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Violet diode laser-induced chlorophyll fluorescence: a tool for assessing mosaic disease severity in cassava (*Manihot esculenta* Crantz) cultivars

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Violet diode laser-induced chlorophyll fluorescence was used in agronomical assessment (disease severity and average yield per plant). Because cassava (*Manihot esculenta* Crantz) is of economic importance, improved cultivars with various levels of affinity for cassava mosaic disease were investigated. Fluorescence data correlated with cassava mosaic disease severity levels and with the average yield per plant.

Keywords: violet diode laser; induction kinetics; fluorescence decrease ratio; disease severity; average crop yield per plant

1. Introduction

Cassava (*Manihot esculenta* Crantz) is an important source of carbohydrates for humans and animals, and has industrial applications, especially as starch for the pharmaceutical industry. The use of processed cassava as filler in comminuted meat products has been achieved [1]. However, an important constraint to cassava cultivation is the cassava mosaic disease (CMD), or Cassava Mosaic Virus (CMV), caused by cassava mosaic geminiviruses (CMGs) (*Geminivirdae*, *Begomevirus*) [2,3], and the disease is difficult to control. This disease can cause yield losses greater than 60%, and no biological or chemical control is currently available to farmers. The use of genetically modified cultivars shows great potential in resisting the effect of CMD [4]. The relationships between cassava mosaic severity in planting material and disease development and the growth and yield of cassava have been studied [5]. A crop development and improvement programme is an important feature for effective evaluation of crop growth and performance [6]. The frequent limitation to crop improvement programmes is the lack of rapid screening techniques to identify plants with improved or impaired metabolism and growth. This need has brought forward many invasive and non-invasive conventional techniques. Assessment of CMD is based on an accurate and reliable visual evaluation of the symptoms. Reliability can be impaired if those responsible lack a thorough understanding of the biotic and abiotic factors that affect cassava growth and symptom expression, thereby leading to erroneous recording of either the presence or absence of CMD [7]. The visual assessment described cannot be used effectively in the screening of a large number of plants, and it is also relatively subjective in nature. Invasive methods are slow and relatively expensive, and the chemicals used destroy the leaf-tissue and might affect plant physiological processes.

Chlorophyll fluorescence spectra of green leaves have proven to be a useful tool in the determination of the state of the plant. Changes in chlorophyll fluorescence emission intensity from photosynthetic tissues provide a non-invasive signal that has been used to determine photosynthetic activity [8–12]. The peak intensity ratio of the red and far-red band of the chlorophyll fluorescence correlates very well with the photosynthetic efficiency of the leaves' photosynthetic apparatus. The use of ratios in establishing variability and applications of chlorophyll emissions of leaves has been reviewed [13]. The relationship between chlorophyll fluorescence and nitrogen deficiency [14–17], and other few studies on pathogen detection [16–19] have also been studied. Changes in chlorophyll fluorescence during the first two weeks of plant growth have been used to ascertain yield of crop varieties [20].

Laser-induced fluorescence can provide rich information about plant machinery (physiology) [21–24], plant tissues [25] and soils [26]. Ultraviolet light-induced chlorophyll fluorescence has been reported to be a good method for plant monitoring in agricultural and plant science applications [18,23]. Much of this information can be extracted by monitoring temporally the yield of chlorophyll fluorescence after a dark-to-light transition. Measuring the chlorophyll fluorescence is a widely accepted *in vivo* method for the investigation of plant conditions. To make quantitative estimates of photosynthetic performance, it is appropriate to

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determine the changes in relative intensity accompanying the induction of photosynthetic activity as a function of time, known as chlorophyll fluorescence induction kinetics (Kautsky effect).

The Kautsky effect has been used to estimate photosystem II activity, which is highly susceptible to conditions such as plant diseases and other stress factors that impair photosynthesis [27–39]. Perturbations of photosynthetic metabolism, which can be induced by biotic and abiotic factors, do modify significantly the characteristics of fluorescence emission kinetics of plants [6]. However, there is also evidence that many metabolic process inhibitors that are not directly involved in photosynthetic metabolism can produce modifications to fluorescence induction kinetics [40–43]. It is possible to quantify the changes in fluorescence induction characteristics resulting from perturbations by using ratios of fluorescence levels during induction [44]. The slow Kautsky effect relies on the relative intensity of two fluorescent levels: maximum fluorescence yield (Fm) and steady state fluorescence yield (Fs). Kautsky effect analysis based on measurements of these two fluorescence parameters can reveal information on the photosynthetic performance of a leaf [38]. Over the past decade, chlorophyll fluorescence decrease ($fd = Fm - Fs$) and thus the chlorophyll fluorescence decrease ratio ($Rfd = [Fm - Fs]/Fs$, or $Rfd = fd/Fs$) have been used as reliable indicators for plant photosynthetic performance and to detect metabolic perturbations [6,42,45–47].

In Ghana there is a Presidential Special Initiative regarding the production of starch for export. The Crop Research Department of the University of Cape Coast and the Crop Research Institute have been working intensely to develop new cultivars that tolerate or are resistant to CMD, which is a major contributing factor to the low yield of most cultivars. Thus, various problems pertaining to new cultivars adaptability, and cultural practices required for optimum utilization of the growing season for good harvest, in comparison with the agronomic performances of four cassava cultivars in the Cape Coast district, are being investigated by applying violet laser-induced chlorophyll fluorescence and studying the fluorescence decrease ratio, with conventional agronomical methods used for reference.

2. Materials and measurement

The studies were carried out at the University of Cape Coast (UCC) teaching and research farm, a site which falls within the coastal savanna zone of Ghana. Intact leaves of four cassava cultivars, namely Capevars, Adehye, Santom and Asamang (AS) were used. The plants were grown without any fertilizer application to the field during the 2007 farming season. Measurements were conducted in the early mornings with average daily temperatures of 29 °C. A portable continuous violet diode laser system (VDLS) [20,48] was used to measure chlorophyll fluorescence induction kinetics.

In the VDLS system, a violet diode laser, emitting at 396 nm at an output power of 3 mW (Nichia NLHV500, Tokyo, Japan), was placed in a tube that has a lens (Geltech C230TM-A, Goteberg, Sweden) for collimating the diverged laser radiation. The output beam was 'cleaned up' for broadband spontaneous emission using a narrowband interference filter (CVI F25-400-4-0.5, Goteberg, Sweden) and was focused by a fibre-port lens assembly (Optics for Research PAF-SMA-6-NUV-Z,) into a 600 μm core diameter fused silica step-index multimode optical fibre via a dichroic beam splitter (CVI, Uppsala, Sweden) placed in front of the spectrometer (Ocean Optics S2000, Ostifildun, Germany). This USB2000 miniature fibre optics spectrometer directly plugs into the USB port of a laptop PC (Tokyo, Japan), from which it draws its power, thus eliminating the need for any A/D interface. Another 1 m, 600 μm fused silica core step-index multimode optical fibre, with a Tefzel jacket of 660 μm cladding diameter, was coupled to the output of the laser beam by an SMA connector fixed on the side of the system (VDLS). The output end of the fibre was supported by a fibre holder and kept in contact with the upper leaf surface, while the lower side of the leaf was sheltered with an aluminium plate for background cover to avoid collection of ambient light coming through the back of the leaf. The use of a fibre holder shades off the laser light intensity incident on the leaf from the ambient light since the irradiated area was selected by the fibre diameter. The use of the single optical fibre facilitates the measurement of fluorescence from leaves attached to the various cassava plants, enabling quick sampling on different cassava cultivars.

The chlorophyll fluorescence returned through the same fibre into the spectrometer, which was equipped with a slit and a grating of 600 lines/mm. The elastically backscattered violet diode laser radiation was effectively blocked by a Schott GG420 coloured glass filter placed behind a dichroic beam splitter. The dispersed radiation was captured on a 2048 element linear CCD (charge-coupled device) array detector.

The sensitivity of the system was checked each day of the measurement using a standard fluorescent material (quinine sulphate) of known concentration and wavelength, which serves as calibration for the system to maintain reproducible recordings.

The laser-induced chlorophyll fluorescence induction kinetics (Kautsky effect) was recorded on the 3rd, 6th and 12th week of the cassava plants' developmental stages. The fourth trifoliate leaf from the shoot apex of the four cultivars was used for the measurements. The optical fibre probe of core diameter 600 μm, with the end housed in a flat disc of diameter 2.5 cm, was in direct contact with the leaf to aid measurements. The small cross-sectional area of the fibre posed as a limitation to the leaf area covered in the measurement.

Complete spectra from leaves of the four cultivars were recorded as an initial test for chlorophyll fluorescence

(ChlF) wavelength selection. The two characteristic peak wavelengths of the ChlF, 685 nm (red) and 740 nm (far red), were selected for Kautsky effect data collection. An initial calibration test for chlorophyll fluorescence induction kinetics was also conducted on the samples for optimizing the exposure time. Initial measurements were done with exposure times of 180 and 300 s. Comparing data from the 180 and 300 s exposure times of violet laser light, it was clear that a steady state can be achieved from 180 s and beyond. Therefore 180 s was sufficiently long enough for analysis and also to reduce the possibility of leaf photo-decolorization. This was done in consideration of the laser intensity, which is a factor in photo-decolorization. Selected leaves were pre-darkened for 20 minutes and placed on a non-fluorescence aluminium plate on top of a vertical adjustable tripod. To obtain statistically reliable data, six measurements were made on the fourth trifoliate leaf. For each cultivar five plants were used in the measurement. The measurements were done for four consecutive days, a cultivar a day, for each period. The diode laser intensity was checked after every leaf measurement using a power meter (NT54-018, Edmund Optics, Nether Poppleton York UK). Conventional agronomical methods were used to obtain disease severity (DS) levels and average yield per plant (AYPP) for each cultivar. Disease severity was assessed by estimating the proportion of total photosynthetic area that was diseased. The DS level measurements were conducted within the same period that induction kinetics data were being taken, but the AYPP was done during the harvest period by the Crop Research Department.

Figure 1. Typical visible effect of CMD on a leaf (Asamang).

3. Results and discussion

In Figure 1 we show a leaf of Asamang depicting the visible effects of CMD across the leaf area. When the dark-adapted cultivar leaves were illuminated with the continuous saturated violet laser light, chlorophyll fluorescence induction (Kautsky effect) signatures rose rapidly to a maximum fluorescence, Fm, then declined to the steady state fluorescence, Fs. The averaged maximum-normalized intensity of the slow component of chlorophyll fluorescence induction

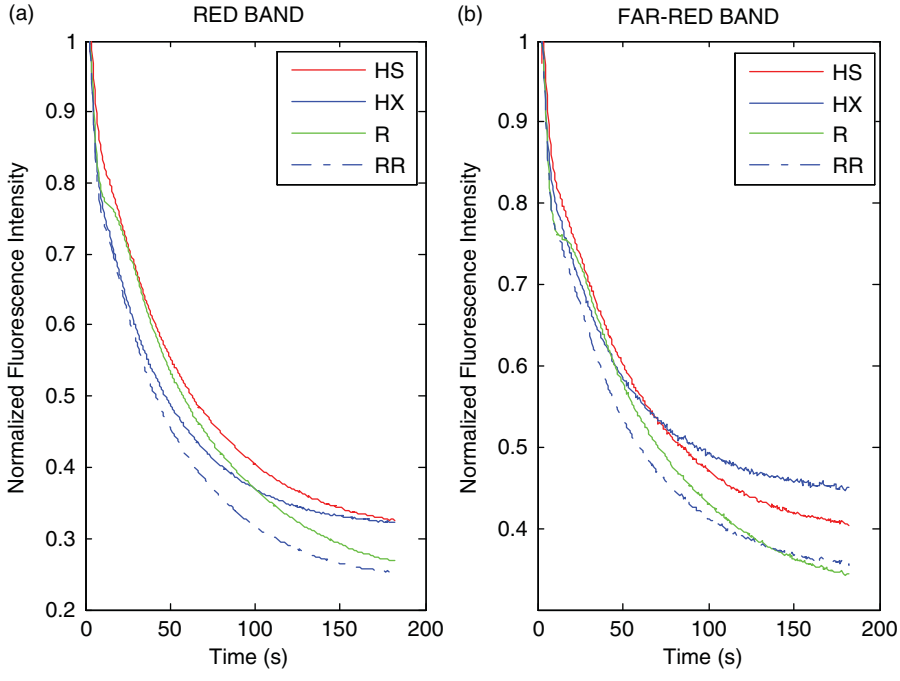

Figure 2. Normalized fluorescence induction curves of the: (a) 685 nm (red band) and (b) 740 nm (far-red band) of the four cassava cultivars: Capevars (RR), Adehye (R), Santom (HX) and Asamang (HS).

Table 1. Cultivars and their respective Rfd-red values against their agronomical assessment.

			Disease		Average yield	
Cultivar	Code	Rfd-Red	Incidence proportion (%)	Severity (DS)	Loss (%)	plant (AYPP) (kg)
Cape Vars	RR	2.97	0.00	1.00	0.00	8.67
Adehye	R	2.73	10.00	1.20	0.00	8.67
Santom	HX	2.10	100.00	2.50	78.00	1.03
Asamang	HS	2.06	100.00	3.70	88.00	1.87

kinetics signatures in the red and far-red bands is shown in Figure 2a and 2b, respectively.

There signatures are similar for the first 15 s, but differences are observed thereafter, implying the cultivars had different steady state chlorophyll fluorescence. This was due to the physiological response of each set of cultivar leaves to the violet light. Since the steady-state fluorescence is related to the actual CO_2 fixation of the photosynthetic apparatus [49], the signatures suggest that the cultivar leaves had different rates of CO_2 fixation. This may be due to the way the cultivar's leaf chloroplast pigments respond to the violet light based on the severity of the CMD. From Thoren *et al.* [50] we can state here that there was a negligible effect of temperature on the laser-induced chlorophyll fluorescence induction kinetics within our measuring temperature range of 28–30 °C.

From the fluorescence maximum and the steady-state fluorescence of the average chlorophyll fluorescence induction kinetics curves, the fluorescence decrease ratio (Rfd) values were determined. The Rfd values of the red band (Rfd-red), which represents the photosynthesis of a whole leaf, is an indicator of the potential photosynthetic quantum conversion capacity of the leaves [49,51]. Table 1 shows cultivars and their respective Rfd-red values against their agronomical assessment. Figure 3 shows the mapping between the Rfd-red values of the four cassava cultivars and their corresponding disease severity (DS) levels. The mapping shows an inverse relationship between Rfd-red and DS. When the DS level is above 2.0 a.u. the corresponding Rfd-red values are below 2.5; for Rfd-red values above 2.5, the DS level is below 2.0 a.u. The highest Rfd-red value shown by Capevars reflects the highest photosynthetic quantum conversion capacity and CO_2 fixation rate, since Rfd is also related to CO_2 fixation [42]. The lowest Rfd value, exhibited by Asamang, indicates that there was a relative decline of photosynthetic apparatus because of the high DS level. Because Rfd values correlate directly with CO_2 fixation, it can be stated that the CMD affected the CO_2 fixation rate and thus the Rfd values. Although Asamang was relatively severely affected by CMD, the Rfd-red value indicates that the photosynthetic functions were still present. Therefore, the higher the DS level, the poorer the photosynthetic efficiency, resulting from a possible low CO_2 fixation and consequently a lower Rfd value.

In order to relate Cassava average yield per plant (AYPP) and Rfd values, Figure 4 shows a plot of Rfd values and AYPP. The graph shows that Rfd values correlate positively with AYPP values. This suggests that a high Rfd value maps well to a high AYPP and thus to a possible high photosynthetic efficiency of the cultivar. Figure 5

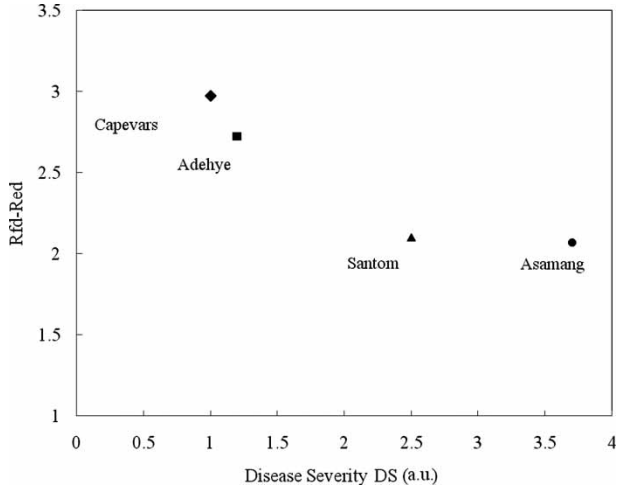

Figure 3. The relationship between the fluorescence decrease ratio (Rfd-red) and the African mosaic disease severity (DS) levels of the various cassava cultivars: Capevars (♦), Adehye (■), Santom (▲) and Asamang (●).

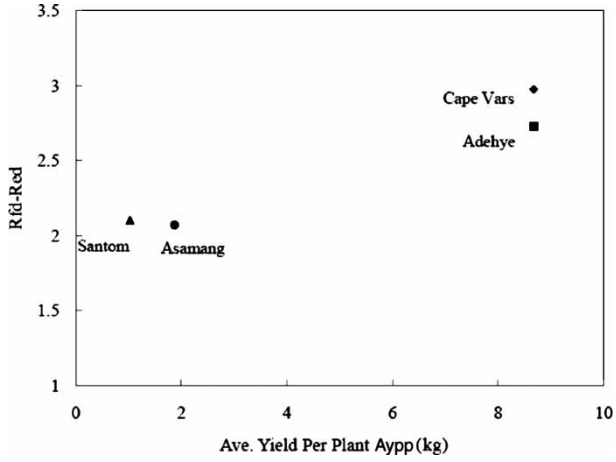

Figure 4. The relationship between the Rfd-red values and the average yield per plant (AYPP) of the various cassava cultivars: Capevars (♦), Adehye (■), Santom (▲) and Asamang (●).

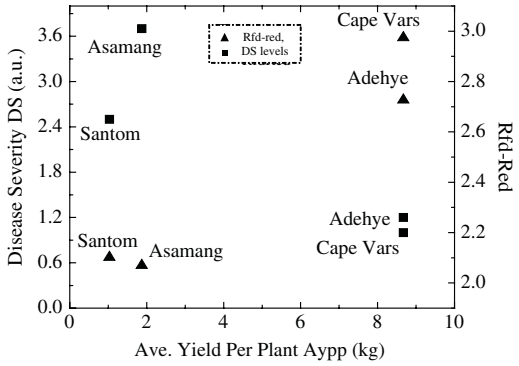

Figure 5. Comparison of the Rfd-red values (▲) and the DS levels (■) of the various cassava cultivars: Capevars, Adehye, Santom and Asamang with their corresponding AYPP.

shows the graph of Rfd-red values and DS levels against AYPP of the cultivars. The pattern depicted in Figure 5 indicates that a low Rfd-Red value corresponds to high DS levels and subsequently a low AYPP of a cultivar. The Asamang cultivar had a higher AYPP than Santom even though Asamang had a higher DS level. This could be attributed to the Rfd-red value of Asamang indicating that photosynthetic functions were still present. The inverse relationship between DS level and crop yield of cassava cultivars has been reported by Bock and Woods [2] and Legg and Fauquet [3]. This suggests that the destructive effect of CMD can be monitored using violet laser-induction chlorophyll fluorescence, thus best cultivar can be ascertained and the AYPP can be forecast.

4. Conclusion

Laser-induced chlorophyll fluorescence induction kinetics (slow Kautsky effect) has been used to map DS levels of four cultivars of cassava (*Manihot esculenta* Crantz) with a portable VDLS. The disease severity level on each cultivar and average yield per plant does show strong correlation with the fluorescence decrease ratio (Rfd-Red). The Rfd-Red values were found to correlate inversely with disease severity levels and positively with average yield per plant. The fluorescence decrease ratio is an integral response to a cultivar influenced by stresses. The work has shown the potential of chlorophyll fluorescence induction kinetics (Kautsky effect) as an alternative and objective technique or method for selecting the best cassava cultivar.

A calibration is necessary for predicting the level of the disease. This work is not exhaustive; however, an approach has been identified that could be used as the basis for future research seeking to improve the disease tolerance of cassava cultivars. From this perspective, the use of the chlorophyll fluorescence imaging technique is being developed.

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