the rate of CO_2 fixation was altered indicating that after herbicide treatment electron transport becomes less efficient at driving CO_2 fixation.

Additional photosynthetic measurements were done for greenhouse and field grown *S. nigrum* and greenhouse grown *P. persicaria* to determine whether the changes observed for climate chamber grown *S. nigrum* could also be detected for plants of another species and for plants grown under more natural conditions (**Chapter 5**). The results showed that also for these plants the application of metsulfuron-methyl resulted in progressive inhibitions of the level of carbon dioxide (CO_2) fixation, the relative quantum efficiency of photosystem I (Φ_{PSI}) and II (Φ_{PSII}) electron transport and a gradual decline in leaf chlorophyll content. Photosynthesis related measurements, conducted 2 to 4 days after treatment at irradiance levels around 400 and 500 μmol m^{-2} s^{-1}, provided valuable information that strengthened the first visual symptoms that appeared 7 to 10 days after application of the herbicide. The quantum efficiency for electron transport by photosystem II appeared to be one of the most practical parameters to use when designing an early detection method to sense the toxicity of metsulfuron-methyl. The use of this parameter would require the comparison of measurements of steady-state Φ_{PSII} for control and treated plants which could be done by either measuring in time (before / after application) or in space (treated / untreated patch).

Finally, the effects of light intensity after application of the ALS inhibitors on the detection of reductions in the quantum efficiency for electron transport by PSII (Φ_{PSII}) were examined (**Chapter 6**). For herbicide treated plants exposed to high light conditions, the losses in Φ_{PSII} were greater than for herbicide treated plants grown under low light. The herbicide treated plants showed increased loss of Φ_{PSII} with time after herbicide application. Whether maintained in high light or shade conditions, the oldest leaves were clearly not affected by the ALS inhibiting herbicide metsulfuron-methyl, whereas the Φ_{PSII} properties of the youngest leaves were most strongly affected by the herbicide. The first significant differences between the mean Φ_{PSII} of the youngest leaves and the mean Φ_{PSII} of the oldest leaves were resolvable at 2 and 4 DAT when treated plants were exposed to light and shaded conditions, respectively. Heterogeneity in loss of Φ_{PSII} was also recorded at the leaf level with the lowest and highest Φ_{PSII} efficiencies recorded for the leaf base and for the area close to the tip of the leaf, respectively. Based on these observations it was concluded that the base of the youngest leaf was the most appropriate position for taking Φ_{PSII} measurements.

In **Chapter 7**, different options for a more efficient use of herbicides are presented with special emphasis on dose optimisation. Some of the crucial factors which make new concepts in weed management more readily adopted by farmers and agricultural contractors are discussed. Finally, an overview of the main conclusions of this thesis is given.

Samenvatting

Onkruidbestrijdingsmiddelen namen in 2000 wereldwijd, uitgedrukt op basis van de hoeveelheid actieve stof, ongeveer 40% van het totale gebruik van alle gewasbeschermingsmiddelen voor hun rekening. De toegenomen bezorgdheid over de negatieve effecten van deze middelen voor het milieu, het veelvuldige optreden van herbicide resistentie en de noodzaak vanuit economisch perspectief om de productiekosten laag te houden, legt op boeren een steeds grotere druk om het gebruik van herbiciden te beperken. Een vermindering van het herbicidengebruik kan langs diverse manieren bereikt worden. Te denken valt aan het verminderen van het aantal toedieningen, de vervanging van volveldsbehandelingen door pleksgewijze toediening op plaatsen met een te hoge onkruiddruk en het gebruik van lage doseringssystemen. Keerzijde van deze methoden is dat het risico op onvolledige onkruidbestrijding wordt verhoogd.

Het Minimale Letale Herbicide Doserings (MLHD) systeem heeft in de praktijk aangetoond dat het in staat is het gebruik van herbiciden te verminderen. Dit systeem is speciaal ontwikkeld voor herbiciden die primair de fotosynthese blokkeren. Op basis van onkruidsoort en grootte wordt een minimale hoeveelheid herbicide geschat die juist voldoende is om het onkruid te bestrijden. Fluorescentiemetingen, uitgevoerd een paar dagen na toediening van het herbicide, maken het mogelijk op korte termijn op een adequate manier de verwachte effectiviteit van het herbicide in te schatten. Deze vroege meting van de effectiviteit is een essentieel onderdeel van de MLHD-methode, omdat het kort na toediening laat zien of een voldoende bestrijdingsresultaat verwacht mag worden. Dit biedt de landbouwer de mogelijkheid, indien nodig, vroegtijdig correctief op te treden. Het in het proefschrift beschreven onderzoek richt zich op een verdere verbetering en verbreding van de MLHD-methode.

In het eerste deel van het proefschrift (**Hoofdstuk 2**) is het belang van weersomstandigheden voorafgaand aan een herbicide toediening op het bestrijdingsresultaat bepaald. In experimenten uitgevoerd in 2002 en 2003 werden planten van perzikkruid (*Polygonum persicaria*) gedurende één tot vier dagen voor herbicidentoediening blootgesteld aan twee lichtniveaus. Van alle gemeten karakteristieken bleek enkel het specifieke bladoppervlak (SLA) en niet de absolute of relatieve groeisnelheid of de plantgrootte in beide jaren een goede correlatie te vertonen met de effectiviteit van het herbicide. De negatieve relatie tussen SLA en ED_{50} maakte duidelijk dat bladkarakteristieken, wellicht via een effect op de opname van het herbicide, een belangrijke bepalende factor zijn voor de herbicide effectiviteit. In het derde jaar van onderzoek (2004) werd deze hypothese bevestigd. Duidelijke verbanden tussen opname en effectiviteit van twee herbiciden (phenmedipham en bentazon) werden gevonden voor alle vier de onderzochte soorten (*Solanum nigrum*, *Senecio vulgaris*, *Chenopodium album* en *Brassica napus*). Voor phenmedipham, een a-polair herbicide, bleek de opname negatief gecorreleerd met globale straling en positief gecorreleerd met relatieve luchtvochtigheid. Voor het polaire herbicide bentazon werd precies het tegenovergestelde gevonden. Deze resultaten waren onafhankelijk van onkruidsoort en bevestigen dat het aan te bevelen is om de weersomstandigheden voorafgaande aan een herbicidenbespuiting mee te nemen in de vaststelling van de adviesdosering.

In het tweede gedeelte van het proefschrift werden de mogelijkheden verkend om het MLHD-systeem uit te breiden met ALS-remmende herbiciden (**Hoofdstuk 3**). Fotosynthesemetingen toonden overtuigend aan dat voor zowel *S. nigrum* als voor *P. persicaria* planten een bespuiting met metsulfuron-methyl, de CO_2-vastlegging, de relatieve kwantumefficiëntie van het fotosysteem I elektronentransport (Φ_{PSI}) en de relatieve kwantumefficiëntie van het fotosysteem II elektronentransport (Φ_{PSII}) aanzienlijk verlaagde vergeleken met onbehandelde controle planten. Al een paar dagen na toediening van het ALS-remmende herbicide werd het fotosynthese apparaat aangetast.

De invloed van metsulfuron-methyl op de werking, regulering en organisatie van het fotosyntheseproces in *S. nigrum* werd in meer detail bestudeerd in **Hoofdstuk 4**. Ondanks het gegeven dat metsulfuron-methyl niet rechtstreeks aangrijpt op het fotosynthese-apparaat, resulteerde de toediening van het herbicide aan 4-week-oude, in een groeikamer opgekweekte, *S. nigrum* planten in een toenemende reductie in fotosynthese. Zowel de CO_2-vastlegging als het elektronentransport werden op 2 en 4 dagen na toediening van het herbicide gereduceerd. Het lineaire verband tussen fotosysteem I en II bleven onaangetast door de behandeling met het herbicide en bleven eveneens intact onder omstandigheden waarin grote veranderingen in pigment samenstelling van de bladeren werden waargenomen. Op basis van deze waarnemingen werd geconcludeerd dat het stress-geïnduceerde verlies in bladchlorofyl niet een catastrofaal proces is, maar veel meer een gevolg van een goed georganiseerde afbraak van componenten. Zowel onder omstandigheden waarin fotorespiratie optrad, als onder omstandigheden waarin dit proces werd onderdrukt, werd de index van elektronentransportsnelheid door fotosysteem I en II en de snelheid van CO_2-vastlegging gewijzigd. Dit duidt erop dat na herbicide toediening het elektronentransport minder efficiënt benut wordt voor het vastleggen van CO_2.

Om na te gaan of de waarnemingen ook van toepassing waren op planten opgegroeid onder meer natuurlijke omstandigheden en voor een andere soort, werden additionele fotosynthesemetingen uitgevoerd onder kas- en veldomstandigheden en met kasgekweekte *P. persicaria* planten (**Hoofdstuk 5**). De resultaten toonden aan dat ook voor deze planten de toediening van metsulfuron-methyl leidde tot een toenemende remming van CO_2 vastlegging, en de relatieve kwantumefficiëntie van het fotosysteem I (Φ_{PSI}) en II (Φ_{PSI}) elektronentransport alsmede een geleidelijke afname van het bladchlorofyl gehalte. Fotosynthesemetingen uitgevoerd op 2 en 4 dagen na behandeling bij stralingsniveaus van 400 en 500 µmol m^{-2} s^{-1} verstrekten waardevolle informatie die de eerste visuele symptomen, zoals waargenomen op 7 en 10 dagen na toediening, versterkten. De kwantumefficiëntie voor het elektronentransport van fotosysteem II bleek één van de meest praktische parameters voor de ontwikkeling van een vroege detectie methode voor het opsporen van de toxiciteit van metsulfuron-methyl. Het gebruik van deze parameter vereist de vergelijking van evenwichts-waardes voor Φ_{PSII} voor zowel behandelde als onbehandelde controle planten. Dit kan gerealiseerd worden door in de tijd (voor en na behandeling) of in de ruimte (behandeld/onbehandelde plek) te meten.

Ten slotte werd het effect van lichtintensiteit na toediening van een ALS-remmend herbicide op het vaststellen van een gereduceerde kwantumefficiëntie van het elektronentransport van PSII (Φ_{PSII}) onderzocht (**Hoofdstuk 6**). Voor met herbicide behandelde planten die blootgesteld werden aan relatief hoge lichtcondities waren de verliezen aan Φ_{PSII} groter dan voor behandelde planten geplaatst onder lage lichtcondities. In de met herbicide behandelde planten werd het verlies aan Φ_{PSII} geleidelijk groter. Ongeacht lichtconditie, werden de oudste bladeren niet aangetast door metsulfuron-methyl, terwijl de karakteristieken van jonge bladeren het sterkst werden aangetast. De eerste significante verschillen in de gemiddelde Φ_{PSII} van de jongste bladeren en de gemiddelde Φ_{PSII} van de oudste bladeren waren aantoonbaar na respectievelijk 2 en 4 dagen na behandeling voor planten blootgesteld aan relatief lichte en relatief donkere omstandigheden. Ook op bladniveau werden er verschillen in het verlies in Φ_{PSII} vastgesteld, met de laagste efficiënties rond de bladbasis en de hoogste efficiënties in de buurt van de top van het blad. Op basis van deze waarnemingen werd geconcludeerd dat de bladbasis de meest geschikte positie is voor het nemen van Φ_{PSII} metingen.

In **Hoofdstuk 7** worden diverse opties voor een meer efficiënt gebruik van herbiciden gepresenteerd, met een speciale nadruk op het optimaliseren van dosering. Factoren van invloed op de acceptatie van nieuwe concepten op het gebied van de onkruidbestrijding door boeren en loonwerkers worden besproken. Verder wordt een overzicht gepresenteerd van de belangrijkste conclusies van het in dit proefschrift gepresenteerde onderzoek.

Résumé

En 2000, les herbicides représentaient près de 40% de la consommation mondiale de pesticides. Une plus grande conscience de l'effet négatif des herbicides sur l'environnement, l'augmentation des cas de résistance aux herbicides et la pression économique visant à réduire les charges de production ont été autant de facteurs qui ont conduit les agriculteurs à réduire leur consommation d'herbicides. La quantité d'herbicide appliquée sur les cultures peut être réduite en privilégiant l'agriculture de précision qui consiste à apporter la bonne dose juste au bon endroit ou en utilisant des doses réduites d'herbicides. Toutefois, l'inconvénient de ces méthodes est d'augmenter le risque d'un contrôle insuffisant des adventices.

Le système d'aide à la décision Minimum Lethal Herbicide Dose a fait ses preuves en tant que système d'aide à la décision permettant l'application de doses réduites d'herbicides. Ce système, initialement développé pour les d'herbicides inhibiteurs de la photosynthèse, permet de calculer la dose minimale d'herbicide recommandée pour contrôler une population particulière d'adventices. Les recommandations sont dépendantes de l'espèce et de la taille des adventices considérées. Pour évaluer l'efficacité du traitement, l'émission de la fluorescence chlorophyllienne est évaluée quelques heures ou jours après application de l'herbicide. Cette dernière composante est un élément essentiel de ce système d'aide à la décision puisqu'elle permet de vérifier rapidement l'effet de l'herbicide alors que celui-ci est utilisé à des doses réduites. Au cours de ces quatre dernières années, des recherches ont été menées pour améliorer le système d'aide à la décision MLHD et étudier la possibilité d'extension cette méthodologie aux herbicides inhibiteurs de l'acétolactate-synthase (ALS).

Dans la première partie de cette thèse (**Chapitre 2**) une attention particulière à été portée à l'influence des conditions climatiques précédant l'application des herbicides sur l'efficacité des herbicides. De jeunes plants de *Polygonum persicaria* ont été exposés à différentes intensités lumineuses durant 1 à 4 jours avant application de l'herbicide inhibiteur de la photosynthèse metribuzin afin de déterminer si la relation entre conditions climatiques et efficacité des herbicides s'effectue principalement au travers de la vitesse de croissance des plantes. La surface spécifique foliaire (SLA), plutôt que la vitesse de croissance des plants (GR) ou la taille des plants (biomasse ou quantité de feuilles) était le seul paramètre étant significativement corrélé avec l'efficacité de l'herbicide metribuzin en 2002 et 2003. La relation négative entre la dose efficace à 50% (ED_{50}) et la SLA a mis en évidence l'importance de la morphologie des feuilles comme un des facteurs déterminant l'efficacité de cet herbicide ; la morphologie de la plante pourrait avoir une influence sur l'absorption

des herbicides. Au cours de la troisième série d'expérimentations (2004), cette possible relation entre l'absorption et l'efficacité des herbicides inhibiteurs de la photosynthèse était confirmé pour quatre espèces végétales (*Solanum nigrum*, *Senecio vulgaris*, *Chenopodium album*, *Brassica napus*) et deux herbicides (phenmedipham and bentazon). Pour Phenmedipham, un herbicide apolaire, l'absorption d'herbicide était négativement corrélée avec la radiation globale et positivement corrélée avec l'humidité relative. Pour l'herbicide polaire bentazon les relations opposées ont été obtenues. Les résultats sont indépendants des espèces végétales étudiées. Ces observations indiquent qu'il serait recommandable de tenir compte des conditions climatiques précédant l'application des herbicides dans le calcul des doses minimales d'herbicide inhibiteurs de la photosynthèse préconisées par le système d'aide à la décision MLHD.

Dans la seconde partie de cette thèse, l'extension du système MLHD aux herbicides inhibiteurs de l'ALS a été considérée. Les mesures des différents paramètres de la photosynthèse obtenus pour de jeunes plants de *S. nigrum* et *P. persicaria* ont montré que la fixation photosynthétique du dioxyde du carbone, et les rendements quantiques du transfert d'électrons par les photosystèmes I et II (Φ_{PSI} et Φ_{PSII}) étaient très nettement inférieurs dans le cas de plantes traitées avec l'herbicide metsulfuron méthyle, un inhibiteur de l'ALS. Ces résultats préliminaires (**Chapitre 3**) ont démontré que l'appareil photosynthétique des plantes traitées était affecté rapidement par la présence de l'herbicide.

Une fois clairement établie que les appareils photosynthétiques des deux espèces végétales étudiées (*S. nigrum* et *P. persicaria*) étaient affectés par un herbicide inhibiteur de l'ALS, l'influence de l'herbicide metsulfuron méthyle sur le fonctionnement, la régulation et l'organisation de la photosynthèse chez *S. nigrum* a été étudié de façon plus détaillée (**Chapitre 4**). Il a été confirmé que l'application de l'herbicide metsulfuron méthyle sur de jeunes plants de *S. nigrum* cultivés en conditions contrôlées entraîne une inhibition progressive des différents paramètres de la photosynthèse listés précédemment. La relation linéaire entre les rendements quantiques des photosystèmes I et II est restée inchangée suite à l'application de l'herbicide et a été maintenue malgré des changements non négligeables au niveau de la composition pigmentaire des feuilles. Il est apparu clairement que la diminution de la concentration en chlorophylle n'est pas la conséquence d'un processus désorganisé mais plutôt le résultat d'une dégradation ordonnée du système. En conditions "photorespiratoires" (21% O_2) et "non photorespiratoires" (1% O_2), la relation entre le rendement quantique du transfert d'électron par les photosystèmes I et II est perturbée, résultant sur une diminution de la proportion du flux d'électron affecté à la fixation photosynthétique du CO_2.

Une série supplémentaire d'expérimentations à été conduite afin de déterminer dans quelle mesure les changements des différents paramètres de la photosynthèse pourraient également être observable pour de jeunes plants de *S. nigrum* cultivés en serres ou en plein champs et pour de jeunes plants de *P. persicaria* cultivés en serre (**Chapitre 5**). Après application de l'herbicide metsulfuron méthyle sur ces jeunes plants de *S. nigrum* et *P. persicaria*, la fixation en dioxyde du carbone, les rendements quantiques des photosystèmes I et II et la concentration de chlorophylle des feuilles étaient très nettement réduits. Ces paramètres, mesurés 2 à 4 jours après application de l'herbicide, à une intensité lumineuse d'environ 400 à 500 μmol m^{-2} s^{-1} fournissent des informations qui corrobore les premiers symptômes visuel qui apparaissent 7 à 10 jours après application de l'herbicide. Le rendement quantique du photosystème II est apparu comme un des paramètres les plus pratique à utiliser comme outils de diagnostic pour prévoir l'efficacité d'un traitement avec l'herbicide metsulfuron méthyle. L'utilisation de ce paramètre nécessite la comparaison dans le temps ou dans l'espace de plantes contrôles et traitées.

Enfin, l'effet de l'intensité lumineuse après application des herbicides inhibiteurs de l'acétolactate-synthase (ALS) sur la perte de rendement quantique du PSII (Φ_{PSII}) a été examiné (**Chapitre 6**). La perte de Φ_{PSII} était plus importante pour les plants traités exposés à une forte intensité lumineuse que pour les plants traités exposés à une intensité lumineuse moindre et devenais de plus en plus dramatique que le nombre de jours après application augmentait. Une étude comparant la perte de Φ_{PSII} pour des feuilles de différents degrés de maturité a montré que seul les plus jeunes feuilles étaient affectées par l'herbicide metsulfuron méthyle. Quelque l'intensité lumineuse soit forte ou moindre, les feuilles les plus âgées n'étaient pas affectées par l'herbicide metsulfuron méthyle. Les premières différences significatives entre la valeur moyenne de Φ_{PSII} pour jeunes et vieilles feuilles ont été observées à 2 et 4 jours après application de l'herbicide selon que les plants étaient exposés à une intensité lumineuse forte ou moindre, respectivement. Un niveau des feuilles les plus jeunes une certaine hétérogénéité a été remarquée. La perte de Φ_{PSII} était maximale pour la base des feuilles et minimale pour l'extrémité. Compte tenu de ces observations il a été conclu que la base des feuilles les plus jeunes était l'endroit le plus adéquat pour mesurer le Φ_{PSII}.

Dans le **Chapitre 7**, différentes options visant à optimiser l'utilisation des herbicides sont présentées. Plusieurs facteurs favorisant l'adoption par les agriculteurs de nouveaux concepts de gestion intégrée des adventices sont discutés. Enfin, les principales conclusions de cette thèse sont présentées.

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PE&RC PhD Education Statement Form

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 22 credits (= 32 ECTS = 22 weeks of activities).

Review of Literature (4 credits)
- Herbicide efficacy and weather conditions (2002)
- Lunch meeting presentations at CWE (2004/2005)

Post-Graduate Courses (6 credits)
- Plant spectrophotometry (2002)
- Ethics in Science (2003)
- Radiation Hygiene 5B (2005)

Deficiency, Refresh, Brush-up and General Courses (7.5 credits)
- Simulation of ecological processes (2002)
- Growth simulations (2002)
- Interactive presentations (2005)
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- EWRS conference (poster presentation) (2002/2005)
- BCPC conference (paper + oral presentation) (2003)
- EWRS / ANPP conference (paper + oral presentation) (2004)
- BCPC seminar (2004)

Curriculum vitae

Ingrid Riethmuller-Haage was born on October 17th, 1977, in Caracas, Venezuela. After completing her academic high school in S^{t} Avold (France), she studied agriculture at ISAB (Institut Supérieur Agricole de Beauvais), in Beauvais (France), and graduated in 2001 with an MSc degree in Agriculture. During her studies at ISAB she did a 6-months ERASMUS exchange at the Agriculture and Forestry University of Cordoba (Spain) and 2 long training periods, one as a production supervisor in Dominican Republic (1997) and one of 8 months in The Netherlands (2000).

She followed an MSc degree in Integrated Pest Management (IPM) in Wageningen University and Research centre (1999-2001). Interested in integrated crop management strategies, she did one research subject on the effects of the mixed-cropping system lima bean / cabbage on the odour response of predatory mites *Phytoseiulus persimilis* and did her major thesis on investigating whether the leek and celery-intercropping systems were technically and economically acceptable for Dutch farmers. She received her MSc diploma with honours in January 2001.

From March to October 2001 she worked at Horticulture Research International (HRI) East Malling (United Kingdom) on developing novel insect pest trapping methods for monitoring and reducing pest populations in strawberry and raspberry crops and was responsible for field trials.

In January 2002, she started as `assistent-in-opleiding (AIO)' at the Crop and Weed Ecology Group under the supervision of Prof. Dr. M.J. Kropff, Dr. ir. L. Bastiaans (Crop and Weed Ecology Group), Dr. ir. C. Kempenaar (Plant Research International) and worked closely with Dr. J. Harbinson (Horticulture Chain Production Group), within the research project that resulted in this thesis.

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