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Metabolic changes in plants as indicator for pesticide exposure

A pilot study

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Metabolic changes in plants as indicator for pesticide exposure

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Preface

This report presents the results of the one-year pilot-project: Metabolic changes in plants as indicator for pesticide exposure. The project aims at investigating the possibilities of developing an indicator based on changes in plant metabolites that can predict exposure of wild plants to pesticides. The project was financed by the Pesticide Researsh Programme (MST J.nr.669-00484) and was carried out by Aarhus University, Department of Bioscience (project leader) and Department of Agroecology. The NMR analysis and following NMR data processing and statistical analysis were performed by the participants from the University of Birmingham.

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Sammenfatning

Der benyttes i dag flere tiltag for at reducere pesticidbelastningen og dermed den negative effekt af pesticider på semi-naturlige habitater, der er naboer til pesticidbehandlede marker. I Danmark er det intentionen at etablere sprøjtefri randzoner langs vandløb og eventuelt også langs levende hegn. Der findes imidlertid ingen brugbar metode til at måle om sådanne tiltag opfylder deres mål.

Det overordnede mål med nærværende pilot-projekt var at undersøge sammenhængen mellem pesticideksponering og ændringer i planteindholdsstoffer, også benævnt plantemetabolitter, i non-target planter, specielt med henblik på at undersøge responset ved lave pesticiddoser samt at undersøge varigheden af responset. Undersøgelsen omfattede den enkimbladede græsart almindelig hvene (Agrostis capillaris) og den tokimbladede urt gåsemad (Arabidopsis thaliana). Begge arter blev eksponeret for fire pesticider, heraf to herbicider (metsulfon-methyl og glyphosat), et fungicid (epoxiconazol), samt en blanding af disse tre pesticider.

Undersøgelserne
Undersøgelsen bestod af fire delelementer:

1) dosis-respons forsøg til bestemmelse af følsomheden af planterne målt som den dosis af de fire pesticidbehandlinger, der skal til for at reducere biomassen med henholdsvis 10 % (ED10), 50 % (ED50) og 90 % (ED90);

2) fremstilling af plantemateriale til brug for senere analyse af planteindholdsstofferne;

3) NMR (Nuclear Magnetic Resonance) screening til identifikation af ændringer i planteindholdsstoffer i eksponerede planter i forhold til kontrol, og

4) identifikation af mønstre i de responderende planteindholdsstoffer. I forbindelse med fremstillingen af plantemateriale til brug for NMR-screening blev der foretaget en visuel bedømmelse af pesticidpåvirkningen. Resultaterne fra denne bedømmelse blev sammenholdt med resultaterne fra dosis-respons forsøg og NMR-screening.

Undersøgelserne blev gennemført ved Institut for Bioscience og Institut for Agroøkologi, begge Aarhus Universitet. NMR screening og efterfølgende identifikation af planteindholdsstoffer blev gennemført af medarbejdere ved Birmingham Universitet.

Hovedkonklusioner
Vi fandt at eksponering overfor de udvalgte pesticider resulterede i tydelige og signifikante ændringer i flere planteindholdsstoffer i de to undersøgte plantearter. Ændringerne i planteindholdsstofferne efter eksponering overfor pesticiderne metsulfuron-metyl, glyphosat og epoxiconazol samt en blanding af disse blev primært fundet indenfor to stofgrupper nemlig aminosyrer og kulhydrater, men der blev også set ændringer i organiske syrer, aminer og alkaloider.

Der blev ikke fundet et ensartet respons i plantemetabolitter i de to plantearter. Kun tre indholdsstoffer, dvs. glutaminsyre, eddikesyre og suberinsyre, viste signifikante ændringer i begge plantearter og for alle tre stoffer var specificiteten af responset modsatrettet. I gåsemad viste aminosyren glutaminsyre således et generelt respons, dvs. at det reagerede på samme måde overfor alle pesticider, her i form af en reduktion af indholdet. Indholdet af glutaminsyre i almindelig

hvene viste derimod et specifikt respons, i form af et øget indhold ved eksponering overfor epoxiconazol og et redeceret indhold ved eksponering overfor de to øvrige pesticider samt pesticidblandingen. Tilsvarende modsatrettede respons blev fundet for to andre organiske syrer.

På artsniveau blev individuelle respons-mønstre af både generel og specifik karakter identificeret. Generelt var responset på pesticideksponeringen større hos gåsemad end hos almindelig hvene. Det gjaldt både effekten på biomasse og ændringerne i indholdsstofferne, hvor såvel antallet af påvirkede plantemetabolitter som omfanget af ændringerne i de enkelte metabolitter var større hos gåsemad end hos almindelig hvene. For almindelig hvene var det således kun muligt at adskille eksponerede planter fra kontrolplanter ved de højeste doser (25 og 100 %) af glyphosat og af pesticidblandingen mens det for gåsemad var muligt at differentiere prøverne både med hensyn til dosering og med hensyn til tid siden eksponering på baggrund af den ændrede sammensætning og mængde af udvalgte planteindholdsstoffer.

Sammenfattende har nærværende pilotprojekt vist, at NMR screening kan anvendes til identifikation af hvilke plantemetabolitter, der påvirkes af pesticideksponering og er dermed et lovende første trin i udviklingen af en potentiel indikator for pesticideksponering. Andre metoder som fx HPLC og HPPC kan muligvis være mere velegnede og billigere ved yderligere undersøgelser af allerede identificerede metabolitter eller stofgrupper som fx aminosyrer eller kulhydrater.

Udvikling af en operationel indikator for pesticideksponering kræver yderligere undersøgelser af respons af flere pesticider på flere arter samt specificitet i relation til andre forhold som kan påvirke plantemetabolitter som f.eks. tørke, gødningsniveau og jordbundsforhold.

Summary

Different actions are made to reduce pesticide exposure of natural and semi-natural habitats bordering agricultural fields. In Denmark one of the the political goals are to establish spray-free border zones along streams and eventually along hedgerows. However, there is no available tool to verify if the various actions meet their goals.

The main objective of the present study was to investigate the potentiale of using changes in plant metabolite composition as an indicator for pesticide exposure. The study was conducted as a pilot-study and aimed at investigating changes in plant metabolites in two non-target plants, one monocot, i.e. Agrostis capillaris, and one dicot, Arabidopsis thaliana, following exposure to four pesticide treatments. The pesticide treatments included two herbicides (metsulfuron-methyl and glyphosate), one fungicide (epoxiconazole), and a mixture of these three pesticides. The study focused at the plant metabolite responses to low pesticide doses. The changes in plant metabolites were determined at different time intervals after exposure in order to examine the durability of the responses.

We used standard NMR (Nuclear Magnetic Resonance) screening to characterize and quantify the content of plant metabolites in exposed and non-exposed control plants. Standard dose-response experiments were used to compare the metabolic responses with visual effects and effects on biomass following the treatments.

We found that pesticide treatments resulted in specific metabolic changes in the two plant species. The responding phytochemical compounds mainly belonged to two groups, the amino acids and the carbohydrates, but also organic acids, amines and alkaloids were affected by the treatments. The induced changes of phytochemical composition and content, however, showed no general uniform pattern across plant species. Only three compounds, i.e. glutamic, acetic and suberic acid, responded significantly in both A. thaliana and A. capillaris and the responses differed among the species. In A. thaliana, glutamic acid showed a general response to all pesticides whereas the response in A. capillaris was specific, i.e. varied between pesticides; and oppositely for acetic and suberic acid, where the responses were specific in A. thaliana and general in A. capillaris.

At the plant species level, individual patterns of both general and pesticide specific metabolites were found. Generally, A. thaliana responded more distinct to the pesticide treatments than A. capillaris. This was true for biomass responses as well as the metabolic changes and the fold changes, i.e. the ratio between the concentrations of a metabolite found in exposed relative to the control samples. In A. thaliana, the collected metabolite signals were structured with regard to both sampling time and dose. In A. capillaris, separation of samples based on metabolic composition was only possible for samples treated with the two highest doses (25% and 100%) of glyphosate and the mixture of metsulfuron-methyl, glyphosate and epoxiconazole).

In conclusion, the NMR-screening seems to be a valuable method for an initial identification of responding plant metabolites (group of compounds). It turned out to be a more sensible method than visual assessments for early identification of pesticide phytotoxicity on A. thaliana. Also metsulfuron-methyl treated A. capillaris was reliable classified by NMR whereas no visual symptoms were present for these treatments. One advantage of the NMR-screening is the possibility to assessment many different compounds at the same time, however, other

methodologies such as HPLC and HPPC may be more appropriate for detailed studies and investigations on selected metabolites.

1. Introduction

1.1 Aim and hypotheses

The main aim of the project was to test if exposure of non-target plants to pesticides changes plant metabolite concentrations in a way that could later on be further developed into an indicator for pesticide exposure of non-target plants. Such an indicator could be used for example to verify the extent to which various actions to reduce pesticide exposure such as spray-free buffer zones meet their goals.

We used standard NMR (Nuclear Magnetic Resonance) screening to characterize and quantify differences in plant metabolites between exposed and non-exposed control plants.

Our main hypothesis was that pesticides even at low dosages induce specific metabolic changes in plants and that the amount and composition of metabolites result in a specific pattern of plant metabolites. We tested the responses of two plant species, one monocot, Agrostis capillaris, and one dicot, Arabidopsis thaliana, to four different pesticide treatments. The pesticide treatments included two herbicides, one fungicide, and a 3-component mixture of these. The durability of the responses was tested for three time intervals following the treatment.

1.2 Rationale

The major contribution of pesticides to the environment arises from agricultural pest control. Measured as treatment frequencies, around 55% of the pesticides use is herbicides (Miljøstyrelsen, 2011). Under normal conditions, 0-25 % of the amount applied in the field have the potential to drift to neighbouring habitats and on average 10 % of the pesticides applied to the field drift off (de Jong et al., 1995; Holterman et al., 1997; de Snoo and de Wit, 1998; Koch et al., 2002). Whereas, these doses are sub-lethal to well-established plants in the surrounding natural and semi-natural habitats they might be lethal to seedlings and young plants within the habitats.

While most animals are mobile and have the possibility to escape pesticide exposure, plants are sessile and therefore also are well-suited for monitoring of exposure to xenobiotics. Thus, plants have been used for monitoring of xenobiotics for many years (e.g. Ernst and Peterson, 1994). This project aims to investigate the potential of plant metabolites as indicators for pesticide exposure.

1.3 Pattern in plant metabolites as putative indicators

Pesticides have the potential to change the biochemical composition and content of the exposed plant. The observed changes depend on the active ingredient of the pesticide and the dose (Cobb and Kirkwood, 2000) and possibly, time of exposure as well as time since exposure affect plant metabolism and the content of specific metabolites within the plant. If the content is significantly different from control plants, the changes can be used as putative indicators for the exposure factor. Several definitions of indicators or biomarkers have been suggested in the literature (Hugget et al., 1992; Ernst and Peterson, 1994; Walker, 1995; Ernst, 1999). In the present project, an extended version of the definition by Ravn et al. (2005a) will be used to include not only herbicides but also fungicides: ‘A biomarker pattern is defined as the changes in the composition and content of phytochemical compounds detected in plants after exposure to herbicides’. The changes can be a reduced content of phytochemical compounds in the plant as well as an increased content.

Changed molecular composition of plants has been shown after exposure to herbicides (Hoagland et al., 1979; Lyndon and Duke, 1988; Ravn et al., 2005a; Ravn et al., 2005b; Hjorth et al., 2006; Petersen et al., 2007) and after exposure to the insecticide 'Fenvalerat', which is a pyrethroid (Eisler, 1992). Previously, common responses of plants exposed to recommended doses of herbicides with similar mode-of-action have been found. Primarily, the reponses have been reported for sugars and amino acids (Ravn, 2009). The investigation by Eisler (1992) of metabolites in crops, such as alfalfa, cotton and cauliflower, after exposure to the pyrethroid 'Fenvalerat' suggests that this insecticide also gives a specific biochemical response in plants.

1.4 The NMR method

With the Nuclear Magnetic Resonance (NMR) Spectroscopy method, the whole spectrum of different compounds and groups of compounds can be detected simultaneously. The method has reliably detected even small changes in the content and composition of phytochemical compounds in response to stressors such as pesticides, metals, cold, drought and patogens (Colquhourn, 2007). Thus, the outcome of the NMR potentially fulfill one of the requirements of a good biomarker (Kjær et al., 2007), i.e. the acuray. Furthermore, it has the advantage of simultaneous identification of a large number of metabolic metabolites belonging to different chemical groups. The NMR method, therefore, is very useful for a first step identification of the most promising compounds that subsequently can be developed as potential indicators of plant exposure to pesticides.

This pilot-project investigates the composition and changes of individual metabolites in pesticide exposed and control plants. We included low pesticide doses to investigate whether specific responses are detectable at doses normally occurring in spray drift, and whether there is a dose-response relationship between degree of exposure (dose) and the content and composition of metabolites. Previous results suggest that the metabolic changes are detectable for a relative long period (up to 21 days) after exposure to the pesticide (Ravn, 2000). However, documentation is needed on the temporal development of the response and therefore temporal responses are included in this study.

1.5 Structure of the project

The project was a one year pilot-project and consists of three main parts including a) dose-response and exposure experiments, b) NMR-analyses and c) identification of patterns in the content of plant metabolites that are candidate indicators for exposure of plants to pesticides.

2. Background

Over time, the flora and fauna within hedgerows and other small biotopes along conventionally cultivated fields have been influenced by the intensive farming practice including the use of pesticides with the result that biodiversity is reduced significantly especially in agricultural areas (e.g. Chamberlain et al., 2000; Donald et al., 2000; Donald et al., 2006; EEA, 2006). Measures to reduce the exposure to pesticides, therefore, are demanded. The establishment of pesticide-free buffer zones is one such action that has the potential to reduce pesticide exposure of neighboring habitats (e.g. Marrs et al., 1989; Marrs et al., 1993) and can thus be expected to have a positive effect on biodiversity. Methods to verify the extent to which various actions to reduce pesticide exposure such as spray-free buffer zones meet their goals are therefore needed.

Three commonly used pesticides were selected for the pilot project. Glyphosate is globally and nationally the most widely used herbicide. Metsulfuron-methyl belongs to the sulfonylureas and this group of herbicides is registered in all major agricultural crops except oilseed rape. Due to the high phytotoxicity of the sulfonylurea herbicides the applied doses are very low and consequently the consumption in weight is low, however based on treatment frequency the sulfonylureas is one of the most frequently used herbicide groups in Denmark. Epoxiconazole is the most important active ingredient among the fungicides. Epoxiconazole was used alone or in mixture with other fungicides on 28% of the fungicide treated areas in Denmark in 2009 (Miljøstyrelsen, 2011). In practice the flora along field edges will be exposed to several pesticides during the growth season and therefore a mixture of glyphosate, metsulfuron-methyl and epoxiconazole was used as the fourth treatment. The plant species Arabidopsis thaliana and Agrostis capillaris were selected as test plants representing a dicotyledon and a monocotyledon species, respectively.

2.1 Plant metabolites and pesticides

2.1.1 Glyphosate

Glyphosate (N-phosphonomethyl) is a systemic herbicide that belongs to the glycines. It is absorbed by all living plant tissue and transported to growth points in roots and shoots (Jensen et al., 2011). It quickly becomes inactive in soil through adsorption to soil minerals and rapid microbial transformation (Gimsing et al., 2004). The herbicide is non-selective and blocks an enzymatic step in the shikimic biosynthesis (Fig. 2.1). The blocked enzyme is 5-enolpyruvylshikimat-3-phosphate synthase (EPSPS). The enzyme condenses shikimat-3-phosphate and phosphatenolpyruvat (PEP) to 5-enolpyruvylshikimat-3-phosphate and inorganic phosphate (Duke, 1988). The inhibition mechanism is complex, but previous studies have shown that glyphosate binds to the portion of EPSPS that is bound to the phosphate part of PEP (Kishore & Shah, 1988).

The three essential aromatic amino acids, phenylalanine, tyrosine and tryptophan, are synthesized via the shikimic biosynthesis (Fig. 2.1). Inhibition of the formation of these three amino acids means that the formation of other plant metabolites, they are part of such as lignin, phenolic compounds, flavonoids and many others, are also inhibited. The shikimic biosynthesis is an important metabolic pathway in plants, fungi and bacteria, but it is not found in animals (Devine et al., 1993).

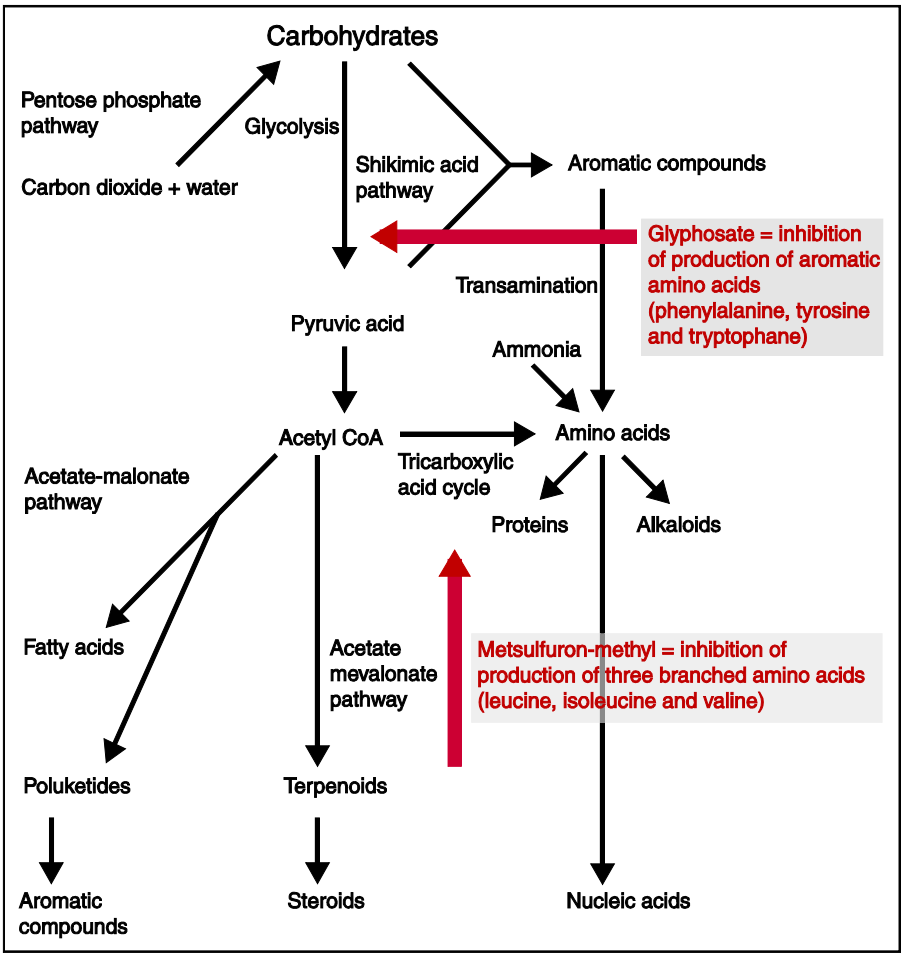

Figure 2.1. Biosynthetic pathways in plants. The red arrows indicate the site of action of the herbicides glyphosate and metsulfuron-methyl (from Vickery and Vickery, 1981).

Apart from the above mentioned biosynthesis of the aromatic amino acids, shikimate is known as biomarker for exposure to glyphosate (Hoagland et al., 1979). Hoagland et al. studied effects of glyphosate on metabolism of phenolic compounds on 3-days-old dark grown soybean seedlings. The amount of extractable phenylalanine ammonia-lyase (PAL) activity increased while the phenylalanine content decreased five-fold. This result indicated that glyphosate could exert its effect through induction of PAL activity and/or inhibition of aromatic amino acid synthesis (Hoagland et al., 1979).

At low exposures to glyphosate, a complex amino acid and glucosinolate pattern was found in young oilseed rape plants (Petersen et al., 2007). Glyphosate effects on hydroxybenzoic acid levels in pigweed (Amaranthus retroflexus L.), ryegrass (Lolium perenne L.), soybean (Glycine max (L.) Merr.), velvetleaf (Abutilon theophrasti Medic.) and yellow nutsedge (Cyperus exculentus L.) have been documented by Lyndon and Duke (1988). Leaves were analysed using High Performance Liquid Chromatography (HPLC). The protocatechuic acid content varied among species and was dependent on the glyphosate dose, the duration of the exposure, and the tissue assayed. Gallic acid levels were higher in four of the five species, and 4-hydroxybenzoic acid was higher in three of the five species treated with glyphosate compared to controls. Vanillic and syringic acids, which were methylated forms of protocatechuic and gallic acids, respectively, were unaffected by the glyphosate treatment (Lyndon and Duke, 1988).

In the grass species Apera spica-venti, four different patterns of compounds encompassing increased levels of glutamine, proline, leucine/isoleucine and reduced chlorophyll content have been identified using High Performance Planar Chromatography (HPPC) (Ravn et al., 2005a).

Glyphosate is a total herbicide and was expected to affect growth of both test plants.

2.1.2 Metsulfuron-methyl

Metsulfuron-methyl is a sulfonylurea herbicide, belonging to the group of acetolactat synthase (ALS) inhibiting herbicides. ALS is the first enzyme that is specific to the formation of three-pronged amino acids (Fig. 2.1). Metsulfuron-methyl is absorbed both through leaves and roots, through which it is transported to the plant's growth points. Metsulfuron-methyl is primarily active against broadleaf dicot species but in high doses it also reduces growth of monocot species (Hageman and Behrens, 1984; Jensen et al., 2011). Aquatic plants as well as terrestrial plants are affected by sulfonylurea herbicides. Ravn et al. (2005a) showed that the content of a number of amino acid changes in eight different aquatic plants that had been exposed to metsulfuron-methyl and they found that these changes may be used as biomarkers of metsulfuron-methyl exposure.

In plants that are sensitive to the herbicide, the formation of the three-pronged amino acids, valine, leucine and isoleucine, is blocked (Chaleff and Mauvais, 1984; Jensen et al., 2011). The inhibition causes plant cell proliferation and the growth to cease (Brown, 1990). A limited translocation of photosyntetat has also been found (Bestman et al., 1990). Due to the inhibition, an increased concentration of toxic alkaloid plant metabolites has been found in tall larkspur (Delphinium exaltatum), and in soybean, anthocyanin synthesis, phenylalanine ammonia lyase activity and ethylene production are affected (Suttle and Schreiner, 1982; Ralphs et al., 1998). These syntheses affected both alkaloid and the phenolic substance formation in the plants.

The dicot test species A. thaliana was expected to be highly susceptible to metsulfuron-methyl while the monocot plant species A. capillaris was expected to be more tolerant.

2.1.3 Epoxiconazole

Epoxiconazole is a systemic fungicide belonging to the group of triazoles. It inhibits the formation of ergosterole, an important compound in the fungal cell membrane (Jensen et al., 2011). Hardly any literature exists on the effects of fungicides on the metabolic processes in plants. Existing literature primarily relates to the metabolism in exposed fungi. The specific fungal toxicity of triazoles is primarily caused by a sterol 14α-demethylation inhibition (Kapteyn et al., 1992).

It was not expected that any of the test species was sensitive to epoxiconazole, however, the fungicide might still affect the plant metabolism and thereby result in significant changes in plant metabolite content and composition.

3. Material and methods

3.1 Generation of plant material and application of pesticides

Two different experiments were conducted with each of the two test species: 1) a dose-response experiment and 2) an exposure experiment to generate plant material for the NMR screening.

The objective of the dose-response experiment was to provide data on the plant responses, i.e. visual symptoms and effects on plant biomass, at different time after exposure to the pesticides. Furthermore, comparison of these results with the results of the NMR screening will indicate if changes in the metabolic composition are a more sensitive measure for pesticide exposure than a visual assessment of plant phytotoxicity or measurement of plant biomass.

The objective of the exposure experiment was to provide plant material for the NMR screening. As we aim at both identifying specific metabolic changes induced in plants exposed to pesticides and at testing the durability of these changes by the screening, groups of plants were exposed to a range of doses of the different pesticides and harvested at different times after pesticide application and later screened by NMR.
Both experiments included four different pesticide treatments including two herbicides, glyphosate (Roundup Bio, 360 g ae/L, Monsanto Crop Sciences Denmark A/S) and metsulfuron-methyl (Ally ST, 500 g/kg, Du Pont Denmark Aps), one fungicide, epoxiconazole (Opus, 125 g/L, BASF A/S, Denmark) and a 3-component mixture of glyphosate, metsulfuron-methyl and epoxiconazole. In addition, the dose-response experiment included a metsulfuron-methyl + epoxiconazole treatment and a glyphosate + epoxiconazole treatment. These treatments were included in order to examine if an effect of the 3-component treatment was related to synergism between the active ingredients or to an increased activity of the herbicides promoted by the adjuvants in the fungicide formulation.

Seeds of Agrostis capillaris and Arabidopsis thaliana were sown in 1 L pots in a potting mixture (field soil, sand and peat 2:1:1 by weight) that contained all necessary micro- and macro-nutrients. A. capillaris were placed in the greenhouse at a temperature of 160 C during the day and 140 C in the night. Higher temperatures and a shorter day were necessary for vegetative growth of A. thaliana and the pots were placed in the greenhouse at 25/150 C (day/night) and a photoperiod of 16 hours. The pots with A. thaliana were covered with polyethylene in order to ensure high soil humidity and promote germination. The pots were sub-irrigated twice a day.

Figure 3.1 Pots with Agrostis capillaris (top) and Arabidopsis thaliana (bottom) in the greenhouse. Photo: S. Mathiassen.

After emergence, the number of plants per pot was reduced to 4 and 6 for A. thaliana and A. capillaris, respectively. The pesticides were applied on 24 January 2011 (A. thaliana) and 1 February 2011 (A. capillaris) using a laboratory pot sprayer fitted with two Hardi ISO F-110-02 nozzles. The nozzles were operated at a pressure of 3 bars and a velocity of 5.5 km/h delivering a spray volume of 154.1 L/ha. At the time of spraying, A. thaliana and A. capillaris had 8-10 leaves and 4-6 leaves, respectively (Fig. 3.2).

Figure 3.2 Growth stages of A. capillaris and A. thaliana at spraying. Photo: S. Mathiassen.

3.2 Dose-response experiments

The dose-response experiments included 8 doses of each pesticide and the mixture. All pesticides were applied as the commercial formulations. The herbicide doses were selected based on the responses in a preliminary dose-response experiment and aimed at covering responses from 0 to 100% reduction of plant biomass. Epoxiconazole is a fungicide and as such it is not phytotoxic and therefore the recommended field dose was used as the maximum dose (100% N). The highest dose (100% N) of the pesticides used in the dose-response experiments are shown in Table 3.1. In the dose-response experiment, a factor 2 was used between the doses for A. thaliana and a factor 2.2 between doses for A. capillaris. The higher doses used on A. capillaris compared to A. thaliana reflect that grass species, in general, are more tolerant to the two herbicides than dicot plant species.

Besides the four pesticide treatments that are common to both experiments, two additional treatments were included in the dose-response experiments: 1) metsulfuron-methyl + epoxiconazole (ME) and glyphosate + epoxiconazole (GE). We included these treatments to test if the formulation adjuvants included in one of the commercial formulations of epoxiconazole (Opus) affected the activity of the herbicides. Previously, such effects have been reported in mixtures of herbicides and fungicides and can be explained by a generally higher content of adjuvants in fungicides which may increase the retention and/ or uptake of herbicide.

The dose-response experiments were carried out with 3 replicates (6 untreated controls) using a complete randomized design. The plants were harvested 21 days after treatment and fresh weight were recorded.

TABLE 3.1
MAXIMUM DOSES (G A.I/HA) USED IN THE DOSE-RESPONSE EXPERIMENTS. MGE IS A MIXTURE OF METSULFURON –METHYL, GLYPHOSATE AND EPOXICANAZOLE

Plant species	Metsulfuron-methyl (g a.i./ha)	Glyphosate (g a.i./ha)	Epoxiconazole (g a.i./ha)	MGE (g a.i./ha)	Metsulfuron-methyl + epoxiconazole (g a.i./ha)	Glyphosate + epoxiconazole (g a.i./ha)
A. capillaris	16.0	360.0	62.5	8+180+31.3	8+31.3	360+31.3
A. thaliana	2.5	90	125	1.25+45+62.5	1.25+31.3	90+31.3

3.2.1 Statistics

The data from the dose-response experiments were subjected to an analysis of variance. For pesticides showing a dose-response, the fresh weight data were subjected to non-linear regression analyses using the log-logistic dose-response model (Seefeldt et al., 1995):

$$U = \frac{D - C}{1 + \exp[b(\log(ED_{50}) - \log(z))]} + C \qquad (1)$$

where, U is the fresh or dry weight, z is the dose, D and C are the upper and lower asymptotes at zero and very high herbicide doses, respectively, ED50 is the dose resulting in a 50% reduction in plant biomass, and b is the slope around ED50. The assumption that logistic dose-response curves could be fitted to the data was assessed by a test for lack of fit comparing the residual sum of squares of an analysis of variance and the non-linear regression.

3.3 Exposure experiments

The pesticide doses used in the exposure experiments for producing plant material to the NMR screening were also decided based on preliminary dose-response experiments. The normal dose (100% N) reflected the dose that was expected to reduce biomass of the test plant by 90%. In case of no or low phytotoxicity of the active ingredient, i.e. epoxiconazole, the 100% N dose was similar to the recommended field dose. In addition to the 100% N dose, three lower doses were applied (1% N, 5% N and 25% N) to investigate the robustness and detection level of pesticide induced metabolic changes in the test plants. The applied doses for each test species are shown in Table 3.2. The metsulfuron-methyl and glyphosate doses used for exposure of A. thaliana were much lower than for A. capillaris reflecting that A. thaliana is more sensitive to herbicides than A. capillaris.

TABLE 3.2
PESTICIDE DOSES (G A.I./HA) APPLIED IN EXPOSURE EXPERIMENTS PRODUCING PLANT MATERIAL OF A. CAPILLARIS AND A. THALIANA FOR NMR ANALYSES. MGE IS A MIXTURE OF METSULFURON-METHYL, GLYPHOSATE AND EPOXICONAZOLE

Plant species	Pesticide	1% N (g a.i./ha)	5% N (g a.i./ha)	25% N (g a.i./ha)	100% N (g a.i./ha)
A. capillaris	Metsulfuron-methyl	0.08	0.4	2	8
	Glyphosate	3.6	18	90	360
	Epoxiconazole	0.63	3.13	15.63	62.50
	MGE	0.04+1.8+0.31	0.2+9+1.57	1+45+7.8	4+180+31.3
A. thaliana	Metsulfuron-methyl	0.01	0.05	0.25	1
	Glyphosate	0.45	2.25	11.25	45
	Epoxiconazole	0.63	3.125	15.6	62.5
	MGE	0.01+0.23+0.3	0.03+1.13+1.57	0.13+5.63+7.83	0.5+22.5+31.3

Plants for the NMR analysis were harvested 7, 14 and 21 days after spraying in order to determine the duration of the metabolic changes. Each treatment included 72 pots, i.e. 24 pots per concentration and 3 harvest times. In each spray event (run of the boom), 6 pots were sprayed and at harvest these pots were pooled into 1 replicate for the NMR analysis. Consequently, the NMR analyses were conducted with 4 replicates that were sprayed separately. The experiment was designed with harvest times as blocks and completely randomized treatments within the blocks. Before each harvest a visual assessment of plant injury was carried out using the scale described by Ravn et al. (2005a) with small modifications (Table 3.3). After harvest the plants were filled in polyethylene bags and immediately frozen on dry ice. The plant material was stored in a freezer at -180C.

TABLE 3.3
RATING SCORE OF VISUAL EFFECTS. THE SCALE IS MODIFIED FROM RAVN ET AL. (2005A)

Rating	Detailed desciption
0	No effect
1	Trace effect. Can be associated with slight growth stimulation (hormesis)
2	Up to 20% growth reduction
3	Up to 30% growth reduction
4	Up to 40% growth reduction
5	Up to 50% growth reduction
6	Up to 60% growth reduction
7	Up to 70% growth reduction
8	Up to 80% growth reduction
9	Up to 100% growth reduction

3.4 NMR-Analysis

Plant samples were analyzed using a Bruker 600 NMR by 1D (one dimensional) and 2D (two dimensional) j-resolved (jres) analysis. 2D jres is an analytical approach which extracts additional information from the data compared to direct analysis of 1D spectrum. Some shifting was found to occur in the data, i.e. NMR peaks moving in relation to the baseline. This can occur for example due to pH changes, matrix differences between samples or extensive metal cations being present in the sample, however, this should have been accommodated by the data processing. Pre-processing was conducted to prepare the samples for analysis by multivariate statistics. The preprocessing was largely able to realign the spectra to compensate for the shifting. All samples were normalized using probable quotients normalization (pqn), transformed using generalized logarithm transformation and mean-centered.

One hudred mg (±5 mg) of each sample was solvent extracted in 6µl/mg methanol and 1.65µl/mg water and homogenized using a Precellys-24 bead based homogeniser (Stretton Scientific, UK). Samples were transferred to clean vials and left on ice for 10 minutes before they were centrifuged at 4°C for 10 minutes at 1800g and left to sit at room temperature for 5 minutes. The supernatant was removed and dried in a centrifugal concentrator (Thermo Savant, Holbrook, NY, US). Samples were stored dry at -80°C until analysis, and then they were resuspended in 100µl of phosphate buffer with 250µM TMSP and 0.03% sodium azide were added. 30µl of each sample was analysed using NMR.

The 2D J-resolved NMR spectra were acquired on a Bruker Avance III 600 MHz instrument equipped with a cryogenically cooled 1.7mm probe. The spectral width was set to 12ppm for the direct and to 50 Hz for the indirect time domain of the 2D spectra. A total of 8 transients per one of the 32 increments were acquired. The solvent resonance was suppressed using NOESY-pre saturation. The free induction decays (FIDs) were apodized using a combination of 0.5 Hz exponential line broadening and an unshifted sine window function for the direct dimension an unshifted sine window function for the indirect time domain prior to Fourier Transformation (Parsons et al., 2009). All spectra were referenced with respect to TMSP.

The 2D J-resolved spectra were analysed with a data mining algorithm using 2D NMR spectra from the Birmingham Metabolite Library (BML) (Ludwig et al., 2011) using a custom written script soon to be published. The 1D projection of the 2D J-resolved spectra was then further processed to prepare them for statistical analysis. They were baseline corrected using a spline based approach (Ludwig and Günther, 2011). The edges of the spectrum, including the TMSP resonance, were removed as was the region around the water signal. Segmental alignment was carried out on a total of 74 manually selected regions. The spectra were scaled using probabilistic quotient normalisation and finally scaled using the glog transform (Parsons et al., 2007). All NMR data processing was carried out in the MATLAB based NMRLab/MetaboLab software package (Ludwig and Günther, 2011).

Methanol and water were HPLC grade from Scientific and Chemical Supplies Ltd (Bilston, UK). Precellys homogeniser tubes were bought ready-filled from Stretton Scientific (Stretton, UK). Sodium azide, sodium phosphate monobasic (NaH_2PO_4) and sodium phosphate dibasic (Na_2HPO_4) were purchased from Sigma-Aldrich (Dorset, UK). D_2O (99.9% purity) and sodium 3-trimethylsilyl-2,2,3,3-d4-propionate (TMSP, 99% purity) were purchased from Goss Scientific Instruments Ltd. (Cheshire, UK).

3.4.1 Statistics, NMR-analyses

Partial least squares regression (PLS regression) was used to analyze the data. PLS regression is an analysis with resemblance to PCA (principal components analysis). While PCA identifies perpendicular planes of maximum variance between the X and Y variables, PLS identifies a linear regression model in a projection of the predicted variables and the observable variables, and thus

describe the fundamental relations between two data set matrices (X and Y). PLS regression is particularly suited when the matrix of predictors has more variables than observations. Partial Least Squares Discriminant Analysis (PLS-DA) used in this report is a variant of PLS which is used when the Y data consists of classes (in this report, sampling time, dose or pesticide). PLSDA is a supervised technique i.e. it is looking to find a difference between classes defined by the investigator, which is why the validation of the models are important. Validation is represented by the classification error rate, which is a measure of how well the regression model described the covariance among the two matrices, and thus, the smaller the error rate the better fit of the model. Classification error rates are important as well as the P values – as a classification error close to 50%, in an analysis of two classes, suggests that the model is not much better than chance.

The PLS was applied to the dataset using two approaches. First, the projected and bucketed jres spectral data (2D jres), which takes into account the signals from all metabolites (including identified as well as unidentified) was applied. For the second "FIMA" approach the 2D jres data was subjected to an automated identification process "FIMA" using a method developed at the University of Birmingham and shortly to be published. FIMA consists of an NMR library of 208 common metabolites. Spectra are matched to the library metabolites and the concentration for each metabolite is calculated relative to Trimethylsilyl propanoic acid (TMSP). TMSP is a chemical compound containing used as internal reference in the NMR analysis. Thus, this approach analyzes the identified metabolites alone.

Individual metabolites identified by the FIMA approach were evaluated by the 'Variable Importance in Projection' (VIP) score which estimates the importance of each variable in the projection and can be used for selecting variables of importance and interest. A VIP Score around one or greater indicates that a variable has an important contribution to the model. Variables with lower VIP scores are less important and are candidates for exclusion.

The relatively small sample numbers in many of the groups will make robust statistical differences difficult to achieve for some subsets of the data. Thus, a reliable factorial analysis of dose and sampling time is not feasible to conduct and we have focused on general effects of sampling times and doses. Unless otherwise stated a cut off value of 5% was used for evaluating test as statistical significant.

4. Results

4.1 Arabidopsis thaliana

4.1.1 Dose-response experiment

The maximum dose of metsulfuron-methyl used on *A. thaliana* was similar to the recommended dose in cereals at stem elongation (BBCH 30). Significant biomass responses were obtained for doses of 0.63 g a.i./ha and higher (Figure 4.1). The maximum glyphosate dose used on *A. thaliana* was 90 g a.i./ha which is much lower than the recommended dose of 720-1080 g a.i./ha for weed control before harvest. The response to glyphosate was highly dose dependent and the efficacy was increased from 10% effect at 11.3 g a.i./ha to >90% effect at 22.5 g a.i./ha (Figure 4.1). Consequently the estimated dose-response curve was very steep. Epiconazole was used at the recommended dose of 125 g a.i./ha as maximum dose. Although epoxiconazole is a fungicide, and therefore not expected to have any phytotoxic effects, it actually reduced the biomass of *A. thaliana* at the highest doses due to scorching of leaves (Figure 4.1).

The dose-response relationship of mixtures indicated that the formulated product of epoxiconazole had a relatively strong synergistic effect on the activity of metsulfuron-methyl and glyphosate and consequently the biomass responses were higher than expected based on the dose-response to the individual pesticides (Figure 4.1).

It was possible to fit the dose-response model to fresh weight data for all treatments except metsulfuron-methyl + epoxiconazole for which the lowest dose gave >90% effect. The ED_{10}, ED_{50} and ED_{90} doses of metsulfuron-methyl and glyphosate were within the dose range applied in the dose-response experiment except the ED_{90} dose of glyphosate at 3.4 g a.i./ha being slightly higher than the maximum dose in the dose response experiment. (Table 4.1) The ED_{90} dose of epoxiconazole was much higher than the maximum dose of 125 g a.i./ha. The ED_{10} to ED_{90} doses of the MGE mixture were within the dose range applied in the dose-response experiment while the applied doses of metsulfuron-methyl + epoxiconazole (ME) and glyphosate + epoxiconazole (GE) were higher than the ED_{10} doses (Table 4.1). The effect of metsulfuron-methyl on plant biomass was increased by a factor 100 or more when applied in mixture with the commercial product of epoxiconazole (Opus) while the activity of glyphosate was increased by a factor 6 (Table 4.1). These responses strongly indicate that adjuvants included in the commercial formulation of the fungicide improve the activity of both herbicides.

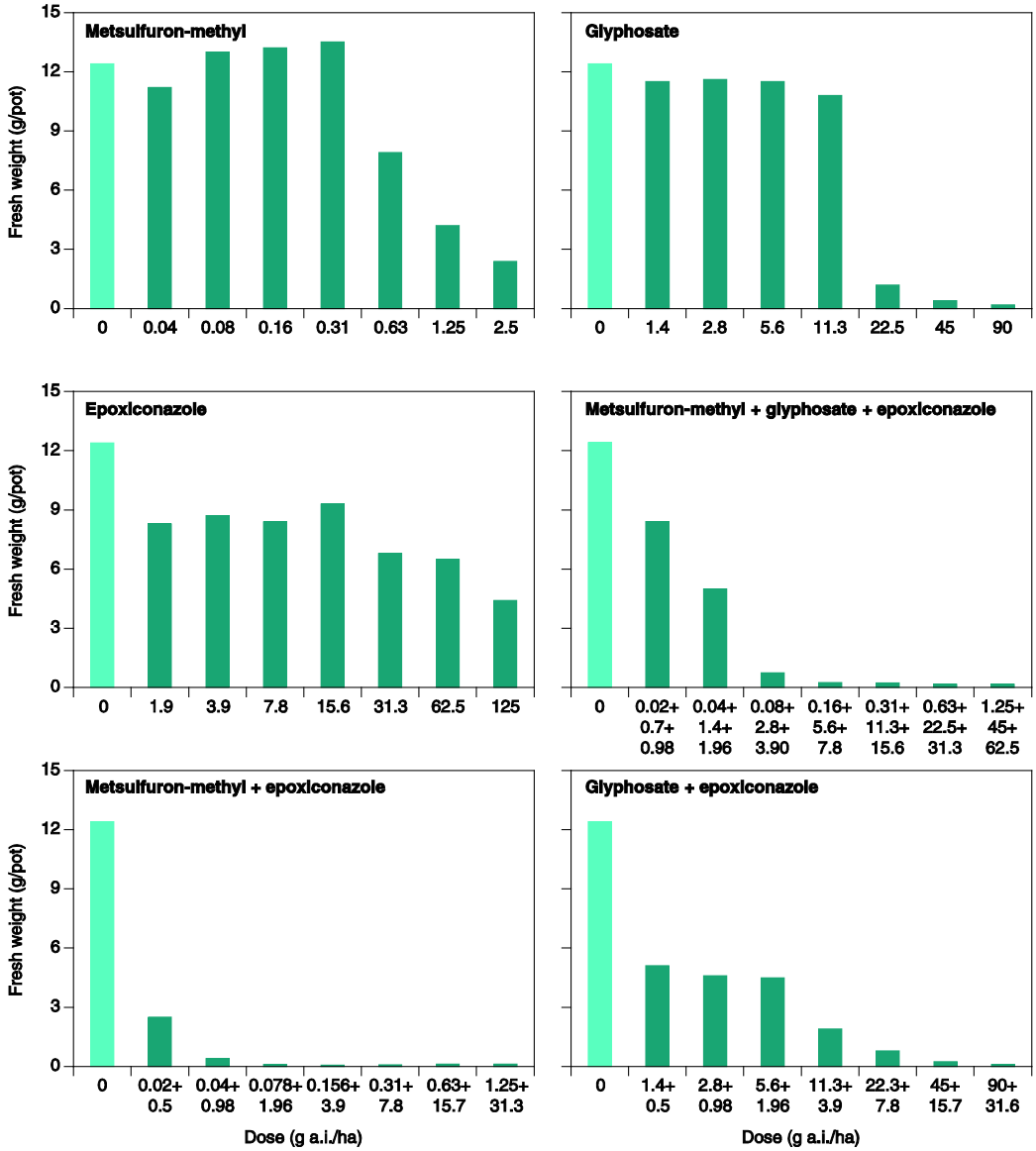

Figure 4.1 Effect of dose ranges of metsulfuron-methyl, glyphosate, epoxiconazole and mixtures of epoxiconazole and metsulfuron-methyl and/or glyphosate on biomass (g fresh weight/pot) of A. thaliana in the dose-response experiments described in section 3.2. Mean values of 3 replicates. All pesticides were applied as commercial products.

TABLE 4.1
ESTIMATED ED_{10}, ED_{50} AND ED_{90} DOSES (G A.I./HA) OF DIFFERENT PESTICIDES ON BIOMASS OF *A. THALIANA* USING THE DOSE-RESPONSE MODEL DESCRIBED IN SECTION 3.3. FIGURES IN PARANTHESES ARE 95% CONFICDENCE INTERVALS. ED DOSES OF METSULFURON-METHYL + EPOXICONAZOLE ARE APPROXIMATED VALUES AS IT WAS NOT POSSIBLE TO FIT THE MODEL TO FRESH WEIGHT DATA OF THIS TREATMENT. THE ED_{90} DOSE OF EPOXICONAZOLE WAS HIGHER THAN THE MAXIMUM APPLIED DOSE. MGE = METSULFURON-METHYL + GLYPHOSATE + EPOXICONAZOLE.

Pesticide	**ED_{10} (g a.i./ha)**	**ED_{50} (g a.i./ha)**	**ED_{90} (g a.i./ha)**
Metsulfuron-methyl	0.30(0.03-0.57)	1.02 (0.63-1.41)	3.42 (1.90-5.00)
Glyphosate	7.40(0.68-14.15)	12.60 (8.80-16.40)	31.63 (23.50-39.71)
Epoxiconazole	5.79(2.56-14.14)	78.80 (43.70-114.00)	>125.00
MGE**	1.05 (0.63-1.49)	2.75(2.14-3.34)	7.10 (5.94-8.36)
Metsulfuron-methyl + epoxiconazole	<0.02	<0.02	0.02 (0.018-0.036)
Glyphosate + epoxiconazole	0.26 (0.01-0.52)	1.94(0.83-3.06)	14.8 (8.5-21.0)

* Doses shown as doses of metsulfuron-methyl and glyphosate, respectively
**Doses shown as sum of doses of metsulfuron-methyl+glyphosate+epoxiconazole (1.1%: 41.4%: 57.5%)

4.1.2 Exposure experiment – plants for NMR analysis

Comparison of the doses used in the exposure experiment for generation of plant material for the NMR analysis (Table 3.2, p. 21) with the estimated ED doses in Table 4.1 shows that the 100% N dose of metsulfuron-methyl resulted in approximately a 50% reduction of the biomass of *A. thaliana* and the 25% N dose gave approximately a 10% reduction. For the 100% N dose the symptoms of plant injury became visible 14 days after spraying as a slight yellowing of the growth point while at 21 days after spraying the plants were partly desiccated and the visual scores suggested higher effects (20% of the 25% N dose and 70% of the 100% N dose. (Table 4.2).

The highest dose of glyphosate used on plants for the NMR analysis was 45 g a.i./ha (Table 3.2 p. 21). According to the dose-response experiment this dose was expected to give more than 90% effect on plant biomass 21 days after spraying, while the 25% N dose (11.25 g a.i./ha) was approximately equal to the ED_{10} of glyphosate on *A. thaliana* (Table 4.1). The plants exposed to 100% N dose of glyphosate showed symptoms 7 days after spraying in form of yellowing of the growth point (Figure 4.2, Table 4.2). At the last harvest the symptoms were distinct for the 25% and 100% N doses.

The highest dose of epoxiconazole used on *A. thaliana* plants for NMR analysis was 62.5 g a.i./ha (Table 3.2). Based on the dose-response experiment this dose was expected to result in a 50% effect on biomass (Table 4.2). Symptoms of slight plant injury became visible 7 days after spraying as scorching of the leaf tips (Figure 4.2). Although the symptoms developed a little by time the plants compensated by growth and the damage was static when assessed as percentage plant injury 21 days after spraying (Table 4.2).

The 25% and 100% N doses of the tank mixture of metsulfuron-methyl + glyphosate + epoxiconazole were expected to reduce biomass by more than 90% while the 5% N dose was expected to give 50% effect on biomass (Table 4.2). Symptoms of plant injury appeared 7 days after spraying for the 25% and 100% N doses and the final scores in the visual assessment 21 days after spraying showed that the plants were more or less desiccated. This is consistent to the responses obtained in the dose-response experiment (Figure 4.1).

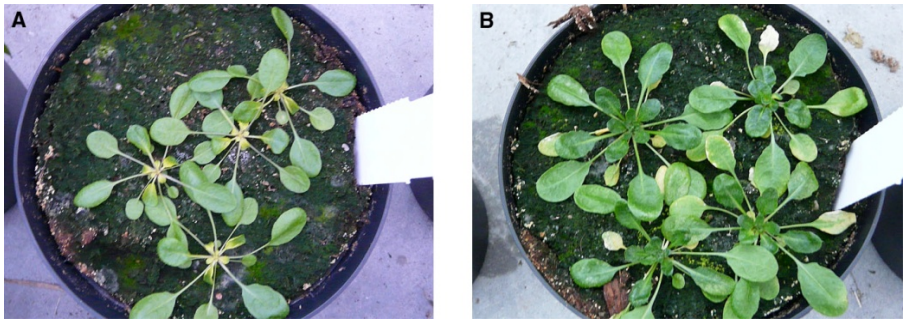

Figure 4.2 Visual symptoms on A. thaliana 7 days after exposure to 45 g a.i./ha of glyphosate (left) and 62.5 g a.i./ha of epoxiconazole (right).

TABEL 4.2
VISUAL ASSESSMENT OF *A. THALIANA* INJURY GIVEN AS RATING SCORES AT DIFFERENT HARVEST TIMES AFTER EXPOSURE TO METSULFURON-METHYL, GLYPHOSATE, EPOXICONAZOLE AND A MIXTURE (MGE) OF THE THREE PESTICIDES. FOR EXPLANATION OF RATING SCORES SEE TABLE 3.3. THE DOSES FOR THE EXPOSURES ARE SHOWN IN TABLE 3.2.

	Metsulfuron-methyl	Glyphosate	Epoxiconazole	MGE
7 DAT				
1% N	0	0	0	0
5% N	0	0	0	0
25% N	0	0	0	2*
100% N	0	2*	1**	3*
14 DAT				
1% N	0	0	0	0
5% N	0	0	0	2
25% N	0	3*	1**	4
100% N	3*	8**	2**	6**
21 DAT				
1% N	0	0	0	0
5% N	0	0	0	5**
25% N	2*	5**	1**	9**
100% N	7**	9**	1**	9**

* yellowing of yougest leaves
** desiccation

4.1.3 NMR results

Arabidopsis thaliana

Using the full dataset, i.e. data on all doses and from all harvest, individual analyses showed that all pesticides could be statistically differentiated from control samples (Table 4.3).

TABLE 4.3
RESULTS OF THE PLSDA ANALYSIS (2D JRES) OF THE FULL *ARABIDOPSIS THALIANA* DATASET. THE MODEL DISTINGUISHING THE TREATMENT FROM CONTROLS WAS STATISTICALLY VALIDATED BY PERMUTATION TESTING WITH THE FOLLOWING P VALUES AND CLASSIFICATION ERROR RATES (CE).

Treatment	CE	P-value
Metsulfuron-methyl	32%	0.009
Glyphosate	16.5%	<0.05
Epoxiconazole	9%	<0.05
MGE	10%	<0.05

Using PCA, samples from plants treated by high doses of MGE, i.e. samples treated by the pesticide mixture, were found to cluster quite distinctly from other samples along the principal component 1 (PC1, Fig. 4.3). Generally, high doses were found in the right half of PC1 in the PCA. This may be a general indicator of death or damage as opposed to detecting specific metabolic responses to pesticides.

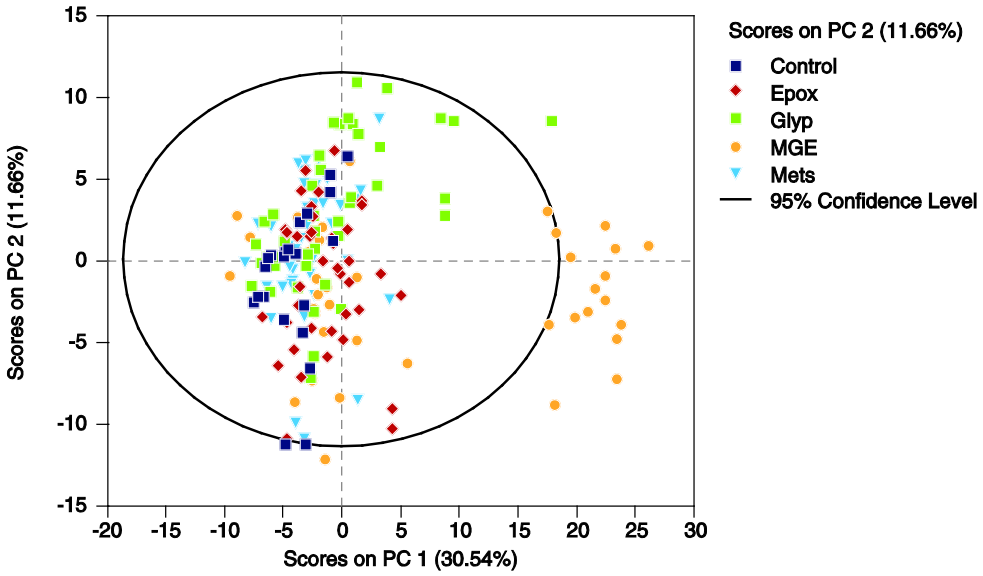

Figure 4.3 Principal component analysis (PCA) of the 2D jres data from all Arabidopsis thaliana samples coloured by pesticide treatment. Numbers in brackets refer to the amount of variantion in the data explained by each of the two first axes. Red triangles: control; blue squares: glyphosate; black stars: epoxiconazole; open diamonds: metsulfuron-methyl; light blue circles: pesticide mix.

When analysis was conducted using the subset of FIMA-identified metabolites slightly less resolution was achieved. In this analysis of the full dataset all pesticides could be distinguished from one another by PLSDA and statistically validated by permutation testing (Figure not shown). Thus, the metabolic profile was able to distinguish among the pesticide treatments. On an individual pesticide level each treatment was contrasted to controls. Here, metsulfuron-methyl treated samples could not be significantly distinguished from controls in a direct comparison while all other pesticides against control comparisons were significant (Tab. 4.3).

TABLE 4.4
RESULTS OF PLSDA ANALYSIS (FIMA) OF THE FULL *ARABIDOPSIS THALIANA* DATASET. THE MODEL DISTINGUISHING THE TREATMENT FROM CONTROLS WAS STATISTICALLY VALIDATED BY PERMUTATION TESTING WITH THE FOLLOWING P VALUES AND CLASSIFICATION ERROR RATES (CE).

Treatment	CE	P-value
Metsulfuron-methyl	-	NS
Glyphosate	29%	0.001
Epoxiconazole	18.3%	<0.05
MGE	24.2%	<0.05

The differences in results obtained by 2D jres and FIMA datasets occur because the FIMA datasets are reduced datasets i.e. only using information on the identified metabolites. Thus, a difference as seen for metsulfuron-methyl can occur if the metabolites in the FIMA dataset are not being significantly important over all sampling times and doses in separating the pesticide treated samples from control. It is worth noting that the classification error rates were quite high for both types of datasets (2D jres and FIMA).

Identified metabolites by FIMA
The FIMA approach allowed the identification of metabolites contributing to the distinction among treatments. A number of metabolites were significantly differently expressed among treatment groups in the full dataset. In total, 24 metabolites were significant after correction for multiple testing (Tab. 4.4). In some cases the regulation (up or down) was similar among pesticides (= general response), however, other cases showed a strong pesticide specific response. The up- or down regulating is expressed by the term 'fold change' which expreses the factor of change compared to untreated control. For example a fold change of 2 shows that the concentration was twice as high as in control.

TABLE 4.5
METABOLITES WITH A SIGNIFICANT CONTRIBUTION TO THE PLSDA MODEL (FIMA) OF ALL *ARABIDOPSIS THALIANA* SAMPLES SEPARATED BY TREATMENT. UNIVARIATE STATISTICAL SIGNIFICANCES AS DETERMINED BY ANOVA HAVE BEEN CORRECTED FOR FALSE DISCOVERY RATES BY USE OF THE BENJAMINI HOCHBERG METHOD. METABOLITES WITHIN THE TOP 50 BY VIP SCORES AND WITH ADJUSTED P VALUES BELOW 10% WERE SELECTED. FC DENOTES 'FOLD CHANGE'. 'INF' DENOTES AN INFINITE FC, I.E. THAT CONTROL LEVEL WAS BELOW DETECTION LIMIT MAKING A DETECTED LEVEL IN A TREATMENT 'INFINITE' HIGHER. A FC OF '0' REPRESENTS THE REVERSE SITUATION, I.E. A METABOLITE LEVEL OF A PESTICIDE TREATED SAMPLE BEING BELOW DETECTION LIMIT WHILE THE METABOLITE WAS DETECTED IN THE CONTROL SAMPLES. 'NA' DENOTES THAT INFORMATION ABOUT THE METABOLITE WAS 'NOT AVAILABLE'. METS: METSULFURON-METHYL, GLYP: GLYPHOSATE, EPOX: EPOXICONAZOLE, MGE: MIX OF THE THREE PESTICIDES.

		FC relative to control samples			
Metabolite	**P value**	**Mets**	**Glyp**	**Epox**	**MGE**
Glycerol	1.6E-04	0.63	0.56	0.28	0.29
Glycine	2.1E-02	1.21	1.42	1.78	2.55
Isoleucine	7.4E-08	0.87	0.93	4.08	3.21
Arginine	6.9E-02	2.69	2.31	3.35	6.92
Leucine	7.4E-08	0.65	1.04	3.15	2.79
Fumaric acid	8.0E-08	0.98	1.01	0.78	0.47
Phenylalanine	2.6E-02	1.49	0.34	10.04	7.31
Maltose	4.9E-03	0.50	0.11	0.13	0.16
Homocystine	4.5E-02	0.96	0.38	0.22	0.24
Phosphocreatine	6.1E-03	0.98	0.35	0.30	0.42
2,3-Butanediol	1.5E-02	2.09	9.05	8.52	14.24
N-Acetylaspartate (NAA)	8.1E-05	0.00	4.94	0.00	30.12
Sucrose-6-phosphate	8.9E-02	6.42	11.80	6.49	8.61
Fucose	8.1E-03	Inf	Inf	Inf	Inf
N-Acetylglucosamine	6.3E-03	NA	Inf	Inf	Inf
b-Alanine	6.3E-03	NA	NA	NA	Inf
Carnitine	5.9E-02	0.75	0.40	0.39	0.70
2-Aminobutyric acid	3.1E-04	6.25	7.17	3.11	37.08
Spermidine	4.5E-02	0.60	0.31	0.40	0.48
Carnosine (beta-alanyl_L-histidine)	8.7E-02	0.78	0.83	0.81	0.64
Fructose	4.7E-04	0.97	1.15	1.39	1.58
Acetic acid	7.4E-02	1.17	0.99	1.22	0.70
Suberic acid	8.0E-02	0.81	2.10	0.67	0.16
Glutamic acid	7.7E-03	0.11	0.00	0.14	0.04

When each pesticide treatment was analyzed separately a number of significant metabolites were found, except for the metsulfuron-methyl treatment which did not show a significant separation from controls. Due to the reduced size of the datasets when making direct comparisons between a single treatment and controls, the number of significant metabolites was strongly affected by correction for multiple testing. There was a relatively large overlap among the metabolites identified from the different treatments. Further, the direction of regulation (up or down) was in all cases the same suggesting a similar response for the shared metabolites (Tab. 4.5 and Fig. 4.4).

TABLE 4.6
METABOLITES WITH A SIGNIFICANT CONTRIBUTION TO THE PLSDA MODEL (FIMA) OF INDIVIDUAL PESTICIDE TREATED SAMPLES OF *ARABIDOPSIS THALIANA* VERSUS CONTROL SAMPLES. METABOLITES THAT ARE STATISTICALLY SIGNIFICANT BY UNIVARIATE STATISTICAL ANALYSIS AS DETERMINED BY STUDENT'S T-TEST BEFORE CORRECTION FOR MULTIPLE TESTING ARE SHOWN. THUS, ALL METABOLITES ARE NOT SIGNIFICANT AFTER CORRECTION FOR MULTIPLE TESTING AND SHOULD BE INTERPRETED WITH CAUTION. P VALUE GIVEN HAS BEEN CORRECTED FOR FALSE DISCOVERY RATES BY USE OF THE BENJAMINI HOCHBERG METHOD. FOLD CHANGES (FC) ARE QUOTED AS MEAN FOLD CHANGE OF PESTICIDE TREATED SAMPLES COMPARED TO CONTROL SAMPLES. 'INF' DENOTES AN INFINITE FC, I.E. THAT CONTROL LEVEL WAS BELOW DETECTION LIMIT MAKING A DETECTED LEVEL IN A TREATMENT 'INFINITE' HIGHER. A FC OF 0 REPRESENTS THE REVERSE SITUATION, I.E. A METABOLITE LEVEL OF A PESTICIDE TREATED SAMPLE BEING BELOW DETECTION LIMIT WHILE THE METABOLITE WAS DETECTED IN THE CONTROL. NA DENOTES THAT INFORMATION ABOUT THE METABOLITE WAS 'NOT AVAILABLE'. GLYP: GLYPHOSATE, EPOX: EPOXICONAZOLEE, MGE: MIX OF THE THREE PESTICIDES (THE TABLE IS SPREAD OVER 3 PAGES).

Treatment: Glyp Metabolite	**P-value**	**FC**
Carnitine	0.04	0.40
Maltose	0.04	0.11
Phosphocreatine	0.04	0.35
Sucrose-6-phosphate	0.05	11.8
Ethanolamine	0.07	0.4
Spermidine	0.07	0.31
Deoxycholic acid	0.07	0.24
Glutamic acid	0.09	0
Glycerol	0.14	0.56
Asparagine	0.27	0.63
Uridine-5-diphosphogalactose	0.34	0.43
Sucrose	0.41	1.72
Pyruvic acid	0.41	0.65
3-Hydroxy-3-methyl butyric acid	0.41	0.08

TABLE 4.6 (CONT.)

Treatment: Epox Metabolite	P-value	FC
Uridine-5-diphosphogalactose	0.01	0.21
Carnitine	0.02	0.39
Phosphocreatine	0.02	0.30
Fructose	0.05	1.39
Maltose	0.06	0.13
Isoleucine	0.1	4.08
Glucose-1-phosphate	0.13	0.09
3-Methylhistidine	0.13	0.23
3-aminoisobutyric acid	0.13	2.55
Glycolic acid	0.13	0.12
Leucine	0.13	3.15
2,3-Butanediol	0.13	8.52
Homocystine	0.16	0.22
Spermidine	0.16	0.4
Methyl isobutyric acid	0.16	0.15
Sucrose-6-phosphate	0.18	6.49
Isocitric acid	0.18	0
Succinic acid	0.23	2.02
Glutamic acid	0.23	0.14
N-Acetylglucosamine	0.23	Inf
Carnosine (beta-alanyl_L-histidine)	0.25	0.81

TABLE 4.6 (CONT.)

Treatment: MGE Metabolite	P-value	FC
Fumaric acid	1.1E-04	0.47
Glycerol	3.8E-04	0.29
Isoleucine	7.9E-04	3.21
Leucine	7.9E-04	2.79
Carnosine (beta-alanyl_L-histidine)	2.0E-02	0.64
Maltose	5.0E-02	0.16
Pyruvic acid	5.4E-02	0.53
2,3-Butanediol	5.4E-02	14.24
Uridine 5-triphosphate	5.7E-02	0.21
Glutamic acid	7.3E-02	0.04
Ethanolamine	7.9E-02	0.45
Sucrose-6-phosphate	8.6E-02	8.61
Glucose-1-phosphate	9.6E-02	0.11
Fucose	9.6E-02	Inf
Glycine	1.0E-01	2.55
2-Aminobutyric acid	1.0E-01	37.08
Arginine	1.0E-01	6.92
Glucose	1.0E-01	2.17
N-Acetylaspartate (NAA)	1.0E-01	30.12
Homocystine	1.0E-01	0.24
Isocitric acid	1.2E-01	0
Glycerol-3-phosphate	1.3E-01	0.57
Homocarnosine	1.3E-01	0.21
Isobutyric acid	1.3E-01	0.29
N-Acetylglucosamine	1.5E-01	Inf
Spermidine	1.6E-01	0.48
Phenylalanine	1.8E-01	7.31

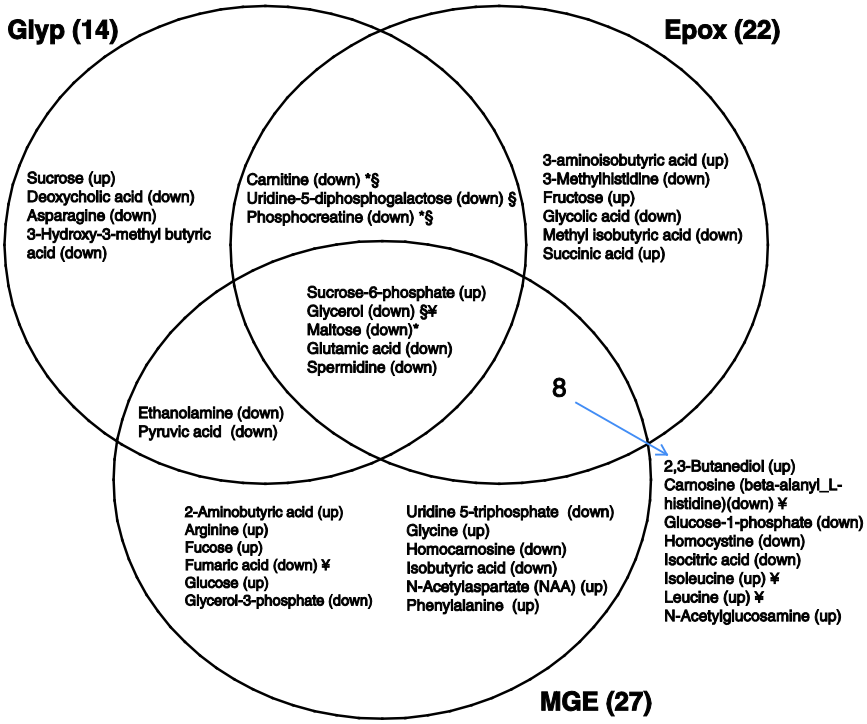

Figure 4.4 Venn diagram showing distribution of metabolites identified in the comparison between individual pesticide treated samples of *Arabidopsis thaliana* and controls and the overlap among treatments. Each circle represents a treatment (Glyp: glyphosate, Epox: epoxiconazole, MGE: mix of the three pesticides) followed by the number of metabolites in brackets. The metabolites significant after correction for multiple testing in each comparison are marked with the following symbols (*: Glyp, Epox: §, MGE: ¥). Metsulfuron-methyl treated samples could not be significantly distinguished from controls in a direct comparison. The direction of regulation in comparison to controls (FC) is indicated by 'up' or 'down' and was in all cases in a similar direction when the same metabolite was identified in several comparisons.

General sampling time and dosage effects

PLSDA analysis was used to investigate the general effects of sampling time and dose further. The following analyzes are done either on sampling time or dose.

Sampling time component

A PLSDA model of all samples (2D jres), separated by time of sampling, was validated by permutation testing (Tab. 4.6). Analysis using individual pesticide data (incorporating all doses) showed that there was a strong and significant sampling time component across doses. Thus, the collected metabolite signal in the data set is structured with regard to sampling time.

TABLE 4.7
RESULTS OF THE PLSDA ANALYSIS (2D JRES) OF INDIVIDUAL PESTICIDE AND CONTROL TREATMENTS WITH ALL DOSES INCLUDED AND SEPARATED ON THE BASIS ON SAMPLING TIME OF *ARABIDOPSIS THALIANA*. THE MODEL DISTINGUISHING AMONG SAMPLING TIMES WAS STATISTICALLY VALIDATED BY PERMUTATION TESTING WITH THE FOLLOWING P VALUES AND CLASSIFICATION ERROR RATES (CE). MGE = MIXTURE OF METSULFURON-METHYL, GLYPHOSATE AND EPOXICONAZOLE.

	Metsulfuron-methyl		Glyphosate		Epoxiconazole		MGE		Control	
Sampling time	**CE**	**P-value**	**CE**	**P-value**	**CE**	**P-value**	**CE**	**P-value**	**CE**	**P-value**
1 Week	0.21	<0.05	0.05	<0.05	0.03	<0.05	0.08	<0.05	0.26	0.014
2 Weeks	0.24	0.001	0.13	<0.05	0.15	<0.05	0.29	0.016	0.29	0.042
3 Weeks	0.19	0.002	0.08	<0.05	0.07	<0.05	0.11	<0.05	0.23	0.013

When the FIMA dataset was analyzed less resolution was achieved. The full dataset still showed significant separation with respect to sampling time and control samples alone could also be separated by PLSDA based on time of sampling but were significant for 3 Weeks only (Tab. 4.7).

TABLE 4.8
TABLE SHOWING RESULT OF PLSDA ANALYSIS OF THE FULL *ARABIDOPSIS THALIANA* DATASET AND CONTROL SAMPLES ONLY (FIMA). THE MODEL DISTINGUISHING AMONG SAMPLING TIMES WAS STATISTICALLY VALIDATED BY PERMUTATION TESTING WITH THE FOLLOWING P VALUES AND CLASSIFICATION ERROR RATES (CE).

	Full dataset		Control samples	
Sampling time	**CE**	**P-value**	**CE**	**P-value**
1 week	24%	<0.05	38%	NS
2 weeks	31%	<0.05	34%	NS
3 weeks	27%	<0.05	13%	0.002

Analysis of individual pesticide treatments also showed the sampling time signal, which in most cases was significant suggesting that metabolites might be used to separate the time course after treatments. The dataset was also analyzed with respect to individual pesticide treatments based on separation by time of sampling by PLSDA models (Tab. 4.8). Differentiation was in most cases significant.

TABLE 4.9
TABLE SHOWING RESULT OF PLSDA ANALYSIS OF THE INDIVIDUAL PESTICIDE TREATMENTS OF ARABIDOPSIS SAMPLES (FIMA). THE MODEL DISTINGUISHING AMONG SAMPLING TIMES WAS STATISTICALLY VALIDATED BY PERMUTATION TESTING WITH THE FOLLOWING P VALUES AND CLASSIFICATION ERROR RATES (CE). MGE = MIXTURE OF METSULFURON-METHYL, GLYPHOSATE AND EPOXICONAZOLE.

	Metsulfuron-methyl		Glyphosate		Epoxiconazole		MGE	
Sampling time	**CE**	**P-value**	**CE**	**P-value**	**CE**	**P-value**	**CE**	**P-value**
1 Week	16%	<0.05	29%	0.01	12%	<0.05	15%	<0.05
2 Weeks	39%	0.103	21%	<0.05	28%	0.009	32%	0.032
3 Weeks	32%	0.036	50%	0.241	14%	<0.05	22%	0.002

Thus, there is an effect of sampling time (plant growth and development) seen in the control group but this effect is most apparent when the pesticide treated groups are also included in the analysis. The FIMA analysis allowed looking for metabolites contributing to the separation. In the controls a small number of metabolites correlated with the sampling times, however, none significantly after correction for multiple testing. When including the pesticide treated samples a larger number of metabolites (19) were significant. Thus, there was a relatively strong signal in the metabolite profile of sampling time suggesting a common change in the metabolite signal with sampling time within the treated plants that was not evident in the control plants alone (Tab. 4.9).

Of the 19 metabolites, fifteen metabolites were up-regulated at '2 Weeks', i.e. 2 weeks after exposure, compared to '1 Week', while only 4 were down-regulated suggesting that the signal did not mainly consist of a generally decreased metabolic signal from dying plants. Furthermore, the intensity of the change in individual metabolites identified to change with sampling time varied, but the majority of metabolites showed a larger fold change deviation at '2 Weeks' than at '3 Weeks' compared to '1 Week'.

TABLE 4.10
METABOLITES WITH A SIGNIFICANT CONTRIBUTION TO THE PLSDA MODEL (FIMA) OF ALL *ARABIDOPSIS THALIANA* SAMPLES (PESTICIDE TREATED AND CONTROLS) SEPARATED BY SAMPLING TIME. UNIVARIATE STATISTICAL SIGNIFICANCES AS DETERMINED BY ANOVA HAVE BEEN CORRECTED FOR FALSE DISCOVERY RATES BY USE OF THE BENJAMINI HOCHBERG METHOD. FOLD CHANGES (FC) ARE QUOTED AS MEAN FOLD CHANGE OF SAMPLES COMPARED TO SAMPLES HARVESTED AT WEEK 1. METABOLITES WITHIN THE TOP 50 BY VIP SCORES AND WITH ADJUSTED P VALUES BELOW 5% WERE SELECTED. 'INF' DENOTES AN INFINITE FC, I.E. THAT CONTROL LEVEL WAS BELOW DETECTION LIMIT MAKING A DETECTED LEVEL IN A TREATMENT 'INFINITE' HIGHER. A FC OF 0 REPRESENTS THE REVERSE SITUATION, I.E. A METABOLITE LEVEL OF A PESTICIDE TREATED SAMPLE BEING BELOW DETECTION LIMIT WHILE THE METABOLITE WAS DETECTED IN THE CONTROL. NA DENOTES THAT INFORMATION ABOUT THE METABOLITE WAS 'NOT AVAILABLE'.

Metabolite	P value	FC Week 2	FC Week 3
Ethanolamine	1.1E-02	2.7	1.8
Succinic acid	1.2E-03	2.8	1.4
Glycine	2.3E-03	0.4	0.9
Homovanillic acid	1.2E-02	1.9	1.0
2-Aminobutyric acid	4.3E-02	0.3	0.3
Carnitine	2.3E-03	1.7	2.9
N-acetylglucosamine-1-phosphate	2.3E-03	22.5	4.2
Glutathione (oxidised)	5.9E-03	3.7	11.0
Carnosine (beta-alanyl_L-histidine)	5.9E-03	1.2	1.5
Leucine	7.5E-03	2.9	1.5
Glutamic acid	1.2E-02	Inf	NA
N-Acetyllysine	3.2E-02	0.6	1.1
Fructose	9.8E-03	1.1	1.4
2-methylbutyric acid	2.0E-02	5.4	1.9
Glycolic acid	4.9E-02	7.3	0.4
Uridine	6.4E-03	1.6	1.1
Isoleucine	6.5E-03	3.0	1.2

Dose component

A PLSDA model of all *A. thaliana* samples (2D jres), separated by treatment dose, could be validated by permutation testing (Tab. 4.10). Analysis using individual pesticide data (incorporating all sampling times) showed that there was a significant dose component when using all sampling times. The dose component was in many cases significant, however, less commonly so for low doses of the pesticide treatments. Thus, the collected metabolite signal in the data set is structured with regard to dose.

TABLE 4.11
TABLE SHOWING RESULT OF PLSDA ANALYSIS (2D JRES) OF THE INDIVIDUAL PESTICIDE TREATMENTS OF *ARABIDOPSIS THALIANA* SAMPLES. THE MODEL DISTINGUISHING AMONG DOSES APPLIED WAS STATISTICALLY VALIDATED BY PERMUTATION TESTING WITH THE FOLLOWING P VALUES AND CLASSIFICATION ERROR RATES (CE). NOTE THAT DOSE 0 REPRESENT CONTROL. MGE = MIXTURE OF METSULFURON-METHYL, GLYPHOSATE AND EPOXICONAZOLE.

	Metsulfuron-methyl		**Glyphosate**		**Epoxiconazole**		**MGE**	
Dose	**CE**	**P-value**	**CE**	**P- value**	**CE**	**P- value**	**CE**	**P- value**
0	0.40	NS	0.23	<0.05	0.13	<0.05	0.13	<0.001
1%	0.49	NS	0.41	NS	0.54	NS	0.25	0.007
5%	0.41	NS	0.41	NS	0.29	0.006	0.20	<0.001
25%	0.39	NS	0.32	<0.05	0.35	<0.05	0.02	<0.001
100%	0.05	<0.05	0.13	0.002	0.28	<0.05	0.15	0.01

Thus, the collected metabolite signal in the data set is structured with regard to both sampling time and dose. However, no single metabolites can be assigned to explain these changes in deeper detail in the present dataset.

4.1.4 Response pattern NMR versus phytotoxic symptoms

For glyphosate and MGE the results of the NMR screening corresponded relatively well with the responses on biomass found in the dose-response experiment. These treatments had large effects on biomass and they affected the metabolites in the plants as well. It is interesting that plants treated with epoxiconazole could be distinguished from untreated control plants by NMR although the effect on biomass of this fungicide was very low. On the other hand the metsulfuron-methyl treatments could not be distinguished from untreated controls although having higher effects on biomass than epoxiconazole. The visual scores showed clear symtoms for highest dose of glyphosate and MGE two weeks after application while a three weeks period was required for significant responses by NMR indicating a faster visual symptom compared to metabolic response.

4.2 Agrostis capillaris

4.2.1 Dose-response experiments

A. capillaris was less susceptible to metsulfuron-methyl and glyphosate than *A. thaliana* and therefore the doses applied to *A. capillaris* are much higher.

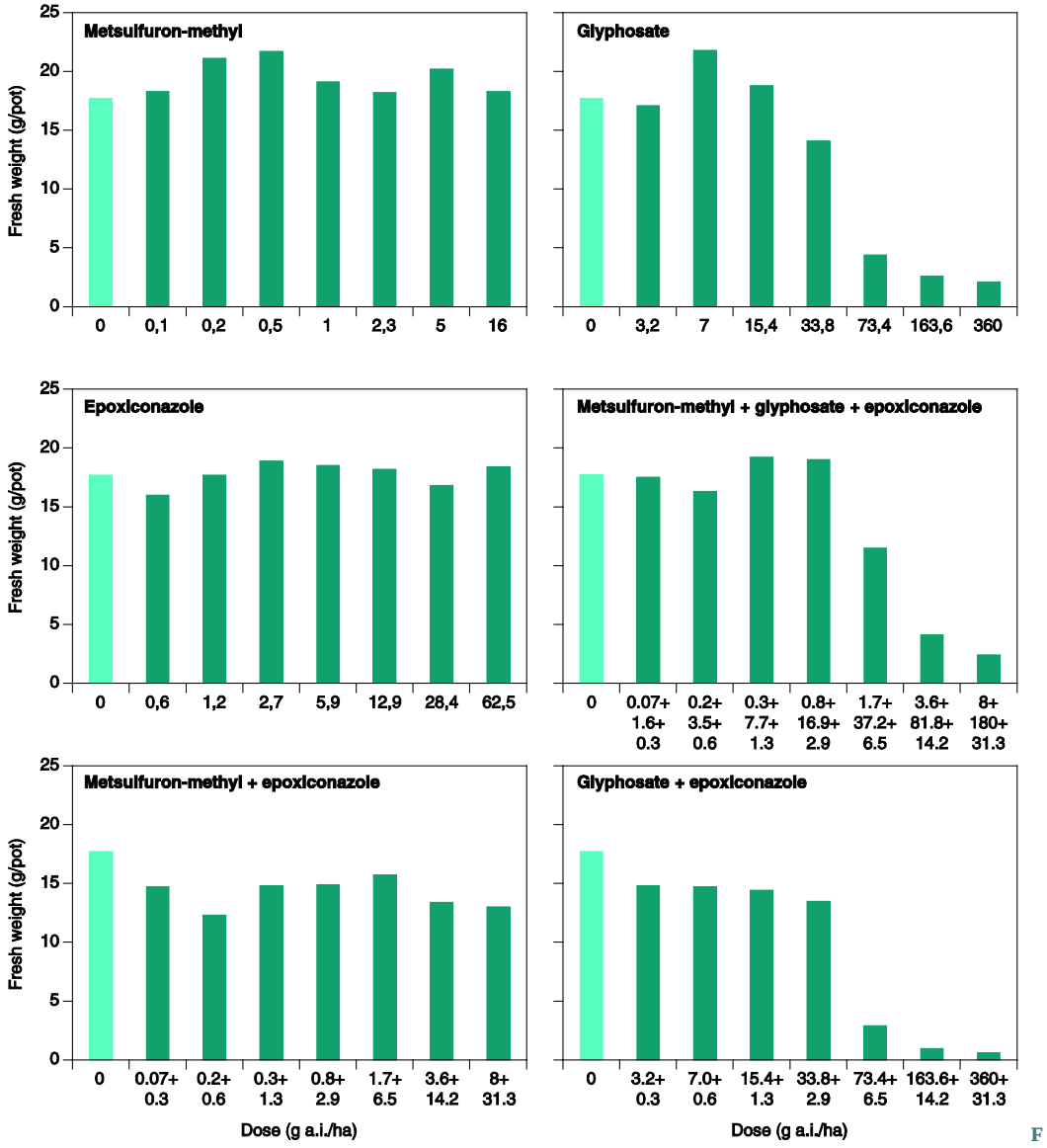

Figure 4.5 The effect of dose ranges of metsulfuron-methyl, glyphosate, epoxiconazole and mixtures of epoxiconazole and metsulfuron-methyl and/or glyphosate on biomass (g fresh weight/pot) of A. capillaris in the dose-response experiments described in section 3.2. Mean of 3 replicates. All pesticides were applied as commercial products.

The maximum dose of metsulfuron-methyl was 16 g a.i./ha which is 3 to 4 times the recommended field dose in winter cereals. Despite the high dose, metsulfuron-methyl had no effect on *A. capillaris* (Fig. 4.5). Tank mix with epoxiconazole did not improve the effect of metsulfuron-methyl, and epoxiconazole applied in normal field rate also had no effect on *A. capillaris* (Fig. 4.5). The applied doses of glyphosate, glyphosate in mixture with epoxiconazole as well as the three component mixture of metsulfuron-methyl, glyphosate and epoxiconazole (MGE) produced responses that allowed fitting the dose-response model (section 3.3) to data. The ED_{10}, ED_{50} and ED_{90} doses were estimated and in contrast to the responses on *A. thaliana* there was no indication that epoxiconazole improved the activity of metsulfuron-methyl and glyphosate on *A. capillaris* (Table 4.11).

TABLE 4.12
ESTIMATED ED_{10}, ED_{50} AND ED_{90} DOSES (G A.I./HA) OF DIFFERENT PESTICIDES ON BIOMASS OF *A. CAPILLARIS* USING THE DOSE-RESPONSE MODEL DESCRIBED IN SECTION 3.3. FIGURES IN PARENTHESES ARE 95% CONFIDENCE INTERVALS. THE ED DOSES OF METSULFURON-METHYL, EPOXICONAZOLE AND METSULFURON-METHYL + EPOXICONAZOLE WERE HIGHER THAN THE MAXIMUM DOSE APPLIED. GLYPHOSATE WAS THE ONLY COMPONENT HAVING AN EFFECT ON THE BIOMASS OF *A. CAPILLARIS* AND THEREFORE THE DOSES OF MGE AND GLYPHOSATE + EPOXICONAZOLE ARE SHOWN AS G A.I./HA OF GLYPHOSATE. MGE = METSULFURON-METHYL + GLYPHOSATE + EPOXICONAZOLE.

Pesticide	ED10 (g a.i./ha)	ED50 (g a.i./ha)	ED90 (g a.i./ha)
Metsulfuron-methyl	>16	>16	>16
Glyphosate	26.2 (16.8-35.2)	51.3 (32.9-69.6)	238.3 (159.3-317.3)
Epoxiconazole	>62.5	>62.5	>62.5
MGE	28.4 (19.8-37.6)*	49.3 (33.6-64.9)*	172.9 (116.1-229.6)*
Metsulfuron-mehyl + epoxiconazole	>8	>8	>8
Glyphosate + epoxiconazole	18.6 (12.8-24.1)*	33.4 (22.9-43.9)*	128.0 (94.1-161.9)*

* in g a.i./ha of glyphosate in the mixture (3.6%: 82.1%: 14.3%)

4.2.2 Exposure experiments - plants for NMR analysis

The maximum doses of metsulfuron and epoxiconazole applied in the exposure experiments were 8 and 62.5 g a.i./ha respectively (Table 3.2). These doses were lower than the ED_{10} doses and consequently no effects were expected on the plants. This was in accordance with the visual assessments showing no symptoms on the plants up to 21 days after spraying (Table 4.12). According to the dose-response experiment the 25% N dose of glyphosate should reduce biomass of *A. capillaris* by more than 50% and the 100% dose should provide more than 90% effect. The visual assessments showed that plant injury was visible in these doses 7 days after treatment and the final effect was approximately 70 and 90% growth reduction. According to the dose-response experiment the effects of glyphosate and metsulfuron-methyl + glyphosate + epoxiconazole were similar at equivalent doses of glyphosate (Table 4.12) and the scores in the visual assessments of the two treatments were also similar.

TABLE 4.13
VISUAL ASSESSMENT OF *A. CAPILLARIS* INJURY AT DIFFERENT HARVEST TIMES. MGE = METSULFURON-METHYL + GLYPHOSATE + EPOXICONAZOLE. FOR RATINGS SEE TABLE 3.3. DOSES ARE LISTED IN TABLE 3.2.

	Metsulfuron-methyl	**Glyphosate**	**Epoxiconazole**	**MGE**
7 DAT				
1 % N	0	0	0	0
5 % N	0	0	0	0
25 % N	0	2*	0	2*
100 % N	0	2*	0	3*
14 DAT				
1 % N	0	0	0	0
5 % N	0	0	0	0
25 % N	0	3*	0	3*
100 % N	0	4*	0	4*
21 DAT				
1 % N	0	0	0	0
5 % N	0	0	0	0
25 % N	0	7**	0	7**
100 % N	0	9**	0	9**

*Yellowing of youngest leaves
**Desiccation

4.2.3 NMR results

The data on *Agrostis capillaris* was analyzed in the same way as the Arabidopsis results (see 4.1.3). Generally, this species showed a much less clear response to pesticides on the metabolomic level, identified as less clear separation among treatments. Using PCA, samples from plants treated by high doses of glyphosate and MGE, i.e. samples treated by the pesticide mixture, were found to cluster quite distinctly from other samples along principal component 1 (PC1, Fig 4.6).

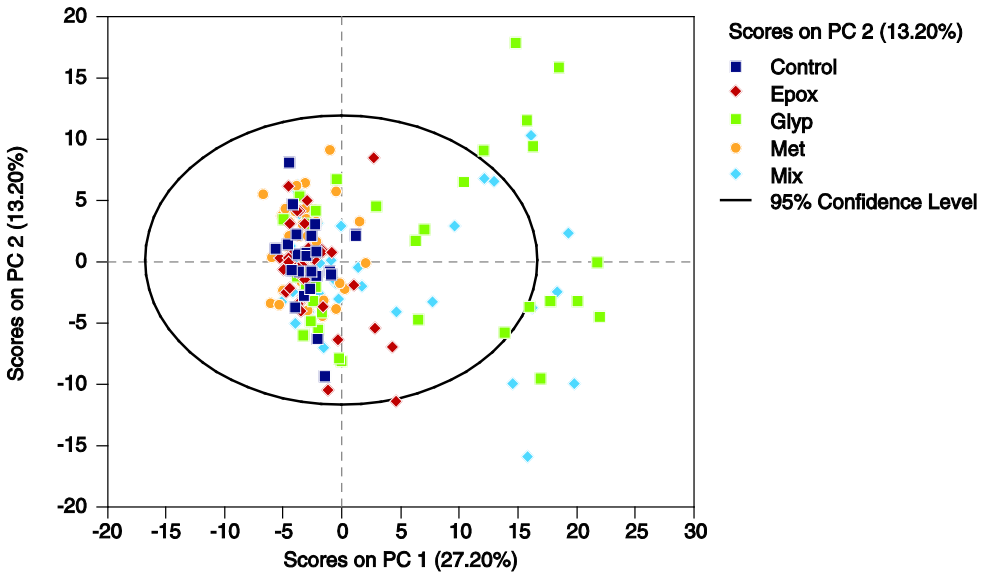

Figure 4.6 Principal component analysis (PCA) of the 2D jres data from all Agrostis capillaris samples coloured by pesticide. Numbers in brackets refer to the amount of variantion in the data explained by each of the two first axes. Red triangles: control; blue squares: glyphosate; black stars: epoxiconazole; open diamonds: metsulfuron—methyl; light blue circles: pesticide mix.

In the 2D jres analysis of the full metabolomics signal from the data set, including all the metabolites, only metsulfuron-methyl, glyphosate and controls were reliably classified and with relatively high classification errors (Tab. 4.13).

TABLE 4.14
RESULTS OF PLSDA ANALYSIS (2D JRES) OF THE FULL *AGROSTIS CAPILLARIS* DATASET. THE MODEL SEPARATING ALL TREATMENT WAS STATISTICALLY VALIDATED BY PERMUTATION TESTING WITH THE FOLLOWING P VALUES AND CLASSIFICATION ERROR RATES (CE).

Treatment	CE	P-value
Metsulfuron-methyl	33%	<0.05
Glyphosate	29%	<0.05
Epoxiconazole	41%	NS
MGE	44%	NS
Control	40%	0.049

Including only identified metabolites (the FIMA analysis) in the model led to a minor improvement in the model resulting in all treatments being significantly separated with the exception of epoxiconazole which remained non-significant (Tab. 4.14). The improved result may be a result of reducing the noise in the model, however, classification error rate remained high suggesting that the separation among treatments was relatively poor.

TABLE 4.15
RESULTS OF PLSDA ANALYSIS OF THE FULL *AGROSTIS CAPILLARIS* DATASET (FIMA). THE MODEL SEPARATING ALL TREATMENT WAS STATISTICALLY VALIDATED BY PERMUTATION TESTING WITH THE FOLLOWING P VALUES AND CLASSIFICATION ERROR RATES (CE).

Treatment	CE	P-value
Control	39%	0.03
Metsulfuron-methyl	38%	0.008
Glyphosate	35%	0.004
Epoxiconazole	44%	NS
MGE	41%	0.04

Still, as the model on the full FIMA data was significant some metabolites (18) could be identified as significantly contributing to the model separating among treatments (Tab. 4.15).

TABLE 4.16
METABOLITES WITH A SIGNIFICANT CONTRIBUTION TO THE PLSDA MODEL (FIMA) OF ALL *AGROSTIS CAPILLARIS* SAMPLES SEPARATED BY TREATMENT. UNIVARIATE STATISTICAL SIGNIFICANCES AS DETERMINED BY ANOVA HAVE BEEN CORRECTED FOR FALSE DISCOVERY RATES BY USE OF THE BENJAMINI HOCHBERG METHOD. METABOLITES WITHIN THE TOP 50 BY VIP SCORES AND WITH ADJUSTED P VALUES BELOW 10% WERE SELECTED. FC DENOTES 'FOLD CHANGE'. 'INF' DENOTES AN INFINITE FC, I.E. THAT CONTROL LEVEL WAS BELOW DETECTION LIMIT MAKING A DETECTED LEVEL IN A TREATMENT 'INFINITE' HIGHER. A FC OF 0 REPRESENTS THE REVERSE SITUATION, I.E. A METABOLITE LEVEL OF A PESTICIDE TREATED SAMPLE BEING BELOW DETECTION LIMIT WHILE THE METABOLITE WAS DETECTED IN THE CONTROL. NA DENOTES THAT INFORMATION ABOUT THE METABOLITE WAS 'NOT AVAILABLE'. METS: METSULFURON-METHYL, GLYP: GLYPHOSATE, EPOX: EPOXICONAZOLE, MGE: MIX OF THE THREE PESTICIDES.

			FC relative to control samples			
Metabolite	**VIP Score**	**P-value**	**Met**	**Glyp**	**Epox**	**MGE**
Proline	20.10	8.3E-04	0.97	35.76	0.59	15.17
Urocanic acid	12.70	1.9E-03	0.67	3.02	0.28	1.14
Glutamic acid	10.90	6.1E-02	0.81	0.60	1.11	0.61
Tyrosine	10.20	6.2E-02	0.49	1.91	0.46	0.98
N-Acetyllysine	10.10	1.7E-05	0.79	5.56	0.80	3.16
trans-Aconitic acid	8.77	7.7E-06	1.42	0.76	1.12	0.74
N-acetylglucosamine-1-phosphate	8.72	1.9E-02	0.82	0.49	0.98	0.77
Suberic acid	8.06	4.6E-03	0.88	0.56	0.94	0.66
Succinic acid	6.59	6.2E-03	0.77	0.47	0.74	0.68
Methionine	6.29	4.5E-02	0.18	1.53	0.20	1.42
5-Aminolevulinic acid	4.41	1.9E-02	1.49	2.88	1.82	3.30
Formic acid	3.51	8.3E-04	0.96	0.61	0.97	0.72
N-Acetylglutamic acid	2.40	1.0E-02	1.00	6.72	1.22	2.17
Methylamine	2.40	2.0E-02	0.74	2.65	0.60	2.86
myo-Inositol	2.28	5.7E-03	1.17	0.86	0.96	0.74
Uridine-5-diphospho-N-acetylglucosamine	1.82	6.2E-03	6.31	49.56	1.15	12.40
Uridine	1.19	4.7E-03	1.09	0.73	0.92	0.89
Acetic acid	1.12	2.3E-02	0.66	0.49	0.70	0.67

The only significant individual comparison pesticide vs. control was for glyphosate. PLSDA analysis on the FIMA dataset identified a number of metabolites contributing significantly to the separation among pesticide treated and control samples (Tab. 4.16).

TABLE 4.17
METABOLITES WITH A SIGNIFICANT CONTRIBUTION TO THE PLSDA MODEL (FIMA) OF INDIVIDUAL PESTICIDE TREATED SAMPLES (GLYPHOSATE) VS CONTROL SAMPLES FOR *AGROSTIS CAPILLARIS*. UNIVARIATE STATISTICAL SIGNIFICANCES AS DETERMINED BY ANOVA HAVE BEEN CORRECTED FOR FALSE DISCOVERY RATES BY USE OF THE BENJAMINI HOCHBERG METHOD. METABOLITES WITHIN THE TOP 50 BY VIP SCORES AND WITH ADJUSTED P VALUES BELOW 5% WERE SELECTED. FOLD CHANGES (FC) ARE QUOTED AS MEAN FOLD CHANGE OF PESTICIDE TREATED SAMPLES COMPARED TO CONTROL SAMPLES. 'INF' DENOTES AN INFINITE FC, I.E. THAT CONTROL LEVEL WAS BELOW DETECTION LIMIT MAKING A DETECTED LEVEL IN A TREATMENT 'INFINITE' HIGHER. A FC OF 0 REPRESENTS THE REVERSE SITUATION, I.E. A METABOLITE LEVEL OF A PESTICIDE TREATED SAMPLE BEING BELOW DETECTION LIMIT WHILE THE METABOLITE WAS DETECTED IN THE CONTROL.

Metabolite	VIP Scores	P-value	FC
Succinic acid	6.1	0.01	0.47
Formic acid	2.6	0.01	0.61
N-acetylglucosamine-1-phosphate	11.5	0.02	0.49
Suberic acid	4.6	0.03	0.56
Acetic acid	1.4	0.03	0.49

High doses (25 and 100%) in contrast to controls

Reducing the dataset to only the higher (25 and 100%) doses increased separation among treatments, so that all treatments, except epoxiconazole treated, could successfully be classified differently from controls by PLSDA analysis of the 2D jres data. The classification error rates were also reduced in this analysis (Tab. 4.17).

TABLE 4.18
RESULTS OF PLSDA ANALYSIS OF THE HIGH DOSIS (25% AND 100%) CONTRASTED TO CONTROL SAMPLES OF THE *AGROSTIS CAPILLARIS* DATASET (2D JRES). THE MODEL SEPARATING ALL TREATMENT WAS STATISTICALLY VALIDATED BY PERMUTATION TESTING WITH THE FOLLOWING P VALUES AND CLASSIFICATION ERROR RATES (CE).

Treatment	CE	P-value
Metsulfuron-methyl	29%	0.012
Glyphosate	<1%	<0.05
Epoxiconazole	-	NS
MGE	4.6%	<0.05

The corresponding analysis of the *Agrostis capillaris* FIMA dataset (control and 25% and 100% doses only) showed also improved seperation and only epoxiconazole treated non-significant similarly to the 2D jres analysis (Tab. 4.18).

TABLE 4.19
RESULTS OF PLSDA ANALYSIS OF THE HIGH DOSIS (25% AND 100%) CONTRASTED TO CONTROL SAMPLES OF THE AGROSTIS DATASET (FIMA). THE MODEL SEPARATING ALL TREATMENT WAS STATISTICALLY VALIDATED BY PERMUTATION TESTING WITH THE FOLLOWING P VALUES AND CLASSIFICATION ERROR RATES (CE).

Treatment	CE	P-value
Metsulfuron-methyl	33.4%	0.033
Glyphosate	0.06%	<0.05
Epoxiconazole	-	NS
MGE	10.0%	<0.05

In this analysis of the FIMA data the contrast between controls and high doses identified a number of significantly contributing metabolites (table 4.19).

TABLE 4.20
METABOLITES WITH A SIGNIFICANT CONTRIBUTION TO THE PLSDA MODEL (FIMA) OF CONTROL AND HIGH DOSES (25% AND 100%) *AGROSTIS CAPILLARIS* SAMPLES SEPARATED BY TREATMENT. UNIVARIATE STATISTICAL SIGNIFICANCES AS DETERMINED BY ANOVA HAVE BEEN CORRECTED FOR FALSE DISCOVERY RATES BY USE OF THE BENJAMINI HOCHBERG METHOD. METABOLITES WITHIN THE TOP 50 BY VIP SCORES AND WITH ADJUSTED P VALUES BELOW 5% WERE SELECTED. FC DENOTES 'FOLD CHANGE'. 'INF' DENOTES AN INFINITE FC, I.E. THAT CONTROL LEVEL WAS BELOW DETECTION LIMIT MAKING A DETECTED LEVEL IN A TREATMENT 'INFINITE' HIGHER. A FC OF 0 REPRESENTS THE REVERSE SITUATION, I.E. A METABOLITE LEVEL OF A PESTICIDE TREATED SAMPLE BEING BELOW DETECTION LIMIT WHILE THE METABOLITE WAS DETECTED IN THE CONTROL. NA DENOTES THAT INFORMATION ABOUT THE METABOLITE WAS 'NOT AVAILABLE'. METS: METSULFURON-METHYL, GLYP: GLYPHOSATE, EPOX: EPOXICONAZOLE, MGE: MIX OF THE THREE PESTICIDES (CONT. NEXT PAGE).

			FC relative to control samples			
Metabolite	**VIP Score**	**P-value**	**Met**	**Glyp**	**Epox**	**MGE**
Proline	26.1	6.3E-07	0.83	82.03	1.39	37.24
Glutamic acid	24.4	4.4E-06	0.88	0.00	0.98	0.09
N-acetylglucosamine-1-phosphate	11.6	1.8E-07	0.66	0.00	1.02	0.33
Isoleucine	10.6	4.5E-04	0.44	4.71	1.52	3.67
N-Acetyllysine	9.8	2.6E-12	0.61	11.69	0.92	6.67
Suberic acid	9.6	1.4E-09	1.01	0.22	0.91	0.17
trans-Aconitic acid	7.6	3.8E-14	1.70	0.27	1.14	0.30
Methionine	6.6	1.1E-02	0.25	3.01	0.21	2.37
Succinic acid	5.8	2.1E-04	0.80	0.30	0.74	0.29
Carnitine	4.3	2.0E-02	0.63	9.10	0.00	6.38
Formic acid	4.1	5.9E-10	1.04	0.28	1.00	0.36
N-Acetylglutamic acid	3.3	2.1E-04	0.61	14.18	0.90	3.71

TABLE 4.20 (CONT.)

			FC relative to control samples			
Metabolite	**VIP Score**	**P value**	**Met**	**Glyp**	**Epox**	**MGE**
5-Aminolevulinic acid	2.7	5.7E-06	1.36	3.80	1.32	5.85
trans-Cinnamic acid	2.2	2.8E-02	0.00	7.47	1.32	5.15
Maleic acid	1.3	4.0E-02	0.00	5.66	0.71	16.66
Tryptophan	1.2	3.7E-02	NA	Inf	NA	Inf
Methylamine	1.1	2.9E-02	1.06	4.33	1.08	2.59
Adenosine	1.1	1.2E-02	1.40	0.24	0.37	0.23
myo-Inositol	1.0	3.7E-02	1.18	0.80	0.98	0.58

When each pesticide treatment was analyzed separately, a number of significant metabolites were found for glyphosate and the pesticide mix (MGE). After correction for multiple testing, 18 and 11 remained significant for the glyphosate and MGE tretments, respectively (Tab. 4.20). There was a large overlap among the metabolites identified from the different treatments, thus, 10 of the 11 metabolites found for the MGE treatment was also present among the metabolites found for glyphosate. Further, the direction of regulation was in all 10 cases in the same direction suggesting a similar response for the shared metabolites (Tab. 4.20).

TABLE 4.21
METABOLITES WITH A SIGNIFICANT CONTRIBUTION TO THE PLSDA MODEL (FIMA) OF INDIVIDUAL *AGROSTOS CAPILLARIS* PESTICIDE TREATED SAMPLES VERSUS CONTROL SAMPLES. UNIVARIATE STATISTICAL SIGNIFICANCES AS DETERMINED BY ANOVA HAVE BEEN CORRECTED FOR FALSE DISCOVERY RATES BY USE OF THE BENJAMINI HOCHBERG METHOD. METABOLITES WITHIN THE TOP 50 BY VIP SCORES AND WITH ADJUSTED P VALUES BELOW 5% WERE SELECTED. FOLD CHANGES (FC) ARE QUOTED AS MEAN FOLD CHANGE OF PESTICIDE TREATED SAMPLES COMPARED TO CONTROL SAMPLES. 'INF' DENOTES AN INFINITE FC, I.E. THAT CONTROL LEVEL WAS BELOW DETECTION LIMIT MAKING A DETECTED LEVEL IN A TREATMENT 'INFINITE' HIGHER. A FC OF 0 REPRESENTS THE REVERSE SITUATION, I.E. A METABOLITE LEVEL OF A PESTICIDE TREATED SAMPLE BEING BELOW DETECTION LIMIT WHILE THE METABOLITE WAS DETECTED IN THE CONTROL (THE TABLE CONTINUES ON THE NEXT PAGE).

Treatment: Glyp Metabolite	**VIP scores**	**P-value**	**FC**
N-acetylglucosamine-1-phosphate	14.0	3.2E-07	0
Formic acid	3.4	6.0E-07	0.28
N-Acetyllysine	6.8	1.9E-06	11.69
trans-Aconitic acid	3.5	5.3E-05	0.27
Glutamic acid	23.2	1.4E-04	0
Suberic acid	7.1	2.2E-04	0.22
Proline	27.5	4.7E-04	82.03
Uridine	1.1	4.0E-03	0.55

TABLE 4.21 (CONT.)

Treatment: Glyp Metabolite	VIP scores	P-value	FC
Methylamine	1.4	5.0E-03	4.33
trans-Cinnamic acid	2.7	5.7E-03	7.47
5-Aminolevulinic acid	2.0	6.0E-03	3.80
Succinic acid	4.8	6.4E-03	0.30
N-Acetylglutamic acid	4.9	8.1E-03	14.18
Isoleucine	8.1	1.3E-02	4.71
Oxoglutaric acid (a-Ketogluturate)	1.0	1.7E-02	0.48
Uridine-5-diphospho-N-acetylglucosamine	1.4	2.1E-02	92.70
Porphobilinogen	2.7	3.5E-02	11.58

Treatment: MGE Metabolite	VIP score	P-value	FC
N-Acetyllysine	7.5	8.2E-05	6.67
Formic acid	4.1	8.4E-05	0.36
Suberic acid	8.2	1.1E-04	0.17
5-Aminolevulinic acid	6.4	2.4E-04	5.85
trans-Aconitic acid	12.6	2.9E-04	0.30
Glutamic acid	24.9	1.3E-03	0.09
N-acetylglucosamine-1-phosphate	8.9	6.8E-03	0.33
Proline	15.4	6.8E-03	37.24
Isoleucine	11.5	6.8E-03	3.67
myo-Inositol	2.6	1.1E-02	0.58
Succinic acid	7.6	1.1E-02	0.29

General sampling time and dosage effects

PLSDA analysis was used to investigate the general effects of sampling time and dose further. The following analyzes are done either on sampling time or dose.

Sampling time component

A PLSDA model of all samples (2D jres), separated by time of sampling, could be validated by permutation testing (Tab. 4.21). Analysis using individual pesticide data (incorporating all doses) showed that there was a strong and significant sampling time component across doses. Thus, the collected metabolite signal in the data set is structured with regard to sampling time.

TABLE 4.22
RESULT OF PLSDA ANALYSIS (2D JRES) OF INDIVIDUAL PESTICIDE AND CONTROL TREATMENTS WITH ALL DOSES INCLUDED AND SEPARATED ON THE BASIS ON SAMPLING TIME OF *AGROSTIS CAPILLARIS*. THE MODEL DISTINGUISHING AMONG SAMPLING TIMES WAS STATISTICALLY VALIDATED BY PERMUTATION TESTING WITH THE FOLLOWING P VALUES AND CLASSIFICATION ERROR RATES (CE). MGE DENOTES MIXTURE OF METSULFURON-METHYL, GLYPHOSATE AND EPOXICONAZOLE.

	Metsulfuron-methyl-		Glyphosate		Epoxiconazole		MGE		Control	
Sampling time	**CE**	**P-value**	**CE**	**P-value**	**CE**	**P-value**	**CE**	**P-value**	**CE**	**P-value**
1 Week	0.04	<0.05	0.07	<0.05	0.16	<0.05	0.09	<0.05	0.18	<0.01
2 Weeks	0.13	<0.05	0.28	<0.05	0.12	<0.05	0.25	<0.01	0.13	<0.05
3 Weeks	0.26	<0.01	0.32	<0.05	0.19	<0.01	0.28	<0.05	0.15	<0.001

Dose component

A PLSDA model of all samples (2D jres), separated by treatment dose, could be validated by permutation testing (Tab. 4.22). Analysis using individual pesticide data (incorporating all sampling time points) showed that there was a significant dose component when using all sampling time points. The dose component was in many cases significant, however, less commonly for low doses of pesticides (only mix at 1%) and not for epoxiconazole at any dose. Thus, the collected metabolite signal in the data set is structured with regard to dose.

TABLE 4.23
RESULT OF PLSDA ANALYSIS OF THE INDIVIDUAL PESTICIDE TREATMENTS OF AGROSTIS CAPILLARIS SAMPLES (2D JRES). THE MODEL DISTINGUISHING AMONG DOSES APPLIED WAS STATISTICALLY VALIDATED BY PERMUTATION TESTING WITH THE FOLLOWING P VALUES AND CLASSIFICATION ERROR RATES (CE). NOTE THAT DOSE 0 REPRESENT CONTROL. MGE DENOTES MIXTURE OF METSULFURON-METHYL, GLYPHOSATE AND EPOXICONAZOLE.

	Metsulfuron-methyl		Glyphosate		Epoxiconazole		MGE	
Dose	**CE**	**P-value**	**CE**	**P-value**	**CE**	**P-value**	**CE**	**P-value**
0	0.38	<0.05	0.26	<0.001	0.44	NS	0.34	<0.01
1%	0.40	NS	0.44	NS	0.55	NS	0.36	<0.05
5%	0.31	<0.05	0.25	<0.01	0.52	NS	0.33	<0.05
25%	0.22	<0.05	0.09	<0.05	0.46	NS	0.21	<0.05
100%	0.43	NS	0.03	<0.05	0.60	NS	0.12	<0.05

Thus, the collected metabolite signal in the data set is structured with regard to both sampling time and doses; however, no single metabolites can be assigned to explain these changes in deeper detail in the present dataset.

4.2.4 Response pattern NMR versus phytotoxic symptoms

In consistence to the very similar responses of glyphosate and MGE on biomass and visual scores the NMR screening also clustered these two treatments together and distinct from epoxiconazole and metsulfuron-methyl which both had low effect on *A. capillaris*. The epoxiconazole treatments did not differ significantly from untreated control in the NMR screening neither did this treatment produce any symptoms on plants or reduction in biomass. Metsulfuron-methyl was reliable classified by NMR screening although having very low effect on plant biomass. In accordance to visual symptoms and biomass the dose component was in many cases significant for high doses by NMR screening.

4.3 Patterns in metabolite composition as response to pesticide exposure

Some patterns in the responses of plant metabolites to pesticide exposure were identified. Depending on the specificity of the responses, they can be described as either general responses (GR), i.e. similar responses, either up- or down, for all pesticides; or specific responses (SR), i.e. different responses to the pesticides (Table 4.23).

TABLE 4.24
SIGNIFICANT CHANGES IN PLANT METABOLITES CONTENT GIVEN AS FOLD CHANGE (FC) BETWEEN EXPOSED SAMPLES AND CONTROLS FOR THE DICOT (**A**) *ARABIDOPSIS THALIANA* AND THE MONOCOT (**B**) *AGROSTIS CAPILLARIS* EXPOSED TO THE HERBICIDES METSULFURON-METHYL (MET), GLYPHOSATE (GLYP), THE FUNGICIDE EPOXYCONACOLE (EPOX) AND A MIX OF THE THREE PESTICIDES (MGE). BLUE COLOUR INDICATES COMPOUNDS THAT RESPONDED SIGNIFICANTLY IN BOTH SPECIES. GR INDICATES GENERAL RESPONCES, EITHER UP- OR DOWN, FOR ALL PESTICIDES. SR INDICATES SPECIFIC RESPONSES, I.E. DIFFERENT RESPONSES TO THE PESTICIDES. SYNERGISTIC RESPONSE (SYN) WAS DEFINED AS WHEN THE PESTICIDE MIXTURE (MGE) RESULTED IN A LARGER RESPONSE THAN EXPECTED BASED ON ADDITION OF THE INDIVIDUAL RESPONSES.

A

Arabidopsis thaliana			Fold change relative to control				Evaluation		
Group of compounds		Potential biomarkers	Met	Glyp	Epox	MGE	GR	SR	Syn
1	Alcohol	'2,3-Butanediol '	20.87	90.51	85.24	142.41	x		x
1	Amino acid	'Glycine '	1.21	1.42	1.78	2.55	x		x
1	Amino acid	'Arginine '	2.69	2.31	3.35	6.92	x		x
1	Amino acid	'2-Aminobutyric acid '	62.49	71.66	31.15	370.77	x		x
1	Amino acid	'Carnosine (beta-alanyl_L-histidine) '	0.78	0.83	0.81	0.64	x		x
1	Amino acid	'Homocystine '	0.96	0.38	0.22	0.24	x		(x)
1	Alcohol	'Glycerol '	0.63	0.56	0.28	0.29	x		(x)
1	Alkaloid	'Carnitine '	0.75	0.40	0.39	0.70	x		(x)
1	Amino acid	'Glutamic acid '	0.11	0	0.14	0.04	x		(x)
2	Amino acids	'Phosphocreatine '	0.98	0.35	0.30	0.42	x		
2	Amine	'Spermidine '	0.60	0.31	0.40	0.48	x		
2	Carbohydrate	'Maltose '	0.50	0.11	0.13	0.16	x		
2	Carbohydrate	'Sucrose-6-phosphate '	64.19	117.98	64.87	86.09	x		
3	Amino acid	'Leucine '	0.65	1.04	31.48	27.89		x	
3	Amino acid	'Isoleucine '	0.87	0.93	4.08	3.21		x	
3	Amino acid	'Phenylalanine '	14.86	0.34	100.39	7.31		x	
3	Amino acid	'N-Acetylaspartate (NAA) '	0	49.35	0	301.21		x	
3	Carbohydrate	'Fructose '	0.97	11.54	13.90	15.80		x	
3	Organic acid	'Fumaric acid '	0.98	10.11	0.78	0.47		x	
3	Organic acid	'Acetic acid '	11.75	0.99	12.17	0.70		x	
3	Organic acid	'Suberic acid '	0.81	21.04	0.67	0.16		x	

B

Agrostis capillaris			Fold change relative to control				Evaluation		
Group of compounds		Putative indicator	Met	Glyp	Epox	MGE	GR	SR	Syn
1	Amino acid	5-Aminolevulinic acid	1.49	2.88	1.82	3.30	x		x
2	Amino acid	N-acetylglucosamine-1-phosphate	0.82	0.49	0.98	0.77	x		
2	Organic acid	Suberic acid	0.88	0.56	0.94	0.66	x		
2	Organic acid	Succinic acid	0.77	0.47	0.74	0.68	x		
2	Organic acid	Formic acid	0.96	0.61	0.97	0.72	x		
2	Organic acid	Acetic acid	0.66	0.49	0.70	0.67	x		
2	Amino acid	N-Acetylglutamic acid	1.00	6.72	1.22	2.17	x		
2	Amino acid	Uridine-5-diphospho-N-acetylglucosamine	6.31	49.56	1.15	12.40	x		
3	Amino acid	Proline	0.97	35.76	0.59	15.17		x	
3	Organic acid	Urocanic acid	0.67	3.02	0.28	1.14		x	
3	Sugar alcohol	myo-Inositol	1.17	0.86	0.96	0.74		x	
3	Amine	Uridine	1.09	0.73	0.92	0.89		x	
3	Amino acid	Glutamic acid	0.81	0.60	1.11	0.61		x	
3	Amino acid	Tyrosine	0.49	1.91	0.46	0.98		x	
3	Amino acid	N-Acetyllysine	0.79	5.56	0.80	3.16		x	
3	Organic acid	trans-Aconitic acid	1.42	0.76	1.12	0.74		x	
3	Amino acid	Methionine	0.18	1.53	0.20	1.42		x	
3	Amine	Methylamine	0.74	2.65	0.60	2.86		x	

All the plant metabolites for which a significant difference between exposed and control plants were found (Tab. 4.23) belonged to the following groups: amino acids, carbohydrates, organic acids, amines, sugar alcohols and alkaloids. All compounds are relatively small with low molecular weight and only few belong to compounds often grouped as secondary compounds such as alkaloids (Vickery and Vickery, 1981).

Three response patterns were identified (Tab. 4.23A and B) for both plant species. Group 1: Similar responses to the three pesticides and the mixture and synergistic response to the mixture; Group 2: Similar responses to the three pesticides and the mixture; and Group 3: Specific responses to the three pesticides and the mixture.

In general, the fold changes for the selected plant metabolites were much lower for *Agrostis capillaris* than for *Arabidopsis thaliana*.

Only three compounds among the identified plant metabolites, marked with blue in Table 4.23, showed significant differences between exposed and control plants in both plant species. These include two organic acids (acetic acid and suberic acid) and one amino acid (glutamic acid). In *A. thaliana* glutamic acid was detected as a general response, while in *A. capillaris* this compound showed a specific response. The two amino acids showed general responses in *A. capillaris* while they were detected as specific responses for *A. thaliana*. Except for *A. capillaris* exposed to epoxiconazole, glutamic acid was found in smaller quantities in exposed compared to control plants in the two plant species. Generally, the differences in glutamic acid content between control and exposed plants were larger for *A. thaliana* than for *A. capillaris*. For the organic acid, acetic acid, *A. thaliana* responded with a 12-fold increase in content compared to controls when exposed to metsulfuron-methyl and to epoxiconazole while there was no effect of glyphosate on the acetic acid content and a reduced content when exposed to the mixture. In *A. capillaris*, exposure both to the three herbicides individually and to the mixture resulted in reduced contents, two-third to half the

concentration found in control plants. Finally, the content of suberic acid was decreased except for *A. thaliana* exposed to glyphosate. Here, a 21-fold increase in the suberic acid content was found compared to control.

5. General discussion

The main objectives of the present study were to examine the changes in plant metabolites in two non-target plants following exposure to four different pesticide treatments and to evaluate the potential of using changes in plant metabolites as an indicator of pesticide exposure of non-target plants. Such indicators are required for testing if different initiatives to reduce exposure of natural and semi-natural habitats to pesticides, such as spray-free buffer zones, have reached their goal.

We found that all pesticides resulted in a metabolic profile that made it possible to distinguish samples exposed to metsulfuron-methyl, glyphosate, epoxiconazole and a mixture of the three pesticides from each other by use of NMR scenning followed by statistical analyses and an automated identification process using the publicly accessibly NMR library at University of Birmingham (Ludwig et al., 2011). However, the changes are not assigned to a single metabolite; they merely consisted of a response patterns. In some cases the regulation - up or down – was similar among pesticides, i.e. general responses. Other cases, however, showed a strong pesticide specific response (Fig 4.4 p. 27).

Similar to previous studies of herbicide exposure and phytochemical responses (Ravn et al., 2005a; Ravn et al., 2005b; Petersen et al., 2007), we found that the responding phytochemical compounds mainly belonged to two groups, the amino acids and the carbohydrates, but also organic acids, amines and alkaloids were affected.

Only two species were included in the present study, one dicot, *Arabidopsis thaliana*, and one monocot, *Agrostis capillaris*. Generally, *A. thaliana* responded much clearer to the pesticide exposures with respect to both growth and metabolic changes identified as clearer separation among treatments. In general, the fold changes of the selected plant metabolites found for *A. capillaris* were much lower than for *A. thaliana*. In *A. capillaris*, separation of samples based on metabolic composition was only possible for samples treated with high doses of glyphosate and the mixture of metsulfuron-methyl, glyphosate and epoxiconazole.

In *A. thaliana*, it was possible to significantly differentiate the responses to the individual pesticides from control samples based on the metabolic profile except for metsulfuron-methyl. In *A. capillaris*, this was only possible for the higher (25 and 100 %) doses. Similarly the dose-response experiments showed that *A. thaliana* was more sensitive to the pesticide treatments than *A. capillaris* measured on biomass reduction.

While epoxiconazole had only little effect on growth of the test species, it resulted in significant changes in the metabolic composition of both *A. thaliana* and *A. capillaris*. Furthermore, the effect on biomass of the herbicides in mixture with epoxiconazole indicated that the adjuvants included in the commercial formulation of epoxiconazole (Opus) altered the responses of plant growth and also resulted in synergistic responses at the metabolic level.

According to the mode-of-action of glyphosate, a decrease in content of the three aromatic amino acids, phenylalanine, tyrosine and tryptophane, and in shikimate, was expected. Such a response was found for *A. thaliana* where a specific response was found for phenylalanine being reduced 0.34 relative to control samples (Table 4.23A). The increased amount of tyrosine (1.91 fold) in *A. capillaris* after exposure to glyphosate (Table 4.23B), however, was unexpected. Possibly tyrosine

was mobilized from other sources to compensate for the supposedly blocked primary production of this amino acid. Unexpectedly, the shikimic acid was not among the compounds that showed significant differences between control and glyphosate exposed plants of the two species. This could be a result of not using the optimal extraction solvent for shikimic acid.

The plant metabolite responses expected for the plant species after exposure to metsulfuron-methyl were a reduction in content of three-branched amino acids such as valine, leucine and isoleucine (Chaleff and Mauvais, 1984; Jensen et al., 2011). In *A. thaliana*, the content of both leucine and isoleucine were reduced (0.65 and 0.87 fold, respectively), whereas no significant changes of these three compounds were found in *A. capillaris*.

Among the metabolic responses to high pesticide concentrations but also to the pesticide mixture (Fig. 4.3) some responses may be more general indications of plant damage or death as opposed to the detected metabolic responses at lower pesticide concentrations.

While earlier studies on changes in plant metabolites following exposure to pesticides have concentrated on effects of herbicides (Suttle and Schreiner, 1982; Lyndon and Duke, 1988; Ravn et al., 2005b; Hjorth et al., 2006; Petersen et al., 2007; Ravn, 2009), the present study included a fungicide as well. The fact that the fungicide induced changes in the metabolic patterns improves the feasibility of the method as an indicator of pesticides exposure.

The NMR screening has been mentioned as a first step towards development of an indicator of pesticide exposure. To be able to develope it further some criteria for the selection of an indicator for pesticide exposure must be established. Heink and Kowarik (2010) reviewed the scientific literature on biodiversity indicators and formulated 19 criteria, which may be used to evaluate a potential indicator. These criteria, which may be used as inspiration for development of criteria for a pesticide exposure indicator, includes relation between indicator and indicandum, feasibility for analysis and interpretation, efficiency, information to be provided by the indicator and perception.

6. Conclusions

The results of this pilot-project confirmed that exposure to pesticides resulted in specific metabolic changes in the two plant species tested and the results, therefore, support the main project hypothesis. However, no general uniform pattern could be defined across plant species based on the results of the induced changes of phytochemical composition and content.

Only three compounds, i.e. glutamic, acetic and suberic acid, responded significantly in both *A. thaliana* and *A. capillaris* and the responses differed among these species. In *A. thaliana*, glutamic acid showed a general response whereas the response in *A. capillaris* was specific; and oppositely for acetic and suberic acid, where the responses in *A. thaliana* was specific and general in *A. capillaris*. However, individual patterns of both general and pesticide specific metabolites were found for the two plant species (Fig. 4.4 and Table 4.23). Further, investigations are needed to confirm if some uniform patterns exist for example for dicotyledons and monocotyledons, respectively.

Generally, *A. thaliana* responded much clearer to the pesticide exposures than *A. capillaris*. This included responses of biomass as well as the metabolic changes; and the fold changes, i.e. the fraction between controls and exposed samples, were much higher for *A. thaliana* than for *A. capillaris*. In *A. capillaris*, separation of samples based on metabolic composition was only possible for samples treated with high doses of glyphosate and the mixture of metsulfuron-methyl, glyphosate and epoxiconazole.

In *A. thaliana*, it was possible to significantly differentiate the responses to the individual pesticides from control samples based on the metabolic profile except for metsulfuron-methyl. In *A. capillaris*, this was only possible for the higher (25 and 100%) doses.

The plant metabolite changes also differed significantly over time in *A. thaliana* suggesting that changes in metabolites might be used to separate the time course after treatments. Although changes in metabolite composition over time as a consequence of plant growth and development was also seen for controls there was a relatively stronger signal in the metabolite profile of sampling time for exposed samples suggesting a common change in the metabolite signal with sampling time within the treated plants that was not evident in the control plants alone.

For *A. thaliana,* there was a significant dose component when using all sampling times. The dose component was in many cases significant, however, less commonly so for low doses of the pesticide treatments. Phytotoxic symptoms on plants were seen from 7 days after application but only for the 25% and 100% dose. The NMR screening was able to detect metabolic changes at all doses and pesticides from 7 days after treatment and therefore it was a more sensible method than visual assessment.

Thus for *A. thaliana*, the collected metabolite signals were structured with regard to both sampling time and dose, however, no single metabolites can be assigned to explain these changes in deeper detail in the present dataset possibly due to the small sample sizes.

The NMR-screening seems to be a useful tool for a first step identification of the most promising compounds that potentially can be developed into an indicator of pesticide exposure of non-target

plants as it allows a simultaneous identification of many different metabolites. For further investigations of the identified metabolites other methodologies such as HPLC and HPPC may be more appropriate as they are more specific and cheaper.

7. Perspectives

Although no general uniform pattern in plant metabolic composition could be defined for the two plant species of this pilot-project, the project confirmed that exposure to pesticides resulted in specific metabolic changes in plants. Especially, the metabolic changes in *Arabidopsis thaliana* look promising for further development of an indicator of non-target plant pesticide exposure. Furthermore, it was possible for this species to significantly distinguish exposed samples from controls over time and even at low doses of pesticides. Finally, the result that both herbicides and a fungicide resulted in significant changes makes it more interesting as indicator of pesticide exposure. The responses of *A. thaliana* is specifically interesting because of an established gene database (TAIR: http:/www.arabidopsis.org) which may facilitate advanced comparisons in the interpretation of the metabolic profiles.

However, the putative indicators, i.e. the patterns of plant metabolite contents, detected by the NMR method in the present project need further investigation to validate the usefulness as indicator. Firstly, the specificity of the responses needs to be further studied. Other stressors than pesticide exposure may result in similar responses or may interfere with the responses. Secondly, the responses of more common non-target species than *A. thaliana* need to be investigated and the responses to a broader range of pesticides including more herbicides, fungicides and insecticides need also to be studied. Finally, the method used for identication of the metabolites also needs to be further developed. While the NMR-screening was valuable for this initial screening, the possibilities of using other methodologies such as HPLC and HPPC also need to be investigated. The NMR-method is time consuming and expensive and therefore less time consuming and less expensive methods are recommended for the next step of developing the identified patterns into indicators. Also the selection of solvents for the extraction may be developed especially for specific groups of compounds that have been identified as important in the initial screening. For example are solvents used for carbohydrates and amino acids not optimal for phenolic compounds or lipids and vise versa (Harborne, 1998) and more specific extractions need to be aimed at the compounds of interest.

Finally, criteria for indicators of pesticide exposure need to be developed before an evalution could be performed. The criteria suggested for biodiversity indicators, see (Heink and Kowarik, 2010), will be a good starting point.

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Summary

The project examines the relationship between pesticide exposure and changes in plant metabolites within of two plant species, specifically focus has been at the response at low pesticide doses and the duration of the response in order to assess the potential for using changes in plant metabolites as an indicator of pesticide exposure. We found that exposure to selected pesticides resulted in significant changes in several metabolites. In general, the response was clearer in Arabidopsis thaliana than in Agrostis capillaris. For Arabidopsis thaliana, it was possible based on the composition of the plant metabolite to separate the samples both in terms of dose and time since exposure.

I projektet undersøges sammenhængen mellem pesticideksponering og ændringer i planteindholdsstoffer i to plantearter; specielt undersøges responset ved lave pesticiddoser og varigheden af responset med henblik på at vurdere potentialet for at benytte ændringer i planteindholdsstoffer som indikator for pesticideksponering. Vi fandt, at eksponering overfor de valgte pesticider resulterede i tydelige og signifikante ændringer i flere planteindholdsstoffer. Generelt var responset tydeligere hos gåsemad end hos almindelig hvene. For gåsemad var det muligt ud fra sammensætning af planteindholdsstofferne at adskille prøverne både med hensyn til dosering og tid siden eksponering.

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