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Assessment of Seed Viability by Laser Speckle Techniques

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This work presents a new technique as a potential methodology to analyse seeds. The technology is known as dynamic speckle, or biospeckle, an optical phenomenon produced when active materials, such as biological tissue, are illuminated by laser light. In the present work, the biological activity of seed tissues has been inferred from quantitative and qualitative measurements of their speckle activity. The aim is to show that the biospeckle technique has a potential as a methodology to assess seed viability. One aspect that needs to be investigated is how the water content in the seeds affects bio-speckle activity. An experiment has been performed to determine the effect of humidity in the results. Seed activity for different levels of humidity was determined using quantitative and qualitative methods. Also, in others experiments, viable and non-viable seeds with different specific humidity levels could be classified using the same technique.

1. Introduction

Viability assessment is an important topic for seed production and commercialisation. Many tests have been developed to determine seed vigour and viability, however, these tests are not consistent among themselves, in the sense that they do not yield exactly the same results (Marcos Filho, 1999). Reliability and equivalence of different tests were the main objectives of recent studies (Hampton *et al.*, 1996; Howarth & Stanwood, 1993).

In this context, the evaluation of laser interferometric techniques as tools for seed analysis is worthy of evaluation. One of them is based on the properties of the dynamic speckle, which is a phenomenon occurring when coherent light is scattered by objects exhibiting some type of activity (Rabal *et al.*, 1996). The intensity in each point of the scattering pattern changes in time in a seemingly random fashion, but those intensity variations are related to, and carry information about, the activity in the object. The activity on the object may result in individual scatterers movement, changes in optical paths due to changes in refractive index, or combinations of both. When the phenomenon is observed in samples with biological activity, it is also known as biospeckle (Aizu & Asakura, 1996).

A similar phenomenon is also present in some industrial processes. Many efforts have been devoted to characterise quantitatively the activity, that is to assign numbers that correlate favourably with measurements of the processes of interest (Briers, 1978, 2000; Oulamara *et al.*, 1989; Okamoto & Asakura, 1995). These methods were applied to some extent to transient phenomena, *i.e.* biological activity of botanical specimens (Xu *et al.*, 1995), evaluation of blood flow (Aizu & Asakura, 1992), and drying of paint (Amalvy *et al.*, 2001).

Similar to the tetrazolium test (Howarth & Stanwood, 1993; AOSA, 1983), the dynamic speckle technique aims to generate a map of the seed, identifying areas with different activities. Since it is a new technique, it is necessary to investigate all possible phenomena which might interfere with the desired results, as for example the effect of humidity.

In this paper, the activity of seeds as inferred from the dynamic speckle patterns generated by them are investigated, with the aim of developing a method for viability assessment.

Two methods are employed in order to evaluate quantitatively and qualitatively the activity of the dynamic speckle patterns. One of them is based on the calculation of the second order moment (moment of inertia) of the co-occurrence matrix in the time history of the speckle patterns (THSP), as proposed by Arizaga *et al.* (1999). The other is a display where the activity of the sample is shown as an image by using a generalised differences (GD) method (Arizaga *et al.*, 2002).

As the seeds must be soaked before testing, the degree of moisture may be an important factor to take into account in the biospeckle measurement. Besides, the presence of water vapour in the path of the light may be a source of error in the measured value of the optical activity. In order to have an estimate of this kind of error, the correlation between dynamic speckle and the moisture level in the seeds was measured.

Then, the information obtained with viable and non-viable biological tissues in presence of moisture was investigated. The studies were based on different experiments that: (a) determined the inertia moment in viable and non-viable seeds during drying; and (b) sorted viable and non-viable seeds into a specific moisture level.

In most cases, different regions of the seeds produce differing levels of activity. Some changes are fast, some are slower and in some regions there are no changes at all. An 'activity image' can be constructed of the sample (the seed in this case) where the dark regions identify low activity and the bright regions denote high activity. The GD method was used to build activity images of seeds and a study of dead (low activity) and live seeds (high activity) is presented. This is convenient for seed viability assessment, since different regions of the seed have distinct importance for its viability.

The paper is organised as follows: Section 2 describes the fundamentals of the speckle phenomenon, and the mathematical tools used in its analysis. In Section 3, we present the experimental procedure, and the application of the method to bean seeds (*Phaseolus vulgaris* L.) is presented. The last section is devoted to discussions and conclusions.

2. Theoretical considerations

2.1. *Fundamentals of the laser speckle phenomenon*

The operation with laser light reveals a perhaps unexpected phenomenon: objects viewed in highly coherent light acquire a peculiar granular appearance. The detailed structure of this granularity bears no obvious relationship to the macroscopic properties of the illuminated object, but rather it appears chaotic and unordered, with an irregular pattern that is best described by the methods of probability theory and statistics. This phenomenon is know as 'laser speckle' (Dainty, 1975). The surfaces of most materials are extremely rough on the scale of an optical wavelength ($\lambda \cong 0{\cdot}6\,\mu m$). When nearly monochromatic light (laser light) is scattered from such a surface, the optical wave resulting at any moderately distant point consists of many components or wavelets, each arising from a different microscopic element of the surface. The distances travelled by these various wavelets may differ by several or many wavelengths if the surface is truly rough. Interference of the coherent wavelets differing in phase results in a granular pattern of intensity that is called speckle. *Fig. 1* shows the physical origin of the

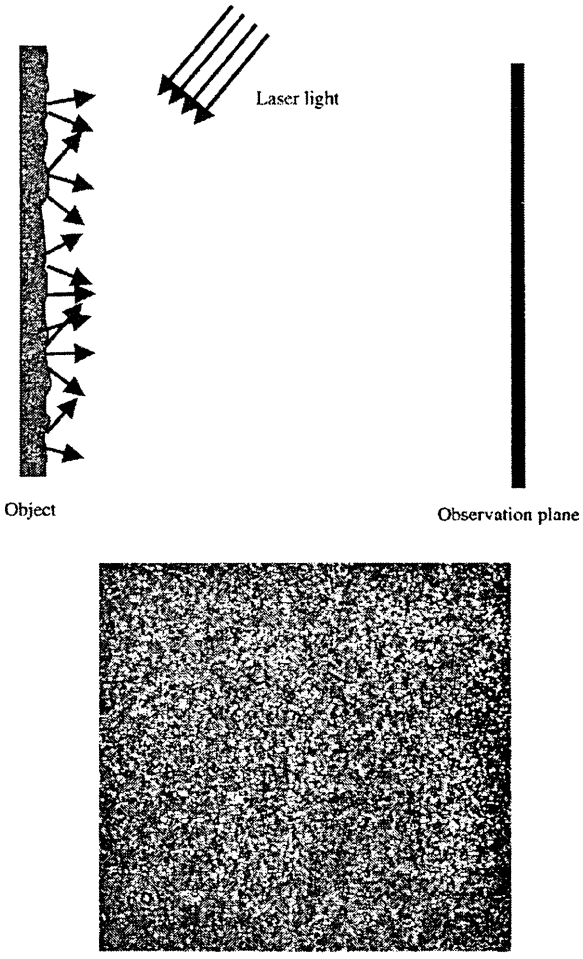

Fig. 1. Speckle pattern

speckle phenomenon and a typical speckle pattern. The object has a rough (unpolished) surface illuminated by laser light and a typical speckle pattern observed at a certain macroscopic distance is also shown.

Dynamic speckle is a phenomenon occurring when laser light is scattered by objects showing some type of activity. It is present in fruits and other biological samples, and some non-biological ones such as the drying of paint. It is due to changes in the phase of light produced by movements of the scatterers, changes in the refractive index, and rotatory power. Contributions from different scatterers beat in the detector producing time variations in local intensity. The visual appearance of the pattern is similar to that of a boiling liquid, so it is sometimes called 'boiling speckle'.

Recently, a method to measure the dynamic activity using the co-occurrence matrix of the time history of speckle pattern (Arizaga *et al.*, 1999), and another one to display the local dynamic activity of the samples was proposed (Arizaga *et al.*, 2002).

2.2. *Moment of inertia of co-occurrence matrix*

A convenient way to show the time evolution of a speckle pattern is that proposed by Oulamara *et al.* For every state of the phenomenon being assessed, 512 successive (digitised) images of the dynamic speckle pattern are registered. A certain column (for example, the middle one) is selected in each of them. Then, a new image is constructed by setting, side by side, the chosen column extracted from the successive patterns. The resulting image is named 'time history of the speckle pattern (THSP)'. Its rows represent different points on the speckle pattern and the columns their intensity in a sequence of regularly spaced time steps. The activity of the sample appears as intensity changes in the horizontal direction, that is, along the rows.

When a phenomenon shows low activity, time variations of the speckle pattern are slow and the THSP shows a horizontally elongated shape. In the limit, when there is no activity, the THSP shows no variation in the horizontal direction. Conversely, when the phenomenon is very active, the THSP shows fast intensity variations that resemble an ordinary spatial speckle pattern.

The THSP is assumed to be representative sample of the state of the phenomenon being assessed when it was registered.

The co-occurrence matrix M_{CO} is defined as

$$M_{CO} = [N_{ij}] \tag{1}$$

where, the entries are the number N of occurrences of a certain intensity value i, that is immediately followed by an intensity value j. This is a particular case of the so-called 'spatial grey level dependence matrices'. It is usually used to characterise texture in images. In the spatial case, its principal diagonal is related to homogeneous regions and the non-zero elements far from it represent high contrast occurrences.

In this work, the variable of interest is time. Then the involved N values are the occurrences of a certain grey value i followed in the next time step by a value j in the THSP as described above.

When the intensity does not change, the only non-zero values of this matrix belong to the principal diagonal. The presence of noise in the illumination source and in the detection precludes this situation.

As the sample shows activity, intensity values change in time, the number N outside the diagonal increases and the matrix resembles a cloud. Nevertheless, this matrix is sparse; it is mostly composed by zero values.

Figure 2 shows the THSP obtained in two extreme cases, high and low activities, and the corresponding co-occurrence matrices. It can be seen that the points in the main diagonal represent no change of intensity while the spread of points out of the diagonal represents time intensity changes. So, if the activity of the biological tissue is low, intensity changes are slow and the only appreciable values of the matrix appear near to the diagonal [*Fig. 2(a)*]. Conversely, if activity is high, the fast intensity changes produce high values far away the principal diagonal of the matrix [*Fig. 2(b)*].

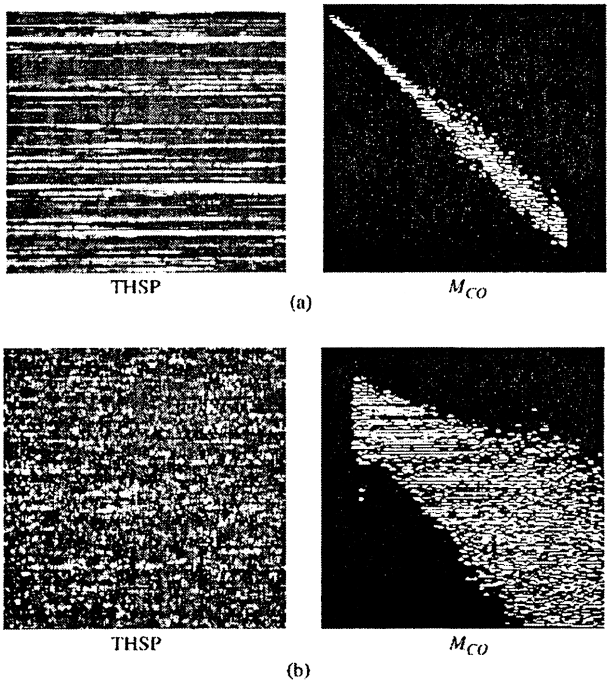

Fig. 2. Time history of the speckle pattern (THSP) and the corresponding co-occurrence matrix (M_{CO}) of: (a) low activity biological material; (b) high activity biological material

In order to obtain a quantitative measure from this matrix, it is necessary first to normalise it. This is done by dividing each row of the matrix by the number of times that the first grey level appeared. The sum of the components in each row then equals to 1.

$$M_{ij} = \frac{N_{ij}}{\sum_j N_{ij}} \tag{2}$$

This modified matrix is named the modified co-occurrence matrix, and it corresponds to a certain generalisation of the histogram. It is an experimental approximation to the transition probability matrix between intensity values in the THSP bearing a certain resemblance to the transition matrix appearing in Markov processes.

A measurement of the spread of the M values around the principal diagonal can be constructed as the sum of the matrix values times its squared row distance to the principal diagonal. This is a particular second-order moment called the moment of inertia M_I of the matrix with respect to that diagonal in the row direction. The name is suggested by the mechanical analogy with that operation. It is

$$M_I = \sum_{ij} M_{ij}(i-j)^2 \tag{3}$$

This measurement is similar to the one currently used in photon correlation spectroscopy, called photon structure function (Chu, 1991).

The occurrences in the diagonal do not contribute to increase the M_I value, while far away M entries add their more heavily weighted values. Similar measurements to this one are used in the discrimination of textures (Allam *et al.*, 1997).

2.3. *Generalised differences method*

When extended regions of a sample are investigated, usually their activity is not uniform. Then, it is sometimes necessary to segment the images according to their activity level. There are several image-processing methods to this end presented by Briers and Webster (1996); Fujii *et al.* (1987), and Konishi and Fujii (1995). Recently, Arizaga *et al.* (2002) proposed that the possibility of intensity changes in different time scales be taken into account by assigning to each pixel in the processed image the value $I'(i,j)$ obtained using the following expression:

$$I'(i,j) = \sum_k \sum_l |I_k(i,j) - I_{k+l}(i,j)| \tag{4}$$

where k and l are indices spanning all the possible numbers of the registered images.

This procedure is called GD. As every $I_k(i,j)$ value is subtracted from all others values in the same location, the result does not depend on the order of appearance of the $I_k(i,j)$ values. With these values, a new image is built where the dark regions identify low activity and the bright regions, the higher ones.

3. Experimental details

The experimental arrangement for both methods is shown in *Fig. 3*, with a low power (10 mW) helium–neon (He–Ne) laser-illuminated sample. By using a charge-coupled device (CCD) camera as detector and a host computer with a frame grabber, the successive sample images were registered, digitised to 8 bits (256 grey levels) and stored. Care was taken so that the speckles were well resolved by the CCD sensor. Very low illuminating intensity was used to minimise the effect of the irradiation on the sample activity. Mean laser illumination was kept constant during all measurements. No appreciable changes in sample reflectivity were observed.

For the co-occurrence matrix analysis, a column of the free propagation speckle pattern was read every 0·08 s. and then, a composite image of 512 by 512 pixels was formed by stacking consecutive columns. Finally, this image was retrieved and the second-order moments of its co-occurrence matrix calculated.

The investigated bean seeds (a total of 450) were kept at five different moisture levels of 13, 20, 30, 37 and 46% wet basis (w.b.), respectively, and were measured with and without a plastic covering film, at three consecutive times. Seeds were treated with an anti-fungi solution of 1:1000 of Captam. The number of replications was three.

Each seed was randomly picked from a set, cut into halves to separate both cotyledons and measured three

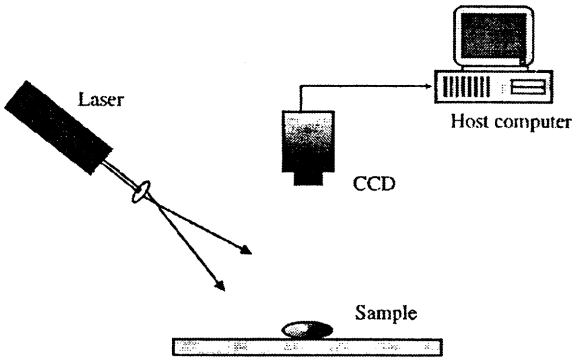

Fig. 3. Arrangement of the seed illumination and image capture: CCD, charge coupled device

consecutive times. The speckle images were then registered, the THSP was constructed and the M_I calculated. Analysis of variance at a probability level of 5% was then applied to the results.

In a further experiment, the seeds were sorted in two types: viable and non-viable. The seeds were verified by the tetrazolium test to confirm the respective batches of non-viable and high vigour bean seeds.

The speckle experiments to sort viable and non-viable seeds were conducted in two ways.

In the first test, two sets of 15 seeds of each kind were soaked for 24 h in germination paper. The THSP was then generated for each one and the M_I of the co-occurrence matrix was obtained.

The second experiment was designed to provide different M_I results during water evaporation in viable and non-viable seeds. Samples were taken from both populations under the same experimental conditions and were soaked in germination paper for 24 h to initiate the germination process. The viable and non-viable seeds were cut into halves and illuminated side by side. The THSP was generated for each one and M_I of the co-occurrence matrix was obtained as before. The initial moisture content of the seeds was 46%, and data were collected during 240 min. The values were averaged from three replicates.

In order to display qualitatively the activity, viable and non-viable seed inspection was also conducted using activity images with the GD method. In this case, the speckled images were not obtained by free propagation but consisted of subjective speckle focused images formed by an objective (focal length $f \equiv 50$ mm). A set of 100 images, each 180 by 480 pixels in size, digitised to 256 grey levels, were registered. After the application of the algorithm described previously, the GD display was obtained. The time required for this process in our image processor (Imaging Technology ITX 151) was less than 2 min.

4. Results and discussion

The analysis of variance (Gomez & Gomez, 1984) was implemented on the M_I results, as transformed by a logarithmic function, to evaluate the influence of the moisture. The results are shown in Table 1. It is possible to observe the significance of the relation between moisture and film at a level of 5%, and also the relation between measurement order and moisture at a level of 1‰.

The analysis of variance of the relation between moisture and film is shown in Table 2. It can be observed that the significance is in the three highest

Table 1
Analysis of variance of ln(inertia moment)

Source of variation	*Degree of freedom*	*Sum of squares*	*Mean square*	*Computed F*	*Significance level*
Moisture	4	52·672	13·168	23·501	0·0000
Film	1	19·901	19·901	35·518	0·0000
Moisture × Film	4	8·733	2·183	3·897	0·0169
Error 1	20	11·206	0·560		
Time	2	0·244	0·122	93·249	0·0000
Time × moisture	8	0·055	0·006	5·265	0·0002
Time × film	2	0·008	0·004	3·128	0·0547
Time × moisture × film	8	0·017	0·002	1·678	0·1340
Error 2	40	0·052	0·001		

Total degree of freedom, 89.
Coefficient of variations 1, % = 21.99.
Coefficient of variations 2, % = 1.06.
Global average, 3.404. Number of observations, 90.

Table 2
Analysis of variance of relation between moisture and film

Source of variation	*Level of moisture*	*Degree of freedom*	*Sum of squares*	*Mean square*	*Computed F*	*Significance level*
Film	13	1	0·000	0·000	0·000	0·9860
Film	20	1	1·050	1·050	1·876	0·1860
Film	30	1	5·343	5·343	9·537	0·0058
Film	37	1	9·511	9·511	16·976	0·0005
Film	46	1	12·727	12·727	22·715	0·0001
Rest		20	11·206	0·560		

levels of moisture (30, 37, and 46% w.b.). This result indicates that evaporation modifies the results in these levels, permitting the conclusion that it is necessary to establish the moisture level before an experiment, and also to determine the best level of moisture required for seed evaluation.

In this case, the results suggest that the levels 23 or 30% w.b. are those where the influence of the evaporation is small or does not exist.

The curves of the averaged results for the relationship between film and moisture are shown in *Fig. 4*. The difference between these curves related to the 'with' and 'without' film conditions can be clearly appreciated. It can be concluded, therefore, that evaporation modifies the M_I results.

Both the viable and non-viable seeds show biospeckle activity after being adequately soaked. It is the moisture that generates an activity measurement not related to the biological sample.

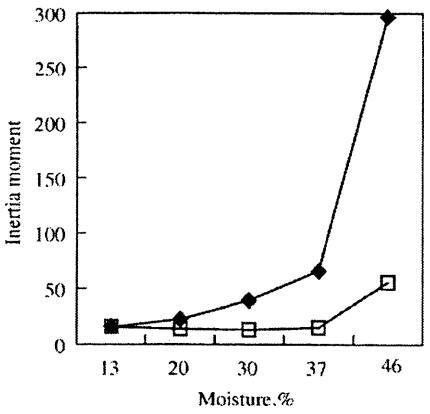

Fig. 4. Curves of the moment of inertia: =□=, with film; =◆=, without film

The results of the first viable/non-viable sorting test are shown in *Fig. 5*. It can be observed that the average of the M_I values for the viable seed set is notably higher than the corresponding value for the non-viable seed set. Only one sample shows anomalous behaviour with respect to the rest in each population. It should be noticed that the 'viable' and 'non-viable' conditions were not assessed by an alternative method and it could be due to misclassification.

The results of the measurement of inertia moment of viable and non-viable seeds as a function of time are shown in *Fig. 6*. In this figure, it can be seen that viable seeds present always greater activity than non-viable seeds. This result shows that the biological activity of the viable beans can be observed even in the presence of water movement and evaporation and could be used to sort the beans according to their viability.

The visual information of viable and non-viable seeds was obtained by using the GD method and the result can be seen in *Fig. 7*. The GD result showed one non-viable seed with a grey level that is clearly lower than that corresponding to the viable seed. This is an indication that viable and non-viable tissues or tissue

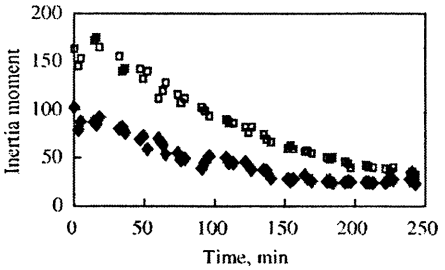

Fig. 6. Time evolution of the inertia moments of: □, viable; and ◆, non-viable beans

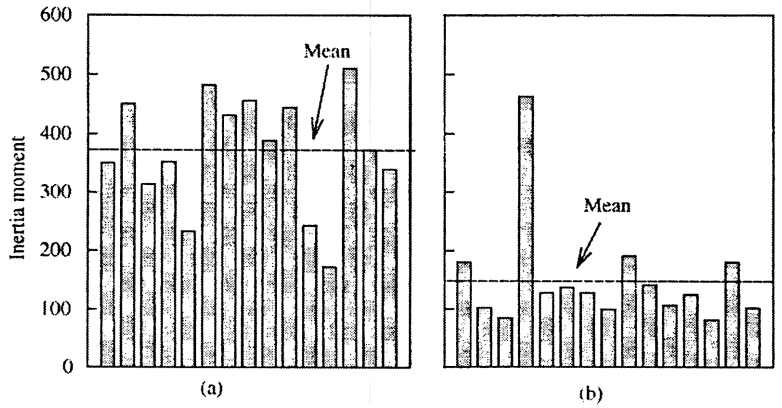

Fig. 5. Activity measurements using the inertia moment method on: (a) potentially viable seeds; and (b) non-viable bean seeds

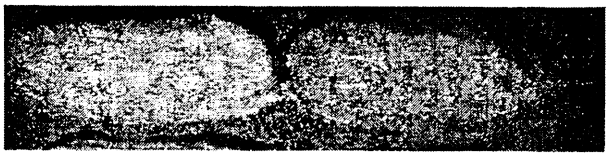

Fig. 7. Viable and non-viable bean seeds display using the generalised differences (GD) method

regions in the same seed with different activities could be distinguished using this type of display.

5. Conclusions

Dynamic speckle techniques were applied to the study of seeds in two series of experiments, one to sort viable and non-viable tissues and the other to investigate the influence of tissue moisture on the measurements.

Two dynamic speckle techniques were employed: the moment of inertia (M_1) of the co-occurrence matrix for the quantitative measurements; and the GD method for qualitative displays.

The experimental results show a significant dependence of the M_1 values on seed moisture content, and this dependence must be taken into account for the application of the dynamic speckle technique. Thus, it was not possible to discriminate between the viable and non-viable material at different moisture contents using the M_1 technique.

It is possible, however, to distinguish between viable and non-viable tissue by quantitative measurements or through a qualitative display, using the GD method.

More extensive validation of the technique is required with greater populations so that statistical tests can be applied to a comparison with actual accepted germination tests. Nevertheless, the proposed technique is simple, relatively cheap and fast, easy to implement and requires only laser and standard digital image processing components. It could be a promising tool to also evaluate other diverse agricultural specimens.

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References

Aizu Y; Asakura T (1996). Bio-speckles. In: Trends in Optics (Consortini A, ed.), pp 27–49. Academic Press, London.

Aizu Y; Asakura T (1992). Bio-speckle phenomena and their application to the evaluation of blood flow. Optics and Laser Technology, **23,** 205–219

Allam S; Adel M; Refregier P (1997). Fast algorithm for texture discrimination by the use of a separable ortonormal decomposition of the co-occurrence matrix. Applied Optics, **36,** 8313–8321

Amalvy J; Lasquibar C; Arizaga R; Rabal H J; Trivi M (2001). Application of dynamic speckle interferometry to the drying of coatings. Progress in Organic Coatings, **42,** 89–99

AOSA (1983). Seed Vigour Testing Handbook. Association of Official Seed Analysts, No 32, pp 82–88. Lincoln, NE, USA

Arizaga R; Trivi M; Rabal H J (1999). Speckle time evolution characterization by co-ocurrence matrix analysis. Optics and Laser Technology, **31,** 163–169

Arizaga R; Cap N; Rabal H J; Trivi M (2002). Display of local activity using dynamical speckle patterns. Optical Engineering, **41,** 287–294

Briers J D; Webster S (1996). Laser speckle contrast analysis (LASCA): a nonscanning, full field technique for monitoring capillary blood flow. Journal of Biomedical Optics, **2,** 174–179

Briers J D (2000). Time varying laser speckles for measuring motion and flow. Workshop on Coherent Optics of Ordered and Random Media. Saratov, Russia, 3–6 October 2000. http://optics.sgu.ru/SFM2000/report/Briers

Briers J D (1978). The statistics of fluctuating speckle patterns produced by a mixture of moving and stationary scatterers. Optics and Quantum Electronics, **10,** 364–366

Chu B (1991). Laser Light Scattering Basic Principles and Practice, Chapter 4. Academic Press, Boston.

Dainty J C (ed.) (1975). Laser Speckle and Related Phenomena. Springer Verlag, Berlin

Fujii H; Nohira K; Yamamoto Y; Ikawa H; Ohura T (1987). Evaluation of blood flow by laser speckle image sensing, part I. Applied Optics, **26,** 5321–5325

Gomez K A; Gomez A A (1984) Statistical Procedures for Agricultural Research, 2nd edn, p 680. John Wiley & Sons, New York

Hampton J G; Kahre L; Van Gastel A J G (1996). Quality seed—from production to evaluation. Seed Science & Technology, **24,** 393–407

Howarth M S; Stanwood P C (1993). Tetrazolium staining viability seed test using colour image processing. Transactions of the ASAE, **36,** 1937–1940

Konishi N; Fujii H (1995). Real time visualization of retinal microcirculation by laser flowgraphy. Optical Engineering, **34,** 753–757

Marcos Filho J (1999). Testes de Vigor: Importância e Utilização. [Vigour testing: importance and application.] In: Vigor de sementes:conceitos e testes (Seeds Vigour: Concepts and Testing) (Krzyzanowski F C; Vieira R D; França Neto J B, eds). ABRATES, Londrina, Brazil

Oulamara A; Tribillon G; Duvernoy J (1989). Biological activity measurement on botanical specimen surface using temporal decorrelation effect of laser speckle. Journal of Modern Optics, **36**, 165–179

Okamoto T; Asakura T (1995). The statistics of dynamic speckle. In: Progress in Optics XXXIV (Wolf E, ed.), pp 185–248. Elsevier Science, Amsterdam

Rabal H J; Arizaga R; Cap N; Trivi M; Romero G; Alanís E (1996). Transient phenomena analysis using dynamic speckle patterns. Optical Engineering, **35**, 57–60

Xu Z; Joenathan C; Khorana B M (1995). Temporal and spatial proprieties of the time-varing speckles of botanical specimens. Optical Engineering, **34**, 1487–1502