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BRIEF COMMUNICATION

Pepper seed germination assessed by combined X-radiography and computer-aided imaging analysis

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Abstract

A lot of pepper seeds having 87 % germination were subjected to X-ray inspection using a non lethal dose of radiation. Seeds with less than 2.7 % (on the basis of total seed area) of free space area, *i.e.* the spaces between embryo and endosperm, were classified as highly viable seeds (97 - 100 % germination) with the lowest level of abnormal seedlings. Seeds X-ray classified as good were subjected to a computerised image analysis to study seed imbibition and radicle elongation. The patterns of seed area increase, chosen as the most accurate indicator of seed swelling, resembled the triphasic curve of water uptake. The first phase was completed at 9 h followed by a second phase that varied widely in time until completion of germination between 52 and 96 h. The proportion of seeds with radicle protrusion between 52 - 56 h and 64 - 72 h assessed with the image analysis was significantly higher than that recorded using a conventional germination test. In addition, the rate of increase of seed area during the third phase of imbibition, mostly due to protrusion of the radicle tip and its growth, was highly correlated with the corresponding radicle elongation rate.

Additional key words: *Capsicum annuum* L., internal seed morphology, seed swelling.

Advances in seed quality evaluation by computer imaging, together with molecular genetics and the new seed enhancement techniques, are allowing to improve conventional seed quality methods (for review see McDonald 1998). Nuclear magnetic resonance (NMR) micro-imaging combined with X-radiography have been applied to the study of pepper seed internal morphology in quiescent and imbibing status (Foucat *et al.* 1993). NMR spectroscopy has been also used in comparing spin-spin relaxation time with germination of soybean and wheat (Krishnan *et al.* 2004). 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.e.* the space comprised between embryo and endosperm), have been related to germination capacity (Van der Burg *et al.* 1994). This type of analysis has been used also to study the internal anatomy of tomato seeds subjected to priming technique (Argerich and Bradford 1989, Downie *et al.* 1999). Recently, the development of appropriate image analysis software packages has allowed to carry out measurements of seed internal structures and to provide more reliable and objective parameters in sorting seed categories according to their germination quality. Since the imbibition phases of germinating seeds can be monitored by an automated vision machine (Dell'Aquila 2004), this technique may give additional information on the degree of physiological variations existing among viable individual X-ray sorted seeeds. The used methodology consists of a computer-aided image analysis system able to capture time-lapsed seed images and to perform seed size and shape measurements. The swelling process in small sized *Brassica* seeds has been assessed in terms of increase in seed volume (McCormac and Keefe 1990) or area (Dell'Aquila *et al.* 2000). Computerized image analysis is reported to be an accurate method to measure also seedling growth rate in lettuce, sorghum and rice seeds (Howarth and Stanwood 1993, Iijima *et al.* 1998). Even if the technique is destructive, its automation feature represents a valid tool

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Abbreviations: TIFF - tagged image file format; G - germination; MGT - mean germination time.
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to monitor several germination characteristics, such as the extension in the first two imbibition phases of the triphasic curve of water uptake (Bewley 1997) and the timing of germination start. The aim of this work is, firstly, to identify viable pepper seeds using a non destructive X-radiography technique and, secondly, to monitor their imbibition by computer-aided image analysis with the aim to defining an imaging parameter as a quality marker in sorting and evaluating high germination seeds.

Fifty seeds of pepper (*Capsicum annuum* L. cv. Sigaretta biondo) for twenty replicates, from an original lot of seeds with 87 % germination at harvest, were placed in a cassette-type holder in direct contact with the film (*Kodak* X-ray film) during the X-ray exposure, according with the method of Van der Burg *et al*. (1994). The carrier was placed 53 cm from the X-ray source, and the photographs were taken at 15 KeV for 5 min exposure time, using *Faxitron 43805* X-ray system (*Hewlett-Packard*, Palo Alto, USA). Developed photographs showing 50 seed images were further scanned with a commercial flat scanner (*Sharp mod 330JX*, Osaka, Japan). Captured seed images were converted into a computer processed format showing the external contours of the whole seed and of internal free spaces, which areas, respectively, were measured by *ImagePro-Plus* (IPP) *v.4.5* software package (*Media-cybernetics*, Siver Spring, USA). Following a X-ray inspection, seeds with extended free space area and showing folded, wrinkled, broken or otherwise deformed embryos were isolated from those with intact internal morphology. Two separate germination tests were carried out: the first on X-ray abnormal classified seeds, and the second on X-ray normal classified seeds. Four replicates of fifty seeds were placed on a 15 cm diameter Petri dish with two sheets of filter papers and 10 cm^3 of distilled water to allow germination at 25 °C in the dark. A seed was scored as germinated when radicle length reached 1.5 - 2 mm. Germinating seeds were counted twice a day, starting on the first day and until the tenth day of imbibition, when the maximum of germination was achieved. The following germination parameters were determined: final cumulative germination percentage; abnormal seedling percentage, according to International Rules for Seed Testing (ISTA 2005); mean germination time (MTG) calculated according to the formula MGT = Σ ($h \times n$)/Σ n, were h is the number of hours from the beginning of the germination test and n is the number of germinating seeds after h hours (Ellis and Roberts 1981). For computerised image analysis experiments, twelve replicates of nine normal pepper seeds were imbibed in a 12 cm diameter Petri dish containing 40 cm^3 of 0.4 % (m/v) polymerized agarose, added with 50 μg penicillin G and 50 μg streptomycin sulphate, at 25 °C. The Petri dish was placed above a small transilluminator connected with a timer which ensured 15 min light every hour for a clear view of the seeds. The computer image analysis unit consisted of the following basic components: a digital camera, *Silicon Video 2112 CMOS*, and a *PIXCI D2X* imaging board (both purchased from EPIX, Buffalo, USA), a commercial personal computer in *Windows* environment and the software package *IPP v. 4.5* for image recording and processing. The software was used to record automatically the successive images of nine seeds every hour and to store them on the hard disk. After a calibration setting to convert pixels into millimeters, the images were analysed to measure the area (*i.e.*, the area of the polygon defined by the object image outline) of the single seed according to software instructions. Data, reported as percentage of increase on dry (not imbibed) seed basis, are the average of twelve replicates of nine pepper seeds. The end of the first phase of rapid increase of seed area as well as that of the second phase of non apparent increase of seed area were determined as the time when the first and the second inflection points, respectively, were detected in the sigmoid curve of seed area increase (Dell'Aquila *et al*. 2000). Radicle length was measured on different time-lapse images of an individual seed within a sample of nine germinating seeds in triplicate from emergence time to 1.5 - 2 mm elongation, using an appropriate measurement tool of the software package. Correlation coefficient and linear regression line between the values of radicle length and those of corresponding seed area, reported as percentage increase of seed area at the beginning of radicle protrusion, were calculated with *Fig. P 2.98* software package (*Biosoft*, Ferguson, USA).

No genetic damages are expected to occur as a consequence of the radiation dose, as previously demonstred by Van der Burg *et al*. (1994), and no difference in G (87 and 88 %, respectively) and MGT (89.3 and 90.3 h, respectively) has been observed between untreated and X-ray treated pepper seeds. Mature pepper seed are laterally flattened, so the resulting X-ray photograph provides a clear view of the internal morphology (Fig. 1*A*-*H*, *on the left*). In addition, the presence of free space within a seed, displayed by the less dense areas of cavities that do not contain cellular protoplasm and do not absorb the radio-energies passing through them, can be further highlighted by a computer processed imaging (Fig. 1*A*-*H*, *on the right*). Following a X-ray inspection, each sample of fifty seeds was divided into four categories: *1*) with a normal embryo and two cotyledons visible in the plane of the photograph, with internal dense structures and lack of free spaces (Fig.1*A*,*B*); *2*) with less dense structure, that allows to observe more distinctly the endosperm, cotyledons and root, with increasing presence of free space (Fig. 1*C*,*D*); *3*) with increasing abnormalities in the embryo (strongly folded and wrinkled) and abundant free spaces (Fig. 1*E*,*F*); *4*) with extended embryo and cotyledons abnormalities (clear signs of morphological degradation, such as broken and deformed structures) and with a free space area exceeding 5 % on the basis of whole seed area (Fig. 1*G*,*H*). Pepper seed sub-groups were manually sorted after X-ray analysis and subjected to germination test. The distribution of seeds in relation to increasing free space area showed that the largest proportion (74 %)

of seeds belonged to X-ray category *1* and *2* and germinated normally, while the remaining seeds with free space area higher than 2.7 % showed increasing abnormal seedlings or did not germinate at all (data not shown). Cumulative germination of the two first pooled X-ray categories of pepper seeds was higher (98 %) and mean germination time was lower (77.3 h) than the original seed sample. Germination of X-ray sorted viable

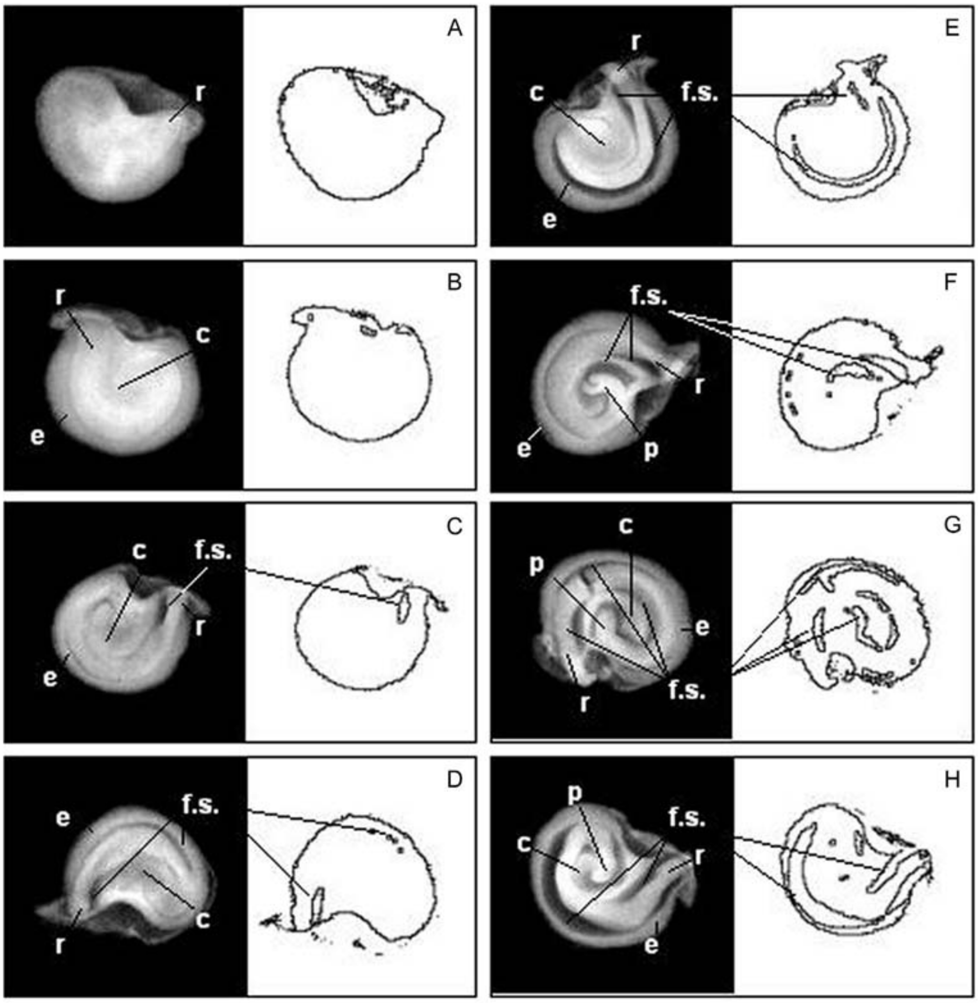

Fig. 1. X-ray photographs (*on the left*) and related computer imaging (*on the right*) of pepper seeds showing different internal morphology (r - root tip, e - endosperm, c - cotyledons, p - plumule, f.s. - free space). For the explanation of *A* to *H* see text.

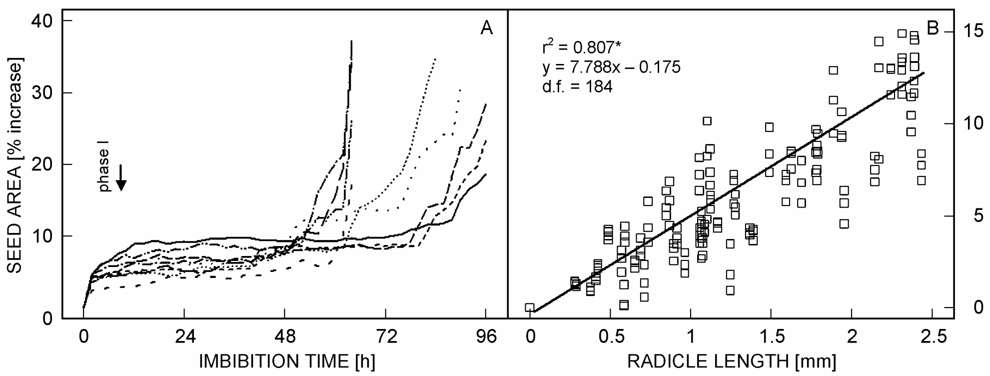

Fig. 2. *A* - seed area increase (on dry seed basis) time courses of nine pepper seeds, as assessed with a computerized image analysis; *B* - linear regression line between pepper seed area increase (on the ungerminated seed basis) and the corresponding radicle length up to 1.5 - 2 mm from three replicates of nine germinating pepper seeds. d.f.= 184; * - significant for $P < 0.001$.

seeds was further tested with an image analysis system allowing the early phases of imbibition to be monitored for each seed within a population of seeds (Fig. 2*A*). The seed area is generally accepted as the most accurate indicator of seed swelling before the completion of the germination process, as already reported in different kinds of seeds (Dell'Aquila 2004). In addition, the start of germination may be estimated in two ways: firstly, by the time when the first radicle protrusion is visible in a set of captured images, and secondly, by the time when the second inflection point of seed area increase occurred. Seed area increase was monitored up to 96 h water imbibition and the appearance of the first radicle protrusion was recorded on twelve replicates of nine seeds each. The patterns resembled the triphasic curve of water uptake (Bewley 1997), where the first phase of rapid area increase was completed at 9 h in all seeds, followed by a second phase of little apparent change of the area that varied widely in time, until completion of germination comprised between 52 and 96 h. A comparison between conventional germination test and image analysis technique showed a different distribution of seeds characterized by radicle protrusion timing in the range of 52 - 56, 64 - 72, 76 - 80 or 84 - 96 h, respectively. Seed proportion on the basis of total seeds for the two first sub-groups, as estimated by image analysis, was significantly ($P \leq 0.05$) higher than that assessed with the other test (data not shown). The last phase of rapid seed area increase, mostly due to protrusion of the radicle tip and its growth, corresponded to third phase of the fast resumption of water uptake. The rate of increase in seed area was correlated with the corresponding radicle elongation rate, both calculated from the time of radicle tip protrusion up to 1.5 - 2.0 mm length, when 'visible' germination can be assessed with a conventional germination test (Fig. 2*B*). A significant correlation was established between radicle length and the corresponding seed area increase, confirming that this image analysis parameter can monitor indirectly early radicle elongation in pepper seeds.

Seed radiography offers seed researchers a non destructive test to evaluate germination potential of economically important crop and tree species. Previous works by Liu *et al*. (1993) and Van der Burg *et al*. (1994) described extensively the presence of internal free space in different parts of tomato seeds, in the dry state or following imbibition and priming treatments. We have used this method to study the distribution of 87 % germination pepper seeds on the basis of free space area. Our data show that the free space area can be taken as an indicator of germination potential and is well related also to the increase of abnormal seedlings, that is a signal of advancing deterioration in a seed population. Apart for X-ray equipment, the used methodology requires a common computer technology: seed X-radiographs can be analyzed with a commercial image analysis software package to easily calculate the area of internal free spaces for a single seed within a population. In addition, the automated recording of the swelling process of most seeds through the detection of size change descriptors, such as the seed area, could be a valid alternative to hand measurement of seed mass and the imprecise timing of germination start, as determined by visual assessment (Dell'Aquila 2004). As expected, differences among seeds were depicted only for the duration of the second phase of area increase, coinciding with phase II of water uptake before radicle protrusion. The potential of image analysis technique is to evaluate the composition of a whole population of seeds in sub-groups according to different times of radicle protrusion also in highly viable seed samples which would apparently have a fast germination as revealed by conventional germination tests. These findings represent a further extension of the technique in studying germination performance of individual seeds, which avoids the assessment of pooled data from an entire seed population masking physiological variations. In addition, since radicle length measurement is difficult to take by hand and is subject to error, image analysis may provide a method to record early radicle elongation rate in terms of corresponding seed area increase. In conclusion, the area of internal free space and that of whole seed may represent valid biological markers in monitoring germination potential of an individual seed in dry state and during different phases of imbibition.

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