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Evolution of seed development, germination performance and chlorophyll fluorescence of chicory achenes

Keywords: fluorescence imaging, chicory, chlorophyll, maturation, germination performance

1. Introduction

Several aspects of seed quality are tested routinely to minimize the risk of sowing seedlots that do not have the capacity to produce the desired crop. Amongst these tests, seed germination is important since it represents the percentage of pure seeds that have the potential to produce established seedlings in the field. The rate of germination corresponds to the reciprocal of the time needed for a given germination percentage to be reached (Halmer, 2008). Accurate procedure of germination tests performed in laboratory is defined by the International Seed Testing Association (ISTA).

Besides these laboratory tests, seed processing lines includes cleaning machines that remove dust and waste material, and conditioning machines performing dimensional sizing, density sorting, and colour sorting. In conventional colour sorting, discoloured seeds are rejected, on basis of inspecting seeds individually to detect differences in reflected colour.

However, even after several sorting operations, some seed batches can contain a large proportion of viable seeds but still not sufficient for commercial use. These batches are lost because the viable and non-viable seeds cannot be separated using the conventional processing methods. The proportion of immature seeds in these lost batches is unknown. It would therefore be useful to provide a new, non-destructive method of distinguishing immature seeds from mature seeds in order to improve sorting processes.

In this context, the potential of fluorescence imaging (Chen et al. 2002; Nedbal & Whitmarsh, 2004) has been examined. The chlorophyll degrades during fruit ripening and the process of degradation was described by Barry (2009). The chlorophyll is also a highly fluorescent molecule. Fluorescence occurs when some of the light absorbed by the chlorophylls is re-emitted at longer wavelength, typically between 650 and 750 nm. The fluorescent properties of chlorophylls have been used to evaluate the maturity of cabbage seeds (*Brassica oleracea* L.) (Jalink et al. 1998; Jalink et al., 1999). A red LED (light emitting diode, wavelength 650 nm) was used as a light source. The seed was illuminated through an interference filter at 656 nm. The emitted fluorescence was filtered at 730 nm and focused by a lens onto a photodiode. The results showed that the magnitude of the chlorophyll fluorescence (CF) signal was inversely related to the quality of seeds. The relationship between the CF and germination performance was studied for tomato (*Solanum lycopersicum* L.) by Jalink et al. (1999) as in their previous study (Jalink et al., 1998) but using a laser light source with 670 nm wavelength. They concluded that seeds with an intermediate CF level were of the best quality, followed by seeds having a low CF signal. Seeds having a high CF signal were the worst. Konstantinova et al. (2002) measured the CF of

barley grains (*Hordeum vulgare* L.) with a SeedScan I Laser Sorter (Satake, Stafford, TX. USA), using the principle developed by Jalink et al. (1998) but including a laser light source instead of a LED. They concluded that sorting a barley seed lot into six subsamples varying in CF values resulted in an optimal quality for the subsamples with low and intermediate CF signals. Suhartanto (2002) thoroughly described the relationships between the fruit CF, seeds CF and germination performance of tomato. A Pulse-Amplitude Modulated method (XEPAM fluorimeter, Heinz Walz GmbH, Germany; method described by Schreiber, 2004) was used to measure the dark fluorescence (CF after adaptation to darkness) of fresh seeds, fruit fluorescence and their photosynthetic activity. The maximum germination percentage and maximum percentage of normal seedlings occurred at 51-54 days after flowering (DAF) when the CF of fresh and dried seeds, as well as seed chlorophyll content, reached a minimum. From these studies, it can be concluded that the performance of CF as a marker for seed performance is dependent upon the species, with a general tendency to a negative correlation between CF and germination performance.

Several difficulties arise in imaging chlorophyll fluorescence of chicory seed. Firstly, the chicory seeds cannot be easily separated from the fruit pericarp. Therefore, it is the whole fruit (achene or *cypsela*) which can be observed non-destructively, and not the seeds as in cabbage, tomato, or barley. Secondly, the chlorophyll concentration in dry chicory seeds is very low, about 1 mg kg^{-1}. (Ooms & Destain, 2011). Thirdly, at the opposite of the radicle tip is a crown of scales, the pappus (the term is more often used to describe the wind dispersion organs which ornate the achenes of other species in the family *Asteraceae*). This latter may be exposed to light during maturation, and consequently contains more chlorophyll than the main body of the pericarp. Analyzing separately the chlorophyll of the pericarp (FPER) and of the pappus (FPAP) may be useful, since their differentiated evolution may be an indicator of seeds maturity.

For all these reasons, the conclusions derived from previous studies on different species may not be applicable. Using a specific imaging system, Ooms and Destain (2011) showed that seed chlorophyll content diminished during maturation following a different logistic trend for the pappus and the pericarp.

The aim of this paper is to identify the stage of the maturation process identifiable using chlorophyll fluorescence.

2. The measurement of chlorophyll fluorescence of chicory *(Cichorium intybus L.)* seeds

2.1.Plant m aterial

Twenty-four chicory plants were grown outdoors (variety: Melci) between 1st August and 30th October 2010, after forced vernalization of the seeds at 4°C from 15th May to 30th June, and one month in a phytotron at 20 °C (1st - 31st July). The capitula were labelled at the day of flowering and harvested at different maturation durations, from 16 days after flowering (DAF) until 44 DAF. The dry weight and water content were evaluated by weighting the seeds (analytical scale: Ohaus Adventurer AR0640) before and after artificial drying (24 h, 30 °C). The CF parameters (FPER and FPAP) were measured using images of the achenes fluorescence obtained with the method described below. Germination tests were performed following the rules of the ISTA (2005) to evaluate the germination percentage (GP) and the germination rate (GR), which is, for a capitula, the inverse of the time needed to observe 25 % of germinated seeds.

2.2. Measurement system

A specific imaging system was designed to measure the chlorophyll-a emitted by the chicory seeds, ensuring to distinguish the emissions provided by the pappus (Ooms & Destain, 2010).

A xenon light source (Hamamatsu Lightingcure L8222, model LC5, 150 W) produced a white light which passed through an interference filter (03FIB002, Melles Griot, Carlsbad, USA) with a central wavelength 410 nm and width at half-maximum 80 nm to excite the chlorophyll-a. The light was conducted to the seeds by an optical fiber. The blue light issued from the filter was absorbed by the seeds chlorophylls, which resulted in fluorescence emission. A high-pass filter (665 nm, 03FCG107, Melles Griot 03FCG107) ensured the selection of the fluorescent signal from the blue light reflected by the object. The images were acquired by a CCD monochrome camera (Hamamatsu C5405-70), with a resolution of 640 x 480 pixels and 256 gray levels. The system was enclosed within a black box to avoid interference from ambient light and was air-conditioned (Tectro TS27, PVG Int. B.V., Oss, The Netherlands) to maintain a temperature of 20 C° (±1 C°). The lamp was optically isolated and white light did not escape inside the box. The blue filter transmitted an unexpected, small amount of infrared light between 770 and 900 nm (detected with spectrometer AVS-SD2000, Avantes, Eerbeek, The Netherlands).

2.3. Image analysis

A specific image analysis code was developed with the GNU Octave language (Ooms & Destain, 2011). After applying background correction (the fluorescence values were divided by the reflectance signal of paper and multiplied by 60), the images were segmented and images of individual seeds were created, each of them being rotated along the main axis of the seed. The

pappus side, which is brighter and larger than the radicle tip, was automatically detected on the basis of the mean width of the left half, its mean fluorescence intensity, the right half width and the right half fluorescence intensity. The accuracy of the detection was greater than 98%. The image was thereafter divided into the "pericarp zone" (Pe, 77% of the seed length) and the "pappus zone" (Pa, 23% of the length). The value of 77% was a compromise based on the observation of 100 seed images. The mean fluorescence values of the two zones were recorded for data analysis. The measurement system and image analysis are summarised in Fig. 1.

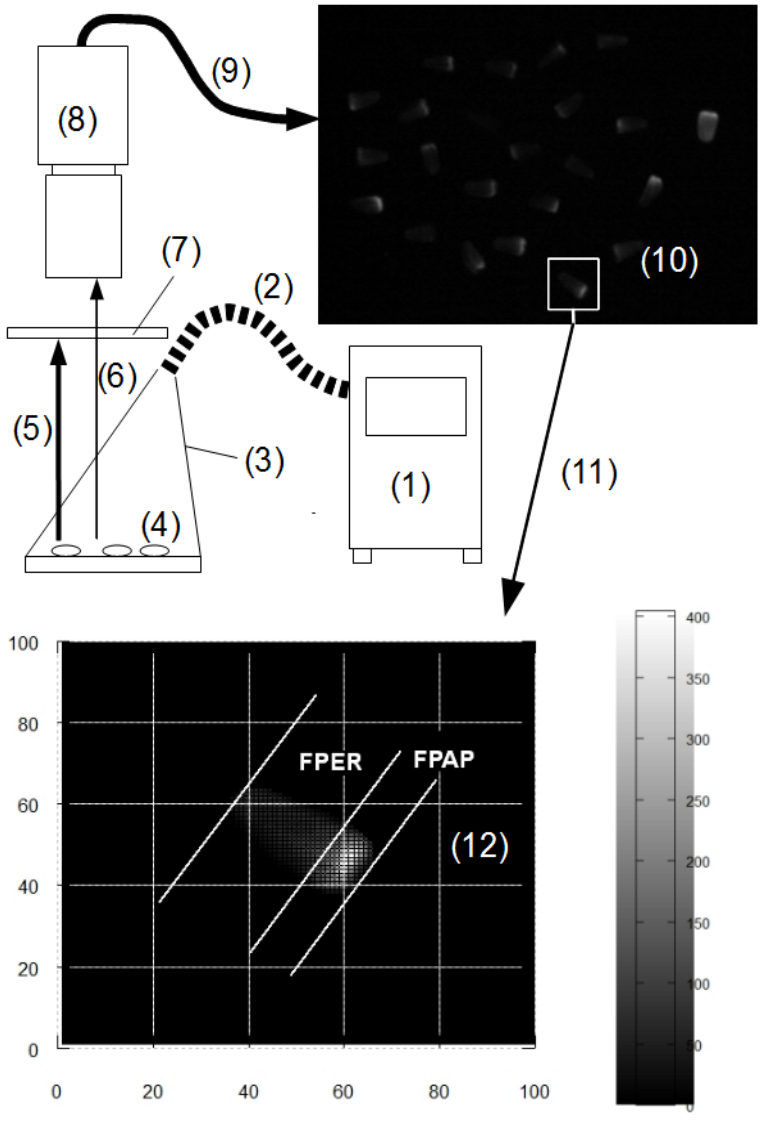

Fig. 1. The component s of the chlorophyll imaging device and the main steps of image analysis. (1) Xenon lamp with blue optical bandpass filter (370 - 450 nm), (2) optical fiber, (3) cone of

blue light, (4) seeds from one capitulum, (5) blue light reflected by the seeds, (6) chlorophyll fluorescence (650 - 730 nm), (7) highpass optical filter (665 nm), (8) CCD camera with zoom objective, (9) connection to the computer, (10) raw image, (11) background correction, then creation of individual images, (12) automated detection of the pappus using dedicated software and estimation of the levels of lfuorescence FPER and FPAP (Ooms & Destain, 2011).

3. Results

Fig. 2 shows the evolution of CF during this phase on the stalk, the weight parameters (dry weight DW and water content WC) and the germination performance (GP and GR). The dry weight increased, while the water content was still high at the end of the observed period (> 45 % on the stalk at 44 DAF, while the WC of stored seeds is about 6 %). According to Egli (2006), the rate of increase of DW is constant during most of the filling phase and a linear regression should be used. Most of the filling occured between 18 and 33 DAF at a rate of 0.048 mg/day per seed. The physiological maturity occurs when the seeds attain their maximum germinability and vigour (Black et al., 2006). It was attained at 39 DAF and the observed period, before 39 DAF, corresponded to the phase of reserve deposition in the seed described in Bewley & Black (1994). The maturation drying should have been the next phase, but it seems to have been delayed and the decrease of WC between 39 and 44 DAF was barely significant.

The experimental data were modeled (Fig. 3).

$$FPER = 120 + 78 \exp(-(t - 16) / 2) \quad (\leq 21 \text{ DAF}) \qquad (1)$$

$$FPER = 120 - 44 / (1 + \exp((26.2 - t) / 0.6)) \quad (\geq 21 \text{ DAF}) \qquad (2)$$

$$FPAP = 255 + 76 \exp(-(t - 16) / 0.97) \quad (\leq 21 \text{ DAF}) \qquad (3)$$

$$FPAP = 255 - 67 / (1 + \exp((25.2 - t) / 0.69)) \quad (\geq 21 \text{ DAF}) \qquad (4)$$

$$DW = 0.048\, t - 0.069 \quad \text{between 18 and 33 DAF} \qquad (5)$$

$$WC = 0.38 + 0.37 \exp(-(t - 16) / 13.4) \qquad (6)$$

$$GP = 0.75 / (1 + \exp((31 - t) / 2.69) \qquad (7)$$

$$GR = 0.4 / (1 + \exp((32.1 - t) / 2.8)). \qquad (8)$$

The evolution of FPAP and FPER followed logistic rules from 21 to 44 DAF, confirming the previous observed tendency (Ooms & Destain, 2011). The decrease of FPER and FPAP observed before 21 DAF may be exponential. The increase of GP and GR also followed logistic rules. The increase of DW may be exponential (asymptote to the right), as was the decrease of WC (Fig. 3).

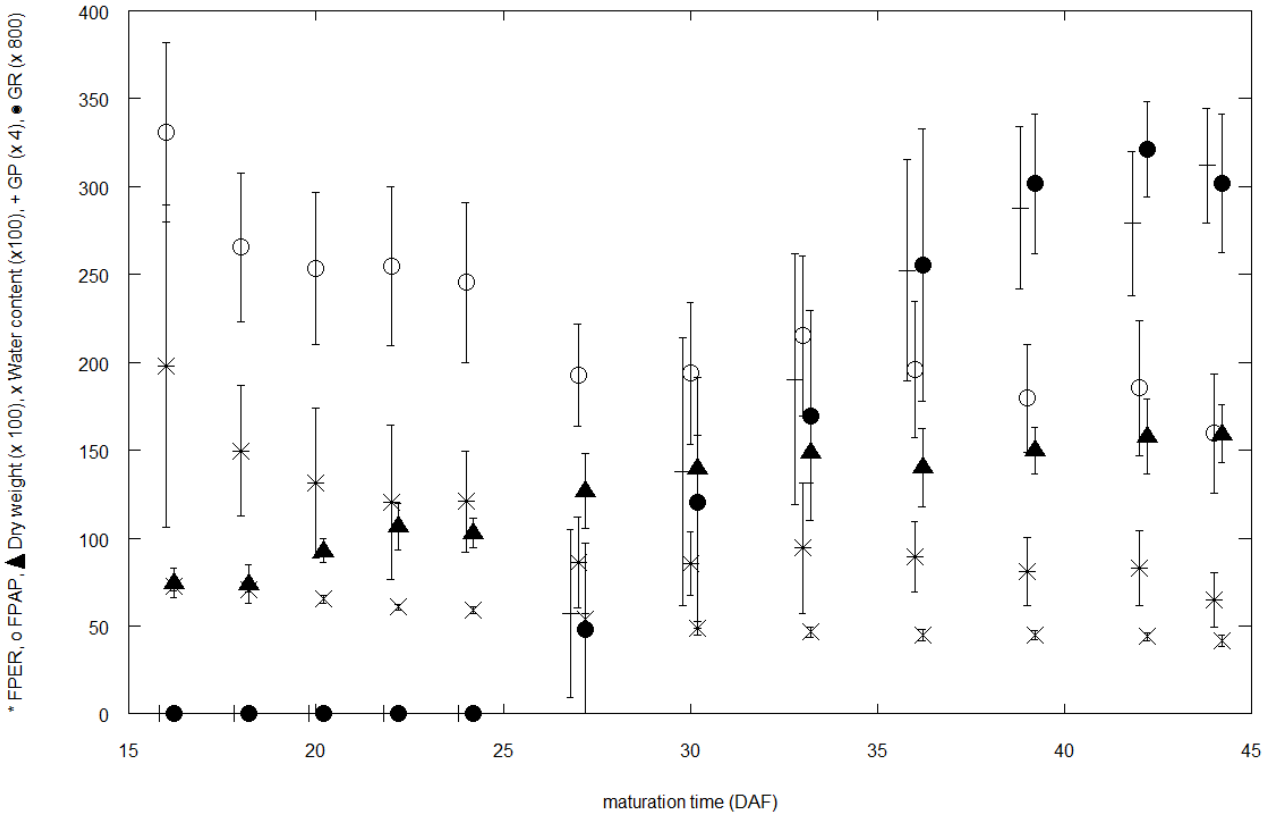

Fig. 2. Evolution of chlorophyll fluorescence (FPER: pericarp, FPAP: pappus), dry weight, water content at harvest, germination percentage and germination rate of chicory seeds at each maturation duration on the stalk from 16 to 44 days after flowering. Means with confidence intervals of the means.

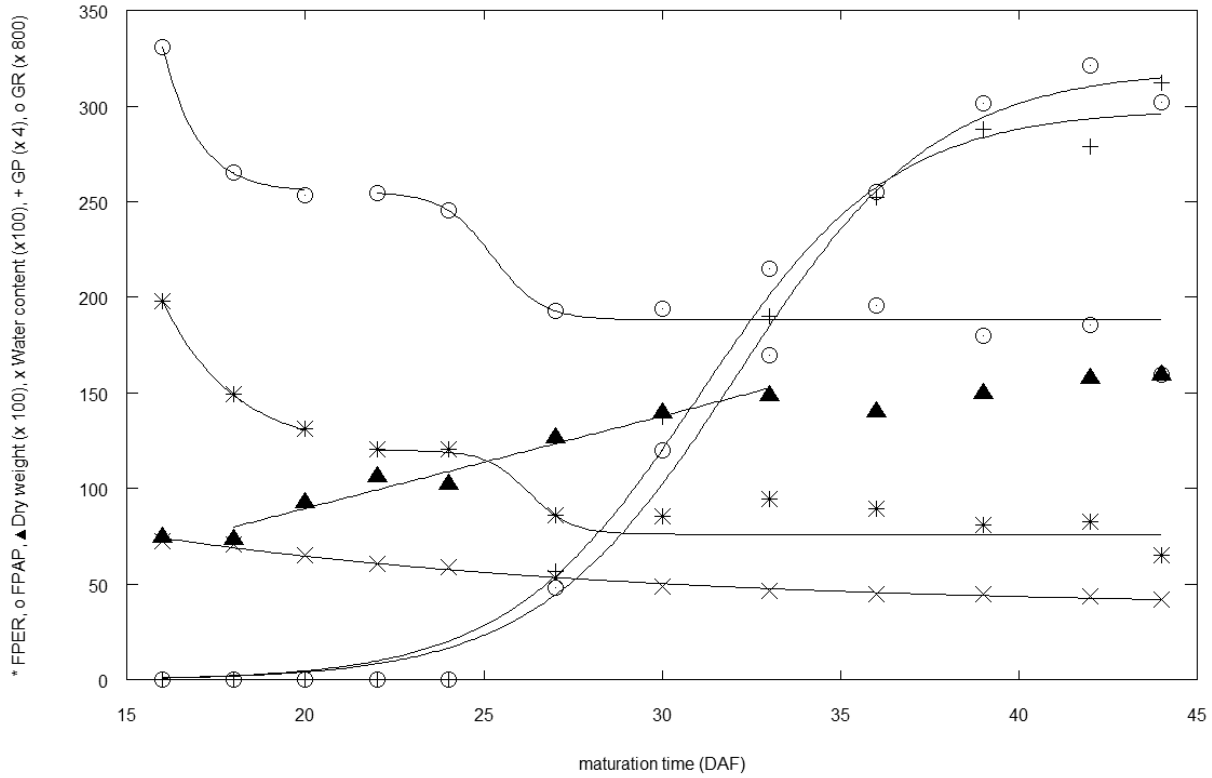

Fig. 3. Modeling of the evolution of chlorophyll fluorescence (FPER: pericarp, FPAP: pappus), dry weight (DW), water content at harvest (WC), germination percentage (GP) and germination rate (GR) of chicory seeds at each maturation duration on the stalk from 16 to 44 days after flowering.

The two following facts are in favor of the use of CF features for the differentiation of immature chicory seeds from mature ones, and as indicators of seed vigor:

- the CF decreased during the filling phase;
- the end of the filling phase corresponded to the physiological maturity, where the maximal germination percentage and vigor is attained (Black et al., 2008).

On the other hand, the efficiency of CF features may be negatively affected:

- the highest decrease of CF did not occur exactly at the same time as the highest increase of GP and GR, but sooner (Fig. 3).
- the large variability of individual measurements (random differences between individuals);

To assess the repeatability, a comparison was made of the evolution of CF features and germination performance indicators for two independant experiments, the one illustrated above, and the data set from Ooms & Destain (2010), obtained in the summer of 2006 using the same

method (Table 1). In 2006, the seeds were grown from cloned plants, while in 2010 the seeds were originated from plants grown from commercial seeds.

Table 1. Comparison of the main events occurring in 2006 and 2010

	Summer 2006 (1)	Autumn 2010
Observed period	18 to 39 DAF	16 to 44 DAF
FPAP at 39 DAF / FPAP at 18 DAF	< 25 % (2)	66 %
Time of highest decrease of FPAP	29 DAF	< 18 DAF and 26 DAF (3)
FPER at 39 DAF/ FPER at 18 DAF	< 38 % (2)	53 %
Time of highest decrease of FPER	22 DAF	< 18 DAF and 25 DAF (3)
GP at 18 DAF	40 %	0 %
GP at 39 DAF	50 %	75 %
Time of highest increase of GP	< 18 DAF	31 DAF
GR at 18 DAF	0.2 d^{-1}	0 d^{-1}
GR at 39 DAF	0.65 d^{-1}	0.4 d^{-1}
Time of highest increase of GR	34.3 DAF	32 DAF

DAF = days after flowering (1) Ooms & Destain (2011) (2) may be 0 %, incertitude due to a positive shift of the CF signal. (3) two-step decrease: < 18 DAF: may be exponential, 25 - 26 DAF: logistic rule as in 2006.

The logistic decrease of CF features observed in 2006 was much weaker in 2010 (highest decrease between 24 and 27 DAF, Fig. 3). An exponential decrease was observed in the interval 16 - 21 DAF in 2010, but could not be recognised on the data of 2006, which started only at 18 DAF. The evolution of the germination percentage was completely different: the maximum GP was attained at 21 DAF in 2006, but only at 39 DAF in 2010, with a much higher value (0.75 instead of 0.5). The GR increased at the same time in 2006 and 2010, but with large difference in absolute value (much higher in 2006). Therefore, great care must be taken before extrapolating the observations made on a particular harvest, and the CF features should not be used for sorting on the sole basis of past experiments.

Current work aims at estimating the correlations between the CF features, the weight parameters and the germination variables in outdoors and greenhouse environments, and to assess the added value of CF features in comparison to weight, size and density features to distinguish between viable and non-viable seeds using sorting simulations.

4. Discussion

Most fruits and seeds have measurable levels of chlorophyll, respectively in their pericarp or testa, and this chlorophyll degrades with time. In the case of chicory, the commercial seed is a fruit which cannot be hulled and the seed is not observable directly. The low amount of chlorophyll implies the use of a highly sensitive device and because of the presence of a distinct pappus, the imaging of fluorescence is favourable.

On basis of experiments performed during two different years, the following observations were made. During the maturation process, which can be characterised by the evolution of dry weight and water content, the chlorophyll fluorescence (CF) decreases according to logistic rules (LR1 and LR2). The germination performance, defined as the germination percentage and germination rate, increases according to other logistic rules (LR3 and LR4). However, the inflexion points of LR1 and LR2 do not occur at the same time as the ones of LR3 and LR4. Furthermore, the amplitude of the decrease of CF is dependent on the weather conditions observed during seed development, while the time at which the inflexion point occurred does not seem to vary. The large differences between the results of experiments made at different years imply reference measurements for each harvest to increase the reliability of the CF measurement technique.

It would be profitable to identify the factors (variety, season, climate, hydric stress, etc.) influencing the evolution of CF to predict the characteristics of the decrease of CF and to predict if its decrease is always concomitant with the increase of the germination performance.

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