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Perspectives in Probing Seed Germination and Vigour

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ABSTRACT

This paper reviews the developments of techniques which are applied in seed quality, in terms of germination and vigour testing. First the definition of seed germination, viability, and vigour derived from evaluation methods over the last four decades will be reassessed to account how new techniques need to satisfy the demand of farmers, seed industries, seed-gene banks and basic seed research. The course of development of seed quality testing techniques is associated with that of basic knowledge and insight into seed morphology, physiology, biochemistry and molecular biology. Related vigour indices, mostly assessed in a destructive manner, are generally used to establish correlations with declining seed viability. The classical tests are widely reviewed and problems inherent to their application are discussed. A new generation of seed quality testing techniques, such as computer imaging analysis, NMR-microimaging and X-ray inspection, chlorophyll fluorescence sorting, infrared-photoacoustic and electrical impedance spectrometry, and micro-calorimetry, contributes to improve relationships between different seed characteristics and viability in economically important crop species. The future perspectives section focuses on the interest of seed researchers and analysts in introducing new automated and computer-aided testing systems in a seed laboratory. The ultimate goal should cover two aspects: First, the definition of non-destructive markers in testing and sorting seed quality, suitable also to be applied on an individual seed within a seed population in automated way, and secondly, the integration of new testing technology with standard methods in a seed laboratory where a large number of seed species may be evaluated for genetic purity analysis, taxonomic variability screening, and viability and vigour prediction.

Keywords: computer imaging, crop seeds, seed ageing, viability and vigour indices
Abbreviations: 2-D, two-dimensional; **AA**, accelerated ageing; **AFLP**, amplified fragment length polymorphism; **AOSA**, Association of Official Seed Analysts; **CCD**, charge-coupled device; **CD**, Controlled Deterioration; **CF**, Chlorophyll Fluorescence; **HPLC**, High-Performance Liquid Chromatography; **i.f.s.**, internal free space; **ISTA**, International Seed Testing Association; **mc**, moisture content; **MM**, Molecular Mass; **NMR**, Nuclear Magnetic Resonance; **PAGE**, Polyacylamide Gel Electrophoresis; **RAPD**, random amplified polymorphic DNA; **RGB**, Red-Green-Blue; **SSR**, simple sequence repeats

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INTRODUCTION

Seed quality is a multiple concept comprising essentially three categories of seed components: species and cultivar purity, health, and potential performance including germination, vigour, field emergence, and storability (Hampton 2002). This review is concerned primary with germination and vigour testing of non-dormant seeds of higher plants that are well adapted to desiccation, the so-called orthodox seeds (as reviewed by Roberts 1989), which are typical of agronomically important species in temperate zones. These tests have been developed much to satisfy the needs of farmers and seed industry, and methods have been improved to meet the required standards. Further, high germination seeds as tested in the laboratory should have maximum potential to emerge in the field, and so seed vigour appears as an additional aspect of seed quality closely related with field performance (Perry 1980; Hampton 1995). Any complete assessment of seed germination and vigour should also be directed to predict the potential storability of

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Invited Review

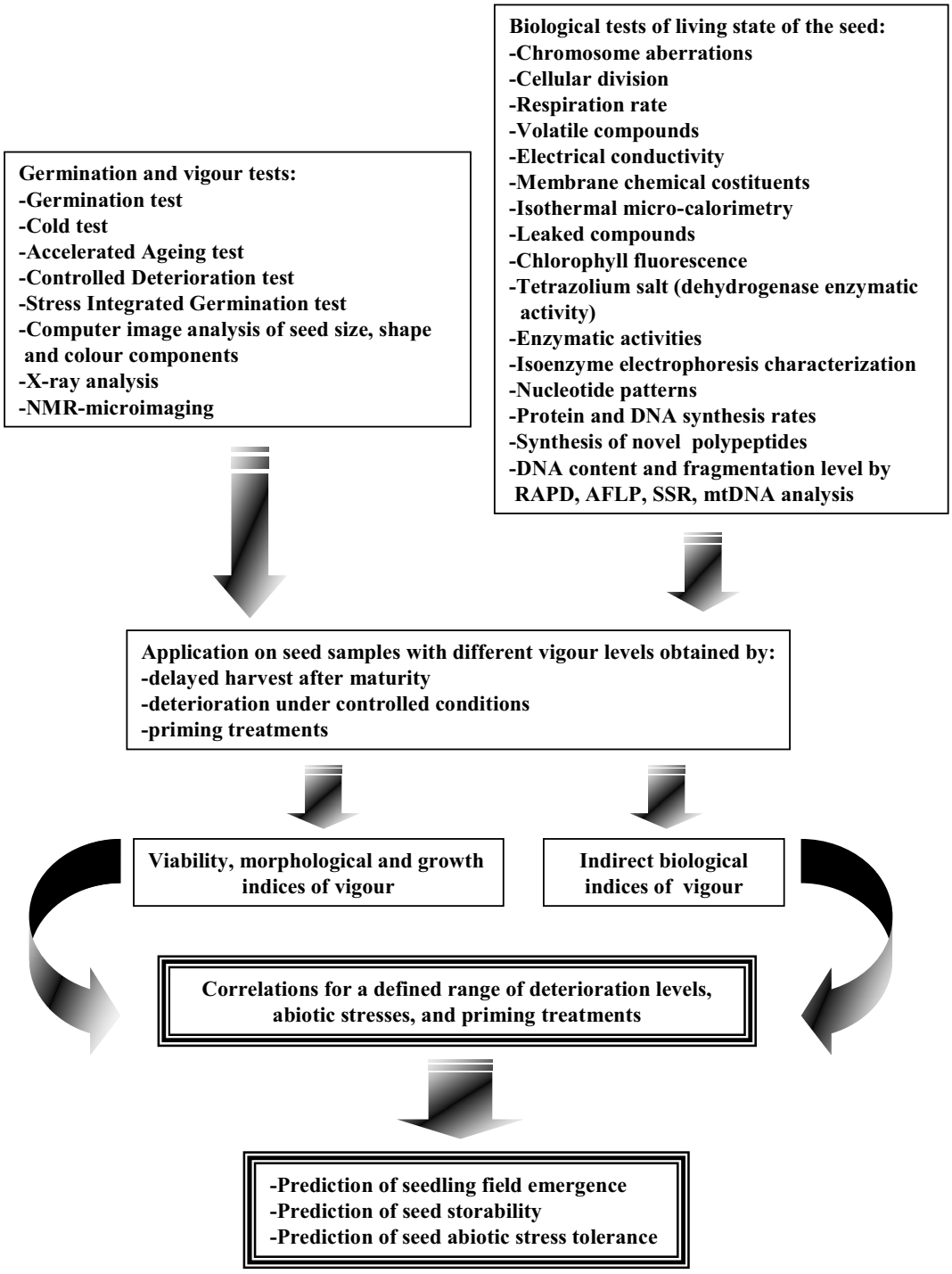

Fig. 1 Schematic flow-chart of viability and vigour tests and their relationships to predict seedling field emergence, seed storability and seed abiotic stress tolerance.

a seed lot, in light of its economical importance if stored in a farm granary or conserved in a seed-genebank to preserve plant genetic resources (Roberts 1989; Chin 1994). To obtain this information, a series of germination and vigour tests are used, and the techniques in seed quality testing continue to evolve. Seed germination test is by definition the estimation of the ability of a seed to produce a normal seedling under optimal environmental conditions (McDonald 1980). According to the International Seed Testing Association (ISTA) and the Association of Official Seed Analysts (AOSA), seed vigour has been defined as the sum total of seed properties which determine the potential of rapid uniform emergence under a wide range of field conditions. A number of criteria to evaluate the performance of vigour tests are related to the development of basic seed research, which involves several types of biological approaches.

Seed biology and germination have interested farmers and scientists since antiquity with the establishment of agriculture. Certain old problems, such as uniformity and loss of germination due to deterioration, have lost none of their actuality. Theophrastus (ca. 372-287 B.C.), who can be considered the first seed physiologist, was the first to study seed quality as an agriculture problem in a scientific way (Evenari 1984). Theophrastus' findings were confirmed by several Greek and Roman authors. After a long period (from the first to seventeenth century B.C.) without any relevant information on the development of seed physiology knowledge, at the beginning of the eighteen century seed germination acquired a new interest among botanists and agronomists (Priestley 1986a). By 1954 ISTA and AOSA defined the demarcation point between germination and vigour, as separate concepts (Woodstock 1973). In a seed laboratory practice, the germination test assesses the emergence from a seed embryo of the essential structures that are indicative to produce a normal seedling under favourable conditions. Despite various concepts and terms to de-

note aspects of vigour, these can be quantified in two ways: (1) by modifying the germination response using stress treatments which imitate field conditions, and so a direct measure of seed vigour as tolerance to stress factors which affect the germination process in different ways can be scored; (2) by measuring certain morphological, cytological, physiological, biochemical, and, more recently, molecular attributes that define an indirect measure of germination performance under the studied growth conditions.

Many of the vigour tests are considered for their standardization and testing one or more factors which represent distinct vigour indices or grouped in a more complex index. Following the maturity stage in which it has been postulated that the seed retains maximum germination and vigour potential (Roberts 1972a), the seed begins to deteriorate with a series of events which culminate in the loss of germination. Many models of seed deterioration have been proposed in the last four decades. Delouche and Baskin (1973) proposed a linear progression of changes in physiological and biochemical processes leading to final impairment of germination. A more complex model was published by Osborne (1980) in which the transition from the viable to the non-viable state in dry seed is related to cumulative and interrelated lesions leading to membrane systems, the synthesis of labile enzymes, and repair system working. A model of seed deterioration that accounts for physiological events that occur during storage and imbibition was defined by McDonald (1999). High seed moisture content would favour free radical production by autoxidation during storage causing mitochondrial dysfunction, enzyme inactivation, malfunctioning membrane systems and genetic damage. During imbibition, a series of cellular lesions is further reinforced by additional free-radical damage, terminating in unsuccessful germination. Although these models remain speculative, seed researchers emphasize that loss of germination is the final consequence of seed deterioration and is preceded by a series of changes in physiological, biochemical and molecular processes. Many of these biological changes have been used as potential indicators of seed vigour which pattern in the 'history' of the seed is distinct from germination, as pointed out by Delouche and Caldwell (1960). A number of experimental approaches have been used to measure indirectly seed vigour levels by correlating them with germination decline in seed lots deteriorated in the laboratory by accelerated ageing techniques (Delouche and Baskin 1973; Matthews 1980). The experimental design in seed quality testing, as generally followed by several seed researchers and analysts, can be schematically represented by the flow-chart of **Fig. 1**. Firstly, delayed harvesting following maturity produces seed lots with different levels of initial vigour. Storage under high temperature and moisture content conditions can provide in a reasonable period of time a series of seed samples with a progressive loss of germination and vigour. In addition, vigour tests could be applied with the same procedures to monitor vigour change screening in seeds which are progressively aged and then exposed to appropriate vigour-restoring treatments, like hydro-soaking or osmo-priming treatments (Dell'Aquila 1987). With this experimental model the hypothesis that the intensity of expression of the majority of ageing symptoms in a seed sample is a continuous rather than a step function may be demonstrated, as pointed out by Ellis and Roberts (1981).

Despite the ease with which a number of different vigour seed lots can be obtained, the majority of proposed tests are carried out by destructive procedures and use a population of whole seeds or their anatomic parts (e.g., the embryo axis), in which highly viable seeds are mixed with those of low viability. Pooled seed samples never reveal the vigour variation among individual seeds, and so average data and statistical errors can mask the ample spectrum of seed quality existing within a seed population interfering with vigour pattern prediction. More recently, sophisticated and mostly non-destructive methods are based on computerized imaging analysis, X-ray inspection, nuclear magnetic resonance (NMR), optical reflectance and transmission, etc. (Chen and Sun 1991). New vigour indices and their fast measurement combined with high-speed sorting systems make new techniques suitable to integrate the standard vigour tests in a seed laboratory. Moreover, interest of seed researchers is also addressed to provide more extensive data bases on morphological and chemical traits of agronomic species with new measurements.

The objective of this review is to describe a broad range of germination and vigour tests developed over the last four decades, with a particular attention on more recent seed quality testing techniques and their potential application in a modern seed testing laboratory.

THE GERMINATION TEST

Probably the first experiments in seed germination testing occurred in the late 'Neolithic age', when man, trying to grow crops, discovered that many seeds do not germinate at all or uniformly or others need specific environmental conditions to germinate. In agronomic terms, a seed can be defined to have germinated once the radicle has been protruded from the seed coat. The term 'viability' is used usually as being synonymous with germination, even if it is applied to a seed that is able to produce a normal seedling and, hypothetically, it is metabolically active in both germination and seedling growth (Copeland and McDonald 2001). So, the occurrence of a visible radicle represents the morphological marker of a germination test, in which the score criterion is essentially a yes or no question. The test is made on an individual seed within a sample with a statistically significant number of seeds, and different germination parameters can be easily calculated: Final cumulative germination percentage, time of start of germination, time to reach maximum germination, and germination uniformity. ISTA (1985a) and AOSA (1988) first published rules which specify substrates, media, promoting environments (i.e., temperature, light, KNO_3, GA_3), and times for the first and final count of germinated seeds for a broad range of species of agricultural, vegetable, flower and tree seeds. More detailed information may be obtained by consulting the web pages of ISTA (http://www.seedtest.org/en/home.html) or AOSA (http://www.aosaseed.com/). The data collected by a complete germination test can be quickly processed and graphically displayed using commercial software packages. Among these, SeedCalculator software package, specifically designed for germination test evaluation and data base implementation, is available as a demo on the Plant Research International (U&R, Wagheningen, The Netherland) URL: http://www.plant.wageningen-ur.nl.

The basic requirements of the germination test are to be rapid, inexpensive and reproducible. The germination test is not objective and it is based on the subjective assessment of a seed analyst, who must decide whether a seed is 'normal' or 'abnormal'. In addition, a seed analyst is subject to a restricted working time, and, so, precision in timing score of germinated seeds can be affected. Another limitation, as pointed out by Hampton (1995), regards the problem that subtle percentage germination differences, statistically non significant, between apparently highly viable seed lots may mask significant differences of vigour components. A number of investigators have suggested using time-course of rate of germination to better describe potential vigour of seed lots. Kotowsky (1926) was the first to introduce the 'coefficient of velocity', defined as the sum of newly emerged seedlings on each day divided by the sum of the number of newly emerged seedling times. Czabator (1962) developed the 'germination value index', which incorporated components for both speed and total germination. Ellis and Roberts (1980, 1981) elaborated the 'mean germination time' index, reciprocal of germination rate, which measures the performance of surviving seeds within a population. Mean germination time has been demonstrated to be a sensitive indicator of the deterioration process rather than viability in durum wheat (*Triticum durum* Desf.) seeds

(Dell'Aquila and Margiotta 1986; Dell'Aquila 1987). A critical review on several single-value germination indices has been reported by Brown and Mayer (1988).

A model for seed germination curves was designed with the assumption that a seed sample may consist of one or more sub-groups in which the probability of a seed germinating in unit time is constant within a sub-group (Bould and Abrol 1981). This method of describing germination data, focused essentially on the degree of germination uniformity, highlighted by some experimental problems, such as the difficulty to identify in a seed population sub-groups with a defined physiological state and the factors which determine the number of seeds which successfully germinate. More recently, the hydrotime concept was introduced which defines a unifying model to describe the patterns of germination that occur in response to the water potential of the seed environment (Bradford 1995). The model can provide several indices of seed quality related to stress tolerance, speed and uniformity of germination, but it needs an extensive rate of germination data. The development of computer automated imaging for scoring germination could facilitate the application of hydrotime analysis (Bradford and Still 2004).

AMPLIFICATION OF GERMINATION RESPONSE BY TREATMENTS MADE ON DRY SEEDS

The first approach in seed vigour testing has been developed in light of its application to study seed deterioration and storability. The germination test alone fails to take in account the progressive decline of deterioration, or, especially in seed lots prior to a large decline in viability. A very popular vigour test, the accelerated ageing (AA) test, was first developed by Delouche and Baskin (1973) for several crop species. The test is mostly based on the following criteria: Dry seeds are subjected to high temperature (40-45°C) and high relative humidity (100% RH) stress for about 48-96 h, and then allowed to germinate in standard conditions. Low quality seeds deteriorate more rapidly than high quality seeds, and so subtle differences of vigour can be estimated by the measure of usual germination parameters. The test was improved and standardized for its applicability to a wide range of crops and for correlations with field emergence (McDonald 1995; TeKrony 1995; Egli and TeKrony 1996), and it was accepted in the ISTA and AOSA rules. Despite a wide use of this method in most seed laboratories, many authors have reported problems of reproducibility, essentially due to the failure to keep seeds at the beginning of ageing under uniform relative humidity. Varying initial seed moisture content can affect the degree of ageing that occurs in a specific period of time, and so variable results can be obtained. The 'controlled deterioration' (CD) method for legume seeds, proposed by Matthews (1980), avoids some AA experimental problems, bringing the seeds to a high and constant moisture content before exposure to high AA temperatures. The CD was tested on a range of species obtaining good correlations with field emergence and commercial storage (Powell and Matthews 1981, 1984). A variation of CD tests included the salt accelerated ageing test first applied to pepper (*Capsicum annuum* L.) seeds, in which water was substituted by salt solutions in an accelerated ageing chamber (Marcos-Filho 1998). Both AA and CD tests can be used to rapidly produce a series of sub-lots that cover the entire range of deterioration under the applied conditions of moisture content, temperature and period of storage. Since storability is one aspect of vigour, the germination response to accelerated ageing may highly correlate with other indices of vigour. This work hypothesis set up the milestone of most basic research on seed ageing studies and testing.

GERMINATION TEST MODIFIED BY INTEGRATING STRESSING FACTORS

For most seeds, imbibition is generally a triphasic process in which the lag-phase with little change in water content is to be considered of primary importance in initiating radicle protrusion from the seed coat (Bewley and Black 1978). Bradford (1990), studying seed water relations, pointed out that the lag-phase can be extended by several non-optimal conditions of germination, such as water and salt stress, low or high temperature, ageing and abscisic acid. Thus, any modification of the germination process, made during imbibition prior to the radicle emergence phase, leads to changes of metabolic patterns and bio-synthesis, and in turn to radicle emergence. Differences in both the tolerance to a given stress factor (usually, a highly concentrated solution of NaCl and cold or high temperatures) and degree of vigour can be amplified and estimated very easily with the facilities of a germination test. McDonald (1980) described the 'cold test' as one of the oldest methods of stressing treatment in estimating vigour in corn (*Zea mays* L.) and sugar beet (*Beta vulgaris* L.). Seeds were placed in soil or paper towels with soil and kept in the cold for an appropriate amount of time. Upon stress removal, the seeds were allowed to germinate under favourable growth conditions to evaluate radicle emergence. The greatest difficulty with the cold test is the standardization which affects correlation with soil emergence (Delouche and Baskin 1973; Lovato and Cagalli 1992). Few efforts have been expended in measuring differences in germination performance by multiple stress laboratory tests which concern thermo-, water- and salt-stress tolerance responses. A complex stressing vigour test, based on a four-day water stress by immersion in 0.15% NaCl solution at two temperatures (25°C for two days followed by 5°C for another two days), has been made to test the vigour of wheat (*T. aestivum* L.) and maize seeds (Barla-Szabó and Dolinka 1988). The new test was compared with the cold test, accelerated ageing and electrical conductivity tests, and was found to be better than the traditional methods in predicting field emergence and in reflecting the general physiological state of a seed lot. In sorghum [*Sorghum bicolour* (L.) Moench] heat shock treatments were applied after 24 h imbibition at 40°C for 30 min followed by a second treatment at 50°C for 2 h (van de Venter and Lock 1992). The heat-shock response has a bearing on seedling emergence under hot and dry, as well as cool and wet sowing conditions. Heat-shock treatments have been used to assess vigour in undeteriorated wheat seed lots (van de Venter *et al.* 1993), while germination obtained by salt imbibition has been reported as a reliable test in evaluating the quality of natural and accelerated aged alfalfa (*Medicago sativa* L.) seeds (Smith and Dobrenz 1987). In durum wheat seeds aged under controlled conditions, to improve the seed vigour pattern prediction of a standard germination test, this was conducted by including stressing factors (40-45°C temperature regimes or exposure to 0.4-0.6 M NaCl solutions) prior to the radicle emergence phase (Dell'Aquila and Di Turi 1996). The progressive variation of germination percentage and mean germination time in deteriorating seeds in comparison with values of a standard germination test were closely correlated with leachate electrical conductivity and embryo protein synthesis rate. The 'stressing integrated germination test' was also applied to predict vigour in fresh and aged lentil (*Lens culinaris* Medik.) (Dell' Aquila 1999), age tolerance of *Brassica* accessions stored in the seed-genebank of the University of Palermo (Palermo, Italy) (Scialabba et *al*. 1999), and tolerance to drying treatments in lentil, hulled wheat (*Triticum dicoccon* L.), white cabbage (*Brassica oleracea* L.) and grass pea (*Lathyrus sativus* L.) (Dell'Aquila and Scialabba 2000).

SEEDLING GROWTH, AND SEED SIZE AND COLOUR TESTING

The seedling growth rate test is based on increasing dry weight accumulation in emerging embryonic axes and is conducted according to the standards for the routine germination test. After germination is evaluated, the growing axis of the embryo is excised from cotyledons or endosperm,

dried at 80°C for 24 h, and the dry weight is determined (McDonald 1980). Since seedling growth rate is correlated with vegetative development in the field, the method is promising although certain standardization factors are needed, as well as attention to genetic traits affecting seedling growth in soybean [*Glycine max* (L.) Merr.] seeds (Burris 1976; Pinthus and Kimel 1979). A simple vigour test for barley (*Hordeum vulgare* L.) was based on the measurement of plumule growth after seven days at 20°C, and data were closely correlated with field emergence performance (Perry 1977). An alternative vigour test, used with vegetable species, is the measurement of root growth, also called the 'slant board test'. This has proved to be a valuable indicator of field vigour in carrot (*Daucus carota* L.) (Gray and Steckel 1983), lettuce (*Lactuca sativa* L.) (Wurr and Fellows 1985), cauliflower (*Brassica oleracea* L.), leek (*Allium porrum* L.) and onion (*Allium cepa* L.) (Finch-Savage 1986).

Differences in mean seed size between and within populations may be the result of a natural selection or genetic drift in different geographical areas, or they may be due to a combination of genetic and environmental factors (Fenner 1991). The hypothesis that seeds of different sizes may have differing quality attributes has been investigated by several authors, obtaining contrasting results. Large seeds of cabbage (*Brassica oleracea* L.) and turnip (*Brassica rapa* L.) are superior to small ones (Hanumaiah and Andrews 1973), or small seeds of red clover (*Trifolium pratense* L.) have better vigour than large seeds (Wang and Hampton 1989). No differences have been found in seed vigour because of varying seed size in corn by Shieh and McDonald (1982) and in kale (*Brassica oleracea* L.) by Komba *et al*. (2007). Another morphological marker of seed vigour may be represented by the darkening of the seed coat due to prolonged storage and deterioration. The effect of lipid peroxidation and the production of free radicals together with non enzymatic reactions, such as Amadori and Maillard reactions, reduce sugars or protein amino groups to form fructosyl derivates or glycated proteins, whose interaction produce polymeric brown products (Wettlauer and Leopold 1991; McDonald 1999). The 'browning effect' has been described in legumes, where colour change can be quite heterogeneous within a seed sample and seeds which maintain their original colour at full maturity tend to preserve high vigour (Priestley 1986b). As a consequence, progressive seed coat browning may indicate decreasing seed quality. This experimental hypothesis has been applied to the study of physiological changes in alfalfa, crimson and white clovers (*Trifolium incarnatum* L. and *Trifolium repens* L., respectively) (West and Harris 1963), and more recently it has been used to discard brown, tan and green fractions of snap bean (*Pisum sativum* L.) seeds by measuring light reflectance differences between off-coloured and white seeds (Lee *et al*. 1998). Light reflectance was determined in rapidly aged bean seeds with a spectrophotometer in the range between 275 and 450 nm, and each single seed was rapidly sorted by an electronic colour sorter in non invasive way.

APPLICATION OF COMPUTER IMAGING TECHNIQUES TO SEED GERMINATION AND SEEDLING GROWTH TESTING

Seed digital images displayed on a monitor or on a printed page are two-dimensional (2-D) in format and can be assumed as a 2-D object having both dimensions placed along the orthogonal axes of a Cartesian plane. As a result, several descriptors of seed size, shape, and colour component density of the individual seed can be easily estimated. The advent of computer-aided image acquisition by video camera, coupled with new algorithms implementing a fast image processing and analysis, has allowed to quantify several seed morphological features, necessary for germination and vigour testing in a wide range of crop species (see Dell' Aquila 2007a for an extensive review). An automated vision system was developed at the National Seed Storage Laboratory (USDA-ARS, Fort Collins, USA) with the aim of introducing new techniques to characterise and preserve seed germplasm (Howarth and Stanwood 1993a, 1993b). The designed system was divided into two basic units: The biological system that allowed seed germination in a chamber with controlled humidity, temperature and lighting, and the computer vision system, including a charge-coupled device (CCD)-camera, a computer and an image analysis software package. The root length for each seed of lettuce and sorghum was automatically assessed and plotted *versus* imbibition time to be statistically compared with measurements made by a seed analyst. Great interest focused on the setting up of an image analysis system to measure linear dimensions of seeds with the aim to correlate their change with the swelling process due to water uptake. The entire imbibition process leading to the germination of *Brassica* seeds was first monitored by McCormac and Keefe (1990), and further in seeds of winter wheat and oat (*Avena sativa* L.) (Kruse 2000). New experimental evidence was produced on the versatility of image analysis in studying the imbibition process up to the 'visible germination' event of white cabbage seeds by Dell'Aquila *et al*. (2000). The measurement software 'ScilImage' (TNO-TPD, The Netherlands) determined area, perimeter, width and length as descriptors of seed size changes during swelling. The above machine vision system was further improved in the laboratory of image analysis of the Institute of Plant Genetics of the National Research Council (CNR, Bari, Italy). A thermostatic chamber was designed to include a colour CCD-camera, a timer depending lighting system and a holder for the Petri dish containing polymerised agarose added with 0.44 M concentrated NaCl, for salt stress imbibition trials on white cabbage seeds (Dell'Aquila 2003). Alternatively, this environment unit was also used to evaluate the germination of broccoli (*Brassica oleracea* L.) and radish (*Raphanus sativus* L.) seeds under different temperature regimes (Dell'Aquila 2005). The computer unit was standardised using a CCD-camera, a commercial imaging board, a 55 mm telecentric lens, a high power computer in XP MS® Windows environments and the more recent version of the commercial software package ImagePro-Plus™ (Media Cybernetics, USA). Alternatively the freeware and open source software package ImageJ (http://rsb.info.nih.gov/ij/) can be adapted for these trials. The morphology and shape of the *Brassica* seed make it suitable for 2-D measurements, assuming that each seed approximates a sphere and that linear expansion is similar along both dimensions. The area increase time-course resembled the triphasic curve of water uptake (Bewley and Black 1978) with a rapid increase in area values characterised the beginning of the third phase, coinciding with a visible radicle growth. When the imbibition process was monitored by seed shape factors, such as roundness, a first phase of no apparent shape change from the start of imbibition to radicle emergence was followed by a second phase of rapid increase, which corresponded to the last phase of seed area increase. Time recording of the second or the first inflection point of seed area or roundness curves, respectively, may provide an objective assessment of germination completion, in physiological terms, and the start of a visible germination, in agronomic terms. The image analysis system was applied to several crop species and an imaging database was developed at the Institute of Plant Genetics (CNR, Bari, Italy) and published on the following web site: http://germimaging.ba.cnr.it, for educational and training purposes. The technique has also been applied to automatically test germination percentage in a large population of seed. van der Heijden *et al*. (1999) used a system controlled by a single computer program to study germination time courses in tomato (*Lycopersicon esculentum* L.), lettuce, *Arabidopsis* and cabbage seeds under different temperature and water stress conditions. The time of radicle tip emergence was used to plot precise germination curves for the thermal time models (Bradford 1995) and to predict seed viability. A more sophisticated image acquisi-

tion system was developed to capture images of different trays, containing plugs in which lettuce, cauliflower and tomato seeds were grown for subsequent transplanting (Ureña *et al*. 2001). This system allowed to classify the degree of seedling development using fuzzy logic and processed data on germination percentage and length of each seedling represented indices of germination quality. More recently, Ducournau *et al*. (2005) elaborated new algorithms based on the idea that the emergence of a radicle tip at a defined time results in a modification of the binary images. The system was tested to study germination of sunflower (*Helianthus annuus* L.) seeds, and detailed germination curves were obtained allowing a perfect fitting in a probit model.

A promising field of application of image analysis is the measure of seedling growth, using CCD-camera or scanner image capturing techniques. An image analysis system, suitable to integrate the slant-board test for the measurement of seedling root length was designed utilizing a moveable gantry that allowed the video camera to scan the length of one group of five carrot or lettuce seedlings at a time (Keefe and Draper 1988; Keefe 1990; McCormac *et al*. 1990). Later on, a more complex biological system, combining temperature and photoperiod control, coupled with a computer unit was developed to assess root elongation rate in rice (*Oryza sativa* L.) and sorghum (*Sorghum bicolor* Moench.) on an hourly basis (Iijima *et al*. 1998). Images of 3 day old lettuce (Sako *et al*. 2001) and soybean seedlings (Hoffmaster *et al*. 2003) were acquired with a special scanner contained in a metal box and processed with an appropriate software package. The generated image analysis parameters represented collectively a vigour index based on morphological features. A similar method to capture digital images of seedlings grown in Petri dishes was also developed to asses vigour of different lots of small sized *Impatiens wallerana* Hook f., petunia (*Petunia* × *hybrida*), lisianthus (*Eustoma grandiflorum* L.), cauliflower, tomato, pepper, vinca (*Catharanthus roseus* L.) and marigold (*Tagetes patula* L.) seeds (Geneve and Kester 2001; Oakley *et al*. 2004). To measure root length and diameter of switch grass (*Panicum virgatum* L.), fescue (*Lolium arundinaceum* [Schreb.]), orchard grass (*Dactylis glomerata* L.) and clover (*Trifolium repens* L.) a more sophisticated system was designed using digital images captured with combined scanner and digital camera (Zobel 2003). An indirect measure of radicle growth was obtained by measuring the rate of increase in area and roundness factor in time-lapsed seed images acquired with a CCD-camera (Dell'Aquila 2004a, 2004b, 2005). More recently, a computer software approach was developed to automatically separate overlapping seedlings of cotton (*Gossypium hirsutum* L.) seeds (Xu *et al.* 2007). A new algorithm was defined to measure each seedling independently, in case of detection on the captured image of overlapped seedlings, and data of seedling growths were related to an overall vigour index.

The new computer imaging technique may be extended to the analysis of Red-Green-Blue (RGB) colour density (ranging from 0-0-0 to 255-255-255 values for black or white colour components, respectively) of 2-D seed images, acquired by a flat-bed scanner. The method is based on measuring the medium RGB index of a single seed, which is then separated into different fractions each having a different RGB range. Lentil seeds, which were deteriorated under controlled conditions of moisture content and temperature, were sorted in three fractions with distinct germination potential over the entire period of ageing (Dell'Aquila 2006). Both studied seed quality parameters (germination percentage, mean germination time and leachate electrical conductivity) and medium RGB index showed correlated changes over deterioration time, showing that the effects of ageing are progressive on sorted seeds. The technique was applied also in sorting high viability fractions in cucumber (*Cucumis sativus* L.), lettuce and tomato seeds (Dell'Aquila 2007a). The seed distribution associated with germination quality changed gradually from unaged to aged seed fractions, confirming experimentally the theoretical hypothesis of the composition in seed sub-samples, differing in physiological quality, of a large seed sample, as described by Bould and Abrol (1981).

X-RAY AND NMR IMAGING ANALYSIS FOR SEED VIABILITY TESTING AND SORTING

Seed radiography offers seed analysts and researchers non-destructive tests to evaluate germination potential in economically important tree and crop species. Inspection of morphology, anatomy, and spatial configuration is related to the absorption of primary radiography energy passing through the less dense area of cellular cavities that does not contain protoplasm (Vozzo 1988). Usually a large number of seeds can be treated at the same time, and non-lethal doses of X-ray ensure no genetic damages to plant material. X-ray imaging has been applied mostly to determine the quality of forest seed species. In *Pinus* genera, tests were compared with other methods such as germination, cutting, and incubation drying separation tests (Simak and Sahlén 1981; Simak 1984; Simak *et al*. 1989). Visual inspection of the radiographs allowed to separate empty seeds, i.e. non mature, insect damaged and dead seeds, from filled seeds, i.e. viable seeds. Nuclear magnetic resonance (NMR) microimaging combined with X-radiography have been applied to the study of pepper seed internal morphology in quiescent and in imbibing status (Foucat *et al*. 1993). The results showed that X-ray technique can be applied for a fast preselection of dry seeds, while NMR-microimaging is a powerful technique for investigating morphology changes during seed germination. Further, a non-destructive NMR spectroscopic technique was applied to separate at least three different fractions of spin-spin relaxation correlated with decreasing viability in soybean and wheat seeds subjected to accelerated ageing treatment (Krishna *et al.* 2003). Tentatively, in tomato seeds different categories of embryo morphology, as detected by visual inspection of X-ray photographs and a subjective analysis of internal free space (i.f.s., the space comprised between embryo and endosperm), have been related to germination capacity (van der Burg *et al*. 1994). More recently, the combined use of a non destructive X-radiography technique and computer-aided image analysis was applied in defining a seed size parameter as a quality index in evaluating and sorting pepper seeds (Dell'Aquila 2007b). Developed X-ray photographs of fifty seeds were scanned with a flat-bed scanner and converted to a digital imaging, and areas of the whole seed and of i.f.s. were measured by ImagePro-Plus software package. Seeds with less than 2.7% (on the basis of total seed area) of i.f.s. area were classified as highly viable seeds with lowest level of abnormal seedlings, whereas those with i.f.s. area ranging between 2.7-5% showed increasing abnormal seedling percentage or did not germinate at all. Using i.f.s. marker manual sorting can be easily carried out to discard defective and dead seeds from a population with apparently uniform germination. To improve the sorting technique, a prototype of an X-ray equipment with application specific to seed quality control was designed by Craviotto *et al*. (2004). The X-ray Seed Analyzer (SEMAX) used a non-destructive method which complied with health security standards and allowed different diagnosis on seed anatomy and physical conditions of seeds. The International Rules for seed Testing (ISTA 1996) and Belcher and Vozzo (1979) defined the rules of X-ray technique for quality control of agricultural and tree species.

CHROMOSOME INTEGRITY AND CELLULAR DIVISION AS MARKERS OF SEED VIABILITY

Within a seed lot the induction of genetic variations involves mutation or alteration of the DNA, RNA, cytoplasm, and chromosomes. This event causes a serious problem for long-term preservation of seed accessions stored in a seed-genebank, because these genetic mutations may be passed on

the succeeding generations and become stabilized in the population (D'Amato and Hoffmann-Ostenhof 1956; Roberts 1972b; Ross 1982). Using root tip squash preparations Abdalla and Roberts (1968) found that the frequency of aberrant cells (anaphase figures with bridges and/or fragments per anaphase cells observed in the first mitosis) increased with storage time and severity of storage conditions in seeds of barley, broad bean (*Vicia faba* L.) and pea (*Pisum sativum* L.). Further support for these data was obtained in durum wheat (Innocenti and Avanzi 1971), in barley (Murata *et al.* 1981) and in lettuce (Rao *et al*. 1987). A classification of both chromosome and chromatid type aberrations observed in seed ageing studies was also tentatively made by Ross (1982). Against accumulation of chromosomal aberrations in seed accessions stored with a genebank facilities, the International Board for Plant Genetic Resources (now, International Plant Genetic Resources Institute; 1976) has made the recommendation that seed stocks should be regenerated to provide the fresh one whenever viability falls to 85%.

Under advancing seed ageing, delay in the occurrence of the first mitosis in root tips was associated with the induction of genetic changes (Orlova *et al.* 1975). Murata *et al.* (1980) extensively studied the effects of deterioration on the time of first mitosis in roots of pea seeds stored under different controlled deterioration conditions. The delay in the occurrence of first mitosis was correlated strongly with the decrease in germination and with root length at which first mitosis occurred. These findings were supported also in durum wheat embryos aged under interdependent combinations of seed mc (between 12 and 18%) and storage temperature (between 25 and 35°C) (Del'Aquila and Margiotta 1986). Ageing was associated with a progressive reduction in the rate of [6- ^{3}H] thymidine incorporation in embryonic tissues (as measure of '*in vivo*' DNA synthesis) and mitotic index (number of cells in mitosis from late profase to early telophase/total number of cells scored) measured in the primary root. The results suggested an indirect test to monitor and predict storability of wheat seeds by the close correlations of radioactive precursor incorporation and mitosis activity with viability and mean germination time. Visual scoring of microscopic observations make the calculation of mitotic index subjective and source of errors, that could be limited with a more modern computer image analysis processing. This possibility, including a standard image processing sequence and by multivariate analysis of image analysis parameters, was investigated for estimation of mitotic index in apical conifer meristems (Sundblad *et al.* 1998).

RESPIRATION ACTIVITY, MEMBRANE INTEGRITY, VOLATILE COMPOUNDS, CHLOROPHYLL FLUORESCENCE, AND ISOTHERMAL MICRO-CALORIMETRY AS MARKERS OF SEED VIABILITY AND VIGOUR

In a series of studies on a wide range of crop seeds it was shown that the adverse effects of several treatments, such as long-term storage, freezing, heating, chilling injury, etc., could be detected by measuring seed respiration using a commercial respirometer (Woodstock 1973). The decrease in respiratory rates, measured as reduction of uptake of O_2 and increase of respiratory quotient, was closely correlated with the decline of germination capacity and subsequent seedling growth in Lima bean (Woodstock and Pollock 1965), corn (Woodstock and Grabe 1967), and soybean (Woodstock *et al*. 1984) seeds. These data were also confirmed in aged seed lots of pea and barley (Carver and Matthews 1975), where the level of oxygen uptake and the respiratory quotient were positively and negatively, respectively, correlated with field emergence. The key respiratory enzyme cytochrome *c* oxidase (EC 1.9.3.1) was also investigated to probe *Phaseolus* respiration using the whole seed or the extracted enzyme, and related them to rate of respiration and vigour (Sowa 1993). The author used also the Fourier transform infrared instrumentation-photoacoustic spectrometry, able to collect all spectral information simultaneously on the functional molecular dipoles that are informative of peptide bonds, membrane lipid side chains and ester linkages, carbohydrates, phosphates and CO_2 production with a variety of sampling techniques in order to utilize intact seeds. Preliminary results indicated a potential use of this technique to examine relationships between biochemical structure and function and the viability of plant germplasm conserved at the National Seed Storage Laboratory (USDA-ARS, Fort Collins, USA).

A wide range of volatile compounds evolve from seeds in storage, and may be either a cause or consequence of seed ageing. By gas chromatography analysis Zhang *et al.* (1994) identified eleven kinds of volatile components emitted by carrot seeds, including propylene, methanol, acetaldehyde, butane, ethanol, acetone, isopropanol, isobutanol, acetic acid and ethyl acetate. The deleterious effect of the volatiles on germination capacity increased with increasing seed moisture content, as measured during 23 and -3.5°C temperature storage in lettuce, soybean, sunflower, carrot, and rice seeds. In order to define some species of volatile compounds as markers of seed vigour loss, Taylor *et al*. (1999) found that the concentration of methanol and ethanol in soybean seeds aged in closed packets increased as germination decreased below 60%. Acetaldehyde was shown to react with non-enzymatic reaction with proteins to form acetaldehyde protein adducts, that are not volatile compounds and can be quantified by immunological techniques. The concentration of this chemical complex was shown to be linearly related to viability in soybean, and so can be relevant as biochemical indicator of seed vigour (Lee *et al*. 2000). Since free radicals may be involved in ageing process (McDonald 1999), their assessment may be useful in testing seed viability. Electronic paramagnetic resonance spectra of individual intact seeds, obtained in a range of pea, soybean, bean, and different *Brassica* species, showed that there is no relationship between free radical content and seed viability or early seedling growth, while prediction of the viability of individual seeds may be established by free radical content of one part of the seed, the testa (Hepburn *et al.* 1986).

Low vigour seeds have been shown to possess poor membrane integrity as a result of mechanical injury and deterioration (Priestley 1986b). When deteriorated seeds are imbibed, cells release cytoplasmic solutes, such as phosphorus and potassium ions, sugars, amino acids, enzymes, into the imbibing medium. Solutes with electrolytic properties carry an electrical charge which is measured by a conductivity meter. Measurement of the conductivity leachates from seeds is rapid, inexpensive and a simple procedure which can be applied on a bulk of 10-100 large size seeds or on individual seeds by an automatic analyzer having a single probe conductivity meter (Steere *et al.* 1981; Hepburn *et al*. 1984). The degree of seed leakage during imbibition is related to the stage of seed maturation, advancing ageing, the frequency of imbibition damages, and the condition of embryo (Matthews and Rogerson 1976; Powell 1986). Using the method in which conductivity is measured on a bulk of lentil, bean and chickpea (*Cicer aretinum* L.) seeds, it has found that conductivity is a good indicator of vigour in untreated and primed seeds, and strongly correlated with germination and seedling emergence (Fernandez and Johnston 1995). Leachate electric conductivity, measured in fresh and aged wheat and lentil seeds following imbibition with different period of salt and high temperature stress, gave a reliable response strongly correlated to modified germination parameters (Dell'Aquila and Di Turi 1996; Dell'Aquila 1999). The test was also taken as an indicator of lentil, hulled wheat, white cabbage and grass pea vigour, when these species were subjected to different drying treatment and stored under CD conditions (Dell'Aquila and Scialabba 2000). Electrical conductivity was used also to measure ion leakage in wheat seeds with high moisture content and stored at -20 to -80°C, as indicator of freezing tolerance and germination capacity (Dell'Aquila and Di Turi 1995). Prob-

lems arise when electrical conductivity is made with a multiprobe automatic seed analyzer meter, as reported in pea and soybean seeds (Hepburn *et al*. 1984; Hamman *et al*. 2001). Measurements of leakage did not differentiate clearly between dead and living seeds, and the range in conductivity of the non-emerged seeds overlapped the range of the emerged seedling, causing a non accurate prediction of germination performance. Also seed size did affect the results, and therefore a standardization could not be applied uniformly within a species (McDonald and Wilson 1979). Despite contrasting results, the conductivity test for pea species has met the requirements to be accepted by ISTA committees (Perry 1984; Fiala 1987). More recently, electrical impedance spectroscopy technique was developed to improve electrical conductivity measurements using a non-invasive methodology (Paine *et al.* 2001). The physiological basis of impedance measurement is based on conductivity depending on the concentration, charge, and diffusion of ions through membrane system in hydrated seed tissues. Snap bean seeds were aged in a manner to provide a range of seed qualities, and subjected to be scanned with a impedance/gain-phase analyser connected to a dedicated computer which was programmed to control the impedance analyser and elaborate the scored data. The low resistance values corresponded to seeds of low quality compared with those having high quality. The test was performed also in soybean seeds, partially imbibed, and related parameters were predictive of normal and abnormal seedling, or dead seed classification, as evaluated by a germination test (Vozáry *et al.* 2007).

The nature of the compounds leaked from deteriorated seed cells may provide a more specific approach in testing seed vigour than the conductivity test. Total sugars and potassium ion were measured in germination medium of pea seeds, and their increase was recorded in seeds whose cotyledons contained dead areas (Powell and Matthews 1977). Pandey (1989) used UV-spectrometric assay to test the exudates of French bean seeds and related them to advancing deterioration. Increasing amounts of alanine, glutamic acid and arginine were found to leak from various poorly germinating vegetable seeds using high-performance liquid chromatography (HPLC) analysis (Taylor *et al.* 1995). Sinapine, a predominant phenolic choline ester which accumulates as a reserve material during seed development on the mother plant and hydrolysed during germination, content was measured in different *Brassica* species by absorbance at 322 nm or with a colorimetric method (Taylor *et al.* 1991, 1993). Although sinapine leakage was found more from deteriorated than non-deteriorated seeds, any correlation with loss of vigour under selected storage conditions has been provided.

At different stages of maturation it has been observed a decrease in total chlorophyll content, as measured by the optical density of seed coat extracts, associated to the increase of germination capacity in carrot seeds (Steckel *et al.* 1989). Because of these relationships, the authors proposed a simple field test using colour cards to assess the chlorophyll content and estimate the optimal maturity time. This process, called 'degreening', can be investigated using chlorophyll fluorescence (CF) analysis, which is resulted to be a highly sensitive method in determining seed maturity and quality in *Brassica*, carrot and tomato (Jalink *et al* 1998, 1999). The method is based on measuring CF signals of intact seeds, by exciting chlorophyll *a* in the seed coat by laser radiation and the resulting fluorescence was measured non-destructively. An electronic ejector pump can separate instantaneously seeds with different CF signals. Three resulting fractions with low, medium or high germination have high, medium and low, respectively, CF content. The technique has been also applied in sorting controlled deteriorated white cabbage seeds, with two sets of experiments in which seeds were sorted before or after the ageing treatment, respectively (Dell'Aquila *et al*. 2002). CF sorting was more effective after the deterioration treatment indicating the potential of this method to be used in a seed-gene bank management, which needs new methods to limit seed accession rejuvenation in open field and to avoid erosion in plant genetic resources.

An exploratory use of isothermal micro-calorimetry to measure directly the heat flow produced as *Ranuculus sceleratus* L. seed age has been reported by Hay *et al*. (2006). Heat flow was recorded in primed and non-primed seeds with a thermal activity monitor at different seed water content. The rate of heat flow and total heat generated was generally greater in control seeds, which aged at a faster rate, than in primed seeds. Short-term experiments indicated the potential of this technique to predict relative longevity of investigated seed lots.

ENZYMATIC ACTIVITIES, PHOSPHOLIPID, SUGAR AND NUCLEOTIDE PATTERNS, PROTEIN AND DNA SYNTHESIS RATE AS MARKERS OF SEED VIABILITY AND VIGOUR

Biochemical indices of vigour derive from methods originally developed for basic research to study seed germination and ageing. In general, fast seed germination and rapid seedling growth are accompanied with high enzyme activity, respiration, ATP pool size, and synthesis of proteins, RNA and DNA, while ageing impairs seed performance and metabolic activities leading to mortality increase (Halmer and Bewley 1984). Many tests have been proposed, having features to be easily carried out during the early hours of imbibition on the whole seed or on the embryo axis, and to permit correlations between biochemical measures and germination or growth parameters. The disadvantageous characteristics of these seed quality markers are those to be destructive and to employ expensive experimental protocols, which complexity may be also source of errors when they are applied on fresh seeds or on those deteriorated. Differences may be due by need to use different extraction buffer, presence of inhibitors that reduce improperly enzyme activity, choice of the analysis on dry or imbibing seeds/embryos, and non significant statistically comparison among average data obtained from small seed samples (Priestley 1986b). A number of biochemical tests of seed vigour have been reviewed by Copeland and McDonald (2001). In this paper we focus the attention on the classic tetrazolium salt test and other biochemical tests that have been developed as single or multiple tests over the last four decades.

The tetrazolium salt test is one of the most valuable seed analysis tools and was firstly introduced by Lakon (1949). The tetrazolium test distinguishes between viable and dead tissues of the seed and/or the embryo on the basis of activity of dehydrogenase enzymes that reduce 2,3,5-triphenyl tetrazolium chloride colourless solution to form insoluble red formazan. Despite the rapidity of the test (few hours), seed viability is interpreted subjectively by a seed analyst according to distribution of staining pattern and the intensity of coloration on a single seed tissues. Howarth and Stanwood (1993c) modified the subjective evaluation of coloured tissue topography using a computer colour image processing. The test is employed also to distinguish, at the end of a germinatation test, ungerminated seeds that are dead from those that are eventually dormant and do not germinate under standard conditions. Instruction handbooks for experimental protocols of tetrazolium test have been published by ISTA (1985b) and AOSA (2000). The test is usually integrated with other tests of vigour. The effects of age and size on seedling vigour of wheat seeds were measured by combining tetrazolium test response, rate of respiration and emergence test (Kittock and Law 1968). A multiple laboratory test for standard germination, seedling vigour classification, seedling length and tetrazolium staining was developed to perform models predicting field emergence of a number of seed lots of soybean (Yaklich and Kulik 1979). An alternative method to quantify spectrometrically the amount of reduction of tetrazolium salt as a measure of viability of wheat and barley seeds was proposed by Harty *et al*. (1972). Using a wide range of deterioration stages, bulk

samples of seeds were ground, incubated with a solution of tetrazolium salt, and colour intensity of formazan solution was measured as absorbance at 480 nm. The modified test avoids subjective evaluation, and gives an indirect measure of dehydrogenase activity as an index of viability prediction of cereal seed samples with different levels of deterioration.

Other enzymatic activities have received particular attention as rapid indicators of seed vigour and viability. Dehydrogenase and glutamic acid decarboxylase (EC 4.1.1.15) activities, measured spectro- or manometrically, respectively, have been used together with osmotic and cool-temperature stress, conductivity, tetrazolium, respiration rate and ATP content tests to determine usefulness and repeatability of laboratory multiple vigour tests as predictors of field seedling emergence for wheat and pepper cultivars (Steiner *et al.* 1989; Trawatha *et al.* 1990). The authors found that, despite measures of seedling growth were the best indicators of field performance, improved predictions could be made by combining selected vigour tests in multiple regression modes, depending on growth locations and years. In contrast, glutamic acid decarboxylase activity test used in a multiple seed vigour test for artificially aged varieties of barley resulted less efficient than the other tests in predicting field emergence, and only if combined with other tests gives reliable results as indicator of seed performance (Kim *et al*. 1994). Differences in alcohol dehydrogenase (EC 1.1.1.1) activity were found in sixteen seed accessions of four *Brassica* species following 5-22 year storage in the germplasm bank of the Universitad Politécnica de Madrid (Madrid, Spain) under long-term (-10°C and 3% moisture content) and short-term (5°C and 8% mc) storage conditions (Ramiro *et al*. 1995). Starch horizontal electrophoresis was used to determine eight enzyme systems in each extract from individual accession: aconitase (EC 4.2.1.3), alchol dehydrogenase, isocitrate dehydrogenase (EC 1.1.1.41), malate dehydrogenase (EC 1.1.1.37), malic enzyme (EC 1.1.1.40), phosphoglucose isomerase (EC 5.3.1.9), phosphoglucomutase (EC 2.7.5.1), and 6-phosphogluconate dehydrogenase (EC 1.1.1.44). The results showed that isozyme analysis can serve as a fast and less expensive economical tool in a seed-gene bank, and alcohol dehydrogenase enzyme system seemed to be related to seed ageing. These data were confirmed also studying germination capacity, activities and electrophoretic patterns of alcohol dehydrogenase and lactate dehydrogenase (EC 1.1.1.27) isoforms in wheat seed samples having different viability degree and imbibed under aerobic and anaerobic conditions (Dinelli and Lucchese 2003). Both enzymatic activities decreased with advancing age, and two additional isoforms, disappearing in aged seeds, were shown in alcohol dehydrogenase pattern when high germination capacity seeds were treated with anaerobic conditions.

Phospholipids as indicators of vigour loss via membrane deterioration have had remarkable attention even if contrasting results make these hypothetical biochemical indices poorly appropriate for seed vigour prediction. Discrepancy bring to the question under which conditions of temperature and relative humidity used for 'accelerated' ageing reproduce the changes of 'slow' ageing or so called 'natural' ageing. A close correlation has been found between membrane phospholipids, measured on dry seeds by spectrometric analysis, composition and loss of viability in both aged and primed tomato seeds (Francis and Coolbear 1984). In contrast, any association between accumulation of peroxides or loss of phospholipids with aged pea seed has been found (Powell and Harman 1985). Two sets of ageing conditions were used to deteriorate cucumber and onion seeds and measure of total phospholipids and fatty acid species or conjugated dienes and malondialdehyde were made with thin layer chromatography and with spectrophotometric methods, respectively (Salama and Pearce 1993). Significant loss of these compounds occurred during both slow and fast ageing, as well as enzymatic activities of phospholipase D (EC 3.1.4.4) and lipoxygenase (EC 1.13.11.12), but lack of correlations between studied enzymes and viability was probably due to the different timing of possible damaging events. Enzymatic activity of peroxidase has been frequently cited as cause of seed deterioration by lipid peroxidation, which may begin with production of free radicals, leading to cell damage and seed death (McDonald 1999). In soybean (Stewart and Bewley 1980), peanut (*Arachis hypogaea* L.) (Sung and Jeng 1994), watermelon (*Citrullus lunatis* Thunb.) (Chiu *et al.* 1995) and sunflower (Bailly *et al.* 1998), loss of viability during accelerated ageing is associated with decrease of total peroxidase (EC 1.11.1.7) enzymatic activity. In radish seeds, polyacryamide gel electrophoresis analysis showed different number of peroxidase isoenzymes in the embryo axis, integument and cotyledons (Scialabba *et al.* 2002). Only the isoform of lowest MM (29.5 kDa) decreased during ageing, and it may suggest their potential to be used as biochemical marker of seed deterioration. More recently, substance (phenolic compounds, α-tocopherol, sterols, ascorbic acid, glutathione and soluble proteins) analysis that could play a role in maintaining seed viability was carried out in beech (*Fagus sylvatica* L.) seed lots, stored for 2-10 years at -10°C (Pukacka and Ratajczak 2007). Viability was strongly and positively correlated with phenolic compound content and UV-absorbing phenols and soluble proteins, while a strong negative correlation was found between viability and superoxide radical, hydrogen peroxide and lipid hydroxyperoxides. These data showed also that beech seed ageing was due to both increase of products of lipid peroxidation and decrease of antioxidative compounds which could play a role to prevent seed deterioration.

Assessment of ATP levels is another approach for predicting vigour. During seed formation, ATP content of seed increase and is utilized for structural growth and biosynthetic processes during germination (Ching 1982). The embryo not only has the enzymes and the substrate for *de novo* synthesis of ATP, but also the savage enzymes to convert adenine and adenosine to ATP. The rate of final percentage of field emergence was measured in several seed lots of carrots, onion and cabbage with rates of ATP synthesis and measure of AMP and malate content (Perl and Kretschner 1988). Contrasting results may be due to differences between both the processes of accumulation or synthesis of ATP: seed quality was strongly correlated with AMP content and ATP synthesis in carrot, with malate content and reduction of ATP synthesis in onion, and with malate and AMP content in cabbage. The possibility to use ATP content as an indicator of seed quality was studied also in onion seeds during 145 weeks of storage at different temperature (Siegenthaler and Douet-Orhant 1994). Following 36 weeks of storage ATP synthesis was greater at 3°C than at 15 or 30°C, indicating that this phenomenon is likely an expression of ageing. Measuring ATP content at 17 h imbibition, a close correlation was established with germination capacity in aged onion seeds. Wheat seeds of reduced vigour ranging between 92-64% can be distinguished from high vigour seeds by decreased levels of nucleotides (ATP, GTP, UTP, CTP, and UDP sugar) measured by HPLC analysis made on imbibing embryo tissue extracts (Standard *et al.* 1983). The analysis of nucleotide levels has the potential to provide a comprehensive diagnosis of the metabolic state of the cereal seeds, but it is not applicable for commercial seed testing needing more standardization and automatism. In cereals, α- (EC 3.2.1.2) and β-amylase (EC 3.2.1.2) activities are essential for providing energy and carbon skeletons to the growing embryo through respiratory breakdown of utilizable ATP, nucleotides and stored lipids at the onset of germination. Amylase enzymatic activities were detected with colorimetric methods in naturally and accelerated aged wheat (Petruzzelli and Taranto 1990) and rice (Nandi *et al*. 1995) seeds, and their decrease in imbibing embryos was correlated with viability.

As seeds age they lose ability to metabolize glucose and maltose to CO_2 during early hours of germination. In wheat and barley, resulting poor correlation between glucose metabolism and deterioration is partially due to the fact that

changes in the endosperm were independent and different of the changes in the embryo (Anderson and Abdul-Baki 1971). A multiple criteria approach was introduced by Abdul-Baki and Anderson (1973) to evaluate 16 lots of soybean by determining in their embryos respiration rate, uptake of ^{14}C-glucose and ^{14}C-L-leucine and their conversion in polysaccharides or proteins and leaching of metabolites through membranes after 5 h of imbibition. The vigour index, calculated by multiplying germination percentage by hypocotyl length at 5 days of imbibition, was strongly correlated with all measured biochemical indices, showing the potential of this laboratory method that in a working day could be able to predict and evaluate seed vigour. In different cultivars of soybean seeds a multiple laboratory test was designed to compare emergence performance with ATP content, conductivity of seed steep water and protein synthesis, using ^{14}C (U)-leucine leakage and incorporation in embryonic axes (Yaklich *et al*. 1979). The correlations of the laboratory tests showed differences between the cultivars, possibly due to genetic differences, sample size, and year to year variation. Another laboratory complex vigour test was designed by Dell'Aquila (1987) to test various degree of vigour in wheat seeds aged and subjected to osmo-priming treatment to recover seed vigour. Germination percentage and mean germination time values were correlated with primary root length and fresh weight, and '*in vivo*' uptake and incorporation of L-[4,5-^{3}H] leucine, as measure of total protein synthesis, and [6-^{3}H] thymidine, as a measure of DNA synthesis, in imbibing embryos. Macromolecule synthesis were strongly correlated with mean germination time, confirming this germination index to be appropriate in determine little differences of vigour in seeds with any apparent change of viability. A complex laboratory test of seed vigour was applied also in wheat seeds differing in vigour degree, as obtained by delayed harvesting after the physiological maturity, ageing under controlled conditions, and osmo-priming treatment to improve germination performance (Dell'Aquila and Tritto 1990, 1991). The relationships among the seed quality components showed that the different physiological states may be reliably monitored by mean germination time closely correlated with protein and DNA synthesis rates. In the case of protein synthesis, 2-D polyacrylamide gel electrophoresis (PAGE) of soluble embryo proteins aided by computer image analysis was used to define specific polypeptides related with the start of germination in wheat seeds (Dell'Aquila and Di Turi 2002). Following '*in vivo*' [^{35}S]-methionine incorporation, some novel polypeptides, probaby belonging to the class of α- and β-tubulin, with pH 5.4-5.9 and MM ranging between 75-54 kDa could be considered as qualitative and quantitative biochemical markers in assessing wheat seed survival to ageing, salt and high temperature stress. A data base of 2-D PAGE patterns of total soluble proteins of wheat embryos, including a computer image analysis elaboration, has been published on the following web page: http://germproteomics.ba.cnr.it.

DNA CONTENT AND POLYMORHPISM IN SEED QUALITY ASSESSMENT

Traditionally, breeders and seed companies determine genetic purity using physical traits assessed in seed or mature plant or biochemical characterizations of seed or seedling enzymes, often separated with electrophoretic techniques (McDonald 1998). One recent advance in genetic purity test has been the application of the technique of polymerase chain reaction (PCR) to perform the random amplified polymorphic DNA (RAPD). The technique provides a great discrimination potential among varieties and has been tentatively applied to monitor DNA polymorphism with seed viability and vigour performance. At the molecular level, increased strand breaks in seed DNA, as a result of advancing ageing, have been demonstred by Cheah and Osborne (1978). Two working hypotheses have been highlighted in studying RAPD profiles in soybean seeds under accelerated ageing treatment (Shatters *et al*. 1995): first, the damage to cellular DNA is randomly localised within the genome, and so any influence on RAPD major bands can be found until the damage became severe; second, the presence of hot-spot regions in the genome more susceptible to ageing damage may produce consistent polymorphism during early seed deterioration. A multiple vigour test has been performed to study the rapid deterioration of soybean seeds, including standard germination, accelerated ageing, electrical conductivity tests, and RAPD analysis on DNA integrity (Marcos-Filho and McDonald 1998). The results showed that soybean seed deterioration can be first detected by germination and vigour decline and later by decrease in DNA concentration for lower quality seed lots. No significant alterations in RAPD profiles associated with advancing ageing were observed regardless of the primer used, suggesting that this analysis is not suitable to be used in seed viability testing. Similar results have been also obtained studying ageing of longleaf pine (*Pinus palustris* Mill.) seeds stored under 4 and 30°C conditions (Tolentino *et al*. 2003). Although DNA content was stable for both storage temperatures, gel electrophoresis revealed slight differences in DNA fragmentation to smaller molecular weight species in relation to two temperature regimes. The major problem with the RAPD technique is the reproducibility regardless of whether the polymophisms arise from microorganism contamination or alteration in seed DNA. An interesting use of more advanced molecular analysis on DNA of 4th and 15th century ancient common millet (*Panicum miliaceum* L.) seeds has been reported by Gyulai *et al*. (2006). Ancient DNA was extracted and analysed by amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) and mitochondrial DNA (mtDNA) and compared with modern millet cultivars. The SSR is optimal for ancient DNA analysis by excluding cross-reactions with contaminant microorganism. Agarose gel electrophoresis showed different degrees of degradation in the 4th and 15th century samples compared to modern common millet, even if results are ambiguous with the detection of high molecular weight fragments in the 15th century DNA. Another application of PCR techniques in studying DNA polymorphism and its integrity is that to study the capacity of seeds that have lost viability to be still valuable as vessels of genetic information. A modern development of seed-gene banks lies in the establishment of related DNA banks. Large quantities of high molecular weight DNA can be extracted from dead seed tissues allowing extension of the useful lifespan non-viable seed accession (Walters *et al*. 2006). DNA extracted from four garden species, with age from 1 to 135 years, was used to examine the early stages of DNA degradation. Seeds that were 70 years old yielded high molecular weight DNA, which was compared with less aged varieties. The results confirmed that DNA degraded more slowly within seeds than in leaf tissues and high-quality DNA can be extracted from old non-viable seeds expanding the utility of seed-gene banks in preserving genetic resources.

FUTURE PERSPECTIVES FOR SEED QUALITY TESTING

Needs for development and improving seed quality tests are various. Farmers require information to expect rapidity and uniformity of seedling emergence, and seed industries need improving specific tests to guarantee the best levels of seed quality for production and trade purposes. Seed-gene banks require non-destructive testing and sorting procedures to store high quality seed accession in cold storage rooms, and seed researchers need seeds with well defined genetic and physiological quality characteristics to have reliable results in seed biology research. Two main aspects predominate in seed germination and vigour evaluation: 1) some vigour tests were designed to identify directly seed germination performance and growth rate by germination test *per se*, or following stressing treatments made before imbibition or during early phase of imbibition prior the emergence phase

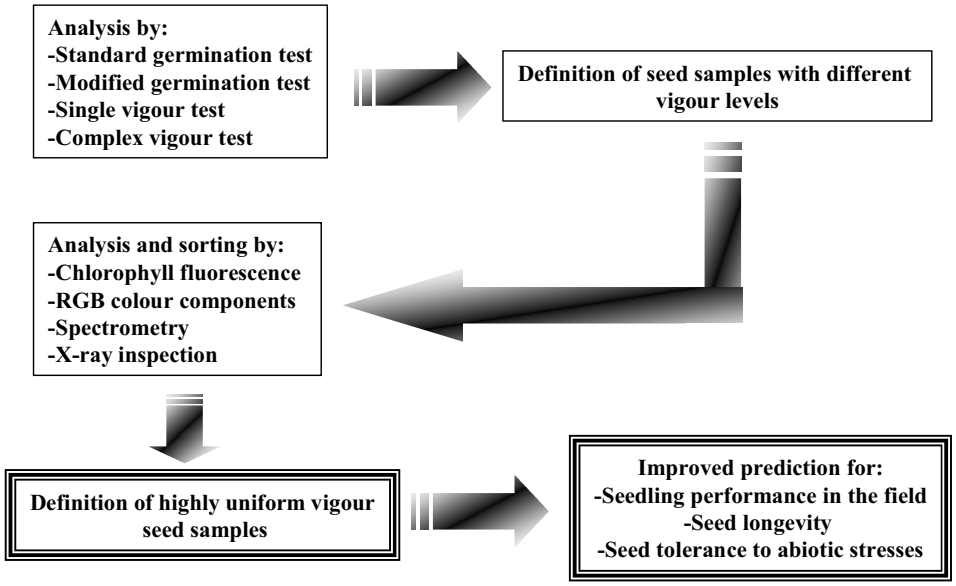

Fig. 2 A schematic model of integration of classical seed quality tests with new techniques improving viability and vigour level prediction and real-time sorting.

with the aim to predict any difference of vigour pattern; 2) a number of seed vigour tests evaluate in destructive way some biological markers which can represent a complex living state of seed. In addition, this approach requires that most relevant tests to be closely correlated with a series of seed vigour levels for viability, emergence and storage prediction. The choice of different types of vigour tests by seed analysts also reflect the continuous development of basic research, methodologies and equipments, as well as the specific demand of farmers and seed industries. Despite the complexity of tests proposed up to now, the main feature is that they are laboratory tests made under controlled environmental and pathogen contamination conditions with limited 'field planting value' but extended 'potential quality value', as pointed out by Hampton (1995).

In the last two decades, the sophistication of non-destructive methods has evolved rapidly with modern technologies. The new features of seed quality testing may provide that the tests should be non-destructive, made on a single seed, rapid, not requiring the use of contaminant chemicals and complex procedures, and giving a series of objective and reliable data that can be easily correlated with those obtained by a standard germination test. In spite of the benefits of modern techniques and non use of hazardous laboratory procedures, the main limitation to use non-destructive technology is related to high cost of instrumentation of new generation, which combine automation, precision, hardware facilities and software packages designed for measurements and data elaboration. Use of computer technology in seed quality assessment will increase in the future with different approaches, including the development of machine vision systems to replace human visual inspection, the maintenance of evaluation records, fast graphical plotting, and statistical elaboration. The declining cost and increasing power of computer hardware as well as the implementation of new algorithms for image processing have made image analysis systems more attractive in automatic examination of different aspects of seed imbibition, germination, and seedling growth (Dell'Aquila 2007a). Computer imaging can also aid the inspection with X-ray and NMR techniques, to provide a non-destructive computer-directed sorting procedure. More recently, X-ray computed tomography has been developed as non invasive approach to three-dimensional visualization and quantification of biological structures (Stuppy *et al.* 2003). Even if this technique has been developed to meet specific problems, it is desirable that it evolves in more reliable evaluation of internal seed structure integrity for viability prediction. As future perspective, the problem of seed viability and vigour testing might be associated with novel technique potential to allow fast selection of an individual seed with high germination capacity from a seed sample with a marginal error. The terms of 'more or less vigorous seed sample', as evaluated by standard methodology, could be replaced by the new concept of 'highly uniform vigour seed sample', based on the potential of a seed laboratory test to select physically seeds with high performance uniformly distributed within a large seed lot. RGB and CF markers have been demonstrated to be effective in sorting high physiological quality seeds during delayed harvesting and ageing (Jalink *et al*. 1998; Lee *et al.* 1998; Dell'Aquila *et al*. 2002; Dell'Aquila 2006), and their application in seed industry production and seed-gene bank management could be promising. A model of integration of classical methods with new methodology in seed quality testing is outlined in **Fig. 2**.

These types of integrated systems may represent one aspect of precision and sustainability systems in agriculture, which include also taxonomic, morphological, and proteomic-genomic features of plant species (Cox 2002). These systems possibly may include: 1) operative system modelling, automation and information technology, 2) digital imaging and new category of chemical-specific sensors, such as functional molecules, enzymes, cell receptors, and nuclei acids with bio-recognition properties, 3) new hypothesis of basic research focused on real-time monitoring of seed quality in non-destructive way. The future target should be a 'new vision' of seed viability and vigour testing systems integrated with other biological systems to define novel traits in economically important crop plants.

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