CHLOROPHYLL FLUORESCENCE OF THE TESTA OF *Brassica oleracea* SEEDS AS AN INDICATOR OF SEED MATURITY AND SEED QUALITY

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ABSTRACT: Chlorophyll fluorescence of the testa of seeds is proposed as a non-invasive method for the determination of maturity and quality of seeds. In this study cabbage seeds (Brassica oleracea) were sorted individually based on the chlorophyll fluorescence signals into four subsamples labeled with respect to their chlorophyll fluorescence signal (low, medium, high and very high). The results show that the magnitude of the chlorophyll fluorescence signal was inversely related to the quality of the seeds, expressed as germination %, normal seedling %, germination rate (T50) and uniformity of germination (T75-T25). The seed lot could be improved from 90 to 97% normal seedlings by sorting out 13% of the seeds with very high chlorophyll fluorescence signals. Advantages of the chlorophyll fluorescence method for sorting seeds are the high sensitivity, the method being fully non-destructive, the high speed at which the fluorescence is generated and measured and the specificity for only chlorophyll. Other pigments or substances which can influence seed colour but do not fluoresce at the specific wavelengths of excitation and emission of chlorophyll, will not contribute to the fluorescence signal. These characteristics make chlorophyll fluorescence highly suitable as a new sorting technique.
Key Words: maturation, chlorophyll fluorescence, cabbage seed, sorting, quality

FLUORESCÊNCIA DA CLOROFILA NO TEGUMENTO DE SEMENTES DE *Brassica oleracea* COMO INDICADOR DA MATURIDADE E QUALIDADE DA SEMENTE

RESUMO: A fluorescência da clorofila do tegumento (testa) de sementes foi proposta como um método não destrutivo para a determinação da maturidade e da qualidade das sementes. Sementes de repolho (Brassica oleracea) foram separadas individualmente, em quatro subamostras, classificadas de acordo com a intensidade da fluorescência (baixa, média ,alta, muito alta). Os resultados indicaram a existência de uma relação inversa entre a magnitude da fluorescência e a qualidade das sementes, expressa pela percentagem de germinação, percentagem de plântulas normais, taxa de germinação (T_{50}) e uniformidade de germinação ($T_{75} - T_{25}$). Verificou-se incremento de 90 para 97% de plântulas normais no teste de germinação quando foram descartadas 13% de sementes classificadas como de emissão muito alta. O método apresenta vantagens por expressar alta sensibilidade, não destruir a semente, pela rapidez com que a fluorescência é avaliada e pela especificidade por um único elemento, a clorofila. Outros pigmentos ou substâncias, que podem alterar a coloração da semente mas não apresentam emissão de fluorescência no comprimento de onda de excitação e emissão da clorofila, não contribuem para a identificação. Estas características tornam a fluorescência pela clorofila um método adequado para a separação de sementes de acordo com a qualidade fisiológica.
Descritores: sementes, maturação, fluorescência de clorofila, repolho, qualidade

INTRODUCTION

An important quality property of seeds is the maturity status. Seeds which are immature or not fully mature are generally speaking of lower quality as mature seeds. In a detailed study on seed development and maturation in bean (*Phaseolus vulgaris* L.), it was found that seed harvested after the end of the seed-filling phase scored higher in a germination test, yielded more normal seedlings and possessed better storability than seeds harvested at a less mature or immature stage (Sanhewe & Ellis,

1996a; 1996b). Steckel *et al*. (1989) harvested seeds of carrot (*Daucus carota* L.) at different stages of maturation and observed an increase in germination performance of the carrot seeds. Coincidently during development the total chlorophyll content of the seed coat (as measured by the optical density of seed coat extracts) decreased. Because of this correlation, Steckel *et al.* proposed a simple field test using colour cards to assess the chlorophyll content of seed extracts to estimate the optimal harvest date. The relation between the amounts of chlorophyll and the maturity has been well studied in seeds of oilseed rape (*Brassica napus* L.) and turnip rape (*Brassica rapa* L.) (Ward *et al.*, 1992; 1995). Using destructive extraction methods (ISO, 1992; Ward *et al*., 1995), it was found that chlorophyll is broken down during the late stages of the ripening process and that the final chlorophyll levels in rape seeds can be affected both by the genotype and the environment. Lower temperatures during seed maturation cause a slower chlorophyll degradation resulting in the so-called 'green-seed' problem. For the majority of seed species the amount of chlorophyll in the seed coat decreases during maturation. At the same time the colour of the seed changes from green to a colour which depends on the species and varies with the cultivar. This process is called degreening. The chlorophyll breakdown in the seed, however, is poorly understood (Ward *et al*., 1995).

In the present study, the chlorophyll fluorescence technique is introduced as a non-destructive and instantaneous method to measure differences in quality by the assessment of the magnitude of the chlorophyll fluorescence signals of individual seeds of cabbage (*Brassica oleracea* L.). Cabbage seeds were sorted on the magnitude of the chlorophyll fluorescence signal in four subsamples and the seeds of each subsample were tested on germination performance. The germination results show that for cabbage chlorophyll fluorescence (CF) can be used as a new sorting technology to improve the quality of cabbage seeds with high efficiency by sorting out the seeds with high chlorophyll fluorescence signal.

MATERIALS AND METHODS

Seed material: Seeds of white cabbage (*Brassica oleracea* L.) of variety Ernando were used for this research, since this particular seed lot contained a small fraction of about 10% of seeds that showed higher values in chlorophyll fluorescence signals compared to the average chlorophyll fluorescence signal of the sample. It was expected that this seed lot could be improved by removing a small fraction of the seeds.

Chlorophyll fluorescence measurement: The equipment used for chlorophyll fluorescence measurements is depicted in Figure 1. The light of the LED-lamp (Hewlett Packard HLMP-8150) which is controlled by a LED power supply (Loep, The Netherlands), is passed through an interference of 656 nm (Edmund Scientific, USA).The beam splitter reflects about half of the intensity of the LED light towards the lens, which focuses the light onto the seed. A part of the chlorophyll fluorescence is captured by the same lens and about 50% of the light is directed towards the filter of 730 nm (Edmund Scientific) and photodiode (PIN-10DP, UDT Sensors, USA). The beamsplitter was used for easy alignment of the two optical light paths: the excitation light of the LED and the fluorescence of the chlorophyll. With the combination of two interference filters of 656 and 730 nm, the background signal of the system due to stray light of the LED was suppressed to a current of about 1 pico ampere (pA), which was low compared to the average fluorescence signal of 12 pA of the seed lot. For sensitive detection of the chlorophyll fluorescence a lock-in amplifier (Stanford Research SR 830, USA) was used which modulated the LED light at 77 Hz and converted the alternating current of the photodiode into a signal that was proportional to the intensity of the chlorophyll fluorescence. The use of a lock-in amplifier had the additional benefit that due to the modulation technique the measurement could be performed at normal room light conditions.

The experimental conditions that predominantly determine the magnitude of the CF signal are the spectral distribution and intensity of the excitation light (Nobel, 1970), the surface area of the seed that is illuminated by the light, the structure of the seed coat, the spectral sensitivity of the filter/ photodiode combination and the solid angle of the lens system. The solid angle is determined by the distance between the seed and the lens, and the diameter of the lens.

CF sorting: Based on the magnitude of the CF signal, 1180 seeds were individually measured and manually separated into four CF subsamples: low, medium,

high and very high CF values. The borders of the four subsamples were chosen in a way that two subsamples, low and medium CF, contained the majority of seeds and the other two subsamples, high and very high CF, the rest of the seeds. The results of the germination test will give information about where the threshold for efficient sorting should be chosen. By setting the borders of the subsamples at 6 pA for the low subsample, between 6 and 10 pA for the medium subsample, between 10 and 25 pA for the high subsample and higher than 25 pA for the very high subsample, resulted in subsample sizes of 41.9, 30.1, 14.8 and 13.2% for the low, medium, high and very high subsample, respectively.

Germination: For each CF subsample, germination of two replicates of 50 seeds was tested on moist filter paper at 20°C and a 12 hour dark/12 hour light cycle (ISTA, 1996). Seeds were visually inspected for root tip emergence, and the germination rate (T_{50}, number of days to reach 50% of maximum germination) and uniformity (T_{75} - T_{25}, number of days between 25% and 75% of maximum germination) were calculated with software called Germination Analysis (Jalink & van der Schoor). The standard deviation (s.d.) of T_{50} and T_{75}-T_{25} was calculated from the T_{50} and T_{75}-T_{25} values of the two replicates. After 5 and 10 days, the seedlings were evaluated and the number of normal seedlings, fresh seeds, dead seeds and abnormal seedlings was scored according to standard ISTA (1996) rules. The data was statistically analysed using the Student's t-test (level of significance: $\alpha = 0.05$).

RESULTS

The results show a clear relation between the chlorophyll fluorescence signal of the four CF subsamples and the germination curves (Figure 2) and germination parameters (TABLE 1). The germination performance was inversely related to the magnitude of the CF signal. Seeds from the subsample with the lowest CF signal germinated at 100% with 100% normal seedlings and had the highest germination rate and showed the most uniform germination behaviour. In comparison to the low, medium and high CF subsample, the very high CF subsample had a significantly reduced performance for almost all aspects, i.e., germination %, normal seedling %, dead seed %, abnormal seedling %, germination rate and uniformity.

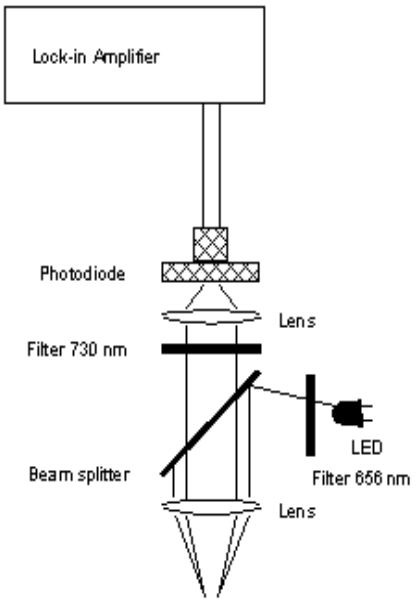

Figure 1 - Schematic representation of the setup used for CF measurements. LED: light emitting diode, λ_{max}=650 nm, full width half maximum (FWHM)=22 nm; LED-PS: LED power supply; filter 656: interference filter, λ_{max}=656 nm, FWHM=10 nm; beamsplitter: 50% transmission, 50% reflection; lens: f=15 mm, ∅=15 mm; filter 730: interference filter, λ_{max}=730 nm, FWHM=10 nm; photodiode: ∅=11.3 mm.

DISCUSSION

For more than a century it is known that chlorophyll shows prompt fluorescence when the molecule is excited at the proper wavelength (Müller, 1874), no one has ever used this effect in order to measure the magnitude of the chlorophyll fluorescence signal in relation to its maturity and quality of individual seeds. By using a combination of lighting technology, narrow bandwidth filters to filter out fluorescence and phase sensitive detection (lock-in amplifier), the chlorophyll a in the testa can be excited and the relative amount of the green pigment can be determined with a much higher sensitivity and selectivity than with a colour or reflection spectrum measurement (Tkachuk & Kuzina, 1982). Small differences in the amount of chlorophyll a in the envelope of individual seeds can thus be

TABLE 1 - Proportion of seed lot after CF sorting and germination performance of the non-sorted cabbage seed lot and the four CF subsamples (see caption Figure 2).

CF subsample	non-sorted	low	medium	high	very high
CF signal [pA]	-	<6	6 -10	10 - 25	>25
Proportion of seed lot [%]	-	41.9	30.1	14.8	13.2
Germination [%]	96±1	100*	100*	97±2	70±9
Normal seedlings [%]	90±2	100*	96±1	90±3	42±14*
Fresh seeds [%]	0	0	0	1±1	0
Dead seeds [%]	4±2	0	0	2±2	30±8
Abnormal seedlings [%]	6±1	0*	4±1	7±4	28±6*
T_{50} [days]	1.38±0.05	1.28±0.05	1.42±0.01	1.7±0.2	2.8±0.2*
T_{75}-T_{25} [days]	0.74±0.03	0.5±0.1	0.74±0.03	1.1±0.2	2.3±0.2*

Data are means±s.d. of 2 replicates of a total of 100 seeds. T_{50}, time to 50% of total germination; T_{75}-T_{25}, time between 25 and 75% of total germination. * Significantly different from the non-sorted controls at α=0.05.

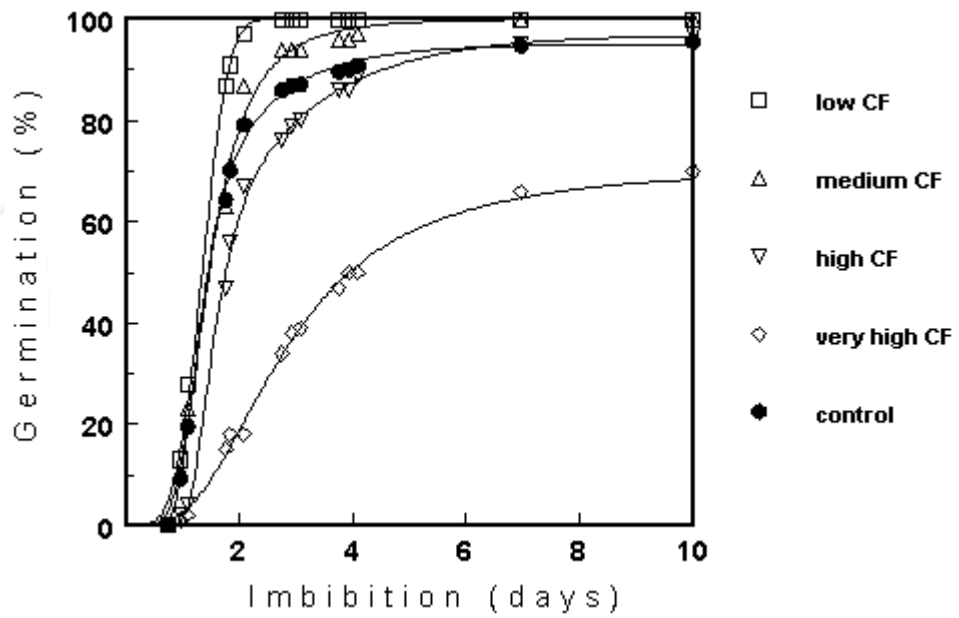

Figure 2 - Germination curves of the four cabbage CF subsamples obtained at a temperature of 20ºC on filter paper. Based on the current of the photodiode, seeds were sorted into four subsamples: 'low CF' with signals lower than 6 pA, 'medium CF' with signals between 6 and 10 pA, 'high CF' with signals between 10 and 25 pA and very high for signals larger than 25 pA.

demonstrated, even in cases when the envelopes are completely uniform in colour for the human eye. The chlorophyll in the seed is photosynthetic inactive, because the metabolism of the seed has stopped at the moment of the acquisition of desiccation tolerance. Hence, contrary to leaves and moist fruit, seeds do not show the so called "variable fluorescence", which is due to photosynthetic activity and which is commonly measured with a pulse amplitude modulation (PAM) fluorometer (Schreiber, 1986).

Seeds from the high CF subsample germinated at a much lower percentage and resulted in a much lower amount of normal seedlings as compared to the seeds with low and medium CF values. Apparently, the quality of the seeds was inversely related to the amount of chlorophyll in the seed coat. The quality of the seed lot could be improved by sorting out seeds with a CF signal higher than 10 pA, meaning seeds belonging to the high and very high subsample. This resulted in an increase in normal seedlings from 90% for the non-sorted control to 98% by removing 28% of the seeds (rejects). The cleaning efficiency = (% improvement/ % rejects) x 100% = (8/28) x 100% = 29%. By sorting out seeds with a CF signal higher than 25 pA the normal seedling percentage resulted in 97% by removing 13.2% of the seeds. In this case the improvement in quality was slightly reduced by 1% as compared to the removal of seeds with CF signals higher than 10 pA, but the cleaning efficiency increased from 29 to 53%.

In this study the number of seeds used for the germination test was low due to the separation of the seeds by hand. In future studies, the research will be focused on automising CF sorting in order to increase the number of sorted seeds. The CF signal will be increased by replacing the LED and 656 filter by a diode laser. This will enhance the CF signals due to the higher intensity of radiation of a laser as compared to the combination of filter and LED-lamp. Furthermore, the 656 filter can be omitted since the laser radiation has a very narrow bandwith. Other cabbage seed lots and other seed species like tomato (*Lycopersicon esculentum* Mill.), pepper (*Capsicum anuum* L.) and cucumber (*Cucumis sativus* L) will be tested with CF sorting.

CONCLUSION

In conclusion, we found that the chlorophyll fluorescence of the cabbage seed coat can be used as a non-destructive marker for the germination performance of cabbage seeds. The presented method is based on a fluorescence measurement which is highly specific for chlorophyll. Other substances which can influence seed colour but do not fluoresce at specific wavelengths of excitation and emission, will not contribute to the fluorescence signal. The presented new method of chlorophyll fluorescence of seeds will work for all seeds of which the chlorophyll is broken down during the maturation process and can be used for the assessment of the maturity and quality of seeds.

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Recebido para publicação em 10/07/98
Aceito para publicação em 03/08/98