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Chlorophyll Fluorescence: A Novel Method to Screen for Herbicide Resistance

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Abstract
The susceptibility to Terbacil of six randomly selected strawberry cultivars ('Aromas', 'Chambly', 'Harmonie', 'Kent', 'La Clé des Champs' and 'Seascape') was analysed using chlorophyll florescence (CF) in comparison with visual observation, in an attempt to develop a method for use in a breeding programme to select herbicide-resistant strawberry lines. Terbacil was applied at one of five rates (0, 0.55, 1.10, 2.20 and 3.30kg/ha), and CF was measured 1, 3, 7 and 14d after application. Visible damage was assessed using a scale of 0 to 10 (where 0 is no damage and 10 is the death of the leaf). All cultivars showed a decrease in CF after herbicide application, but this decrease was not the same for all cultivars. 'Chambly' and 'Harmonie' had the lowest CF changes, and both appeared to be tolerant or resistant to Terbacil, in keeping with previously reported results. In contrast, 'Kent' and 'Aromas' showed a significant decrease in CF on the third day after treatment with Terbacil at 2.20 and 3.30 kg/ha, an indication that their chlorophyll system had been damaged by herbicide application without any visible signs of leaf damage. It is concluded that CF can be used as an alternative and more accurate method to evaluate seedlings in a strawberry breeding programme aimed at selecting herbicide-resistant lines. This method could be very useful, especially for those lines that do not show any visible leaf damage from herbicide application, even though their chlorophyll system is damaged enough to cease plant growth and development.

Key words: breeding programme, chemical, strawberry cultivars, Terbacil.

Introduction

Canada is one of the world's largest consumers of strawberries (*Fragaria* × *ananassa* Duch.), and in 2005 roughly 85,000t of the fruits were eaten across the country. However, Canada produces only 22,000t annually, and most of the berries that are consumed (nearly 82,000t) are imported from the United States and Mexico. Imports, which are worth roughly US$170 million annually, therefore represent four times the domestic production (Food and Agriculture Organization of the United Nations, 2007).

The major strawberry-growing regions in Canada are Quebec (36% of national production) and Ontario (32%), followed by British Columbia (15%), Nova Scotia (7.9%) and New Brunswick (2.8%). These areas have particularly harsh winters; for

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example, temperatures in Quebec may drop as low as −45 C during the winter (Khanizadeh and DeEll, 2005). Since strawberries are important to Canadians, increasing production by adapting this fruit to local conditions is crucial.

Newly selected cultivars at Agriculture and Agri-Food Canada's Horticulture Research and Development Centre, located in Quebec, are developed for certain characteristics such as cold-hardiness and resistance to disease and mechanical harvesting. The characteristics of these selected cultivars are analysed, and their resistance to various diseases, pests and herbicides is evaluated annually during the selection process.

Only Terbacil herbicide is licensed for strawberries and it is commonly used in the first planting year to protect against grasses and broadleaf weeds (Centre de référence en agriculture et agroalimentaire, 2007; McCully and Jensen, 2004; Ontario Ministry of Agriculture, Food and Rural Affairs, 2012).

The measurement of chlorophyll fluorescence (CF) is a new method used in recent years to determine the response of leaves to different types of stress such as cold and drought (DeEll and Toivonen, 2003) as well as to aid in fruit breeding (Khanizadeh and DeEll, 2003). The CF method is based on the fact that the light energy absorbed by chlorophyll molecules can be spent in three different ways: it can be used to fuel photosynthesis, be dissipated as heat, or be re-emitted as light (fluorescence). In the first process, the de-excitation of chlorophyll occurs through a photochemical pathway that allows the chlorophyll reaction centre (P680) of Photosystem II to reduce an electron acceptor, plastoquinone Q_A, and thus complete the electron chain; this process accounts for most de-excitation. In the second process, the de-excitation of chlorophyll is accomplished by the dissipation of energy through heat. The first two processes compete with the third process, the de-excitation pathway involving the emission of fluorescence. Therefore, an increase in the first two parameters results in a decrease in CF. In contrast, increased fluorescence occurs when the Photosystem II reaction centres are closed (i.e., are reduced) at the plastoquinone Q_A level (Maxwell and Johnson, 2000). This increase occurs because chlorophyll can no longer transmit its energy to plastoquinone Q_A if the latter has already been reduced; instead, de-excitation occurs through the emission of fluorescence. The advantage of using CF measurement is that not only is it very effective but also it is non-destructive and can be carried out in the field. Recent studies have shown that when a Photosystem II inhibitor herbicide such as Terbacil is applied to leaves, fluorescence decreases, demonstrating the role of the herbicide in inhibiting the photosystem (Christensen *et al.,* 2003). However, to date no study has focused on demonstrating the potential correlation between visible damage and decreased CF after herbicide application.

The purpose of the present study was, therefore, to determine if there is a correlation between visible damage and decreased leaf fluorescence after treatment with herbicide. Should such a relationship be demonstrated, the herbicide resistance of cultivars or new lines in a breeding programme could be determined simply by measuring their CF, without having to wait for visible symptoms to appear.

Marerials And Methods

Experimental set-up

The experiments were carried out at the Horticulture Research and Development Centre, in Saint-Jean-sur-Richelieu, QC, Canada. Six strawberry cultivars ('Aromas', 'Chambly', 'Harmonie', 'Kent', 'La Clé des Champs' and 'Seascape') were grown in a greenhouse under natural light. 'Aromas' and 'Seascape' are ever-bearing, whereas 'Chambly', 'Harmonie', 'Kent' and 'La Clé des Champs' are June-bearing. Bare-rooted plants of these cultivars were harvested in the late autumn and kept in a cold room between −1 and 0 C until they were planted in six-inch pots containing Premier Pro-Mix BX soil mixture. The potted plants were then transferred to the greenhouse and watered daily as needed, until the plants were seven weeks old, at which point they had enough leaves for the experiment to begin.

A completely randomized block design was used in both experiments, and five plants were used in each block.

Herbicide application

The herbicide used was Terbacil (3-*tert*-butyl-5-chloro-6-methyluracil), for which the recommended maximum application rate is 0.55kg ha^{-1} for seven-week-old plants during the first planting year (Centre de référence en agriculture et agroalimentaire, 2007). Five different rates of Terbacil (0, 0.55, 1.10, 2.20 and 3.30kg ha^{-1}) were applied, and the control group received only water.

The sprayer used was a 1-L hand sprayer calibrated to apply 11.5mL of solution per 10 squirts, with an accuracy of roughly 2µg. The solutions for the test rates were obtained by mixing 0.48, 0.96, 1.92 and 2.88g of Terbacil (wettable powder) in 10L of water to obtain rates of 0.55, 1.10, 2.20 and 3.30kg ha^{-1}, respectively.

After herbicide application, the plants were not watered for 48h to avoid washing off the herbicide (Polter *et al*., 2005), but daily watering was resumed afterward.

Fluorescence measurements

Measurements of CF were taken on all plants before the application of the herbicide, and were repeated 1, 3, 7 and 14d post-application. Measurements were taken on opposite leaves on the same plant, and the most representative leaves were selected on the basis of the same age, development stage, side, exposure to light and chlorophyll content. Measurements of CF were always taken on the same two leaves, which were tagged with coloured labels.

In addition, a specific dark period was used throughout the experiment before CF was measured, as described previously (DeEll and Toivonen, 2003). The CF was measured after the plants had been kept in the dark for 2h in a Conviron RGV36 plant growth chamber (Winnipeg, MB, Canada) with a temperature of 25 C and relative humidity of 60%.

An Opti-Sciences OS30p chlorophyll fluorometer (Opti-Sciences, 2004) was used to measure CF, and the fluorescence rate (Fv/Fm, the ratio of variable fluorescence to maximum fluorescence) was calculated using the method described by DeEll and Toivonen (2003).

Foliar phytotoxicity symptoms

Each leaf that was selected was checked after the herbicide application to assess visible damage. Damage was recorded on a scale from 0 to 10 (where 0 is no damage and 10 is the death of the leaf). Photos of the plants were taken before herbicide application as well as 3, 7 and 14d afterward to monitor the evolution of visible symptoms.

Statistical tests

The data were subjected to the analysis of variance (ANOVA) and general linear model (GLM) procedures of the SAS software package (SAS Institute, 1989) and were then used to determine the differences between cultivars and the effect of the herbicide rate. In addition, a regression analysis was carried out for each cultivar to determine if there was a linear relationship between the treatment and the Fv/Fm ratio.

Results

All cultivars showed a linear response to an increase in the herbicide rate (Table 1). However, not all cultivars reacted in the same way. ‘Aromas’ and ‘Kent’ appeared to be the most sensitive to Terbacil, whereas ‘Chambly’ and ‘Harmonie’ seemed to be more resistant. These findings are in keeping with previously published data on the ‘Chambly’ cultivar (Khanizadeh *et al*., 1990).

Table 1. Effects of the application of five different rates of Terbacil on the fluorescence rate (Fv/Fm) and the appearance of visible damage in six strawberry cultivars (seven-week-old plants).

Cultivar	Day	Fv/Fm 0 kg/ha	0.55 kg/ha	1.10 kg/ha	2.20 kg/ha	3.30 kg/ha	LSD[†]	Effect	Equation	R^2
	0	0.820 a	0.812 a	0.827 a	0.812 a	0.826 a	0.02*			
	1	0.808 a	0.780 a	0.742 a	0.575 b	0.725 a	0.12*			
'Aromas'	3	0.837 a	0.797 a	0.714 a	0.493 b	0.515 b	0.15*	L[††]	0.840 − 0.097x***	0.28***
	7	0.833 a	0.824 a	0.814 a	0.448 b (1)[†††]	0.361 b (7)	0.25*			
	14	0.832 a	0.822 a	0.741 a	0.424 b (5)	0.357 b (10)	0.29*			
	0	0.822 a	0.821 a	0.823 a	0.831 a	0.820 a	0.02*			
	1	0.802 ab	0.805 ab	0.822 a	0.768 bc	0.740 c	0.05*			
'Chambly'	3	0.835 a	0.803 a	0.807 a	0.794 a	0.702 b	0.06*	L	0.831 − 0.019x***	0.12***
	7	0.834 a	0.828 a	0.830 a	0.807 a	0.824 a	0.04*			
	14	0.825 a	0.824 a	0.822 a	0.802 a	0.700 a	0.14*			
	0	0.833 a	0.819 ab	0.817 b	0.830 ab	0.827 ab	0.02*			
	1	0.833 a	0.825 a	0.804 ab	0.808 ab	0.746 b	0.07*			
'Harmonie'	3	0.835 a	0.824 ab	0.821 ab	0.794 ab	0.764 b	0.07*	L	0.831 − 0.009x***	0.07***
	7	0.832 ab	0.838 a	0.833 ab	0.816 b	0.831 ab	0.02*			
	14	0.823 a	0.834 a	0.814 a	0.834 a	0.832 a	0.02*			
	0	0.832 a	0.819 a	0.803 a	0.809 a	0.823 a	0.05*			
	1	0.841 a	0.783 bc	0.790 b	0.742 c	0.740 c	0.05*			
'Kent'	3	0.843 a	0.794 a	0.716 a	0.768 a	0.474 b	0.13*	L	0.847 − 0.076x***	0.26***
	7	0.837 a	0.747 ab	0.792 ab	0.695 ab	0.631 b (1)	0.19*			
	14	0.835 a	0.803 a	0.747 ab	0.638 b	0.134 c (4)	0.16*			
	0	0.818 ab	0.818 ab	0.835 a	0.827 ab	0.815 b	0.02*			
	1	0.782 a	0.807 a	0.766 a	0.759 a	0.608 b	0.09*			
'La clé des Champs'	3	0.836 a	0.744 ab	0.786 a	0.611 bc	0.543 c	0.13*	L	0.832 − 0.043x***	0.22***
	7	0.829 a	0.828 a	0.824 a	0.753 ab	0.650 b	0.11*			
	14	0.831 a	0.826 a	0.820 a	0.762 a	0.780 a	0.09*			
	0	0.825 a	0.824 ab	0.807 b	0.818 ab	0.817 ab	0.02*			
	1	0.828 a	0.762 b	0.790 ab	0.733 bc	0.681 c	0.06*			
'Seascape'	3	0.831 a	0.811 a	0.806 a	0.740 ab	0.673 b	2.07*	L	0.833 − 0.038x***	0.19***
	7	0.825 a	0.826 a	0.816 a	0.765 a	0.710 a	0.14*			
	14	0.820 a	0.824 a	0.798 a	0.727 ab	0.616 b	0.17*			

[†] LSD, least significant difference. [††] L, linear.

[†††] The number in parentheses represents the score for visible damage on the leaf when damage was observed (where 1 is slight damage and 10 is the death of the leaf).

a–c On a given line, values followed by the same letter are not significantly different at $P<0.05$.

*, statistically significant at 0.05; **, statistically significant at 0.01; ***, statistically significant at 0.001.

Figure 1 shows the effect of herbicide application on the fluorescence rate. Healthy plants generally have Fv/Fm values between 0.75 and 0.8 (Khanizadeh and DeEll, 2003). The cultivar 'Harmonie' seemed to be resistant to Terbacil, given that at an application rate of 3.3kg/ha, the Fv/Fm value remained above 0.8 (Table 1). 'Chambly' was also resistant, since its Fv/Fm ratio remained at or above 0.75. 'Seascape' and 'La Clé des Champs' showed a slight sensitivity to Terbacil.

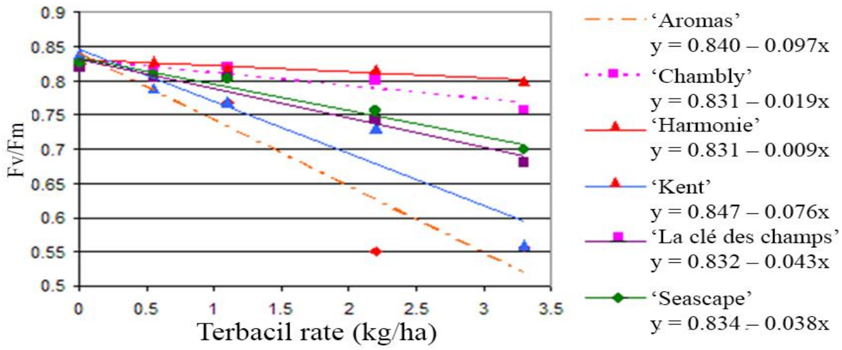

Fig. 1. Effects of the application of five different rates of Terbacil on the fluorescence rate (Fv/Fm) in seven-week-old plants of six strawberry cultivars.

Beginning on day 3, the response of 'Aromas' to the treatments at 2.2 and 3.3kg ha^{-1} was strikingly different from that of the other cultivars, and a significant difference was observed between the treatments at 1.1 and 2.2kg ha^{-1}. On day 14, the difference in the values of the Fv/Fm ratio between the control plants and the plants receiving the treatment at 1.1 kg ha^{-1} was only 11%. The disparity was much greater (close to 50%) between the control plants and the plants treated with 2.2 kg ha^{-1}. 'Aromas' seemed to be the most susceptible cultivar, followed by 'Kent'.

Foliar phytotoxicity symptoms

The only cultivars with visible symptoms from herbicide application were 'Aromas' and 'Kent', which displayed progressive leaf necrosis. In 'Kent', these symptoms appeared mainly in plants receiving 3.3kg ha^{-1}, whereas in 'Aromas', the symptoms also appeared in plants receiving 2.2kg/ha. The symptoms began to appear on the fifth day after application.

Figure 2 shows that in 'Kent', the Fv/Fm ratio decreased faster than the symptoms appeared. On day 3, no symptoms were visible, yet fluorescence values were already very low. The same observation applies to 'Aromas'. Fluorescence can therefore detect a cultivar's susceptibility before the appearance of visible symptoms. Damage was visible only on 'Aromas' and 'Kent' on days 7 and 14, with symptoms worsening on day 14, and these symptoms were present only at rates of 2.2kg ha^{-1} or above.

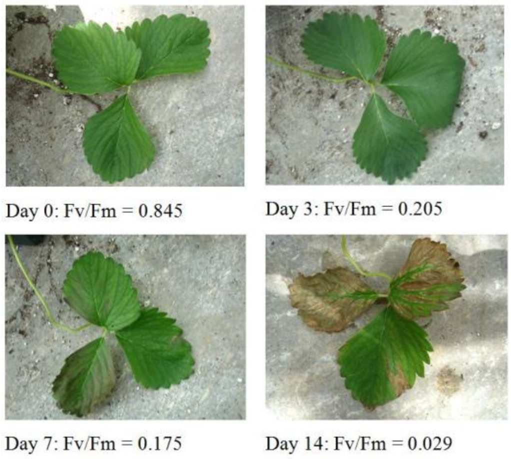

Fig. 2. Evolution of visible damage appearing on the 'Kent' cultivar over a 14-d period after the application of TTerbacil at 3.30kg ha^{-1}.

The same trend was seen in the evolution of visible damage. The cultivars 'Aromas' and 'Kent' seemed to be much more susceptible to Terbacil than 'Harmonie', 'Chambly', 'Seascape' and 'La Clé des Champs' were, and this sensitivity increased with the application rate.

Discussion

The results of the experiment show that the cultivars 'Harmonie' and 'Chambly' were resistant to Terbacil, 'Seascape' and 'La Clé des Champs' were moderately sensitive, and 'Aromas' and 'Kent' were very susceptible.

For ‘Aromas’ and ‘Kent’, the decrease in fluorescence values was accompanied by the appearance of visible symptoms. Although a drop in fluorescence values was also observed in the other cultivars, their leaves appeared to be undamaged. Therefore, the value of Fv/Fm must drop below a certain threshold before symptoms are observed. At values over 0.6, it can be assumed that no symptoms will appear, and the cultivar can be considered resistant to the herbicide.

Therefore, a relationship exists between decreased fluorescence and the appearance of visible damage. Fluorescence measurements provide a quantitative value of the stress experienced by the plant and are a more objective method than visual observations. Since the assessment of visible damage is very subjective, the CF measurement technique allows a more rigorous interpretation of the results. However, it is necessary to determine a threshold below which the fluorescence value is sufficiently low that the cultivar should be considered sensitive to the herbicide. As Table 1 shows, on day 1 no difference can be observed between the cultivars regardless of the treatment, and not until day 3 do the actual effects of the herbicide on fluorescence begin to appear, even though most cultivars have similar fluorescence values. However, by day 7, at high rates of the herbicide, significant differences in fluorescence can be seen, and visible symptoms begin to appear.

Herbicide-sensitive cultivars such as ‘Kent’ show little or no visible damage in the field even though their leaves are damaged internally. According to Polter *et al.* (2004), when Terbacil is applied solely on the leaves of greenhouse-grown strawberries, the injuries observed are greater than those seen when the herbicide is applied to the roots. Barrentine and Warren (1970) reported that Terbacil absorption by leaves is negligible in comparison with root uptake. In the present experiment, the roots were not affected by the herbicide, whereas in the field, a portion of the herbicide reaches and penetrates into the soil, where it can be absorbed by the roots. As a result, the herbicide’s phytotoxic effect is increased.

In this type of experiment, it would be preferable to use younger plants (four weeks old), which are more sensitive to Terbacil, since herbicide tolerance increases as the layers of epicuticular wax develop and as the plant grows and its root system becomes larger and deeper. The composition and morphology of the cuticular layers also change with the age of the leaves, and the cuticles of the mature leaves limit the foliar uptake of Terbacil (Polter *et al.*, 2004; Genez and Monaco, 1983).

In conclusion, CF measurements do provide good estimates of herbicide tolerance and can be used in a breeding programme to select for resistant lines.

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