Rapid Determination of Rice Seed Vigor by Ultraweak Chemiluminescence During Early Imbibition

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Abstract: With high-sensitivity single-photon counter, ultraweak chemiluminescence (UCL) study on rice (*Oryza sativa* L.) seeds stored for different years was carried out. The intensity of UCL was observed to be related to the degree of aging of rice seeds during early imbibition (0 - 30 min). Rice seeds with longer storage time had lower intensity of UCL during early imbibition. The germination rate of rice seeds showed a significant positive correlation with the intensity of UCL. Singlet oxygen ($^1O_2$) in rice seeds during early imbibition was investigated by chemiluminescence (CL) method using a sensitive CL probe MCLA (2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo [1,2-a] pyrazin-3-one). The output of $^1O_2$ showed a high positive correlation with the germination rate. Analysis based on the experimental results demonstrated that UCL in rice seeds during early imbibition could be attributed mainly to singlet oxygen ($^1O_2$). The UCL technique is a potential way in elaborating a fast, quantitative and non-invasive method for rapid determination of the degree of aging rice seed.

Key words: rice seeds, early imbibition, ultraweak chemiluminescence, MCLA, singlet oxygen

The study of ultraweak chemiluminescence (UCL) of plant and its applications in agriculture has had very significant achievements. In recent years, many evidences have been accumulated that interactions between water and cereal significantly affect the processing, storage and the quality of cereal products (Slawinska *et al*. 1998, Gorski and Slawinska 1998). Chemiluminescence (CL)-based methods have recently been developed for monitoring the deterioration of food and investigation of reactions of water with cereal products. It has been shown that the interaction between water and dry cereal grains, flour and bread products results in much stronger CL (Slawinska *et al*. 1998; Slawinska and Slawinski 1997, 1998a, b). However, no research on its nature and properties and mechanisms of formation of the substances with such properties and possible analytical methods of the water-induced CL from rice seeds during early imbibition has been done.

The study of seed quality has become one of hot spots in seed sciences recently. There are a number of reports and reviews on seed aging and the loss of viability, resulting from accumulation of free radicals (George and Hendry 1993). One of the UCL of living systems results from $^1O_2$ generated in biological metabolism. The aims of the present work are to determine (i) whether the UCL in rice seeds during early imbibition is correlated to $^1O_2$ of the free radical reactions, and (ii) whether rapid evaluation of the aging degree of rice seed can be done by measuring the UCL.

In the present study, the vigor of rice seeds was examined by measuring the differential characters of the seed UCL intensity during early imbibition (0 - 30 min). We first observed that the aging degree of rice seeds was correlated to the intensity of UCL during early imbibition. The germination rate of rice seeds showed a significantly positive correlation with the intensity of UCL. 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, 2-a] pyrazin-3-one (MCLA) (Nakano 1998, Wang and Xing 2002). Additional evidence for $^1O_2$ came from the quenching effect of sodium azide (NaN_3) on MCLA-mediated CL, which showed a high positive correlation with the germination rate. Analysis based on the experimental results demonstrated that UCL in rice seeds during early imbibition could be attributed to singlet oxygen ($^1O_2$).

We suggested that UCL technique could be a potential way in elaborating a fast, quantitative and non-invasive method for the rapid determination of the

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degree of aging of rice seed.

1 Materials and Methods

1.1 Materials

Regular rice (*Oryza sativa* L.) seeds of the 8072-2 were taken from Guangdong Academy of Agricultural Sciences. They were harvested in July of 1996, 1998, 1999 and 2001. All the samples were taken in separate cloth bags, stored in a desiccator with silica gel and kept at room temperature. Healthy seeds in all experiments were selected and prepared carefully. MCLA, purchased from Tokyo Kasei Kogyo CO., Ltd., concentrations were based upon $\varepsilon_{430\ nm} = 9.6 \times 10^3 (mol/L)^{-1} cm^{-1}$.

1.2 Determination of CL

CL measurements were made with a low noise and high-sensitivity single photon counting (SPC) device developed by ourselves (Fig. 1). The system consists of a temperature-controlled lightless sampling box, a single photon counting photomutiplier tube (PMT; MP962, Perkin Elmer Optoeletronics, Wiesbaden, Germany) and a computer-controlled photon counter module. The sensitive part of the spectrum of the PMT's photocathode was 185 - 850 nm and the typical quantum efficiency was 20 %. The dark count rate was about 25 counts per second (cps). Before the measurement of CL, equal number of rice seeds were weighed, put in a quartz cuvette and kept in sample ponds of the dark box and kept in the dark for 20 - 30 min to avoid photo-induced delayed luminescence, then the measurement began. After the average photon count rate from the dry samples had stabilized, an appropriate amount of distilled water or analytical reagents were injected equably into the cuvette through a light-tight auto-injector 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 per gram dry weight (cps/g DW).

All operations were performed in thiplicates measurements at 25 ℃, and a relative humidity 65 % in complete darkness. The results of measurements presented in the text are the average CL intensity of three replicates samples after deduction of the dark counts (scattering background and dark current).

1.3 Measurement of free radical production

Free radical production was determined by using CL probe MCLA. MCLA can selectively react to both $O_2^{\cdot -}$ and $^1O_2$ generated in biological systems (Nakano 1998). MCLA has been used to measure $O_2^{\cdot -}$ or $^1O_2$ formation *in vivo* and *in vitro* (Wang and Xing 2002, Uehara *et al*. 1991, Masuda *et al*. 2001, Koga *et al*. 1991). The measurements were performed in deionized doubly distilled water. The background signal

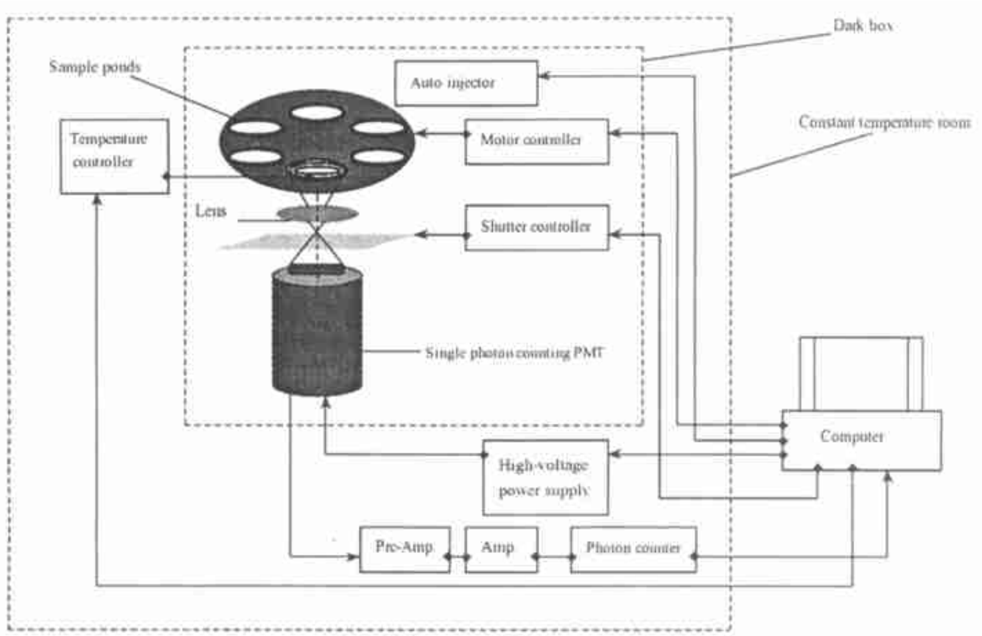

Fig. 1 A schematic representation of the single photon counting system for measurements of CL
Constant temperature room was 25 ℃, relative humidity was 65 %.

was about 45 cps. The method of measurement was the same as that of 2. 2, the quantity of $^1O_2$ was measured by the quenching effect of sodium azide (NaN_3) on MCLA-mediated CL.

1.4 Germination tests

Germination tests were carried out on three replicates of 50 seeds each, transferred to filter paper moistened with 10 ml distilled water in each of Petri dish. Germination data were recorded after 14 d of seed soaking. The germination rates of the seeds harvested in 2001, 1999, 1998 and 1996 were mean of three replicates.

2 Results

2.1 UCL kinetic curves and relationship between UCL and germination rate

The UCL kinetic curves were shown in Fig. 2. The dry rice seeds exhibited low UCL intensity with a signal-to-noise (S/N) ratio of 1. 5. Addition of water resulted in a rapid increase of light emission from seeds, with the S/N value exceeding 5. The emission had a rapid ascending stage in the first few minutes, as the ascending part of an curve. Then the emission reached a stationary state and decreased very slowly. The shape of kinetic curves and the intensity of CL depended on the degree of aging of rice seeds. The longer the seed was stored, the lower the intensity of the UCL in the early imbibition period (Fig. 2).

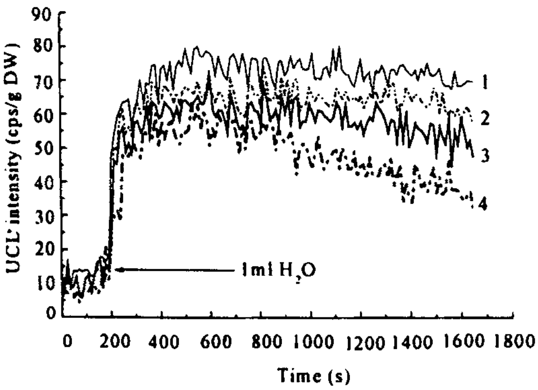

Fig. 2 Kinetics of UCL from seeds of rice cv. 8072-2 of the different aging degree upon treatment with distilled water (arrow)

Curves 1, 2, 3 and 4 stand for the years of harvesting 2001, 1999, 1998 and 1996 respectively.

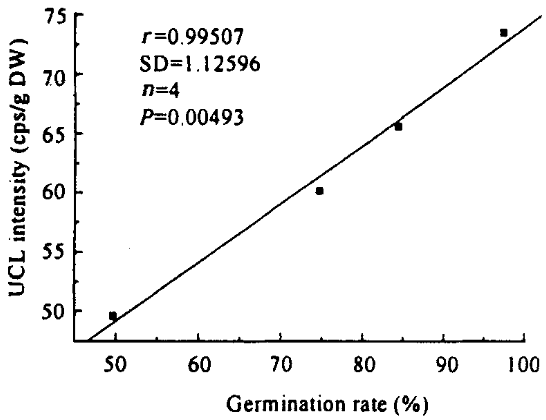

Fig. 3 Relationship between UCL during early imbibition and germination rates

The germination rates of the seeds harvested in 2001, 1999, 1998 and 1996 were 97. 4 %, 84. 3 %, 74. 7 % and 49. 6 %, respectively, mean of three replicates. The intensity of UCL was the mean of values taken during 800 - 1 200 s.

The germination rate of rice seeds had a high significant positive correlation to the average intensity of UCL which was the mean of values taken during 800 - 1 200 s (Fig. 3), with a correlation coefficient of 0.99.

2.2 MCLA-mediated CL kinetic curves and relationship between $^1O_2$ production and germination rate

MCLA can selectively react with both $O_2^{\cdot -}$ and $^1O_2$ generated in biological systems, and has been widely used as a sensitive method for the assessment of the capacity of biological systems to produce $O_2^{\cdot -}$ and $^1O_2$ (Wang and Xing 2002, Uehara *et al*. 1991, Masuda *et al*. 2001, Koga *et al*. 1991). Fig. 4 showed that the background light emission of MCLA was about 45 cps. Dry rice seeds exhibited low UCL intensity with an S/N ratio of 1.5. The addition of MCLA resulted in an immediate and sharp increase in light emission from seeds, with S/N ratios reaching 25 - 75. The intensity of CL became lower with an increase in storage time (Fig. 4A). $^1O_2$ came from the quenching effect of sodium azide (NaN_3) on MCLA-mediated CL (Fig. 4B). The output of $^1O_2$ of rice seeds showed a high positive correlation to the germination rate, with a correlation coefficient of 0. 92 (Fig. 5).

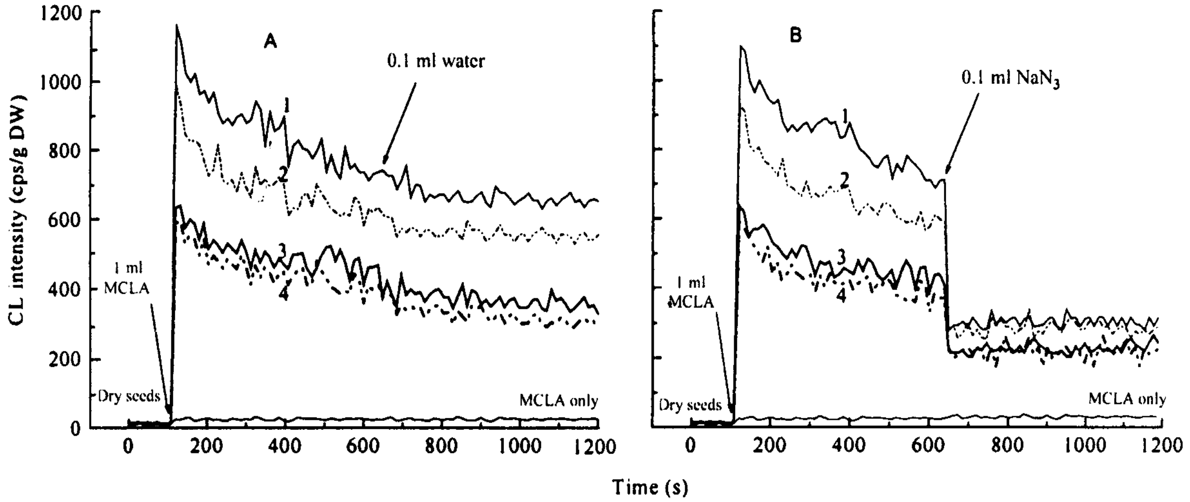

Fig. 4 Kinetic curves of MCLA-mediated CL from seeds of rice cv. 8072-2 with different aging degrees of aging
MCLA (1 mmol/L), deionized doubly distilled water and NaN_3 (1 mmol/L) were added at the point indicated by the arrow. Curves 1, 2, 3 and 4 stand for harvesting year 2001, 1999, 1998 and 1996 respectively.

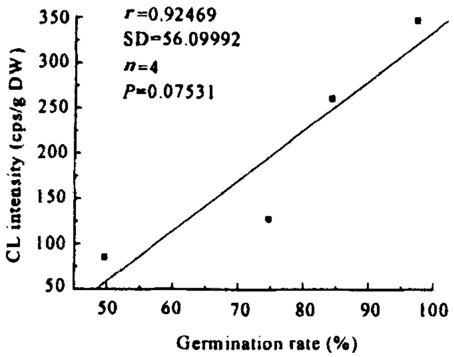

Fig. 5 Relationship between the output of $^1O_2$ during early imbibition and germination rates
The quenching effect of NaN_3 on MCLA-mediated CL is due to the output of $^1O_2$. The germination rates of the seeds harvested in 2001, 1999, 1998 and 1996 were 97.4%, 84.3%, 74.7% and 49.6% respectively, each the mean of three replicates. The intensity of CL was the mean of values taken during 800 - 1 200 s.

3 Discussion

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

Our results showed that the UCL in rice seeds during early imbibition gradually decreased with the increase in years of storage (Fig. 2). The intensity of UCL showed a significant positive correlation to the germination rate of rice seeds with germination test, $r \cong 0.99$ (Fig. 3). The uptake of water by seeds is an essential, initial step toward germination. Water probably very rapidly enters the peripheral cells of the seed and the tissues as small as the radicle. Metabolism can commence during this first phase, within minutes after introduction of the seed to water (Bewley and Black 1985). Deamination and transamination of amino acid begin in the first few minutes of imbibition (Bewley and Black 1985). The product of oxidative deamination is α-ketoacid and ammonia. In fact, this reaction consists of two steps: dehydrogenate and hydrolysis. Amino acid oxidase of catalytic dehydrogenation reaction is a flavoprotein, which receives hydrogen from amino acid to form reduced flavoprotein. Then the hydrogen of reduced flavoprotein combines with oxygen directly to produce hydrogen peroxide (H_2O_2) which can be a source of hydroxyl radical (·OH) when transition metal ions such as ferric and cupric ions are present. ·OH results to peroxidation of lipid in the cell membranes of dry seeds. Excited carbonyl and singlet oxygen arise from lipid peroxidation. CL is usually thought to be produced during the deexcitation (directly or indirectly) of high-energy excited carbonyl and singlet oxygen. The higher the seed vigor is, the stronger the seed metabolism will be.

So the generation of $^{1}O_2$ from seeds of high viability is more than that from those of low viability, and the UCL in high vigor seeds is stronger than that in low vigor ones. However, $^{1}O_2$ can be generated through other metabolic pathways in rice seeds during early imbibition, the mechanism of which need be elucidated.

The singlet oxygen ($^{1}O_2$) with lowest excitation plays an important role in biological system, but direct detection of the highly reactive short-lived $^{1}O_2$ in biological system is extremely difficult because the quantum yield of its infrared emission is 10^{-6} at best (Wang and Xing 2002). CL has been widely used as a method to assess the capacity of biological systems to produce reactive oxygen species (ROS) (Raha *et al*. 2000). MCLA, as a sensitive CL probe, can selectively act on $^{1}O_2$ generated in biological systems and magnify the signal. MCLA has been used to measure the formation of $O_2^{\cdot-}$ or $^{1}O_2$ *in vivo* and *in vitro* (Nakano 1998, Wang and Xing 2002, Uehara 1991 *et al*., Masuda *et al*. 2001). Our results showed that the intensity of MCLA-mediated CL gradually decreased with an increase in number of years of storage (Fig. 4A). The determination of $^{1}O_2$ is based on the quenching effect of NaN_3 on MCLA-mediated CL (Fig. 4B), $^{1}O_2$ production in rice seeds during early imbibition showed a high positive correlation with the germination rate with $r \cong 0.92$ (Fig. 5).

Nandi *et al*. (1997) found that a significant decrease in stable free radical accumulation in rice seeds during aging under natural conditions with electron paramagnetic resonance response (EPR) technique. In fact, it is remarkable that there have been so many positive correlations between EPR responses, lipid analyses and viability (Bajpai *et al*. 1991). Working on various fresh and aged seeds of plant species (barley, wheat, tomato, onion and pepper), Conger and Randolph (1968) 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.

In conclusion, it has been shown in our study that there is a clear relationship between UCL and seed vigor during natural aging. The germination rate of rice seeds have a significant positive correlation with the intensity of UCL during early imbibition (0 - 30 min). The generation of $^{1}O_2$ in rice seeds during early imbibition was detected by MCLA which showed a high positive correlation with germination rate. Analysis based on the experimental results demonstrated that the UCL in rice seeds during early imbibition can be attributed mainly to singlet oxygen ($^{1}O_2$).

The correlation between the intensity of UCL during early imbibition and degree of aging of rice seeds reported here led us to the conclusion that the UCL technique is a potential way in elaborating a fast, quantitative and non-invasive method for rapid determination of the degree of aging rice seed.

References

Bajpai RP, Bajpai PK, Roy D (1991). Ultraweak photon emission in germinating. *J Biolum Chemilum*, **6**(4): 227 - 232

Bewley JD, Black M (1985). Seeds Physiology of Development and Germination. New York: Plenum Press, 136 - 139

Conger AD, Randolph ML (1968). Is age-dependent genetic damage in seeds caused by free radical? *Radiation Bot*, **8**: 193 - 196

George A, Hendry F (1993). Oxygen, free radical processes and seed longevity. *Seed Sci Res*, **3**: 141-153

Gorski Z, Slawinska D (1998). Imaging of the water-induced chemiluminescence in various grains and cereals. In: Lewicki PP (ed). Properties of Water in Foods. Warsaw: Warsaw Agricultural University Press, 210 - 220

Koga S, Nakano M, Uehara K (1991). Mechanism for the generation of superoxide anion and singlet oxygen during heme compound-catalyzed linoleic acid hydroperoxide decomposition. *Arch Biochem Biophys*, **289**: 223 - 229

Masuda H, Tanaka T, Tateishi M, Naito M, Tamai H (2001). Detection and cytotoxicity of cisplatin-induced superoxide anion in monolayer cultures of a human ovarian cancer cell line. *Cancer Chemother Pharmacol*, **47**: 155 - 160

Nakano M (1998). Detection of active oxygen species in biological systems. *Cellul Mol Neurobiol*, **18** (6): 565 - 579

Nandi S, Sen-Mandi S, Sinha TP (1997). Active oxygen and their scavengers in rice seeds (*Oryza sativa* cv. IET 4094) aged under tropical environmental conditions. *Seed Sci Res*, **7**: 253 - 259

Raha S, Mceachern GE, Myint AT, Robinson BH (2000).

Superoxides from mitochondrial complex III: the role of manganese superoxide dismutase. *Free Radical Biol Med*, **29**(2): 170 - 180

Slawinska D, Gorski Z, Slawinski J (1998). Chemiluminescence imaging of oxidative changes in plant food. *Pol Food Nut Sci*, **48**(7): 67 - 72

Slawinska D, Slawinski J (1997). Chemiluminescence of cereal products I. Kinetics, activation energy and effect of solvents. *J Biolum Chemilum*, **12**: 249 - 259

Slawinska D, Slawinski J (1998a). Chemiluminescence of cereal products II. Chemiluminescence spectra. *J Biolum Chemilum*, **13**: 13 - 19

Slawinska D, Slawinski J (1998b). Chemiluminescence of cereal products III. Two-dimensional photocount imaging of chemiluminescence. *J Biolum Chemilum*, **13**: 21 - 24

Uehara K, Hori K, Nakano M, Koga S (1991). Highly sensitive chemiluminescence method for determining myeloperoxidase in human polymorphonuclear leukocytes. *Anal Biochem*, **199**: 191 - 196

Wang J (王 娟), Xing D (邢 达) (2002). Detection of vitamin C-induced singlet oxygen formation in oxidized LDL using MCLA as a chemiluminescence probe. *Acta Biochim Biophys Sin* (生物化学与生物物理学报), **34**(1): 15 - 20

用吸胀初期超微弱化学发光的方法快速检测水稻种子活力

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摘要: 用高灵敏度的单光子计数系统探测了不同贮藏年份的水稻(*Oryza sativa* L.)品种8072-2吸胀初期的超微弱化学发光(ultraweak chemiluminescence, UCL)。观测到水稻种子吸胀初期(0～30 min)UCL强度与其老化程度呈负相关,水稻种子贮藏时间越长,萌发率越低,UCL的强度越弱,水稻种子UCL强度与萌发率呈极显著正相关。用灵敏的能与单线态氧($^1O_2$)反应产生化学发光的发光试剂MCLA (2-methyl-6-(*p*-methoxyphenyl)-3, 7-dihydroimidazo[1,2-a]pyrazin-3-one),检测到吸胀初期水稻种子有单线态氧($^1O_2$)的生成,产生$^1O_2$的量与其萌发率呈高度正相关。水稻种子吸胀初期单线态氧的生成是超微弱化学发光的重要诱发因素之一。种子吸胀初期UCL的差异有望成为一种快速、定量、无损伤检测水稻种子活力的新方法。

关键词: 水稻种子, 吸胀初期, 超微弱化学发光, MCLA, 单线态氧
学科分类号: Q947

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