An easy and rapid method using microscopy to determine herbicide effects in Poaceae weed species

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Abstract: A new, easy, rapid and relatively inexpensive method using microscopy has been developed for the detection of herbicide effects in leaves of grass weed species displaying no visual signs of damage. The method has potential to be used as a tool to indicate future death of grass species due to herbicide exposure by observing phytochemical effects, i.e. early-warning effects. In the present study, *Apera spica-venti* (L.) Beauv., *Bromus hordeaceus* L., *Alopecurus myosuroides* Huds., *Lolium perenne* L. and *Poa annua* L. were exposed to lethal rates of four herbicides with different mode of action. The herbicides investigated were the thiocarbamate: prosulfocarb, the sulfonylurea: iodosulfuron, the aryloxyphenoxypropionate: fenoxaprop-P-ethyl and the organophosphate glyphosate. Autofluorescence of leaves was studied under a microscope using ultraviolet and blue light. The fluorescence of leaves treated to enhance flavonoids was also examined. To confirm the results, microspectrofluorometry was performed. Effects indicating future death of the grasses were observed in visually healthy leaves following treatment with prosulfocarb, glyphosate and iodosulfuron. No changes were detected following treatment with fenoxaprop-P-ethyl. After exposure to glyphosate or iodosulfuron, changes in the content of flavonoids and other compounds with a conjugation system and rigid structure and a decrease in the content of chlorophyll were detected in the leaves. Prosulfocarb treatment resulted in changes in the content of flavonoids and other compounds with a conjugation system and rigid structure and an increase in the content of chlorophyll in the leaves. The results obtained from microspectrofluorometry indicated that exposure to prosulfocarb caused a reduction in the flavonoids quercetin, naringenin and/or naringin.
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Keywords: herbicide metabolism; flavonoids; chlorophyll; microspectrofluorometry; early warning

1 INTRODUCTION

In the Poaceae family, 44% of the plant species are among those termed the worst weeds of the world.[1] These include loose silkybent (*Apera spica-venti* (L.) Beauv.), soft brome (*Bromus hordeaceus* L.), blackgrass (*Alopecurus myosuroides* Huds.), perennial ryegrass (*Lolium perenne* L.) and annual bluegrass (*Poa annua* L.), which are common in crops in northwestern Europe.

In order to control Poaceae weed species in crops, a wide range of herbicides are available. Iodosulfuron, prosulfocarb and fenoxaprop-P-ethyl are selective herbicides that are recommended for control of certain grass weed species, while glyphosate is a non-selective herbicide used for total weed control on non-cropped areas or in transgene glyphosate-resistant crops. The four herbicides represent different modes of action. Iodosulfuron is a sulfonylurea and

inhibits the synthesis of branched chain amino acids.[2] Prosulfocarb is a thiocarbamate and inhibits the synthesis of long-chain fatty acids.[3] Fenoxaprop-P-ethyl is an aryloxyphenoxypropionate and inhibits fatty acid synthesis,[4] while the organophosphate glyphosate inhibits the synthesis of aromatic amino acids.[5] The phytochemical changes in composition and content due to exposure are therefore expected to be different for the four herbicides.

Phytochemical changes in Poaceae due to herbicide exposure appear before visual signs are observed on the plants in the field.[6] Detection of phytochemical changes can consequently be used to show early-warning signals indicating future death of plants.

A wide range of analytical methods is available to detect phytochemical changes, such as chromatographic techniques coupled online with various detectors.[7–10] These methods are time-consuming

owing to sample preparation, including extraction and filtration, are complicated to perform and require expensive apparatus. Often lower precision is acceptable and, in these cases, microscopy can be utilised instead. The many advantages of microscopy used to detect phytochemical changes include high speed, low cost and low maintenance. Microscopy is therefore an ideal method, useful where a fully equipped laboratory is not available but a quick test is needed. Microscopy has previously been used for a wide array of studies, such as easy and early detection of environmental stress in plant secreting cells,[11] rapid detection of production of recombinant insecticidal proteins in plants[12] and rapid, simple and inexpensive identification of herbicide-tolerant transgenic rice seeds.[13]

In this paper we focus on a new microscopy method for detecting herbicide effects in pieces of leaves of grass weed species displaying no visual signs of damage. Five Poaceae species exposed to four herbicides with different modes of action were studied using three different modes of microscopy.

2 EXPERIMENTAL METHODS
2.1 Plant cultivation and herbicide application
Plants of loose silkybent, soft brome, blackgrass, perennial ryegrass and annual bluegrass were grown in 2 litre pots with 15 plants per pot. A potting mixture of soil, sand and peat ($2 + 1 + 1$ by weight) containing all necessary macro- and micronutrients was used. The pots were subirrigated automatically twice a day with deionised water. Plants of loose silkybent and annual bluegrass were also grown in 1 litre pots in field soil with 15 plants per pot. The field soil contained 1.6% organic matter. The 1 litre pots were subirrigated automatically twice a day with a nutrient solution containing all necessary macro- and micronutrients. All the pots were placed in a heated glasshouse ($14\,°C$) with supplementary artificial light (16/8 h light/dark photoperiod).

The following herbicides were applied to the plants: iodosulfuron $50\,g\,kg^{-1}$ WG (Hussar, Bayer Crop-Science A/S, Kgs. Lyngby, Denmark) at 5 g a.i. (active ingredient) ha^{-1} in tank mixture with 0.5 litre ha^{-1} of the oil adjuvant Renol (Bayer CropScience A/S); pro-sulfocarb $800\,g\,litre^{-1}$ EC (Boxer EC, Syngenta Crop Protection A/S, Copenhagen, Denmark) at 2400 g a.i. ha^{-1}; fenoxaprop-P-ethyl $69\,g\,litre^{-1}$ EC (Primera Super, Bayer CropScience A/S) at 69 g a.i. ha^{-1} in tank mixture with $2\,g\,litre^{-1}$ of the anionic surfactant Isoblette (Bayer CropScience), except on blackgrass where the fenoxaprop-P-ethyl rate was 55.2 g a.i. ha^{-1}; and glyphosate isopropylamine $360\,g\,AE\,litre^{-1}$ SL (Roundup Bio, Monsanto Crop Sciences Denmark A/S, Copenhagen, Denmark) at 360 g AE ha^{-1}. The rates applied are those currently applied in farming in Denmark, which are known by experience to be lethal.

Glyphosate, fenoxaprop-P-ethyl and iodosulfuron are primarily taken up through foliage and consequently were applied to plants grown in the potting

mixture containing peat. Prosulfocarb, which also exerts soil activity, was applied to plants grown in field soil in order to avoid herbicide inactivation by binding to organic matter.

The herbicides were only applied to the weed species for which their use is recommended in Denmark.

Herbicide application was carried out at the relevant leaf stage, i.e. the two/three-leaf stage for prosulfocarb and the four/five-leaf stage for glyphosate, fenoxaprop-P-ethyl and iodosulfuron. The herbicides were applied in deionised water in a spray volume of 145 litre ha^{-1} using a laboratory pot sprayer fitted with two Hardi-ISO F-110-02 flat fan nozzles (Hardi International, Taastrup, Denmark).

Fourteen days after exposure, herbicide damage to the plants was evaluated visually, the plants were harvested and immediately freeze-dried and thereafter kept dry and protected from light until analysis.

2.2 Microscopy
For each species a 4–6 mm piece of the centre of the leaf was cut from ten randomly selected plants which were either untreated or had been treated with herbicide. Care was taken to use only healthy, normal-looking leaves.

First the autofluorescence of the leaf pieces was studied directly without any reagent treatment. The fluorescence of the leaf pieces was then studied after treatment with Neu solution,[14] which enhances the flavonoids. The Neu solution contained 2-aminoethyl diphenylborinate ($10\,g\,litre^{-1}$) and polyethylene glycol 4000 ($50\,g\,litre^{-1}$) in 95% ethanol. The leaf pieces were placed on a microscope slide, and 5 µl of the Neu solution was distributed evenly on each of the leaf pieces.

An epi-fluorescence microscope (Optiphot 2, Nikon, Champigny-sur-Marne, France) with a superhigh-pressure mercury lamp was used to study the leaf pieces. The microscope was equipped with both an ultraviolet filter with an excitation filter at 365 nm and a barrier filter at 400 nm, and a blue filter with an excitation filter at 450–490 nm and a barrier filter at 520 nm. A digital camera (Coolpix 4500, Nikon) stored the pictures. The camera was set on normal image adjustment, focus mode at infinity and aperture 5.0. The shutter speed was varied between 0.001 and 1s, using the same shutter speed for pictures that were to be compared. To ensure picture comparability, the camera was focused on the same standard leaf for every picture, then, before the picture was taken, the standard leaf was replaced by a leaf piece of the sample without refocusing.

Three modes of microscopy were used. Autofluorescence of sample leaves was studied using both ultraviolet and blue light, while Neu-treated leaves were observed using only ultraviolet light. For each mode a 40× magnification was used.

The difference between control and herbicide-exposed samples was evaluated. The colour of the sample was established using each of the three modes

of microscopy, and the variation in colour within the samples was determined as little, medium or large. The difference between a pair of control and exposed samples using each of the three modes of microscopy was determined as a 'yes' or a 'no'.

2.3 Microspectrofluorometry

For loose silkybent and annual bluegrass a 4–6 mm piece of the centre of the leaf was cut from ten randomly selected plants which were either untreated or had been treated with the herbicide prosulfocarb. Care was taken to use only healthy, normal-looking leaves. A Neu solution was prepared and applied to leaf pieces as described in Section 2.2.

A microspectrofluorometer (Jobin-Yvon, Chilly-Mazarin, France) was used to examine the leaf pieces. Using a xenon lamp (450 W), an excitation monochromator (Triax 180, CCD camera) and a filter barrier at 400 nm, the wavelengths 361–368 nm were selected to excite the compounds in the sample placed on an Olympus microscope (BX 60, Rungis, France). A pinhole allowed the selection of an area of analysis with a diameter of 300 μm. The fluorescence of the compounds in the sample was diffracted by an emission monochromator (Triax 320, CCD camera) and detected with a charge-coupled device (CCD) camera. The software Spectra Max, a part of the microspectrofluorometry equipment (Jobin-Yvon) displayed the fluorescence emission spectrum.

Fluorescence spectra were obtained at four different positions on each of the five leaf pieces.

The difference between control and herbicide-exposed samples was evaluated. The mean spectrum of each sample was calculated and the difference in the spectra between a pair of control and exposed samples was determined using a t test ($P \leq 0.05$).

3 RESULTS

3.1 Visual evaluation at harvest

At harvest of the grasses, no visual signs of damage were observed on the leaves of plants exposed to prosulfocarb. On plants exposed to fenoxaprop-P-ethyl, small yellow spots were observed. Plants exposed to iodosulfuron and glyphosate displayed visual signs of leaf damage as yellow spots. Only leaf pieces without visual damage were used for further analysis.

3.2 Microscopy

Using microscopy and ultraviolet light, colour changes due to prosulfocarb exposure were seen in loose silkybent (Fig. 1) and annual bluegrass both before and after the leaf pieces were treated with Neu solution (Table 1). With annual bluegrass, colour changes were also seen under blue light before Neu treatment (Fig. 2).

Iodosulfuron exposure induced differences in colour between control and exposed plants with all the applied modes of microscopy.

With fenoxaprop-P-ethyl, none of the modes of microscopy showed any differences between control and treated plants.

A difference in colour due to glyphosate exposure was detected with the microscope under blue light before Neu treatment of the leaves, and a colour change was also detected with the microscope under ultraviolet light for all plant species except blackgrass when treated with Neu solution.

Chlorophyll autofluorescence is red under blue light. Autofluorescence under ultraviolet light arises from a wide range of compounds possessing a conjugation system and rigid structure,[15] such as aromatic amino acids, flavonoids and phenolic acids. After treatment with Neu the fluorescence of flavonoids is enhanced and they become yellow or orange when examined under ultraviolet light.

The effect of iodosulfuron viewed with the microscope under blue light was seen as a reduced red fluorescence, indicating the disappearance of chlorophyll. Upon treatment with Neu solution an increase in yellow and orange fluorescence under ultraviolet light indicated changes in flavonoids. A change from blue to bright blue/green autofluorescence under ultraviolet light indicated a change in compounds possessing a conjugation system and rigid structure, e.g. flavonoids.

Fenoxaprop-P-ethyl exposure displayed no effect on flavonoids and other compounds possessing

Table 1. Phytochemical changes in leaves of five Poaceae species exposed to four types of herbicide compared with leaves of control plants observed using three modes of microscopy[a]

Herbicide	Apera spica-venti			Bromus hordeaceus			Alopecurus myosuroides			Lolium perenne			Poa annua		
Iodosulfuron	1	2	3	NC			NC			1	2	3	NC		
	+	+	+							+	+	+			
Prosulfocarb	1	2	3	NC			NC			NC			1	2	3
	0	+	+										+	+	+
Fenoxaprop-P-ethyl	1	2	3	NC			1	2	3	NC			NC		
	0	0	0				0	0	0						
Glyphosate	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	+	0	+	+	0	+	+	+	0	+	+	+	+	+	+

[a] Key: 1, autofluorescence at excitation with 450–490 nm ($n = 10$); 2, autofluorescence at excitation with 365 nm ($n = 10$); 3, fluorescence after Neu treatment at excitation with 365 nm ($n = 10$); +, a difference was detected; 0, no difference was detected; NC, not cultivated.
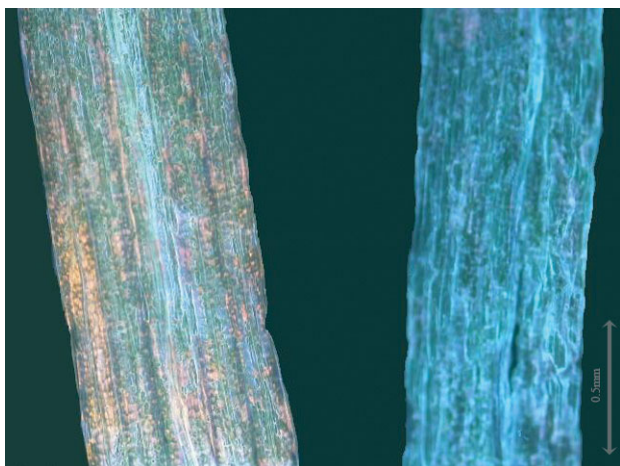
Figure 1. Fluorescence after Neu treatment of *Apera spica-venti* leaves excited with 365 nm: left, control leaf; right, prosulfocarb-exposed leaf.
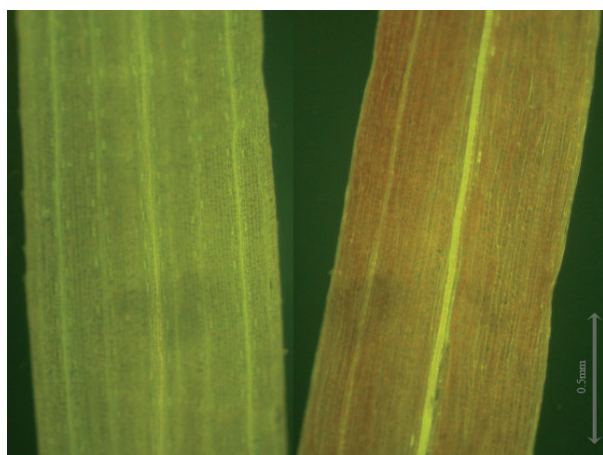
Figure 2. Autofluorescence of *Poa annua* leaves excited with 450–490 nm: left, control leaf; right, prosulfocarb-exposed leaf.

a conjugation system and rigid structure, or on chlorophyll.

Glyphosate exposure produced effects on chlorophyll and flavonoids similar to iodosulfuron in all plant species except blackgrass. It also produced a colour change from blue to bright blue/green in blackgrass, perennial ryegrass and annual bluegrass, indicative of alterations in compounds possessing a conjugation system and rigid structure.

On the other hand, leaf pieces of plants exposed to prosulfocarb showed an increase in the red fluorescence of chlorophyll (Fig. 2) except in loose silkybent, a decrease in the orange/yellow fluorescence of flavonoids (Fig. 1) and a change from bright blue/green to blue in the fluorescence originating from compounds possessing a conjugation system and rigid structure.
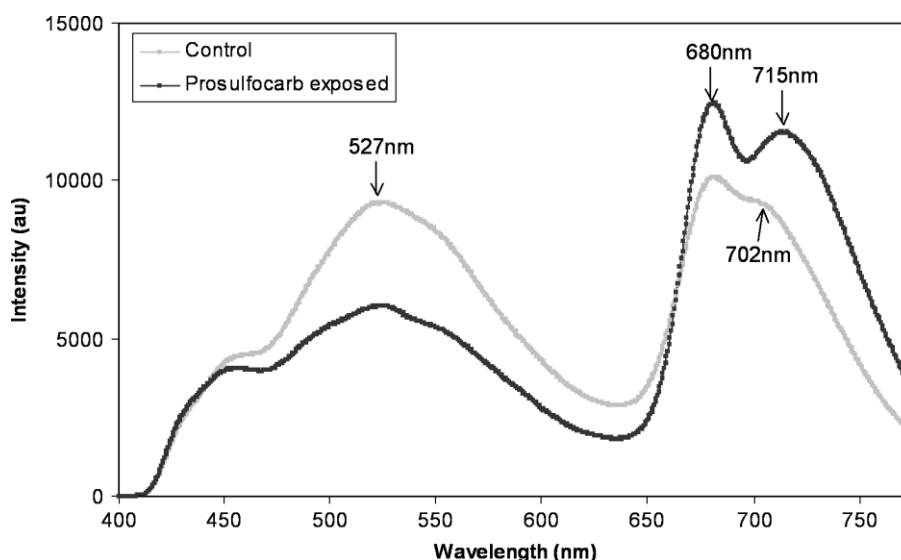
3.3 Microspectrofluorometry
For both loose silkybent and annual bluegrass the phytochemical differences produced by exposure to prosulfocarb were evaluated qualitatively using the fluorescence emission spectra produced by the microspectrofluorometer.

The fluorescence spectrum of control plants of loose silkybent revealed a significantly larger intensity in the wavelength areas 457–650 and 680–775 nm compared with the fluorescence spectrum of the prosulfocarb-exposed loose silkybent observed under the same conditions of measurement (Fig. 3). At 558 nm the intensity of the peak in the control was twice that in the herbicide-treated leaf. At 677 nm the spectra of both control and exposed leaves showed a peak, but the control peak was again larger. The intensity of the peak at 722 nm in the herbicide-treated leaf was lower than that in the control.

For annual bluegrass a difference between the spectra of control and prosulfocarb-exposed leaves was also observed (Fig. 4). At 525–646 nm the intensity of the unexposed annual bluegrass was significantly larger than that of the prosulfocarb-exposed annual
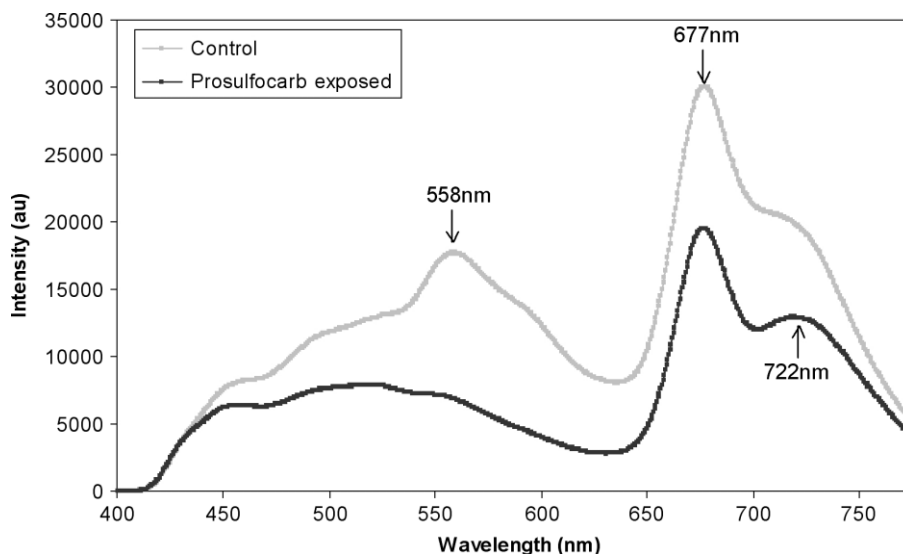
Figure 3. Fluorescence emission spectra for leaves of *Apera spica-venti* unexposed (control) and exposed to prosulfocarb, treated with Neu and excited with 361–368 nm.

Figure 4. Fluorescence emission spectra for leaves of *Poa annua* unexposed (control) and exposed to prosulfocarb, treated with Neu[14] and excited with 361–368 nm.

Table 2. Peak maxima in microspectrofluorometry of flavonoid standards on dissolution in methanol

Flavonoid	Neu[14] derivatisation peak (nm)	No derivatisation		
		First peak (nm)	Second peak (nm)	Third peak (nm)
Apigenin	562	670	630	458
Apigenin-7-glucoside	562	655	628	464
Catechin	444[a]	447	524	673
Eriodychol	533	534	447	–
Genistein	550	581	449	–
Genistin	549[b]	524	772	458
Hyperoside	590	648	510	493
Kaempferol	536	547	663	–
Luteolin	574	648	450	–
Luteolin-7-glucoside	595	647	448	–
Morin	546	572	–	–
Myricetin	583	557	–	–
Myricitrin	584	666	621	453
Naringenin	535	526	666	–
Naringin	530	526	–	–
Quercetin	564	549	668	–
Rutin	573	670	528	–
Taxifolin	563	563	454	–

[a] Plus a second peak at 507 nm.
[b] Plus a second peak at 670 nm and a third peak at 453 nm.

bluegrass, and at 667–688 and 699–775 nm the intensity of the exposed plants was significantly larger than that of the control. At 527 nm the intensity of the peak in the herbicide-treated leaf was two-thirds that in the control. At 680 nm the spectra of both control and exposed leaves showed a peak, but that of the exposed leaf was larger. The intensity of the peak at 702 nm in the control was lower than that in the exposed leaf, and the intensity of the peak at 715 nm in the herbicide-treated leaf was larger than that in the control.

Tests with standards dissolved in methanol using the microspectrofluorometric method showed that 90% of flavanoids are fluorescent at 500–600 nm upon Neu derivatisation (Table 2). Chlorophyll *a* produces a peak between 676 and 680 nm *in vivo*.[16] In certain cases a second red-shifted peak of chlorophyll *b* is observed.[17]

The intensity of the flavonoids in loose silkybent and annual bluegrass was significantly higher in the control than in the exposed leaves. In annual bluegrass the intensity of chlorophylls *a* and *b* was significantly higher in the exposed plants than in the control.

One of the flavonoids expected to decrease in content in loose silkybent following prosulfocarb exposure was the flavonol aglycone quercetin. A single peak was observed at 564 nm for a quercetin standard dissolved in methanol when treated with Neu. Small differences in peak wavelengths occurred between the standard and the sample owing to non-equivalent matrices, thus this matched the finding of the peak at 558 nm in the control sample on treatment with Neu. To confirm the expected identity, tests of the samples were made using the method described in Section 2.3 but without the Neu solution. The autofluorescence of the quercetin standard had its maximum peak at 549 nm, matching a peak at 551 nm in the control sample. A second peak at 668 nm was observed in the quercetin standard, which, if present, would have been hidden by the three times larger chlorophyll peak in the spectrum of the sample. Thus quercetin was very likely present in the sample.

The flavanone aglycone naringenin and/or naringin, naringenin-7-*O*-neohesperidoside, were expected to be among the flavonoids whose content was reduced in annual bluegrass after prosulfocarb exposure. The peak at 527 nm in the fluorescence spectrum for the control samples treated with Neu matched the only peak of the naringenin standard at 525 nm, and it matched the only peak of the naringin

standard at 530 nm. Both the naringenin and naringin standards without Neu treatment revealed a single peak positioned at 526 nm, which matched the finding of a peak at 523 nm in the control sample when it was not treated with Neu.

4 DISCUSSION AND CONCLUSIONS

The present study revealed that effects of herbicides on Poaceae weeds could be detected by a simple microscopy method, and this was confirmed on selected plant samples by microspectrofluorometry. In general, all the modes of microscopy were valuable in detecting the effects of herbicides on Poaceae.

The herbicide-treated grass weed species provided information concerning phytochemical responses, detectable before visual responses appeared, due to the lethal rates of glyphosate, iodosulfuron and prosulfocarb, and thus the phytochemical changes induced by herbicide treatments could provide the basis for early detection of lethal effects.

Prosulfocarb was studied the most thoroughly of the four herbicides. The phytochemical effects of prosulfocarb were expected to be the most difficult to observe, because the visual symptoms following exposure to prosulfocarb appeared slowly compared with those of the other herbicides.

The results from microscopy and microspectrofluorometry demonstrated that prosulfocarb reduced the fluorescence intensity of flavonoids in the leaves. The reduced intensity indicated either that prosulfocarb decreased the content of flavonoids or that the fluorescence intensity of the disappearing flavonoids was larger than that of the emerging flavonoids. Prosulfocarb is a carbamate herbicide known to inhibit the elongase enzyme, which facilitates the synthesis of very-long-chain fatty acids.[3] In previous experiments by Tanigaki *et al.*,[18] with the carbamate herbicide propyl *N*-(3,4-dichlorophenyl)carbamate (5091), inhibition of fatty acid synthesis was shown to affect products of the shikimate pathway, supporting the results reported in this study. Microscopy indicated changes in the composition of compounds possessing a conjugation system and rigid structure, such as aromatic amino acids, flavonoids and phenolic acids. It could be the flavonoids, as suggested by microspectrofluorometry and microscopy, or it could be other compounds observed as shoulders or hidden beneath other peaks in the fluorescence spectra of the microspectrofluorometry. Which compounds were affected cannot be determined on the basis of the results of this study. Microspectrofluorometry showed that the content of the flavonoid quercetin decreased in loose silkybent. This was in accordance with the findings of Johnson *et al.*,[19] who identified quercetin-glycones in Poaceae. Microspectrofluorometry suggested that the content of naringenin and/or naringin was reduced in annual bluegrass, a finding supported by previous experiments on Poaceae.[20] Isolation and identification would have to be performed to verify the presence of these compounds. Microscopy and microspectrofluorometry determined that the content of chlorophyll increased in certain Poaceae species. Similar observations were made in the alga *Anabaena sphaerica* following exposure to the thiocarbamate herbicide molinate.[21]

The aryloxyphenoxypropionate herbicide fenoxaprop-P-ethyl inhibits fatty acid synthesis by targeting the enzyme acetyl CoA-carboxylase facilitating the synthesis of malonyl CoA.[4] Even though the plants were exposed to lethal rates of fenoxaprop-P-ethyl, microscopy indicated no effect after 2 weeks on the content of chlorophyll, flavonoids or other compounds containing a conjugation system and rigid structure, which was supported by results from thin layer chromatography studies (Lauridsen L, unpublished).

The mode of action of the sulfonylurea herbicide iodosulfuron is inhibition of acetolactate synthase and consequently the inhibition of branched amino acid synthesis.[2] Many essential enzymes are synthesised from branched amino acids, so disturbance on several biosyntheses can be expected. This could explain the observed changes in the content of compounds with a conjugation system and rigid structure, e.g. the flavonoids, detected by microscopy. The content of chlorophyll decreased, as reported in experiments on pea (*Pisum sativum* L.), lentil (*Lens culinaris* L.)[22] and grasses such as *Taeniatherum caput-medusae* (L.) Nevski and *Bromus tectorum* L.[23] after exposure to sulfonylurea herbicides.

Microscopy demonstrated that the organophosphate glyphosate changed the content of flavonoids in most of the Poaceae species. Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase in the shikimate pathway and thus the production of aromatic amino acids.[5] Aromatic amino acids are precursors of flavonoids, explaining the observed effects on this group of phenolic derivatives. For three of the five plant species the content of compounds containing a conjugation system and rigid structure changed. The observed changes could be in the flavonoids and/or in the aromatic amino acids. The content of chlorophyll also decreased, as observed previously in the weed *Chenopodium album* L. following exposure to glyphosate.[24]

All the effects detected in microscopy are in agreement with the results obtained through microspectrofluorometry, comparable to previously performed experiments, or can be related to the mode of action of the herbicides. Hence, it is reasonable to conclude that the results concerning herbicide effects provided by microscopy are reliable.

The study has demonstrated that microscopy can be used to predict the future death of herbicide-stressed plants before macroscopic symptoms appear. The speed and simplicity of the method suggest that it might be used to confirm whether a herbicidal application has been effective or an additional dose should be applied to protect crop yield.

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