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Phytochemical responses to herbicide exposure and effects on herbivorous insects

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Preface

The project presented in this report was carried out in the period from 1996 to 2000 as a collaborative work involving both The National Environmental Research Institute, Department of Terrestrial Ecology and The Royal Veterinary and Agricultural University, Chemistry Department.

The report consists of a summary in both Danish and English, an introductory chapter that presents the background and the scope of the report, a range of chapters presenting the experimental data and finally the results are discussed.

Two master students (Kristine Krogh and Annette Salomonsen) have written their thesis in connection with the project. Dr. Gerard A. J. M. Jagers op Akkerhuis took part in designing the experiment of Chapter 9. Their contribution is appreciated.

Results from the project has have been presented at the Tenth International Symposium on Insect-Plant Relationships in Oxford 4-10 July 1998 and at a research seminar organised by the Danish Environmental Protection Agency on May 28 1998 in Copenhagen.

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The project was overseen by a steering committee. The members have made valuable contributions to the project. The committee consisted of:

Inge Vibeke Hansen, Environmental Protection Agency (chairman in the period between January 1996 and July 2000).
Claus Hansen, Environmental Protection Agency (joined the committee as Chairman in July 2000).
Thomas Secher Jensen, The Natural History Museum, Aarhus.
Jens Kvist Nielsen, The Royal Veterinary and Agricultural University, Chemistry Department.
Christian Kjær, The National Environmental Research Institute, Department of Terrestrial Ecology.

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Sammenfattende artikel

Baggrund

Det er velkendt, at indholdet af sekundære planteindholdsstoffer spiller en afgørende rolle for, om en plante angribes af insekter. Stofferne kan påvirke insekters adfærd og kan ligeledes påvirke insekters vækst og formering, oftest i negativ retning på grund af giftvirkning. Hvis indholdet af sekundære plantestoffer ændres, for eksempel som følge af herbicidpåvirkning, kan der derfor ske betydelige ændringer i samspillet mellem herbivore insekter og deres værtplanter. Ændringerne kan favorisere planten eller insektet afhængigt af, om det er indholdet af hæmmende eller stimulerende stoffer, der ændres, og afhængigt af, om der sker en stigning eller en reduktion af indholdet.

Hvis herbicider har en effekt på planters kemiske forsvar, kan det have stor betydning. For afgrødeplanter vil en øget koncentration af planteforsvarsstoffer kunne reducere behovet for insekticidsprøjtninger, hvorimod en reduceret mængde forsvarsstoffer kan øge behovet for insekticid behandling. På udyrkede arealer kan ændringer i planternes kemi påvirke artssammensætningen via ændringer i enkelte arters trivsel og dermed grundlaget for herbivorerne.

Undersøgelser har vist, at sulfonylurea-herbicider (også kaldet minimidler) kan påvirke herbivore insekter negativt. Pileurtsbladbillens larver havde en forringet overlevelse, når værtplanterne havde været eksponeret for minimidlet chlorsulfuron. Den forringede overlevelse var afhængig af herbiciddosis og af herbivortæthed. Det er projektets hovedhypotese, at ændringer i planteindholdsstoffer er drivkraften bag den forøgede dødelighed. Det er endvidere vores forventning, at de fenoliske stoffer er vigtige i denne sammenhæng.

Formål

I projektet undersøgtes de fenoliske stoffers rolle i interaktionen mellem snerle-pileurt, pileurtsbladbillen og ukrudtsmidlet chlorsulfuron. Målet var at identificere og kvantificere de fenoliske stoffer der forekommer i høje koncentrationer i bladene af snerle-pileurt og/eller respondere på herbivori og behandling med ukrudtsmidlet chlorsulfuron. Der blev opstillet forsøg for at beskrive effekten af en række ukrudtssprøjtemidler samt en række biotiske og abiotiske faktorer på de kvantitativt mest betydende fenoler i snerle-pileurt. Nærværende projekt undersøgte endvidere effekten af behandling med minimidler i lave doser på tre plante – herbivor-systemer.

Undersøgelser

Der blev isoleret seks fenoliske stoffer fra bladene af snerle-pileurt, 3-O-E-caffeoylquinonsyre (neochlorogen syre)(1), 1-O-E-caffeoyl-beta-D-glucose (2), 3-O-E-p-coumaroyl-beta-D-glucose (3), caffeoyl-tartronsyre (4), caffeoyl-meso-tartronsyre (5), quercetin-3-O-beta-D-glucuronide (6) (Figur 2.1). Stof 3 blev isoleret fra blade behandlet med ukrudtsmidlet chlorsulfuron samt i feltplanter. De øvrige hovedkomponeneter blev fundet både i behandlede og ubehandlede planter. Stofferne nummereret 1, 2, 4 og 5 er estere af kaffesyre, mens stof nummer 3 er en coumarin-syre-ester, og stof 6 er et flavonoid.

Otte ukrudtsmidler med seks forskellige virkemåder blev testet for deres effekt på koncentrationen af de seks fenoliske planteindholdsstoffer. Alle de testede ukrudtsmidler inducerede en reduktion i indholdet af stofferne 1, 4 og 6 i de øverste blade (Tabel 3.2). Denne respons kan være et mere generelt stress fænomen. Indholdet af stof 2 steg i blade placeret i bunden eller midt på planten, hvis planterne var blevet behandlet med de to minimidler metsulfuron og chlorsulfuron. Der var ingen signifikante effekter i topbladene. Koncentrationen af stof 3 steg kun i de nederste og i de midterste blade af planter behandlet med minimidler. Koncentrationen var upåvirket af de øvrige herbicider.

Effekten af tid, herbivori, UV-lys og dosis blev undersøgt mere indgående for chlorsulfuron.

Koncentrationen af stofferne 2, 3 og 6 steg i det første 4 dage efter sprøjtning (Tabel 4.1). Herefter faldt koncentrationen igen . Efter 30 dage var koncentrationen sammenlignelig med koncentrationen i ubehandlede planter.

Intensiteten af ultraviolet lys er stærkt reduceret under væksthusforhold sammenlignet med friland. Samtidigt vides det, at UV-lys kan have betydning for koncentrationen af sekundære stoffer i planter. Væksthusforsøg, hvor planter blev eksponeret for UV-B lys, viste, at usprøjtede snerle-pileurtsplanter havde et forøget indhold af stofferne 1, 2 og 6 når de blev eksponeret for UV-B. Indholdet af stof 4 var uændret og stof 5 forekom i lidt lavere koncentration. Stof 3 forekom ikke i usprøjtede planter. I sprøjtede planter resulterede bestråling med UV-B lys i et forøget indholdet af stofferne 1, 2, 3 og 6. Koncentrationen stof 4 var uændret og koncentrationen af stof 5 var reduceret ved UV-B bestråling.

Forsøg med varierende antal herbivorer viste, at indholdet af stofferne 1, 2, 3 og 4 generelt faldt med stigende tæthed af planteædende insekter. Dette fald var dog primært udtrykt ved den højest tæthed af larver (Tabel 5.4).

Stofferne 2 og 3 var positivt korreleret med sprøjtemiddeldoseringen. Dosis-respons-relationen kunne bedst beskrives med en sigmoid funktion (Fig. 5.1).

De mange undersøgelser gennemført på forskellige tidspunkter viste generelt god overensstemmelse mellem koncentrationen af de fenoliske stoffer i planter, der har været udsat for de samme betingelser i form af sprøjtedosis, tidspunkt for prøvetagningen etc.

Et laboratorieforsøg viste en svag sammenhæng mellem indholdet af stofferne 2 og 3 og pileurtsbladbillelarvers overlevelse. For bedre at kunne beskrive larvernes indtag af stofferne 2 og 3 gennem udviklingen, blev der opstillet en matematisk model til at beskrive koncentrationen af stofferne i bladene over tid og under forskellige betingelser. Denne model blev brugt til at beregne larvernes samlede indtag af disse stoffer, som igen blev relateret til observeret dødelighed af larver på både kontrol planter og planter sprøjtet med chlorsulfuron. Denne beregning resulterede i et meget bedre sammenhæng mellem larvedødelighed og koncentrationen af planteindholdsstofferne 2 og 3.

Forskelle mellem planter dyrket inden- og udendørs

Alle de hidtil omtalte resultater er udført under laboratorie- eller væksthus-betingelser. Der blev også udført forsøg udendørs. Disse viste, at planteind-holdsstofferne var fordelt i planten som under laboratoriebetingelser, men koncentrationerne var noget højere, undtagen for stof 4. (Figur 4.1).

Når planterne blev sprøjtet med chlorsulfuron, var der en vis forskel mellem planter dyrket inde og ude. Stof 2 blev generelt fundet i højere koncentrationer i planter dyrket udendørs. Denne forskel kan til dels tilskrives de ændrede lysforhold, eftersom stof 2 steg, når planten blev eksponeret for UV-B.

Koncentrationen af stof 3 var også påvirket af vækstbetingelserne. I planter dyrket indendørs blev der fundet en induktion eller et forøget indhold i blade, der var placeret i bunden eller i midten af planten. Planter dyrket udendørs havde et forøget indhold af stof 3 i topbladene. Igen kan en del af forklaringen være forskelle i lysforholdene i de to situationer

Alt i alt kan man sige, at planternes vækstbetingelser påvirkede såvel fenolers fordeling og koncentration i usprøjtede planter, som herbicideffekten. Forskelle i lysforhold (UV-B) kan forklare en del, men ikke alle forskelle observeret mellem planter dyrket inde og ude.

Indikator for eksponering

Stof 3 kan anvendes som indikator for eksponering af snerle-pileurtplanter med sulfonylureaherbicider (Fig. 3.3). Stoffet kan detekteres i chlorsulfuron-sprøjtede planter allerede ved en koncentration der svarer til 0.067 gange (7%) den anbefalede markdosering (0.25 g a.i. ha^{-1}). Endelig er det tilstede i planten i mindst 16 dage efter den faktiske eksponering. Dette tidsforløb gælder for planter der er dyrket under meget gunstige forhold. Under feltforhold ville dette stof højst sandsynligt være tilstede i længere tid.

Effekter på herbivore insekter

Ud over snerle-pileurt og pileurtsbladbillen blev det også undersøgt, om der var effekt på to andre plante-insekt systemer, nemlig den store kålsommerfugl på raps samt kornbladlus på hvede. Minimidlerne chlorsulfuron, metsulfuron og tribenuron blev testet. Det var formålet at undersøge, om andre minimidler havde effekter som de observeret med chlorsulfuron.

Der var en tendens til reduceret overlevelse af pileurtsbladbillen på snerle-pileurt, når planten var behandlet med metsulfuron og tribenuron. Dette indikerer, at effekter, som de der er observeret for chlorsulfuron, må forventes for disse minimidler, hvis højere doser benyttes.

Den store hvide kålsommerfugls larver blev ikke direkte påvirket af ukrudtsmidlerne, men værtsplanten tabte sine blade ved meget lave doser. Det må derfor forventes, at disse insekter vil være påvirket i omkringliggende naturarealer på grund af reduceret fødegrundlag. Undersøgelserne viser endvidere, at sommerfuglen højst sandsynligt ikke vil have en forøget skadevirkning efter sprøjtemiddelafdrift ind i nærliggende marker, og at reducerede sprøjtemiddeldoser af ukrudtsmiddel højst sandsynligt ikke vil give nogen fordel for denne art i marken.

Der var ingen indikation af, at metsulfuron påvirker værtplantekvaliteten for kornbladlus på hvede. Planten er i øvrigt tolerant overfor det valgte sprøjtemiddel.

Konklusioner

- Det er sandsynligt, at fenoler spiller en rolle i de biologiske interaktioner mellem snerle-pileurt og pileurtsbladbillen

- Minimidler har en specifik effekt på fenoliske indholdsstoffer i snerlepileurt samt på pileurtbladbillens trivsel, som ikke kunne genfindes for seks andre herbicidtyper og to andre plante-insekt systemer
- Det er sandsynligt, at lignende effekter vil forekomme i andre plantearter, men reaktionen er altså ikke generel for alle herbicider og plante-insekt-systemer.

Summary and conclusions

Background

Secondary plant metabolites are known to affect herbivorous insects. Effects of increased/induced metabolites on insects may be positive, through stimulation of consumption or negative, due to toxicity of the compound(s) or deterrent effects owing to changes in odour or taste.

Phenolic compounds are often involved in plant defence against herbivores, and the level of these compounds in plants may change after herbivore damage or herbicide treatment. Some phenolic compounds have also been shown to stimulate feeding or oviposition of insects.

The content and composition of phytochemicals may change as a consequence of e.g. chemical treatment, climatic stress, or herbivory. Consequently, not only herbicide treatment itself, but also the activity of herbivores as well the conditions under which testing takes place may affect the internal response in plants.

It has been observed that the food quality of Black bindweed (*Fallopia convolvulus)* to larvae of the leaf beetle *Gastrophysa polygoni* may be affected by spraying with sulfonylurea herbicides. It was shown that *G. polygoni*- larvae had an increased mortality on sprayed plants and that mortality was dependent on chlorsulfuron dose and herbivore density. It is our hypothesis that a herbivore-induced chemical defence, which was enhanced by the chlorsulfuron treatment, caused the observed increase in mortality. Furthermore, we suggest that phenolic compounds are active in this relationship.

Aim

The work presented in this report was initiated in order to investigate the possible role of phenolic compounds in the interactions between *F. convolvulus*, *G. polygoni* and the herbicide chlorsulfuron. The aim was to identify and quantify phenolic compound occurring in significant concentrations in leaves of *F. convolvulus* and/or compounds for which the concentrations responded to herbivore or herbicide stress. The impact of growth stage, herbivore load, herbicide dose and growth conditions were included in the different experiments. Furthermore, the project aimed at describing the effect of sulfonylurea herbicides on other plant-insect relationships and if other types of herbicides could have a similar effect.

Experimental data

Six phenolic compounds were isolated and identified from *F. convolvulus* leaves 3-O-E-caffeoylquinic acid (neochlorogenic acid)(1), 1-O-E-caffeoyl-beta-D-glucose (2), 3-O-E-p-coumaroyl-beta-D-glucose (3), caffeoyl tartronic acid (4), caffeoyl meso-tartronic acid (5), quercetin-3-O-beta-D-glucuronide (6) (Fig. 2.1). Compound 3 was isolated from leaves treated with the herbicide, chlorsulfuron, other compounds 1, 2, 4, 5, and 6 were isolated from untreated leaves. Compounds 1, 2, 4 and 5 were esters of caffeic acid while 3 was an ester of p-coumaric acid, and 6 was a flavonoid.

Eight herbicides with different modes of action were tested for their impact on the concentrations of the selected phenolic compounds. It was found that all tested herbicides induced a significant reduction of concentrations of compounds 1, 4 and 6 in top leaves (Table 3.2). This reaction may be a more general herbicide stress response.

The content of compound 2 increased 2 to 3 times in bottom and middle leaves of plants treated with tribenuron and chlorsulfuron. The effect of these two herbicides on top leaves was not significant but seemed to be opposite to the impact found in the lower leaves. Compound 3 was found to increase only in the bottom and middle leaves of plants treated with the sulfonylurea herbicides. It was only found in trace amounts in plants treated with other types of herbicides.

A closer examination was made of the impact of chlorsulfuron in combination with a range of other influential factors. These factors encompass time, UV-B radiation, herbivory and chlorsulfuron dose.

One to 4 days after herbicide application the content of compounds 2, 3 and 6 increased (Table 4.1). Hereafter a gradual decrease followed until day 30 after spraying when levels were comparable with the content in unsprayed plants.

Supply of UV-B light in the laboratory increased the similarity with field plants for compounds 1, 2 and 6 in middle leaves. Thus, light conditions are an important parameter for these compounds. UV-B light did not affect the concentrations of compounds 3 and 4 in unsprayed plants.

Experiments with varying numbers of herbivores per plant showed that the content of compounds 1, 2, 3 and 4 in general was reduced with increasing density of herbivores. The decrease was, however, mainly observed at the highest density (Table 5.4).

The dose-response reaction of the concentration of compounds 2 and 3 in the leaves described a sigmoid response within the investigated concentration range of chlorsulfuron (Fig. 5.1). The reduction of the content of compounds 1, 4, and 6 in *F. convolvulus* leaves was consistent with what has been observed for several other herbicides. The herbicide-induced depression of these compounds caused a significant difference between herbicide-exposed plants and unexposed plants.

The many experiments conducted generally showed a large degree of comparability in the concentration of phenolic compounds for plants grown under similar conditions, such as dosage, time since spraying, UV-radiation etc.

An experiment carried out in controlled environment showed a weak correlation between the concentrations of the compounds 2 and 3 and the survival of *G. polygoni* larvae. In order to improve this correlation and describe larval intake for these compounds a mathematical model was developed. The model aims at describing the leaf concentration of compounds 2 and 3 over time and under different environmental conditions. The model was used to estimate the cumulated larval intake of the selected phenolic compounds during development from egg to pupae. Intake was then related to the observed mortality of the larvae on both chlorsulfuron-sprayed

and unsprayed plants. This calculation improved the correlation between larval survival and the concentrations of compounds 2 and 3 considerably.

Differences between greenhouse- and field-grown plants

Phytochemicals were generally quite similarly distributed within unsprayed plants grown under laboratory and field conditions (Figure 4.1). However, concentrations were higher in field plants, except for compound 4, indicating an effect of growth conditions on most of the phytochemicals.

When the plants were sprayed with chlorsulfuron, the phytochemical response differed somewhat between laboratory and field plants (Figure 4.1): For compound 2, the content was generally higher in field plants. This may partly be due to differences in light conditions, since compound 2 is increased by UV-B light, and this part of the light spectrum was absent under the standard laboratory conditions.

The response in concentrations of compound 3 to spraying also differed between growth conditions: In laboratory plants, an increase/induction was found in middle and bottom leaves, whereas field plants responded by an increase in top leaves. Part of the explanation may be differences in light conditions between the two situations.

All in all, it was shown that growth conditions affected not only concentrations and distribution of phytochemicals in unsprayed plants, but also the effect of herbicides on concentrations of the phytochemical compounds. Light conditions (UV-B) could explain some, but not all of the phytochemical difference between plants grown under laboratory and field conditions.

Indicator of herbicide exposure

Compound 3 seems applicable as indicator for exposure of *F. convolvulus* to sulfonylurea herbicides. The compound is only present in trace amounts in control plants, and the concentration increases in plants sprayed with 0.067 times the recommended field rate of chlorsulfuron (0.25 g a.i. ha^{-1}) or more. The compound is present at least until 16 days after actual exposure. Considering the growing conditions during the experiment, which made the plants grow very fast, it may be difficult to find any indicator substance with a longer lifetime. Under field conditions with lower average temperatures the indicator is expected to persist for a longer time. Finally, concentration of the compound was unaffected in plants treated with other types of herbicides.

Herbicide treatment and the performance of herbivorous insects

In addition to the *Fallopia-Gastrophysa* test system two other plant-herbivore systems were tested, namely the large white butterfly on oilseed rape and the cereal aphid on winter wheat plants. The following sulfonylurea herbicides were tested on the three plant-herbivore-systems: chlorsulfuron, metsulfuron and tribenuron. It was the intention to study whether other sulfonylurea herbicides had similar effects as those observed for chlorsulfuron.

A tendency of reduced survival was observed for *G. polygoni* feeding on hosts treated with sulfonylurea herbicides other than chlorsulfuron. This indicates that the effects observed for chlorsulfuron may be expected also for these herbicides, if higher dosages are used.

Larvae of the large white butterfly larvae (*Pieris brassicae*) were not directly affected by any of the herbicides in the dosages tested, but the host plant lost the leaves at very low dosages. An effect is therefore expected for specimens

in adjacent non-crop habitats due to reduction of food resources. The experiments furthermore suggest that that the butterfly will not increase its pest status following spray drift from adjacent fields and that the use of reduced dosages of herbicide are unlikely to benefit insects associated with *Brassica napus* in the fields.

Furthermore, there were no indications of that metsulfuron treatment changed the quality of the *winter wheat* plants, as the cereal aphid (*Sitobion avenae*) developed and reproduced equally well on treated and untreated plants. Winter wheat is tolerant to sulfonylurea herbicides.

Conclusions

- It is likely that phenolic compounds play a role in the interactions between *F. convolvulus* and *G. polygoni*
- The phytochemical changes observed in *F. convolvulus* are specific to the sulfonylurea herbicides
- It is, however, plausible that similar effects will occur for other species. The test with two other insect-plant-systems revealed that the response is not a general phenomenon.

1 General introduction

Herbicides are widely used in conventional agriculture. On average, a Danish agricultural field is sprayed with herbicides 1.37 times a year (Miljøstyrelsen, 2000). Spraying a field with herbicides may increase pest populations. This is found especially for synthetic auxins (review by Campbell (1988)). Most of these studies concerns aphid species ((Hintz and Schultz, 1969; Oka and Pimentel, 1974; Oka and Pimentel, 1976; Oka, 1979)) or species living in the meristematic tissues of the plant (Ingram and Charpentier, 1947; Ishii, 1963). For other feeding guilds, like foliar feeders, the response is more variable, but with a tendency to reduced performance of the herbivore (Agnello *et al*., 1986a; Agnello *et al*., 1986b; Agnello *et al*., 1986c; Holopainen *et al*., 1991; Meisner *et al*., 1987). The positive effects on aphids has been attributed both to a reduced predation in field studies, probably due to toxic effects of the herbicide on predators (Adams and Drew, 1969; Adams and Drew, 1965)), and to an improved food quality of treated host plants (Maxwell and Harwood, 1960; Oka and Pimentel, 1974).

Plants present in the spray drift zone (adjacent crops as well as wild plants) may be affected by the herbicide. If the food quality of the plants for herbivorous insects is changed, unintended and unwanted effects may occur. In adjacent crops, an improved food quality may result in an increased pest population with the possible need of additional insecticide spraying. For plants present in natural habitats near to the sprayed field, negative effects on herbivorous insects may disturb ecosystems or may have negative effects on species of public concerns (e.g. butterflies).

The proportion of the herbicide-sprayed area treated with sulfonylurea herbicides is increasing. One study (Kjær and Elmegaard, 1996) has indicated that this group of herbicides may have detrimental effects on leaf chewing herbivores. The food quality of *Fallopia convolvulus* to *Gastrophysa polygoni* larvae may be affected by spraying with (low dosages of) sulfonylurea herbicides (Kjær and Elmegaard, 1996), which, under field conditions, may result from herbicide drift from the sprayed fields into adjacent areas. Furthermore, *G. polygoni* has been found to thrive better on *F. convolvulus* plants grown in laboratory than on plants grown under field conditions (unpublished data). The phenoxy acid dichlorporp has been shown to reduce the survival of *G. polygoni* at low dosages, i.e. 0.167 times the recommended field rate (Madsen, 1995).

The sulfonylurea herbicides are a class of herbicides that act by inhibiting the first enzyme specific to the branched chain amino acid biosynthetic pathway (Chaleff and Mauvais, 1984). Furthermore, it has been shown that this is accompanied by a reduced translocation of photosynthetate (Bestman *et al*., 1990; Devine *et al*., 1990; Vanden Borne *et al*., 1988). As a consequence of this inhibition (Ralphs *et al*., 1998; Suttle and Schreiner, 1982; Suttle *et al*., 1983) have found an increased concentration of secondary plant compounds in tall larkspur, soybean and sunflower. The compounds affected encompassed both phenolics and alkaloids. These herbicides are selective against broad-leaved species, i.e. dicotyledons. The differences in susceptibility of plants have been found to rely primarily on differences in the

capacity for metabolism of the herbicides (Hageman and Beherens, 1984; Hall *et al*., 1992).

Secondary plant metabolites are known to affect herbivorous insects. Effects of increased/induced metabolites on insects may be positive, through stimulation of consumption or negative, due to toxicity of the compound(s) or deterrent effects owing to changes in odour or taste. These types of impact have been described for a range of compounds including both primary and secondary plant metabolites (Harborne, 1993; Rosenthal and Berenbaum, 1992). The secondary plant compounds with these properties involve, among others, the alkaloids, terpenes, phenols and glucosinolates. We have chosen to focus on the phenolic compounds in this report because they are often found to be involved in plant defence against herbivores (Horwath and Stamp, 1993; Shaver and Lukefahr, 1969). Furthermore, the level of these compounds in plants may change after herbivore damage (Dixon and Paiva, 1995; Watermann and Mole, 1994) or herbicide treatment (Devine *et al*., 1993; Duke and Hoagland, 1978; Lydon and Duke, 1989; Lydon and Duke, 1993). Some phenolic compounds have also been shown to stimulate feeding or oviposition of insects (Feeny *et al*., 1988; Harborne and Grayer, 1993; Shaver *et al*., 1998) including a Japanese *Gastrophysa*-species that is stimulated to feeds on another Polygonaceous by certain phenolic compounds (Matsuda, 1976; Matsuda, 1978; Matsuda, 1981).

The content and composition of phytochemicals may change as a consequence of e.g. chemical treatment, climatic stress, or herbivory as describe above. Consequently, not only herbicide treatment itself, but also the activity of herbivores as well the conditions under which testing takes place (laboratory versus field) may affect the internal response in plants (Watermann and Mole, 1994).

It is our hypothesis that the observed increase in mortality of *G. polygoni* larvae with herbivore density and dosage of the herbicide chlorsulfuron is caused by a herbivore induced chemical defence, which is enhanced by the chlorsulfuron treatment. This hypothesis is based on the reduced translocation in plants treated with sulfonylurea herbicides and the density dependent response of the larvae. Furthermore, we suggest that phenolic compounds are active in this relationship. This suggestion is based on the phytotoxic potential of phenolic compounds (Shaver and Lukefahr, 1969) and their role in host recognition processes of herbivores.

The present report was initiated in order to investigate the possible role of phenolic compounds in the interactions between *F. convolvulus*, *G. polygoni* and the herbicide chlorsulfuron. The aim was to identify and quantify phenolic compound occurring in large concentrations in leaves of *F. convolvulus* and/or responding to herbivore and herbicide stress of the plant. The impact of growth stage, herbivore load, herbicide dosage and growth conditions were included in the different experiments.

The interactions between herbicide treatment, phytochemical changes in plants and herbivore response were addressed and the main focal points were:

- The effect of sulfonyl-urea herbicides on selected crops and weeds
- Indirect effects of herbicide treatment on selected insects utilising the above plants as hosts
- The possible relationship between any effects on plants and insects following herbicide treatment and the phenolic profile of the plants

- The effect of herbivory on selected phytochemicals
- Comparison of laboratory and field observations
- Risk assessment of herbicide drift to non-target organisms in areas adjacent to treated fields
- The potential of phytochemicals as indicators of plant exposure to sulfonyl-urea herbicides

1.1 Organisms studied

Three plant – herbivore systems representing different feeding guilds were included in the project in order to study how common side-effects of herbicides are on herbivores. The chosen organisms are all likely to be exposed to sulfonylurea herbicides due to spray drift into adjacent crops or semi-natural areas. Butterflies were represented by the *Brassica napus – Pieris bassicae* system, aphids by the *Triticum aestivum – Sitobion avenae* system, and leaf beetles by the *F. convolvulus – G. polygoni* system. The former two are described in more detail in Chapter 10, whereas the *F. convolvulus – G. polygoni* system is used in several chapters and therefore is presented below.

F. convolvulus (L.) A. Löve is one of the main host plants for the leaf beetle *G. polygoni* (Coleoptera: Chrysomelidae) (Sotherton, 1982). *F. convolvulus* is a strict annual weed species. It often climbs adjacent crop plants. Under greenhouse conditions the plant reproduces mainly by self-fertilisation. It has no leaf loss during development, but continues to grow until senescence, when all leaves dies almost synchronously and the seeds ripen. Details of the life cycle of *F. convolvulus* are presented by (Hume *et al.*, 1983).

G. polygoni L. is a chrysomelid beetle utilising mainly two food plants, *viz. F. convolvulus* and *P. aviculare* L. It eats the foliage of the plants in all three larval instars and as adults. The larvae pupate in the soil.

F. convolvulus is an important weed in agricultural fields, and herbicides are used in order to control it. On the other hand, seeds of this plant as well as all stages of the beetle may serve as important protein sources for young chicks of the partridge (Southwood, 1969) and other farmland birds (Potts, 1986). Numbers of the farmland birds have declined dramatically during the last 20-40 years, and attempts have therefore been made to use reduced amounts of herbicides in field margins in order to increase the food sources available to farmland birds. The success of these attempts depends on the food quality of plants treated with sublethal dosages of herbicides. *F. convolvulus* plants sprayed with low dosages of the herbicide chlorsulfuron and at the same time heavily loaded with herbivores have been shown to provide food of lowered quality to young *G. polygoni* larvae (Kjær and Elmegaard, 1996). These results suggest that combined effects of herbicide and herbivore stress may induce a defensive reaction in the plant, while there are only minor effects when the two factors act alone.

1.2 Types of experiments performed

In the course of the project period a range of experiments has been performed. The main factors included in the experiments presented are outlined schematically below in Figure 1.1.

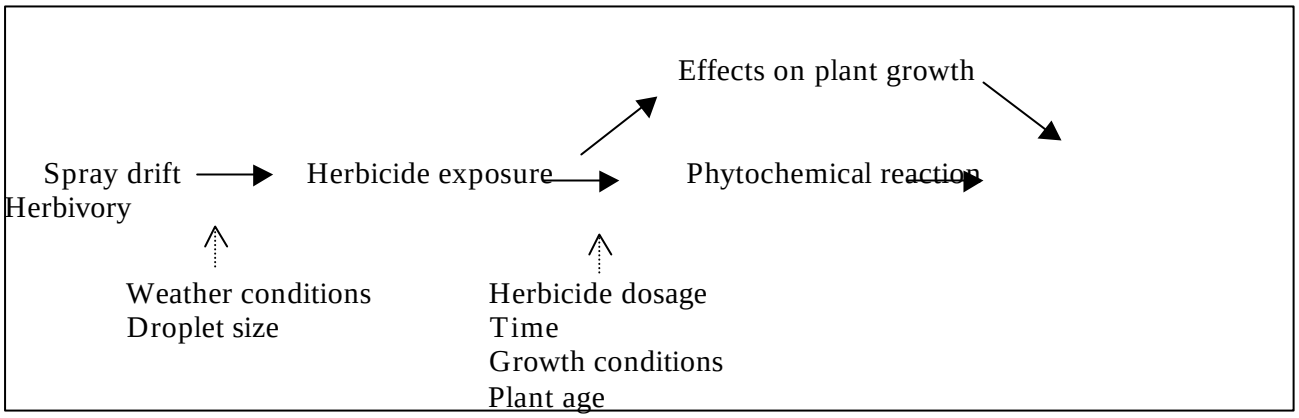


Figure 1.1
Schematic outline of the "study system" involved in the report.

In order to assess the phytochemical reaction of the plants, separation, identification and quantification of the quantitative most important phenolic compounds in *Fallopia convolvulus* were performed, as presented in Chapter 2.

Plants were sprayed with herbicide(s), and the resulting phytochemicals were measured to describe the correlation between herbicide treatment and changes in the phytochemical profile. First, different herbicides with different modes of action were applied to identify phytochemicals that may be unique to plants treated with sulfonylurea herbicides (Chapter 3). Secondly, a dose-response relationship between chlorsulfuron dosage and the content of selected phytochemicals was aimed at (Chapter 5). Thirdly, the time-course of the observed phytochemical changes following chlorsulfuron treatment was studied by harvesting leaves at different time intervals after spraying, to get an idea of induction and persistence (Chapters 2, 4, 5 and 7). Fourthly, studies of phytochemical concentrations in leaves at different positions (i.e. of different age) were conducted separately (Chapters 3 and 4) in order to examine whether old or young plant parts were more affected by herbicide treatment, and thus to find indications of possible mechanisms the observed effects on phytochemicals.

The possible differences in phytochemical response to herbicide treatment between plants grown under laboratory conditions and plants grown under outdoor conditions were studied in Chapter 4 by comparing plants grown indoor and outdoor after chlorsulfuron spraying. Since one of the major factor differing between the mentioned growth conditions and known to affects phytochemicals is UV light, this factor was included in the laboratory studies.

The influence of herbivory on the phytochemical profile of *F. convolvulus* was studied in Chapter 6. The experiments comprised both natural (*Gastrophysa polygoni)* and simulated herbivory.
Subsequently, effects on beetle survival of the changes in phytochemicals induced by chlorsulfuron treatment of the plant were studied by introducing the larvae on chlorsulfuron treated leaves (Chapter 7). Furthermore, the (possibly indirect) effects of sulfonylurea herbicide treatment of three different host plants on the associated herbivorous insects were studied by applying three herbicides in different dosages before introduction of the herbivore (Chapter 10). Both short-term and chronic effects on the herbivores were studied.

Since herbicide drift into adjacent field or natural areas is a likely exposure route for both plants and herbivorous insects, this aspect was studied by exposing *F. convolvulus* to chlorsulfuron in the field at different distances to the spray track (Chapter 9). Subsequently, the plants were transferred to the greenhouse and toxic effects as well as effects on the phytochemical profile were quantified. The influence of differences in spray droplet size on growth effects was studied in a greenhouse experiment (Chapter 9).

In many experiments a range of variables were varied simultaneously, because this allows for comparisons between experiments. Identification and quantitative analysis of selected phenolic compounds
The aim of this chapter is to identify the most important phenolic compounds in *F. convolvulus* leaves and to develop a reliable quantitative method for detection of these compounds in plant tissues.

2 Identification and quantitative analysis of selected phenolic compounds

The aim of this chapter is to identify the most important phenolic compounds in *F. convolvulus* leaves and to develop a reliable quantitative method for detection of these compounds in plant tissues.

2.1 Materials and methods

2.1.1 Plant material

F. convolvulus was grown in a greenhouse. Leaf material was lyophilised and stored dry at - 20°C until extractions were made. The compounds extracted, identified and quantified in this chapter are numbered from 1 to 6.

2.1.2 General techniques

HPLC was run initially on a Pharmacia chromatograph equipped with a Pharmacia LKB VWM 2141 dual wavelength UV detector. Reversed phase C18 columns (5µm particle size, 4.6 × 250 mm) were used, and the final column choice was a Phenomenex Prodigy ODS3 column, since it could tolerate the low pH values which were necessary for the analyses. Solvents were: 0.5% aqueous trifluoracetic acid (A) and acetonitrile (B). The following gradient was used: 0-5.0 min 5% B isocratic, 5.0 - 25.0 min: linear gradient 5 - 20% B, 25.0 - 40.0 min: linear gradient 20 - 35% B; 40.0 - 55.0 min: linear gradient: 35 - 60% B; 55-60 min: linear gradient 60-80% B; 60-85 min: 80% B isocratic; 65-75 min: 80-5% B; 75-85 min: 5% B isocratic. The flow rate was 0.8 ml min^{-1}. In 1999 and 2000, HPLC was run on a Shimadzu LC-10AT liquid chromatograph equipped with a Shimadzu SPD M10 AVP Diode array detector. The Prodigy C18 column was used and solvents were: 0.5% aqueous trifluoracetic acid (A) and acetonitrile (B). The following gradient was used: 0-5.0 min 5% B isocratic, 5.0 - 25.0 min: linear gradient 5 - 30 % B, 25.0 - 40.0 min: linear gradient 30 - 50% B; 40.0 - 50.0 min: linear gradient: 50 - 80% B. The flow rate was 0.8 ml min^{-1}. High voltage electrophoresis was run on Whatman No. 3 chromatography paper in a buffer, Pyridin-HOAc-H_2O (25:1:500) at pH 6.5; 50 min at 5 kV and 90 mA.

2.1.3 Hydrolysis

Acid hydrolysis was performed in 1 M HCl in 50% methanol for 2 hours (reflux). Each hydrolysate was evaporated to dryness. N-trimethylsilyl (TMSi) imidazole was added to hydrolysates of compounds 2, 3 and 5, and the resulting TMSi ethers were analysed by GC-MS. TMSi ethers of meso and racemic dl tartaric, as well as glucose, galactose and mannose were used as reference compounds. The hydrolysate of compound 6 (2 mg) was redissolved in H_2O (1 ml) and extracted with ethylacetate (3 × 2 ml) in a separation funnel. The aqueous and ethylacetate phases were concentrated to

small volumes and analysed by paper chromatography together with authentic compound 6 (unhydrolysed), kaempferol, quercetin, glucuronic, galacturonic acid and glucuronolactone formed by acid treatment of glucuronic acid.

Enzymatic hydrolysis of compound 6 (2 mg) was performed in 500 µl H_2O and 2.5 mg glucuronidase (Sigma) was added. The mixture was left at room temperature for 3 hours. The hydrolysate was then evaporated to dryness, redissolved in H_2O (1 ml) and extracted with ethylacetate (3 × 2 ml) in a separation funnel. The aqueous and ethylacetate phases were concentrated to small volumes and analysed by paper chromatography together with authentic compound 6 (unhydrolysed), kaempferol, quercetin, glucuronic, and galacturonic acid.

2.1.4 Extraction and isolation

Lyophilised leaves (100 g $batch^{-1}$) were transferred to boiling 70% aqueous ethanol (4 l) and homogenised for 5 minutes with an Ultra-Turrax homogeniser. After filtration, the residue was extracted × 2 with 70% aqueous ethanol (2.5 l) at room temperature. The filtrates were combined, evaporated to a small volume (ca. 400 ml) and extracted in a separation funnel with equal amounts of chloroform (× 3) and later with ethylacetate (× 3). The aqueous phase was further concentrated, centrifuged for 10 minutes at 15000 rpm before it was transferred to a column (10 × 23 cm) containing Polygosil C18 60-4063 (Macherey Nagel). The column was rinsed first with H_2O and later with increasing concentrations of aqueous ethanol. Fractions (200 - 500 ml) were collected according the UV-absorption of the effluent. All compounds were finally purified on MCI GEL CHP20P (2.6 × 100 cm, Mitsubishi Chemical Co.). Fractions containing compounds 4 and 5, which eluted early from the C18 column were separated on the MCI GEL column using 5% acetic acid in 15% aqueous ethanol. Fractions containing compound 1, which eluted later from the C18 column were treated similarly. Fractions containing compounds 2, 3 and 6 were purified on the MCI GEL column rinsed with 10 - 25% aqueous ethanol. The purity of the compounds and fractions was controlled by HPLC.

2.1.5 Quantitative analysis

Lyophilised plant material (25 - 100 mg) was homogenised in 70% aqueous ethanol (5 ml) in a centrifuge tube at room temperature. After centrifugation, the supernatant was removed and the pellet was extracted twice with 70% aqueous ethanol using the same procedure. Kaempferol-3-O-J-D-[J-D--glucopyranosyl(1→2)glucopyranoside]-7-O-J-D-glucopyranoside isolated from cabbage leaves (Nielsen *et al.*, 1993) was added as an internal standard during the first homogenisation step. 0.50 µmole internal standard was added to samples of leaves of 60 to 100 mg, while 0.25 µmole was added to smaller samples. The combined extracts were evaporated to a small volume and transferred to a volumetric flask (5.0 ml). 3.0 ml from the volumetric flask was purified by solid phase extraction using a 500 mg C18 column (International Sorbent Technology). The effluent from the C18 column (ca. 3 ml) was collected and combined with the effluent from the same column using 40% aqueous ethanol to a total volume of 10.0 ml (volumetric flask). The effluent from the C18 column (100 µl $sample^{-1}$) was analysed by HPLC. Response factors (at 330 nm) were 1.00 for the flavonoid (compound 6) and 0.72 for the hydroxycinnamoyl esters. Response factors for the hydroxy-cinnamoyl esters were determined by comparison of published **e-**values for

caffeic acid derivatives (18500 at 330 nm) (Clifford 1999) with those for flavonol glycosides (14.000 at ca. 350 nm ≈ 13.000 at 330 nm).

2.2 Results

2.2.1 Identification of the phenolic compounds

Six phenolic compounds were isolated and identified from *F. convolvulus* leaves (Fig. 1). Compound 3 was isolated from leaves treated with the herbicide, chlorsulfuron, while other compounds were isolated from untreated leaves. Compounds 1, 2, 4 and 5 were esters of caffeic acid while compound 3 was an ester of p-coumaric acid, and compound 6 was a flavonoid. The identification of these compounds involving FAB mass spectrometry and 1D and 2D NMR and various chemical techniques is unequivocal (Nielsen, Olsen & Kjær, unpublished).

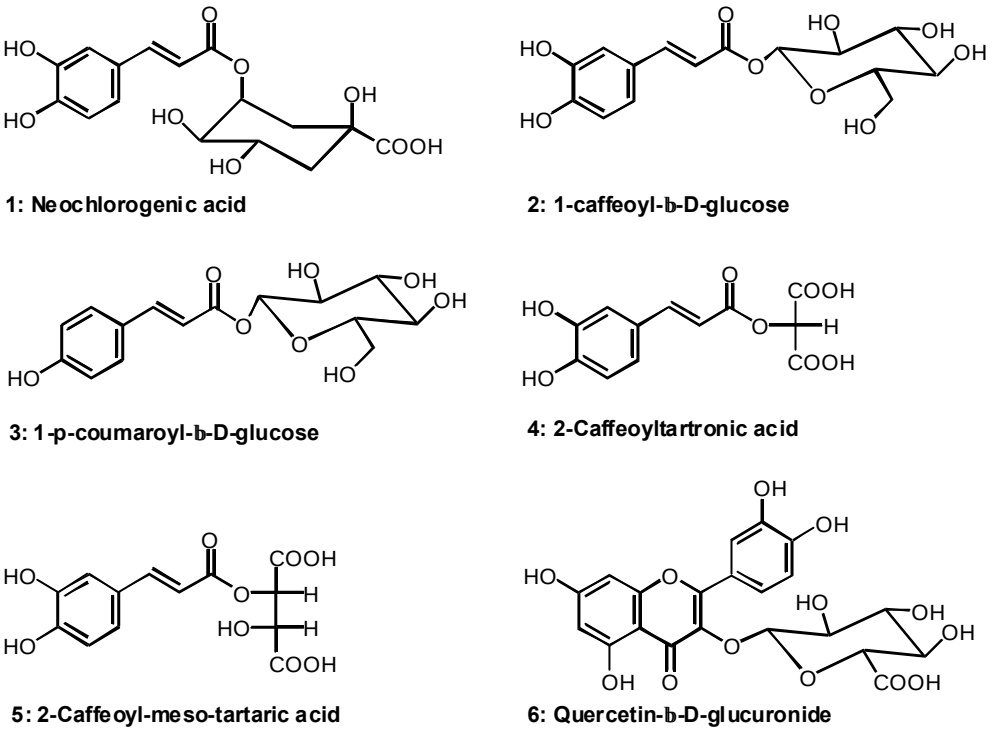


Figure 2.1
Phenolic compounds isolated from leaves of *F. convolvulus*

The compound 1 was found to be a caffeoylester of quinic acid, but retention times on HPLC as well as NMR spectra demonstrated that the compound was different from chlorogenic acid, which is the most common caffeoyl quinic acid ester. Comparison of 1D and 2D NMR of 1 with those published previously for this compound and its isomers (Corse *et al*., 1966; Flores-Parra *et al*., 1989; Scholz-Böttcher *et al*., 1991) demonstrated that esterification had occurred in the 3-position of quinic acid in 1, while it occurs in the 5-posion in chlorogenic acid (5-CQA). Compound 1 is therefore neochlorogenic acid (3-CQA) which is also a rather common plant constituent. The naming of the caffeoylquinic acids follow the new conventions from (IUPAC, 1976) which is opposite to the one used in earlier investigations. As an example

neochlorogenic acid is 3-CQA according to the new conventions, while is was named 5-CQA in older literature, for example in (Corse *et al*., 1966).

Compound 2 was found to be 1-caffeoyl-β-D-glucose. The glucose moiety was identified after acid hydrolysis of the parent compound, while the attachment between caffeic acid and glucose was determined by NMR. Similar analyses demonstrated that compound 3 was 1-p-coumaroyl-β-D-glucose.

Mobility studies using high voltage electrophoresis demonstrated that compounds 4 and 5 contained two free dicarboxylate groups suggesting that they were caffeoyl esters of hydroxydicarboxylic acids (Fig 2.1). Compound 4 was found to be 2-caffeoyltartronic acid, while compound 5 was found to be 2-caffeoyl-meso-tartaric acid. The identification of the acyl moiety as meso-tartaric acid as opposed to one of the optically active forms of tartaric acid was confirmed by GC-MS after acid hydrolysis.

UV- and NMR-data demonstrated that compound 6 is a flavonoid, and identified the aglycon as quercetin. Mobility studies using high voltage electrophoresis and several chemical techniques demonstrated the presence of a glucuronic acid moiety. The attachment between quercetin and glucuronidc acid was confirmed by NMR. Compound 6 could therefore be identified as quercetin-3-O-β-D-glucuronide. None of the compounds had previously been found in leaves of *F. convolvulus*, but they had been reported from other plant species (see later). The compounds constituted more than 90% of the phenolic found in a typical sample from *F. convolvulus* leaves (Fig. 2.2).

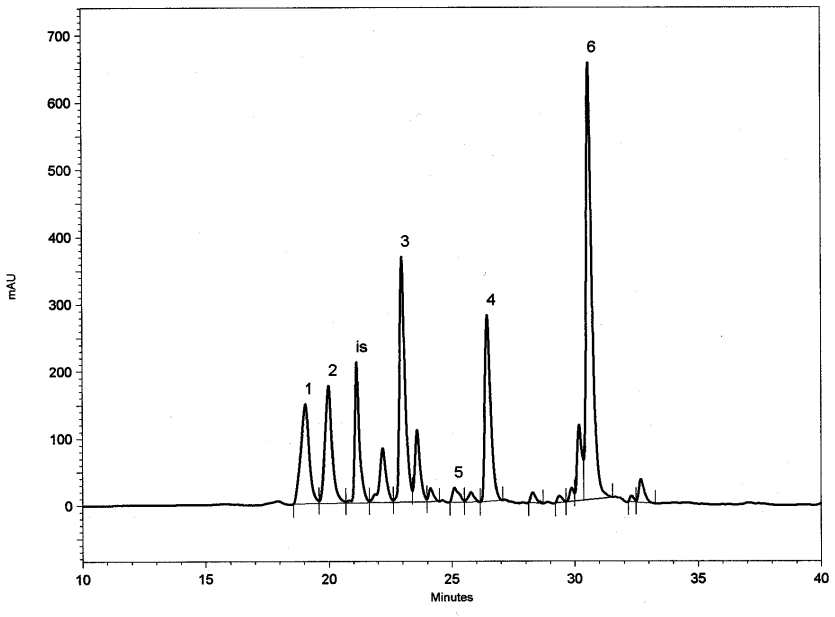


Figure 2.2
HPLC chromatogram showing peaks at 330 nm originating from six phenolic compounds isolated from *F. convolvulus* leaves. Numbers are according to Fig. 1; is: internal standard.

2.2.2 Quantitative analysis of phenolic compounds in *F. convolvulus* leaves

Quantitative analyses have been based on HPLC throughout the studies, but considerable developments have occurred in the actual methods used. When the project started, the Pharmacia HPLC equipped with a dual wavelength detector was available. This system was more or less satisfactory for identification of the major compounds although severe overlap between the identified compounds and some minor compounds occurred from time to time. With the increasing interest in detection of sometimes trace amounts of compound 3, this system proved to be insufficient, since it was not possible to distinguish between trace amounts of compound 3 and other minor phenolic compounds. It was therefore a big improvement that a Shimadzu HPLC equipped with a diode-array detector became available in 1999. The advantage of the diode-array detector is that it is possible to obtain full UV spectra of all the peaks. Since UV spectra of compound 3 are clearly different from UV spectra of caffeoyl derivatives (compounds 1, 2, 4, 5 and several minor compounds), the identification of 3 could be made with much higher certainty (Fig 2.3).

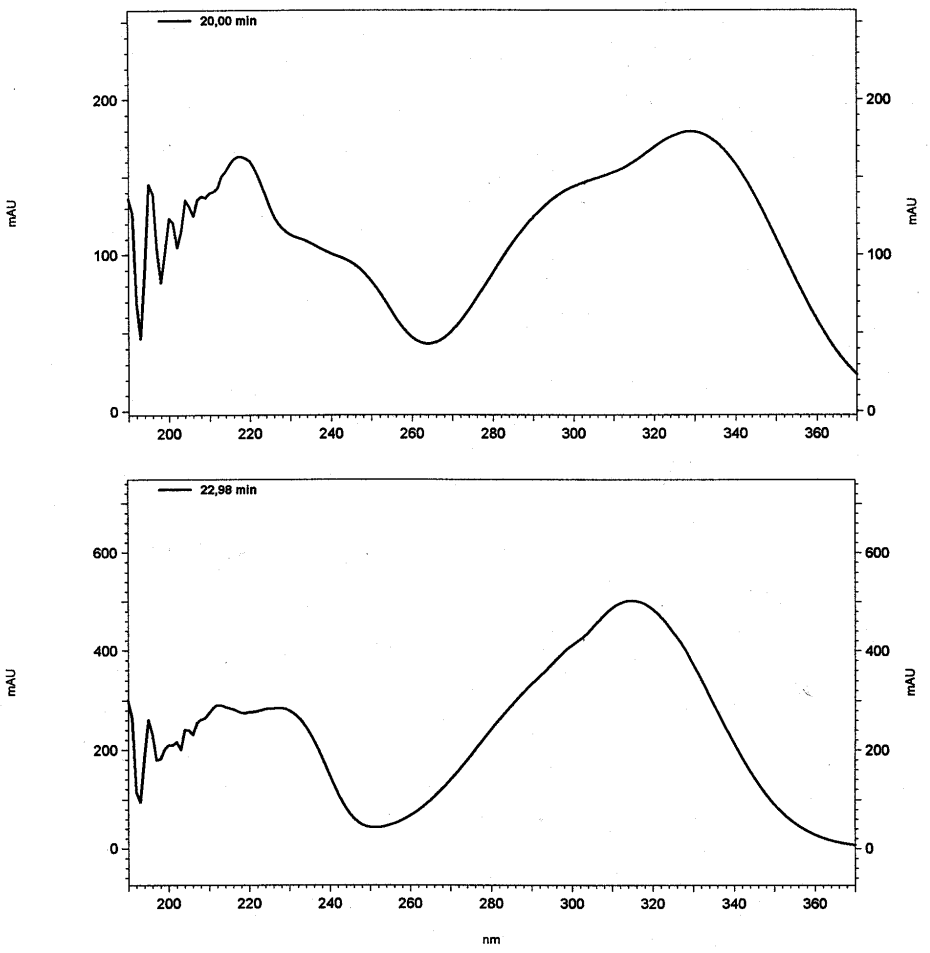


Figure 2.3
UV spectra of compounds 2 (top) and 3 (bottom) demonstrating the typical difference between caffeoyl esters (UVmax higher than 320 nm) and p-coumaroyl esters (UVmax below 320 nm).

2.3 Discussion

None of the compounds identified from *F. convolvulus* are new natural products, but they have not previously been found in Polygonaceae. Neochlorogenic acid (compound 1) is less common than its isomer, chlorogenic acid, but has nevertheless been identified from a variety of plants including cabbage and coffee beans (Clifford, 1999) and a variety of ripe fruits (Möller and Herrmann, 1983). The glucose esters, compounds 2 and 3, are also widely distributed in the plant kingdom (Mølgaard and Ravn, 1988), and they are often precursors for biosynthesis of other hydroxycinnamic acid esters (Strack *et al*., 1987). 2-caffeoyl-tartronic acid (compound 4) seems occur more rarely in plants, since it has only been reported from three plant species, *Vigna radiata* (Fabaceae) (Strack *et al*., 1985), *Chondrilla juncea* (Asteraceae) (Terencio *et al*., 1993) and *Nepeta cataria* (Lamiaceae) (Snook *et al*., 1993). 2-caffeoyl-meso-tartaric acid (compound 5)has previously been reported from *Equisetum arvense* (Hohlfeld *et al*., 1996), while the isomeric compound 2-caffeoyl-L-tartaric acid (caftaric acid) has been reported from grapes and wine as well as from grapevine leaves, where it occurs together with quercetin-3-O-β-D-glucuronid acid (compound 6) (Goetz *et al*., 1999; Singleton *et al*., 1978). Caffeoyl esters of D-tartaric acid have been reported from *Cichorium* species (Compositae) (Wöldecke and Herrmann, 1974). The mixture of hydroxycinnamoyl derivatives found in *F. convolvulus* has not been found previously in other plant species. A characteristic mixture of different natural compounds may be more important than any single compound for the ability of phytophagous insects to recognise their host plants (Feeny *et al*., 1988). The phenolic compounds isolated from *F. convolvulus* may therefore be important for the ability of the leaf beetle, *G. polygoni*, to recognise *F. convolvulus* as one of its major host plants.

Some of the compounds from *F. convolvulus* as well as their isomers have previously been reported to be involved in biological interactions. Neochlorogenic acid (compound 1) inhibited growth of Spodoptera *litura* larvae in the same way as its more common isomer, chlorogenic acid (Stevenson *et al*., 1993). Chlorogenic acid has been reported as a deterrent or growth reducing compound for several insects (Hoover *et al*., 1998; Stamp and Yang, 1996), but at the same time it is part of a behavioural active mixture which stimulate oviposition by black swallowtail butterflies (*Papilio polygenes*) on carrot leaves. Other caffeoyl quinic acid derivatives are important oviposition stimulants or synergists for other swallowtail butterflies (Carter *et al*., 1999; Haribal *et al*., 1998). Typically, there is a tight linkage between chemical structure and biological activity in these interactions, and it is characteristic that the caffeoylquinic acids are more active in combination with other host plant compounds for example flavonoids (Carter *et al*., 1999; Feeny *et al*., 1988). Flavonoids are often involved in biological interactions and deterrent as well as stimulatory effects on insect behaviour and growth have been reported depending on the type of insect and the particular chemical structure (Harborne and Grayer, 1993). However, no effect on insects has yet been attributed to quercetin glucuronid (compound 6), but this compound may be involved in resistance to grey mould in grape berries together with caffeoyl-L-tartaric acid (caftaric acid) (Goetz *et al*., 1999). Caffeoyl-tartronic acid (compound 4) has previously been identified as a precursor for catechol in a grasshopper, *Romalea guttata* (Snook *et al*., 1993). The grasshoppers obtain the precursor from its host plant, catnip, and metabolise it into a component of their own defensive secretion.

3 Impact of herbicides with different modes of action on phenolic compounds

The changes in composition of phenolic compounds in *F. convolvulus* leaves, observed after application of chlorsulfuron (Chapter 2), may be caused by general stress reactions of the plant and/or specific actions induced by this specific herbicide. The same responses may be induced by other herbicides with the same or different mode of action. An assay including herbicides representing six different modes of action was carried out in a greenhouse and in controlled-environment-chamber to further investigate how specific the response is. Two herbicides of the same family as chlorsulfuron were included in the experiment to test if the responses seen for chlorsulfuron are general for all three sulfonylurea herbicides.

3.1 Methods

3.1.1 Herbicides

Eight different herbicides were selected in order to represent six different modes of action and three sulfonylurea compounds (Table 3.1).

Table 3.1
Herbicides used in the study. Active ingredient, trade name and manufacturer, label rate, dosage used and mode of action.

Active ingredient	Trade name (manufactor)	Label rate g.a.i./ha	Dosage used g. a.i./ha	Mode of action
Pendimethalin	Stomp SC (Cyanamid)	800-2000	200	Cell division inhibitor
Metolachlor	Dual Gold (Novartis)	2500	2500	Lipid biosynthesis inhibitor
Dicamba	Banvel 4S (Novartis)	200	88	Growth hormone (Auxin)
Bromoxynil	Saxo (Rhone Poulenc)	400	160	Photosynthetic inhibitor
Glyphosate	RoundUp (Monsanto)	1260	200	Aromatic amino acid biosynthesis inhibitor
Tribenuronmethyl	Express (Dupont de Nemours)	7.5	7.5	Aliphatic amino acid biosynthesis inhibitor
Metsulfuron	Ally (Dupont de Nemours)	4-6	2	Aliphatic amino acid biosynthesis inhibitor
Chlorsulfuron	Glean 20 DF (Dupont de Nemours)	4	4	Aliphatic amino acid biosynthesis inhibitor

The dosage applied was determined in a pilot study and chosen to be sufficient to produce a clear effect without killing the plant during the experimental period.

3.1.2 Plants

For each herbicide, three replicates of three *F. convolvulus*-plants were sprayed and two plants were sprayed with water. For the sulfonylurea herbicides, the detergent Citowett were added to all replicates including control.

Plants were sprayed at the five leaves stage in a pot sprayer (manufacturer Christensen, Slagelse, DK). The pot-sprayer was calibrated to deliver 200 l ha^{-1} at 2 bar and 4.7 km h^{-1}, nozzle Hardy No 16. The plants were kept in a greenhouse at 12-20 °C until spraying. After spraying, plants treated with herbicide were left for 24 h in a separate compartment of the greenhouse and subsequently all plants were moved to a growth chamber where the environment was set to 20 °C, 16 h light, 70% RH. Seven days after spraying three leaves were cut of each plant, one leaf from the top, one from the middle, and one from the bottom of the plant. The leaves were freeze-dried for 24 h and stored in a freezer until chemical analyses were performed. Due to loss of leaves, a few replicates of bottom leaves and one middle leaf could not be sampled from plants sprayed with the chlorsulfuron.

3.1.3 Chemical analysis

The leaves were analysed for six phenolic compounds i.e. 3-O-E-caffeoylquinic acid (neochlorogenic acid) (compound 1), 1-O-E-caffeoyl-beta-D-glucose (compound 2), 3-O-E-p-coumaroyl-beta-D-glucose (compound 3), caffeoyl tartronic acid (compound 4), caffeoyl meso-tartronic acid (compound 5), quercetin-3-O-beta-D-glucuronide (compound 6). Chemical analysis and numbering of the compounds follows Chapter 2. Compound number 5 was only found in trace amounts.

3.1.4 Statistics

Differences in mean concentration of the phenolic compounds in leaves were tested in a Tukey ‘s Studentized Range (HSD) Test by use of the SAS statistical Software Program assuming p=0.05 as level of significance.

3.2 Results

The content of compound 1 was highest in top leaves and decreased downward (Table 3.2). For all the herbicides tested application significantly reduced the concentration of compound 1 in the top leaves by 70-100% (Fig. 3.1). In the middle leaves the concentration was reduced significantly by c. 30-60% for pendimethalin, metolachlor, tribenuron methyl and chlorsulfuron. For dicamba, glyphosate, bromoxynil, and metsulfuron no changes were observed in middle leaves. In the bottom leaves no clear trends in changes of concentration could be registered for any of the tested herbicides.

Table 3.2
Phenolic compounds (µmol g^{-1} dw) in *F. convolvulus*-plants sprayed with different herbicides. Leaves sampled from top, middle and bottom of plants seven days after spraying. Concentrations are given as means ± one standard error of mean.

Compound	Herbicide	Bottom	Middle	Top
1	Water	1.3 ± 0.39	4.3 ± 0.67	16.7 ± 2.71
	Pendimethalin	1.6 ± 0.83	1.9 ± 0.27	4.5 ± 0.47
	Metolachlor	0.0 ± 0.00	1.6 ± 0.25	3.4 ± 0.38
	Dicamba	1.5 ± 0.49	3.1 ± 0.49	7.4 ± 1.58
	Bromoxynil	0.5 ± 0.47	4.7 ± 0.53	2.2 ± 1.13
	Glyphosate	2.4 ± 0.41	4.6 ± 0.31	2.7 ± 0.95
	Tribenuronmethyl	0.2 ± 0.22	2.4 ± 0.19	2.5 ± 1.10
	Metsulfuron	0.8 ± 0.19	2.9 ± 0.14	5.9 ± 0.76
	Chlorsulfuron	0.0 ± 0.00	1.9 ± 0.27	0.0 ± 0.00
2	Water	3.3 ± 0.26	7.9 ± 0.70	5.3 ± 1.73
	Pendimethalin	3.0 ± 0.35	3.6 ± 0.34	5.2 ± 0.58
	Metolachlor	1.3 ± 0.64	4.5 ± 0.34	0.5 ± 0.36
	Dicamba	4.0 ± 0.38	6.0 ± 0.50	7.7 ± 1.95
	Bromoxynil	2.0 ± 0.75	3.7 ± 0.84	3.0 ± 1.36
	Glyphosate	3.1 ± 0.31	5.8 ± 1.20	2.9 ± 1.11
	Tribenuronmethyl	8.8 ± 1.54	15.7 ± 1.14	0.8 ± 0.53
	Metsulfuron	4.6 ± 0.53	7.9 ± 1.00	3.6 ± 0.87
	Chlorsulfuron	8.6 ± 2.81	15.3 ± 2.35	2.0 ± 1.03
3	Water	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	Pendimethalin	0.0 ± 0.00	0.2 ± 0.11	0.0 ± 0.05
	Metolachlor	0.1 ± 0.12	0.4 ± 0.12	0.0 ± 0.00
	Dicamba	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	Bromoxynil	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
	Glyphosate	0.0 ± 0.00	0.3 ± 0.19	0.2 ± 0.13
	Tribenuronmethyl	13.5 ± 2.35	3.3 ± 0.84	0.0 ± 0.00
	Metsulfuron	6.6 ± 1.30	0.5 ± 0.20	0.0 ± 0.00
	Chlorsulfuron	11.4 ± 1.60	3.9 ± 1.51	0.7 ± 0.70
4	Water	13.9 ± 0.92	17.9 ± 1.24	27.7 ± 1.43
	Pendimethalin	16.8 ± 1.34	14.1 ± 0.93	16.8 ± 2.18
	Metolachlor	9.1 ± 1.96	13.1 ± 0.78	10.1 ± 0.86
	Dicamba	14.9 ± 1.51	16.7 ± 1.43	17.2 ± 3.19
	Bromoxynil	17.3 ± 1.60	19.8 ± 1.73	8.3 ± 2.47
	Glyphosate	14.2 ± 1.27	16.8 ± 0.89	6.3 ± 1.06
	Tribenuronmethyl	7.6 ± 0.71	14.7 ± 0.94	13.9 ± 0.90
	Metsulfuron	8.3 ± 0.79	16.1 ± 1.03	15.8 ± 2.29
	Chlorsulfuron	8.7 ± 1.02	13.0 ± 1.84	10.8 ± 1.10
6	Water	0.3 ± 0.08	2.4 ± 0.63	21.7 ± 2.22
	Pendimethalin	0.8 ± 0.28	1.1 ± 0.30	4.3 ± 1.16
	Metolachlor	0.3 ± 0.14	1.0 ± 0.23	3.5 ± 0.76
	Dicamba	0.3 ± 0.08	0.9 ± 0.31	5.4 ± 1.20
	Bromoxynil	0.4 ± 0.21	2.4 ± 0.77	0.4 ± 0.43
	Glyphosate	0.1 ± 0.05	1.8 ± 0.23	8.5 ± 1.36
	Tribenuronmethyl	0.6 ± 0.36	1.3 ± 0.32	4.7 ± 1.30
	Metsulfuron	0.1 ± 0.06	1.5 ± 0.20	9.7 ± 1.32
	Chlorsulfuron	0.1 ± 0.05	0.5 ± 0.23	2.5 ± 0.58

The concentration of compound 2 was highest in middle and top leaves (Table 3.2). Tribenuron methyl and chlorsulfuron treatment increased concentration of this compound 2 to 3 times in bottom and middle leaves respectively (Fig. 3.2). In top leaves the same herbicides apparently reduced concentration of compound 2, although not significantly. Metsulfuron-treated plants followed the same pattern for top and bottom leaves but the reaction was weaker.

Compound 3 was only found in significant amounts in bottom and middle leaves sprayed with sulfonylurea herbicides (Fig. 3.3). The effect of metsulfuron was weaker than for the other two sulfonylureas.

Compound 4 occurred generally in high concentrations, highest in the top leaves (Table 3.2). All tested herbicides reduced the concentration of compound 4 in the top leaves (Fig. 3.4). In bottom and middle leaves the changes due to herbicides were insignificant except for tribenuron methyl, which reduced the amount of compound 4 in bottom leaves as well. The two other sulfonylurea compounds induced the same changes in concentration pattern in the plants, but the changes were not statistically significant.

The concentration of compound 6 compared to compound 4 is approximately the same in the top leaves but much lower in middle and bottom leaves (Table 3.2). All tested herbicides reduced concentration of compound 6 in the top leaves (Fig. 3.5). Differences in concentrations in the lower leaves are uncertain because of the small amounts present there (Table 3.1).

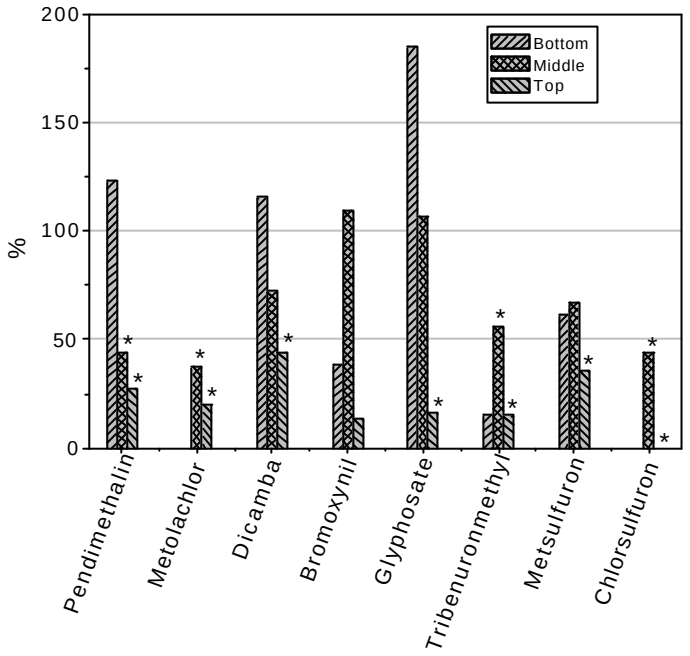


Figure 3.1
Relative content of compound 1 seven days after spraying in leaves from plants sprayed with different herbicides. 100%= control, i.e. water treatment. * indicates significant difference between treatment and control.

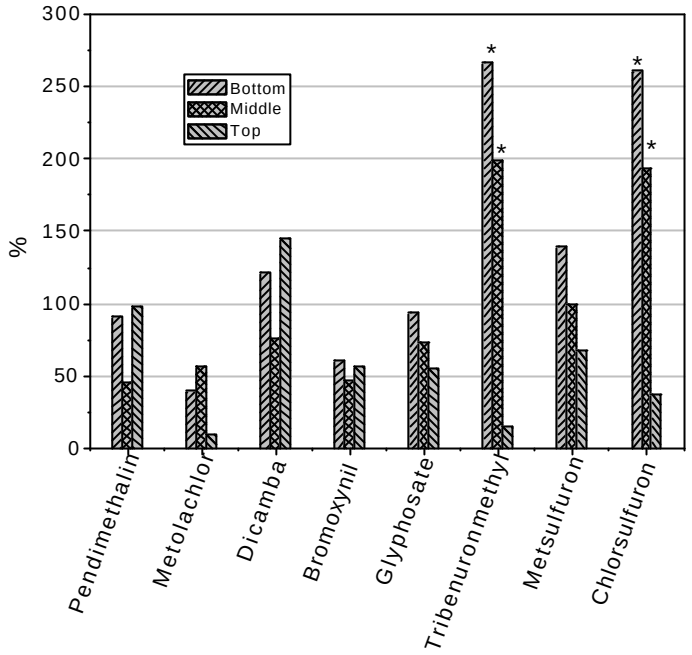


Figure 3.2
Relative content of compound 2 seven days after spraying in leaves from plants sprayed with different herbicides. 100%=water. * indicates significant difference between treatment and control.

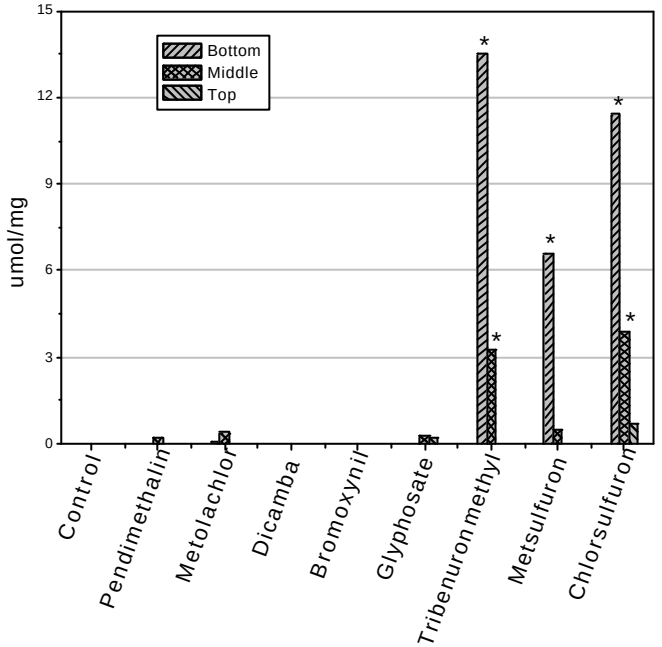


Figure 3.3
Compound 3 (µmol mg-1 dw) seven days after spraying in leaves from plants sprayed with different herbicides. * indicates significant difference between treatment and control.

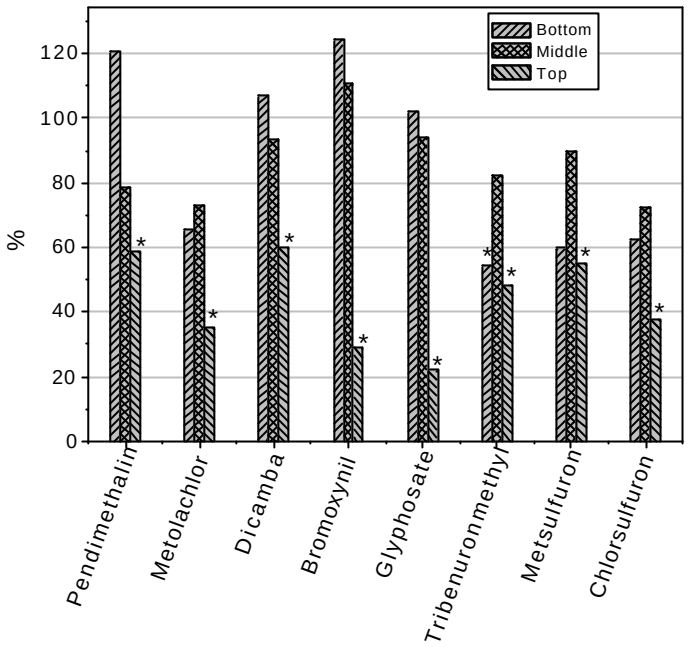


Figure 3.4
Relative content of compound 4 seven days after spraying in leaves from plants sprayed with different herbicides. 100%=water. * indicates significant difference between treatment and control.

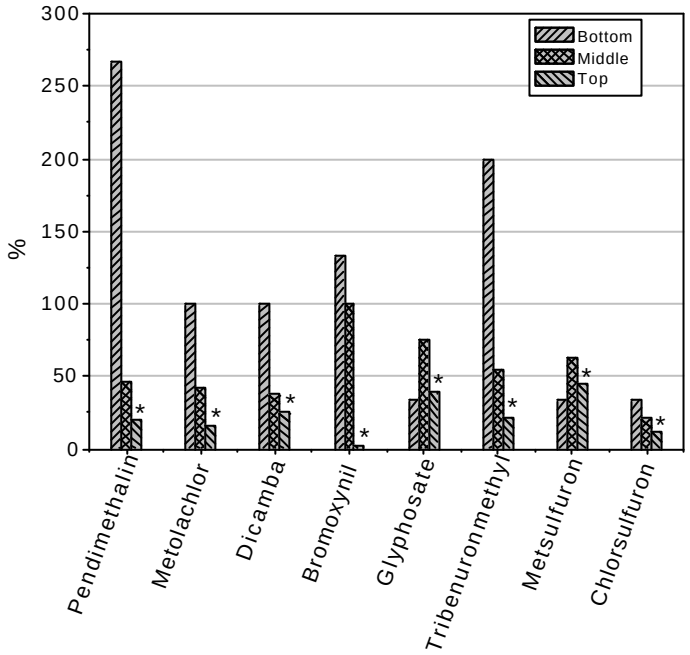


Figure 3.5
Relative content of compound 6 seven days after spraying in leaves from plants sprayed with different herbicides. 100%=water. * indicates significant difference between treatment and control.

3.3 Discussion

All the tested herbicides induced a significant reduction of concentrations of compounds 1, 4 and 6 in top leaves. This reaction may be a more general herbicide stress response. The occurrence of the response solely in the top leaves may be explained by the age of the leaves, young leaves having a higher metabolism as they grow faster etc. It may also be influenced by the position of the leaves in relation to light intensity. The exposure of the top leaves compared to the lower leaves is probably higher when plants are sprayed from a nozzle above. Consequently, there may also be a dose – response relationship involved in the observed effect of leaf position. This should be combined with the different sensitivity of the plant to different herbicides.

The content of compound 2 increased 2 to 3 times in bottom and middle leaves of plants treated with tribenuron and chlorsulfuron. The effect of these two herbicides on top leaves was not significant but seemed to be opposite to the impact found in the lower leaves. Of the five phenolic compounds identified compound 2 was the most evenly distributed in control plants. This difference in distribution in the plant suggests that the compound have a different route of synthesis.

Compound 3 was only found in significant amounts in bottom and middle leaves from plants treated with the sulfonylurea herbicides. The importance of the sulfonylurea compounds mode of action is not clear to us and the effect may be linked to other characteristics of this herbicide group.

4 Phytochemical responses to chlorsulfuron treatment under laboratory and field conditions

The apparently reduced food quality of *F. convolvulus* to *G. polygoni* larvae when the plant is sprayed with sulfonylurea herbicides or grown under field-like conditions compared to control plants grown in the laboratory may be a result of changes in the secondary metabolites of the plant.

The aim of this chapter was to study whether the phytochemical profile of *F. convolvulus* changes as a result of growth conditions and herbicide treatment. This was done by exposing the plant to herbicide spraying under laboratory as well as field-like conditions and analysing the resulting phytochemical contents.

4.1 Materials and methods

F. convolvulus plants were exposed to different dosages of chlorsulfuron under laboratory and field-like conditions to see if differences in growth conditions and herbicide treatment caused differences in selected phytochemicals.

4.1.1 Phytochemical responses to chlorsulfuron treatment under greenhouse and field conditions

F. convolvulus plants were grown in two different set-ups, i.e. in greenhouse and under semi-field conditions outside the greenhouse. The plants were sprayed with different dosages of chlorsulfuron. At the end of the experimental period, the content of phytochemicals in leaves was estimated.

For both exposure situations, seed dormancy was broken by at least three months storage in humid Sphagnum medium at 5°C. The seeds were then placed under light conditions at approximately 20°C to produce seedlings, and the seedlings were transplanted to 11-cm pots containing a standard growth medium (SM vækstmuld, Stenrøgel). After transplantation, the pots were kept in a greenhouse at approximately 22°C, and a daily light period of 16 h. Sunlight was supplemented with artificial light (300 $\mu E\ m^{-2}\ s^{-1}$) when light intensity dropped below 5 klux. If light conditions exceeded 25 klux, the artificial light was shut off. Plants for the field experiment were grown on tables outside, and climatic information was used to calculate their physiological age (Physiological age = (measured temperature - 8°C) $\times$ time, calculated on basis of hourly climatic data).

Plants were sprayed with Glean 20 DF (Dupont) (20 % chlorsulfuron) plus the detergent Citowett (BASF AG) when they had 5-7 leaves. Dosages of 0, 0.5 and 1.0 times the recommended field rate (4 g a.i./ha) were applied. The spraying equipment was a pot sprayer (Christensen) with Hardi 411014 flat-fan nozzles, calibrated at 200 l/ha. There were three replicates, each consisting of 3 plants. Plants for the laboratory experiment were placed in a controlled-

environment chamber at a photo flux density of 350 μE m^{-2} s^{-1}, 20°C, 70% RH and photoperiod of 16 h. Plants for the field experiment were returned to the outdoor tables after spraying.

After 1, 2 and 4 days laboratory plants were harvested, and field plants were cut off after 4 and 7 days (physiological time). Leaves from top, middle and bottom of plants were freeze-dried for phytochemical analyses.

For determination of the phytochemical profile of the leaves, extraction, separation and characterisation were performed as described in Chapter 2. Middle leaves were analysed for all collection times, whereas top and bottom leaves were only analysed when collected after 4 days and 7 days (field plants only).

4.1.2 Effects of UV-B light on phytochemical response

A further laboratory experiment was set up to study if the differences in phytochemical profile between laboratory and field conditions observed in the above experiments (see results below) could be explained by differences in light conditions.

F. convolvulus plants were produced as described for the above experiment and covered with 1 mm mesh. For plants receiving UV-B light, this was supplied by Phillips TL 12/ 40 W lamps placed 0.5 m apart and 1 m above the plants. The lamps were turned on for a period of 5 h every day, imitating the daily UV-B influx on sunny days in the beginning of July. A cellulose acetate filter was used to absorb light below 290 nm (not UV light).

UV-B light (+/-) was combined with chlorsulfuron spraying in a 2×2 factorial design, with herbicide dosages of 0 and 0.125 times the recommended field rate, and treatments were replicated six times. Spraying took place when the plants had approximately 8 leaves and was performed with the pot sprayer described above. Spray solutions were not replicated. Four days after spraying, the third (middle) leaf of every plant was collected for analyses of phytochemicals, which were performed as described in Chapter 2.

4.1.3 Statistics

Effects of the various variables on phytochemical content were analysed by analysis of variance (ANOVA or GLM procedures of the SAS Stat programme). First, tests of interactions were performed, and in case of no significance, analyses for main effects alone were carried out. Means were compared by Tukey's HSD t-test. All analyses were evaluated at the 5 % significance level.

4.2 Results

4.2.1 Phytochemical responses to chlorsulfuron treatment under greenhouse and field conditions

Greenhouse experiments

Five phytochemicals were identified: three hydroxycinnamoyl derivatives (compounds 1, 2 and 4), one coumaroyl derivative (compound 3) and one flavonyl glucoside (compound 6) (cf. Chapter 2). The content of the single substances in the middle leaves at the three collection times is presented in Table 4.1.

Table 4.1
Phenolic content (µmoles g^{-1} dry weight, means ± s.e.) in *F. convolvulus* leaves from the middle of the plants at different harvest times (days) after herbicide treatment under laboratory conditions. p values indicate whether there is a significant effect of herbicide dosage.

Compound	Time	Herbicide dosage (field rate units)			p
		0	0.5	1.0	
1	1	3.2 ± 0.33	2.5 ± 0.28	2.7 ± 0.22	ns
	2	3.6 ± 0.39	3.2 ± 0.21	2.7 ± 0.17	ns
	4	3.4 ± 0.28	2.4 ± 0.18	2.4 ± 0.15	< 0.01
2	1	1.0 ± 0.34	0.7 ± 0.38	1.2 ± 0.28	ns
	2	1.4 ± 0.48	3.9 ± 0.91	2.4 ± 0.47	< 0.05
	4	1.9 ± 0.55	8.9 ± 1.34	9.0 ± 1.05	< 0.001
3	1	0	0	0.1 ± 0.08	ns
	2	0	0.3 ± 0.16	0.5 ± 0.15	ns
	4	0	7.3 ± 1.71	5.6 ± 0.81	< 0.001
4	1	12.2 ± 0.76	10.0 ± 1.08	12.2 ± 0.86	ns
	2	12.6 ± 0.83	12.7 ± 0.67	11.8 ± 1.04	ns
	4	11.6 ± 1.12	10.6 ± 0.61	10.6 ± 0.55	ns
6	1	2.4 ± 0.11	2.0 ± 0.19	2.3 ± 0.29	ns
	2	2.8 ± 0.27	2.9 ± 0.39	2.0 ± 0.24	ns
	4	1.7 ± 0.21	1.8 ± 0.04	2.0 ± 0.14	ns

Collection time

The concentration of compound 1 was unaffected by herbicide treatment after 1 and 2 days. When harvested 4 days after herbicide treatment, the content was significantly lower in treated leaves than in controls. The content of compound 2 in treated plants rose already at day two after herbicide treatment, and this effects was even stronger after 4 days. Compound 3 was never found in unsprayed plants. Induction of this compound started at day 2 after spraying, and was significant after 4 days. Compounds 4 and 6 were unaffected by herbicide treatment at all harvest times.

The analysis of variance showed significant effects of herbicide dosage on compounds 1, 2, and 3 (Table 4.2). Time of leaf harvest significantly affected the content of compounds 2, 3 and 6, and there was an interactive effect of herbicide dosage and harvest time on compounds 2, 3 and 6 (Table 4.2). The

effects of herbicide treatment increased with time for compounds 2 and 3, whereas the interactive effect for compound 6 is ”artificial”, since this compound was not affected by herbicide dosage (Tables 4.1 and 4.2).

Table 4.2
Effects of herbicide dosage and harvest time on the phenolic content of middle leaves of *F. convolvulus* grown under laboratory conditions, as tested by analysis of variance.

Com-pound	Variable	Degrees of freedom	F	p
1	Time	2	2.93	ns
	Dosage	2	8.38	< 0.001
	Time * dosage	4	0.63	ns
2	Time	2	45.81	< 0.0001
	Dosage	2	15.14	< 0.0001
	Time * dosage	4	8.64	< 0.0001
3	Time	2	42.26	< 0.0001
	Dosage	2	12.96	< 0.0001
	Time * dosage	4	11.2	< 0.0001
4	Time	2	2.12	ns
	Dosage	2	1.15	ns
	Time * dosage	4	0.95	ns
6	Time	2	9.02	< 0.001
	Dosage	2	0.45	ns
	Time * dosage	4	2.64	< 0.05

Leaf position

The content of phenols at day four in leaves at different position of the plant is presented in Table 4.3 and Figure 4.1. Compounds 1, 2, 4 and 6 occurred in highest concentrations in the top leaves. Herbicide treatment caused the content of compound 1 to decrease in all types (ages) of leaves, whereas the content of compounds 2 and 3 rose in the lower and middle leaves, but not in the top (young) leaves. The level of compound 6 decreased in the top leaves decreased as a consequence of herbicide treatment. These trends are confirmed by the analysis of variance (Table 4): All compounds were significantly affected by leaf position, and all except compound 2 were affected by herbicide dosage. Furthermore, the interactive effect of dosage and leaf position on compounds 1, 3 and 6 confirms the different effects of herbicide treatment on phytochemicals in leaves in different positions.

Field experiment

The plants were generally much more compact/stunted than plants grown under laboratory conditions.

The same five phenolic compounds as described for laboratory studies were identified, i.e. compounds 1-4 and 6 (cf. Chapter 2). Generally, the phytochemical content of field-grown plants was higher than that of laboratory plants, but not for compound 4 (Table 4.5, Figure 4.1). In contrast to the laboratory plants, small concentrations of compound 3 were found in unsprayed leaves.

Figures for 4 and 7 days (physiological time) are not directly comparable, since they were analysed on two different occasions.

With a few exceptions, the vertical distributions of the phytochemicals followed the same pattern 4 and 7 days after herbicide treatment (Figure 4.1). Herbicide dosage had only few significant ($p < 0.05$) effects on phytochemical content when tested for the different substances, harvest times and leaf positions (Table 4.5).

Compound 1 was found in higher concentrations in the top than in the bottom of control plants. Chlorsulfuron treatment depressed this compound in top parts of the plant, both after 4 and 7 days, and the same herbicide effect was also found in the middle parts after 7 days.

Table 4.3
Phenolic concentrations (μmoles mg^{-1} dry weight, means ± s.e.) in bottom, middle and top leaves of *F. convolvulus* 4 days after chlorsulfuron treatment. The plants were grown under laboratory conditions. p-values indicate result of analysis of variance on effects of herbicide dosage.

Com-pound	Leaf position	Herbicide dosage (field rate units)			p
		0	0.5	1.0	
1	bottom	1.7 ± 0.12	1.0 ± 0.07	1.0 ± 0.08	< 0.001
	middle	3.4 ± 0.28	2.4 ± 0.18	2.4 ± 0.15	< 0.01
	top	19.4 ± 1.83	8.0 ± 0.93	7.6 ± 0.91	< 0.0001
2	bottom	1.1 ± 0.21	2.1 ± 0.41	2.3 ± 0.36	< 0.05
	middle	1.9 ± 0.55	8.9 ± 1.34	9.0 ± 1.05	< 0.001
	top	6.6 ± 3.64	5.5 ± 1.33	6.3 ± 1.89	ns
3	bottom	0	3.6 ± 0.56	3.6 ± 0.56	< 0.0001
	middle	0	7.3 ± 1.71	5.6 ± 0.81	< 0.001
	top	0.1 ± 0.08	0.4 ± 0.13	1.2 ± 0.62	ns
4	bottom	6.8 ± 0.79	4.3 ± 0.36	4.8 ± 0.45	< 0.05
	middle	11.6 ± 1.12	10.6 ± 0.61	10.6 ± 0.55	ns
	top	15.3 ± 2.52	9.9 ± 0.87	13.8 ± 1.53	ns
6	bottom	0.7 ± 0.07	0.6 ± 0.09	0.6 ± 0.09	ns
	middle	1.7 ± 0.21	1.8 ± 0.04	2.0 ± 0.14	ns
	top	29.3 ± 2.89	11.4 ± 1.02	10.9 ± 1.87	< 0.0001

Table 4.4
Results of analyses of variance of the effect of leaf position (bottom, middle, top) on *F. convolvulus* plants harvested 4 days after herbicide treatment. Plants were grown under laboratory conditions.

Com-pound	Variable	Degrees of freedom	F	p
1	Position	2	162.33	< 0.0001
	Dosage	2	33.67	< 0.0001
	Position * dosage	4	22.04	< 0.0001
2	Position	2	8.01	< 0.001
	Dosage	2	2.42	ns
	Position * dosage	4	2.21	ns
3	Position	2	20.68	< 0.0001
	Dosage	2	25.18	< 0.0001
	Position * dosage	4	6.21	< 0.001
4	Position	2	33.08	< 0.0001
	Dosage	2	4.63	< 0.05
	Position * dosage	4	1.12	ns
6	Position	2	174.17	< 0.0001
	Dosage	2	24.37	< 0.0001
	Position * dosage	4	24.94	< 0.0001

Compound 2 concentrations were higher in top and middle than in bottom leaves of control plants after 4 days, but tended to accumulate in the middle parts after 7 days. Four days after chlorsulfuron treatment, there was a strong tendency of an increased content in the bottom parts of the plants. After 7 days, however, there was a strong tendency of a decrease in the middle leaves.

Compound 3 was found in small concentrations in control plants. After chlorsulfuron treatment, the content was increased, and the main proportion was found in the top leaves at both harvest times.

Compound 4 was rather evenly distributed in control plants. Following chlorsulfuron treatment, no significant change was seen, but there was a tendency of decline in top leaves.

The content of compound 6 increased from bottom in control plants. Herbicide treatment with 0.5 and 0.25 times the field rate was followed by a decrease in leaves at all positions (for 4 days only significant for bottom leaves). At full field rate, this effect was less evident, especially in bottom leaves.

Table 4.5

Phytochemical concentrations (μmoles g^{1} dry weight, means ± s.e.) of leaves at different positions, 4 and 7 days (physiological time) after chlorsulfuron treatment of *F. convolvulus* plants grown under field conditions. p value indicates outcome of analysis of variance on effect of herbicide dosage on phytochemical content. N = 3 for 4 days and N = 6 (8) for 7 days.

Com-pound	Time	Leaf position	Herbicide dosage (field rate units)				p
			0	0.25	0.50	1.00	
1	4	Bottom	7.4 ± 2.35	3.1 ± 0.58	4.2 ± 0.8	4.6 ± 0.46	0.21
		Middle	8.3 ± 0.87	9.1 ± 1.2	7.8 ± 0.90	6.1 ± 1.1	0.23
		Top	16.3 ± 3.7	7.1 ± 0.74	5.1 ± 1.08	4.7 ± 1.6	0.0011
	7	Bottom	6.2 ± 0.69	6.1 ± 0.44	4.1 ± 0.49	6.5 ± 0.97	0.093
		Middle	14.4 ± 1.20	9.3 ± 1.06	8.3 ± 0.98	9.5 ± 0.76	0.0018
		Top	18.8 ± 3.91	11.6 ± 1.58	7.7 ±2.14	4.9 ± 1.04	0.0023
2	4	Bottom	6.8 ± 0.59	5.0 ± 0.51	4.5 ± 0.33	11.6 ± 3.1	0.051
		Middle	7.3 ± 0.64	10.4 ± 1.2	8.9 ± 0.57	8.6 ± 1.01	0.13
		Top	11.1 ± 1.7	11.6 ± 1.8	9.7 ± 1.8	8.6 ± 1.5	0.61
	7	Bottom	5.9 ± 0.67	5.9 ± 0.40	5.0 ± 0.93	5.8 ± 1.45	0.90
		Middle	15.7 ± 1.72	10.8 ± 1.45	8.7 ± 1.81	9.7 ± 2.19	0.059
		Top	9.3 ± 2.46	16.1 ± 1.29	12.5 ± 4.83	12.4 ± 2.10	0.56
3	4	Bottom	1.2 ± 0.28	0.37 ± 0.088	0.43 ±0.13	4.1 ± 2.0	0.10
		Middle	0.84 ± 0.39	1.9 ± 0.35	3.3 ± 1.7	2.2 ± 0.88	0.33
		Top	0.76 ± 0.52	3.6 ± 1.4	3.2 ± 0.88	3.6 ± 0.64	0.13
	7	Bottom	0.5 ± 0.17	0.2 ± 0.14	0.4 ± 0.12	0.3 ± 0.19	0.53
		Middle	0.0 ± 0.00	0.4 ± 0.26	1.0 ± 0.37	0.3 ± 0.29	0.13
		Top	0.0 ± 0.00	3.8 ± 0.39	3.9 ± 0.60	3.2 ± 0.97	0.093
4	4	Bottom	7.8 ± 3.3	5.1 ± 0.73	4.3 ± 0.52	6.3 ± 1.68	0.59
		Middle	7.6 ± 1.3	7.2 ± 0.53	6.2 ± 0.54	5.9 ± 0.53	0.46
		Top	7.5 ± 2.04	5.5 ± 1.0	5.4 ± 0.82	5.3 ± 1.0	0.58
	7	Bottom	5.9 ± 0.47	4.8 ± 0.84	4.2 ± 0.44	7.0 ± 1.34	0.13
		Middle	9.8 ± 1.19	7.6 ± 1.24	6.7 ± 0.79	8.0 ± 0.62	0.18
		Top	7.8 ± 1.25	6.9 ± 0.97	6.5 ± 1.74	4.3 ± 1.07	0.35
6	4	Bottom	14.0 ± 1.45	7.0 ± 0.68	7.8 ± 0.76	13.7 ± 2.36	0.017
		Middle	18.9 ± 1.8	20.3 ± 2.4	15.5 ± 1.5	13.8 ± 1.6	0.082
		Top	30.2 ± 6.4	23.1 ± 1.7	20.5 ± 2.5	20.0 ± 3.3	0.23
	7	Bottom	13.8 ± 0.84	10.6 ± 0.84	9.6 ± 0.50	12.9 ± 0.98	0.0049
		Middle	33.5 ± 2.61	21.9 ± 3.27	20.5 ± 2.06	22.9 ± 2.99	0.013
		Top	43.8 ± 6.00	29.3 ± 3.84	31.1 ± 5.10	39.7 ± 3.43	0.12

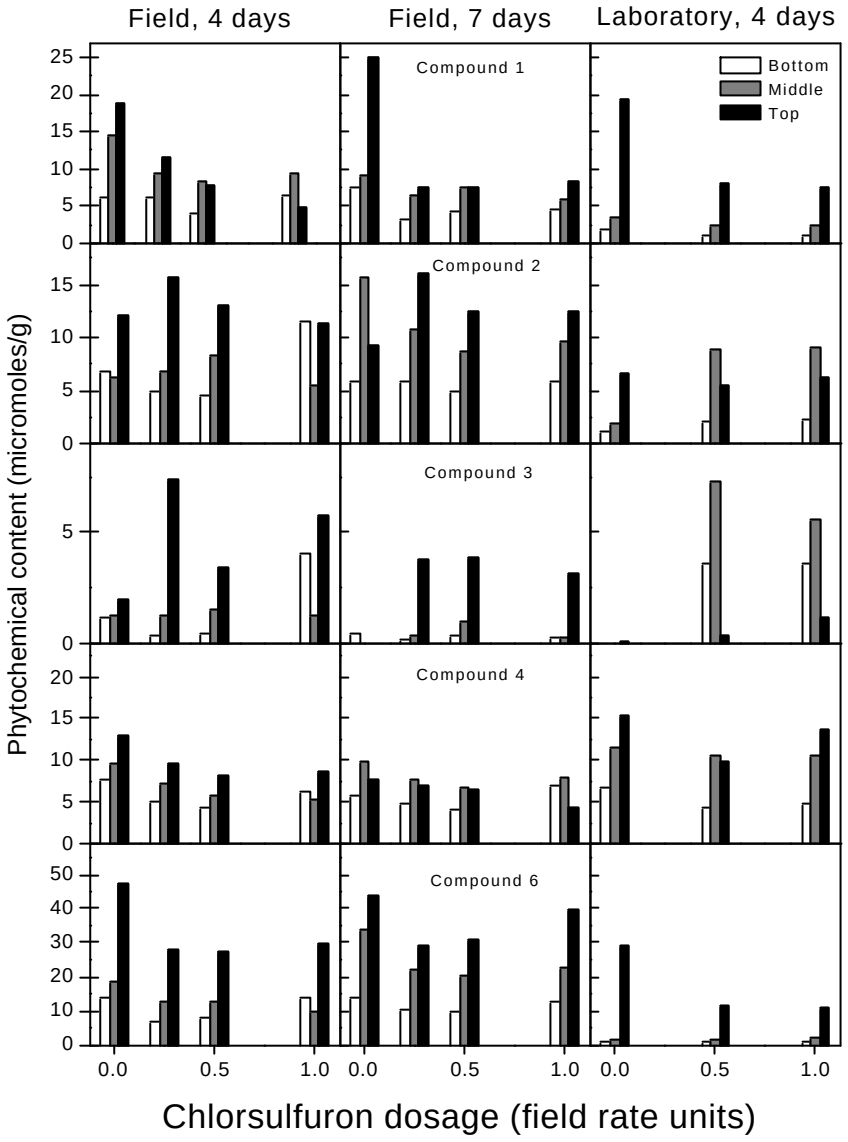


Figure 4.1
Content (µmoles g^{-1} dw) of 5 phenolic compounds in *F. convolvulus* grown under field conditions (middle and right) and in laboratory (left), measured 4 and 7 days (field, physiological time) and 4 days (laboratory) after chlorsulfuron treatment as function of herbicide dosage (times field rate) in bottom, middle and top leaves. N.B. different scales on y-axes.

4.2.2 Effects of UV-B light on phytochemical response

The effects of combinations of UV-B light and chlorsulfuron treatment on the content of phytochemicals in leaves sampled from the middle of the plants are presented in Table 4.6. Six phenolic compounds were identified and quantified (compounds 1-6, cf. Chapter 2).

Unsprayed plants

In unsprayed plants, UV-B increased the content of compounds 1, 2 and 6; the content of compound 4 was unaffected (but very high compared to the above results), and compound 5 was slightly suppressed. Compound 3 was not found in unsprayed plants.

Table 4.6
Concentrations of phytochemicals (µmoles g^{-1} dry weight, means ± s.e.) in *F. convolvulus* 4 days after chlorsulfuron treatment. Plants were grown under laboratory conditions, with or without supply of UV-B light. p values indicate effects of UV-B supply as evaluated by analysis of variance.

Com-pound	Herbicide dosage (field rate units)	No UV-B	With UV-B	p
1	0	4.0 ± 0.24	5.2 ± 0.30	0.012
	0.25	3.3 ± 0.43	3.9 ± 0.18	0.29
2	0	0.6 ± 0.59	7.6 ± 1.90	0.0055
	0.25	1.6 ± 1.05	7.9 ± 2.88	0.066
3	0	0.0 ± 0.00	0.0 ± 0.00	-
	0.25	0.5 ± 0.19	2.0 ± 0.73	0.071
4	0	26.3 ± 2.15	28.1 ± 2.70	0.61
	0.25	23.7 ± 2.37	21.8 ± 2.03	0.57
5	0	4.9 ± 0.32	3.2 ± 0.77	0.069
	0.25	5.3 ± 0.94	2.3 ± 0.75	0.031
6	0	1.9 ± 0.41	6.0 ± 0.58	0.0002
	0.25	1.9 ± 0.59	5.9 ± 0.39	0.0002

Herbicide treatment

There was an interactive effect ($p \leq 0.015$) of herbicide treatment and supply of UV-B light for all compounds, except compound 4 ($p = 0.27$). For compound 1, the effects of herbicide treatment and UV-B were contrary, the herbicide decreasing the content and UV-B increasing it. For compound 2, the increase in content caused by herbicide treatment was slightly diminished when UV-B was supplied. For compound 3, the induction by herbicide treatment was enhanced by UV-B. For compound 5, UV-B reversed the effect of herbicide treatment from positive to negative. For compound 6, the interactive effect seems artificial, since there was hardly any difference between sprayed and unsprayed plants, but a large positive impact of UV-B.

Herbicide treated plants only showed significant UV-B effects for compounds 5 and 6 (Table 4.6). In plants not receiving UV-B light, there was a significant effect of herbicide treatment on compound 3. All other compounds were unaffected by herbicide treatment. In UV-B treated plants, herbicide effects occurred for compounds 1 and 3 ($p = 0.0032$ and 0.022).

4.3 Discussion and conclusions

The observed effect of the rather short time-span of 1 to 4 days on the content of compounds 2, 3 (increase) and 6 (increase followed by decrease) (Table 4.1) shows that the occurrence (and distribution) of these compounds within *F. convolvulus* is very dynamic. Hence, the chosen time-window may be crucial for results and conclusions drawn from the various studies presented in the present report.

Phytochemicals were generally quite similarly distributed within unsprayed plants grown under laboratory and field conditions (Figure 4.1). However, levels were higher in field plants, except for compound 4 (Figure 4.1), indicating an effect of growth conditions on the selected phytochemicals.

Supply of UV-B light in the laboratory increased the similarity with field plants for compounds 1, 2 and 6 in middle leaves – most successfully for compound 2 (Table 4.6). Thus, light conditions seem an important factor for those compounds. UV-B did not affect compounds 3 and 4 in unsprayed plants.

When the plants were sprayed with chlorsulfuron, the phytochemical response differed somewhat between laboratory and field plants (Figure 4.1): For compound 2, the content was generally higher in field plants. This may partly be due to differences in light conditions, since compound 2 is increased by UV-B light, and this part of the light spectrum was absent under the standard laboratory conditions. The vertical distribution of compound 2 differed between sprayed field plants and sprayed laboratory plants, with an increase in middle leaves in laboratory plants, whereas the distribution in field plants was hardly affected. The response in compound 3 to spraying also differed between growth conditions: In laboratory plants, an increase/induction was found in middle and bottom leaves, whereas field plants responded by an increase in top leaves. Part of the explanation may be differences in light conditions between the two exposure situations. From Table 4.6, it is obvious that UV-B light increases the response to spraying in compound 3 in middle leaves, but effects on the vertical distribution is unknown. Levels of compound 6 were generally higher in field plants than in laboratory plants (Figure 4.1), and the vertical distribution was more homogeneous in field plants. Differences in light conditions may be part of the explanation for the differences in level and distribution, since compound 6 is increased by UV-B (Table 4.6).

All in all, it was shown that growth conditions affected not only concentrations and distribution of phytochemicals in unsprayed plants, but also the effect of herbicides on the same phytochemical compounds. Light conditions (UV-B) could explain part, but not all of the phytochemical difference between plants grown under laboratory and field conditions.

The main conclusions that may be drawn from the described experiments are:

- The vertical/age distribution of phytochemicals was fairly similar for control plants grown under laboratory and field, but concentrations were generally higher in field plants.
- For compounds 2, 3 and 6, the effects of chlorsulfuron treatment on phytochemicals differed between plants grown under standard laboratory conditions and plants grown under field conditions.
- Introduction of UV-B supply in the laboratory increased the content of compounds 1, 2 and 6 in unsprayed plants, and this increased the similarity in phytochemical content between laboratory and field plants.
- In chlorsulfuron treated plants, UV-B seemed to work contrary to the herbicide on compounds 1, 2 (slightly) and 5, whereas the inductive effect of the herbicide on the content of compound 3 was enhanced by UV-B.
- For compounds 2, 3 (to some degree) and 6, the supply of UV-B caused the content of phytochemicals in chlorsulfuron sprayed laboratory plants to approach that of field plants in middle leaves.

- The total influence of UV-B on phytochemical response following chlorsulfuron treatment remains unresolved, since only middle leaves were analysed for UV-B effects.

5 Dose dependence and persistence of chlorsulfuron-induced phytochemical changes

Changes in phytochemical composition of a plant may have impact on the fitness of the plant, for example in terms of plant growth and reproduction or changes in palatability of the plant to herbivores. The dose-response relationship and the durability of the response are of paramount importance to the potential impacts of the phyto-chemical changes. Therefore, a dose-assay and a time-assay were set up to investigate the changes in concentration of some phenolic constituents after spraying with chlorsulfuron, as a function of dosage and time, respectively.

5.1 Methods

F. convolvulus plants were grown in 11 cm plastic pots filled with standard pot soil. At the 5-7 leaves stage, the plants were sprayed in a pot-sprayer (Christensen, Slagelse, DK) with the herbicide Glean 20 DF (20% chlorsulfuron) and the detergent Citowett (0.05 v%). The pot-sprayer was mounted with Hardi nozzles no. 16 and adjusted to deliver 200 l ha^{-1} at 2 bar. Six dosages were applied, viz. 0.03125, 0.0625, 0.125, 0.25 0.5, and 1 times the recommended field rate, which is 4 g chlorsulfuron ha^{-1}. For each dosage, five replicates (plants) were made. Two control groups were sprayed with water and Citowett. In total, 40 plants were used. After five days, one middle leaf (third leaf from the bottom) was abscised, freeze-dried and analysed for phenolic constituents.

In the time assay, two dosages corresponding to 0.25 and 0.5 times the recommended field rate were used. As controls we used plants sprayed with water and Citowett. Three replicates, each with three plants, were run per treatment per harvest time, and in total 108 plants were included in the assay. Harvest was carried out at four points in time, i.e. 4 days, 8 days, 16 days and 30 days after spraying.

At harvest, one leaf from the middle of each harvested plant was removed, freeze-dried and analysed for phenolic compounds. The surface area of all leaves was measured by use of an area meter (Li-Cor 3100, Lincoln, Nebraska) and dry weight of the plant not used for chemical analysis was obtained after freeze drying for 24 h.

The experiments were carried out from March to May 1998. Plants were grown in a green house. Twenty-four hours after spraying, the plants were moved to a controlled-environment chamber and kept at 20 C, 16:8 h light:dark, and 70% RH.

5.1.1 Statistics

Differences in mean concentration of the phenolic compounds in leaves were tested in a Tukey 's Studentized Range (HSD) Test by use of the SAS statistical Software Program assuming p=0.05 as level of significance.

5.1.2 Chemical analysis

The leaves were analysed for six phenolic compounds, i.e. 3-O-E-caffeoylquinic acid (neochlorogenic acid) (compound 1), 1-O-E-caffeoyl-beta-D-glucose (compound 2), 3-O-E-p-coumaroyl-beta-D-glucose (compound 3), caffeoyl tartronic acid (compound 4), caffeoyl meso-tartronic acid (compound 5) and quercetin-3-O-beta-D-glucuronide (compound 6). Chemical analysis and numbering of the compounds follow Chapter 2. Compound 5 was not identified in any samples from the present study.

5.2 Results

5.2.1 Dose-assay

Only the results dealing with compounds 2 and 3 are presented here. At 0.03125 times the recommended field rate, were there is no significant changes in concentration of compounds 2 and 3 in the leaves, although concentration of compound 2 was elevated compared to the control. At 0.0625 times the recommended field rate, a clear increase was observed for both compounds. Compound 2 reached its maximum leaf concentration in plants sprayed with 0.125 – 0.5 times the recommended field rate and no further increase was seen (Fig. 5.1). Compound 3 followed the same pattern and reached maximum concentration at 0.25 times recommended field rate with a tendency to further increase at higher dosages.

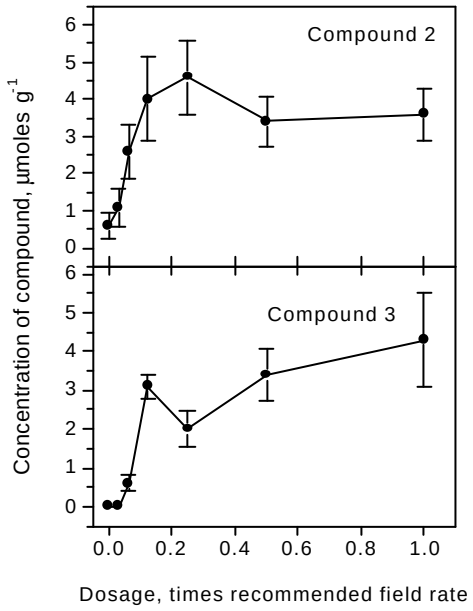


Figure 5.1
Concentration of compounds 2 and 3 in middle leaves of *F. convolvulus* as a function of chlorsulfuron dosage (dosage in multipla of recommended field rate).

5.2.2 Time-assay

Results for all detected compounds are presented. Concentration of compounds 1, 4, and 6 in middle leaves decreased significantly with age in control plants. During the first 4 days of our observations, the concentration of compound 1 drops from 4.2 to 1.3 μg g^{-1} dw. Compound 4 drops from 19.0 to 6.3 μg g^{-1} dw and concentrations of compound 6 decreases from 4.5 to 1.2 μg g^{-1} dw (Figure 5.2). The content of compound 1, 4 and 6 was reduced in leaves from sprayed plants but there was no difference between 0.25 and 0.5 tims the recommended field rate (Fig.5.2). The effect was just significant 4 days after spraying. After 8 days differences between treatments could not be detected anymore.

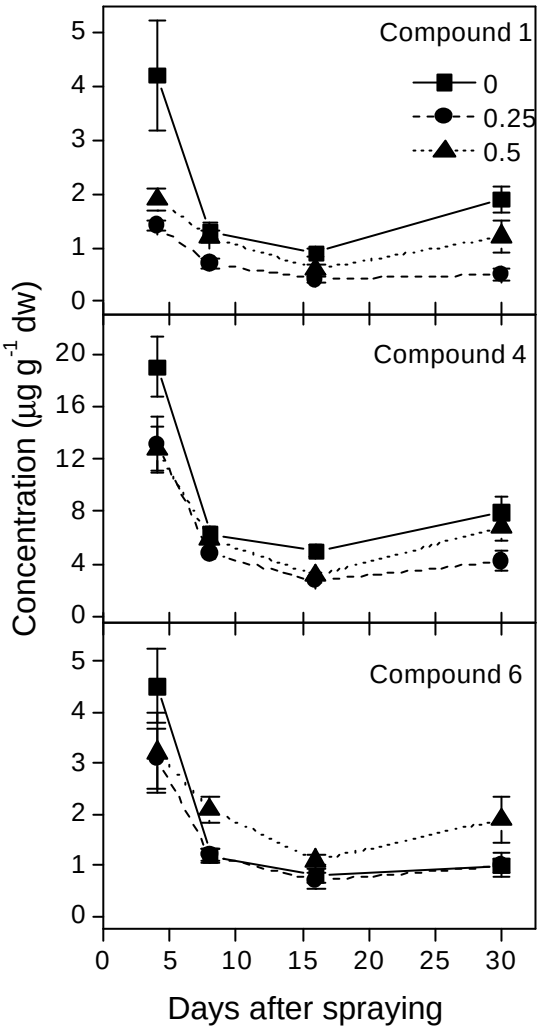


Figure 5.2
Concentrations (μg g^{-1} dw) of compounds 1, 4 and 6 in middle leaves of *F. convolvulus* as a function of time (days) after spraying for two chlorsulfuron dosages, 0.25 and 0.5 times the recommended field rate.

The concentration of compound 2 was considerably higher in leaves from plants sprayed with 0.5 times the recommended field rate than in the control leaves (Fig. 5.3). This effect could not be detected 8 days after spraying. 0.25 of recommended field rate did not have any effect on the concentration of compound 2.

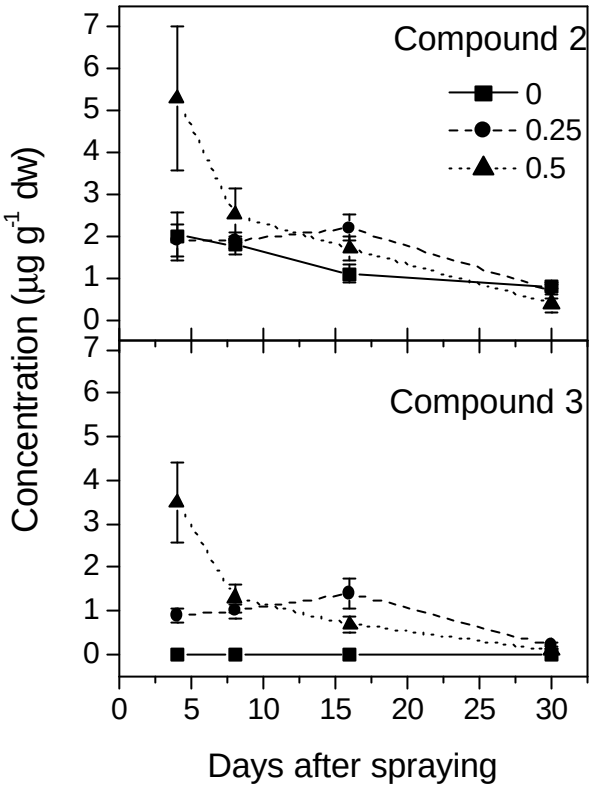


Figure 5.3
Concentrations (µg g^{-1} dw) of compounds 2 and 3 in middle leaves of *F. convolvulus* as a function of time (days) after spraying with two chlorsulfuron dosages, 0.25 and 0.5 times the recommended field rate.

Compound 3 was only found in sprayed plants and the concentration in the leaves was dosage dependent (Fig. 5.3). The mean middle leaf concentration 4 days after spraying was 3.5 µg g^{-1} dw (s.e = 0.91) in leaves sprayed with 0.5 times the lable rate dosage and 0.9 µg g^{-1} dw (s.e =0.18) in leaves sprayed with 0.25 times the recommended field rate. After 8 days, the concentration of compound 3 in leaves treated with 0.5 times the recommended field rate decreased to the same level as in leaves treated with 0.25 times the recommended field rate. This level was approximately the same after 16 days. At day 30, compound 3 could still be detected in some but not all of the plants, and only in trace amounts.

The mean area of middle leaves from control plants increased steeply until day 16, whereafter growth ceased or some of the leaves started to loose area (Fig. 5.4). The growth of leaves exposed to chlorsulfuron was low and insignificant

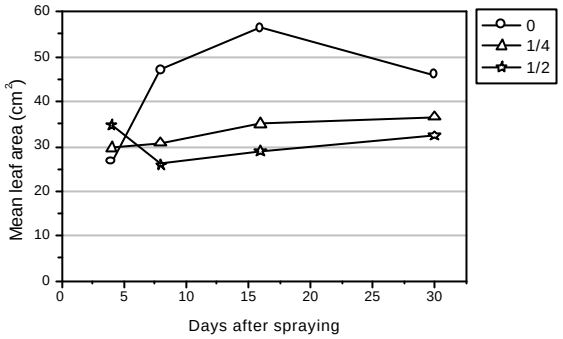


Figure 5.4
Mean leaf area (cm^2) of middle leaves from *F. convolvulus* as a function of time (days) after spraying with chlorsulfuron at three dosages: 0, 0.25, and 0.5 times the recommended field rate.

5.3 Discussion

The dose-response reaction of the concentration of compounds 2 and 3 in the leaves seems to be dominated by a sigmoid response mechanism within the investigated concentration range of chlorsulfuron (Fig. 5.1).

The reduction of the content of compounds 1, 4, and 6 in *F. convolvulus* leaves was consistent with what has been observed in the experiment with several different herbicides (Chapter 3). The herbicide-induced depression of these compounds made a significant difference between herbicide-exposed plants and unexposed plants. The difference disappeared with time and was not detectable after 8 days (Fig. 5.2) because the level of these compounds dropped in the control plants.

In leaves subjected to chlorsulfuron treatment at the highest dosage the concentration of compound 2 was considerably higher than in the control plants 4 days after spraying. Four days later this effect disappeared. Compound 3 was only found in chlorsulfuron-sprayed plants as seen before (Chapters 2 and 3). The concentration was dependent of chlorsulfuron dosage. At the highest dosage, concentration of compound 3 dropped steeply from day 4 to day 8 (Fig. 5.3). The decline was exactly parallel to the decline of compound 2 at the highest chlorsulfuron dosage. The concentration curves describing compounds 2 and 3 at the low chlorsulfuron dosage follow similar tracks.

Sixteen days after spraying, the chlorsulfuron treated plants still contained significant amounts of compound 3, but 30 days after spraying not all plants contained detectable amounts. Compound 3 seems very applicable as indicator for exposure to chlorsulfuron at 0.065 times the ideal recommended field rate (0.25 g per ha) until 16 days after actual exposure. Considering the ideal growing conditions during the experiment, which made the plants grow very fast and finish there life cycle fast, it may be difficult to find any indicator substance with a longer lifetime. Under field conditions with lower average temperatures the indicator may even live longer. It is surprising that 0.25 times the recommended field rate of chlorsulfuron appeared to induce a different concentration development of compound 3 in time in the middle leaves compared to 0.5 times the recommended field rate. Again, the pattern

was consistent with compound 2. At 0.5 times the recommended field rate the concentrations of compounds 2 and 3 were relatively high after 4 days but then the major part of this peak concentration was metabolised during the next 4 days. However, at the low dosage (0.25 times the recommended field rate) there were no signs of a net degradation from day 4 to day 16 of compound 3! It should be mentioned that at the applied herbicide dosages, the growth of the plants is severely inhibited (Fig. 5.3). Thus, there is hardly any dilution of herbicide or constituents taking place in the sprayed plants.

6 The effect of herbivory on phytochemical profile

The present experiments were conducted to test if herbivores and herbicide together cause a (consequent) change in the content of selected phenolic compounds. It was observed in Chapter 4 that UV-B-radiation in general increases the amount of phenolic compounds in *F. convolvulus*. Rousseaux *et al*. (1998) observed that herbivorous insects eat more on plants that were not exposed to UV-B radiation. If this is the case for *G. polygoni* it may imply that an interaction between effects of UV-B and herbivory exists. It is therefore of interest to study if such interactions exist for compounds shown to increase due to UV-B and chlorsulfuron treatment.

6.1 Materials and methods

The effect of herbivore load was assessed by means of artificial defoliation in order to reduce the variability of the data due to for example mortality of the larvae or abnormal feeding behaviour. However, first a trial was conducted to verify that comparable responses are found in plants exposed to artificial and natural defoliation, respectively. The combined effect of herbivory and UV-B radiation was assessed in a greenhouse experiment.

6.1.1 Comparison of phytochemical responses to natural and simulated herbivory

Thirty *F. convolvulus* plants possessing approximately five leaves each were placed in a controlled-environment-chamber administered at 20°C, 16 h photoperiod, and a relative humidity of 70%. The following day, 10 larvae were placed on each of 10 plants. Ten other plants were exposed to simulated herbivory equal to the feeding activity by 10 *G. polygoni*-larvae (Table 1 presents the leaf area removed during the experiment based on a pilot experiment) and another 10 plants were left without leaf damage. Both natural and simulated herbivory were initiated on the third leaf counted from the bottom of the plant. After 6 days, the damaged leaves were harvested. Those exposed to natural herbivory were gently cleaned for faecal deposits with water before they were freeze-dried singly. The other leaves were freeze-dried without further handling. Early in development, larvae are more or less aggregated. As they grow, they become more dispersed on the leaves. Therefore, the artificial defoliation was conducted as follows (Table 6.1). On day 1, only one hole was made, and thereafter the number of holes created increased to 10 on day 3 (equal to the number of larvae). Hereafter, the size of the holes increased. If specimens died or disappeared (probably dead) they were replaced with specimens of the same size and age.

Table 6.1
Schematic presentation of the procedure for simulated leaf damage.

Day	Area removed per day (cm^{-2})	
1	0.125	(1 hole)
2	0.65	(4 holes)
3	1.70	(10 holes)
4	3.35	(10 holes)
5	5.73	(10 holes)

6.1.2 Effects of herbivore density on the content of selected phenolic compounds

As described above, there is a marked effect of herbivore density on the quality of the host plant to the herbivore when sprayed with chlorsulfuron. Therefore, an experiment was set up to measure the content of selected phenolic compounds in plants when manipulating the herbivore density and chlorsulfuron treatment.

Ninety-six *F. convulvulus* plants with five true leaves each, were placed in a controlled-environment-chamber. The conditions in the chamber were set at 20 °C, 16 h photoperiod and 70% RH. Half of the plants were sprayed with chlorsulfuron in a dosage of 0.5 times the recommended field rate in cereals (10 g ha^{-1} formulated chlorsulfuron, i.e. 2 g a.i. ha^{-1}). The other half was sprayed with water. The day after treatment the plants were exposed to artificial defoliation. The defoliation (herbivory) was simulated by cutting out pieces of leaf each day equal to the feeding activity of 0, 10, 20, and 40 *G. polygoni*-larvae placed on the same leaf. Removal of leaf material was done on the third leaf from the bottom on plants with five leaves and was initiated on the first day after spraying. On day 7 after spraying, the treated (”eaten”) leaf was harvested. All harvested leaves were freeze-dried singly and analysed for the selected phenolic compounds. All treatments were made as three independently prepared spray solutions with two samples per replicate.

6.1.3 Effects of herbivory and UV-B-light in combination

One hundred and twenty *F. convulvulus* seedlings were transplanted into pots and placed on watering tables in a greenhouse. Sixty potted plants were placed under UV-B exposure and another 60 pots placed without supplementary radiation. When the plants possessed eight leaves, 36 plants of similar size were selected from each treatment. The plants were then sprayed with chlorsulfuron in the following dosages: 0, 0.125 and 0.25 times the recommended field rate. Every treatment was replicated six times.

Four days after spraying, 10 newly hatched larvae were placed on the lower side of the third leaf counted from the bottom of the plant. The larvae were confined on the plant by a fine 1-mm mesh net formed as a bag. Seven days after spraying the leaf with larvae was harvested and freeze-dried for subsequent chemical analysis.

For plants receiving UV-B light, this was supplied by Phillips TL 12/ 40 W lamps placed 0.5 m apart and 1 m above the plants. The lamps were turned on for a period of 5 h every day, imitating the daily UV-B influx on sunny

days in the beginning of July. A cellulose acetate filter was used to absorb light below 290 nm.

During the experiment, plants were watered from the bottom, the temperature in the greenhouse was maintained at a target temperature of 22°C with variations due to external weather, and the photoperiod was 16h daylight. The plants were supplied with additional light if light intensity dropped below 5 klux; light was switch off when intensity raised above 25 klux. The artificial light source gave an approximate photo flux density of 300 $\mu E\ m^{-2}\ sec^{-1}$ at the soil surface. The plants were moved around during the experiment to avoid position effects.

6.1.4 Spraying procedure

Plants were sprayed with a pot sprayer designed for automatic and controlled spraying of larger plants (Kristensen pot sprayer, Ringsted, Denmark; $l \times w \times h = 120 \times 100 \times 170$ cm). Trials with chlorsulfuron were conducted with the surfactant Citowett (BASF) added to the spray solution (0.5% v/v). Control plants were sprayed with water. The sprayer was equipped with two Hardi flat fan nozzles type 411014 separated by 53 cm and used at a working pressure of 2 bar. The sprayer was calibrated to deliver a spray volume of 200 $l\ ha^{-1}$.

6.1.5 Chemical analyses

Six phenolic compounds have been identified from *F. convolvulus*, as described in Chapter 2. We analysed the plant material for these six compounds. The compounds identified were: 3-E-caffeoylquinic acid (compound 1), 1-E-caffeoyl-β-D-glucose (compound 2), 1-E-p-coumaroyl-β_D-glucose (compound 3), 2-E-caffeoyl-tartronic acid (compound 4), 2-E-caffeoyl-meso-tartaic acid (compound 5), and quercetin-3-O-β-D-glucuronide (compound 6). For determination of the phytochemical profile of the leaves, extraction with ethanol and kampfeol triosid was performed (Chapter 2). The phenolic compounds were separated by HPLC, and characterised by negative ion FAB-MS as well as 1- and 2-dimensional NMR techniques.

6.1.6 Statistical analyses

Natural and artificial defoliation was compared by testing the concentration of each compound in a t-test. A two-way ANOVA was performed to test for effects of herbivory and herbicide treatment on the content of each compound. The first test was done with interactions, but as the interaction was non-significant, tests with only main effects were used. Regression analysis was performed to describe the effect of herbivory on compound 2 and 3. The effect of UV-B radiation and herbivory in combination were tested by means of two-way ANOVA. Single comparisons were made with Tukey t-tests.

6.2 Results

6.2.1 Comparison of phytochemical responses to natural and simulated herbivory

No significant effects on the content of selected phenolic compounds were found of damaging the leaves by either artificial or natural herbivory. None of the treated leaves analysed for phenolic compounds were different from the control plants. (Table 2).

Table 2
Statistical analyses of the impact of type of leaf damage on the concentration of phenolic compounds (One-way ANOVA).

Compound	DF	F	p
1	2	0.47	0.6389
2	2	0.66	0.5329
3	2	3.30	0.0722
4	2	0.49	0.6235
5	2	3.22	0.0759
6	2	0.11	0.8963

6.2.2 Effects of herbivore density on the content of selected phenolic compounds

The content of all analysed compounds changed significantly in response to herbicide treatment. Compounds 1, 4 and 6 decreased significantly (Tukey-t-test), and compounds 2 and 3 increased (Table 3 and 4). Only compound 2 showed significant changes in relation to herbivory. The concentration of compound 2 decreased with increased herbivory for both sprayed and unsprayed specimens (Fig 6.1A). Compound 3 was only found in detectable concentration in sprayed plants. For the sprayed plants, the concentration decreased with increasing herbivory (Fig 6.1B).

Table 3
Statistical analyses of the impact of herbicide treatment and artificial defoliation on the concentration of phenolic compounds (Two-way ANOVA without interactions).

Compound	Effect	DF	F	p
1	Herbicide	1	27.98	<0.0001
	Herbivory	3	1.91	0.1310
2	Herbicide	1	32.92	<0.0001
	Herbivory	3	4.78	0.0076
3	Herbicide	1	70.86	<0.0001
	Herbivory	3	0.32	0.8098
4	Herbicide	1	25.30	<0.0001
	Herbivory	3	2.63	0.0556
6	Herbicide	1	4.84	0.0339
	Herbivory	3	0.13	0.9441

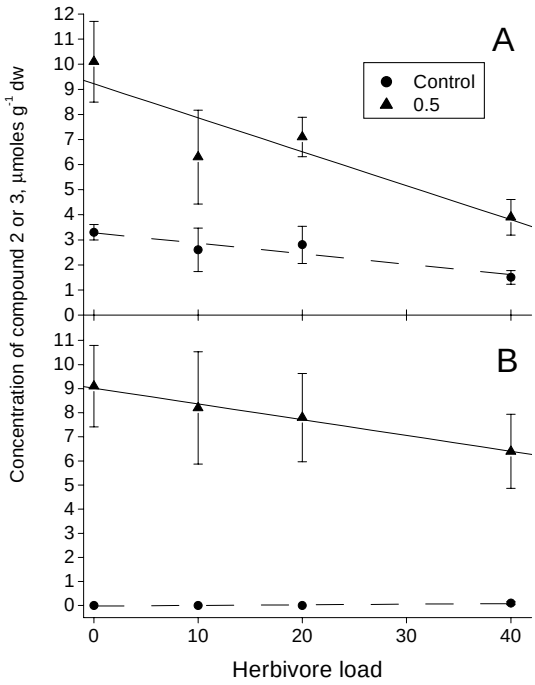


FIGURE 6.1
Relationship between the degree of artificial defoliation and the concentrations of compound 2 (Fig. A) and compound 3 (Fig. B). The herbivore load is artificial defoliation equally the feeding of a specified number of *G. polygoni* larvae. Circles represent data from unsprayed plants and triangles represent plants treated with chlorsulfuron at a dosage of 0.5 times the recommended field rate. The concentration of the compounds is given in µmoles g dry weight^{-1}. Error bars represent Standard Error of Mean

TABLE 4
The effect of chlorsulfuron treatment and simulated *G. polygoni* herbivory on the content of selected phenolic compounds in black bindweed (*F. convolvulus*). The analyses were made on the artificially damaged third leaf counted from the bottom of the plant.

		Defoliation equal to feeding by a specified number of larvae			
Compound	Dosage	0	10	20	40
1	0	3.0 ± 0.38	3.1 ± 0.56	3.0 ± 0.61	2.1 ± 0.34
	0.5	1.9 ± 0.21	1.5 ± 0.19	1.2 ± 0.07	1.1 ± 0.22
2	0	3.3 ± 0.31	2.6 ± 0.87	2.8 ± 0.74	1.5 ± 0.27
	0.5	10.1± 1.61	6.3 ± 1.87	7.1 ± 0.79	3.9 ± 0.71
3	0	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.03	0.1 ± 0.06
	0.5	9.1 ± 1.69	8.2 ± 2.33	7.8 ± 1.83	6.4 ± 1.54
4	0	15.2 ± 2.50	14.3 ± 2.48	11.7 ± 1.78	9.4 ± 1.73
	0.5	8.6 ± 1.00	6.9 ± 0.60	5.7 ± 0.58	5.8 ± 0.91
6	0	2.1 ± 0.52	2.1 ± 0.26	1.7 ± 0.11	1.9 ± 0.30
	0.5	1.3 ± 0.23	1.5 ± 0.22	1.7 ± 0.16	1.7 ± 0.05

6.2.3 Effects of herbivory and UV-B-light in combination on the phenolic compounds

Two-way ANOVAs were conducted for each compound and none showed significant interactions. Therefore, test were made with main effects only (i.e. herbicide treatment and UV-B radiation).

UV-B radiation and spraying with chlorsulfuron had a significant impact on the concentration of both compound 1 ($p = 0.0063$ and $p < 0.0001$ respectively in a two-way ANOVA) (Fig. 6.2) and compound 2 ($p = 0.0091$ and $p = 0.0005$; two-way ANOVA). Spraying caused a lower concentration of compound 1, but the two herbicide dosages were not different ($p > 0.05$; Tukey). However, the concentration was higher in plants exposed to UV-B radiation than in control plants. For compound 2, only the highest spray dosage caused a significant increase compared to the control ($p < 0.05$; Tukey t-test). The UV-B-radiation caused higher concentrations in exposed plants.

Compounds 3 and 4 were not affected by UV-B radiation ($p = 0.0824$ and $p = 0.0890$ respectively; two-way ANOVA). Herbicide treatment affected the concentration of compound 3 ($p = 0.0074$; two-way ANOVA). There was a tendency to reduced concentration of compound 4 with increasing herbicide treatment.

Exposure to UV-B radiation and herbicide spraying with chlorsulfuron had no effect on the concentration of compound 5 ($p = 0.3901$; $p = 0.1016$; two-way ANOVA).

UV-B-radiation had a significant effect on the concentration of compound 6 ($p = 0.0012$; two-way ANOVA). The concentration was higher in UV-B treated plants. Spraying with chlorsulfuron did not change the concentration of compound 6 ($p = 0.1167$; two-way ANOVA).

6.3 Discussion

The phytochemical response of the plants exposed to herbicide and to UV-B radiation showed the same trends as described for in Chapter 4, except that the herbicide caused a decrease in compound 4. The difference may be due to differences in timing of harvesting.

The absolute concentration of compound 2 was high in the experiment with UV-B radiation compared to the previously presented data from controlled environmental chambers. The first experiment was conducted in the greenhouse, which both have higher temperature fluctuations and probably also experience higher light intensities. The content of the phenolic compounds in this study with UV-exposure and herbivory were comparable to the UV study without herbivory (Table 4.6). It was also found that the levels for the phenolic compounds in the experiment with artificial defoliation were comparable to the control plants with herbivores in the ”UV-B experiment”, except again for compound 4 (Table 6.3 and Figure 6.1).

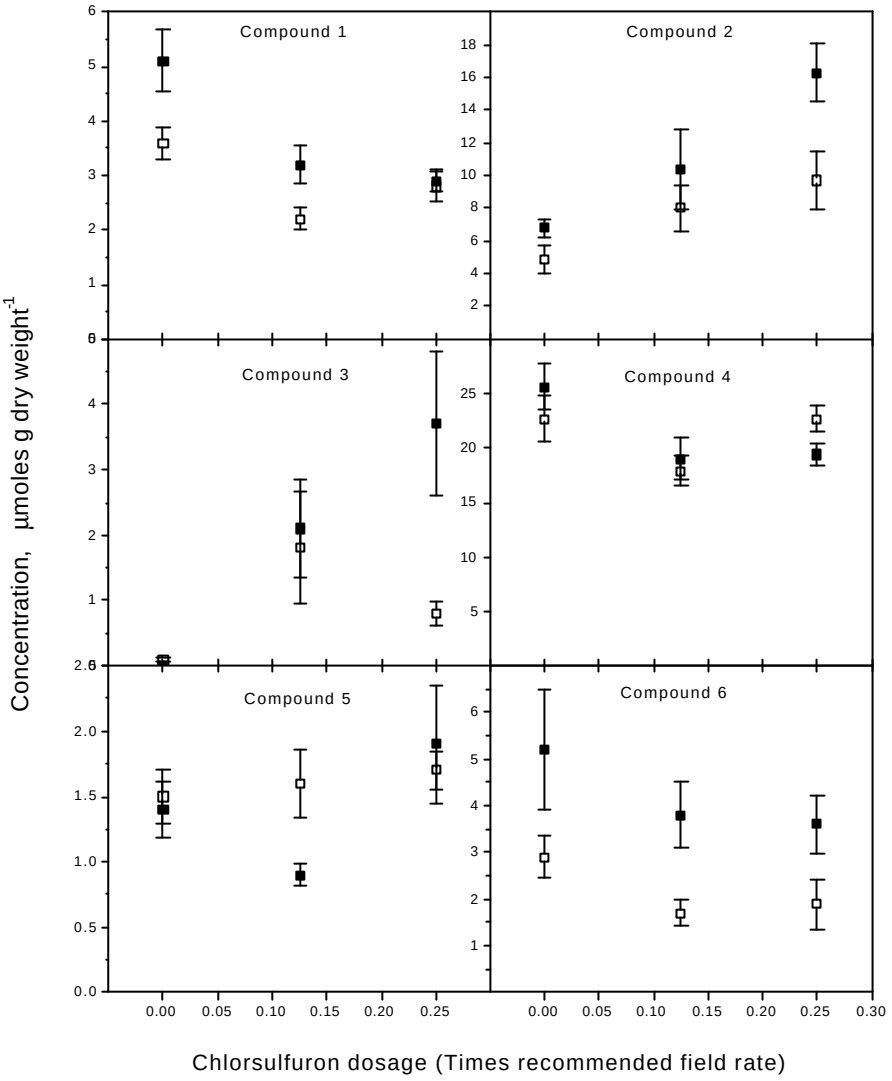


Figure 6.2
Relationship between herbicide dosage, UV-B radiation and the content of selected phenolic compounds. The open symbols represent plants were unexposed to UV-B radiation and closed symbols represent UV-B exposed plants. Bars represent standard error of mean.

It was the aim of this study to find if any of the selected compounds increased with herbicide dosage and herbivore load. No such combination was found. However, if we should proceed along the working hypothesis that phenolic compounds elicit the observed effects only two compounds are likely candidates i.e. compounds 2 and 3, which increase with herbicide treatment.

7 Phenolic compounds and mortality of herbivorous larvae

The observed increased in mortality of *G. polygoni* larvae with herbivore density and dosage of the herbicide chlorsulfuron has led us to the suggestion that *F. convolvulus* possesses a herbivore-induced chemical defence, which is enhanced by the chlorsulfuron treatment. It is our hypothesis that phenolic compounds could be active in this relationship. We take a starting point in those compounds identified in Chapter 2 which in Chapter 4 have been shown to increase with herbicide treatment, i.e. compound 2 and compound 3. For these compounds we will establish correlations between the concentration and the number of surviving *G. polygoni* larvae. Therefore, this chapter presents an experiment designed to document correlation between these phenolic compounds and larval survival.

7.1 Materials and methods

The content of selected phenols in *F. convolvulus* plants treated in four different ways was measured for the third leaf on day 1, 4, 7 and 10 after treatment. The treatments encompassed: 1) herbicide treatment 2) herbivory, 3) both herbicide treatment and herbivory 4) no treatment plants. Herbicide treatment implies that plants were sprayed with 0.5 times the recommended field rate of chlorsulfuron (i.e. 2 g ha^{-1}). Herbivory was introduced to adding 20 newly hatched *G. polygoni*-larvae to the plant one day after spraying. The larvae were placed on the lower side of the third leaf counted from the bottom of the plant. Untreated plants were sprayed with water.

The experiment was conducted as a controlled-environment-chamber experiment. Seedlings of black bindweed (*F. convolvulus*) were transplanted singly to pots and placed in the greenhouse until they possessed five true leaves. Each treatment was replicated three times. All plants were confined in polyurethane cylinders until the adult beetles emerged from the soil.

Simultaneously with the chemical analysis, the survival of the larvae were registered. Upon harvest, the larvae residing on the leaves were moved to another leaf. The harvested leaves were cleaned for faecal deposits and freeze-dried before the chemical analysis. A schematic presentation of the experiment is given in Table 1.

Table 7.1
Schematic presentation of the experimental design

Treatment	Day 1	Day 4	Day 7	Day 10
Control plants	3 plants	3 plants	3 plants	3 plants
Sprayed	3 plants	3 plants	3 plants	3 plants
Herbivory (20 larvae)	none	3 plants	3 plants	3 plants
Sprayed and herbivory	none	3 plants	3 plants	3 plants

7.1.1 Spraying procedure

Plants were sprayed with a pot sprayer designed for automatic and controlled spraying of larger plants (Kristensen pot sprayer, Ringsted, Denmark, l × w × h = 120 × 100 × 170 cm). Trials with chlorsulfuron were conducted with the surfactant Citowett (BASF) added to the spray solution (0.5% v/v). Control plants were sprayed with water. The sprayer was equipped with two Hardi flat fan nozzles type 411014 separated by 53 cm and used at a working pressure of 2 bar. The sprayer was calibrated to deliver a spray volume of 200 l ha^{-1}.

7.1.2 Chemical analyses

Six phenolic compounds have been identified from *F. convolvulus*, as described in Chapter 2. We analysed the plant material for these six compounds. The compounds identified were: 3-E-caffeoylquinic acid (compound 1), 1-E-caffeoyl-β-D-glucose (compound 2), 1-E-p-coumaroyl-β-D-glucose (compound 3), 2-E-caffeoyl-tartronic acid (compound 4), 2-E-caffeoyl-meso-tartaic acid (compound 5), and quercetin-3-O-β-D-glucuronide (compound 6). For determination of the phytochemical profile of the leaves, extraction with ethanol and kampfeol triosid was performed (Chapter 2). The phenolic compounds were separated by HPLC, and characterised by negative ion FAB-MS as well as 1- and 2-dimensional NMR techniques.

7.1.3 Statistical analyses

An ANOVA was performed to test for effects of herbivory and herbicide treatment and time on the content of each compound. The first test was done with interactions, but as interactions were non-significant, a test with only main effects was used. Regression analysis was performed to describe the realtionship between compound 2 or compound 3 and larval survival. Single comparisons were made with Tukey t-tests.

7.2 Results

7.2.1 Phytochemical responses of *F. convolvulus* to chlorsulfuron, herbivory and time

All compounds were significantly affected by the herbicide treatment. However, only the concentration of compound 2 and compound 3 increased with herbicide treatment (Tukey t-test), whereas all the other compounds (i.e. compounds 1, 4 and 6) decreased when treated with chlorsulfuron (Tukey t-test) (Table 7.2). There was a significant effect of herbivory on the content of compound 4, which increased with herbivory.

7.2.2 Relationship between content of phenolic compounds and insect survival

The data presented in Table 7.2 and Figure 7.1 are combined of leaves harvested in intervals of three days after spraying. In order to ensure that the data were not biased due to harvesting date, the whole data set was tested in a one-way anova with the principal factor being time and the concentration of compounds 2 and 3 as covariates. The time variable was not significant and the regression analysis also showed that only compound 3 had significant impact on the survival of *G. polygoni* larvae, i.e. the rate of decrease with

dosage was significant different from 0 (t-test, p=0.0367). Despite this result, we chose to look at both compounds 2 and 3. These two compounds have repeatedly been shown to increase with herbicide dosage/treatment, and their structure is very similar. Thus, they may have the same mode of action if they are responsible for the observed reduction in larval survival. The regression analysis of survival as a function of concentration of compound 3 revealed the following equation: {Compound 3 = 13.32 – 0.725 × Dosage, r = 0.635, p = 0.005}. Compound 2 did correlate with the survival of the larvae, but not in the same degree as compound 3 {Compound 2 = 14.11 – 0.441 × Dosage, r = 0.505, p = 0.033}. A regression analysis of larval survival and the total amount of both compound 2 and 3 also showed a significant correlation between these two parameters, i.e. {Compound 2+3 = 14.53 – 0.366 × Dosage, r = 0.644, p = 0.004}. The total amount has a slightly better correlation than that for compound 3 and a much better one than that for compound 2.

Table 7.2
Concentration of phenolic compounds in black bindweed (*F. convolvulus*) in relation to time (days) since herbicide treatment, herbicide treatment with chlorsulfuron and feeding by 20 *G. polygoni* larvae.

Compound	Time	Control	Herbicide	Herbivory	Herbivory and herbicide
1	1	4.2±0.33	2.3±0.16-	-	-
	4	6.0±2.18	2.1±0.40	4.8±1.82	2.9±0.22
	7	2.2±0.29	1.9±0.50	5.8±2.89	2.3±0.83
	10	2.2±0.31	1.3±0.10	3.6±0.13	1.2±0.24
2	1	0.6±0.59	0.0±0.02	-	-
	4	2.3±1.79	7.8±3.59	2.1±1.83	6.1±3.12
	7	0.4±0.27	3.0±2.12	5.2±4.28	8.2±2.26
	10	0.1±0.06	7.4±1.95	5.1±0.54	6.6±1.89
3	1	0	0	-	-
	4	0	7.9±3.96	0	5.8±5.55
	7	0	1.3±0.20	0	7.4±1.60
	10	0	3.8±1.18	0	3.9±0.40
4	1	21.5±1.44	13.9±0.57	-	-
	4	19.4±8.10	7.5±4.28	29.8±6.88	15.2±5.95
	7	13.9±0.63	10.8±1.22	24.8±5.18	10.0±0.67
	10	15.6±1.44	7.9±1.44	26.2±2.87	10.9±0.96
6	1	1.8±0.20	0.7±0.25	-	-
	4	1.8±0.86	0.8±0.18	1.9±0.75	0.6±0.09
	7	0.8±0.36	1.1±0.40	2.4±1.42	0.9±0.27
	10	0.9±0.047	0.9±0.12	1.4±0.22	0.8±0.17

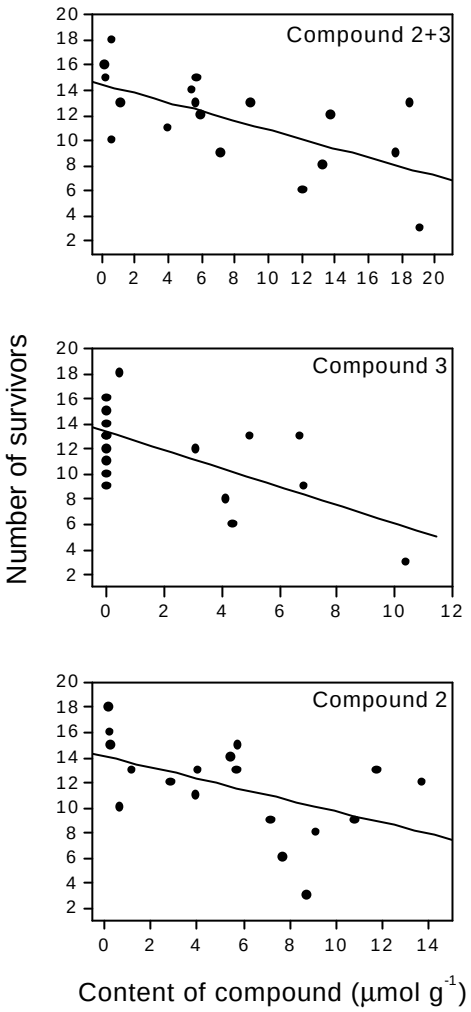


Figure 7.1
The relationship between the number of surviving *G. polygoni* larvae and the concentration of the compounds 2, 3 and both added. The initial number of larvae was 20.

7.3 Discussion

There is some variation in the dose-response relationship, because the larvae probably did not stay on the third leaf for their entire development and there are differences in phytochemical concentrations between different strata of the plant (see Chapter 4 for example). Furthermore, the relation between the compounds and the survival of the larvae expresses effects on the larvae population over a longer period, whereas the chemical data represent spot checks of the content over the development. In order to adjust for this, a model for the intake of the compounds is needed. The large variation may also express that these compounds co-vary with some unidentified compounds that are the main causal reason for the mortality of the larvae.

The slightly better performance of the linear model when both compounds 2 and 3 are used is presumable due to the fact that compound 3 was not found in untreated plants, but *G. polygoni* did die to some degree in the controls which all contained compound 2. If bioassays cannot verify that these two

compound are active in the increased mortality one should look for compounds that are found in detectable concentration in control plants and changes with chlorsulfuron treatment or for compounds that alter the host recognition behaviour.

The mortality found in this experiment,s was lower than that reported earlier (Kjær and Elmegaard, 1996). A possible explanation of this discrepancy is differences in design. In the former experiment, the beetles were added as eggs. This means that the larvae hatch and start feeding later, when the amounts of compounds 2 and 3 are higher due to herbicide treatment. This hypothesis is supported by the fact that compound 3 is not found in measurable quantities in sprayed leaves until day 4 after spraying (see Chapter 4 and Table 7.1).

Finally, other mortality factors not necessarily related to the herbicide treatment and not accounted for in the present set-up may influence the variation. This includes the presence of tolerant ecotypes of plants. Kjær *et al.* (1998b) observed that plants exposed to high concentrations of copper resulted in a differentiation of the growth rate and the mortality of *F. convolvulus.* This indicates that tolerant ecotypes might exist in *F. convolvulus.*

The data presented in this chapter therefore shows that the mortality of *G. polygoni* larvae are positively correlated to the concentration of compounds 2 and 3 in the leaves of *F. convolvulus*.

8 Modelling changes in the content of two phenolic compounds

It was observed in Chapter 7 that the survival of *G. polygoni* larvae was correlated to the concentration of two phenolic compounds, namely those denominated as compound 2 and compound 3 in Chapter 2. The simple linear regression analysis showed that the concentration of the two compounds explained between 25 and 41% of the variation in larval survival. The concentration in the plant was measured over time (time intervals of 3 days), whereas the survival data integrate the impact throughout development or over longer periods. However, a larva feeding on a certain leaf at a certain point in time carries the history of earlier uptake unless the metabolism of the compounds are so fast and efficient that the compound is irrelevant and practically non-existing in the insect.

It is evident from the previous chapters that a range of factors affects the concentration of these two phenolic compounds. They were correlated both to chlorsulfuron treatment (Chapters 4 and 5), to herbivory and UV-B radiation (Chapter 6), time (Chapters 4 and 5) and for compound 2 also development (Chapter 2).

The complexity of the system governing the concentration of these phytochemicals in the plant makes it attractive to develop a model which calculate the concentration as a function of the aforementioned parameters. The model can be used to improve the description of herbivore mortality from time related content of these compounds and the consumption and growth of the herbivorous larvae.

8.1 Materials and methods

8.1.1 Plant model

The model, which will be presented below, is built on the assumption that the impact factors governing the concentration of compounds 2 and 3 are independent of each other. However, we involved an interaction coefficient for dosage and time because it has been suggested that many herbicides rejuvenate the treated plants. In the present study, it most likely means that the developmental progression of the phytochemical profile is slowed down or stopped and the effect of dosage is altered. It has, for example, been observed that *F. convolvulus* stopped producing leaves after treatment with chlorsulfuron, but after a period of ten days it resumed the previous exponential growth (Kjær, 1994).

The overall structure of the model is therefore as follows:

$$Concentration = (UV + Herbivory + Time + Intercation + Const) \times Dosage$$

where *Concentration* is the concentration of either compound 2 or 3 in the leaves of *F. convolvulus*. The model was parameterised by using the data on

effects of herbivory, UV-B radiation, chlorsulfuron dosage and time on the concentration of compounds 2 and 3 presented in previous chapters.

Based on the data in Chapter 5 we assumed the effect of ***Dosage*** could be described by means of a sigmoid dose-response curve:

$$Dosage: \quad \frac{(ddose - adose) \times \left(1 + e^{-bdose \times X_0 dose}\right)}{1 + e^{bdose \times (x - x_0 dose)}},$$

where x is the herbicide dosage, *ddose* is the response at dosage = 0, *adose* is the asymptotic response. $X_0 dose$ is the dosage at the point of inflexion of the curve and *bdose* is the slope parameter at the inflexion point.

The change in concentrations of compounds 2 and 3 with time was described by a parabolic function, because the concentration in the single leaf first increase and then decrease (Chapters 4 and 5), and a similar pattern has been seen in the studies of Suttle *et al.* (1983). Further, there was an initial increase in the concentration after spraying, which declined after some time either due to metabolism of the compounds or due to growth dilution (Chapter 5). ***Time*** in the equation below is the age of the single leaf.

$$Time: \quad atime \times t^2 + btime \times t,$$

where t is the leaf age in days, *atime* and *btime* are rate coefficients of the curve.

The effects of ***Herbivory*** and ***UV****-B* radiation was described as simple linear relationships i.e.:

$$Herbivory: \quad aherb \times herbivores$$
$$UV\text{-}B\ radiation: \quad aUV \times UV$$

where aUV and *aherb* are rate coefficients.

The ***Interaction*** between time and dosage was described by:

$$atime \times dose \times x \times t,$$

where *atime ´ dose* is the rate constant, x is the herbicide dosage, t is leaf age (e.g. time).

The data presented in Chapter 7 on relationship between content of compounds 2 and 3 and herbivore survival was not used for parameterisation, but saved to validate the output of the model.

In order to homogenise the variance the data were transformed by a Box-Cox transformation where the power parameter was found by a maximum likelihood approach (Seber and Wild, 1989 p.71). The transformed model was fitted to the transformed data assuming normal distributed residuals using FindMinimum in Mathematica (Wolfram, 1996). The significance of the different effects included in the model was tested using log-likelihood tests.

Regression analysis was used on the relationship between estimated intake of the phenolic compounds selected and the survival of *G. polygoni* larvae.

8.1.2 Intake of phenolics by *G. polygoni* larvae over time

There was a large variation in the regression between observed survival and the concentration of compound 2 and compound 3 in Chapter 7. It was hypothesised that this was a consequence of the fact that survival expresses effects on the larvae population over a longer period, but the chemical data only represent spot check of the content over the development. In order to adjust for this, a model for intake of the compounds was needed. The plant model together with unpublished data on consumption and larval growth were used to calculate the body burden, i.e. the consumed amount of the compound per unit body weight, per day. The equation for the body burden of a larva at a certain point of development is:

$$Body\ burden = \frac{\sum_{1}^{day} Concentration_{day} \times Consumption_{day}}{weight_{day}},$$

where *Concentration* is the concentration on a specific day as predicted by the Plant model, *Consumption* is the food intake on a daily basis (mg DW) and *weight* is the weight of the larvae on that specific day. By this equation it is assumed that the compounds are not metabolised at all or that metabolism is weight dependent.

The Body burden was calculated for the conditions in the experiment described in Chapter 7 and related to the average survival of the larvae.

8.2 Results

8.2.1 Plant model

The following parameters were tested in the log likelihood ratio test: *ddose*, *atime*, *aherb*, *aUV*, and *atimedose*. Only the interaction equation (*atime´ dose*) was non-significant for the description of content of compound 2 (Table 8.1). There was an interaction between time and dosage for compound 3. *ddose* was significant for compound 2, which mean that the concentrations in the untreated plants were significantly different from zero. For compound 3 *ddose* was not significant, i.e. control plants do not contain detectable amounts of this compound. *atime* was significant for both compounds, indicating that the parabolic function described the effect of time better than a linear response. Both *atime* and *aherb* were significant, that is both UV-B and herbivory have an effect on the content of compound 2 and compound 3.

Table 8.1
Test values and limits of significance for the parameters in the mathematical model produced

	Compound 2		Compound 3	
test	c^2 – value	*p*	c^2 -value	*p*
ddose = 0	28.77	<0.0001	0.116	0.2662
atime = 0	22.147	<0.0001	113.64	<0.0001
aherb = 0	9.9920	0.0016	35.13	<0.0001
aUV = 0	15.634	<0.0001	3.997	0.046
atime´ dose = 0	2.258	0.132911	9.074	0.0026

Table 8.2 presents the parameter value to the model estimated in the fitting process.

Table 8.2
Parameter estimates for the model describing plant content of compound 2 and compound 3.

Parameter	Compound 2	Compound 3
ddose	0.38888	0
adose	1.60474	0.66799
X_0dose	0.119072	0.06319
bdose	0.975425	188.979
atime	-0.01051	-0.01004
btime	0.3251	0.38182
aherb	0.1106	0.04416
aUV	8.7654	-0.67484
atimedose	0	0.02598
k	2.38811	-1.18065

8.2.2 Validation

The parameterised model was validated against the data presented in Chapter 7 by visual inspection of the residual plot of the predicted values (Figure 8.1). The residuals were homogeneously distributed around zero, which means that the predicted values did not depart systematically from the observed values.

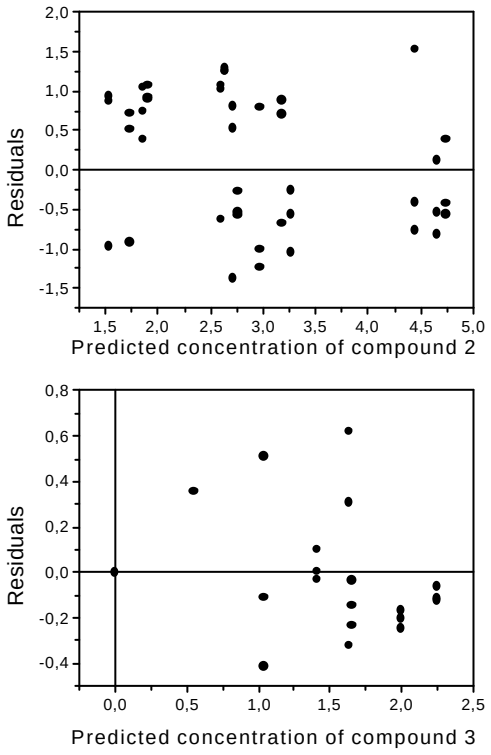


Figure 8.1
Residual plots of the predicted concentration of compound 2 and 3 in the experiment presented in Chapter 7.

8.2.3 Body burden estimates as descriptor for larvae mortality

Regression analysis of the calculated body burden and the average survival of *G. polygoni* larvae gave a clear correlation (Table 8.3 and Figure 8.2). The relationships involving compound 2 have by far the best correlation.

Table 8.3
Regression analysis of body burden of combination of compound 2 and 3, and survival of *G. polygoni* larvae. The relationship is of the form: Number of survivors = a × Compound concentration + b.

Compound	a	b	N	p	r^2
2	-0.953	19.53	12	<0.0001	0.835
3	-1.482	17.09	12	0.019	0.440
2+3	-0.640	18.86	12	0.00027	0.750

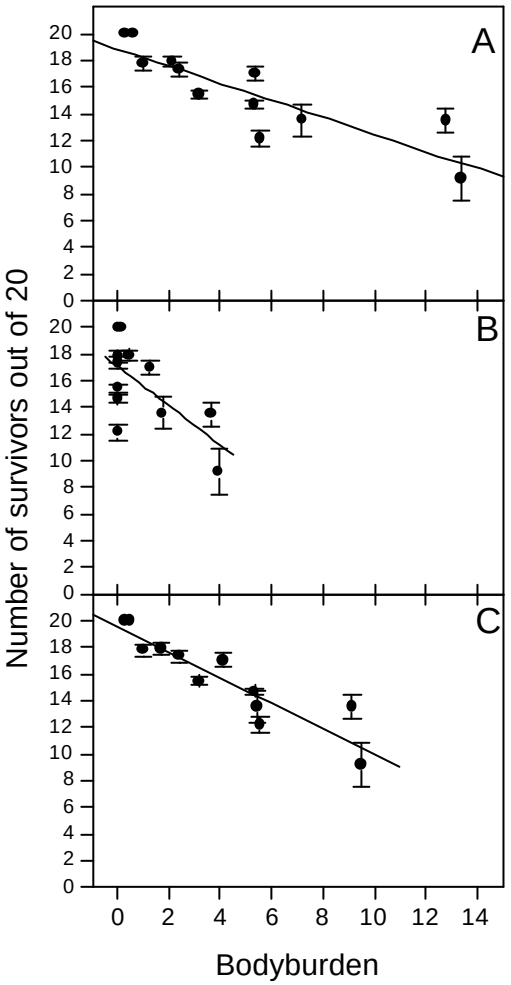


Figure 8.2
Mean survival of larvae as a function of calculated body burden of compounds 2 and 3 in three situations: A: Body burden of both compound 2 and 3, B: Body burden of compound 3, C: Body burden of compound 2. Bars represent Standard Error of Mean.

8.3 Discussion

The plant model clearly fitted best for compound 2. The strong correlation between the modelled concentration and the survival of the larvae clearly suggest that the compound is connected to the effect mechanism. Furthermore, it suggests that inclusion of the cumulative uptake of these compounds increased the confidence in the relationship. It is therefore tempting to assume that compound 2 or the sum of compounds 2 and 3 are the prime elicitor of effects because the variation in compound 2 also explains the high mortality in the control treatments. However, a strict demonstration of the effect of these compounds is lacking before a cause – effect relationship is established. Due to the fit of the regression analysis we do not reject our working hypothesis that these two compounds are likely to elicit toxic effects or repellence.

The analysis included both control plants and herbicide treated plants. As there was a high mortality of herbivores on the control plants and compound 3 is only found in treated plants this can not explain all mortality.

The models fitted for both compound 2 and compound 3 showed that herbivory causes a slight increase in the concentration of these compounds (i.e. the rate parameter was positive). This is contradictory to the data presented in Chapter 6, which showed that the concentration decreases with increasing herbivory. The only explanation to this is that the negative relationship between herbivore load and the compounds 2 and 3 was weak and largely governed by the highest herbivore load i.e. defoliation equal to 40 larvae per plant. Most of the experiments included for the fitting only employed 20 larvae, and consequently the weight and variation at this level will determine the sign of the slope. According to the regression lines presented in Chapter 6. Plants exposed to artificial herbivory equal to 0 and 20 larvae differ in the concentration between 11 and 22% and for actual differences between 14 and 30%.

The presented model could potentially be used to calculate risks for defined scenarios, e.g. various herbicide drift scenarios. This issue will be pursued later.

9 Drift of herbicides, and the importance of droplet size - Field and laboratory studies

Drift of herbicides may potentially cause detrimental effects on non-target organisms, including indirect effects on insects, birds and small mammals through effects on their food sources. Deposition caused by drift is a function of the distance to the spraying device (e.g. Davies and Gilbert, 1985; Nordby and Skuterud, 1975). However, effects of drifting pesticides may also be a function on the droplet size distribution of the spray, since small droplets may be taken up more easily and may be more concentrated due to higher evaporation of water/solvent than of the active ingredient (Nordby and Skuterud, 1975). Furthermore, droplet size distribution may change with distance from spraying device.

The aim of the present experiments was to study effects of herbicide drift under realistic conditions in the field in relation to measured exposure. This was supplemented with laboratory studies of the impact of droplet size distribution on effects of equal herbicide dosages.

9.1 Materials and methods

9.1.1 Laboratory studies of impact of differences in nozzle choice on the effect of herbicides on plants

F. convolvulus plants were grown in the greenhouse for 4 weeks at min. 20°C, 16 h light and 70 % RH, achieving an average initial dry weight of 0.45 ± 0.11 g (5-7 leaves). The plants were sprayed with a mixture of chlorsulfuron and glycine. Spraying was performed with a pot sprayer, using three different nozzles with different droplet size distribution (small, medium, large average droplets). Chlorsulfuron concentrations were 0, 0.03125, 0.0625, 0.125, 0.25 and 0.5 times the recommended field rate (4 g ha^{-1}), and glycine concentration in the same relative propotions (37.5 g/l at 0.5 times the recommended field rate). There were 4 plants per nozzle size and concentrations, except for 0.125 times the field rate with the medium nozzle (12 plants).

After spraying, the plants were allowed to grow for 7 days. Then they were harvested, and dry mass was determined for above ground plant parts.

The deposition on glass plates (90 cm^2) was measured. All measures of spray deposition were done by spectrometric determination of the glycine present in the spraying mixture (Babcock *et al*., 1990). A colour reagent consisting of 95 g KH_2PO_4, 43 g NA_2HPO_4, 5 g ninhydrin and 3 g fructose dissolved in 1 l water was prepared, together with a KIO_3 solution made of 4 g KIO_3, 1.2 l water and 0.8 l 96 % ethanol. For calibration purposes, glycine standards of 2.0, 4.0, 8.0 and 16 mg/l were prepared. For analysis, 3 ml sample achieved by washing the glass plates with water was mixed with 2 ml colour reagent and boiled for 10 minutes in a water bath. Thereafter, the samples were

cooled in another bath, 5 ml KIO_3 solution was added, and the samples were mixed. After 10 minutes, the samples were measured in a spectrophotometer at 570 nm.

9.1.2 Field study of drift of herbicide and related effects on plants

F. convolvulus plants grown in the greenhouse (see above) were placed in a barley field (3-5 leaves, growth stage 13-15 (Zadoks *et al*., 1974), which was sprayed with the herbicide Glean 20 DF in recommended field rate (4 g chlorsulfuron ha^{-1}). Glycine (75 g l^{-1}) was added to the spraying mixture (200 l ha^{-1}) for measurements of deposition. The field was sprayed on May 18th 1999, at eastern-south-eastern wind (*i.e.* at right angle to the tractor track) of approximately 2.5 m s^{-1}. The plants were placed at different distance from the spraying track (0, 1, 1.5, 2.25, 3.38, 5.06, 7.59, 11.39 and 17.09 m from the tip of the sprayer) in 8 columns in the field. Control plants were placed approximately 40 m upwind from the spraying track. After spraying, some plants (1-3 per distance and column) were transferred to the greenhouse to allow effects of the spraying to occur, while other plants (1 per distance and column) were analysed for deposition of the herbicide by measuring the deposition of the glycine mixed into the spraying solution. Beside the plants, also glass plates and hair curlers were placed on racks in the field at different heights (10, 30, 60 and 90 cm above the ground, curlers only) and distances (0, 1, 1.5, 2.25, 3.38, 5.06, 7.59, 11.39, 17.09 and 40 m from the tip of the sprayer) to get estimates of herbicide deposition.

In the laboratory, plants, glass plates and curlers were washed with water, and the deposited glycine measured by spectrometry, as described above. Plants for estimates of herbicide effects were harvested after 7 days at min. 20°C, 16 h light and 70 % RH. Above-ground biomass was dried at 80 °C, and dry weight was determined.

9.1.3 Statistics

Effects of nozzle size on growth and of vertical and horizontal position on deposition were analysed by analysis of variance (ANOVA). Means were compared by Tukey t-test. Results were evaluated at the 5 % significance level.

9.2 Results

9.2.1 Laboratory study

Choice of spraying nozzle had no impact on the effect of chlorsulfuron on the growth of *F. convolvulus* (Figure 9.1). Growth was depressed at chlorsulfuron dosages of 0.0625 times the recommended field rate and higher (Figure 9.1).

Measures of deposition on glass plates corresponded to 2.3, 1.9 and 1.7 g a.i. ha^{-1} for the small, medium and large nozzle size, respectively, at 0.5 times the field rate (nominally 2 g a.i.ha^{-1}). Comparison is difficult, since no replication was performed. Consequently, no experimental background for the field studies was obtained from the greenhouse study concerning actual exposure.

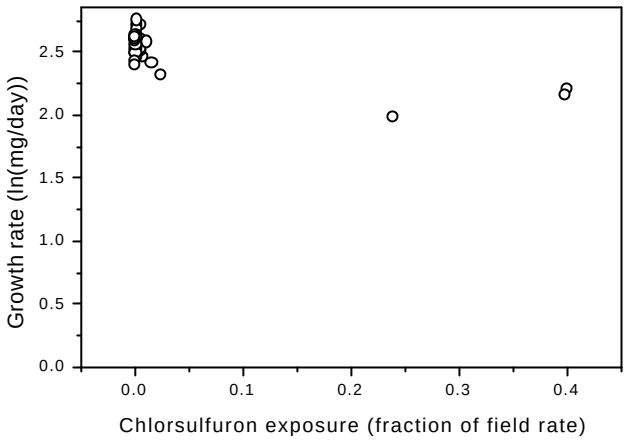


Figure 9.1
Impact of nozzle size on chlorsulfuron effects on *F. convolvulus* grown under laboratory conditions.

9.2.2 Field study

Results from four rows were excluded due to failure of the spraying procedure (water in device, i.e. no deposition of herbicide). Correlations between the different measures of chlorsulfuron deposition (curlers, glass plates, plants) were high (correlation coefficients of 0.94-0.98). In the following, deposition on glass plates is used, since that deposition measure is the only one expressed on an area basis (g ha^{-1}).

Deposition decreased with increasing distance to the sprayer (Figure 9.2), but was almost unaffected by their vertical position (data not shown). The chlorsulfuron deposition on glass plates in the drift zone (1 m or more from the spraying track) was less than 5% of the nominal recommended field rate, compared to 25-40% under the spray nozzles. Actual deposition in the drift zone was thus ≤ 7% of the actual deposition under the sprayer (Figure 9.2), i.e. at full field rate. Effects on plant growth were small or absent: only plants placed right beneath the sprayer were significantly smaller than the control plants (and all other plants), whereas plants sprayed at 3.38 m distance to sprayer, corresponding to an estimated exposure of 1.1% of the deposition at recommended field rate (i.e. right under the nozzles), were significantly larger than the control plants (Figure 9.3).

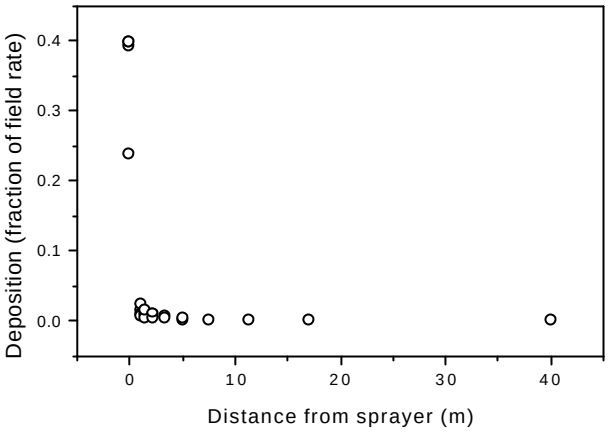


Figure 9.2
Deposition of chlorsulfuron on glass plates, expressed as fraction of the average deposition at recommended field rate.

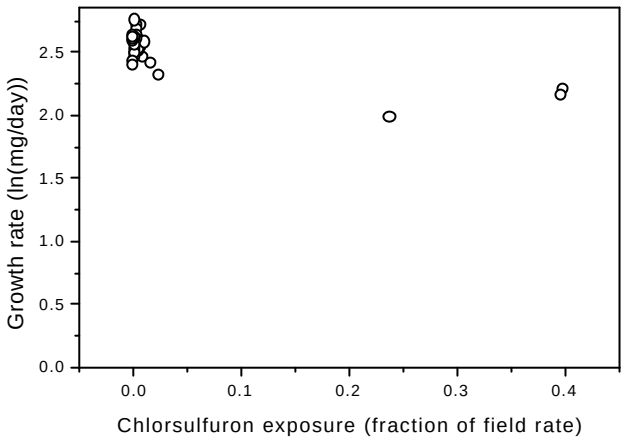


Figure 9.3
Growth of *F. convolvulus* as function of chlorsulfuron exposure, expressed as fraction of average actual exposure at recommended field rate.

9.3 Discussion

Effects on the growth of *F. convolvulus* occurred at comparable chlorsulfuron dosages in laboratory and field studies (at 0.0625 times the recommended field rate and higher). For plants exposed in the field, an indication of a hormetic effect was seen at a distance of 3.38 m from the sprayer, corresponding to c. 1.1% of the actual exposure at the recommended field rate. Kjær (1994) found a similar effect at 0.1 times the field rate in a laboratory experiment. No such effect was seen in the present laboratory experiment, possibly due to the choice of test dosages. For plants exposed in the field, negative effects on growth only occurred on plants placed directly under the sprayer. The drift zone where effects occurred was thus less than 5 m wide. However, the wind speed was low in the field (c. 2.5 m s^{-1}), and the drift zone may be wider under more windy conditions.

The measured deposition on e.g. glass plates may not exactly mimic the exposure of plants, but the relationship between distance and deposition is probably well described, due to the high correlation between the various deposition measures (see above).

The lack of effect of droplet size on toxicity in the laboratory study does not necessarily imply that no droplet size effect exists in the field. In the laboratory, there was hardly any evaporation, whereas in the field evaporation of water may increase the concentration of chlorsulfuron, especially within the smallest droplets, leading to higher concentrations in small droplets than in large ones.

The conclusions drawn from the present study are:

— At a wind speed of 2.5 m/s, drift effects of chlorsulfuron treatment only occurred at distances of less than 5 m from the sprayer.
— Negative effects of chlorsulfuron were found at dosages of c. 0.25 times the recommended field rate, under both laboratory and field exposure conditions.
— Field exposed plants experiencing an estimated chlorsulfuron dosage of c. 0.3% of the recommended field rate (1.1% of the measured exposure at field rate) displayed signs of a hormetic effect of the herbicide.
— No impact of droplet size distribution of herbicide effects was detected.

10 Relationships between herbicide treatment of host plants and the performance of herbivorous insects

The present chapter tests whether the negative effects on leaf chewing herbivores found in the literature are a general phenomenon or it is characteristic for the specific plant-herbivore pair and the compound tested. Therefore, the present study evaluates three insect/plant interaction systems for their susceptibility to sulfonylurea herbicides. The three test systems are representative of species which could be exposed to sulfonylurea herbicides due to spray drift into other crops or into semi-natural habitats. The organism pairs used are *Pieris brassicae/Brassica napus* (hereafter Pieris), *G. polygoni/F. convolvulus* (hereafter Gastrophysa), and *Sitobion avenae/Triticum aestivium* (hereafter Sitobion), respectively. The test design differs between systems due to their different feeding guilds.

10.1 Methods and Materials

10.1.1 Pieris-Brassica test system

Brassica napus is an annual plant (either spring or winter annual). This species occurs both as crop, weed, and wild species with scattered distribution in disturbed habitats. *Pieris brassicae* is a pierid butterfly. The larvae feed mainly on plants of the *Brassica* family (Feltwell, 1982) and prefer foliage for consumption in all life stages. The egg clutches are normally so large that the host plant is totally defoliated and the larvae are forced to migrate to other host plants in order to complete larval development (Davies and Gilbert, 1985). This species pair may experience herbicide effects both in natural habitats and in crops exposed to spray drift from adjacent fields. Both acute and chronic tests were conducted with the insects.

10.1.1.1 Insect bioassay

Brassica napus in the vegetative stage (i.e. possessing 4 true leaves) was sprayed with herbicide. Four days after spraying, 20 newly hatched larvae of *Pieris brassicae* were placed on each of the plants. After four days, the weight gain and survival of the larvae were assessed. Each dosage consisted of three independently prepared replicates of three samples each (spray dosages and used pesticides are presented in Table 1). At the day of placing larvae on the plants, a randomised sample of 40 larvae was collected in order to measure the average size of the experimental animals. The fresh and dry weights of these animals were measured as eight samples of five individuals each. The experiments were conducted in a controlled-environment-chamber administered at 20°C, 70% RH, and 16 h photoperiod. Living specimens were counted daily, and on day 8 after spraying, the weight of the larvae was measured. This time span was chosen because a pilot experiment had shown that plants at the higher dosages loose their leaves shortly after this point in time. Survival was estimated as the fraction of larvae remaining on the plant on day 4 after placing the larvae on the plant in relation to the numbers present after one day. This was done because a single experiment

(chlorsulfuron) lost a good deal of specimens over the first 24 hours even in the control treatment. Subsequently, this was examined in a small experiment, which showed that within a short time span of 1-2 hours after hatching the larvae are vulnerable to handling. The procedure of applying larvae to the plants was hereafter altered, and larval disappearance was avoided in the remaining experiments.

In a long-term (chronic) experiment, larvae were allowed to complete larval development. *Brassica napus* plants were sprayed in the vegetative stage (i.e. possessing 4 true leaves) with the sulfonylurea-herbicide metsulfuron-methyl at different dosages, i.e. 0, 0.05, 0.1 and 0.2 times the recommended field rate. Four days after spraying, 20 newly hatched larvae of *Pieris brassicae* were placed on the sprayed plants. The experiments was conducted in a controlled-environment-chamber administered at 20°C, an RH of 70%, and a 16 h photo period. At the time when the larvae started to move on the plant these were moved to cages in a greenhouse cell and remained here for the rest of their development.

The survival of the larvae was registered throughout development, and developmental time from egg hatch to adult stage was measured. Hereafter, biomass of the adults was determined.

10.1.1.2 Plant bioassay

Simultaneously with the acute insect bioassay, host plants without larvae were treated with the herbicides to assess effects on the plants. The set-up was similar with respect to soil type, temperature, etc., but the plants were treated with a broader range of dosages, in order to a establish dose - response relation for the plants. The plants were treated when they possessed four leaves. Each treatment dosage was sprayed in three independently prepared replicates with three plants per replicate. At the time of spraying, 20 plants of similar size as the experimental plants were weighed, i.e. measures of root and shoot biomass (both dry and fresh weight). On the eighth day after spraying, the experimental plants were harvested, both shoot and root biomass (fresh and dry weight) was measured.

10.1.2 Gastrophysa-Fallopia test system

F. convolvulus is a strictly annual weed species. It often climbs adjacent crop plants. Under greenhouse conditions, the plant reproduces mainly by self-fertilisation. *F. convolvulus* has no leaf loss during development, but continues to grow until senescence, when all leaves die almost synchronously and the seeds ripen. Details of the life cycle of *F. convolvulus* are presented by (Hume *et al.*, 1983). Plants for experiments and insect food were grown in pots in a greenhouse. Seeds were harvested and stored cold to break dormancy before sowing. *G. polygoni* is a chrysomelid beetle utilising mainly two food plants, viz. *F. convolvulus and Polygonum aviculare* L. It eats the foliage of the plants in all three larval instars and as adults. The larvae pupate in the soil. The beetles were kept in culture on *F. convolvulus* plants at 20°C and 16 h photoperiod.

10.1.2.1 Insect bioassay

A controlled environment experiment was conducted to measure the suitability of herbicide-sprayed *F. convolvulus* plants as hosts for *G. polygoni*. Newly hatched larvae of *G. polygoni* were placed on leaves of plants subjected to different herbicide dosages. After spraying, the plants were caged singly in

polystyrene cylinders and placed in a controlled-environment-chamber (photoperiod of 16 h, constant temperature of 20°C, relative humidity of 60%, and a photo flux density of 350 μE m^{-2} sec^{-1}). One day after herbicide treatment, 20 newly hatched *G. polygoni* larvae were placed on the leaves of the plants. Each treatment was replicated four times. The larvae were placed on the lower side of leaves in the middle section of the plant according to the behaviour of ovipositing females (Kjær *et al*., 1998). Subsequently, the number of larvae and adults emerging after pupation were recorded every second or third day. When the adult beetles emerged, they were removed and weighed.

Plant bioassay
Simultaneously with the insect bioassay host plants without insects were treated with the herbicides to assess effects on the plants. The set-up was similar to the insect bioassay. At the time of spraying, shoot dry weight of seven plants of similar size as the experimental plants were measured. On day 16 after spraying, the experimental plants were harvested and shoot biomass was measured.

10.1.3 Sitobion-Triticum test system

Sitobion avenae is an aphid pest of cereal crops and sucks from the phloem of the host plants. This aphid prefers to feed in the upper part of the host plant, due to a better food quality (El-Sayed in Klingauf (1987)). Unlike broad-leaved weeds, cereal crops are tolerant to sulfonylurea herbicides. Therefore, no plant bioassay was performed. The difference between sensitive and tolerant species is based on their ability to metabolise the pesticide (Ustimenko, 1990). It is possible that this metabolism evokes a reallocation of resources from storage organs to the leaves to the potential benefit of the aphids. It is therefore important to test if the use of herbicide may improve the performance of the aphids.

10.1.3.1 Insect bioassay
The effects of the sulfonylurea herbicide metsulfuron on the performance of the aphid *Sitobion avenae* were assessed in a feeding experiment in a controlled-environment-chamber (20°C, 16 h photoperiod and 20°C). Seeds of *Triticum aestivium* (var. Lambros) germinated in compost soil at 20°C. The day following herbicide treatment, one 0-24 h old aphid nymph was applied to each plant. Prior to application the nymphs were weighed. Plants and aphids were enclosed in glass cylinders (diameter of 5 cm) which were closed in the top with a 0.1 mm mesh. Hereafter, the aphids were observed two times a day to register developmental time until adult stage (D±0.5 day). When the aphids had reached adult stage, they were weighed and placed on the plant again. Hereafter, the fecundity of the aphids were assessed in a period equal to two times the developmental time of the specific individual, i.e. D. Approximately halfway in this period all nymphs produced were killed so that the original adult could be recognised and nymph production from the F1-generation was avoided. Replicates in which the adult had disappeared were excluded from the measures of fecundity.

10.1.4 Spraying procedure

Pieris
Plants were sprayed with a pot sprayer designed for automatic and controlled spraying of larger plants (Kristensen pot sprayer, Ringsted, Denmark; l × w ×

h = 120 × 100 × 170 cm). Table 10.1 gives the tested herbicides and the actual dosages. The trial with chlorsulfuron was conducted with the surfactant Citowett (BASF) added to the spray solutions (0.5% v/v). Control plants were treated with water. The sprayer was equipped with two Hardi flat fan nozzles type 411014 separated by 53 cm and used at a working pressure of 2 bar. The sprayer was calibrated to deliver a spray volume of 200 l ha^{-1}.

Table 10.1
Presentation of the dosages and herbicides used on the Pieris/Brassica system.

Herbicide	Spray dosages, insect bioassay (times recommended field rate[a])	Spray dosages in plant bioassay (times recommended field rate[a])
Chlorsulfuron	0-0.025-0.05-0.1-0.2	0-0.025-0.05-0.1-0.2-0.4-0.8
Metsulfuron	0-0.025-0.05-0.1-0.2	0-0.0125-0.025-0.05-0.1-0.2-0.4-0.8
Tribenuron	0-0.25-0.5-1	0-0.25-0.50-0.75-1

[a]list of recommended field rates in Denmark for cereals: Chlorsulfuron 4 g a.i. ha^{-1}; Metsulfuron 4 g a.i. ha^{-1}; and Tribenuron 7.5 g a.i. ha^{-1}.

10.1.4.1 Gastrophysa

Plants were sprayed with a pot sprayer at rates of 0-0.067-0.125-0.25-0.5 times the field rate. In Denmark, the recommended field rates for the selected sulfonylurea herbicides in cereals are as follows: Metsulfuron-methyl (Ally®) 4 g a.i. ha^{-1}; tribenuron (Express®) 7.5 g a.i. ha^{-1}. The trial with metsulfuron-methyl was conducted with the surfactant Citowett (BASF) added to the spray solutions (0.5% v/v). Control plants were treated with water. The sprayer was equipped with Hardi flat fan nozzles type 411014 and used at a work pressure of 2 bar. The sprayer was calibrated to a spray volume of 200 l ha^{-1}. The plants were treated when they had 4-6 true leaves. Each treatment dosage was sprayed in four independently prepared replicates with three plants per replicate, i.e. one for the insect bioassay and two for the plant bioassay.

10.1.4.2 Sitobion

The plants were treated with metsulfuron-methyl (Gropper® DuPont (179 g metsulfuron kg^{-1})) and Citowett (BASF) (0.5% v/v) with a hand held spray atomizer when the plants were in growth stage 12 (Zadoks *et al.*, 1974) with dosages of 0 and 7.16 g a.i. ha^{-1} (recommended field rate). Twenty-five replicates were established for each treatment. The spray atomiser was calibrated to give a specified amount of water per time unit, and the speed of the sprayer was adjusted so that the specified spray volume was delivered over the whole surface and the point of run-off was reached.

10.1.5 Statistical analyses

10.1.5.1 Pieris and Gastrophysa

Effects of herbicide treatment on biomass, Relative Growth Rate (RGR), and survival were tested in an ANOVA, (GLM-procedure SAS, Type III SS) (SAS, 1989). If no first order interactions appeared significant, the test was repeated omitting interactions. Differences between means were tested in a Tukey HSD t-test. Comparisons between control treatment and single dosages were done by a Dunnett's T test. The overall level of significance was 0.05.

Sitobion

Effects of metsulfuron-methyl treatment on developmental time (D), growth rate and fecundity were compared by means of paired t-tests. The overall level of significance was 0.05.

10.2 Results

10.2.1 Pieris

Acute test

The relative growth rate for oilseed rape plants treated with metsulfuron-methyl was significantly reduced for both shoots (One-way anova, df=7, F=8.26, p<0.0001) (Fig 10.1B) and roots (One-way anova, df=7, F=17.82, p<0.0001). The same was observed for shoots (One-way anova, df=4, F=3.25, p=0.021) and roots (One-way anova, df=4, F=15.48, p<0.0001) (Fig 10.1D) when plants were treated with tribenuron. Chlorsulfuron treated plants failed to show any significant reduction in shoot growth rate (One-way anova, df=6, F=0.38, p=0.8863), while root growth rate was significantly reduced with application rate (One-way anova, df=6, F=3.19, p=0.0092).

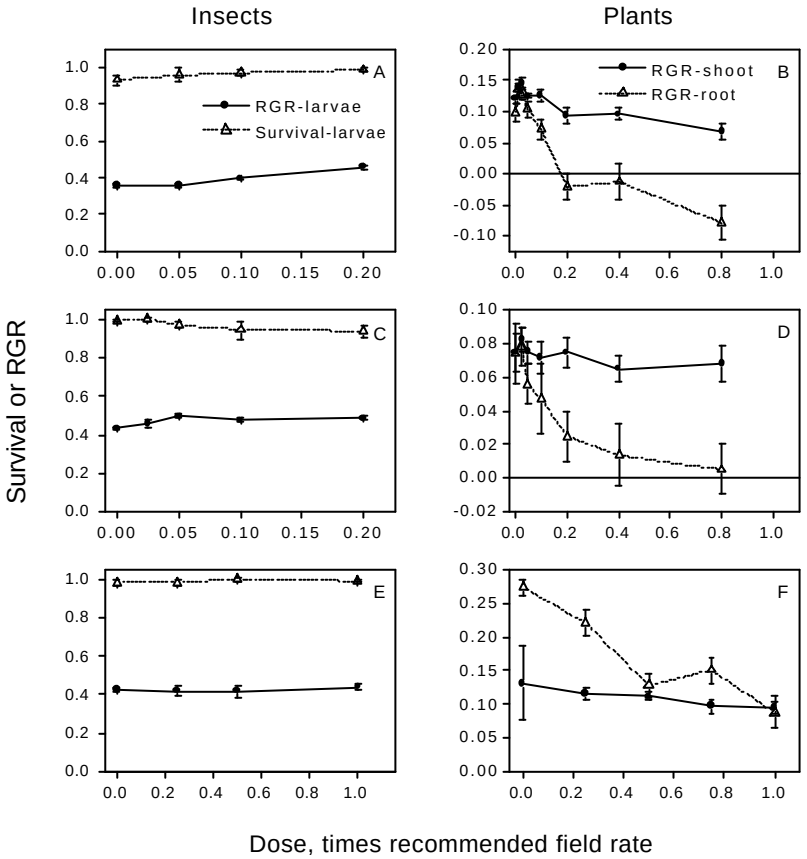


Figure 10.1

Response of *Pieris brassicae* (RGR and survival) and the host plant *Brassica napus* (RGR of roots and shoot) to treatment of the plant with selected sulfonylurea herbicides. Fig. A and B: Metsulfuron, Fig. C and D: Chlorsulfuron, and Fig. E and F: Tribenuron.

Insects

Survival was not affected by replicate, dosage or trial, i.e. no significant mortality/disappearance was observed for any of the tested herbicides (Two-way anova, df=19, F=0.71, p=0.8099) (Figure 10.1A, C, and E). The relative growth rate of the catepillars was significantly increased with dosage in the trial with metsulfuron-methyl-treated host plants (One-way anova, df=3, F=9.55, p<0.0001). No significant changes were observed for *Pieris brassicae* on plants treated with either chlorsulfuron (One-way anova, df=4, F=2.16, p=0.0917) or tribenuron (One-way anova, df=3, F=0.128, p=0.2168). However, a trend toward increased growth rate was observed when the host plant was treated with low dosages of chlorsulfuron.

Chronic test

Plants treated with 0.1 and 0.15 times the recommended field rate of metsulfuron-methyl lost all leaves before larval development was completed. The larvae placed on plants treated with 0.05 times the recommended field rate performed equally well as those on plants treated with water. No significant changes were observed for either developmental time, larval growth or hatching weight of the adults (t-test, Table 10.2).

Table 10.2

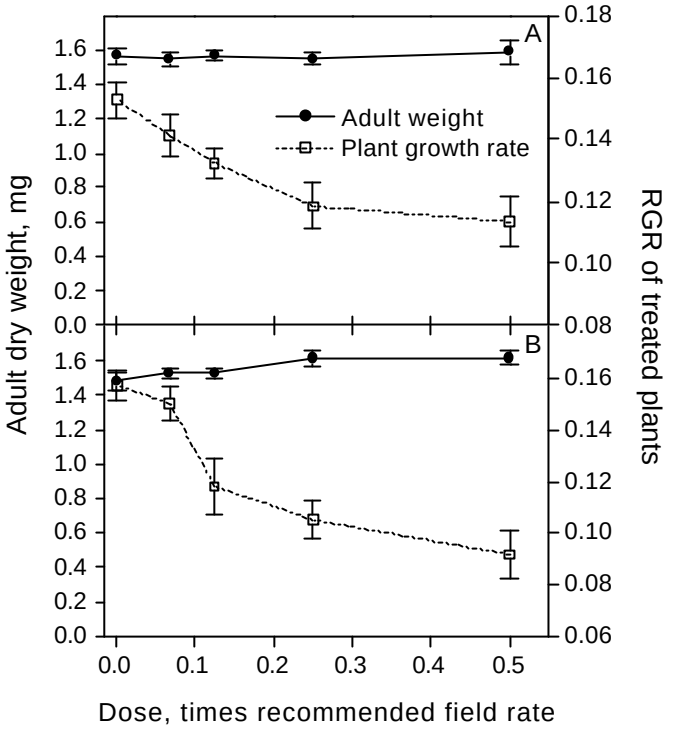
Performance of *Pieris brassica* feeding on *Brassica napus* sublethally treated with metsulfuron-methyl at a rate of 0.05 times the recommended field rate. Fresh weight of the larvae was measured on day 12 after being placed on the plants. Values given are mean ± Standard Error of Mean.

Variable	N	Untreated plants	N	Treated plants
Developmental time, days	77	25.88 ± 0.14	56	25.85 ± 0.20
Larval fresh weight, mg	80	451.2 ± 8.3	58	441.9 ± 13.3
Adult dry weight, mg	77	55 ± 3	56	52 ± 2

No significant differences between sprayed and unsprayed host plants were observed (t-test)

10.2.2 Gastrophysa

Black bindweed plants were negatively affected by the herbicide treatment (Fig. 10.2). A one-way anova revealed the following results for tribenuron (One-way anova, df=4, F=5.58, p=0.0008) and for metsulfuron (One-way anova, df=4, F=11.29, p<0.0001). Leaf beetle larvae developing on the treated plants did not show changes in developmental rate (larvae to adult) with treatment. At least, a regression analysis showed that developmental rate was not correlated to dosage (Tribenuron: Rate = $0.0005 \times$ dosage + 19.35, $r^2 = 2 \times 10^{-6}$. Metsulfuron: Rate = $21.35 - 1.39 \times$ dosage, $r^2 = 0.057$) The size of the hatched adult was not different between treated and control animals (One-way anova, Tribenuron: df=4, F=0.12, p=0.974, R^2=0.0042, and Metsulfuron df=4, F=1.86, p=0.12, r^2=0.048). Regression analysis revealed that survival of larvae to adult stage showed a non-significant trend towards reduced survival (tribenuron: Number survivors = $7.4 - 5.95 \times$ dosage, $r^2 = 0.071$, and metsulfuron: Number survivors = $9.03 - 7.07 \times$ dosage, $r^2 = 0.168$).



Figur 10.2

Effects of tribenuron (A) and metsulfuron (B) on the size of adult beetles (*G. polygoni*) upon pupal hatch and on the relative growth rate (RGR) of host plants (*F. convolvulus*).

10.2.3 Sitobion

The treatment of the host plant with metsulfuron did not change the performance of *Sitobion avenae*. A t-test between values for treated and untreated plants, respectively, did not reveal any significant changes in developmental speed, growth rate, size of adult or fecundity (Table 10.3).

Table 10.3

Aphid (*Sitobion avenae*) performance on herbicide treated and untreated host plants (*Triticum aestivium*). Values given are mean ± Standard Error of Mean. No significant differences were found between aphids on sprayed and unsprayed host plants (t-test)

Variable	N	Untreated plants	N	Treated plants
Developmental rate, D days	29	9.297±0.140	32	9.258±0.150
Relative growth rate	26	0.0476±0.0029	33	0.0464±0.0030
Fresh weight of adults, mg	26	0.486±0.025	33	0.466±0.025
Fecundity, # of nymphs	27	13.56±0.76	33	15.33±0.65

10.3 Discussion

It was the aim of this study to test if treatment with sulfonylurea herbicides can be expected to cause effects on herbivorous species living on treated plants. None of the insect/plant relationships studied showed a reduced performance of the herbivore on treated plants, and *P. brassicae* even had an incereased growth rate on plants treated with metsulfuron-methyl. Consequently, it must be concluded that the palability of the hosts was unchanged. The observed tendency to a reduced survival for *G. polygoni* indicates, however, that the effects observed for chlorsulfuron (Kjær and Elmegaard, 1996) may be expected also for these herbicides, if higher dosages are used. In Chapter 3 it was observed that the content of compounds 2 and 3 in plants treated with chlorsulfuron and with tribenuron were comparable. Therefore, similar effects were expected on the leaf beetle for the two herbicides. This was not the case, probably because the comparison was made on basis of full field rate in Chapter 3 whereas in this study the dosage was 0.5 times the field rate at maximum.

The butterfly larvae were not directly affected in the dosage range tested for any of the herbicides, but the host plant lost the leaves at very low dosages. This observation suggests that that the butterfly will not increase its pest status following spray drift from adjacent fields and that the use of reduced dosages of herbicide are unlikely to benefit insects associated with *Brassica napus.* These experiments measured the end-points after 4 days, and it may be argued that sublethal effects would show up at a later stage. However, the plants were so affected that the leaves were lost shortly after this point in time even at dosages as low as 0.1 times the recommended field rate. This was also observed in the chronic test.

Further, there were no indications that the quality of the treated *Triticum* plants was changed, as the aphids developed and reproduced equally well on treated and untreated plants. This observation, first of all, expresses that the metabolism of the herbicide to compounds without herbicidal activity is so fast and inexpensively that an altered allocation is not seen, or that the insects suck on single cells rather that the conductive tissue.

So, on the basis of the present data set the conclusion is that sulfonylurea herbicides are unlikely to cause widespread effects on herbivorous insect other than the probable disappearance of the host plant.

11 General summary and discussion

11.1 General phytochemical trends in relation to biotic and abiotic factors

11.1.1 Growth conditions

Three different experimental set-ups were used, resulting in different growth conditions for plants (and herbivores), i.e. controlled-environment chamber (after treatment) (Chapters 3, 4, 5, 6, 7 and 10), greenhouse with or without supply of UV-B light (Chapters 4 and 9) and field-like conditions on tables outside the greenhouse (Chapter 4). Since phytochemicals were not measured in plants from Chapters 9 and 10, they are excluded from this part of the discussion. Here only results of growth conditions are presented, i.e. phytochemical concentrations in control plants not subjected to herbivory. The collective term'laboratory' is used for greenhouse and controlled-environment chamber.

The concentration of compound 1 was higher in plants grown under field conditions than in plants grown in greenhouse or controlled-environment chamber (Chapters 2, 4, 5, 6). Concentrations consistently increased from bottom to top leaves, i.e. was largest in younger leaves (Chapters 2, 3, 4) both in controlled-environment-chamber and under field conditions. Supply of UV-B caused a slight increase in middle leaves of greenhouse plants (Chapter 4), but not enough to approach the content to that of field plants. In Chapter 4, time had no effect on the vertical distribution of compound 1, but when the same leaf position was followed over time, the concentration of compound 1 decreased (Chapter 2). In Chapter 7, in which the plants are followed for a longer period than in Chapter 4, the concentration of compound 1 in the middle (third) leaf first increased and then decreased. Chapter 5 was not comparable. Thus, it seems likely that compound 1 can be found in higher concentrations in rather young leaves than in older leaves, as a consequence of either dilution due to growth, transport out of the ageing leaves, or formation of the compound mainly taking place in growing (younger) leaves.

For compound 2, concentrations were also higher in field plants than in laboratory and greenhouse plants (Chapters 2, 3, 4, 6, 7, 10). UV-B had a large impact on greenhouse plants, increasing the concentration to the same level as seen in field plants (Chapter 4). This strongly indicates that light is one of the major factors affecting compound 2. Vertical distribution within the plants was similar for field and laboratory plants, with the highest concentrations in the top leaves, except for Chapter 3, where concentrations tended to be equal or higher in middle leaves. Concentrations in the middle (third) leaf tended to increase with time and then decrease (Chapters 2, 4 and 7). This was somewhat opposite to the effect in field plants (Chapter 4), in which the content in the middle leaf did not decrease with time, but then again the time-span was shorter for field plants than for laboratory plants.

Compound 3 was never found in untreated laboratory plants, whereas (only small) concentrations were found in plants grown under field conditions

(Chapter 4). Supplying greenhouse plants with UV-B light (Chapter 4) could not mimic this difference.

Compound 4 occurred in comparable concentrations in field and controlled-environment-chamber plants (Chapters 2, 4, 6, 7), whereas the corresponding data for controlled-environment-chamber in Chapter 3 and greenhouse plants in Chapter 4 were about twice as high. Since effects of time (Chapters 2, 4 and 7) were small (decrease) or absent, and UV-B light (Chapter 4) had no effect on compound 4, the differences between the various experiments cannot be explained. Concentrations increased from bottom to top (or middle) leaves for both field and laboratory plants.

Compound 5 was found in much larger concentrations in greenhouse plants (Chapter 4) than in plants grown in controlled-environment chamber (Chapter 2). We have no obvious explanation for this discrepancy, since neither time nor UV light seemed to have any major impact on this compound, which was not identified in the other experiments.

Compound 6 was found in larger concentrations in field plants than in laboratory plants (Chapters 2, 3, 4, 6, 7, 10). UV-B supply increased the similarity in concentrations of compound 6 in middle leaves between greenhouse and field plants, but there were still considerable larger concentrations in field plants, indicating that other factors differing between laboratory and field conditions have a major impact on this compound. In laboratory plants, compound 6 was found almost exclusively in the top leaves (Chapters 2, 3, 4), whereas the vertical distribution in field plants was more uniform, although concentrations still were highest in top leaves (Chapter 4). Concentrations decreased slightly with time in laboratory plants (Chapters 2, 4, 7), whereas no time effect was seen in field plants (Chapter 4).

In conclusion, the occurrence and distribution of the selected phytochemical compounds in control plants were fairly similar in experiments performed under laboratory conditions. With the exception of compound 4, concentrations were higher in field plants than in laboratory plants, and the vertical distribution also was somewhat different from plants grown indoor. Light conditions proved to be an important factor in the phytochemical difference between growth conditions for compounds 1, 2 and 6, which has also been found for phenolic compounds in e.g. birch (Lavola, 1998), rice (Ambasht and Agrawal, 1997) and several other species, as reviewed by Waterman and Mole, 1989).

11.1.2 Chlorsulfuron treatment

Concentrations of compound 1 decreased in both laboratory and field plants after treatment with the herbicide chlorsulfuron, especially in top leaves, but the same trend was seen in middle leaves (Chapters 3, 4, 6 and 7). There were hardly any effects of time in laboratory plants (Chapters 4 and 7), whereas for field plants the herbicide effect increased with time (Chapter 4). Supply of UV-B did not change the general picture in greenhouse plants (Chapters 4 and 7).

Chlorsulfuron treatment generally increased the content of compound 2 in laboratory plants (Chapters 3, 4, 6, and 7), whereas herbicide effects were almost absent in field plants (Chapter 4). Effects were most pronounced in bottom and middle leaves of laboratory plants, whereas concentrations in top leaves were unaffected or reduced (Chapters 3 and 4).

As already mentioned, in laboratory plants compound 3 only occurred after herbicide treatment, whereas in field plants the compound was also found in untreated plants. In laboratory plants, the highest concentrations of compound 3 following herbicide treatment was found in bottom (Chapter 3) or middle leaves (Chapter 4), whereas in field plants the highest concentrations in herbicide treated plants were found in top leaves (Chapter 4). The herbicide effects on compound 3 in middle leaves of greenhouse plants were enhanced by UV-B light (Chapter 4).

The content of compound 4 was almost unaffected in both laboratory and field plants in Chapter 4, but there was a tendency of a decrease at chlorsulfuron dosages up to 0.5 time the recommended field rate, whereas at full dosage the concentration increased again. A similar effect of time on phytochemical response following chlorsulfuron treatment was found in sunflower seedlings by (Suttle *et al*., 1983). In contrast to this, both 0.5 times the field rate (Chapters 5, 6 and 7) and full field rate (Chapter 3) resulted in a decrease in compound 4 in laboratory plants. Since time does not seem to have an effects on the effect of herbicide treatment on compound 4, an explanation of the mentioned discrepancies seems difficult on basis of the presented studies.

Compound 5 was only observed after herbicide treatment in one experiment (Chapter 4), and consequently comparisons between studies are not possible.

Concentrations of compound 6 were generally reduced following chlorsulfuron treatment (Chapters 3, 5 and 6), particularly in top leaves of laboratory plants (Chapters and 4) and middle leaves of field plants (Chapter 4). As was the case for control leaves, UV-B supply caused the concentration of compound 6 in middle leaves of laboratory plants to approach that of field plants (Chapter 6).

All in all, consistency is good between the different laboratory experiment concerning the effects of chlorsulfuron treatment on the selected phytochemical compounds, except for compound 4. Herbicide effects on phytochemicals were generally less evident in field plants than in laboratory plants. Differences in herbicide effects between growth conditions were largest for compounds 2, 3 and 6. Supply of UV-B light in the laboratory reduced the difference between laboratory and field. This may be a consequence of induced changes in the plant, as discussed above, or a result of UV- mediated changes of the chemistry of the herbicide (Gold *et al.*, 1994).

However, part of the difference between field and laboratory plants is due to differences in vertical distribution of the phytochemicals within the plants, and the effect of UV-B on this aspect remains unsolved. Other possible explanations of the observed differences in levels and distribution between laboratory and field plants may include the habitus of the plants. Field plants were very compact compared to laboratory plants, and as a consequence herbicide deposition may be more uneven in field plants, assuming that the top leaves exerted a “shadowing” effects on the lower leaves. This may have led to a lower exposure of middle and bottom leaves, which may explain the tendency of compounds 2 and 3 to concentrate more in the top leaves of field plants than in laboratory plants. Furthermore, field plants are expected to have a thicker wax layer than laboratory plants, and especially the lower (older) leaves of field plants may thus have experienced a lower internal

concentration of the herbicide, which may also add to the difference in vertical distribution compared to laboratory plants (Schreiber and Schönherr, 1992). On the other hand, wind and heavy rainfall may damage the wax layer, resulting in an increased penetration of the herbicide (MacKerron, 1976) as referred in Bonnet and Bossharert, 1994).

11.2 Implications

11.2.1 Laboratory versus field

From the above it follows that laboratory experiments may provide a fairly good background for predictions of the effects of chlorsulfuron on the selected phytochemicals under field conditions concerning the general pattern. However, numerical values are generally underestimated in laboratory, and there seems to be a tendency to overestimate herbicide effects on concentrations of phytochemicals. The UV experiments revealed that laboratory conditions may approach field condition if UV-B light is supplyed in combination with ordinary lamps. There are, of course, other parameters differing between laboratory and field, which may affect the phytochemical profile both in control plants, and in herbicide treated plants. It was noted in Chapter 4 that field plants have a different growth form than laboratory plants, i.e. are more compact and therefore experience a different herbicide and light exposure. In addition, colour differences are often seen. Both characteristics are likely to stem from differences in light intensity, possibly combined with wind and precipitation. Furthermore, these parameters may also affect other plant characteristics, such as the thickness of the wax layer (as discussed above) and the persistence and translocation of herbicides within the plants (Shaner, 1994), which may in turn affect the activity of the herbicide. A solution for approaching the mentioned parameters to the situation in the field may be semi-field experiments, like the ones described in the present report, where plants are grown outside, but with controlled supply of water etc., and sprayed under controlled conditions. However, this limits the experimental season greatly compared to laboratory experiments. Furthermore, the remaining discrepancies from real field conditions (soil characteristics, water supply and exposure conditions) may also indirectly affect the phytochemical response. This may happen through their effects on physical plant characteristics and metabolism, but also because of differences in actual exposure, as indicated in Chapter 9, where spraying with dosages at the recommended field rate resulted in c. 40 % deposition right beneath the sprayer.

11.2.2 Indicator of exposure

In Chapter 3 we found that treatment with all the tested herbicides, i.e. both sulfonylurea herbicides and herbicides with other modes of action, caused a response in the concentrations of compounds 1, 4 and 6 in *F. convolvulus*. These compounds may thus be general herbicide stress indicators, and for compounds 1 and 6 this is supported by the rather uniform reaction in the different laboratory studies (see start of this chapter).

Concentrations of compounds 2 and 3 in *F. convolvulus* leaves only changed when the plants were sprayed with sulfonylurea herbicides (Chapter 3). In the presented laboratory experiments, compound 3 was only found in sprayed plants, not in the control plants. However, in field plants compound 3 was found in low concentrations in controls (Chapter 4). Consequently, the use of

this compound as an indicator of plant exposure to sulfonylurea herbicides may not be as promising as indicated by the laboratory experiments. However, if the simple relationship between chlorsulfuron dosage and leaf concentrations of compound 3 found for laboratory plants in Chapter 5 also holds for the field situation, this compound may still possess an indicator potential.

In Chapter 5 we found that there was a ”window” of 2-3 weeks under laboratory conditions of 20°C in which the effect of chlorsulfuron on compound 3 was detectable and the response linear with herbicide dosage. Under field conditions, this would correspond to a window of approximately 1.5 months in field. We also found that the phytochemical response took at least 4 days to be induced (Chapter 2 and 4), which would correspond to a delay of approximately one week under field conditions. The lowest chlorsulfuron dosage at which the response of compound 3 was induced was 6.25 % (1/16) of the field rate (Chapter 5). At the lowest dosage tested, i.e. 3.125 % of the field rate, no response could be detected. In relation to spray drift (Chapter 9), this means that a phytochemical response in the plant tested here can only be expected within the first meter or so from the spray boom, assuming the sensitivity under field conditions equal the one found under laboratory conditions.

11.2.3 Indicator of effects on herbivores in agricultural fields and in the spray drift zone

In Chapter 8 a model for the concentration of compounds 2 and 3 was presented. The model mediated calculations of body burden with respect to compounds 2 and 3were related to the survival of the insect larvae. Compound 2 was highly correlated with the survival of the larvae. The phenolic compounds we have focused on may co-vary with other compounds, which are the actual elicitors of the observed effects. Such a general response would be caused by the general side effect of sulfonylurea herbicides, i.e. reduced transport out of the leaves (Bestman *et al*., 1990; Vanden Borne *et al*., 1988). Other compounds that might be relevant encompass both primary (e.g. nitrogenous compounds) and secondary plant metabolites. It is intriguing that compound 2 explains not only the mortality of insects on treated plants but also the high mortality of insect placed on control plants. Compound 3 also responded to herbicide dosage, but the control plants weakened the correlation, because compound 3 is not present in control plants.

Another explanation for the observed disappearance/mortality of *G. polygoni* is that a feeding attractant is reduced so that the beetle stops eating. For this to happen it is a prerequisite that the insect does not recognise the plant as a host even under the no-choice conditions of starvation.

In the project, two field experiments was carried out in which the survival of *G. polygoni* larvae was followed over time without any measures of phenolic compounds. In the first experiment, no herbicide treatment was involved, whereas in the other, herbicide treatment was included in three dosages. In all experiments, irrespective of herbicide dosage, only 2 to 4 individuals per replicate (out of 20) survived a full larval development. In order to confirm if this high mortality should be expected from the concentrations of phenolic compounds, we modelled the mortality from the content of compound 2 on the basis of the established regression line between survival and concentration

of compound 2 in laboratory experiments (Chapter 8). The content of compound 2 was modelled with the assumption of 20 larvae from the start, all larvae present on the third leaf counted from the bottom of the plant and UV-B radiation present. The computations revealed that only minor effects of chlorsulfuron dosage are predicted (Figure 1.1), with the number of survivors approximately the same as observed in the experiments.

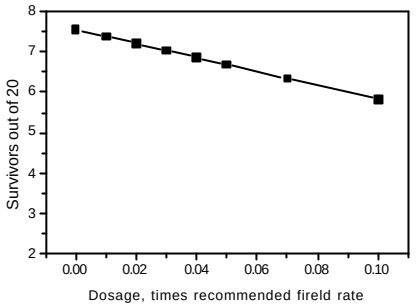


Figure 11.1
Model estimates of number of larvae surviving as a function of chlorsulfuron dosages applied to host plant.

The calculations presented above may also be used in a prediction of *G. polygoni*- survival on plants placed in the spray drift zone. In Chapter 9 it was measured that plant in a 5 m zone downwind of the spray swath would receive between 0.01 and 0.06 times the recommended field rate. A slight reduction in the number of surviving larvae should therefore be expected (Figure 11.1).

The calculations presented above suggest that nearly all *G. polygoni* would die under normal conditions in the agricultural field. This is, however, not the case (Kjær *et al*., 1998a). The reason for the discrepancy could be that normally *F. convolvulus* plants, unlike our test plants, are growing in the shadow of the crop plants. Therefore, they do not receive the same amount of UV-B light. The larvae are primarily found on the lower parts of the plant, i.e. on parts receiving low UV-B radiation. Consequently, the UV parameter in the model is too high for these conditions, but not for the test condition. To make this model more precise and relevant for prediction of field effects, an experiment should be performed with a range of light intensities and qualities, incorporating the behaviour of the larvae.

The spray drift measures reported in Chapter 9 were performed under low wind conditions and with only one swath. The use of multiple swaths has been observed to increase the downwind deposition with a factor approximately 1.4 (Dobson *et al*., 1983; Gilbert and Bell, 1988), and the boom height and wind speed also affect the spray drift (Nordby and Skuterud, 1975). The volume of spray solution as predictor of effect can be questioned, because droplets evaporate and the resulting drop can be more concentrated with respect to the spray and smaller droplet also tend to be better withheld by diverse structures. This may explain the 10 times difference in effect of the same volume spray solution in the spray zone compared to observation under the sprayer observed by (Nordby and Skuterud, 1975). If this observation is valid a safety factor of 10 should be added to the actual deposition measures in order to estimate biological effects. The study, unfortunately, had different effect assessments for the two treatment conditions, i.e. lab and field assessment, rendering the estimate controversial. But if the measure in fact does express an ecotoxicological difference the spray drift zone is greatly expanded.

11.3 Conclusion

In general terms, the implications of the present study are:

- The use of phytochemical exposure indicators specific of single herbicides or groups of herbicides seems promising for plants treated with low-dosage herbicides.
- For herbivorous insects in and outside the field, the main effect of low-dosage herbicides is likely to be the possible loss of food source. The effects so far documented for one herbicide-group out of six and one insect-plant system out of three, is not a general phenomenon, how ever, it is likely to be found in other systems as well.
- Drift effects of low dosage herbicides are likely to occur within a distance of 5 m from the sprayed field on plants in the nearby hedge/field margin and in adjacent crops.
- The study has underlined the difficulties in making reliable predictions of the concentration of phenolic compounds in plants grown in the field on basis of laboratory data. The inclusion of UV-light in laboratory set-ups reduce the difference between lab and field significantly

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