QUALITY STANDARDS FOR SEED GERMINATION: THE USE OF NUCLEAR DNA AMOUNTS TO PREDICT STORAGE BEHAVIOR AND GERMINATION RATE

Raoul J. Bino, Steven P.C. Groot
and R.D. de Castro
Department of Reproduction Technology
Centre for Plant Breeding
and Reproduction Research (CPRO-DLO)
POB 16, 6700 AA Wageningen
the Netherlands

Yongqing Liu
Department of Horticulture
Hunan Agricultural University
Changsha 410128
P.R. China

1. High quality seed production

Production of high quality seed is the basis for a durable and profitable agriculture. After production, seed is processed, conditioned, stored, shipped and used throughout the world. Seed quality, as typified by general condition, purity and viability has to be controlled at all steps of the production chain until it is used for the next year' crop. Seed quality of many species is improved by preconditioning, cleaning and disinfection. Nevertheless, seed lots with a high germination capacity under laboratory conditions, may produce low amounts of normal seedlings under practical conditions. This difference causes financial losses through waste of plant material and inefficient use of facilities and labor. These losses are especially high in the labor- and cost-intensive cultivation of vegetables. New ways to control seed quality are therefore especially developed for the expensive vegetable seeds.

2. Tomato seeds

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important annual vegetables in the world. Many new varieties are created each year by intensive breeding efforts to meet the different requirements all over the world. Common tomato plants are diploid and reproduced by seeds. Newly bred varieties are almost all hybrids normally produced by artificial crossing. Therefore, high-quality seeds are required to match these high-cost hybrids to ensure their authentic value. Considerable efforts have been made to improve seed quality during development and after harvest (Heydecker and Coolbear 1987, Khan 1991). More profound knowledge on the physiological and biochemical changes and their interactions during development, pre-treatment and germination will certainly contribute to the improvement of seed quality (Khan 1991). A tomato seed contains a fully developed embryo that is enclosed by a considerable amount of endospermal tissue and a thin seed coat with seed hairs (Smith 1935). Compared with the seed of *Arabidopsis thaliana*, a model plant species for scientific research, a tomato seed has the advantage of a fairly large size which enables the study of the location of physiological and biochemical activities in different seed parts during development and germination (Groot 1987).

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3. Embryogenesis

In tomato, the zygote starts to divide into an embryo proper and a suspensor at 5 days after pollination (DAP). Then the embryo proper grows very fast whereas suspensor cells almost cease to proliferate, resulting in a ball-shaped embryo at 10 DAP. The young embryo continuously grows and reaches the heart-shape stage at 15 DAP and the torpedo-shape or cotyledon-embryo stage at 20 DAP. The suspensor handle which anchors at the micropylar end, never disappears until the embryo has fully developed with long curved cotyledons at 30 DAP. At the same time, the fertilized central cell or the endosperm nucleus starts to divide and occupies the space where the nucleus tissue originally exists. Then, the developing seed starts to dehydrate. The dry weight of the developing seed continuously increases until 40 DAP when the milky endosperm becomes solid. At the same time, the seed gains its desiccation-tolerance and germinability. Thereafter, seed maturation continues until the acquisition of full germinability at 50 DAP or even later (Berry and Bewley 1991, Demir and Ellis 1992). However, under some climatic conditions, and in certain genotypes, tomato seeds may have primary dormancy at the end of the maturation period (Groot and Karssen 1992).

4. Dormancy

Dormancy is defined as the disability of seed to germinate under favorable environmental conditions. Dormancy is of ecological significance for seed species to survive in the wild (Hilhorst 1995). Although in seed physiology many terms are in use to classify dormancy, a simple, generally accepted classification factually distinguishes two types of dormancy: primary or secondary dormancy (Karssen 1982). This classification is meaningful because primary dormancy is essentially related to seed development and maturation whereas secondary dormancy can only occur after the seed is dispersed and is often subject to the annual dormancy cycle in the seed bank and is reversible. When primary dormancy is relieved and suitable conditions are present, germination may occur. If germination does not occur, secondary dormancy may have been induced. Secondary dormancy can be relieved and induced again many times from year to year. In the dormant state, seeds have low metabolism and are insensitive to the environment, thereby, prolonging the seed's life-span (Bewley and Black 1994).

In different seed species, dormancy may be induced through different mechanisms (Hilhorst 1995). The mechanism of dormancy is not fully understood. General speaking, dormancy is a consequence of the action of the internal factors like plant hormones, interacting with external factors such as light, temperature, moisture and inorganic chemicals. The presence of the hormone abscisic acid (ABA), as a general growth inhibitor, has long been associated with the induction of primary dormancy during seed development (Hilhorst and Karssen 1992). Characteristically, the ABA content increases during the first half of fruit development and to decline at the later phase of maturation, when the seed water content decreases (Bewley and Black 1994). ABA is produced in leaves and transported into fruit tissues and seed parts (Goldbach and Goldbach 1977, Dowdney and McWha 1979). Apart from the induction of dormancy, ABA is thought to prevent precocious germination (Farrant *et al.* 1993, Finch-Savage *et al.* 1992). As an alternative hypothesis it was suggested that ABA may be indirectly involved in the

prevention of viviparous germination by increasing the sensitivity of seeds to the osmotic environment (Groot and Karssen 1992).

In some cultivars like Moneymaker, tomato seeds may manifest primary dormancy after maturation under certain cultivation conditions (Groot 1987). The dormancy in tomato seeds is not a true dormancy (no germination at any set of environmental conditions), but a relative dormancy (only germinate at a restrict range of environmental conditions). Studies with the ABA-deficient tomato mutant (*sit*^{w}) showed that ABA plays an important role in the induction of primary dormancy (Groot and Karssen 1992). This primary dormancy in wild type tomato seeds can be relieved by a dry storage period (Groot 1987). Since secondary dormancy in tomato seeds can be induced by far-red light and retained in the dark, this particular type of dormancy is related to phytochrome action (Lercari 1991). Dormancy in many seed species can be relieved or broken by these physiological treatments (Khan 1991).

5. Osmotic priming

After harvest, seed quality in many crops can be increased by physiological treatments that are based on seed hydration such as presoaking, wetting, humidification, osmotic priming, solid matrix priming and pre-germination (Khan 1991). Of all the physiological treatments, osmotic priming, i.e. seed hydration at a low water potential while preventing radical protrusion, followed by dehydration, has been most intensively studied (Heydecker *et al.* 1973). Since tomato seeds are of a great interest to the vegetable seed industry, many efforts have been made to optimize the priming procedures (Alvarado and Bradford 1988, Argerich and Bradford 1989, Dahal and Bradford 1990) in combination with a low temperature (Coolbear *et al.* 1987), growth regulators (Finch-Savage and McQuistan 1991), irradiation and seed coating (Khan 1991). Many approaches have been used to reveal the 'priming' mechanism that allows the seed to achieve the beneficial changes that are consistent with the improvements of rate and uniformity of seedling emergence. One of the hypotheses to explain the positive effects of priming on germination performance is the induction of biochemical repair mechanisms during the pre-imbibition. This repair activation may relieve the damages to some macromolecules, which are acquired during storage (Rao *et al.* 1987). Unfortunately, these positive results may be followed by a reduction in storage life (Argerich and Bradford 1989). The recorded effects of priming on the storability of seeds are somewhat contradictory (Argerich and Bradford 1989). For instance, Georghiou *et al.* (1987) reported that in sweet pepper (*Capsicum annuum* L.), osmoconditioning in a 0.4 M mannitol solution for 4 days considerably delayed ageing and increased seed longevity. One of the possible reasons for this contradiction is that the mechanisms behind the priming procedure are far from well known. It is rational to hypothesize that the improvement of storability after priming should be related to the biochemical repair mechanism, while a decline of storability may be paralleled with the progression of the germination processes during the pre-imbibition period. Therefore, in fact, the priming mechanism(s) can not be fully understood without understanding the germination mechanism(s).

6. Germination

Upon maturation, the quiescent seed is a complex of old and new generations. The tissues from the fertilized cells (embryo and endosperm) are from the new generation and are entirely protected by the maternal tissues (seed coat and pericarp). After shedding, the quiescent seeds start to germinate under the required environmental conditions. In many species, seed germination is determined by the maternal tissue. Seed manifests some sorts of sensors that can perceive and respond to the environmental changes (Bewley and Black 1994). The variation in response to the natural conditions within a seed population results in a wide range of germination times. However, modern agriculture demands a fast and uniform stand establishment with a high germination. Therefore, studies on the mechanisms and regulation of the germination response form an essential basis for agricultural practice.

Seed germination encompasses the initiation of growth of a previously quiescent or dormant embryo. For most seeds, germination begins with the imbibition of water and ends with radicle protrusion. Imbibition is generally a triphasic process, with an initial rapid water uptake, followed by a plateau phase with a little change in water content, and a subsequent increase in water content coincident with radicle growth (Bradford 1986). Together with imbibition, the seed metabolism is soon activated (Osborne 1983). At the beginning of imbibition, an amount of solutes leak out of the imbibing seed into the imbibition medium due to the disorganization of cell ultrastructure and the disruption of the cell membrane during its transition from a crystalline phase to a gel phase (Bewley and Black 1994). This disorganization or disruption may seriously deteriorate the seed (Bewley and Black 1994). However, the leachate that contains some germination inhibitors like ABA, may facilitate germination as well (Abdul-Baki and Stoner 1978). The first enzymatic activity which is provoked by the seed is directed towards the repair of damages which have accumulated during maturation, storage and imbibition (Osborne 1983). Gradually, the syntheses of proteins, RNAs and DNA, and nuclear replication or cell division in some seed species occur before visible germination (Bino *et al.* 1993, Elder and Osborne 1993, Weges 1987). All these metabolic activities in combination with water uptake by the seed, result in the growth of embryo. Visible germination occurs when the potential of the embryo growth exceeds the restraint as imposed by the surrounding tissues.

In tomato seeds, the completion of germination is considered to be dependent both upon the expansion force of the embryo and the occurrence of endosperm weakening (Bradford 1990, Karssen *et al.* 1989). The mechanical restraint of the endosperm layer opposite the radicle tip can be measured by an Instron 1122 Universal testing instrument (Groot and Karssen 1987). Using this method it was found that the endosperm is weakened prior to visible germination, which can be ascribed to the activities of a series of hydrolytic enzymes (Groot *et al.* 1988). The hydrolytic enzyme activity is stimulated by GAs excreted from the radicle tip The degree of reduction in the restricting force against the embryo growth, imposed by the endosperm, is the key for control of seed germination. However, Liptay and Schopfer (1983) suggested that the difference in germinability between two genotype lines resided in the germinating embryo, or in other words, the embryo growth mainly determines seed germination. Apparently, embryo

growth and endosperm weakening may play different roles in germination, and their relationship needs further clarification.

7. The expression of phytohormone responsive genes

In recent years a great effort has been made in our knowledge on the functions of phytohormones at the molecular level. Indeed, many phytohormone response genes have been identified. In particular, the expression of ABA and gibberellin (GAs) responsive-genes has been intensively studied in respect to seed maturation and germination. During seed maturation, the expression of certain ABA response genes was found to be correlated with the development of desiccation tolerance. For instance, in maize (*Zea may* L.), viviparous (nondormant) mutant seeds, which lack ABA or do not respond to it, neither fully desiccate nor accumulate certain ABA response gene *lea* (late embryogenesis abundant) products (Kriz *et al.* 1990). Some ABA-responsive genes are also responsible to environmental stresses such as osmotic stress, high temperature and desiccation etc. As ABA levels increase in the tissue subjected to the stress, it is often considered that the expression of the ABA-responsive genes induced by the stress, is mediated by the increase of ABA (Henson 1984). During germination in cereal seeds, GA induces the expression of genes necessary for utilization of the stored seed reserves and for seedling growth. At this time the effects of ABA on gene expression are generally antagonistic to those of GA. For example, application of exogenous ABA to germinating cereal seeds sharply reduces levels of GA-responsive hydrolase mRNAs and proteins. This is presumably due to the ABA-mediated induction of factors that inhibit hydrolase transcription and/or translation (Nolan and Ho 1988, Rogers 1988). This inbibition may also occur at the level of hydrolase activity: in some cereals, ABA promotes the accumulation of a protein that inhibits germination-specific amylase isozymes (Leah and Mundy 1989). Recent studies have shown that some GA-responsive genes also respond to ABA (Hattori *et al.* 1992, Urao *et al.* 1993). The mutually antagonistic effects of these two hormones may be interpreted at the level of gene expression (Gubler *et al.* 1995).

8. Hormone-deficient mutants

Cytokins, auxins, gibberellins, abscisic acid and ethylene all play important roles both in plant growth and development and in seed maturation and germination (Bewley and Black 1994). In the past decades, the majority of studies on hormonal regulation of seed development and germination were based on correlations between either the application of exogenous growth regulators, or the simultaneous occurrence of peak value of the endogenous hormone content, and the morphological, physiological and biochemical changes. Factually, these types of correlations did not exclude the possibility that the application of exogenous growth regulators artificially changed the normal growth and developmental processes. The application of exogenous hormone neither did avoid the possibility that hormones are accidentally involved in the specific aspects of seed development and germination. Therefore, to deal with the regulation of plant hormones in seed development and germination, two other approaches, are frequently adopted in recent years: (1) the use of hormonal synthesis inhibitors to reduce the hormone content *in situ*, and (2) the use of hormone deficient or responsiveness mutants. Particularly, the

latter approach offers a deliberate means of elucidating the regulatory functions of plant hormones. This method has been successfully applied in studies on the roles of endogenous GAs and ABA in the development and germination of *Arabidopsis thaliana* and tomato seeds (Groot and Karssen 1987, Groot and Karssen 1992, Karssen and Laçcka 1986, Ooms *et al.* 1993). In recent years, seeds in several plant species were chemically mutated and some mutants which lack the capacity to synthesize, or are insensitive to specific hormone, were selected by screening the botanical traits specific to the hormone deficiency (Reid 1990). Using the mutants it was found that in seeds *of Arabidopsis thaliana* and tomato, GAs are absolutely required for the germination (Koornneef and Van der Veen 1980, Karssen and Laçcka 1986, Groot and Karssen 1987), while ABA is mainly responsible for the induction of primary dormancy and the prevention of vivipary (Groot and Karssen 1992, Ooms *et al.* 1993). Based on the germination of the GA and the GA/ABA double mutant, Karssen and Laçcka (1986) formulated a revision of the hormone-balance theory of seed dormancy and germination, in which the role of ABA is restricted to the induction of dormancy during seed development, and in which it is postulated that GAs are active in the stimulation of germination.

In the background of the tomato cultivar Moneymaker, the GA-deficient mutant (*gib-1*) and the ABA-deficient mutant (*sit*^{W}), were selected after treating the seeds with ethylmethanesulfonate (Koornneef *et al.* 1981). GA-deficient *gib-1* seeds were selected in the M_2 population when they were not able to germinate in water but did germinate in 10 TM GA_{4+7}. GA-deficient plants are extreme dwarfs with dark green leaves, short internodes and abnormal sterile flowers. The application of GA_{4+7} promotes plant growth and restores the fertility resulting in fruit development and seed set (Groot and Karssen 1988). No significant GA-like activity was detected in extracts from *gib-1* immature fruits, though the plants were sprayed with exogenous GA_{4+7} before flowering, whereas high levels of GAs were found in the immature wild type fruits (Koornneef *et al.* 1990). The *sit*^{W} genotype was selected for wilting in the M_2 population. In the turgid leaves, the ABA level of *sit*^{W} plants is about 10% of that of wild type plants (Cornish and Zeevaart 1985). In the developing seeds, ABA levels of *sit*^{W} are strongly reduced as compared to the wild type seeds. The peak of the ABA content in wild type seeds occurs around 30 DAP whereas no obvious changes in ABA levels in *sit*^{W} seed have been detected throughout development. Significantly, even in the mature stage, ABA levels in seeds of the wild type are still 10-fold higher than in the *sit*^{W} genotype (Groot and Karssen 1992).

9. Cell cycle

Seed germination is the start of the growth of a new plant. Growth simply depends upon cell division, enlargement and differentiation. The sequence of processes occurring during cell division is often referred to as the cell cycle. Within the cell cycle four different phases can be distinguished. DNA replication occurs during interphase, known as S phase. Before and after the S phase, interphase cells are engaged in growth and metabolic activities. These two phases are respectively called the G_1 and G_2 phases of the interphase. G_2 is normally followed by a cell division or mitosis (metaphase or M phase). In diploid somatic cells at G_1 phase, the nuclei contain the 2C DNA value, whereas the nuclei in G_2 phase have 4C DNA value. Here, 'C' stands for the 'constant value' of the DNA content in the haploid tissue. After mitosis, divided cells re-enter the G1 phase.

Little is known about cell cycle activities during seed formation, physiological priming and germination processes. In most orthodox seed species, after maturation or shedding, the water content of a dry seed is too low for the cell cycle to progress (Bino *et al.* 1992). In dry seeds, nuclear replication stages have been identified using autoradiography and Feulgen staining, and it was found that embryos of some species contained both 2C and 4C nuclei, while others solely comprised 2C nuclei (Bewley and Black 1994). Flow cytometry with the use of double-strain DNA-specific fluorescent dyes has offered possibilities to quickly determine the DNA amount of large numbers of nuclei. Bino and colleagues (1992, 1993) have used this technique to quantify the amounts of nuclear DNA with high accuracy in various seed species and seed parts. In radicle tips of tomato embryos from mature seed of the wild type, it was found that most nuclei were arrested in the G_1 stage with the 2C amount of DNA (Bino *et al.* 1992). Apparently, during maturation of the seed inside the fruit the cell cycle is silenced in the G_1 stage. ABA-deficient *sit*^{w} seeds contained a significantly higher proportion of G_2 cells compared to the wild type. Abscisic acid has been shown to be an inhibitor of cell division and may play an important role in the arrest of cell cycle activities during seed maturation. During development of wild type seed, the endogenous ABA induces dormancy, arresting the germination of the seeds within the wet fruit, whereas such dormancy is not induced in the ABA deficient *sit*^{w} seeds (Groot and Karssen, 1992). Possibly, the absence of ABA in the *sit*^{w} causes a reduced dormancy state of the seed resulting in a less stringent arrest of the cell cycle which is reflected by a higher proportion of cells with 4C amount of DNA.

10. Nuclear DNA amounts to predict seed performance

In the cascade of germination, the activation of DNA synthesis is a crucial step leading to visible germination (Osborne 1983). In tomato, upon imbibition no cell cycle activities were found in dormant seeds. Seeds will only complete germination when dormancy is broken, and DNA synthesis is initiated in the radicle tips prior to visible germination. About 18 hours after imbibition of tomato seeds in water the 4C signal of tissues at the radicle tip started to increase (Bino *et al.* 1996). Therefore, it was concluded that the activation of cell cycle in seeds upon imbibition can be considered as an indication of the breakage of dormancy and the induction of germination. Apart from the water content, cell cycle activities in seeds are regulated through hormones (Liu *et al.* 1994). Gibberellin-deficient *gib-1* tomato seeds do not show DNA replication activity and do not germinate in water. When exogenous GAs are added to the imbibition solution, or when the endosperm layer opposite the radicle tip is removed, DNA replication activity in the radicle tips of *gib-1* seeds is started, and, subsequently, the seeds germinate (Liu *et al.* in press).

The appearance of 4C DNA amounts in radicle cells of wild type tomato seeds is a clear indication that the germination process has started. The rate at which 4C DNA levels arise is, among others, influenced by the osmotic potential of the imbibition solution (Lanteri *et al.* 1993), the temperature (Bino, unpublished results) and the quality of the seed lot. High quality tomato seed lots manifest a more rapid increase of 4C signals compared with lots with a low quality in terms of germination rate and uniformity. This indicates that the advancement in the germination process of tomato seeds can be followed through the changes in the amounts of DNA. The increase in 4C levels gives a

clear indication about the response of a seed to imbibition. Therefore, the nuclear replication stage can be used as a rapid test for seed performance.

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