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ORIGINAL RESEARCH

Rapid determination of rice seed vigour by spontaneous chemiluminescence and singlet oxygen generation during early imbibition

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ABSTRACT: Using a highly sensitive single photon counter, a spontaneous chemiluminescence (CL) study on rice (*Oryza sativa* L.) seeds stored in different years was carried out. We first observed that the degree of ageing in rice seeds was related to the intensity of spontaneous CL during early imbibition (0–30 min). Rice seeds stored for a shorter time had a stronger intensity of CL in early imbibition. The germination rate of rice seeds showed an obvious positive correlation with the intensity of spontaneous CL. Singlet oxygen ($^1O_2$) in rice seeds during early imbibition was investigated by a CL method using a *cypridina* luciferin analogue, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo [1,2α] pyrazin-3-one (MCLA), as a selective CL probe. Additional experimental evidence for the formation of $^1O_2$ came from the quenching effect of sodium azide (NaN_3) on MCLA-mediated rice seeds' CL. Analysis based on the experimental results demonstrated that spontaneous CL in rice seeds during early imbibition was mainly contributed by singlet oxygen ($^1O_2$). Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: rice seeds; early imbibition; chemiluminescence; MCLA; singlet oxygen

INTRODUCTION

The study of spontaneous chemiluminescence (CL) of plant and its applications in agriculture is a very significant task. In recent years, compelling evidence has accumulated that interactions of water and food significantly affect the processing, storage and the quality of food products (1–2). Nowadays, CL-based methods have been developed for monitoring of deterioration of food and investigation of reactions of water with cereal products. It has been shown that the interaction of water with dry cereal grains, flour and bread products results in much stronger CL (3–5). However, no research on CL properties, mechanisms and possible analysis of the water-induced CL from rice seeds in early imbibition has been done.

The study of seed quality is one of the most important factors in seed sciences. There are a number of reports and reviews on seed ageing and the loss of viability, both of which result from accumulation of free radicals (6). The aims of the present work were to determine whether the rapid evaluation of the aging degree of rice seed could be determined by the spontaneous CL of rice seeds and (6) whether the spontaneous CL in rice seeds during early imbibition is correlated to $^1O_2$ of the free radical reactions.

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In the present study, the vigour of rice seeds was examined by measuring the differential characters of the spontaneous CL intensity of the seed during early imbibition (0–30 min). We observed that the degree of ageing of rice seeds was related to the intensity of spontaneous CL during early imbibition. The germination rate of rice seeds showed an obvious positive correlation with the intensity of spontaneous CL. Singlet oxygen ($^1O_2$) in rice seeds during early imbibition was detected by a sensitive CL probe 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo [1,2α] pyrazin-3-one (MCLA) (7–8). Additional evidence for $^1O_2$ came from the quenching effect of sodium azide (NaN_3) to MCLA-mediated CL, which showed a high positive correlation with the germination rate. Analysis based on the experimental results demonstrated that spontaneous CL in rice seeds during early imbibition was mainly contributed by singlet oxygen ($^1O_2$).

We believe that the spontaneous CL technique is a potential way of elaborating a fast, quantitative and non-invasive method for the rapid determination of the degree of ageing of rice seed.

MATERIALS AND METHODS

Materials and reagents

Regular rice 8072-2 seeds were obtained from Guangdong Academy of Agricultural Sciences. They were

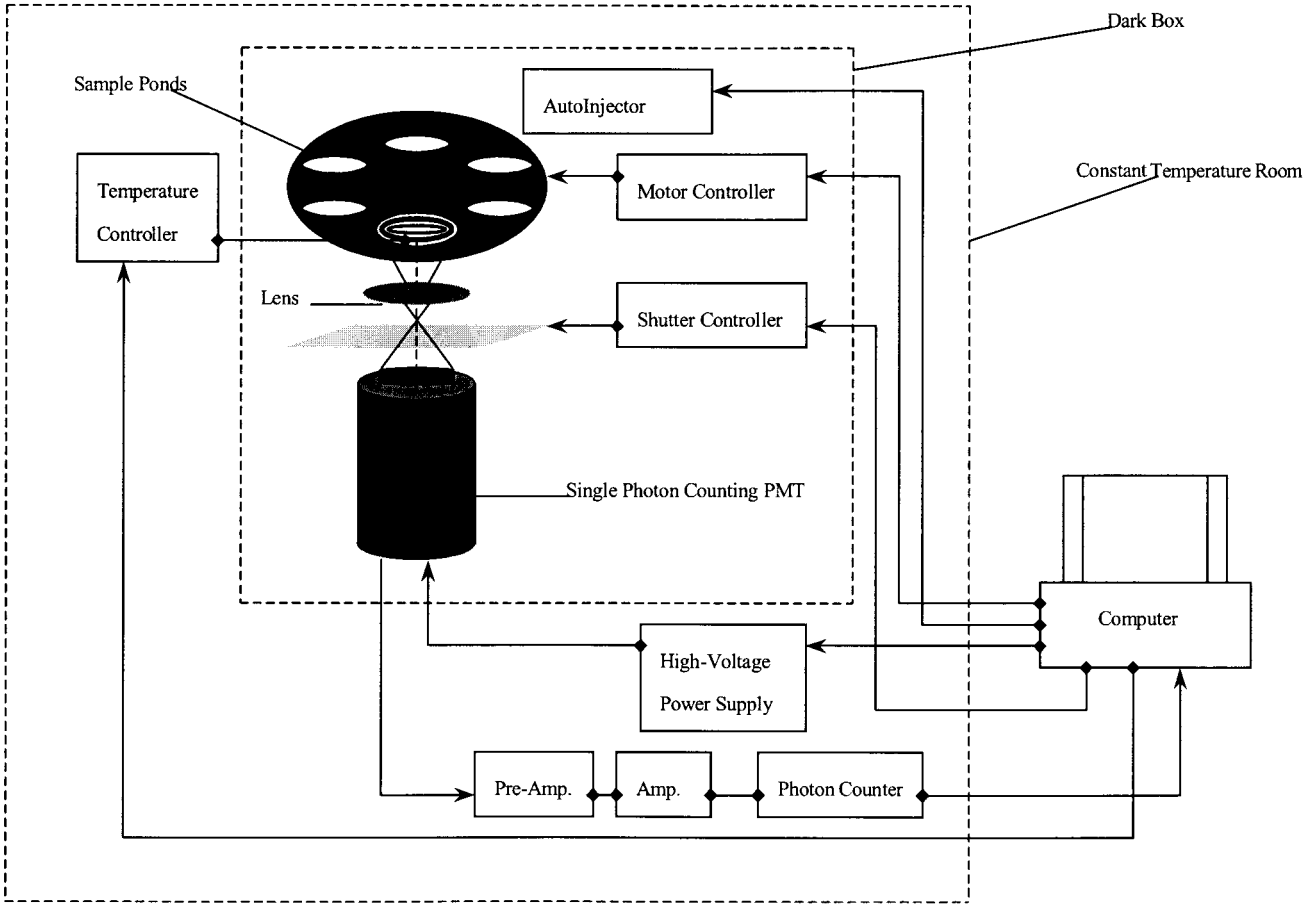

Figure 1. A schematic representation of the single photon counting system for measurements of CL: constant temperature room was 20°C, relative humidity was 65%.

harvested in July 1996, 1998, 1999 and 2001. All the samples were taken in separate cloth bags, stored in a desiccator with silica gel and kept in room temperature (15–28°C). Seeds in all experiments were selected and prepared carefully. MCLA was purchased from Tokyo Kasei Kogyo Co. Ltd; MCLA concentrations were based upon $\varepsilon_{430nm} = 9.6 \times 10^3$ (mol/L)/cm. NaN_3 was AR grade and made in China. Cu–Zn superoxide dismutase (SOD) was obtained from Sigma. MCLA, NaN_3 and SOD were performed by deionized double-distilled water just before experiments.

Determination of CL

CL measurements were made with a low noise and highly sensitive single photon counting (SPC) device developed by ourselves (Fig. 1). The system consists of a temperature-controlled light-tight sample chamber, a single photon counting photomutiplier tube (PMT; MP962, Perkin Elmer optoelectronics, Wiesbaden, Germany) and a computer-controlled photon counter module. The spectral sensitivity of the PMT's photocathode was 185–850 nm and the typical quantum efficiency was 20%. The dark count rate was about 25 counts/s (cps). Before the measurement of CL, rice seeds of equal number were weighed, put in a quartz cuvette and kept in sample ponds of the dark box for darkening 20–30 min, in order to avoid photo-induced delayed luminescence; then the measurement began. After the average photon counts rate from the dry samples had stabilized, the appropriate amount of distilled water or analytical reagents were injected equably into the cuvette through a light-tight autoinjector controlled by a computer. The whole data acquisition time of each experiment was about 30–50 min. The intensity of CL was normalized to cps/g dry weight (cps/g dw).

All operations were performed in three parallel measurements at 20°C, 65% of the relative humidity in complete darkness. The results of measurements presented in the text were the average CL intensity of the sample of three replicates, which took out the dark counts (scattering background and dark current).

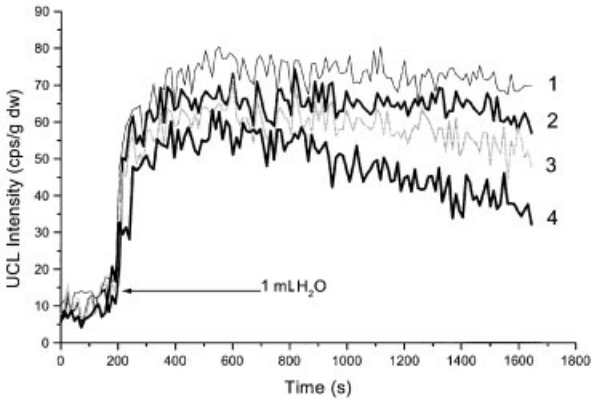

Figure 2. Kinetics of spontaneous CL from rice seed 8072-2 of the different degrees of ageing upon treatment with distilled water (arrow): curves 1, 2, 3 and 4 stand for year of harvest 2001, 1999, 1998 and 1996, respectively.

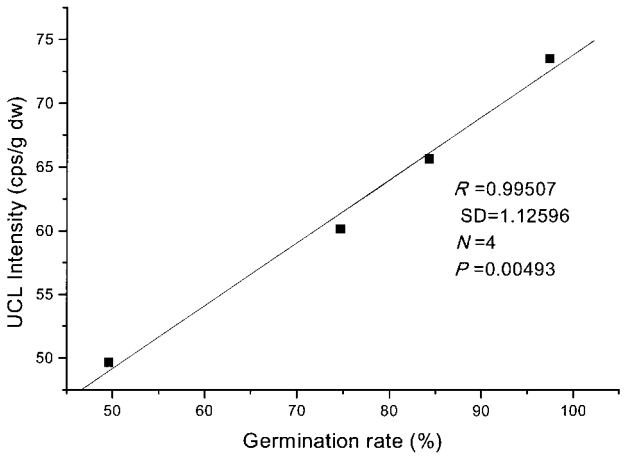

Figure 3. Relationship between spontaneous CL during early imbibition and germination rates. The germination rates of the seeds harvested in 2001, 1999, 1998 and 1996 were 97.4%, 84.3%, 74.7% and 49.6%, respectively (means of three replicates). The intensity of spontaneous CL was the mean value during 800–1200 s.

Measurement of reactive oxygen species production

CL has been widely used as a method for assessing the capacity of biological systems to producing reactive oxygen species (ROS) (9). MCLA can selectively react to both superoxide anion ($O_2^{\cdot -}$) and $^1O_2$ generated in biological systems (7). The measurements were performed in deionized double-distilled water. The background signal was about 45 cps. The method of measurement was as above (see Determination of CL). The $^1O_2$ generations were confirmed further by the quenching effect of sodium azide (NaN_3) to MCLA-mediated CL. The $O_2^{\cdot -}$ generations were measured by the quenching effect of SOD.

Germination tests

Germination tests were carried out on three replicates of 50 seeds each, and transferred to Petri dishes containing filter paper moistened with 10 mL distilled water. Germination data were recorded after 14 days of seed soaking. The germination rates of the seeds harvested in 2001, 1999, 1998 and 1996 were 97.4%, 84.3%, 74.7% and 49.6% respectively (mean of three replicates).

RESULTS

Relationship between spontaneous CL and germination rate

The spontaneous CL kinetic curves, $I = f(t)$, were shown in Fig. 2. The dry rice seeds exhibited low spontaneous CL intensity (I) with the signal-to-noise (S/N) ratio 1.5. The addition of water resulted in a rapid increase of light emission from seeds, with the S/N value reaching greater than 5. During the first several minutes, the emission clearly had a rapid ascending stage, as $I = f(t)$ curves were composed of an ascending stage. Then the emission reached to a stationary state and decreased very slowly. The shape of kinetic curves and the intensity of CL depended on the degree of ageing of rice seeds; the longer the storage period of the seed, the lower the intensity of the spontaneous CL in the early imbibition period (Fig. 2). The germination rate of rice seed had an obvious positive correlation to the average intensity of spontaneous CL (Fig. 3), the correlation coefficients (R) $R \cong 0.99$.

MCLA-mediated CL kinetic curves and quenching effect of NaN_3 and SOD

MCLA can selectively react with both $O_2^{\cdot -}$ and $^1O_2$ generated in biological systems. Fig. 4 shows that the MCLA background light emission was about 45 cps. The dry rice seeds exhibited low spontaneous CL intensity, with S/N ratio = 1.5. The addition of MCLA resulted in an immediate and sharp increase of light emission from seeds, with the S/N ratio reaching 25–75. The intensity of CL was gradually reduced with the increase of storage year (Fig. 4A).

NaN_3 can be used as a quencher of $^1O_2$. In order to confirm the $^1O_2$ formation further, the effect of NaN_3 on the MCLA-mediated CL was observed. The intensity of MCLA-mediated CL of rice seeds harvested in 2001, 1999, 1998 and 1996 were inhibited markedly by 56.2%, 51.5%, 43.2% and 37.8%, respectively (Fig. 4B).

SOD can be used as a quencher of $O_2^{\cdot -}$. The intensity of MCLA-mediated CL of rice seeds harvested in 2001, 1999, 1998 and 1996 were inhibited by 8.23%, 7.07%,

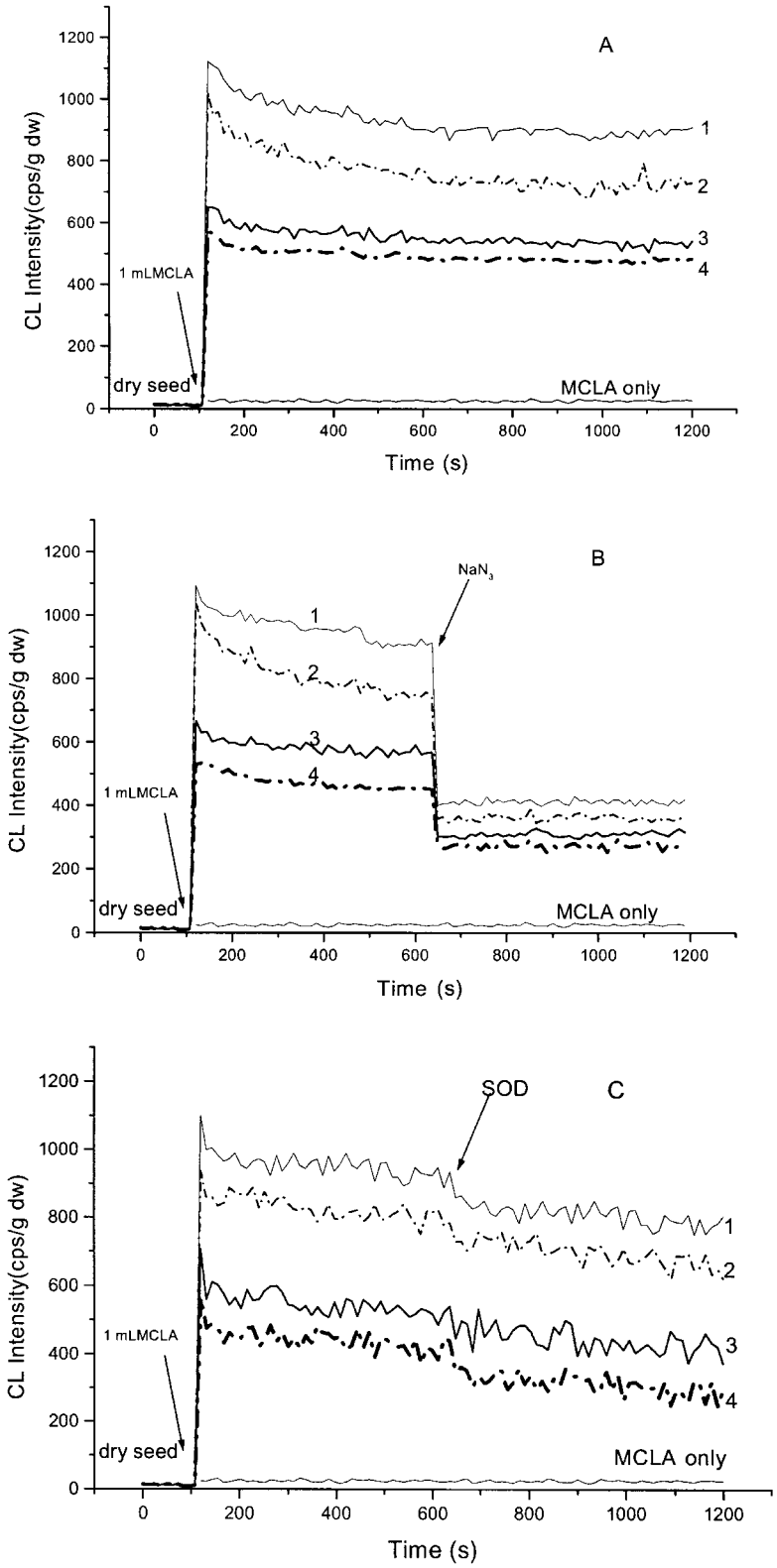

Figure 4. Kinetics curves of MCLA-mediated CL from rice seed 8072-2 of different degrees of ageing (A) and the quenching effect of NaN_3 (B) and SOD (C): MCLA (1 μmol/L), 0.1 ml NaN_3 (1 mmol/L) and 0.1 ml SOD (1 μmol/L) were added at the point indicated by the arrow. Curves 1, 2, 3 and 4 stand for years of harvest 2001, 1999, 1998 and 1996, respectively.

7.79% and 8.12%, respectively, which indicated that $O_2^{\cdot -}$ output in early imbibition is little.

DISCUSSION

It is commonly accepted that the spontaneous CL of living systems results from the electronically excited triplet state in peroxidation of unsaturated fatty acids, or from $^1O_2$ and the excited triplet state carbonyls of the dismutation of peroxyradicals generated in biological metabolism.

Dimole $^1O_2$ emissions at 634 nm and 704 nm have often been used for the identification of $^1O_2$ in biological systems, but unfortunately the red emission is extremely inefficient in water (10). On the other hand, most of the generated $^1O_2$ could be trapped by MCLA at the rate constant of 2.9×10^9 (mol/L)/s to produce an excited dioxetane analogue, which then emits light at 465 nm with high efficiency (11). The present study clearly indicated that MCLA-mediated CL is a very useful and sensitive method for detecting $^1O_2$ generated in biological systems.

Our results showed that the spontaneous CL in rice seeds during early imbibition was gradually reduced with longer storage (Fig. 2). Compared with the general germination test, the intensity of spontaneous CL showed an obvious positive correlation with the germination rate of rice seeds, $R \cong 0.99$ (Fig. 3). The uptake of water by seeds is an essential, initial step toward germination. The water probably very rapidly enters into the peripheral cells of the seed and into tissues as small as the radicle. Hence, during this first phase, metabolism can commence within minutes of introduction of the seed to water (12). Deamination and transamination of amino acids begin within a few minutes of early imbibition (12). The product of oxidative deamination is α-ketoacid and ammonia. In fact, this reaction consists of two steps: dehydrogenation and hydrolysis. Amino acid oxidase of catalytic dehydrogenate reaction is a kind of flavoprotein. Flavoprotein receives the hydrogen from amino acid and is transformed into reduced flavoprotein, then the hydrogen of reduced flavoprotein combines with oxygen directly to produce hydrogen peroxide (H_2O_2). H_2O_2 can be a source of hydroxyl radicals ($^{\cdot}OH$) when transition metal irons in rice seeds, such as ferric and cupric ions, are present. $^{\cdot}OH$ resulted in lipid peroxidation in the cell membranes of the seeds. The generation of excited carbonyl and singlet oxygen arise from the interaction of lipid peroxidation. CL is mostly thought to be produced during the de-excitation (directly or indirectly) of high-energy excited carbonyl and singlet oxygen; the higher the seed vigour, the stronger the seed metabolism. Therefore, the generation of $^1O_2$ from high-viability seed is more than that from low-viability seed, and the spontaneous CL in high-vigour seed is stronger than that in low-vigor seed. Our results showed that the intensity of MCLA-mediated CL was gradually reduced with the increase of storage year (Fig. 4A). The effect of NaN_3 on the MCLA-mediated CL further confirmed the $^1O_2$ formation in rice seeds during early imbibition (Fig. 4B). However, $^1O_2$ might be generated through the other metabolic pathways in rice seeds; its mechanism should be researched continuously.

Nandi *et al.* (13) found a significant decrease in stable free radical accumulation in rice seeds during ageing under natural conditions, using an electron paramagnetic resonance response (EPR) technique. In fact, it is remarkable that there have had so many positive correlations at all between EPR responses, lipid analyses and viability (14). Working on various kinds of fresh and aged seeds (barley, wheat, tomato, onion and pepper), Conger and Randolph (15) also suggested that the endogenous free radical concentration in fresh, dry seeds might decay with age, causing cell damage through a series of radical–molecule reactions as it decays.

CONCLUSION

In conclusion, it was shown in our study that a clear relationship between spontaneous CL and seed vigor during natural ageing. The germination rate of rice seeds had an obvious positive correlation with the intensity of spontaneous CL during early imbibition (0–30 min). The generation of $^1O_2$ in rice seeds during early imbibition was detected by MCLA. Analysis based on the experimental results demonstrated that spontaneous CL in rice seeds during early imbibition was mainly contributed by singlet oxygen ($^1O_2$).

We believe that the spontaneous CL technique is a potential way in elaborating a fast, quantitative and non-invasive method for the rapid determination of the ageing degree of rice seed.

REFERENCES

1. Slawinska D, Gorski Z and Slawinski J. Chemiluminescence imaging of oxidative changes in plant food. *Pol. J. Food Nutr. Sci.* 1998; **7/48**(2): 67–72.
2. Gorski Z and Slawinska D. Imaging of the water-induced chemiluminescence in various grains and cereals. In *Properties of Water in Foods* Lewicki PP. ed. Warsaw Agricultural University Press: Warsaw, 1998; 210–220.
3. Slawinska D and Slawinski J. Chemiluminescence of cereal products. I. Kinetics, activation energy and effect of solvents. *J. Biolumin. Chemilumin.* 1997; **12**: 249–259.
4. Slawinska D and Slawinski J. Chemiluminescence of cereal products. II. Chemiluminescence spectra. *J. Biolumin. Chemilumin.* 1998; **13**: 13–19.
5. Slawinska D and Slawinski J. Chemiluminescence of cereal products. III. Two-dimensional photocount imaging of chemiluminescence. *J. Biolumin. Chemilumin.* 1998; **13**: 21–24.
6. George A and Hendry F. Oxygen, free radical processes and seed longevity. *Seed Sci. Res.* 1993; **3**: 141–153.

7. Nakano M. Detection of active oxygen species in biological systems. *Cell. Mol. Neurobio.* 1998; **18**(6): 565–579.
8. Wang J and Xing D. Detection of vitamin C-induced singlet oxygen formation in oxidized LDL using MCLA as a chenmiluminescence probe. *Acta Biochim. Biophys. Sinica.* 2002; **34**(1): 15–20.
9. Raha S, Mceachern GE, Myint AT and Robinson BH. Superoxides from mitochondrial complex III: the role of manganese superoxide dismutase. *Free Rad. Biol. Med.* 2000; **29**(2): 170–180.
10. Koga S, Nakano M and Uehara K. Mechanism for the generation of superoxide anion and singlet oxygen during heme compound-catalyzed linoleic acid hydroperoxide decomposition. *Arch. Biochem. Biophys.* 1991; **289**: 223–229.
11. Mashiko S, Suzuki N and Koga S *et al.* Measurement of rate constants for quenching singlet oxygen with a *Cypridina* luciferin analogue (2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroi midazo [1,2 α]pyra-zin-3-one) and sodium azide. *J. Biolumin. Chemilumin.* 1991; **6**: 69–72.
12. Bewley JD and Black M. *Seeds: Physiology of Development and Germination* Plenum: New York, 1985; 136–139.
13. Nandi S, Sen-Mandi S and Sinha TP. Active oxygen and their scavengers in rice seeds (*Oryza sativa* cv. IET 4094) aged under tropical environmental conditions. *Seed Sci. Res.* 1997; **7**: 253–259.
14. Bajpai RP, Bajpai PK and Roy D. Ultraweak photon emission in germinating seeds: a signal of biological order. *J. Biolumin. Chemilumin.* 1991; **6**(4): 227–232.
15. Conger AD and Randolph ML. Is age-dependent genetic damage in seeds caused by free radical? *Radiat. Bot.* 1968; **8**: 193–196.

