Chlorophyll in tomato seeds:
marker for seed performance?

Mohamad Rahmad Suhartanto

Promotor :   Prof. Dr. L. H. W. van der Plas
                     Hoogleraar in de Plantenfysiologie
                     Wageningen Universiteit

Co-promotoren: Dr. H. W. M. Hilhorst
                     Universitair docent
                     Laboratorium voor Plantenfysiologie
                     Wageningen Universiteit

                     Dr. H. Jalink
                     Senior onderzoeker
                     Business Unit Plant Development and Reproduction
                     Plant Research International

Oppositie: Dr. R. J. Bino, Plant Research International
                     Prof. Dr. C. M. Karssen, Wageningen Universiteit
                     Prof. Dr. O. van Kooten, Wageningen Universiteit
                     Prof. Dr. Ir. P. Struik, Wageningen Universiteit

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Cover illustration: "Chlorophyll fluorescence in a developing tomato embryo"

Propositions (Stellingen)

1) Chlorophyll in seed is required during seed development, but undesirable during maturation (*this thesis*).

2) Both the maternal genotype and light conditions play a critical role in determining the chlorophyll content of tomato seeds (*this thesis*).

3) Chlorophyll fluorescence of dry tomato seeds is a sensitive indicator of the physiological maturity of tomato seed (*this thesis*).

4) Seed quality is a complex concept, consisting of a number of components that each represent different physiological principles, e.g. germinability, viability, vigour and storability.

5) The most powerful method to study life processes in seeds is non-destructive and instantaneous.

6) A thesis which is written in English should be defended in English.

7) If the doctor makes a mistake, the patient will go to the soil. But, if the 'seed doctor' makes a mistake, the effect can only be seen when the seedlings emerge from the soil. Therefore, can we use a *"stethoscope"* to detect seed quality?

8) The best human being is somebody who always gives a valuable contribution to nature (Muhammad SAW).

These propositions belong to the PhD thesis entitled: "Chlorophyll in tomato seeds: marker for seed performance?"

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M. R. Suhartanto

Wageningen, June 24, 2002

The Netherlands.

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Preface

First of all, I deeply praise God for His blessing during my hard time and His gift to fill my life with little knowledge about His unique creativity, seeds. A special and essential contribution is of my parents (H. Soeharto and Hj. Sri Soemirah [late]), the first teachers who taught me how to live and love nature. Many other teachers have taught me invaluable knowledge since my play group in Indonesia until my PhD in Wageningen the Netherlands. This thesis could never have been completed without a full collaboration between the Laboratory of Plant Physiology of Wageningen University and the Department of Reproduction Technology, Centre for Plant Breeding and Reproduction Research (CPRO-DLO, now Plant Research International) in the Netherlands.

I gratefully acknowledge Dr. Henk Hilhorst, who opened my eyes to the broad world of seed science and who gave me a lot of examples of his international relationships. *Henk, I never forget your invaluable support in my difficult time of bringing my family to the Netherlands, after the long and lonely period I lived in a different continent without my family.* Dr. Henk Jalink, I would like to thank you for your support and input since the early stages of my study. *I learned from you how to be a brave researcher and never worry to try new methods and make an error.* I would like to express my gratitude to my daily supervisor Dr. Peter Toorop for showing me how to be a good seed scientist. He was also the first Dutch person that I talked to at the Ede-Wageningen Station and who showed me how I could survive in Wageningen *(thanks a lot for giving me a warm orange blanket).* Not to forget to mention your wife, Rose Toorop, who was so understanding and who spent a lot of time with me discussing about life in The Netherlands. *Henk, Henk and Peter, thank you very much for your guidance, support and patience to correct my English writing which was 'confusing' with my Indonesian-English.* Their close supervision and generous support in all scientific aspects greatly improved the value of this thesis.

I am much indebted to Professor Linus van der Plas for being my promotor and being able to find some time and dedicate it to my Ph.D. research project. I always remember a wise professor who mostly went to the Lunteren Annual Meeting by bike. My special thanks go to Dr. Raoul J. Bino, who has accepted me at the Department of

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Contents

List of abbreviations

ABA   abscisic acid

AsA   ascorbic acid (reduced form)

au   *aurea* mutant

CD test   controlled deterioration test

CFI   chlorophyll fluorescence imaging

CF   chlorophyll fluorescence

DAF   days after flowering

DHA   dehydroascorbic acid

D value   the difference between the amount of chlorophyll at the start of seed
            development and at the end of seed maturation

F_0 minimal chlorophyll a fluorescence

F_m maximum chlorophyll a fluorescence

GAs   gibberellins

gib1   gibberellin-deficient mutant

GSH   glutathione (reduced form)

hp-1   high pigment mutant

HPLC   high performance liquid chromatography

LIF   laser induced fluorescence

LIIF   light Induced fluorescence

PAM   pulse amplitude modulation

Rubisco   Ribulose 1,5-Bisphosphate carboxylase/oxygenase

sit^w abscisic acid-deficient mutant

T_{50} germination rate, time (days) when 50% of total seeds are germinated

T_{75-25} uniformity of germination, time (days) between 25% and 75% of total
            seeds are germinated

Chapter 1

General introduction

The tomato seed

Seed is not only essential for plant production but also the main material for the transfer of technology to farmers. The lifes of millions of people depend on seed, which makes it important biologically, sociologically and economically. The level of seed usage indicates the stage of development in any agricultural community.

As an important annual vegetable, tomato (*Lycopersicon esculentum* Mill.) has become of high economic value and many studies have been undertaken to improve its quality, which is commonly transferred by the seed. As a model plant species for scientific research, the tomato seed has been used most extensively to study the physiology and biochemistry of seed development, germination and dormancy. Over the last 15 years a number of processes has been identified that are strictly correlated with tomato seed performance, such as DNA replication and other cell cycle-related events, endosperm weakening and hydrolytic enzyme activity, and water relations during seed development and germination (Hilhorst and Toorop, 1997).

Tomato seed development, maturation and performance

Developmental processes influence the biochemical and physiological behaviour of seeds and, hence, also seed quality, including germinability, uniformity of seedling growth and storability (Liu, 1996). The tomato seed develops in fully hydrated tissue; this development is accompanied by specific changes in physical appearance and biochemical properties which are related to seed performance.

Seed maturity is the stage of development at which there is no further increase in dry weight (Valdes and Gray, 1998). Tomato seeds exhibit a diversity in size and weight after maturation. The size and weight of the mature seeds may influence seed performance. Both genetic and environmental factors may affect these physical properties. There are indications that inheritance of germination rate is related to seed size (Whittington and Fierlinger, 1972; Nieuwhof *et al.*, 1989). The bigger seeds had higher weight, germination percentage and vigour as compared to the smaller seeds (Palanisamy and Karivaratharaju, 1990). However, tomato seed weight does not

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influence subsequent fruit yield (Nieuwhof _et al._, 1989). Also the maternal environment plays an important role in determining seed weight (Pet and Garretsen, 1983; Nieuwhof _et al._, 1989). Reduction of the light level during plant growth caused a significant decrease in seed weight (Baevre, 1990). Seeds from plants grown in weedy conditions had a higher vigour than those from weed-free plants (Liptay and Friesen, 1982); it was proposed that the high temperature in non-weedy conditions affected seed vigour (Liptay and Moore, 1989). Thus, both heritable and environmental factors during seed development and maturation on the mother plant govern ultimate seed performance. Fluctuations in germinability may occur at times during development, due to endogenous rhythms (Fig. 1). The underlying mechanism of these rhythms are not known (Maguire, 1977). Differences in growth conditions of the parent plant, especially light, at the time of seed development may generate a wide variation in germination responses to light. This variability has led to conflicting germination interpretations by various researchers, and causes fundamental problems in obtaining reproducible results with seeds of different batches (Vidaver, 1977).

Biochemical changes during seed development influence seed performance. Reduction in non-protein N and oil level, and increase of sugar and total N content were closely related with the increase in germinability and germination rate at the milky, brown and mature stages of tomato seed development. Decreases in total N and sugar at the overmature stage were suspected to reduce tomato seed quality (Alekseev _et al._, 1986). According to Demir and Ellis (1992), tomato seeds of the third truss reached physiological maturity earlier than those of the first and second truss. Using similar batches, Jalink _et al._ (1999) have shown that seeds of the third truss show lower chlorophyll fluorescence than seeds of the first and second truss upon development and maturation. However, a clear difference in seed quality among trusses could not be determined.

In relation to fruit colour, Valdes and Gray (1998) have demonstrated that the quality of seeds harvested from red fruits was higher compared with those from mature green and overripe fruits (dark red). However, Chaudhari _et al._ (1992) and Baruah _et al._

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(1996) found that maximum seed quality was attained in fully ripe-red fruits. Moreover, Dharmatti *et at.* (1989) showed that nutrition during plant growth influenced both the stage at which maximum germinability was attained and the change of fruit colour.
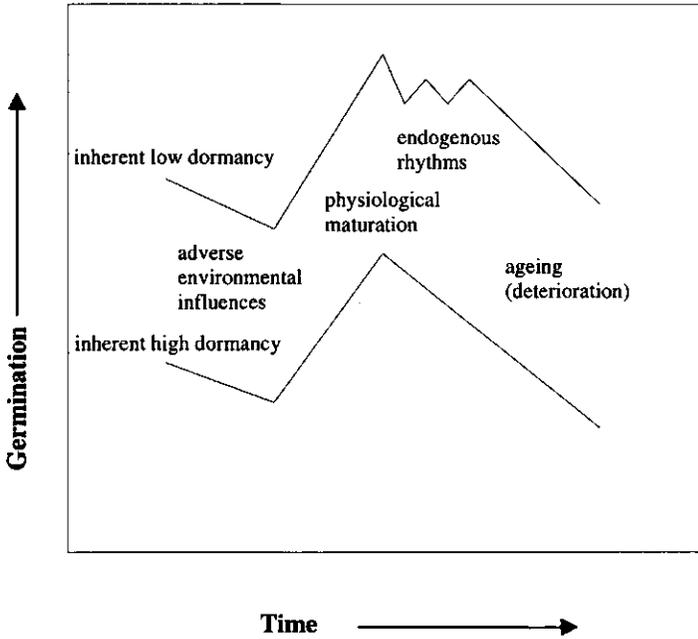
Fig.1. *Hypothetical diagram of effects of inherent and environmental factors on germination and performance of seed (Maguire, 1977).*

Chlorophyll in seeds

The development of the Laser Induced Fluorescence (LIF) technique by Jalink *et. al.* (1996), which is able to measure chlorophyll in seeds instantaneously and non-destructively, has initiated attempts to explore the role of chlorophyll in seed quality. The high sensitivity of this technique makes it possible to assess the maturation level of individual tomato seeds based on their chlorophyll level and sort the seeds into different classes of chlorophyll content. Based on this relatively novel approach, it is supposed

that chlorophyll fluorescence of the seed can be harmonised with other established physiological parameters to further identify seed quality components.

Commonly, the disappearance of chlorophyll is one of the criterions that crops are ripening. For seeds, the disappearance of the green colour is not widely used as a criterion for the maturation stage, except for rapeseed in view of the importance of removing green seeds in the oil industry. The influence of chlorophyll pigments on oil quality has been extensively studied, and from these studies it has become apparent that chlorophyll in the seed is degraded during maturation. However, only little information is available about the possible roles of chlorophyll in seed development, maturation and germination. In carrot, geranium and soybean seed, chlorophyll content of the seed is negatively and linearly related to seed germination (Steckel *et al.*, 1989; Kwong, 1991; Illipronti, 1997), but this relationship is still unexplained.

In cabbage, seed quality increased as the signal of the chlorophyll fluorescence in the seed coat decreased (Jalink *et al.*, 1998). However, experiments on tomato seeds sorted by LIF into several classes of chlorophyll content showed that maximum seed quality was attained when the chlorophyll fluorescence reached a near-minimum. Subsequently, both seed quality and chlorophyll fluorescence appeared to decrease further (Jalink *et al.*, 1999). This disappearance of chlorophyll might be important because chlorophyll is a primary source of singlet oxygen ($^1O_2$), a very powerful oxidising agent (Thomson *et al.*, 1987). This free radical formation is an overflow mechanism, allowing the chlorophyll to dispose of electrons. Therefore, the low germination of matured green seeds may be caused by oxidative deterioration by free radicals that leads ultimately to cell death.

The functionality of chlorophyll in the seed

Little is known about the function of chlorophyll in the seed. Quebedeaux and Chollet (1975) found that the chlorophyll concentration in soybean (*Glycine max*) pod declined steadily with increasing seed weight between anthesis and seed maturation. Sugimoto *et al.* (1987) showed that developing cotyledons of immature soybean seeds

have photosynthetic activity. They also suggested that the intensity of light that reaches the chloroplasts is quenched due to the accumulation of storage substances during maturation, resulting in a gradual loss of photosynthetic activity. The function of chlorophyll in the pod has been investigated by Andrew and Svec (1975). They found that, although the gross photosynthetic rate of the pods per gram fresh weight was slightly lower than that of the leaves, much higher chlorophyll concentrations occurred in the leaves and, thus, the photosynthetic rate in pods was greater than in leaves when expressed on a mg chlorophyll basis. The increasing rate of gross photosynthetic rate on a chlorophyll basis, observed as pods increase in size, may be important during the rapid seed-filling period. Bewley and Black (1994) stated that in some legumes, e.g. certain cultivars of soybean and field and garden pea, the translocated sucrose produced by photosynthesis in the leaves and pods may be stored temporarily as starch in the pod prior to remobilisation and transfer to the developing seeds.

The photosynthetic activity within seeds can be measured by means of pulse amplitude modulation (PAM) fluorimetry. In 1986, the PAM fluorometer was introduced, which can be used for a thorough analysis of fluorescence in comparison with other photosynthetic signals. The analytical power of this instrument has been further increased by the development of a P700 probe which can be operated in conjunction with the fluorometer (Schreiber *et al.*, 1988). Schreiber *et al.* (1996) reported on a new measuring system, based on a modified PAM fluorometer, which is capable of detecting chlorophyll fluorescence with an extremely high accuracy and enabling a high spatial resolution within leaves using a fiber-optic microprobe. These specifications made this system particularly useful in the present study, considering the dimensions of the tomato seed.

Ultrastructural changes during development and maturation

In order to understand more clearly the significance of the presence of chlorophyll in the seed, several microscopic studies have been carried out concentrating on special stages of seed development. Saio *et al.* (1985) reported that at about 40 days

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after flowering (DAF) in soybean seeds the number of chloroplasts was maximal. After 45 to 50 DAF, chloroplasts rapidly decreased in number, accompanied by the disappearance of the starch grains and the small oil droplets near the vacuole-like structure. Johnson-Flanagan and Thiagarajah (1990) showed that canola embryos at 65% moisture content contained mature chloroplasts that were oval and could be characterised by the presence of large starch grains. The thylakoids were depressed to the outer membrane by the starch, but both granal and stromal thylakoids could be identified. As the moisture content decreased there was an increase in the liquid bodies and a loss of starch from the chloroplasts. Embryos at 56% moisture content contained plastids with lipid bodies and a vestigial inner membrane system. Mature non green seeds did not contain such chloroplasts.

Electron microscopy revealed an abnormal chloroplast ultrastructure in ABA (abscisic acid) mutants (*aba3, aba1, and aba4*) of *Arabidopsis thaliana*. The mutants contained significantly more chloroplasts per cell than the wild type, as well as significantly more grana stacks per chloroplast, bus less lamellae per granum (Rock *et al.*, 1992). In mature soybean seeds (cv. Clark and Dare) which exhibited seed degreening, no internal membrane structures were observed. In contrast, the plastids observed in two chlorophyll-retention lines (*dld2* and *cyt-G1*) had large, stacked thylakoid membranes (Chao *et al.*, 1995).

Chlorophyll degradation

Several researchers have studied chlorophyll degradation during development of canola seeds. Johnson-Flanagan and Thiagarajah (1990) demonstrated that chloroplast ultrastructure changed and degradation of chlorophyll-protein complexes occurred as the seed moisture content decreased and the seed degreened.

The chlorophyll degradation process involves two types of reaction (Hendry *et al.*, 1987):

a) Type I degradation includes the loss of magnesium and phytol, and modifications of the side chains of the chlorophyll's tetrapyrrole structure yielding phaeophytins and

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phaeophorbides due to the action of up to five enzymes. The sequence in which these enzymes act on the chlorophyll is still unclear.

b) Type II degradation involves the cleavage of the macrocyclic ring system and subsequent degradation to smaller carbon/nitrogen fragments. The type II reactions require both light and oxygen.

Effect of abiotic stresses on chlorophyll degradation

A number of abiotic stresses may interfere with the breakdown of chlorophyll. Wards *et al.* (1992) reported that the chlorophyll content decreased as seeds matured, and that the rate was affected by temperature. A low rate of chlorophyll degradation occurred at low temperatures during the ripening period. Johnson-Flanagan *et al.* (1994) showed that humidification of canola seeds at 97% relative humidity for 10 d had the potential to decrease total pigment content to 25%.

The mechanism of light action in controlling leaf senescence is still unclear, since light has been known either to stimulate or retard this process (Biswal and Biswal, 1984). In leaves, chlorophyll was degraded rapidly when kept in total darkness. The loss of chlorophyll was well correlated with the breakdown of chlorophyll-carrying proteins. The breakdown of chlorophyll was strongly retarded by continuous illumination with white light of intensities as low as 0.5 μmol photon.$m^{-2}.s^{-1}$, but at an intensity of more than 10 μmol photons. $m^{-2}.s^{-1}$ the retardation of chlorophyll breakdown was decreased (Okada *et al.*, 1992).

Effect of hormones on chlorophyll degradation

In leaves, growth regulators (ethylene, auxin and ABA) increase chlorophyllase activity, the enzyme which is involved in chlorophyll degradation, whereas others (cytokinins and GA) inhibit it (reviewed by Drazkiewicz, 1994). Although in general GA acts as a senescence retarding hormone (Abeles *et al.*, 1989, reviewed by Drazkiewicz, 1994), Rodriguez *et al.* (1987) showed that GA_3 increased chlorophyll degradation, possibly by the induction of other enzymes such as peroxidase,

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lipoxygenase or chlorophyll oxidase. It is also possible that the enhancement of chlorophyll breakdown by GA_3 is due to an increment in accessibility of the enzymes involved in chlorophyll degradation to their respective substrates.

Seed chlorophyll breakdown and ethylene evolution were positively correlated during seed ripening, but higher ethylene levels did not appear to control the rate of chlorophyll breakdown (Ward *et al.*, 1995). Johnson-Flanagan and Spencer (1996) reported that chlorophyllase activity is not affected by ethylene in a direct manner.

Hormone mutants

In Arabidopsis seeds severe ABA-insensitivity correlates with the absence of chlorophyll breakdown during seed maturation. One mutant (*abi3*) remains green during maturation, and is characterised by a reduced desiccation tolerance and/or longevity (Leon-Kloosterziel, 1997). The embryo of the *en3* mutant (an allele of *ABI3*) remains green throughout seed development and has a large reduction in seed reserve protein. The seeds are desiccation intolerant, and the M3 generation of these seeds is dark green shrivelled phenotypically (Nambara *et al.*, 1992).

It was shown that chlorophyll content in the fruits and leaves of monogenic *hp-1* tomato mutants (exhibiting exaggerated phytochrome response) is higher than in the wild type. The *au* mutant has less chlorophyll in leaves and fruits compared to wild type (Kerckhoffs, 1996). These mutants may also have different levels of chlorophyll in the seed, which makes them valuable in the present study.

Laser Induced Fluorescence

Jalink *et al.* (1996) designed the prototype of the Laser Induced Fluorescence (LIF) equipment to measure and analyse chlorophyll fluorescence in the seed instantaneously and non-destructively (Fig. 2). The use of LIF makes it possible to perform physiological and biochemical assays after chlorophyll fluorescence measurement in the same seeds. By a combination of lighting technique (laser diode, Coherent), narrow bandwidth filters (half bandwidth of 10 nm; Edmund Scientific),

sensitive photodiodes (UDT sensors, PIN-10DP) and phase sensitive detection (lock-in amplifier; Stanford Research SR 830), chlorophyll *a* can be excited and measured sensitively. For seed chlorophyll fluorescence analysis, the chlorophyll fluorescence signal of the lock-in amplifier was fed into a computer to obtain a frequency histogram. The computer is also controlling the ejector mechanism for sorting seeds according to the intensity of the fluorescence. The ejection process is regulated by a pulse of air, depending on the threshold value of the chlorophyll fluorescence signal.

The choice of tomato as a model plant

Tomato (*Lycopersicon esculentum* Mill.) seed is a convenient research object because of its simple structure. Its size allows for easy manipulation and dissection (Hilhorst *et. al.*, 1998). Mutants of tomato are available, which makes it possible to better understand the roles of hormones and phytochrome in the degradation of chlorophyll. In addition, being an economically important crop, tomato is normally reproduced by seed. The availability of various cultivated and wild Lycopersicon accessions may give extra information on the degradation of chlorophyll.

Aims and frame of the thesis

The present thesis aims at explaining the presence of chlorophyll in tomato seed during development and maturation by studying the relation between chlorophyll synthesis and breakdown and seed performance. Two hypotheses are tested; the first is that active chlorophyll in young tomato seed is positively correlated with seed filling and, thus, that the amount of chlorophyll in young seed is correlated with increased seed performance. The second hypothesis is that high chlorophyll content near the end of maturity will result in low seed performance due to the involvement of free radicals.

Some preliminary results on chlorophyll degradation during development and maturation and its relation to seed performance are presented in **Chapter 2**. To study the presence and the functionality of chlorophyll in tomato seeds, we applied a number
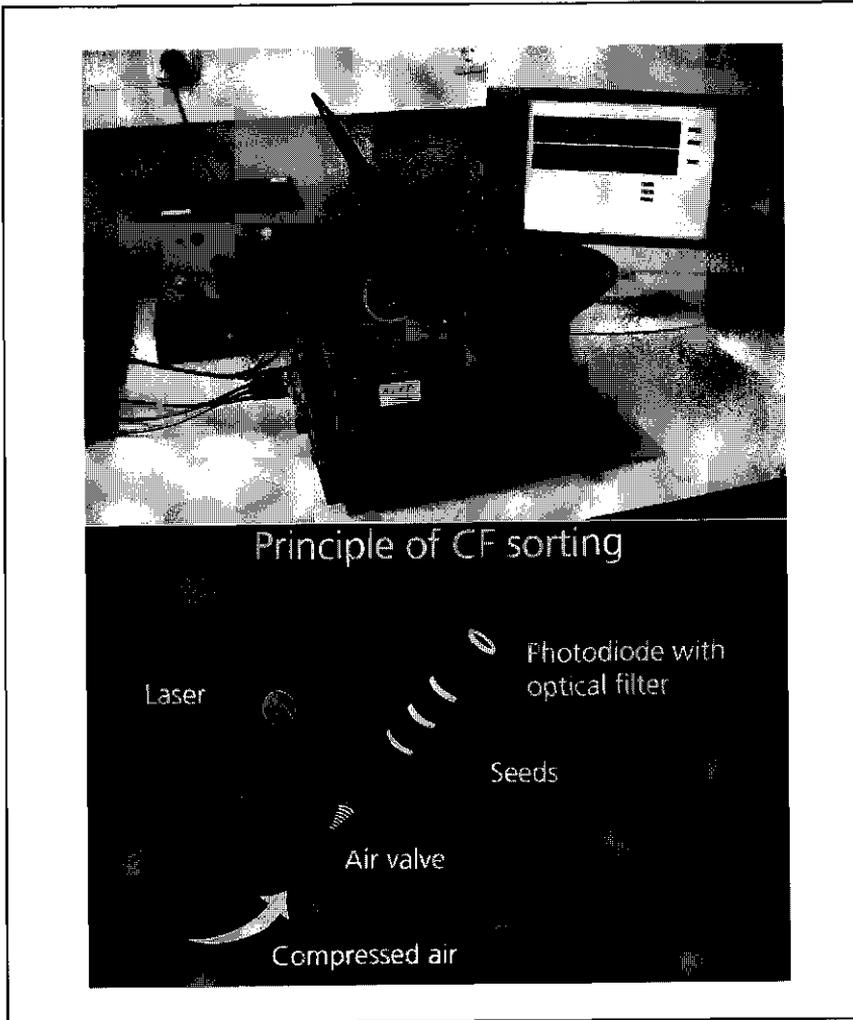
Fig. 2. The prototype of the laser induced fluorescence equipment (LIF) and its principle sorting mechanism

of photosynthetical approaches as described in **Chapter 3**. In addition, genotypes with different fruit sizes were compared in relation with seed chlorophyll content and photosynthetic activity during development and maturation.

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Based on this comprehensive information, we attempted in **Chapter 4** to manipulate the chlorophyll level in tomato seeds by covering fruits during the growing period, and correlated this to seed performance. **Chapter 5** deals with the influence of endogenous ABA, GA and phytochrome on seed chlorophyll content and performance, using some mutants (*sit^w^*, *gib1, au* and *hp-1*). Finally, to test the hypothesis that the level of chlorophyll in mature seeds is related to oxidative damage, we performed controlled deterioration experiments, which are described in **Chapter 6**. The general discussion **(Chapter 7)** presents our view of the role of chlorophyll in tomato seed in relation to seed performance.

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Chapter 2

Chlorophyll fluorescence as a biophysical marker for physiological
maturity of tomato (*Lycopersicon esculentum* Mill.) seeds

Mohamad R. Suhartanto, Peter E. Toorop, Henk Jalink, Raoul J. Bino and Henk W.M.
Hilhorst

Abstract

Chlorophyll fluorescence was studied as a parameter for physiological maturity of tomato seeds. Maximum germination and maximum percentage of normal seedlings was achieved at 51-54 days after flowering (DAF) when chlorophyll fluorescence of fresh and dried seeds, as well as seed chlorophyll content, reached a minimum. Between 39 and 54 DAF, chlorophyll content and fluorescence of the seeds was negatively correlated with germinability (maximum germination and normal seedling percentage). Chlorophyll fluorescence of the seeds decreased earlier than chlorophyll fluorescence of the fruits. There were two phases of chlorophyll degradation in tomato fruit, the first phase occurred between 21-36 DAF and the second phase between 48-60 DAF. The onset of the plateau period (39-42 DAF) coincided with the start of the period that seeds became germinable. Fruit fluorescence and photosynthetic yield reached a minimum at 57-60 DAF, 3 days after seed physiological maturity. At this stage fruit colour has turned to red (hue angle 70-80 degrees).

Fluorescence microscopy showed that the majority of chlorophyll is located in the seed coat, whereas chlorophyll fluorescence imaging (CFI) analysis located low levels of chlorophyll in the embryo, mainly in the radicle tip. Chlorophyll fluorescence of both fresh and dried seeds as well as fluorescence of the fruit appeared to be sensitive indicators of physiological maturity of tomato seeds.

Key words

Introduction

Information about seed maturity is essential when studying seed physiology. Seeds from different maturation stages may display different biochemical activities, which may lead to different physiological behaviour (Liu, 1996). It has been widely reported that the maximum seed dry weight is a reliable parameter to detect physiological maturity in some species, guaranteeing maximum quality (Rasyad *et al.*, 1990; Valdes and Gray, 1998). This hypothesis has been the subject of some controversy as Demir and Ellis (1992) reported that maximum seed quality occurred after maximum seed dry weight was reached. Fruit colour has also been used as a parameter to detect seed quality. Using this qualitative parameter, Valdes and Gray (1998) showed that tomato seeds from red fruits yielded a higher germination performance than seeds from mature green or overripe (dark red) fruits.

In most cases, the degradation of chlorophyll in fruits and seeds is related to the maturation process. Maturation of the tomato embryo was found to be completed at 40 days after pollination when maximum embryo protein content, size and seed dry weight was attained (Berry and Bewley, 1991). In this state, the fruit colour was still green-orange, while the seed testa had already turned brown. Kwong (1991) found that green seeds of geranium (*Pelargonium x hortorum*) did not germinate well, but most of the excised embryos germinated normally when placed on a nutrient medium. Steckel *et al.* (1989) have also investigated the relationship between chlorophyll content and germination in carrot (*Daucus carota*) seed, and showed that the percentage of germination was negatively and linearly related to seed moisture content, and chlorophyll a+b content in the seed coat. It was suggested that a chlorophyll test using colour cards may give equally good prediction of the optimum harvest date of carrot seed as a germination test. Unfortunately, seeds from different maturation stages are very difficult to remove from a batch since they usually are similar in shape, size and mass. Jalink (1996) developed a method to measure chlorophyll fluorescence of seeds by means of light induced fluorescence (LIIF) and laser induced fluorescence (LIF; Jalink *et. al.,* 1999), and created a fluorescence separator which made it possible to

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distinguish maturation levels of tomato seed batches based on the chlorophyll level of the seed. Jalink *et al.* (1998) also showed that LIIF could be used to improve the quality of a *Brassica oleracea* seed batch by identifying and removing less mature seeds from the batch. Thus, chlorophyll fluorescence has been used as a non-destructive marker for seed quality.

Recently, Jing *et al.* (2000) showed that chlorophyll fluorescence appeared to be an appropriate marker for optimal harvesting of cucumber seeds. In the present study we measured the chlorophyll fluorescence and content of tomato seeds during development and maturation using LIF, and assessed its relationship with physiological maturity. Similarly, the quantitative changes in fruit fluorescence and fruit colour were related to physiological maturity. The localisation of chlorophyll in tomato seed was also investigated using both fluorescence microscopy and chlorophyll fluorescence imaging (CFI) analysis, a new technique to detect chlorophyll in seeds.

Materials and methods
Plant material

Tomato plants (*Lycopersicon esculentum* Mill. cultivar Moneymaker) were grown in a greenhouse under natural daylight at an average temperature of 25°C/20°C day/night. Self-pollinated flowers were tagged every 3 days, and seeds were harvested after 21, 24, 27, etc. until 75 DAF.

Chlorophyll fluorescence measurement
Fluorescence of fresh seeds

The XE-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) was used to measure fluorescence of fresh seeds. Samples of 12 seeds, which covered a 1 cm^2 area within the cuvet (3 replicates of 12 seeds each), were excited repetitively at frequencies of 4 Hz by μsec light pulses from a Xenon lamp light. The excitation light passed through a short-pass filter (2 x 3 mm BG 39) to eliminate the long wavelength component. Long-pass filters, 1 x R 65 (Balzers) + 1 x RG645 (Schott), in front of the

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photodiode prevented stray measuring light reaching the detector. The dark fluorescence (F_0) was used as the parameter for fluorescence of fresh seeds (Schreiber *et al.*, 1993).

Fluorescence of dry seeds

Seeds with different levels of maturity were dried over saturated $CaCl_2$ for 4 days at 20 °C and 32% RH (moisture content 6.5% ± 0.1, fresh weight basis) and stored in a moisture-proof container at 2-3°C until further use. The fluorescence of individual dry seeds was measured with LIF using a combination of red diode laser and narrow bandwidth filters as described by Jalink *et al.* (1999). The chlorophyll fluorescence was calculated as the average of 500 to 1500 seeds at each maturation stage.

Fruit fluorescence and photosynthetic activity

Chlorophyll fluorescence of fruits with different levels of maturity was measured with LIIF (Jalink *et al.*, 1998). This method uses the unique property of chlorophyll of showing fluorescence at 730 nm when excited by light of the proper wavelength (670 nm). At each level of maturation the fluorescence of ten fruits was measured randomly on 10 diagonal locations of each fruit. Additionally, photosynthetic activity of these fruits was measured with the MINI-PAM (Heinz Walz GmbH, Effeltrich, Germany) after keeping tomato fruits in the dark for at least 10 minutes (dark-adapted samples), according to the manufacturers manual. Photosynthetic activity expressed as yield parameter (quantum yield of photochemical energy conversion) was calculated by:

$$\text{Yield} = (F_m - F_0)/F_m$$

with: F_m = maximal fluorescence yield

F_0 = dark fluorescence

spectrophotometer. Using this method, we observed the relative light transmission through fruit pericarp tissue at wavelengths ranging from 450 nm to 900 nm at 3 stages in fruit development and maturation (40 DAF, 45 DAF and 57 DAF). Using the same fruits (plus 75 DAF), a lightmeter (Model LI-189, LI-COR, USA) was used to quantify white light transmission through 0.5 cm thick fruit pericarp tissue.

Fluorescence microscopy

The localisation of chlorophyll in tomato seed was studied using fluorescence microscopy. Fresh seeds at 40 DAF were hand-sectioned longitudinally with a razor blade and seed coat tissues were carefully separated from endosperm and embryo tissues. Observations were made with a Nikon Labophot epifluorescence microscope equipped with 100 W Hg lamp and FITC filter. Images were made with Sony 3-CCD video camera (10X FL 0.5 NA) model DKR 700 P.

Chlorophyll fluorescence imaging

A comparative study on the localisation of chlorophyll in young tomato seed was carried out using CFI. Chlorophyll was excited as described previously by Jalink *et al.* (1998). Detection was done with a cooled CCD camera with a Sony ICX084AL chip linked to a computer and analysed with software developed by R. van der Schoor (personal communication).

Results

Seed

Fresh and dry seeds showed a similar trend in their chlorophyll fluorescence. The chlorophyll fluorescence decreased sharply between 30 and 51 DAF and remained constant afterwards (Figs. 1a and 1b). Seed chlorophyll content decreased sharply from 21 until 51 DAF; after 51 DAF the decrease was slow but distinct (Fig. 1c and inset).

Maximum seed dry weight in our experiment was achieved at 45 DAF and maintained constant thereafter (Fig. 1d). Achievement of maximum seed dry weight was 6 d before germinability and percentage normal seedlings attained their maximum.

Seeds became germinable from 42 DAF and a maximum germination was reached between 51 DAF and 54 DAF (Fig. 1e). The maximum percentage of normal seedlings was achieved at the same developmental stage, although some fluctuation was observed afterwards (Fig. 1f). The onset of germinability and achievement of full germinability were delayed after a CD test, and this delay was similar for the percentage normal seedlings. Maximum germinability was still achieved after 54 DAF, upon which seed vigour decreased and some fluctuation occurred (66-75 DAF; Figs. 1e and 1f). Percentage germination, between 39-54 DAF, was negatively correlated to fresh seed fluorescence, dry seed fluorescence and seed chlorophyll content (Fig. 2a and 2b). The same correlation was found between percentage normal seedlings and fresh seed fluorescence, dry seed fluorescence and seed chlorophyll content (data not shown).

Germination rate (T_{50}) and uniformity (T_{75-25}) were lower upon a CD test of mature seeds. Before the CD test, from 54 DAF onward, germination rate and uniformity remained more or less constant, whereas after a CD test germination was slower (or T_{50} increased) transiently (Fig. 3). A correlation was found between the level of germination and the percentage normal seedlings ($r^2 = 0.84$, not shown). Although a similar trend was found with germination rate, no significant correlation was found between either percentage germination or the percentage normal seedlings and germination rate ($r^2 = 0.28$ and $r^2 = 0.39$ respectively, not shown). Thus, the fluctuation of seed quality between 66 and 75 DAF could not be proven conclusively.

Fruit

Fruit fluorescence declined gradually until 36 DAF, levelled off between 36 and 48 DAF, and declined rapidly between 51-54 DAF. From 60 DAF onward, fruit fluorescence could not longer be detected (Fig. 4a). Until 51 DAF fruits were still

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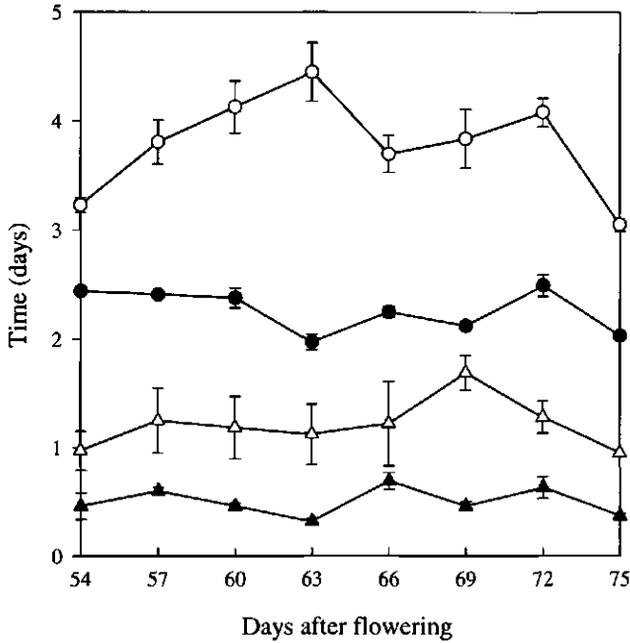
Fig. 3. *Seed vigour expressed as T_{50} (circles) and T_{75-25} (triangles) between 54 DAF and 75 DAF before (closed symbols) and after (open symbols) a CD test.*

green (hue angle approximately 115°); between 54 and 57 DAF the colour turned to red (hue angle reaching 70-80°). After 60 DAF the dark red colour (hue angle 60°) remained unchanged (Fig. 4b).

Fruit photosynthetic activity remained high until 48 DAF, and began to decrease at 51 DAF. Fruit photosynthesis dramatically decreased until 57 DAF and decreased further to negligible values afterwards (Fig. 4c). The maximum light transmission of 0.5 cm pericarp tissue (approximately 10-11%) was reached around 57 DAF (Fig. 3d). Additionally, transmission of light between 650-700 nm clearly increased when fruit developed from an immature (40 DAF) to a mature stage (57 DAF; Fig. 5).

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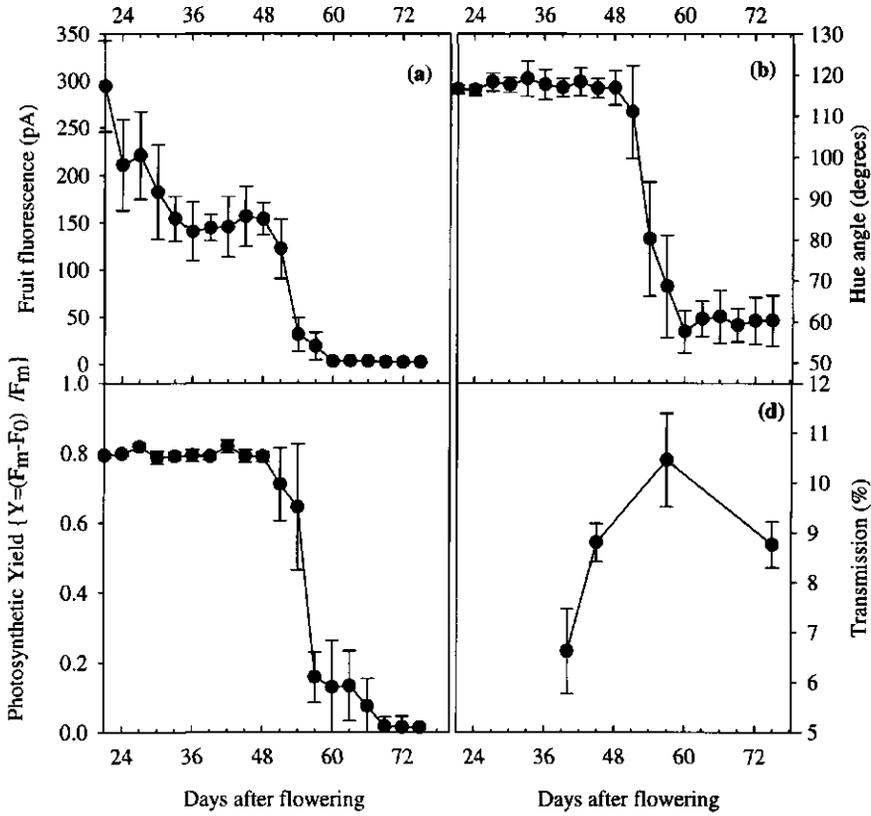
Fig. 4. *Changes in tomato fruit fluorescence (a), tomato fruit colour (b), tomato fruit photosynthesis (c), and percentage of light penetration through 0.5 cm tomato pericarp tissue (d) during development and maturation. Values are means $\pm$ sd (n $\geq$ 3)*

quantitative determination of high fluorescence values, since a fast drop in chlorophyll content 21-30 DAF is not paralleled in the determination of fluorescence (Figs. 1a, 1b and 1c). Moreover, a linear and negative correlation between chlorophyll fluorescence or content of the seed and its germinability could be seen in the period between 39 DAF and 54 DAF (Figs. 2a and 2b). The onset of germinability took place after 39 DAF and germinability was maximal between 51 and 54 DAF (Fig. 1e and 1f). Maximum seed dry weight, a common parameter for seed physiological maturity, was achieved at 45 DAF, 6-9 d before maximum germinability. This result shows that chlorophyll fluorescence can be used as a good marker for physiological maturity of tomato seed.

Based on germination percentage and percentage normal seedlings, seed vigour declined somewhat after 54 DAF, whereas germination rate (T_{50}) uniformity (T_{75-25}) remained largely unchanged. This confirms the finding of Valdes and Gray (1998) that tomato seeds show a decline in seed quality after the achievement of maximum quality in overripe tomato fruits. Demir and Ellis (1992) found an increase in seed quality after maturation in overripe fruits. However, they did not break dormancy before testing. Tomato seeds lose dormancy when left in overripe fruits.

Both a chilling and a KNO_3 treatment were applied to all of the maturation stages in our experiments, so that dormancy could not interfere with the results. Most of the seeds that did not germinate were dead. We can not exclude a possible role of desiccation intolerance during seed drying, since we did not germinate fresh seed in this experiment. However, Demir and Ellis (1992) showed that desiccation tolerance is not involved, since drying before testing increased the germination ability of tomato seeds during development. Valdes and Gray (1998) also found that the final percentage of normal seedlings from fresh and dried seeds showed very similar responses to changes in fruit maturity and seemed not to be significantly different.

There were two phases of chlorophyll degradation in tomato fruit. The initial decrease (Fig. 4a, 21-36 DAF) might be caused by a dilution of the constant amount of chlorophyll in the growing fruit, whereas the second decrease (48-60 DAF) was

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probably caused by net chlorophyll degradation which corresponded to the fast drop in photosynthetic activity (Fig. 4c). The fastest growth of tomato fruit occurred between 20 and 40 days after anthesis (Liu *et al.*, 1996). During the plateau period (36-48 DAF) germinability of seeds started to develop (Figs. 1e and 1f) and achieved its maximum at 54 DAF, 3d before fruit fluorescence disappeared and photosynthetic activity became negligible. In this stage (57 DAF), fruit colour had turned to red (hue angle reaching 70-80 degrees; Fig. 4b). These simple and visible parameters may be valuable for quick prediction of seed quality. Valdes and Gray (1998) also reported that seeds with optimum quality were harvested from red tomato fruits.

The decrease in seed chlorophyll fluorescence occurred earlier than that of fruit chlorophyll fluorescence, before the fruit colour turned red and photosynthetic activity became negligible (Figs. 1a, 1b, 4a, 4b and 4c). Figure 4d shows that the amount of light which was able to reach the seeds increased from 40 DAF until 57 DAF. This increase of transmitted light might accelerate chlorophyll breakdown of the seeds during maturation resulting in a high germination performance (Figs. 1e, 1f and 2).

Using fluorescence microscopy, chlorophyll appeared to be located in the seed coat of immature seeds only (Figs. 6a and 6b). At 60 DAF, chlorophyll in the seed coat could no longer be detected (data not shown). The decrease in chlorophyll fluorescence in the mature seed coat seemed to be correlated with cell death in the testa (Werker, 1997; Berry and Bewley, 1991). Using CFI, a far more sensitive method, we were able to detect chlorophyll in the embryo, specifically in the area of the radicle tip (Fig. 6d). Still, this chlorophyll fluorescence in the radicle tip is of minor importance compared to the chlorophyll fluorescence in the testa (Fig. 6c).

The question that remains is; what is the role of chlorophyll in developing seeds? Presumably, the contribution of seed photosynthesis to seed filling is low, compared to that of fruits and leaves (Chauhan and Pandey, 1984). Up to 11% of the light passed through 0.5 cm of pericarp tissue when fruits were irradiated with white light. From this we may conclude that light can reach the developing seeds (Fig. 4d). This light penetration might explain the presence and activity of chlorophyll (Chapter

al., 1993). Photosynthetic activity expressed as yield parameter (quantum yield of photochemical energy conversion) was calculated by:

$$Yield = (F_m-F_0)/F_m$$

with: F_m = maximal fluorescence yield

F_0 = dark fluorescence (minimal chlorophyll *a* fluorescence)

Rubisco activity

A spectrophotometric assay was performed based on the reduction of D-3-PGA formed in the carboxylation reaction by the combined action of phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase in which the reaction velocity is measured by directly recording the NADH consumption as a decrease in absorbance at 340 nm in a spectrophotometer (Di Marco and Tricoli, 1983, with slight modifications). The fresh seeds (0.33 g, equal to 29 seeds of 30 DAF, 27 seeds of 40 DAF or 44 seeds of 50 DAF) were homogenised in a mortar in 2 ml of ice cold extraction buffer (pH 8.2) containing 0.1 M Tris-HCl, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM β-mercaptoethanol, 0.5 mM EDTA and 2% PVP. After centrifugation at 15000 rpm for 15 min, the supernatant was taken for the carboxylation reaction. For the carboxylation reaction at 25°C, 100 µl of 0.5 µM RuBP in 1 ml of the reaction buffer minus $NaHCO_3$ was added to the supernatant The reaction was stopped after 1 min by adding 100 µl 1 N HCl and neutralised with 100 µl 1 N NaOH. To this mixture was added 85 µl 4 nM NADH, 125 µl 50 mM ATP, 125 µl 50 mM phosphocreatine and 125 µl creatine phosphokinase. After recording the absorbance at 340 nm of this solution against a blank containing the same amount of NADH, 25 µl phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase were added and the absorbance read again after 30 min.

Starch content

Tomato seeds (3 x 20 seeds of 30 DAF, 3 x 15 seeds of 36 DAF and 2 x 10 seeds of 40 DAF - 75 DAF) were ground in liquid nitrogen, and the powder was mixed with 40% (v/v) methanol at a powder to solvent ratio of 1:10 (w/v) and shaken for 2 h. The extraction was repeated four times at room temperature (25-28 °C) until a polyphenol-free powder was obtained (Sripad and Rao, 1992). To remove glucose, the sample was heated in 80% (v/v) methanol to 76 °C for 15 min. Remaining methanol was evaporated in a Speedvac for 2 h. After washing 3 times with 1.0 ml of water, the pellet was used for starch analysis. Solubilization of the pellet was done by adding 20 μl 8N HCl and 100 μl DMSO to the sample which was then placed in a water bath of 60° C for 90 min. 150 μl water, 40 μl 5N NaOH and 185 μl citrate buffer (pH 4.6) containing 0.1 M citric acid and 0.2 M $Na_2HPO_4.2H_2O$ were added to the samples, after which they were centrifuged at 14 000 rpm for 5 min. The supernatant was analysed enzymatically using a starch assay kit (Boehringer Mannheim). Starch content was determined based on seed dry weight after calculation of the starch concentration according to a standard curve.

Oxygen evolution

Photosynthetic oxygen evolution was measured with a Hansatech oxygen electrode (Hansatech Ltd., King's Lynn, UK). Oxygen evolution of developing seeds at 30 DAF (88 seeds), 40 DAF (93 seeds) and 50 DAF (106 seeds) was measured at 25°C at different light intensities, ranging from 0 to 200 $μmol/cm^2/s$.

Chlorophyll fluorescence imaging

A study of the localisation of chlorophyll in developing tomato seeds was carried out using CFI. Chlorophyll was excited as described previously by Jalink *et al.* (1999). Detection was done with a cooled CCD camera with a Sony ICX084AL chip linked to a computer and analysed with software developed by R. van der Schoor.

among wild type, *hp-1* and *au* (Figs. 3A and 7A). The minimal chlorophyll fluorescence of the seeds was influenced by mutations in the phytochrome pathway as shown with the *hp-1* and *au* mutants. The phytochrome chromophore mutant *au* seeds exhibited the lowest chlorophyll fluorescence during seed development. Compared with the wild type, the peak of chlorophyll fluorescence of *hp-1* seeds occurred later during development, which might be caused by a slower development (Figs. 3B and 7B).

Seed photosynthetic activity of the small fruit varieties (Cherry Bush, Cerasiforme, Cherry Yellow and *L. pimpinellifolium*) decreased faster than that of seed of big fruit varieties (Moneymaker, Tropic VF and Fito) during development and maturation (Figs. 8A and 8B). Qualitative and quantitative methods were employed to investigate the presence and degradation of chlorophyll in tomato seeds derived from fruits of different sizes. Qualitatively, chlorophyll fluorescence of embryos of small fruit varieties appeared to decrease somewhat faster than that of embryos of big fruit varieties (Fig. 9). These data were supported by quantitative measurements (Figs. 10A and 10B) which show that seed chlorophyll fluorescence of small fruit varieties decreased mainly between 30 DAF and 50 DAF, whereas seed chlorophyll fluorescence of big fruit varieties decreased between 50 DAF and 60 DAF.

Within small fruit varieties, the cultivated tomato (Cherry Bush and Cherry Yellow) showed a more rapid decrease in photosynthetic activity than seed of wild Lycopersicon accessions (*L. esculentum* var. *cerasiforme* and *L. pimpinellifolium*; Figs. 8B). Especially the chlorophyll fluorescence of *L. cerasiforme* was higher than that of the other small fruit varieties.

Environmental conditions, including light intensity and day length, might also influence the level of chlorophyll in tomato seeds. Tomato seeds of cv. Moneymaker grown in spring exhibited a lower chlorophyll fluorescence during development (Figs. 1, 3B and 10A) than seeds grown in autumn. The maximum chlorophyll fluorescence of the autumn-seeds was shifted to a later stage (50 DAF) of development compared with the maximum fluorescence of spring-seeds (30 DAF) during the developmental period studied.

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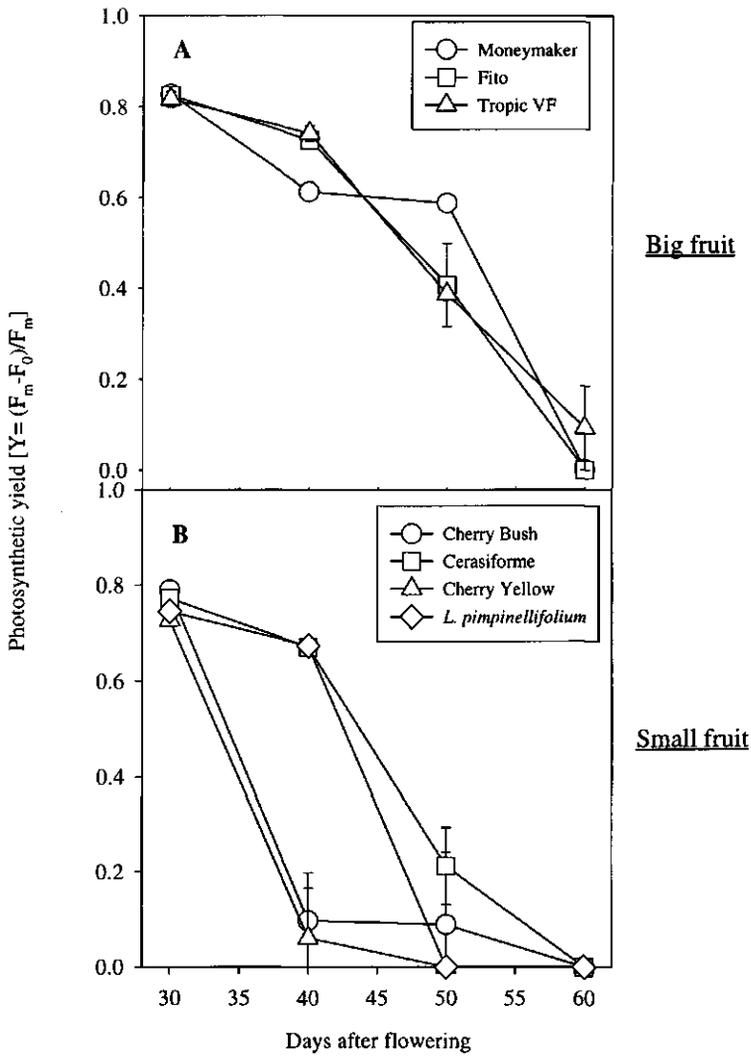
Fig. 8. _Photosynthetic yield of big (A: Moneymaker, Tropic VF and Fito) and small fruit (B: Cherry Bush, Cherry Yellow, Cerasiforme and Lycopersicon pimpinellifolium) varieties during development and maturation. Values are means ± sd (n=3). Seeds harvested in autumn 2000._
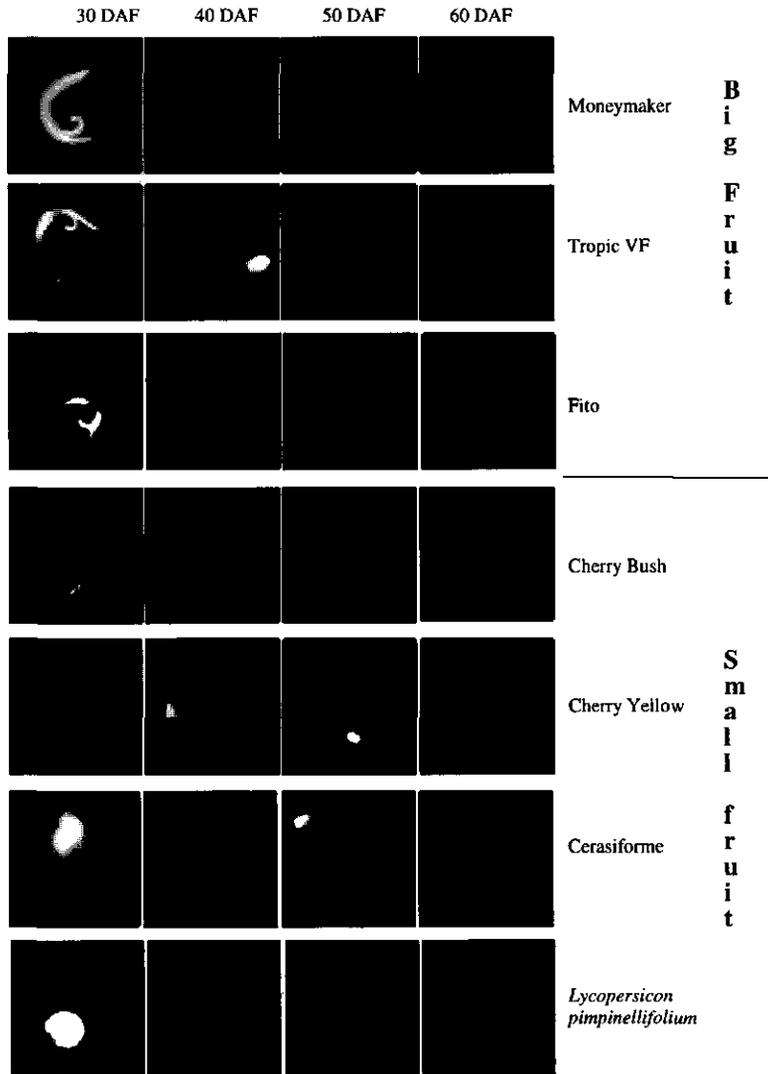
Fig. 9. *Chlorophyll fluorescence images of embryos of big-fruit varieties (Moneymaker, Tropic VF and Fito) and small-fruit varieties (Cherry Bush, Cherry Yellow, Cerasiforme and Lycopersicon pimpinellifolium) during development and maturation. Embryos from seeds harvested in autumn 2000.*
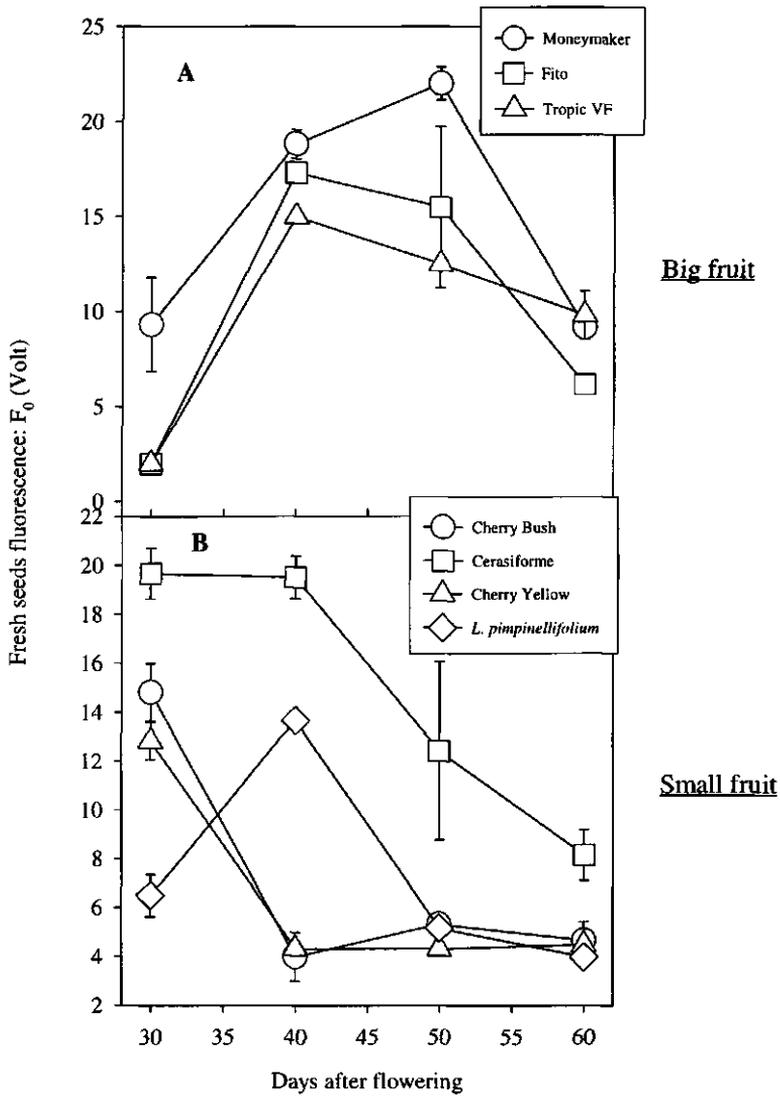
Fig. 10. _Fresh seed chlorophyll fluorescence of big (A: Moneymaker, Tropic VF and Fito) and small fruit (B: Cherry Bush, Cherry Yellow, Cerasiforme and Lycopersicon pimpinellifolium) varieties during development and maturation. Values are means $\pm$ sd (n=3). Seeds harvested in autumn 2000._

Discussion

Using HPLC, it was shown that in young tomato seeds chlorophyll was the predominant pigment. Carotenoid pigments (neoxanthine, violaxanthine, luteine, zeaxanthine and β-carotene) were also present in young tomato seeds but at substantially lower levels (Table 1 and Fig. 2). An important function of carotenoids is to act as photoprotective agents, preventing photo-oxidative damage (Cogdell, 1988; Rau, 1988). It seems likely that these pigments may also protect the developing embryo against harmful effects of radiation.

The majority of chlorophyll is located in the seed coat, but it was also present in low amounts in the radicle tip at the end of seed development (Fig. 9). It appeared that in young tomato seeds chlorophyll is not only present, but is also active as judged from the maximum quantum efficiency (yield) data (Fig. 3A). We may conclude that photosystem II is functional in the chloroplasts of young tomato seeds. The potential to assimilate CO_2 was proven by the slight increase of Rubisco activity between 30 DAF and 40 DAF, coinciding with the transient peak in starch content around 42 DAF (Figs. 4 and 5). We showed that photosynthetic oxygen evolution occurred, resulting in net oxygen evolution at a light intensity of more than 40 $\mu mol.cm^{-2}.s^{-1}$ in 30, 40 and 50 DAF seeds (Fig. 6). In many oily seeds such as legumes, soybean and rape, there is an initial increase in starch content in the cotyledons during development, followed by a decline to a low amount of starch in the mature seed (Bewley and Black, 1994). It appears that starch accumulation and degradation in the tomato seed is similar to other oily seeds. In the later stages of development (older than 40 DAF), when there was a negligible level of Rubisco activity, photosynthetic activity and seed chlorophyll fluorescence (Figs. 3A, 3B, 4 and 5), starch is likely to be mobilised to provide carbon skeletons for oil and protein synthesis (Bewley and Black, 1994).

However, our previous studies showed that less than 11% of the incident white light passed through 0.5 cm of pericarp tissue, and maximum light transmission occurred at 57 DAF when seed chlorophyll content is very low (Chapter 2). It is therefore questionable if there is net photosynthetic activity in developing tomato seeds

that are still in the fruit since respiratory oxygen uptake dominated at light intensities lower than 40 μmol.cm^{-2}.s^{-1}. However, young _Arabidopsis_ seeds require functional chloroplasts for normal embryo development (Apuya _et al.,_ 2000) and chlorophyllous seeds may use the light reactions to generate ATP and NADPH required for the conversion of maternally supplied sucrose to fatty acids used in oil synthesis and storage (Singal _et.al,_ 1987; Asokanthan _et al.,_ 1997). Moreover, seed development of soybean, _Arabidopsis thaliana_ and _Brassica rapa_ is controlled by oxygen (Quebedeaux and Hardy, 1973; Porterfield _et al.,_ 1999). It was concluded that O_2 apparently controls the partitioning between vegetative and reproductive biomass (Quebedeaux and Hardy, 1973).

In the mutant seeds the highest chlorophyll fluorescence was found in _hp-1_ at 40 DAF and the lowest values in the _au_ mutant (Fig. 7B), which has also been shown in the fruits (Kerckhoffs, 1996). The reduction of chlorophyll level in the _au_ leaves had only a limited effect on photosynthetic performance, especially when the plants reached the flowering state (Becker _et al.,_ 1992). It seems that also in tomato seeds phytochrome deficiency mainly influences the amount of chlorophyll, but that the overall photosynthetic yield is not affected. Compared to the wild type, the _au_ mutant has a low dark germination level because it has a phytochrome deficiency (Georghiou and Kendrick, 1991). In our experiments, the _au_ mutant did not germinate after seed drying, even in the presence of GA$_{4+7}$ and KNO$_3$, either in the dark or under continuous red light (results not shown), although a treatment with gibberelic acid (GA$_3$) should result in high germination of this _au_ mutant (Koornneef _et al,_ 1985; Georghiou and Kendrick, 1991). The lack of germination in the present study may be due to dormancy which was not (fully) broken during the short period of storage leading to impaired germination even in the presence of GA. Georghiou and Kendrick (1991) showed that germination of the _au_ mutant in darkness increased with the period of post-harvest storage at laboratory temperature. It is also possible that _au_ mutant seeds have a high ABA content. In the phytochrome chromophore mutant of _Nicotiana plumbaginifolia,_ the amount of ABA in matured seeds was higher than in its wild type, also leading to a low

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Abstract

The chlorophyll fluorescence and dry weight of tomato (*Lycopersicon esculentum* Mill) cv. Moneymaker seeds was reduced when the seeds were harvested from dark-grown fruits. These seeds germinated slower than seeds from control (uncovered) fruits, and produced less normal seedlings than seeds from control (100% light) or from fruits grown under 11.5% of control light intensity. Seeds from dark-grown fruits produced often abnormal seedlings which were stunted in hypocotyl and primary root while their cotyledons remained trapped in the seed coat. During controlled deterioration after 6 months of storage at 2-3 °C there was only a slight decay in germinability and percentage of normal seedlings in the seeds from fruits grown under 100% light. In general, seeds from fruits grown under 11.5% of the control light intensity or grown in the dark showed lower performance than seeds from control fruits after controlled deterioration.

Ascorbic acid levels did not differ among the treatments and increased during controlled deterioration, whereas non-protein thiols strongly decreased. Non-protein thiol content of the seeds from dark-grown fruits and fruits grown at 11.5 % light intensity was slightly lower than that of seeds from the 100% light control during the first two weeks of controlled deterioration.

Storage of green maturing fruits (45 DAF) in white light for 10 d lowered the seed chlorophyll fluorescence, but this did not result in improved germination performance. When green maturing fruits (45 DAF) were stored in the dark for 10 d, germination was improved up to 25% compared to that before storage. Seed chlorophyll degradation was more pronounced when fruit was stored in white light than in darkness, but the amount of chlorophyll was not correlated with seed performance.

Key words

Lycopersicon esculentum, chlorophyll fluorescence, controlled deterioration, ascorbic acid, non-protein thiols, tomato seed germination.

Introduction

Developing seeds of angiosperm plants often contain chlorophyll, which is degraded towards the completion of development. The role of chlorophyll during seed development is poorly understood. Commonly, seeds with a high chlorophyll content by the end of the maturation phase will show a low performance (Steckel *et al*, 1989; Jalink *et al*, 1998).

It is known that chlorophyll synthesis and breakdown are influenced by light (Biswal and Biswal, 1984; Von Wettstein *et al.*, 1995). Liptay and Friesen (1982) showed that tomato plants grown in weed infested plots produced seeds with higher vigour than those from weed-free plots. It was suggested that stress during growth promotes physiological conditions that increase seed vigour. Yanagi *et al.* (1995) demonstrated that ascorbic acid and reducing sugar contents of tomato fruit decreased with increased shading. Moreover, Baevre (1990) showed that yield, fruit-set and seed weight were reduced significantly by shading. Recently, we found that young tomato seed contains chlorophyll of which the majority is located in the seed coat. Furthermore, we showed that chlorophyll is also present in the embryo and is maintained in low amounts in the radicle tip towards the end of seed development (Chapter 2). However, only little information is available about the possible role of chlorophyll in seed development, maturation and germination.

In seeds of some weedy species, chlorophyll in the investing structure (the structures surrounding the seeds) reduced the R:FR ratio of sunlight and induced light requirement for germination. Thus, when seeds mature within the green tissues they would have most of their phytochrome in the inactive (Pr) form, and require light to stimulate germination (Cresswell and Grime, 1981). Derkx and Karssen (1993) showed that sensitivity to light was high in Arabidopsis seed lots harvested between September and March and significantly lower in those harvested between April and June. Currently, we found that tomato seed chlorophyll content during development and maturation was lower when seeds were grown and harvested in the spring compared to the autumn (Chapter 3). Although Derkx and Karssen (1993) stated that it is not likely

that the photoperiod could account for the differences in light requirement of the seeds, we still assume that environmental factors such as the spectral quality of light during seed maturation may influence seed germination, as reported previously (Hayes and Klein, 1974; McCullough and Shropshire, 1970). Moreover, Hayes and Klein (1974) found, in relation to the induction of phytochrome controlled germination responses, that developing seeds of *Arabidopsis thaliana* appear to act independently of the parent plant. Since tomato seeds develop in fleshy hydrated tissues, it was of interest to know how large the influence of the parent plant and light environment is on seed chlorophyll degradation and germination performance.

Here, two approaches were used to study the effect of light on chlorophyll degradation and the relation with seed performance. Firstly, we manipulated the seed chlorophyll level during development and maturation by covering fruits with black cotton cloth of different thickness, and, subsequently, seed performance was evaluated in relation with seed chlorophyll content. The second approach was to study the effect of light and the contribution of the mother plant to seed performance at the end of seed development, by storing green maturing fruits under different light conditions, measuring seed chlorophyll and evaluating seed performance.

The storability of seeds harvested from covering fruits was also evaluated in this paper by measuring germinability and anti-oxidants (ascorbic acid and non-protein thiols) of seeds during 8 weeks of controlled deterioration. It has been reported that shading in tomato, Satsuma mandarin and pear reduced ascorbic acid content of the fruits (Endo, 1975; Izumi *et al.*, 1990; Izumi *et al.*, 1992; Yanagi *et al.*, 1995). We hypothesise that covering fruits would also reduce antioxidant levels in the seeds and may lead to a reduction in its storability. During storage, an anti-oxidant protection system may be required in seeds to counteract the toxic events of free radicals. Ascorbic acid can react with various forms of active oxygen including $^1O_2$, $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$, whereas glutathione is an effective scavenger of $\cdot OH$ and $O_2^{\cdot-}$ radicals (Dalton, 1995).

Material and methods

Performance of seeds from covered fruits

Plant and seed material

Tomato (_Lycopersicon esculentum_ Mill.) plants were grown in a glasshouse with a temperature range of 20-29°C in the summer of 2000. Flowers were tagged and fruits at 14 days after flowering (DAF) were covered with black cotton cloth of different thickness and harvested at 60 DAF. The percentage of light intensity penetrating through the cloth was measured with a lightmeter (Model LI-189, LI-COR, USA). Of the incident light 11.5 % could reach the fruits with the thin cover and 0.01% with the thick cover. The maximum temperature difference between inside and outside of the cover was 2-3°C. The seeds were extracted from the fruits in 2% HCl for 1 h, washed with running tap water and dried over a saturated $CaCl_2$ solution for 4 days at 20 °C and 32% RH, yielding a seed moisture content of $6.5 \pm 0.1\%$ (fresh weight basis) and stored in a sealed aluminium seed bag at 2-3°C until further use.

Fluorescence of dry seeds

The fluorescence of individual dry seeds was measured by laser induced fluorescence (LIF), using a combination of a red diode laser and narrow bandwidth filters as described by Jalink _et al._ (1998). The chlorophyll fluorescence was calculated as the average of 3 replicates of 700 to 1200 seeds for each covered fruit treatment.

Seed dry weight

Three replications of 30 seeds per maturation stage were dried at 80°C for 48 hours and then weighed after cooling in a desiccator with silica gel for at least 30 minutes.

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Germination

Germination analysis was conducted by placing 3 replicates of 30 seeds on 1 layer of germination paper (Schut paper T 300, diameter 80 mm) in 90-mm petridishes moistened with 6 ml 0.2% KNO_3 (ISTA, 1996). The dishes were sealed with parafilm and incubated in the dark at 25°C. Seeds were considered germinated when radicle protrusion (>1 mm) was visible. Germination was scored until 96 h after the start of imbibition, after which length of hypocotyl and primary root were measured. Normal seedlings and vigour were evaluated when the length of hypocotyl and primary root was longer than 10 mm. The percentage of normal seedlings was plotted as percentage of the total number of seeds in the test (germinated + ungerminated).

Seed storage performance of covered fruits

Storage performance was evaluated after 6 months of storage at 2-3°C. The effects of controlled deterioration were examined in seeds which were stored in plastic containers at 35-36°C and 75% RH using a saturated NaCl solution, for 8 weeks.

Physiological parameters, such as moisture content, germinability, percentage of normal seedlings, germination rate (T_{50}, days to reach 50% germination of total seeds) and uniformity (T_{75-25}, the days between 25% and 75% germination of total seeds) were measured after 0, 2, 4, 6 and 8 weeks. Germination parameters were calculated with the Seed Calculator software. After germination, seedlings were transferred to a phytotron set at 22°C, 60% RH with 8h dark and 16h white light (75 W/m^2). Seeds were evaluated as normal or abnormal according to International Seed Testing Association rules (ISTA, 1996). Normal seedlings were scored daily from 7d until 14 d after imbibition. Ascorbic acid and non-protein thiols (see below) of the seeds were also measured after 0, 2, 4, 6 and 8 weeks of controlled deterioration.

Analysis of non-protein thiols (SH-groups)

Using the method of De Vos *et al.* (1994), non-protein thiols were extracted by homogenising 10 seeds in 1 ml of 5% (w/v) 5-sulfosalicylic acid + 5 mM

diethylenetriamine-pentacaetic acid (SSA + DTPA) at 0-4°C using a Mikro-Dismembrator U (Braun Biotech International GmbH, Germany). The extracts were placed on ice for at least 10 min to allow proteins to precipitate, then centrifuged in an Eppendorf centrifuge at maximum speed for 10 min. Total non-protein thiols in the extract were determined with 6 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent). Cold supernatant (300 µl) was mixed with 630 µl of 0.5 M K_2HPO_4 and 25 µl of DTNB solution, and the absorbance was read after 30s at 412 nm in a spectrophometer (DU-64, Beckman Instruments, Inc., USA). Analysis was done in 3 replicates. The content of total non-protein thiols (including glutathione) was calculated on the basis of seed dry weight and using an absorption coefficient = 13,600 cm^{-1}.

Ascorbic acid (AsA) and dehydroascorbic acid (DHA) determination

Fifteen tomato seeds were ground with liquid N_2 using a Mikro-Dismembrator U (Braun Biotech International GmbH, Germany) to which 1.5 ml 6% (w/v) trichloroacetic acid (TCA) was added. AsA and total ascorbate were determined in the supernatant according to Rao and Dubey (1993). Total ascorbate was determined after reduction of DHA to AsA by 10 mM dithiothreitol, and the DHA amount was calculated from the difference between total ascorbate and reduced ascorbate (AsA). The absorbance was read at 525 nm in a spectrofotometer (DU-64, Beckman Instruments, Inc., USA), and concentrations were calculated using a standard curve.

Fruit storage experiment

Fifteen green maturing fruits (45 DAF) were randomly harvested from tomato plants and stored for 10 d in a phytotron at 22°C, 60% RH under an 8h dark and 16h white light (75 W/m^2) regime. The same number of fruits was also stored in black cloth at the same storage conditions, whereas another 15 fruits were processed and seeds were dried and stored at 2-3°C. Tomato fruits at 55 DAF were harvested and used as a control. Germination performance and seed chlorophyll fluorescence were measured after fruit storage. Normal seedlings and vigour were characterised when the length of

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Abstract

In developing seeds of tomato (*Lycopersicon esculentum* Mill.), chlorophyll fluorescence of wild type, and *sit^w*, *gib1*, *au* and *hp-1* mutants was studied. Compared to wild type and *gib1*, *sit^w* seeds showed considerably lower fluorescence during development, which increased after maturation. Seed chlorophyll fluorescence of wild type and *gib1* decreased similarly during maturation (50-60 DAF). Together with the increase of seed chlorophyll fluorescence and the decrease of seed performance (germination and percentage normal seedlings), the number of seeds with free space of *sit^w* also increased upon maturation, confirming the occurrence of viviparous germination. A higher germination rate (T_{50}) and uniformity (T_{75-25}) were observed in *sit^w* mutant seeds compared with wild type. The low fluorescence of *sit^w* may be related to both chlorophyll and carotenoid deficiency. *Sit^w* seeds attained maximum performance between 50 DAF and 60 DAF when seed chlorophyll fluorescence was minimal. Maximum performance of wild type and *gib1* was achieved at 60 DAF, which coincided with their minimum seed fluorescence.

There was no clear difference in chlorophyll fluorescence between wild type and *hp-1* seeds, whereas seed chlorophyll fluorescence of the *au* mutant was slightly lower than that of wild type and *hp-1*. Seeds of *hp-1* reached their maximum performance at 63 DAF, when their seed chlorophyll fluorescence reached a minimum value, at the same time as wild type seeds. Maximum performance of *au* seeds was attained at 53 DAF, when their seed chlorophyll fluorescence was also minimal, after which dormancy became deeper upon post maturation (63-73 DAF).

Key words

Lycopersicon esculentum Mill., tomato, chlorophyll fluorescence, ABA mutant, GA mutant, seed development, germination, maturation, aurea mutant, high pigment mutant.

Introduction

Abscisic acid (ABA) and gibberellins (GAs) are essential for seed development and germination (Bewley and Black, 1994). In tomato seed, endogenous ABA plays an important role in the induction of dormancy during seed maturation (Groot and Karssen, 1992). Development of fertile flowers and seed germination are absolutely dependent on the presence of endogenous GAs (Groot _et al._ 1987). The absence of ABA or GAs during tomato seed development may influence seed chlorophyll content, which could affect the role of chlorophyll in the developing seed. It has been widely reported that _gib1_ mutants show a dwarf growth habit and a darker leaf colour (Groot _et al._, 1987; Koornneef _et al._, 1990), whereas most ABA mutants are altered in endosperm and seedling pigmentation (Robertson, 1975; Fambrini _et al._, 1993; Maluf _et al._, 1997; Holding _et al._, 2000).

It has been shown that young tomato seeds contain chlorophyll (Jalink _et al._ 1999). In immature seeds, the majority of chlorophyll is located in the seed coat and a low amount in the embryonic axis (Chapter 2). With laser induced fluorescence (LIF), chlorophyll in seeds can be detected non-destructively (Jalink _et al._ 1999). We used this technique to study the role of chlorophyll in developing tomato seed, using the hormone deficient mutants _sit^w_ (ABA-deficient) and _gib1_ (GA-deficient). Using X-ray analysis, Liu (1996) showed an increase of seeds with free space and viviparous germination in over-mature seeds of the _sit^w_ mutant. We used the same method to observe viviparous germination, and correlated it with the presence and breakdown of chlorophyll during late development and maturation.

Using a tomato mutant deficient in the biosynthesis of the phytochrome chromophore (_au_), and the high pigment-1 (_hp-1_) mutant, exhibiting an exaggerated phytochrome response, it has been shown that chlorophyll content in the fruits and leaves in monogenic _hp-1_ is higher than in the wild type and the _au_ mutant. The _au_ mutant had less chlorophyll in leaves and fruits compared to the wild type (Kerckhoffs, 1996). It is possible that these mutants also have different levels of chlorophyll in their seeds, which makes them useful in this study. Koornneef _et al._ (1985) have shown that

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the *au* mutant showed poor seed germination compared to wild type. High germination of this mutant in the dark could be obtained by gibberellic acid treatment (Koornneef *et al.*, 1985; Georghiou and Kendrick, 1991) or nitrate treatment (Georghiou and Kendrick, 1991).

Material and methods

Plant material

Tomato (*Lycopersicon esculentum* Mill.) plants of the homozygous GA-deficient (*gib1*) and ABA-deficient (*sit^w*) mutants and their isogenic parent wild type (cv. Moneymaker) were grown in a greenhouse with a temperature range of 20-29°C in the summer of 2000. Each of the *sit^w* plants was sprayed once a week with 2 ml of a 10 μM ABA solution to reduce wilting. The top and flower bud regions of the *gib1* plants were sprayed with 10 μM GA_{4+7} once a week until fruit set to stimulate shoot growth and fertile flower development (Groot and Karssen, 1992). Flowers were self-pollinated by vibration and tagged to obtain seeds of defined stages of development (days after flowering, DAF).

Tomato seeds from the *aurea* (*au*) and *hp-1* mutants, and their wild type isogenic parent (cv. Moneymaker) were obtained from tomato plants grown in a greenhouse with a temperature range of 21-31°C in the spring of 1999.

Seeds with different levels of maturation were dried over saturated $CaCl_2$ for 4 days at 20 °C and 32% RH, resulting in a moisture content of 6.5% ± 0.1 (fresh weight basis) and stored in sealed aluminium bags at 2-3°C until further use.

Fluorescence of dry seeds

The fluorescence of individual dry seeds was measured by LIF, using a combination of a red diode laser and narrow bandwidth filters as described by Jalink *et al.* (1999). The chlorophyll fluorescence of wild type, *sit^w* and *gib1* was calculated as the average of 300 to 800 seeds at each maturation stage and measured three times (3 x 300 to 800 seeds). Due to the low amount of spring harvested seeds of *au*, *hp-1* and its

wild type, the presented chlorophyll fluorescence data of each developmental stage for these lines was an average from individual seeds from batches of 75 to 300 seeds, and measured once.

Germination

Germination analysis of wild type, sit^w and $hp-1$ was conducted by placing 3 replicates of 30 seeds on 1 layer of germination paper (Schut paper T 300, diameter 80 mm) in 90-mm Petri dishes moistened with 6 ml water, or 10 µM GA_{4+7} for the *gib1* seeds. Seeds of the *au* mutant were incubated in 0.2% KNO_3. The dishes were sealed with parafilm and incubated in the dark at 25°C. Seeds were considered germinated when radicle protrusion (>1 mm) was visible. Germination was scored until 5 d after the start of imbibition, upon which seedlings were transferred to germination conditions recommended by the International Seed Testing Association (ISTA), at an alternating temperature regime of 20/30°C with 16 h dark and 8 h light. Seedlings were evaluated as normal or abnormal according to ISTA rules (ISTA, 1996). The percentage of normal seedlings was plotted as percentage of the total number of seeds in the test (germinated + ungerminated). Normal seedlings were scored daily from 7 d until 14 d after imbibition.

Germination rate expressed as T_{50} (days to reach 50% of total germination) and uniformity (T_{75-25}, days between 25% and 75% of total germination) were calculated using the Seed Calculator Programme (H. Jalink and R. van der Schoor, personal communication).

X-ray photography

For X-ray analysis, two replicates of 20 seeds from each maturation stage of wild type, sit^w and *gib1* were placed 25 cm from the X-ray source window (Liu, 1996). The X-ray photograph was made at 15 keV and 7 min exposure time, using a 43805N X-ray System (Faxitron™ series, Hewlett Packard, USA).

Results

Seed chlorophyll fluorescence

Chlorophyll fluorescence of developing seeds of wild type and *gib1* differed from that of *sit^w* seeds. *Sit^w* seeds had a lower fluorescence than wild type and *gib1* until 50 DAF, after which their fluorescence clearly increased. In contrast, seed chlorophyll fluorescence of the wild type and the *gib1* mutant decreased from 40 DAF and reached a minimum at 60 DAF (Fig. 1a).

Seed chlorophyll fluorescence of *au* was generally slightly lower than that of wild type and *hp-1* upon development and maturation (Fig. 2a). A sharp decrease occurred between 43 DAF and 53 DAF for all three genotypes, after which minimal fluorescence was achieved at about 63 DAF. There was no clear difference between seed chlorophyll fluorescence of wild type and *hp-1*.

Germination performance

Sit^w seeds were germinable at 40 DAF and reached a maximum at 60 DAF (Fig. 1b). Although also reaching their maximum germinability at 60 DAF, both wild type and *gib-1* seeds were not able to germinate at 40 DAF. The best performance of wild type seeds, which was expressed as the percentage of normal seedlings, was achieved at 60 DAF, whereas the best performance of *gib1* and *sit^w* seeds occurred between 50 DAF and 60 DAF. A slight decline in seed performance was observed at 70 DAF for both wild type and *gib1*. The decline of *sit^w* seed performance, observed at 70 DAF was much larger (Fig. 1c). Based on germination rate (T_{50}) and uniformity (T_{75-25}), *sit^w* seeds germinated faster than wild type seeds, and slightly faster than *gib1* seeds (Figs. 1d and 1e).

Seeds of *hp-1* reached maximum germinability at 63 DAF, and produced a maximal percentage of normal seedlings at the same stage of maturation as the wild type (63-73 DAF) (Figs. 2b and 2c), whereas *au* seeds reached maximum germinability at 53 DAF; a strong decline in germinability occurred after 53 DAF. Most of the *au* seeds at 63-73 DAF were dormant, and insensitive to KNO_3 and GA_{4+7}. The *au* mutant
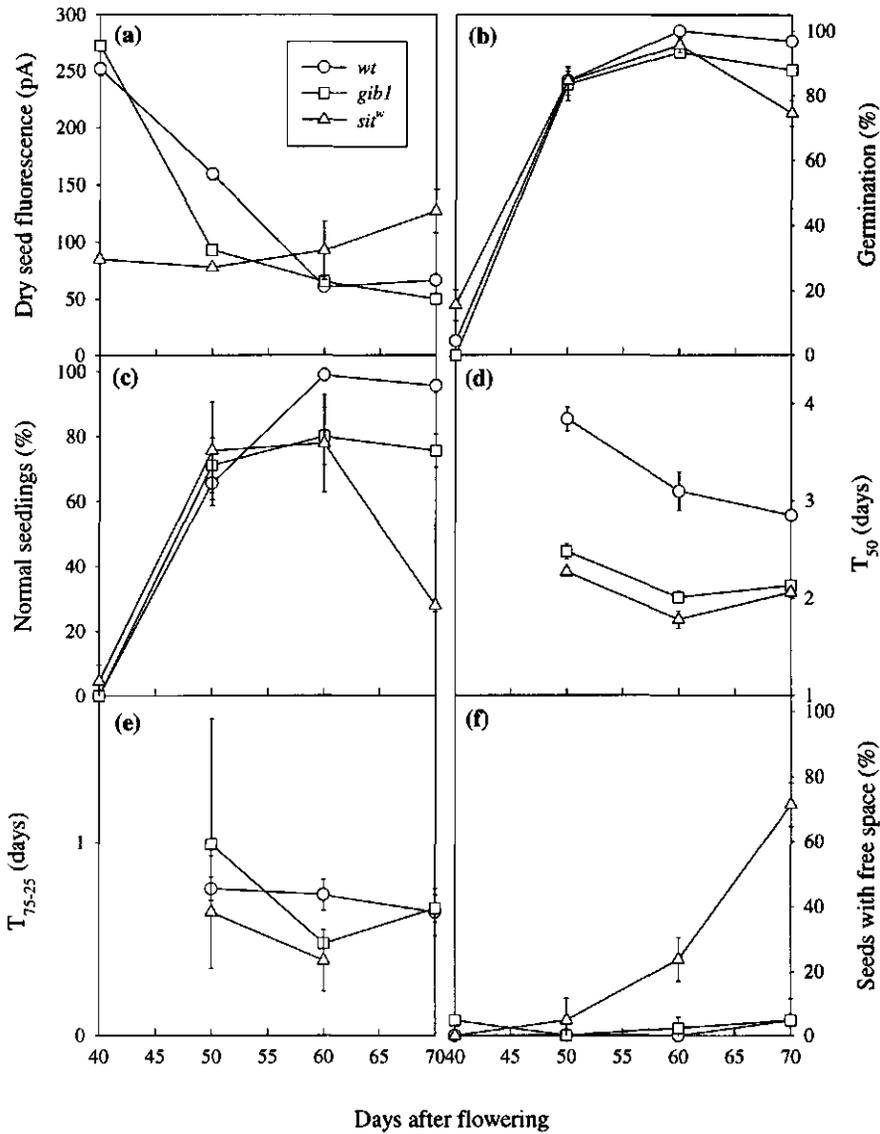
Fig. 1. *Seed chlorophyll fluorescence (a), germinability (b), percentage normal seedlings (c), T_{50} (d), uniformity T_{75-25} (e), and seeds with free space (f), during seed development and maturation of wild type, gib1 and sit^w seeds (harvested in the summer of 2000). Values are means $\pm$ sd ($n \leq 3$).*

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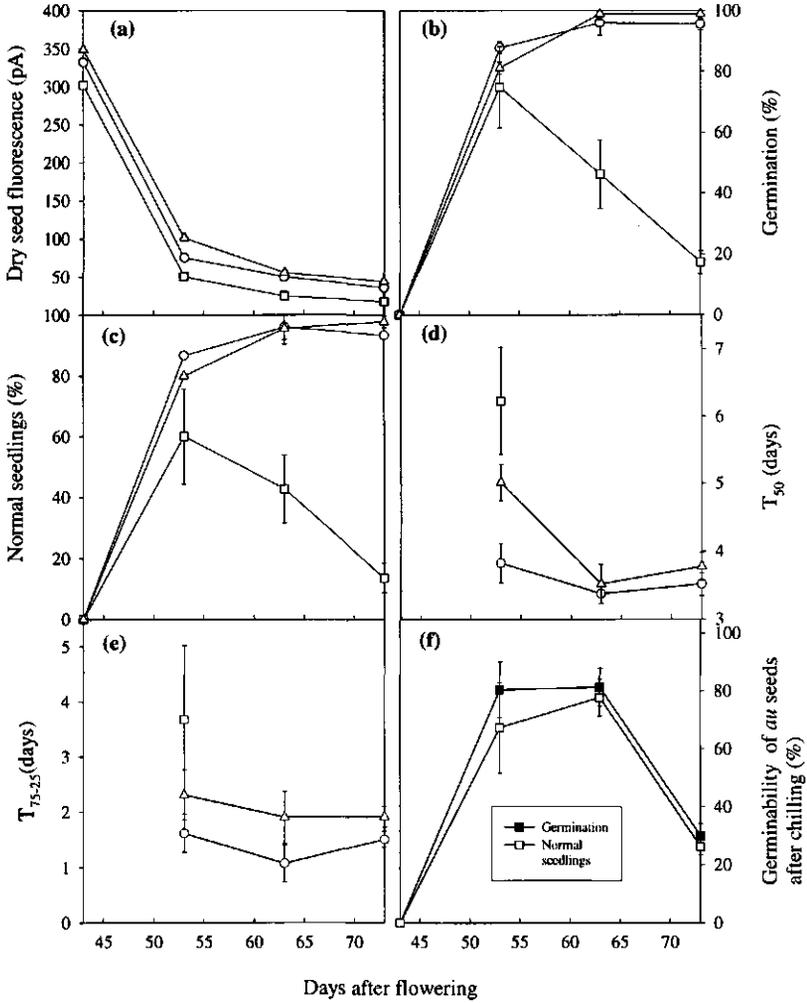
Fig. 2. *Seed chlorophyll fluorescence (a), germinability (b), percent normal seedlings (c), T_{50} (d) and uniformity T_{75-25} (e), during development and maturation of wild type, au and hp-1 seeds (harvested in the spring of 1999). After chilling at 5 °C for 3 d, au seeds were germinated again in continuous white light at 25 °C and germinability and percentage normal seedlings were determined (f). Values are means ± sd (n = 3). Fig. 1a, values are means from 75-300 seeds and measured once.*

not only showed poor germination, but also germinated slower than the other two genotypes (Fig. 2d). After 14 d in the germination test conditions, the non-germinated seeds were chilled at 5°C for 3 d and germinated again in continuous white light at 25°C. Maximum germination and normal seedling percentage increased significantly at 63 DAF, although most seeds of 73 DAF were still dormant (Fig. 2f); these seeds were still viable until 30 d of imbibition as they remained firm and the embryos were still white (not shown). Apparently, the chilling treatment was not enough to break their dormancy.

Seed with free space

In seeds of wild type, sit^w and *gib1* free space was visualised by X-ray photography after drying upon seed maturation (Fig. 3). In parallel with the increase in chlorophyll fluorescence, the number of sit^w seeds with free space also increased upon maturation (Figs. 1a and 1f). No more than 5% of the wild type and *gib1* seeds showed a free space and no clear increase was observed during maturation.

Discussion

Using LIF, a sensitive technique to detect the presence of chlorophyll in tomato seeds, we have shown that chlorophyll fluorescence of sit^w seeds was low during the final part of development (40 DAF) and increased upon maturation as compared to wild type and *gib1* seeds (Fig. 1a). Apparently, in this ABA-deficient mutant chlorophyll was synthesised during viviparous germination and could be detected by LIF. The number of sit^w seeds with free space was also positively related to the increase of seed chlorophyll fluorescence (Figs. 1a and 1f), corroborating the occurrence of viviparous germination. During viviparous development, the endosperm is degraded and free space remains (Liu, 1996). Apparently, the viviparously germinating seeds also became desiccation intolerant. Seed performance of these three genotypes could not be evaluated based on germination rate alone, since sit^w seeds germinated faster than the other two genotypes (Figs. 1d and 1e), but at later developmental stages most of their seedlings developed

Seed with free space   Seed without free space
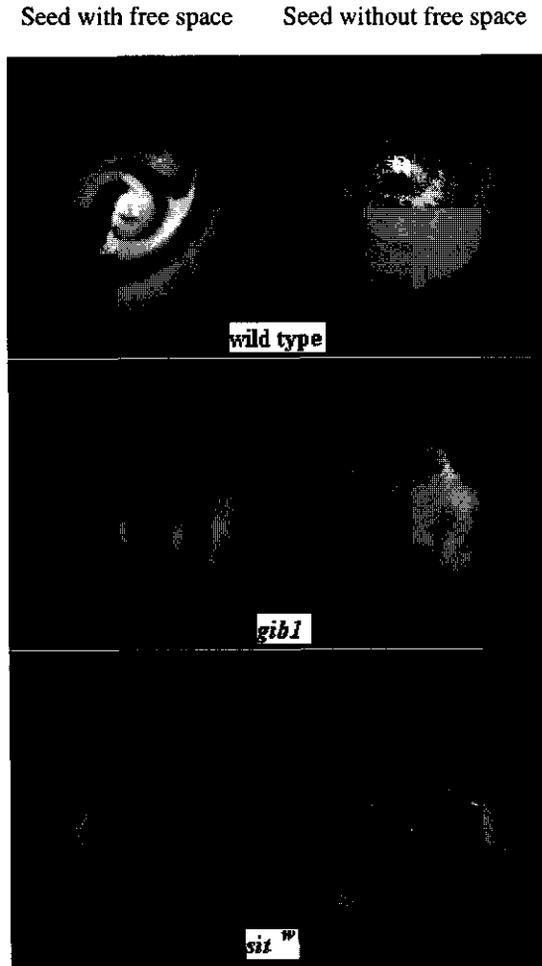
Fig. 3. *X-ray images of wild type, gib1 and sit^w seeds with and without free space upon maturation (70 DAF).*

abnormally (Fig. 1c). Unlike Downie *et al.* (1999), we found that the number of seeds with free space was markedly increased during the later stages of seed maturation and we observed significant vivipary, as judged from the increase in seed chlorophyll fluorescence.

The low amount of *sit*[w] seed chlorophyll by the end of development (40 DAF) could be explained by the results of Maluf *et al.* (1997) who demonstrated a deficiency in chlorophyll and carotenoid synthesis in the viviparous maize (*Zea mays* L.) mutant (*vp12*). This mutant showed a lower expression of geranylgeranyl pyrophosphate synthase (GGPPS), the enzyme that is responsible for the synthesis of geranylgeranyl pyrophosphate, the immediate precursor of the terpenoid pathway. Hence, a deficiency in GGPPS will affect chlorophyll as well as carotenoid- and ABA-synthesis (Britton, 1988; Rudiger and Schoch, 1988; Maluf *et. al.*, 1997). Since LIF only detects chlorophyll fluorescence mainly in the seed coat, the low fluorescence of *sit*[w] seeds may also be due to their thinner testa compared with the wild type. *Sit*[w] seed has only 1-2 cell layers in the testa, whereas the wild type seed has 5 layers (Hilhorst and Downie, 1995).

Koornneef *et al.* (1990) stated that the dark-green leaf colour of the *gib1* mutant was not so much due to a higher chlorophyll content compared to the wild type, but was correlated with a changed leaf morphology, characterised by wrinkled and thicker leaves. Using *gib1* seed, which is not morphologically different from the wild type, we showed that *gib1* seeds indeed have a similar chlorophyll content and show a similar decline in seed chlorophyll content as the wild type. In previous experiments we found that seed chlorophyll fluorescence and content of *gib1* was always slightly higher than that of the wild type during seed development and maturation (data not shown), especially at the early stages of seed development (younger than 45 DAF).

Seed chlorophyll fluorescence is an accurate marker for seed maturation. The best seed performance of wild type and *hp-1* was achieved when their seed chlorophyll fluorescence reached a minimum during seed maturation (Figs. 2a, 2b and 2c). Maximum performance of *au* seeds was attained at 53 DAF, after which their dormancy became deeper. In contrast with the observations of Georghiou and Kendrick (1991) who could break this dormancy by KNO_3, GA_{4+7}, and dry storage of *au* mutant seeds, we were only able to break the dormancy of *au* seeds with KNO_3 at 53 DAF. However, after chilling at 5°C for 3 d followed by germination in continuous white light at 25°C, germination and normal seedling percentage increased significantly in seeds at 63 DAF

(Fig. 2f). Seeds of 73 DAF remained dormant and only 30% germinated. Induction and subsequent (partial) release of dormancy during maturation is a common phenomenon in tomato seeds (Hilhorst, 1995; de Castro *et al.*, 1998). This type of dormancy is gradually released during a few months of dry storage (Groot and Karssen, 1992). Clearly, this also occurred in seeds of the *au* mutant but the period of storage of the seeds used in our experiments was not sufficient to break dormancy of the *au* seeds, contrary to the wild type and *hp-1* seeds, which had lost their dormancy during dry storage. Tomato seeds usually do not require light to induce germination. It is believed that the amount of pre-existing active phytochrome (P_{fr}) in the seeds is sufficient to overcome the threshold for germination (Kendrick and Cone, 1985), making the seeds independent of light (but not of phytochrome) for germination. During induction of dormancy seeds become less sensitive to light: more light (more P_{fr}) is required for germination (Hilhorst, 1990). As the *au* mutant seeds have virtually no active phytochrome it is plausible that germination becomes increasingly problematic when the sensitivity to light decreases during induction of dormancy.

Wild type tomato seeds grown in summer exhibited higher performance than spring seeds. Although germinability and normal seedling percentage (Figs. 1b, 1c, 2b and 2c) are similar, the summer-grown seeds germinated faster and more uniform than the spring-grown seeds (Figs. 1d, 1e, 2d and 2e). Chlorophyll fluorescence of dry seeds harvested from both growing conditions seemed to be comparable. However, using the Xe-PAM fluorometer, chlorophyll fluorescence of fresh seed grown in spring was lower than in autumn (Chapter 3). It is likely that chlorophyll content during development and maturation is influenced by day length and light intensity.

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Chapter 6

The relationship between the amount of chlorophyll in tomato (*Lycopersicon esculentum* Mill.) seeds and oxidative stress during controlled deterioration

Mohamad R. Suhartanto, Peter E. Toorop, Henk Jalink, and Henk W.M. Hilhorst

Abstract

The effect of seed chlorophyll content on the oxidation of non-protein thiols and ascorbic acid (AsA) during slow controlled deterioration (35°C and 75% RH) was studied in seeds of tomato (*Lycopersicon esculentum* Mill.), under continuous red light and in darkness. The decline in germinability, percentage of normal seedlings, germination rate (T_{50}), and uniformity (T_{75-25}) was faster in darkness than under red light. A decrease of chlorophyll fluorescence was observed after controlled deterioration in red light. The relation between chlorophyll degradation and seed performance during controlled deterioration in red light is discussed. The involvement of phytochrome was also discussed to explain the faster deterioration in the dark than in red light. Controlled deterioration for 8 weeks resulted in a marked loss of non-protein thiols and a distinct increase of AsA. The possible operation of the ascorbate-glutathione cycle in dry seed is discussed and related to seed deterioration. A decay of all physiological parameters during the controlled deterioration period was clearly observed after 12 weeks, but the effect of the chlorophyll content on these parameters remained unclear. It is hypothesized that carotenoids are involved as antioxidants in the improvement of seed storability.

Key words

Lycopersicon esculentum, tomato, chlorophyll fluorescence, controlled deterioration, antioxidant, non-protein thiols, ascorbic acid, red light, tomato seed germination.

Introduction

Seed deterioration during storage has been proposed to be related to oxidative injury, which may include loss of antioxidants (Senaratna _et al._ 1988). Free radicals may damage membranes and other macromolecules, resulting in a loss of seed viability. To counteract these toxic events, antioxidant protection systems are present in plant tissues. These protective systems consist of both enzymatic and non-enzymatic reactions. One of the most efficient detoxification mechanisms is the ascorbate/glutathione cycle in which hydrogen peroxide is scavenged (Foyer and Halliwell, 1976). This cycle seems important in photosynthetic tissues, but only little information is available about this mechanism in dry seeds (Senaratna _et al._, 1988; Pukacka, 1991; De Paula _et al._, 1996; Tommasi _et al._, 1999). From this information it appears that dry seeds lack ascorbic acid (AsA) whereas dehydroascorbate (DHA) is only present in small quantities and is hardly of any importance as antioxidant.

Ascorbate is present in all subcellular compartments, including the apoplast, chloroplast, cytosol, vacuoles, mitochondria and peroxisomes (Foyer and Lelandais, 1996; Jimenez _et al._, 1997). In the chloroplast the concentration of glutathione is high (Wolosiuk and Buchanan, 1977) and predominantly present in the reduced form (GSH; Halliwell and Foyer, 1978).

Dry seeds contain chlorophyll, which is easily detectable by chlorophyll fluorescence techniques. Generally, high seed performance is associated with a low chlorophyll content (Jalink _et al._, 1998). We hypothesise that the presence of chlorophyll during seed maturation is undesirable since it is associated with lower quality, particularly lower seed longevity. Chlorophyll may also be a primary source of $^1O_2$ (singlet oxygen) which is formed from O_2^- (superoxide radical) (Thomson _et al._, 1987), when chlorophyll is excited by light (Cogdell, 1988). Furthermore, singlet oxygen can oxidise chlorophyll, lipids, proteins and nucleic acids. In the present study the relationship between the amount of chlorophyll in seeds and oxidative stress during controlled deterioration at 35°C and 75% relative humidity was studied to mimic practical storage conditions in the tropics. Under these stress conditions, especially in

light, extensive oxidation may occur in seeds with higher chlorophyll content and the presence of antioxidants (AsA and GSH) may be associated with the extent of oxidative injury.

Materials and methods

Seed material

Seeds of tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) were obtained from fruits of plants grown in a glasshouse in the summer of 2000. Seeds were extracted from red fruits, treated with 2% HCl for 1 h, washed with running tap water and dried over saturated $CaCl_2$ for 4 days at 20 °C and 32% RH until a seed moisture content of 6.5% ± 0.1 (fresh weight basis) and stored in sealed aluminium bags at 2-3°C until further use. Three levels of chlorophyll content (low, medium and high) were obtained by sorting the seeds by means of Laser Induced Fluorescence (LIF; Jalink *et al.*, 1999).

Controlled deterioration condition

The effects of controlled deterioration were examined in seeds which were stored in plastic containers at 35-36°C and 75% RH over a saturated NaCl solution. Two light conditions were compared: darkness and red light generated by LEDs (Kingbright[R], Taiwan Part No. BL0307-50-44; wavelength 652 nm with half-band width 26 nm and intensity 240-245 μmol or 26-27 W/m^2).

Chlorophyll fluorescence of seeds

The fluorescence of individual dry seeds before, during and after 8 weeks of controlled deterioration was measured with LIF using a combination of a red diode laser and narrow bandwidth filters as described by Jalink *et. al* (1999).

Germination test and determination of seed moisture content

Germination analysis was conducted on 3 replicates of 30 seeds, placed on top of 1 layer of germination paper (Schut paper T 300, diameter 80 mm) in 90 mm Petri

dishes moistened with 5 ml H_2O. The dishes were sealed with parafilm and incubated in the dark at 25°C. Seeds were considered germinated when radicle protrusion was visible (> 1 mm). Seed viability was expressed as percentage germination, germination rate (T_{50}, number of days to reach 50% germination of total seeds) and uniformity (T_{75-25}, the number of days between 25% and 75% germination of the total seed population) and scored until 5 d. After this period, seedlings were transferred to a phytotron set at 22°C and 60% RH, with an 8h dark and 16 h white light (75 W/m^2) cycle. Seedlings were evaluated as normal or abnormal according to ISTA rules (ISTA, 1996). Normal seedlings were scored daily from 7d until 14 d after the start of imbibition. Percentage normal seedlings was derived from the whole seed batch (ungerminated + germinated seeds).

Seed moisture content (percentage of fresh weight) during controlled deterioration was determined by weighing two replicates of 30 seeds before and after drying for 1 h at 130 °C (ISTA, 1996).

Analysis of non-protein thiols (SH-groups)

In most plant species the major non-protein thiol is GSH. In tomato seed extracts more than 70% of the total acid-soluble thiol is GSH (De Vos *et al.*, 1994). Using their method, non-protein thiols were extracted by homogenising 10 seeds in 1 ml of 5% (w/v) 5-sulfosalicylic acid + 5 mM diethylenetriamine-pentacaetic acid (SSA + DTPA) at 0-4°C using a Mikro-Dismembrator U (Braun Biotech International GmbH, Germany). The extracts were placed on ice for at least 10 min to allow proteins to precipitate, then centrifuged in an Eppendorf (5415C) centrifuge at maximum speed for 10 min. Total non-protein thiols in the extract were determined with 6 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent). Cold supernatant (300 µl) was mixed with 630 µl of 0.5 M K_2HPO_4 and 25 µl of DTNB solution, after which the absorbance was read after 30 s at 412 nm (absorption coefficient = 13,600 cm^{-1}). Analysis was done in 3 replicates. The content of total non-protein thiols (including GSH) was calculated on the basis of seed dry weight.

AsA and DHA determination

Fifteen tomato seeds were ground with liquid N_2 using a Mikro-Dismembrator U (Braun Biotech International GmbH, Germany) and homogenised in 6% (w/v) tricloroacetic acid. AsA and total ascorbate were determined in the supernatant according to Rao and Dubey (1993). Total ascorbate was determined after reduction of DHA to AsA by 10 mM dithiothreitol and the DHA level was estimated on the basis of the difference between total ascorbate and reduced ascorbate (AsA). The absorbance was measured at 525 nm and concentrations were determined by comparison with a standard curve.

Statistical analysis

The experiment was performed as a splitplot experiment. The light condition was the main plot and the chlorophyll level of the seeds was the splitplot factor. Physiological parameters, such as percentage germination, percentage of normal seedlings and T_{50}, were assessed after 0, 2, 4, 6, 8, and 12 weeks of light treatment, while the uniformity (T_{75-25}) was determined until 8 weeks. Ascorbic acid and non-protein thiol contents of the seeds were measured after 0, 2, 4, 6 and 8 weeks of storage. Analysis was performed by using the statistical programme Genstat 5 (Windows version release 4.1, Lawes Agricultural Trust, Rothamsted Experimental Station U.K.). If the F-test was significant, differences among the treatments were further investigated using the Least Significant Difference (LSD) test ($P<0.01$). The effect of a single-factor was not further evaluated when the interaction between factors was significant. This experimental design was primarily aimed at the interaction between factors. However, if the interaction between factors was not significant, the evaluation was done from the effect of the single factor (Steel and Torrie, 1981).

Results

Chlorophyll fluorescence

Three tomato (cv. Moneymaker) seed lots, each containing seeds with their chlorophyll fluorescence falling within a distinct class were obtained by sorting the seeds in an LIF set up with adjustable fluorescence threshold values (Jalink *et al.*, 1996; Chapter 1). The 3 sub-lots sorted by this method are indicated as low-, medium- and high chlorophyll fluorescence (CF). These 3 seed lots were each subjected to a CD treatment, both under red light and in darkness.

After 8 weeks of controlled deterioration under red light, chlorophyll fluorescence of all individual seeds shifted to lower levels and the frequency distributions of fluorescence of the 3 sorted seed lots became more narrow as compared to the start of controlled deterioration (0 weeks; Figs. 1a and 1c), while the distribution of chlorophyll fluorescence of the seeds aged in darkness remained largely unchanged (Fig. 1b).

Seed moisture content

Seed moisture content of the seeds of the 3 sub-lots stored in the dark increased between 0 and 2 weeks and remained stable at 9-10% until 6 weeks of controlled deterioration. Between 6 and 8 weeks the moisture content of the low- and medium CF sub-lots decreased to below 9% and that of the high CF sub-lot to below 8%. Seeds stored under red light had an approximately 1.5% lower moisture content than seeds stored in darkness (Fig. 2).

Summary of the analysis of variance

Table 1 lists the effects of light (L) and seed chlorophyll content (C) during controlled deterioration (T) on seed performance parameters (non-protein thiols, ascorbic acid, germinability, T_{50}, T_{75-25} and percentage normal seedlings).
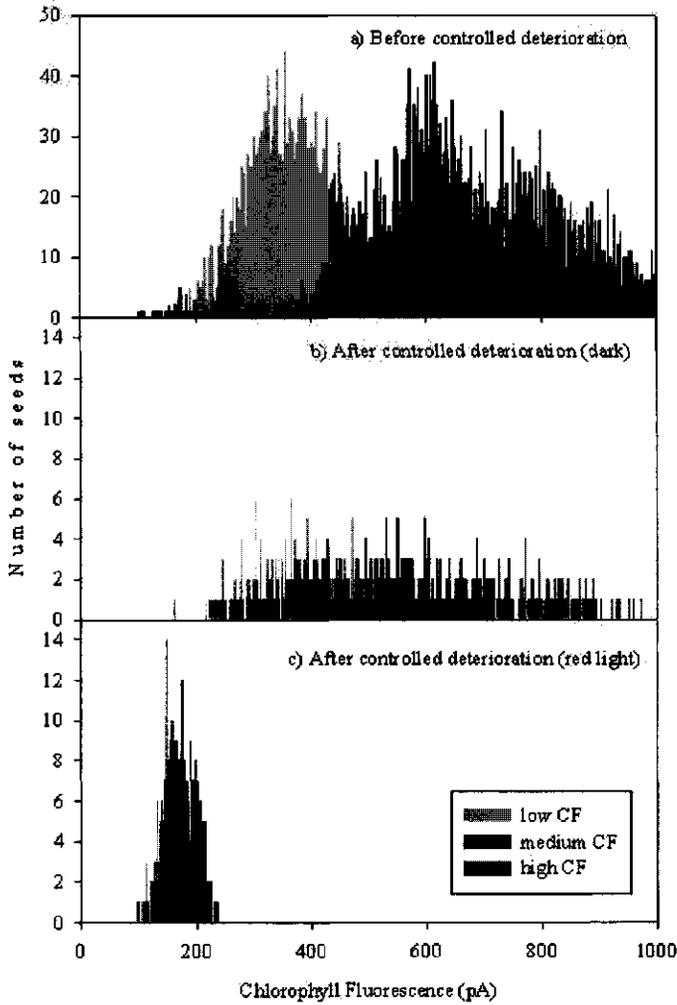
Fig. 1. *Changes in chlorophyll fluorescence before (a) and after 8 weeks of controlled deterioration under red light (c) or in darkness (b). The chlorophyll fluorescence at the start of the controlled deterioration was measured in 2200 seeds at each chlorophyll fluorescence level (low, medium and high). The chlorophyll fluorescence of the seeds stored under red light and in darkness (about 250 seeds per treatment) was measured after 8 weeks.*
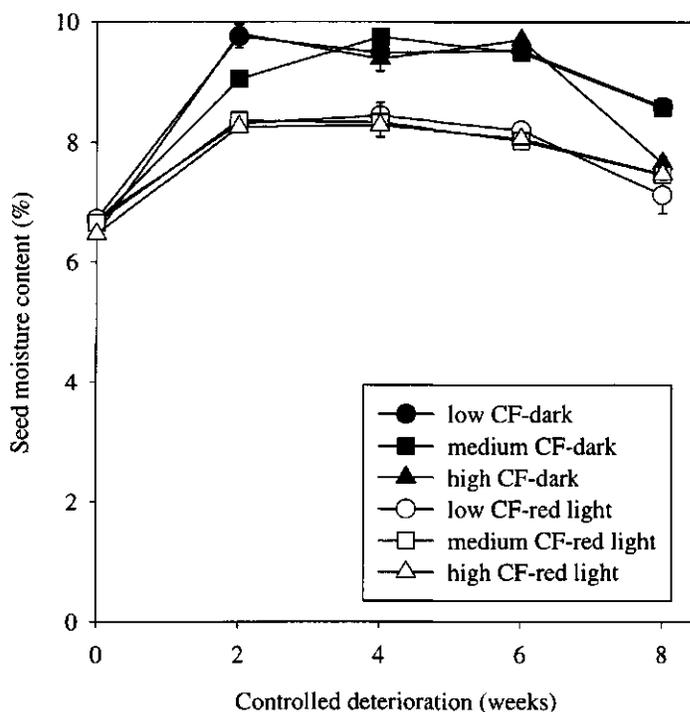
Fig. 2. *Seed moisture content during controlled deterioration under red light and in darkness. Values are means ± sd (n=2).*

Non-protein thiols

There was a significant linear negative effect of the controlled deterioration period ($P<0.001$) on the content of non-protein thiols but interactions were not significant. Although there was some effect of the chlorophyll level ($P< 0.05$) we ignored the chlorophyll level effect, since testing against the residual mean square of the time stratum resulted in $P>0.05$.

Ascorbic acid

There was a strong significance (P<0.001) for the positive effect of the controlled deterioration period on AsA content as well as interaction with the duration of controlled deterioration and the light condition. However, the interaction effects could only be explained by higher than quadratic terms. The biological meaning of this effect is not clear, so we ignored this interaction.

Table 1. *Interactions of light, chlorophyll content and controlled deterioration time, with non-protein thiol and ascorbic acid content, germinability, T_{50}, T_{75-25} and percentage of normal seedlings during the controlled deterioration period. LC, LT, CT and LCT refer to interactions between light (L), chlorophyll content and CD time (T).*

Treatments	Non-protein thiol	Ascorbic acid	Germination	T_{50}	T_{75-25}	Normal seedlings
Light (L)	ns	ns	*	ns	ns	**
Chlorophyll content (C)	*	ns	***	ns	**	**
Controlled Deterioration time (T)	***	***	***	***	***	***
Interaction:						
LC	ns	ns	***	ns	ns	ns
LT	ns	***	***	***	ns	***
CT	ns	ns	ns	**	ns	ns
LCT	ns	ns	ns	ns	ns	ns

ns   not significant
***   significant at P < 0.001
**   significant at 0.001≤ P < 0.01
*   significant at 0.01≤ P < 0.05

Germinability parameters

A linear negative effect of duration of controlled deterioration on percentage germination, germination rate (T_{50}), uniformity (T_{75-25}) and the percentage of normal seedlings was significant (P<0.001). Also there was evidence for a significant interaction between the controlled deterioration period and the light condition (P<0.001) on percentage germination, germination rate (T_{50}) and percentage of normal seedlings (cf. Tables 2 and 5).

The interaction between light condition and seed chlorophyll level was significant (P<0.001) on percentage germination. An indication (P<0.01) of interaction effects between chlorophyll level and time of controlled deterioration on germination speed (T_{50}) was also observed, as well as between chlorophyll level and uniformity (T_{75-25}) and percentage of normal seedlings (cf. Table 3).

The differences among significant treatments were evaluated with the LSD test (Tables 2-5).

The effect of seed chlorophyll level during controlled deterioration

During the first two weeks the relief of residual dormancy by the storage conditions resulted in a decrease of T_{50}. After two weeks, the germination rate started to slow down (T_{50} increased). Seeds with high chlorophyll content seemed to be somewhat more vigorous than seeds with low chlorophyll level after 8 weeks of controlled deterioration (Table 2). Moreover, based on uniformity (T_{75-25}) and the percentage of normal seedlings, seeds with higher chlorophyll content appeared to be more vigorous (Table 3). Seeds with the lowest chlorophyll content, which deteriorated more in darkness had lower germinability than in red light (Table 3).

Table 2. *The effect of chlorophyll level of seeds during the controlled deterioration period on germination rate (T_{50}).*

Chlorophyll level	Controlled deterioration period (weeks)					
	0	2	4	6	8	12
	T_{50} (days)					
low	2.25 cd	1.63 h	1.86 g	2.10 f	2.41 c	3.57 a
medium	2.25 d	1.68 h	1.84 g	2.03 f	2.29 cd	3.43 b
high	2.33 e	1.65 h	1.87 g	2.04 f	2.36 de	3.45 b

Means followed by the same letter within a column and/or a row are not statistically different (P>0.01) according to the LSD test

Table 3. *The effect of chlorophyll level in seeds on percentage of normal seedlings and uniformity (T_{75-25}). Both light condition and seed chlorophyll level influenced germinability.*

Parameter	Chlorophyll level		
	low	medium	high
Normal seedlings (%) [1]	87.4 b	89.1 ab	91.1 a
T_{75-25} (days) [1]	0.40 a	0.35 b	0.34 b
Germination (%) [2]			
dark	89.4 b	93.5 ab	93.4 ab
red light	96.9 a	97.3 a	97.2 a

1) Means followed by the same letter within a row in the same parameter are not statistically different (P>0.01) according to the LSD test

2) Means followed by the same letter within a column and/or a row are not statistically different (P> 0.01 according to the LSD test

Table 4. *The effect of the length of the controlled deterioration period on non-protein thiol and ascorbic acid content of seeds.*

Parameter	Controlled deterioration period (weeks)				
	0	2	4	6	8
Non-protein thiols (nMol g^{-1} seed DW)	232.2 a	74.3 b	21.9 c	9.4 d	9.1 d
Ascorbic acid (μMol g^{-1} seed DW)	12.8 d	21.3 c	24.4 bc	29.9 b	56.8 a

Means followed by the same letter within a row in the same parameter are not staristically different (P>0.01) according to the LSD test

Antioxidant content during controlled deterioration

Non protein thiols and ascorbic acid showed an opposite response to controlled deterioration. The content of non-protein thiols decreased significantly after 2 weeks and in the same time the ascorbic acid content increased until the end of the controlled deterioration period (Table 4). Amounts of dehydroascorbic acid (DHA) remained constant at a negligible amount during controlled deterioration (data not shown).

The effect of light condition during controlled deterioration

In darkness, the percentage germination decreased somewhat between 6 and 8 weeks, while the percentage of normal seedlings had already decreased at 6 weeks of controlled deterioration. However, under red light the decrease of germination and percentage of normal seedlings only started to decrease between 8 and 12 weeks of controlled deterioration (Table 5). After 12 weeks of CD treatment, germinability and percentage normal seedlings was much lower in the dark treated seeds. Moreover, after 12 weeks of controlled deterioration in darkness, seeds germinated slower (T_{50} higher) than seeds deteriorating under red light. Also the uniformity of germination (T_{75-25}) increased significantly (less uniform) during the deterioration period, although there were no differences between the treatments.

Table 5. *The effect of light condition and controlled deterioration period on germinability, percentage of normal seedlings and germination rate (T_{50}). Uniformity (T_{75-25}) was only influenced by the period of controlled deterioration.*

Parameter	Controlled deterioration period (weeks)					
light condition	0	2	4	6	8	12
Germination						
(%)						
dark	99.6 a	98.9 a	98.9 a	97.4 ab	95.2 b	62.6 d
red light	99.6 a	98.2 ab	100.0 a	99.3 a	100.0 a	85.7 c
Normal seedlings						
(%)						
dark	95.2 a	94.1 a	93.3 ab	88.5 b	86.3 cd	60.9 e
red light	95.2 a	95.6 a	94.8 a	93.0 ab	90.0 abc	83.5 d
T_{50} (days)						
dark	2.28 d	1.65 g	1.84 f	2.05 e	2.33 cd	3.65 a
red light	2.28 d	1.66 g	1.87 f	2.06 e	2.38 c	3.33 b
T_{75-25} (days)	0.47 a	0.26 c	0.32 bc	0.34 b	0.44 a	

Means followed by the same letter within a column and/or a row in the same parameter are not statistically different (P> 0.01) according to the LSD test

Discussion

In leaves, a low fluence of red light may retard chlorophyll breakdown (Okada *et al.*, 1992; Jordi *et al.*, 1994; Kappers, 1998). However, the mechanism of light action in the control of leaf senescence remains unclear, since light has also been shown to accelerate this process (Biswal and Biswal, 1984).

Chlorophyll fluorescence of tomato seeds in all three classes was clearly shifted to a lower level when seeds had been stored under red light (245 μmol photons.m^{-2}.s^{-1}), as compared to seeds stored in darkness (Fig. 1), indicating an increased degradation of chlorophyll in the light. The chlorophyll degradation process occurred in a dry system (moisture content around 8%) at 35 °C within a period of 8 weeks or less and in high (red) light intensity. This process may be related to the slower decrease of germinability after storage under red light than after storage in darkness.

The most obvious reason for the better seed storability under red light is the lower moisture content which was approximately 1.5% lower than of the seeds deteriorated in darkness (Fig. 2). The temperature under red light conditions was maximally 1°C higher than in darkness and this is expected to enhance the deterioration process. Chlorophyll degradation of developing canola seeds (*Brassica napus*) increased as the seed moisture content decreased and the activities of peroxidase and chlorophyllase (enzymes that are involved in chlorophyll degradation) reached a maximum between 65-55% moisture content and decreased thereafter (Johnson-Flanagan and McLachlan, 1990 a,b). It was not reported whether the activity of these enzymes was still detectable at seed moisture contents below 45%. We have shown here that under red light irradiation chlorophyll degradation occurred at a seed moisture content of 8%. Thus, chlorophyllase and peroxidase may still be active during chlorophyll degradation at this low moisture content. These enzymatical reactions require water and, thus, it may be argued that the lower seed water content during controlled deterioration under red light may be caused by these enzymatic processes. However, since controlled deterioration occurred in a closed system with a relatively large buffering capacity of the saturated salt solution, as compared to the mass of seeds in the container, we must assume that the capacity of this system was sufficient to keep the water activity of the atmosphere constant and that throughout the controlled deterioration period there was equilibrium between atmospheric and seed moisture content. An alternative explanation, therefore, is that under red light the seeds took up less water from the atmosphere between 0 and 2 weeks. However, this can only be

explained by assuming that the red light stored seeds have an altered chemical composition or have been subjected to morphological changes. The latter possibility warrants further study.

It is possible that phytochrome is involved in controlling seed performance during controlled deterioration. Vertucci *et al.* (1987) have hypothesised that photoreactions do not require thermal energy and likely occur at all moisture contents. Moreover, photoreactions were enhanced by an increase of seed hydration from 8 to 18%. Kendrick (1974) already found that irradiation with red light of freeze-dried *Pisum* epicotyl tissue in which phytochrome is in the P_r form resulted in the production of intermediates in the $P_r \rightarrow P_{fr}$ pathway. Kendrick and Spruit (1977) stated that seeds dried under continuous red light should contain a large proportion of total phytochrome "frozen" as intermediates, and these seeds germinated in darkness. In many seeds stored in the dark, a large proportion of the total phytochrome measured in the dry seed was found to be in the P_{fr} from (Kendrick and Spruit, 1977). We hypothesis that this P_{fr} keeps reactions that contribute to chlorophyll degradation proceeding, resulting in a loss of chlorophyll and, hence, in better seed performance.

It is also possible that the better seed performance after storage under red light is due to an increase in the amount of carotenoids. Carotenoids may play a role in scavenging singlet oxygen, which is generated by an excited state of chlorophyll by red light. The protective mechanism operates by energy transfer from the excited chlorophyll and singlet oxygen to the carotenoid, which absorbs and dissipates it without chemical change (Rau, 1988; Diplock, 1994). Synthesis of carotenoids still occurred, albeit at a slow rate, in the presence of light during the drying of pepper fruits and maximum concentrations were reached between 24 and 90 hours, after which a significant loss appeared (Minguez-Mosquera *et al.*, 1994). In our previous experiments, using high performance liquid chromatography, we detected the synthesis of carotenoids after chlorophyll degradation in tomato seeds (Chapter 3). A similar process was found by Ikoma *et al.* (2001) in *Citrus unshiu* Marc. fruits.

The mechanisms of free radical production in ageing seeds are still unclear. In general, thiols are among the cellular compounds that are firstly affected during oxidative stress, because of the susceptibility of their sulfohydryls group(s) to oxidation. However, it seems unlikely that this system is similarly active in hydrated and dry seeds (De Vos *et al.*, 1994). The red light may enhance oxidative stress during storage, due to the occurrence of a photoinhibition process. Powles (1984) stated that photoinhibition is induced by visible light (400-700 nm) and not the consequence of bulk pigment destruction, but rather of bleaching that occurs only after a certain degree of photoinhibition has been attained. In this experiment we have demonstrated the occurrence of chlorophyll disappearance in red light, but there was no significant difference of the antioxidant level (ascorbic acid and non-protein thiols) between seeds stored under red light and darkness.

GSH of tomato seeds decreased during storage as reported by De Vos *et al.* (1994) and in sunflower seeds (De Paula *et al.*, 1996). The opposite trend in ascorbic acid content was detected in this experiment (Table 4). Studying spinach (*Spinacia oleracea* L.) chloroplasts Foyer and Halliwell (1976) proposed that glutathione functions to stabilise enzymes of the Calvin cycle, and it may also act to keep ascorbic acid in the chloroplast in the reduced form, according to the reaction:

dehydroascorbate + 2 GSH → GSSG + ascorbate

As the GSH level declines, the resynthesis of GSH from GSSG (requiring photosynthetic NADPH) apparently was not occurring or not efficient enough.

The results obtained in the present study are not in agreement with observations obtained with dry seeds of *Acer platanoides*, *Vicia faba* L., *Pinus pinea*, *Avena sativa*, and wheat in which ascorbic acid was not present and only small amounts of dehydroascorbate (DHA) were detected (Pukacka, 1991; Arrigoni *et al.*, 1992; De Gara *et al.*, 1997; Tommasi *et al.*, 1999). In sunflower seeds neither ascorbic acid nor DHA were detected, and the major detoxifying mechanism was the GSH system (De Paula *et*

al., 1996). In dry seeds, ascorbate may be oxidised during the desiccation phase of seed maturation and cannot be generated when the moisture content becomes too low for the operation of the ascorbate-glutathione cycle (Smirnoff and Wheeler, 2000). Moreover, in a storage experiment with soybean seeds, ascorbate levels were estimated as an ascorbate/dehydroascorbate ratio and there was an indication of a smaller proportion of ascorbate occurring in the reduced form after storage. The ratio was 1.6 in axes from control seeds and 1.0 in axes from naturally aged seed (Senaratna *et al.*, 1988). We were able to detect very low amounts of ascorbic acid at the beginning of the storage period, which increased by the end of the storage period, whereas DHA could not be detected. Apparently, in the presence of glutathione, there was a reduction of DHA to ascorbic acid.

Tomato seed deterioration seems not directly related to changes in the levels of GSH. De Vos *et al.* (1994) demonstrated that ageing in tomato seeds involved GSH oxidation to GSSG, which is indicative of oxidative stress, although the lowered viability of aged seeds could not directly be attributed to the decreased GSH pool or to the highly oxidised glutathione redox status. They proposed that if the observed oxidation of GSH in ageing tomato seeds was generated from metal-catalysed 'autoxidation', GSH and other cellular thiols could both act as antioxidant and sources of free radicals.

We could not detect a significant effect of the chlorophyll level on ascorbic acid and non-protein thiol contents of the seeds. The lower performance of seeds with the lowest chlorophyll levels may have resulted from harvesting overmature fruits. Overripe tomato fruits may yield less vigorous seeds (Valdes and Gray, 1998). The lack of relationship between chlorophyll level in seeds with antioxidant levels in this present study may be due to the small range of chlorophyll levels that were used, and to harvesting seeds towards the end of maturation. For future experiments seeds with a larger range of chlorophyll levels at the beginning of the maturation stage should be used.

In conclusion, chlorophyll fluorescence of tomato seeds decreased during the controlled deterioration period in continuous red light, and seeds displayed a better performance as compared to seeds stored in darkness. A decrease of non-protein thiol content accompanied by an increase in the content of AsA upon controlled deterioration suggest the presence of the AsA/GSH cycle in tomato seeds of low moisture content. There was no clear relation between the activity of this cycle and the (changes in) chlorophyll content during controlled deterioration.

Acknowledgement

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Chapter 7

General discussion

Introduction

The presence of chlorophyll in seeds and its relation with the progress of seed maturation has gained renewed interest after the development of LIF by Jalink *et al.* (1996, 1998, 1999). An exponential relationship between the amount of chlorophyll and chlorophyll fluorescence ($P<0.001$) in developing tomato seed was observed during our preliminary studies (Fig. 1). This made the analysis of seed chlorophyll content in relation with seed performance easier and faster. However, most important is the non-destructive character of the LIF technique which makes it possible to relate chlorophyll content with performance of single seeds or classes of seeds.
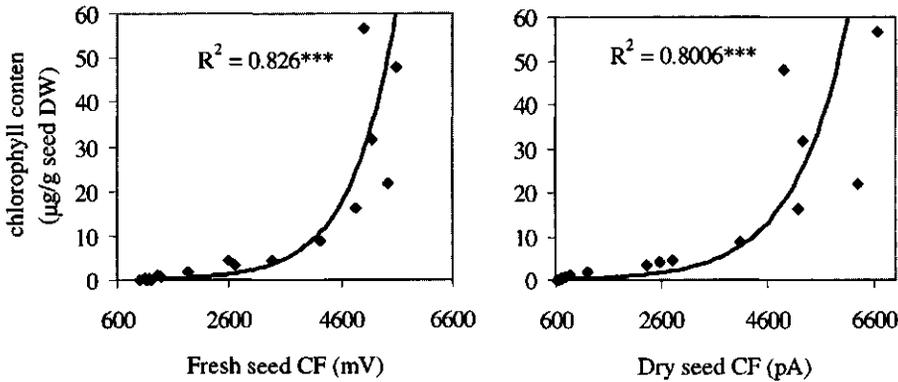
Fig. 1. *Correlation plots of fresh (using Xe-PAM) and dry seed (using LIF) chlorophyll fluorescence (CF) and seed chlorophyll content in developing tomato seeds (21 DAF until 75 DAF). *** = significant (P<0.001).*

There is controversy concerning the moment when tomato seeds attain physiological maturity or maximum seed quality. In Chapter 2 we showed that the maximum quality of tomato seeds was achieved when the chlorophyll fluorescence reached a minimum and levelled off afterwards. In this chapter it was also shown that it is essential to use the same stage of seed maturation in studying physiological and

biochemical activities. There were various levels of seed maturation at the same stage of fruit development, which is corroborated by the large standard deviation in seed chlorophyll fluorescence within the same fruit maturation stage (Fig. 1b, Chapter 2). The standard deviation became smaller when seeds attained maximum quality. This result indicates that only assessment of a maturation marker of each individual seed will lead to unequivocal establishment of a relationship between maturation stage and seed performance (quality). Because of its non-destructive character and the possibility to measure LIF in individual seeds, as well as the consistent relationship between chlorophyll content and progress of seed maturation we used seed chlorophyll fluorescence to analyse seed performance in relation to developmental processes

Chlorophyll in tomato seed is physiologically active

Using fluorescence microscopy we showed that most of the chlorophyll was located in the seed coat (Chapter 2), and applying a sensitive chlorophyll fluorescence imaging technique, we were able to detect the presence of chlorophyll in the embryo as well (Chapter 3). Not much is known about the functionality of chlorophyll in tomato seeds. Most information of the function of chlorophyll in seeds has been obtained in *Brassica* spp., soybean and pea (Chapter 1). In the present study we have demonstrated that chlorophyll in tomato seeds is physiologically active and may be functional. *In vitro*, photosynthetic oxygen evolution and Rubisco activity was detected in young seeds (30 DAF until 40 DAF; Chapter 3). Photosynthetic activity was high in 30 DAF seeds and lower at 40 DAF (Chapter 3). Also, carotenoid compounds were present in 30 DAF and 40 DAF seeds, suggesting active photosynthesis at this stage of development. Cogdell (1988) stated that carotenoids act as photoprotective agents and as accessory light-harvesting pigments. The first function is essential; in the absence of carotenoids there would be no photosynthetic activity in the presence of oxygen. Since Rubisco activity and photosynthetic oxygen evolution were observed in young tomato seeds, we may conclude that there is photosynthetic activity in tomato seeds, although it is difficult to imagine to what extent this use of energy has led to an evolutionary

advantage. Li *et al.* (2000) hypothesised that during the evolution of oxygenic photosynthesis, members of the light-harvesting complex (LHC) protein family with roles in photoprotection appeared before those involved in light harvesting. Since chlorophyll is degraded dramatically during maturation, this protection system might be prolonged by carotenoids (Chapter 3). Although further studies are needed, it has been shown that chlorophyll content and photosynthetic activity of seeds harvested from small fruit varieties decreased faster than from big fruit varieties, and also seed chlorophyll content of Cherry Bush and Cherry Yellow was lower than that of its wild related species (Chapter 3), indicating a deterioration of chlorophyll content and functionality during domestication.

Tomato seed contains about 18-20% crude oil (Lazos and Kalathenos, 1988; Liadakis *et al.*, 1995) and more than 75% of the fatty acids from this oil are unsaturated (Abdel-Rahman, 1982; Lazos and Kalathenos, 1988; Roy *et al.*, 1996). Tomato seeds also accumulated starch around 42 DAF (Chapter 3). After starch accumulation around mid-development of oleogenic seeds, the starch is mobilised, possibly to be used for triacylglycerol synthesis, although this has not been demonstrated (Bewley and Black, 1994). Moreover Apuya *et al.* (2001) showed that embryos of Arabidopsis require functional chloroplasts for normal development. Chlorophyll in developing seeds might be used for photosynthetic reactions to generate ATP and NADPH required for the conversion of maternally supplied sucrose to fatty acids for oil synthesis and storage (Singal *et al.*, 1987; Asokanthan *et al.*, 1997).

Seed chlorophyll content and performance

Experiments were executed to answer the question whether low seed chlorophyll content is associated with high performance. This relationship appears to hold true prior to the attainment of physiological maturity (Chapter 2). However, after physiological maturity there is no clear evidence for this negative correlation. In Chapter 6, for example, chlorophyll degradation appeared to occur in red light-controlled deterioration, but not in the dark-controlled deterioration. Seed performance in the

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former condition was better than in the latter. On the other hand, there were indications that matured seeds with high chlorophyll content have better performance than overmature seeds with low chlorophyll content.

Figure 2 presents schematically the relations proposed between chlorophyll content and seed performance. In general, chlorophyll content of tomato seed builds up during early seed development and decreases during maturation (Chapters 2, 3, 4 and 5). After physiological maturity, seed chlorophyll content slightly decreased in
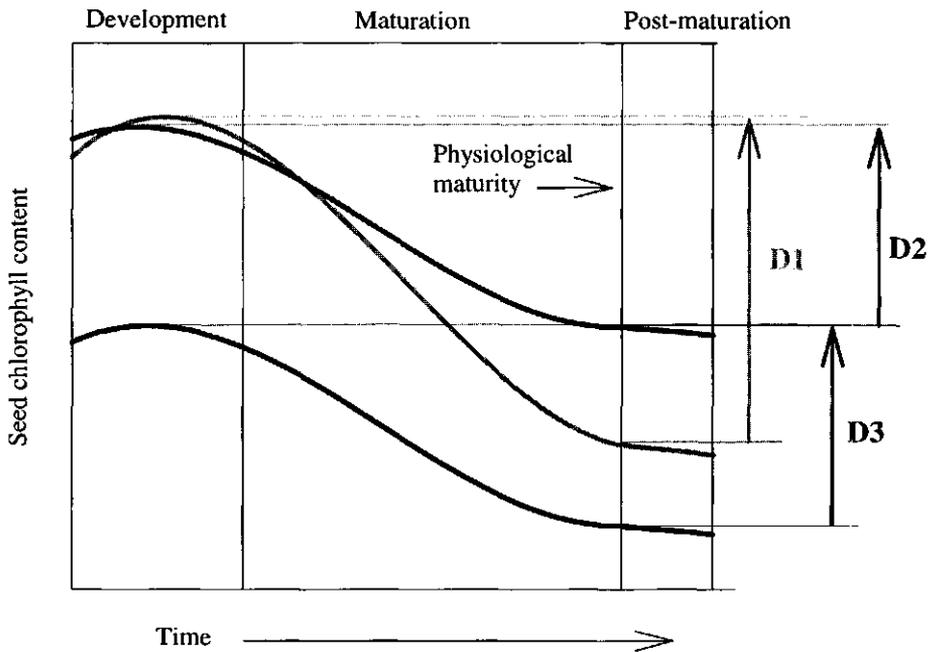
Fig. 2. *Schematic model for the relationship between the amount of chlorophyll and seed performance upon development and maturation*

Table 1. *The relationship between the amount of chlorophyll and seed performance*

Experiment	Delta chlorophyll (D)	Germination performance	Dormancy
Seeds harvested from covered fruit versus control (Fig. 2, Chapter 4)			
- control	D1	High	no dormancy
- covered	D3	Low	dormant
Seeds harvested from stored fruits versus control (Fig.5, Chapter 4)			
- control	D1	High	no dormancy
- storage fruits	D2	Low	dormant
Seeds of wild type, *hp-1* and *au* (Fig. 2, Chapter 5)			
- wild type	D1	High	no dormancy
- *hp-1*	D1	High	no dormancy
- *au*	D3	Low	dormant

overmature seeds (post-maturation; Chapter 6). The difference between maximum chlorophyll content and chlorophyll content at the stage of physiological maturity (Delta Chlorophyll = D) may describe the status of seed performance. Seeds with a high D (D1) value will have a better performance than seed with a low D value (D2; Chapter 4, D3; Chapter 4 and 5). Seeds with low D values, such as D2 and D3 may also possess dormancy. In fruit covering and fruit storage experiments we found the D2 and D3 phenomenon, whereas the _au_ mutant displayed the D3 phenomenon (Table 1). There were indications that the slight decrease in chlorophyll content during post-maturation reduced seed performance (Chapter 6). This schematic model could not be applied to _sit^w_ mutant seeds, because they developed in the D3 condition but performed as well as seeds in D1 conditions (Chapter 5). Besides, after reaching physiological maturity, _sit^w_ seed chlorophyll content decreased, due to the occurrence of viviparous germination.

Light influences chlorophyll content during seed development

It has been shown in Chapter 3 that seed chlorophyll content during development and maturation (30 DAF until 60 DAF) in tomato seeds grown in spring was lower than in seeds grown in autumn. Light intensity and day length have been suggested to influence this difference in chlorophyll content. It was shown in Chapter 4 that seeds that had developed in darkness contained lower amounts of chlorophyll. As mentioned in Chapter 2, the decay of chlorophyll of seeds harvested from small-fruit varieties was faster than of seeds harvested from big-fruit varieties, suggesting the involvement of light during chlorophyll synthesis and breakdown. Small fruit has thinner fruit pericarp tissue than big fruit, allowing light to penetrate to the seeds and accelerate chlorophyll degradation. Day length may also be involved in the accumulation of chlorophyll during seed development and maturation but we do not have data to support this statement.

It is well known that light controls the development of chloroplasts. The changes of light quality affect the balance of chloroplast gene expression of photosystem I and II (Pfannschmidt _et al._, 1999). In _Arabidopsis thaliana_ leaves, high light intensity reduced

the amount of granal thylakoids per chloroplast, the proportion of chlorophyll *b* relative to chlorophyll *a*, and the accumulation of the major Lhcb polypeptides (Weston *et al.*, 2000). Under natural conditions tomato seeds develop in relatively low light intensity. Less than 11% of the incident white light reached the developing seeds (Chapter 2). However, also tomato seeds appear to require light since their development in the dark resulted in seeds with low performance. The light may provide the energy to engage photoprotective mechanisms for proper embryo development. Moreover, Li *et al.* (2000) showed that the PsbS protein (a member of the chlorophyll *a/b*-binding, LHC family of protein) contributes to photoprotective energy dissipation rather than photosynthetic light harvesting.

Abscisic acid and gibberellins

Abscisic acid and gibberellins (GAs) play an important role in seed development (Bewley and Black, 1994). In comparison with wild type and the *gib1* mutant, *sit^w* seeds accumulated a lower chlorophyll content upon development and maturation (Chapter 5) although a post-maturation increase could be observed, in this mutant which is presumably related to the occurrence of viviparous germination. In contrast, ABA-insensitive mutants of *Arabidopsis thaliana* seeds failed to degrade chlorophyll during maturation and showed poor longevity and desiccation tolerance (Ooms *et al.*, 1993). Koornneef *et al.* (1984) also have shown that the ABA content of ABA-insensitive seeds was higher than that of the wild type and ABA-deficient mutants. ABA-deficient *viviparous 12* maize mutant seeds are also deficient in carotenoids and chlorophyll synthesis (Maluf *et al.*, 1997). They also showed that this mutant has a lower expression of geranylgeranyl pyrophosphate synthase (GGPPS), the enzyme that is responsible for the synthesis of geranylgeranyl pyrophosphate, the precursor of ABA, carotenoids and chlorophyll.

There is no clear information on the effect of endogenous GA on chlorophyll synthesis and degradation in seeds (Chapter 5), although we found that the chlorophyll level in young *gib1* seeds tended to be higher than in young wild type seeds

(unpublished results). In the early stages of carotenoid-, chlorophyll-, ABA- and GA-biosynthesis, mevalonic acid is the precursor that is converted into the C5 compound isopentenyl diphosphate (IDP; Fig. 3; Britton, 1988). Some of the IDP isomerized to dimethylallyl diphosphate. From these precursors, the isoprenoid chain is built by means of prenyl transferase enzymes synthesizing geranyl diphosphate (GDP), farnesyl diphosphate (FDP) and geranylgeranyl diphosphate (GGDP), a key step in the terpenoid pathway, resulting in the formation of a precursor for the synthesis of the chlorophyll side chain phytol and GA (Britton, 1988; Tudzynsky, 1999). In the *gib1* mutant, the activity of ent-kaurene synthetase A is reduced (Bensen and Zeevaart, 1990). It is possible that the lack of an adequate level of GA after blocking at this specific step of the GA biosynthesis pathway leads to the increase of pigmentation, both chlorophyll and carotenoids (Fig. 3). However, the ABA content of *gib1* mutant seeds was not higher than in wild type (H.W.M. Hilhorst, personal communication).

Is oxidative stress related to seed chlorophyll content?

To prevent oxidative stress, seeds have several antioxidant systems, including non-enzymatic antioxidant compounds, such as glutathione (GSH), tocopherol and ascorbic acid (Hendry *et al.*, 1992). In contrast with ascorbic acid content, non-protein thiols of seeds decreased during controlled deterioration (Chapter 6). It is questionable whether the ascorbate/glutathione cycle occurs in dry seeds; most authors found this cycle to occur in wet systems (leaves) only. However, because of the limited information, further experiments are needed to confirm this result.

The results presented in Chapter 6 also show that there was no clear indication that mature seeds with higher chlorophyll content were more stressed during controlled deterioration than mature seed with lower chlorophyll content. However, there was strong evidence that seeds subjected to red light-controlled deterioration (RD) contained lower chlorophyll content than seeds from dark-controlled deterioration (DD). The RD-seeds displayed better germination performance than DD-seeds during 12 weeks of controlled deterioration. The question remains whether the better performance of RD-

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Summary

Seed quality is a complex concept, consisting of a number of components that each represent different physiological principles, e.g. germinability, viability, vigour and storability. Because of this complexity it has been problematic to find physical, biochemical or molecular markers that unequivocally and reproducibly predict seed performance in the field or during storage. Seed quality is acquired during the maturation phase of seed development and it has been shown recently that chlorophyll can be used as a marker for seed maturity and, thus, indirectly for seed quality.

The aim of this thesis was to (i) determine whether chlorophyll fluorescence could also be used as a marker for seed maturity in tomato and (ii) to address the question whether chlorophyll or its degradation products could influence seed quality.

We show that chlorophyll can also be used as a marker for physiological maturity of tomato seeds. The chlorophyll content of the seeds was negatively correlated with germinability towards the end of maturation. Maximum germinability and maximum normal seedling percentage were achieved at 51-54 days after flowering (DAF) when the chlorophyll fluorescence of both freshly harvested and dried seeds, as well as that of the fruit reached a minimum (Chapter 2). It was concluded that chlorophyll fluorescence appeared to be a sensitive indicator of physiological maturity of tomato seeds.

Fluorescence microscopy showed that the majority of chlorophyll is located in the seed coat, whereas chlorophyll fluorescence imaging analysis also showed low levels of chlorophyll in the embryo, mainly in the radicle tip (Chapter 3). Besides chlorophyll a and b, young tomato seeds (30-40 DAF) contained the carotenoids neoxanthine, violaxanthine, lutein and zeaxanthine, and β-carotene. In freshly harvested seeds chlorophyll was active and a significant quantum yield, Rubisco activity and photosynthetic oxygen evolution could be determined _in vitro_ until 40 DAF (Chapter 3).

Chlorophyll fluorescence of tomato seeds was influenced by both the size of the fruit and the light conditions during the growth period. Chlorophyll fluorescence of

seeds from small-fruit varieties declined faster during maturation than chlorophyll fluorescence of seeds from big-fruit varieties, presumably because seeds in the small-fruit varieties were exposed to a higher light intensity than those in the big fruits. Also, spring-grown seed chlorophyll fluorescence was lower than that of seeds grown in autumn, presumably because of conditions of lower light intensity during (early) spring.

Seeds from the phytochrome-deficient *au* mutant displayed lower chlorophyll fluorescence than seeds from the wild type and the high-pigment *hp-1* mutant, but their photosynthetic potentials were similar, which indicated that the level of chlorophyll did not influence its functionality or efficiency. It was concluded that chlorophyll fluorescence was influenced by the level of active phytochrome in the seed and that the rate of its degradation depended on the amount of light that could penetrate the fruit tissues (Chapter 3).

To further explore the influence of light intensity on the formation and degradation of chlorophyll during seed development, fruits were grown under full light, reduced light intensity and in the dark, by covering the fruits with black cloth. Seeds from fruits grown in darkness or under reduced light intensity produced higher numbers of abnormal seedlings and deteriorated faster in a controlled deterioration test than seeds from fruits grown under full daylight. As lower light intensities resulted in lower chlorophyll fluorescence, both during early development and maturation, it was concluded that the presence of chlorophyll is a prerequisite for normal seed development (Chapter 4).

Storage of mature green fruits under full daylight or in darkness for 10 days showed that degradation of seed chlorophyll proceeded faster in the light than in the dark but that seeds from the dark-stored fruits had considerably better germination performance. These results made clear that the correlation between low chlorophyll content and high seed quality is not always evident and may depend on growth and (fruit) storage conditions (Chapter 4).

Seed chlorophyll fluorescence was also affected by abscisic acid and gibberellins. Compared to wild type and the gibberellin-deficient *gib1* mutant, seeds

from the ABA-deficient sit^w mutant displayed lower chlorophyll fluorescence upon development and maturation. This could be explained by the fact that the biosynthetic pathways of ABA and chlorophyll overlap to some extent. However, chlorophyll fluorescence of the sit^w seeds increased during maturation. This increase in chlorophyll fluorescence confirmed the occurrence of viviparous germination of the sit^w seeds. Viviparous germination was accompanied by an increase in the number of seeds with free space, indicating precocious degradation of the surrounding endosperm (Chapter 5).

The presence of chlorophyll during maturation appears to be undesirable since it is generally associated with lower seed quality, particularly with respect to seed longevity. The relationship between the amount of chlorophyll in seeds and the level of oxidative stress during controlled deterioration (at 35°C and 75% relative humidity) was studied, both in darkness and under red light, to mimic practical storage conditions in the tropics. Under these stress conditions higher levels of oxidation are expected in seeds with higher chlorophyll content. Levels of the anti-oxidants ascorbic acid and non-protein thiols were monitored since these compounds are claimed to be associated with the regulation of oxidative injury in seeds. In general, ascorbic acid levels increased during controlled deterioration whereas the non-protein thiol content decreased sharply (Chapter 6).

In darkness the decay in seed performance was faster than that of seeds that deteriorated under red light. A decrease of chlorophyll fluorescence was observed after controlled deterioration in red light, but not in darkness. Performance of seeds after deterioration under red light was significantly better than that of seeds after deterioration in darkness. This result confirmed the earlier conclusion that chlorophyll fluorescence and seed quality are negatively correlated. It is clear that chlorophyll is undesirable for the maintenance of seed quality, although we could not provide conclusive evidence that the differences in chlorophyll content of the mature seeds was related to differing levels of oxidative stress during controlled deterioration (Chapter 6).

Finally, a general discussion of the role of chlorophyll in developing tomato seeds is presented, based on the results from this thesis as well as the literature. A

descriptive model is proposed to indicate the possible links between the amount of chlorophyll and seed performance and quality. The difference between the chlorophyll content during development and during maturation may reflect the quality status of tomato seed (Chapter 7). The overall conclusion is that chlorophyll is required during seed development but undesirable during maturation.

Samenvatting

Zaadkwaliteit is een complex begrip omdat het een aantal onderdelen bevat die elk gebaseerd zijn op verschillende fysiologische principes, b.v. kiemkracht, levensvatbaarheid, 'vigour' en bewaarbaarheid. Vanwege deze complexiteit bestaat er tot op heden geen fysische, biochemische of moleculaire merker die ondubbelzinnig en reproduceerbaar het gedrag van zaden in het veld of tijdens opslag kan voorspellen. Zaadkwaliteit wordt opgebouwd tijdens de afrijpingsfase van de zaadontwikkeling. Recent is aangetoond dat chlorofyl gebruikt kan worden als indicator van de voortgang van de afrijpingsfase en dus indirect van de zaadkwaliteit.

Het doel van dit proefschrift was (i) vaststellen of chlorofyl fluorescentie ook gebruikt kon worden als een merker voor de kwaliteit van tomatenzaad en (ii) de vraag beantwoorden of chlorofyl, of afbraakprodukten ervan, de zaadkwaliteit kunnen beïnvloeden.

Aangetoond werd dat chlorofyl inderdaad gebruikt kan worden als een merker voor fysiologische rijpheid van tomatenzaad. Het chlorofylgehalte van de zaden was negatief gecorreleerd met de kiemkracht tegen het einde van de afrijpingsfase. Maximale kiemkracht en maximum percentage normale zaailingen werden 51-54 dagen na de bloei bereikt. Op dat moment was de chlorofylfluorescentie van zowel vers geoogste als tussentijds gedroogde zaden alsmede van de vruchten, tot een minimum gedaald (Hoofdstuk 2). Geconcludeerd werd dat chlorofylfluorescentie een gevoelige indicator is voor de fysiologische rijpheid van tomatenzaad.

Fluorescentiemicroscopie liet zien dat het grootste deel van het chlorofyl zich in de zaadhuid bevindt. Met behulp van chlorofyl fluorescentie 'imaging' werd echter aangetoond dat er ook een kleine hoeveelheid chlorofyl in het wortelpuntje van het embryo aanwezig is (Hoofdstuk 3). Naast chlorofyl a en b bleken jonge tomatenzaden (30-40 dagen na de bloei) ook de carotenoïden neoxanthine, violaxanthine, luteine en β-caroteen te bevatten. Chlorofyl bleek in vers geoogste zaden actief te zijn. Tot 40 dagen

na de bloei kon een significante quantumopbrengst, Rubisco activiteit en fotosynthetische zuurstofafgifte waargenomen worden (Hoofdstuk 3).

De chlorofylfluorescentie van tomatenzaad werd beïnvloed door zowel de grootte van de vrucht als de lichtcondities tijdens de groeiperiode. De chlorofylfluorescentie van zaden afkomstig van rassen met kleine vruchten daalde sneller tijdens de afrijping dan die van rassen met grote vruchten. Waarschijnlijk worden de zaden in de kleine vruchten blootgesteld aan een hogere lichtintensiteit dan die in grote vruchten. Ook bleek de chlorofylfluorescentie van zaden die in het voorjaar waren geproduceerd lager te zijn dan die van zaden afkomstig van een zomerteelt, waarschijnlijk omdat de gemiddelde lichtintensiteit tijdens het (vroege) voorjaar lager was dan in de late zomer en het vroege najaar.

Zaden afkomstig van de fytochroom-deficiente *au* mutant lieten een lagere chlorofylfluorescentie zien dan zaden van het wild type en van de high-pigment *hp-1* mutant. Niettemin was de fotosynthetische potentie van de verschillende genotypen vergelijkbaar, wat aanleiding gaf tot de conclusie dat het chlorofylgehalte geen invloed heeft op de functionaliteit of efficiëntie ervan. Tevens werd geconcludeerd dat chlorofylfluorescentie beïnvloed wordt door de hoeveelheid actief fytochroom in het zaad en dat de snelheid van de chlorofylafbraak afhangt van de hoeveelheid licht die de vruchtweefsels kan passeren (Hoofdstuk 3).

Voor nader onderzoek van de invloed van de lichtintensiteit op de vorming en afbraak van chlorofyl tijdens de zaadontwikkeling, werden vruchten gekweekt onder vol daglicht, gereduceerd daglicht en in volledig donker, door de vruchten te bedekken met zwart doek. Zaden van vruchten die waren gegroeid in het donker of met gereduceerde lichtintensiteit produceerden grotere aantallen abnormale zaailingen en verloren sneller hun vitaliteit in een versnelde verouderingstest dan zaden van vruchten die onder volle lichtomstandigheden waren gegroeid. Aangezien lagere lichtintensiteit leidde tot lagere chlorofylfluorescentie, zowel tijdens de vroege ontwikkeling als tijdens de afrijping, werd geconcludeerd dat de aanwezigheid van chlorofyl een voorwaarde is voor normale zaadontwikkeling (Hoofdstuk 4).

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Bewaring gedurende 10 dagen van volgroeide groene vruchten onder vol daglicht of in volledig donker liet zien dat de afbraak van chlorofyl sneller verliep in het licht dan in het donker. Wel bleken de zaden van de in het donker bewaarde vruchten beter te kiemen. Hieruit blijkt dat de relatie tussen laag chlorofylgehalte en hoge zaadkwaliteit niet altijd opgaat en afhankelijk is van groei- en bewaarcondities van zowel vruchten als zaden (Hoofdstuk 4).

De chlorofylfluorescentie van het zaad werd eveneens beïnvloed door abscisinezuur (ABA) en gibberellinen (GAs). Vergeleken met het wild type en de gibberelline-deficiënte _gibl_ mutant, lieten zaden van de ABA-deficiënte _sit^w_ mutant een lagere chlorofylfluorescentie zien tijdens zaadontwikkeling en -afrijping. Dit kon worden verklaard uit het feit dat de biosyntheseroutes van ABA en chlorofyl deels gelijk zijn. Echter, de chlorofylfluorescentie van de _sit^w_ zaden nam toe tijdens de afrijping. Deze toename bevestigde het voorkomen van vivipare kieming van de _sit^w_ zaden. De vivipare kieming ging ook gepaard met een toename van het aantal zaden met een zg. 'free space', een indicatie van een voortijdige afbraak van het endospermweefsel rondom het embryo (Hoofdstuk 5).

De aanwezigheid van chlorofyl tijdens de afrijping lijkt ongewenst te zijn omdat die in het algemeen wordt geassocieerd met een lagere zaadkwaliteit, vooral in relatie tot de levensduur van het zaad. De relatie tussen de hoeveelheid chlorofyl in het zaad en de mate van oxidatieve stress tijdens de versnelde veroudering (35 °C en 75% relatieve luchtvochtigheid) werd onderzocht, zowel in het donker als onder rood licht, om de praktische bewaaromstandigheden in de tropen na te bootsen. Onder deze stressvolle omstandigheden kunnen hogere niveaus van oxidatie verwacht worden in zaden met een hoger chlorofylgehalte. De gehalten aan de anti-oxidanten ascorbinezuur en niet-eiwit thiolen werden gevolgd omdat deze componenten betrokken zouden zijn bij de regulatie van oxidatieve stress. In het algemeen steeg de hoeveelheid ascorbinezuur tijdens de versnelde veroudering, terwijl het gehalte aan niet-eiwit thiolen sterk daalde (Hoofdstuk 6).

De afname van de zaadkwaliteit verliep sneller in het donker dan die van zaden die versneld verouderden onder rood licht. Een afname van de chlorofylfluorescentie werd waargenomen tijdens veroudering onder rood licht maar niet in het donker. De kwaliteit van de zaden die waren verouderd onder rood licht was aanmerkelijk beter dan die van zaden na veroudering in het donker. Dit resultaat bevestigde de eerdere conclusie dat chlorofyl fluorescentie en zaadkwaliteit negatief aan elkaar gerelateerd zijn. Het is duidelijk dat chlorofyl ongewenst is bij het handhaven van de zaadkwaliteit ofschoon geen doorslaggevend bewijs geleverd kon worden dat de verschillen in chlorofylgehalte de oorzaak waren van de verschillen in de mate van oxidatieve stress tijdens de versnelde veroudering (Hoofdstuk 6).

Gebaseerd op de resultaten beschreven in dit proefschrift alsmede resultaten uit de literatuur wordt tenslotte een algemene discussie over de rol van chlorofyl in zich ontwikkelende tomatenzaden gevoerd. Er wordt een beschrijvend model voorgesteld waarin de mogelijke verbanden tussen chlorofylgehalte en zaadkwaliteit worden aangegeven. Het verschil tussen het chlorofylgehalte tijdens de (vroege) zaadontwikkeling en dat tijdens de afrijping lijkt de kwaliteitsstatus van het zaad weer te geven (Hoofdstuk 7). De uiteindelijke conclusie is dat chlorofyl benodigd is voor normale zaadontwikkeling maar dat het ongewenst is tijdens de afrijpingsfase.

Ringkasan

Kualitas benih merupakan suatu konsep yang kompleks, mencakup sejumlah komponen yang mewakili prinsip-prinsip fisiologi, misalnya daya berkecambah, viabilitas, vigor dan daya simpan. Hal ini menimbulkan kesulitan untuk menemukan penciri (marker) fisik, biokimia maupun molekuler yang tepat dan dapat diulang yang dapat memprediksi kualitas benih di lapangan maupun di penyimpanan. Benih berkualitas diperoleh dan ditentukan oleh tingkat kemasakan selama perkembangan benih dan telah ditunjukkan bahwa klorofil dapat digunakan sebagai penciri kemasakan benih, atau secara tidak langsung merupakan penciri kualitas benih juga.

Tujuan penelitian ini adalah (i) menentukan apakah fluoresen dari klorofil (klorofil fluresen) dapat digunakan sebagai penciri tingkat kemasakan benih tomat dan (ii) menjawab pertanyaan apakah klorofil dan atau produk dari proses peluruhan klorofil dapat mempengaruhi kualitas benih.

Hasil penelitian ini menunjukkan bahwa klorofil dapat digunakan sebagai penciri masak fisiologi benih tomat. Kandungan klorofil benih berkorelasi negatif dengan daya berkecambah sampai tahap akhir periode pemasakan benih. Maksimum daya berkecambah dan persentase kecambah normal tercapai saat 51-54 hari setelah berbunga (HSB) ketika klorofil fluresen dari benih segar maupun benih kering mencapai minimum, demikian juga fluresense dari buah (Bab 2). Dapat disimpulkan bahwa fluresen dari klorofil merupakan indikator yang sensitif untuk menetapkan masak fisiologi benih tomat.

Pengamatan dengan mikroskop fluresen menunjukkan bahwa klorofil sebagian besar terletak di kulit benih, namun dengan _teknik imaging_ dapat ditunjukkan bahwa embrio benih tomat juga mengandung klorofil. Pada akhir periode pemasakan benih, klorofil masih dapat dideteksi di area radikel (Bab 3). Selain klorofil a dan b, benih tomat muda (30 sampai 40 HSB) juga mengandung carotenoids (neoxanthine, violaxanthine, lutein, zeaxanthine dan ß–caroten). Klorofil dalam benih tomat muda (sampai dengan 40 HSB) masih aktif terlibat dalam proses fotosintesis _in vitro,_

tercermin dari parameter potensi photosintesis (yield), aktivitas Rubisco dan oksigen evolution (Bab 3).

Klorofil fluresen benih tomat dipengaruhi oleh ukuran buah dan kondisi cahaya selama pertumbuhan benih. Klorofil fluresen benih dari varietas-varietas berbuah kecil meluruh lebih cepat daripada varietas-varietas berbuah besar. Diduga bahwa benih dari varietas-varietas berbuah kecil tumbuh dalam kondisi dengan intensitas cahaya yang lebih tinggi daripada benih dari varietas-varietas berbuah besar. Demikian pula benih yang ditanam saat musim semi memiliki klorofil fluresen yang lebih rendah daripada benih hasil pertanaman pada musim gugur. Hal ini menguatkan dugaan bahwa kondisi cahaya mempengaruhi klorofil fluresen benih.

Benih mutan dari *aurea* (phytochrome defisiensi) memiliki klorofil fluresen yang lebih rendah daripada tetuanya (wild type) maupun dari *hp-1* (mutan yang memiliki photorespon berlebihan atau *exaggerated photoresponse*). Namun, benih dari ketiga genotipe tersebut memiliki potensi fotosintesis yang tidak jauh berbeda. Hal ini mengindikasikan bahwa tingkat kandungan klorofil benih tidak mempengaruhi fungsi dan efisiensinya. Kesimpulan dari Bab 3 adalah bahwa klorofil fluresen dipengaruhi oleh kandungan phytochrome benih, dan laju peluruhan klorofil benih tergantung dari jumlah cahaya yang dapat melalui pericarp buah dan mencapai benih.

Untuk mengungkap pengaruh intensitas cahaya dalam pembentukan dan peluruhan klorofil selama pertumbuhan benih, dilakukan percobaan penutupan buah dengan kain hitam untuk memperoleh perbedaan tingkat pencahayaan. Benih dari buah yang ditumbuhkan dalam kondisi gelap dan intensitas cahaya rendah memiliki kualitas (persen kecambah normal) dan daya simpan yang lebih rendah daripada kontrol (buah ditumbuhkan dalam kondisi cahaya alami). Karena intensitas cahaya rendah menghasilkan benih dengan tingkat klorofil rendah baik selama pertumbuhan maupun pemasakan, maka disimpulkan bahwa klorofil sangat diperlukan dalam pertumbuhan benih (Bab 4).

Penyimpanan buah hijau masak (45 HSB) dalam kondisi cahaya penuh atau dalam gelap selama 10 hari menunjukkan masih terjadinya peluruhan klorofil dalam

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benih. Laju peluruhan klorofil benih lebih tinggi bila buah disimpan dalam kondisi cahaya penuh daripada dalam gelap. Namun kualitas benih dari buah yang disimpan dalam gelap akan lebih baik. Hasil ini menunjukkan bahwa hubungan antara rendahnya klorofil dalam benih tidak selalu menghasilkan benih berkualitas tinggi. Nampaknya kondisi pertumbuhan dan penyimpan buah juga mempengaruhi kualitas benih (Bab 4).

Klorofil fluresen benih juga dipengaruhi oleh asam abscisic dan gibberellin. Dibandingkan dengan tetuanya dan mutan gibberellin atau *gib-1* (gibberellin-deficient mutant), benih dari mutan ABA atau *sit^w* (ABA-deficient mutant) memiliki klorofil fluresen lebih rendah selama pertumbuhan dan perkembangan benih. Hal ini mungkin dapat menunjukkan fakta bahwa terdapat hubungan antara biosintesis klorofil dan ABA. Klorofil fluresen benih dari *sit^w* meningkat setelah periode pemasakan. Hal ini menunjukkan adanya sifat vivipari dari mutan tersebut. Sifat vivipari ini juga diperkuat dengan meningkatnya jumlah benih yang memiliki ruang bebas (free space) antara embrio dan endosperm. Munculnya ruang bebas dalam benih merupakan indikasi terjadinya peluruhan endosperm (Bab 5).

Kehadiran klorofil nampaknya tidak diinginkan dalam proses pemasakan benih karena berhubungan dengan rendahnya kualitas benih, khususnya dalam kaitannya dengan daya simpan. Hubungan antara tingkat kandungan klorofil dengan tingkat stres oksidasi selama proses penuaan benih (dalam kondisi 35°C dan kelembaban udara 75%) dituangkan dalam Bab 6.   Percobaan ini dilakukan di dua kondisi penuaan, yaitu dalam gelap dan cahaya merah.   Dalam kondisi stres ini diharapkan proses oksidasi terhadap benih berkandungan klorofil tinggi akan lebih besar, sehingga daya simpannya cepat menurun. Tingkat asam ascorbat dan non-protein thiol terus diamati selama penuaan, karena senyawa tresebut sangat berhubungan dengan proses oksidasi yang menimbulkan kemunduran benih. Selama penuaan nampak bahwa asam ascorbat dalam benih meningkat, sedangkan non-protein thiols menurun dengan tajam.

Kualitas benih menurun lebih cepat bila penuaan dilakukan dalam gelap dibandingkan dalam cahaya merah. Klorofil fluresen benih nampak jelas menurun setelah penuaan dalam cahaya merah, namun dalam proses penuaan dalam kondisi gelap

nampak tidak berubah. Hasil ini memperkuat kesimpulan sebelumnya bahwa klorofil fluresen dari benih berkorelasi negatif dengan kualitas benih. Nampak jelas bahwa klorofil sangat tidak diharapkan dalam mempertahankan kualitas benih, meskipun penelitian ini tidak membuahkan bukti yang sangat kuat bahwa tingkat kandungan klorofil benih akan menghasilkan perbedaan tingkat stres oksidasi selama proses penuaan (Bab 6).

Akhirnya, sebuah pembahasan umum tentang peranan klorofil dalam pertumbuhan benih disajikan dalam Bab 7, berdasarkan hasil dari penelitian-penelitian ini dan dari studi pustaka. Sebuah hipotesis diajukan dalam bentuk model tentang hubungan antara kandungan klorofil dalam benih dengan kualitas benih. Perbedaan (delta) antara kandungan klorofil benih dalam tahap pembentukan benih dan tahap pemasakan benih mungkin mencerminkan status kualitas benih tomat. Kesimpulan akhir adalah bahwa klorofil dibutuhkan dalam proses pertumbuhan (pembentukan) benih, namun sangat tidak diharapkan dalam periode pemasakan.

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Curriculum vitae
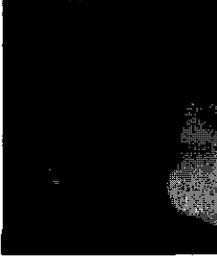
Mohamad Rahmad Suhartanto was born in Jember, a small city in the East Java province of the Republic of Indonesia on the 23rd of September 1963. In 1982 he obtained his certificate for secondary level education in Malang, a beautiful city in the East Java Province where he has spent more than 15 years living with his lovely parents and family. In June 1982, he was accepted to study in the Department of Agronomy of Bogor Agricultural University (IPB) in Bogor, a rainy city in the West Java Province, about 60 km from Jakarta. IPB is the only Indonesian higher education institution dedicated exclusively for agricultural, natural resources and environmental development. He chose seed science and technology as his major subject and he graduated in March 1987, and was immediately employed by the same university as a lecturer. The most exciting period was between 1992-1994, when he received his master degree in Seed Science from IPB (1994), and he found the person who would follow him on his journey in life, **Dyah Santi Puspitasari**, to whom he married (1992), and his son (**Azka Afuza Faris Nugroho**) was born (1993). In November 1997, two weeks after his daughter (**Abkarin Tara Nadhira**) was born, he came to the Netherlands to study at the Laboratory of Plant Physiology of Wageningen University in collaboration with the Centre for Plant Breeding and Reproduction Research (now Plant Research International) in Wageningen.

After finishing his Ph.D, his plan is to continue his task as a lecturer at IPB and to develop a seed laboratory using a biophysics approach. He intends to make a collaboration on seed science research with both the Laboratory of Plant Physiology of Wageningen University and Plant Research International.

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