

Linking fluorescence induction curve and biomass in herbicide screening

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Abstract: A suite of dose–response bioassays with white mustard (*Sinapis alba* L) and sugar beet (*Beta vulgaris* L) in the greenhouse and with three herbicides was used to analyse how the fluorescence induction curves (Kautsky curves) were affected by the herbicides. Bentazone, a photosystem II (PSII) inhibitor, completely blocked the normal fluorescence decay after the P-step. In contrast, fluorescence decay was still obvious for flurochloridone, a PDS inhibitor, and glyphosate, an EPSP inhibitor, which indicated that PSII inhibition was incomplete. From the numerous parameters that can be derived from OJIP-steps of the Kautsky curve the relative changes at the J-step [$F_{vj} = (F_m - F_j)/F_m$] was selected to be a common response parameter for the herbicides and yielded consistent dose–response relationships. Four hours after treatment, the response F_{vj} on the doses of bentazone and flurochloridone could be measured. For glyphosate, the changes of the Kautsky curve could similarly be detected 4 h after treatment in sugar beet, but only after 24 h in *S. alba*. The best prediction of biomass in relation to F_{vj} was found for bentazone. The experiments were conducted between May and August 2002 and showed that the ambient temperature and solar radiation in the greenhouse could affect dose–response relationships. If the Kautsky curve parameters should be used to predict the outcome of herbicide screening experiments in the greenhouse, where ambient radiation and temperature can only partly be controlled, it is imperative that the chosen fluorescence parameters can be used to predict accurately the resulting biomass used in classical bioassays.

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Keywords: Kautsky curve; bentazone; flurochloridone; glyphosate; fluorescence

1 INTRODUCTION

Screening for herbicide efficacy in the greenhouse or in the field is usually a lengthy process.¹ The experiments take up much space and the evaluation of results, either quantitatively (biomass) or qualitatively (scoring systems), can usually first be done more than a week after treatment. Several novel methods have been implemented in an attempt to shorten the evaluation time, such as the measurement of herbicide-linked accumulation of intermediates in the plant.² If it were possible to apply a cost-effective method shortly after exposure to a herbicide, the costs of screening experiments would be greatly reduced. An option could be to use the shape and form of the fluorescence transition curve, or Kautsky curve.^{3–7} The use of various fluorescence parameters, derived from the Kautsky curve, is common in photosynthesis research and in plant stress research.^{4,8–11} It is well documented that the shape of the Kautsky curve is affected by various stress factors, such as temperature and water stress, pathogens and herbicides.^{8,12–16} Measurement of fluorescence is a rapid, non-invasive and simple method to measure a number of parameters linked to the physiological

status of the plant. However, as pointed out by Govindjee,⁴ the wealth of information contained in the Kautsky curves can be so overwhelming that it may become an ambiguous signal. Consequently, a direct cause-and-effect relationship is sometimes difficult to identify.

The most popular parameter derived from the Kautsky curve is that of maximum relative fluorescence [$(F_m - F_0)/F_m$], which measures the relative magnitude of the fluorescence curve between the O and P steps (see Fig 1). This parameter is effective in providing a snapshot of the physiological status of a plant being exposed to various stress factors, and has been used to measure the effect of photosystem II (PSII) inhibitors.^{7,17–19} Fluorescence has also been used for the study of herbicides with other modes of action than the inhibition of PSII.^{5,16,20} The most consistent results have been obtained under controlled conditions in the laboratory or in growth chambers where ambient temperature and light regimes were strictly monitored.¹⁹ If the evaluation of screening experiments for herbicides should utilise the Kautsky curve, then it is imperative to establish well-documented dose–response relationships for the

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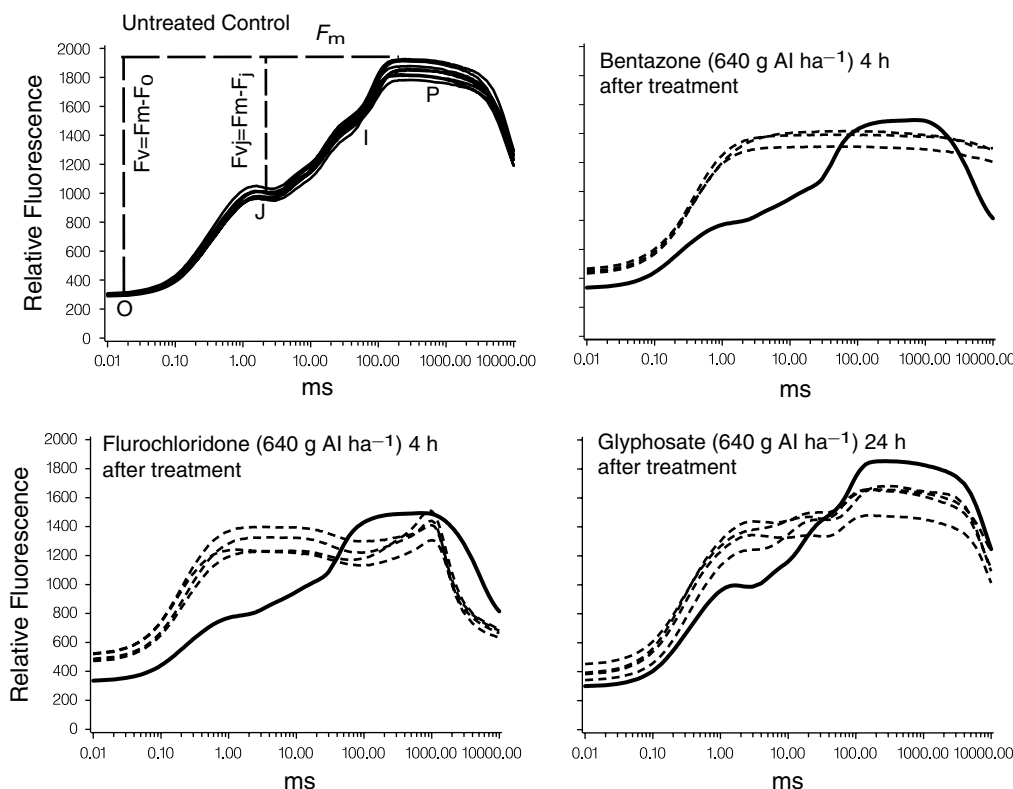


Figure 1. Kautsky curves for control (eight replicates) and herbicide treated white mustard (*Sinapis alba*) (four replicates). The average transition curves for the untreated plants (—), measured at the same time as for the treated plants (- - -) are included for comparison. The OJIP-steps are based upon the definitions given by Govindjee.⁴ Illumination of dark adapted leaves for 10 s with a wavelength of 650 nm and an intensity of 3000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

selected fluorescence parameter and link these measurements to the resulting biomass of the test plants.

The objectives of this paper are threefold: (1) to analyse how three herbicides with different sites of action affect the shape of the Kautsky curve, (2) to find a common fluorescence parameter that can describe dose–response relationships, and (3), to link the fluorescence measurement to biomass production in response to herbicide dose. The goal is to link the chosen fluorescence parameter to the final biomass outcome of a suite of assays, even when we do not have full command of the ambient light and temperature regime.

2 MATERIALS AND METHODS

White mustard (*Sinapis alba* L) and sugar beet (*Beta vulgaris* L) were grown in the greenhouse in pots with sphagnum during a three-month period from May to August 2002. White mustard plants were thinned to six plants per pot a week after sowing and sprayed at their fourth true leaf stage with either bentazone (Basagran 480 g AI litre⁻¹ SL, dose range 10–640 g AI ha⁻¹), glyphosate-isopropylamine (Cheminova non-commercial 360 g AE litre⁻¹ SL, 10–640 g AE ha⁻¹) or flurochloridone (Rainbow 250 g AI litre⁻¹ EC, dose range 10–640 and 5–320 g AI ha⁻¹). Sugar beets were thinned to four plants per pot and sprayed at the two true leaves stage with glyphosate-isopropylamine (dose range of

20–1280 and 10–640 g AE ha⁻¹). One week after spraying, plants were harvested and their dry weight determined.

There were two independent experiments for each herbicide, whose dose–response curve consisted of seven doses plus an untreated control. The experimental layout was a complete randomised design with four replicates for each herbicide dose and eight replicates for the untreated control. The spraying was done in a spray-chamber a Hardi 4110-12 hydraulic nozzle at a pressure of 4 bar to give a rate of 150 litre ha⁻¹.

Chlorophyll fluorescence was measured at 4, 24 and 48 h after spraying, using a portable chlorophyll fluorometer (Handy-PEA, Hansatech Instruments, King’s Lynn, Norfolk, UK), which emits a light of 650 nm wavelength with an intensity of 3000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 10 s. The measurements were taken on dark-adapted leaves, (30-min adaptation), at approximately the same stage of development.

The Kautsky curves for different doses and time intervals were visually examined for the effects of time and dose. Parameters from the Kautsky curve were obtained using the BIOLYZER program developed by R Rodriguez and R Strasser, University of Geneva, Switzerland, available at <http://www.unige.ch/sciences/biologie/bioen/bioindex.html>, with OJIP steps as fix points.^{21,22}

Dose–response effects were analysed using biomass as well as the parameter F_{vj} , defined as $[(F_m - F_j)/F_m]$,

where F_j is fluorescence measured after about 2 ms (the J-step of the Kautsky curve) and F_m is the maximum fluorescence intensity (P-step) (see Fig 1). A logistic curve was used to describe the response of fluorescence measurements and dry matter against dose:²³

$$U = \frac{D - C}{1 + \exp\{b[\log(z) - \log(ED_{50})]\}} + C, \quad b > 0,$$

where U is the response at the dose z . D is the upper limit where the dose is zero, and C is the lower limit at infinite doses. ED_{50} is the dose that gives a response half way between D and C , and b describes the slope around ED_{50} . The method of stabilizing the variance of responses by using Transform-Both-Sides on the non-linear regression and the tests for lack of fit of the models have been described elsewhere.²³

3 RESULTS AND DISCUSSION

The Kautsky curves for all plant species, doses and times were inspected, and Fig 1 illustrates the effect of the three herbicides on the Kautsky curve. It should be noted that the Kautsky curves for the untreated control plants were consistent, whilst for the herbicide-treated plants the variation was somewhat larger, probably because of non-uniform spray coverage. Uneven coverage will also be reflected in the subsequent translocation of the herbicides into newly developed leaves. Except for flurochloridone 24 h after treatment, the effect of the herbicides on the Kautsky curves were visible before any herbicidal symptoms emerged on the plants.

Bentazone is an inhibitor of PSII, and is known to dramatically change the shape of the Kautsky curve, as has been shown for many PSII inhibitors.^{5,16} In Fig 1 the effect of bentazone on the J-step (inhibition of the transfer of electrons from Q_A to Q_B) is obvious. The lack of fluorescence decay after 1000 ms suggests that PSII is totally inactivated, and that PSII-mediated fluorescence quenching cannot take place.

Flurochloridone, a phytoene desaturase (PDS) inhibitor, causes rapid bleaching of susceptible pigments and thus disrupts the protective effect of the carotenoids that play an important role in light harvesting as well as the protection of chlorophyll from active oxygen species.²⁴ Compounds that inhibit the biosynthesis of carotenoids are classified as inhibitors of photosynthesis *sensu lato*, but, in contrast to the PSII inhibitors, visual symptoms (bleaching) develop rapidly following herbicide exposure. It is therefore expected that PDS herbicides will have a marked effect on the Kautsky curve soon after herbicide application. In Fig 1, the observed effect of flurochloridone on the J-step can be explained by the photo-inhibition of PSII, following the bleaching of the protective carotenoids. In contrast to bentazone, the level of fluorescence quenching at 1000 ms suggests that some PSII activity remains.

Glyphosate is an inhibitor of EPSP synthase and blocks the synthesis of the aromatic amino acids. It is

not a photosynthetic inhibitor *per se*, but binding of glyphosate to EPSP synthase in the cytosol leads to unregulated diversion of erythrose-4-phosphate (E4P) into the shikimate pathway, and thus to an inhibition of the reduction of ribulose biphosphate (RBP).²⁵ This diversion of carbon as E4P from the Calvin cycle lowers the level of RBP, leading ultimately to the over-reduction of the photosynthetic electron chain and to the photo-inhibition of PSII. At the same time, inhibition of EPSP synthase leads to the inhibition of homogentisic acid production, a metabolite required for the reduction of plastoquinone, a component of the electron transfer chain in photosynthesis responsible for mediating the flow of electrons from PSII to the cytochrome b6-f complex. In addition, reduced plastoquinone is required for the activation of newly synthesised carotenoids. Thus, glyphosate can mediate the over-reduction and photo-inhibition of PS II, while at the same time inhibiting the activation of the vital carotenoids required to protect PSII from photo-inhibition. In Fig 1, the observed effect of glyphosate at the J-step can thus be explained by the photo-inhibition of PSII, following the over-reduction of the photosynthetic apparatus as well as a restriction in the activation of protective carotenoids. In contrast to bentazone, the level of fluorescence quenching at 1000 ms suggests that at least some PSII activity remains. Madsen *et al*²⁰ found a notable effect of glyphosate after the P-step by using the slope of the transition curve, which suggests that the PSII mediated fluorescence quenching is somewhat affected. Kirkwood *et al*¹⁷ used the $F_v/F_m [(F_m - F_o)/F_m]$ parameter to measure the effect of glyphosate on the fluorescence and detected some effect one day after treatment, whilst Ralph²⁶ did not find any effect of glyphosate on fluorescence even at two orders of magnitude higher doses of glyphosate than those used for PSII-inhibiting herbicides.

It is particularly relevant to note that a complete lack of fluorescence decay after 1000 ms is only observed for the PSII inhibitor, bentazone (Fig 1). In contrast, fluorescence quenching is present after 1000 ms for the non-PSII-inhibiting herbicides glyphosate and flurochloridone; suggesting that PSII photo-inhibition by these herbicides is not complete. While this phenomenon can be explained by the specificity of bentazone contra the intrinsic radical-detoxifying mechanisms of the chloroplast, it is an exciting observation that the changes in the OJ-steps and after the P-step make it possible to discern between differing modes of action in PSII photo-inhibition. The availability of an assay of this sensitivity will enable scientists to increase their knowledge of the mechanisms of primary and secondary photo-inhibitory herbicides, while at the same time providing plant physiologists with a vital tool to broaden their understanding of photosynthetic processes.

On the basis of the changes in the Kautsky curves (Fig 1) we decided to find a common parameter, which could be used to describe the dose-response

of the tested herbicides. Numerous parameters can be derived from the form and shape of the Kautsky curve in response to herbicide dose, many of which are probably more or less logically correlated. The most common one is that of $F_v/F_m [(F_m - F_0)/F_m]$, which basically is a scaled measurement of the magnitude of the variable fluorescence from the O to the P-steps. In Fig 1 it is obvious that dramatic changes occur at the J-step for all three herbicides. Klem *et al*¹⁹ used the relative variable fluorescence, $V_j [(F_j - F_0)/(F_m - F_0)]$, for the analysis of isoproturon effect on *Matricaria perforata* Merat grown in petri dishes. For screening purposes we decided to use the parameter $F_{vj}[(F_m - F_j)/F_m]$ because this parameter is most likely to detect changes in electron transport and thylakoid energization. Percival and coworkers^{3,5} have earlier suggested the use of F_{vj} .

Table 1 summarises the dose–response regressions for the herbicides and Figs 2–5 illustrate some of the response curves. For bentazone the biomass ED_{50} was rather consistent for both experiments, but in experiment 2, the slope of the response curves was not well described because of a very steep slope and a consequently poorly estimated ED_{50} that also resulted in a significant lack of fit (Table 1). The effect of

the fluorescence could be detected four hours after treatment and yielded ED_{50} values in the vicinity of those for the biomass (Table 1 and Fig 2).

For flurochloridone the biomass dose–response relationships showed that the doses were rather high, but already after 4 h and before any visible symptoms appeared, the fluorescence gave a clear dose–response relationship (Table 1, Fig 3). The dry matter dose responses for the second experiment could not be described by the regression because of too high doses and it is therefore not included in Table 1. Fluorescence at 24 h yielded an ED_{50} value close to that of dry matter (Table 1), but at that time the visual symptoms were already emerging.

For glyphosate the biomass of white mustard gave consistent ED_{50} values for the two experiments and the same applies for fluorescence 24 h after treatment, but in both instances ED_{50} values were substantial higher than those for biomass (Table 1, Fig 4). There was no notable effect on fluorescence after four hours (data not shown). For sugar beets a slight effect on fluorescence was seen already after 4 h, but the range of the fluorescence was restricted to the upper end of the response curve, with a consequent poorly described response curve (Table 2, Fig 5). After 24 h ED_{50}

Table 1. Summary of dose–response regressions for dry matter and fluorescence of white mustard (*Sinapis alba*)^a

	D (g per pot or F_{vj}/F_m)	C (g per pot or F_{vj}/F_m)	b (slope)	ED_{50} (g Al ha ⁻¹)	Lack of fit ^{b,c}
Bentazone					
Expt 1, dry matter	3.79 (3.46–4.12)	0.67 (0.61–0.73)	4.98 (2.69–7.29)	29 (24–34)	0.38 ^{ns}
Expt 2, dry matter	4.14 (3.77–4.50)	0.66 (0.56–0.76)	17.6 (–)	19 (–)	4.30 ^s
Expt 1, 4 h F_{vj}/F_m	0.45 (0.44–0.47)	0.07 (0.04–0.10)	8.88 (1.71–16.06)	31 (24–38)	0.74 ^{ns}
Expt 2, 4 h F_{vj}/F_m	0.48 (0.44–0.52)	0.08 (0.05–0.10)	5.11 (0.47–9.76)	18 (15–21)	3.51 ^s
Flurochloridone					
Expt 1, dry matter	3.95 (3.71–4.18)		0.22 (0.12–0.32)	3.2 (–)	0.18 ^{ns}
Expt 1, 4 h F_{vj}/F_m	0.47 (0.45–0.49)		0.89 (0.70–1.09)	211 (160–262)	3.22 ^s
Expt 2, 4 h F_{vj}/F_m	0.46 (0.44–0.47)		0.68 (0.55–0.82)	191 (145–236)	0.55 ^{ns}
Expt 1, 24 h F_{vj}/F_m	0.39 (0.35–0.43)		0.37 (0.17–0.57)	4.3 (–)	4.48 ^s
Expt 2, 24 h F_{vj}/F_m	0.42 (0.31–0.53)		0.37 (0.04–0.70)	0.03 (–)	0.84 ^{ns}
Glyphosate					
Expt 1, dry matter	3.75 (3.31–4.20)	0.97 (0.81–1.13)	1.21 (0.65–1.77)	27 (15–38)	0.89 ^{ns}
Expt 2, dry matter	3.91 (3.62–4.19)	0.81 (0.70–0.92)	2.26 (1.54–2.98)	62 (52–73)	0.78 ^{ns}
Expt 1, 24 h F_{vj}/F_m	0.45 (0.41–0.49)		1.11 (0.52–1.69)	782 (472–1093)	3.82 ^s
Expt 2, 24 h F_{vj}/F_m	0.41 (0.38–0.45)		2.71 (0.84–4.57)	399 (292–506)	0.34 ^{ns}

^a Lack of fit is based upon an analysis of variance. Numbers in parentheses are approximate 95% confidence intervals. (–) means confidence intervals cover zero.

^b ^{ns}Lack of fit test is non-significant at 95% level, meaning that data are well described by the estimated curve.

^c ^sLack of fit test is significant, meaning that data are not well described by the curve.

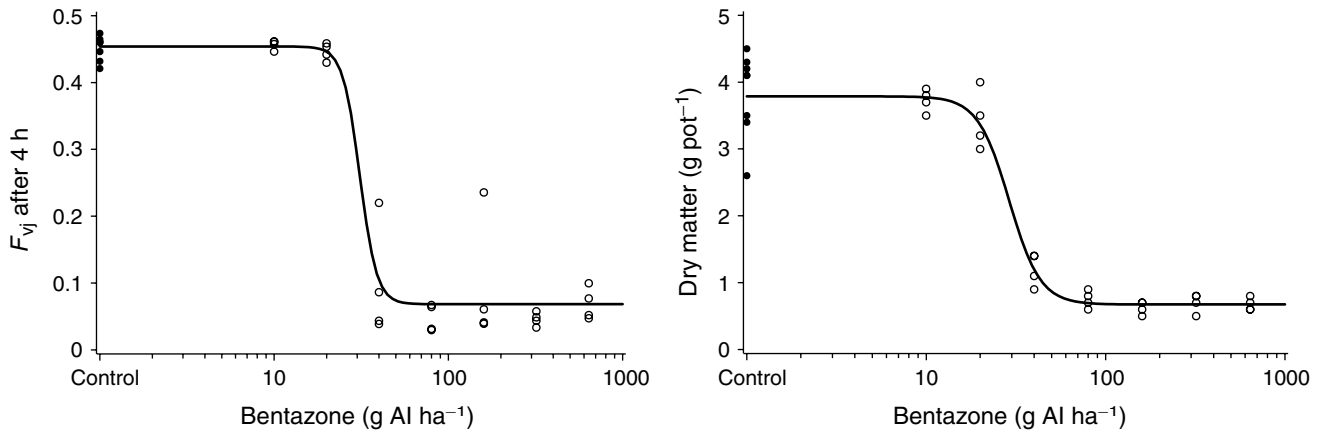


Figure 2. Dose–response curves for fluorescence 4 h and dry matter 7 days after treatment of white mustard (*Sinapis alba*) with bentazone (experiment 1).

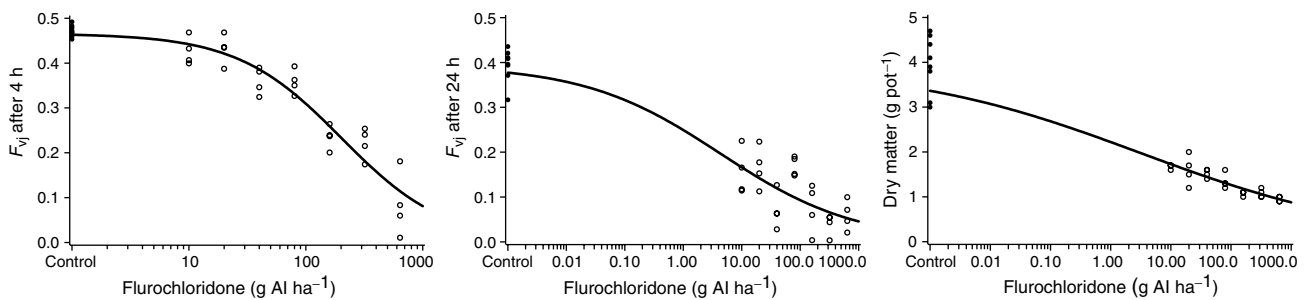


Figure 3. Dose–response curves for fluorescence 4 and 24 h after treatment and dry matter 7 days after treatment of white mustard (*Sinapis alba*) with flurochloridone (experiment 1).

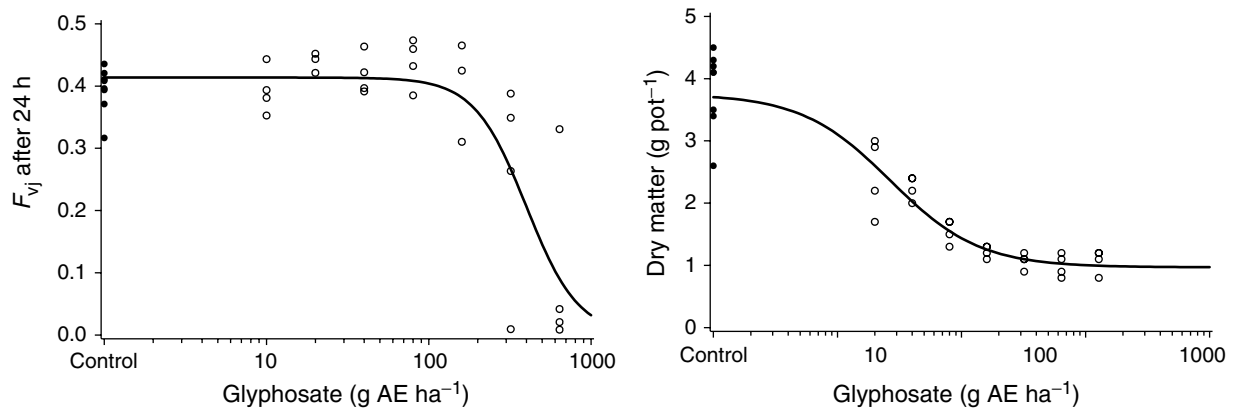


Figure 4. Dose–response curves for fluorescence 24 h and biomass 7 days after treatment of white mustard (*Sinapis alba*) with glyphosate (experiment 2).

values were more precisely estimated but substantially higher than those for biomass.

Figure 6 illustrates the link between biomass and fluorescence responses after the untreated controls have been omitted from the graphs. For bentazone this relationship is consistent for the two independent experiments with white mustard (Fig 6). Due to the high potency of flurochloridone, the range of biomass in this experiment was rather restricted (Figs 3 and 6). After 4 h there were no clear-cut relationships between fluorescence and resulting biomass, these appearing only after 24 h (Fig 6), that is, after symptoms became visible. For glyphosate the relationship

was less pronounced because the distribution of biomass responses differed somewhat between the two experiments (Fig 6). However, Binder *et al*⁹ compared visible needle damage of *Picea glauca* [Moench] Voss at various latitudes with reference to freezing intensities, as a function of F_v/F_m and found a consistent sigmoid relationship, as did Lindgren and Hällgren.²⁷

It is obvious by comparing Figs 2–6 that most information (and subsequently the best link between fluorescence and resulting biomass) is in the mid-range of the response curves. The variation between experiments, particularly with flurochloridone and glyphosate, may be alleviated if the ambient

Table 2. Summary of glyphosate dose response regressions for dry matter and fluorescence of sugar beet (*Beta vulgaris*)^a

	<i>D</i> (g per pot or F_{vj}/F_m)	<i>C</i> (g per pot or F_{vj}/F_m)	<i>B</i>	<i>ED</i> ₅₀ (g AE ha ⁻¹)	Lack of fit ^b
Expt 1, dry matter	1.16 (1.04–1.29)	0.38 (0.33–0.43)	2.08 (0.96–3.19)	16 (11–21)	0.52 ^{ns}
Expt 2, dry matter	1.9 (1.59–2.22)	0.11 (0.07–0.15)	3.65 (1.58–5.71)	29 (22–37)	0.96 ^{ns}
Expt 1, 4 h F_{vj}/F_m	0.45 (0.44–0.46)	0.31 (–)	2.63 (–)	1216 (–)	0.63 ^{ns}
Expt 2, 4 h F_{vj}/F_m	0.44 (0.42–0.45)		0.90 (0.52–1.28)	1508 (723–2293)	2.16 ^{ns}
Expt 1, 24 h F_{vj}/F_m	0.41 (0.39–0.43)	0.15 (0.10–0.21)	4.67 (0.10–9.24)	105 (71–140)	0.75 ^{ns}
Expt 2, 24 h F_{vj}/F_m	0.40 (0.38–0.42)		1.04 (0.60–1.48)	762 (496–1027)	1.01 ^{ns}

^a Lack of fit is based upon an analysis of variance. Numbers in parenthesis are approximate 95% confidence intervals. (–) means confidence intervals cover zero.

^b ^{ns}Lack of fit test is non-significant at 95% level, meaning that data are well described by the estimated curve.

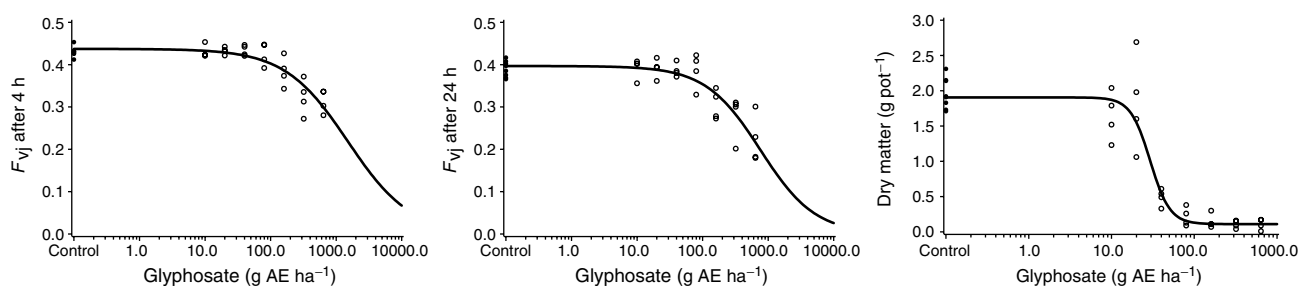


Figure 5. Dose–response curves for fluorescence 4 and 24 h after treatment and dry matter 7 days after treatment of sugar beets with glyphosate (experiment 2).

temperature and radiation can be controlled. During spring, summer and early autumn, light saturation (1000–1500 μmol photons m⁻² s⁻¹) would probably be the rule at the parallels under which the experiments have been conducted, and most variation would be experienced under ambient greenhouse temperatures that depend heavily upon cloud cover.

Using specific and non-specific herbicide inhibitors of photosystem II, we demonstrate that, in contrast to the complete inhibition of PSII by bentazone, the Kautsky curve is affected by flurochloridone and glyphosate at the J-step, while retaining some fluorescence quenching after the P-step. This indicates that photosystem II inhibition by flurochloridone and glyphosate is not complete. However, whatever the mode of action of the three herbicides, the J-step is relatively the most affected by the herbicides.

The influence of time on symptom development on the Kautsky curve (Figs 1–5) and biomass reduction (Figs 2–6) depends on the herbicide mode of action and herbicide dose as well as plant species. Which of those three factors are the more important is difficult to say at this stage and requires more experimentation. Certainly, herbicide mode of action is a factor that plays an important role. The direct PSII inhibitors and the PDS inhibitor, affecting PSII indirectly, developed changes in the Kautsky curve much faster than did glyphosate in *S. alba*. In *B. vulgaris* we could,

however, detect changes in the Kautsky curve 4 h after treatment with high doses of glyphosate (Fig 5). The visual symptoms for PDS inhibition develop rapidly, and become pronounced after one or two days, depending of the environment and the dose. Consequently, if fluorescence should be of interest for PDS inhibitors in a screening procedure, the fluorescence symptoms should necessarily develop within the first 24 h after treatment. For glyphosate the detection of changes in the Kautsky curve could still be useful as much as 24 h after treatment and perhaps even later, because of the slow development of visual symptoms. Thus, it is evident that there are species differences in the development of changes in the Kautsky curve in response to glyphosate treatment, the reason for which could be attributed to many factors.

In this paper we have been concentrating on a common fluorescence parameter for all three herbicides. Accumulation of more data may reveal other or a combination of parameters from the Kautsky curve that can specifically be used for herbicides which do not directly affect the PSII system.²⁸ If the fluorescence parameter F_{vj} , or any other parameter or combination of parameters, should be used as an early response parameter of herbicide action, it must be linked to the biomass production used in classic assays. Clearly, by deriving information from the Kautsky curve in response to herbicide application, it

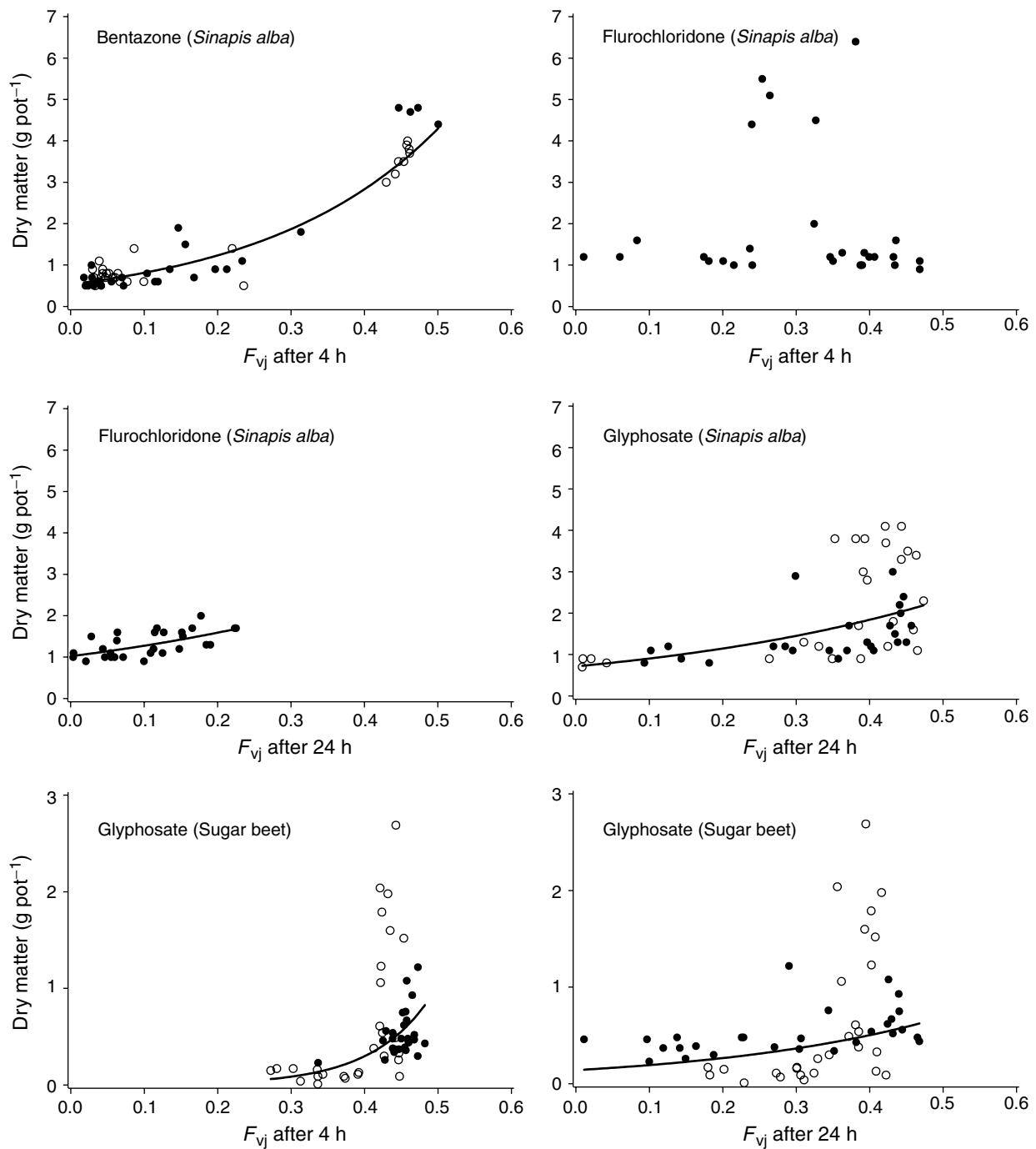


Figure 6. The relationship between biomass and fluorescence. ○ and ● denote different experiments.

is possible to discern between differing modes of action of PSII photo-inhibition. In this way, the analysis of the Kautsky curve could enable scientists to increase their knowledge of the mechanisms of primary and secondary photo-inhibitory herbicides, while at the same time providing plant physiologists with a vital tool to broaden their understanding of photosynthetic processes.

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