

Imaging of ultra-weak bio-chemiluminescence and singlet oxygen generation in germinating soybean in response to wounding

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ABSTRACT: Ultra-weak bio-chemiluminescence (UBC) from germinating soybean (*Glycine max* L. Merr) cotyledon under mechanical wounding was observed using a high-sensitivity imaging system based on an ICCD detector and a highly sensitive single photon counter (SPC) device. The UBC imaging showed that the intensity at the injury location on a wounded cotyledon was obviously enhanced as compared with that at the non-injured point. The UBC intensity of wounded cotyledons was initially very high and reached a stationary state after about 5 min. Wounding-induced emission could be suppressed by wounding in the presence of sodium azide. Deuterium oxide amplified the emission intensity. It was concluded that singlet oxygen ($^1\text{O}_2$) was the main cause of the emission during the wounding phase. Copyright © 2002 John Wiley & Sons, Ltd.

KEYWORDS: ultra-weak bio-chemiluminescence; soybean cotyledon; wounding; defence response; singlet oxygen

INTRODUCTION

Historically, early stress-induced changes in plants have been mainly detected after destructive sampling followed by biochemical and molecular determinations. Ultra-weak bio-chemiluminescence (UBC) imaging techniques that allow immediate detection of stress situations, before visual symptoms appear and adverse effects become established, are emerging as promising tools for crop yield management. UBC, as a common phenomenon in all living things, is closely correlated with the physiological conditions and biochemical processes occurring in an organism (1–5). It is known for animal and plant matter that when their bodies are stimulated by external injuries, they respond in order to defend themselves from harm, e.g. polymorphonuclear leukocytes emit photons during the process of phagocytosis (6). A similar phenomenon in plants was discovered by Takahiro *et al.* in 1996 (7). They found that ultra-weak bio-chemiluminescence was generated from sweet potatoes that were infected by non-pathogenic *Fusarium oxysporum*.

It is well known that enhanced low-level chemiluminescence is produced by wounded plants (8). Abeles and Salin (8,9) reported that the photon emission by wounded plants during destruction of the cell wall and membrane was caused by reactions involving chemi-

luminescent substances such as peroxidase, hydrogen peroxide and unknown endogenous cellular substances. But to our knowledge there are no reports of mechanical wounding inducing singlet oxygen generation. In our experiments, we observed the UBC response to mechanical wounding of soybean cotyledon in the presence of NaN_3 and D_2O . The results of the studies on mechanical wounding show enhanced UBC from the D_2O solvent and reduced UBC from the NaN_3 solvent.

Mechanical wounding is an environmental stress factor that influences all higher plants. Wounds can directly disrupt physiological function and, furthermore, wounds are potential infection sites for opportunistic pathogens (10). Recent studies have revealed that reactive oxygen species (ROS) might play an important role in inducing protection mechanisms during both biotic and abiotic stresses (11,12). Consequently, the avenues of antioxidant production and its regulation have become an active research field. Singlet molecular oxygen ($^1\text{O}_2$) is a highly reactive form of molecular oxygen that may harm living systems by oxidizing critical organic molecules. From *in vitro* studies it is clear that $^1\text{O}_2$ oxidizes many organic molecules, including membrane lipids, proteins, amino acids, nucleic acids, nucleotides, pyridine nucleotides, carbohydrates and thiols (13).

The aim of the present work is to image UBC from soybean cotyledons in response to wounding, and to determine whether the UBC under wounding conditions is correlated to $^1\text{O}_2$. In the present study, using a high-sensitivity imaging system and a high-sensitivity single photon counter detector, we have studied the UBC from wounded germinating soybean cotyledons. The UBC

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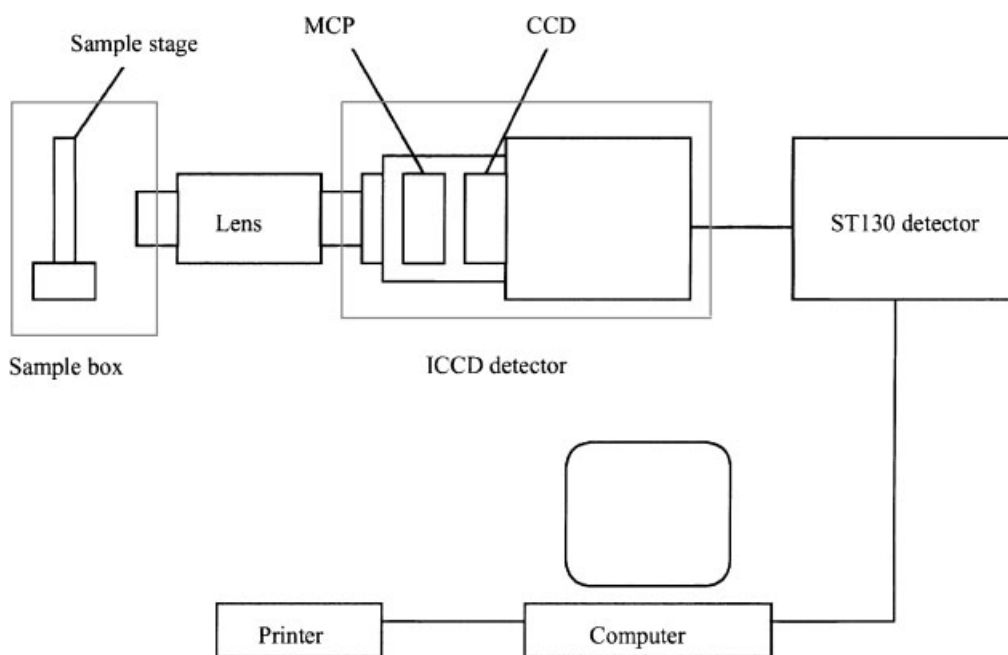


Figure 1. Schematic diagram of the imaging experimental setup. (ICCD: Intensified Charge Couple Device, MCP: Micro-Channel Plate).

image and the time-dependent intensity show clearly that wounding can lead to an obvious increase of the UBC intensity. Singlet oxygen is the main cause of the emission during the wounding phase.

MATERIALS AND METHODS

Materials

Healthy soybean (*Glycine max* L. Merr) seeds were used as experimental samples. The seeds were surface-sterilized with 1% NaOCl for 1 min and thoroughly rinsed with water (first with sterilized distilled water, and then with sterilized deionized water at least four times). Then they were kept immersed in deionized water for 6 h. Finally, the seeds were placed over moist germination paper and cultured in an artificial climate box (LRH-250-GS) at $25 \pm 2^\circ\text{C}$ and $65 \pm 5\%$ relative humidity. The light regime was 12 h dark/12 h light (3000 Lx). After 4 days, the skin of the seed had shed, the radical emerged and the plumule was still hidden between the cotyledons. Deuterium oxide (D_2O , 99.9 atom % D, from Aldrich Chemical Company Inc.) was stored under nitrogen until needed. NaN_3 was AR grade and made in China.

Sample treatment

Before each experiment, soybean sprouts were placed in a dark room for dark adaptation for 0.5–1 h to avoid photo-induced delayed luminescence. The cotyledon cuts

were about 0.5 mm deep and 4 mm long and made with a knife. The wounded or intact soybean cotyledons were put in quartz sample dishes and immersed in pure deionized water or reagents, then measured immediately. The time from wounding to the start of measurement was controlled strictly at 2 min. All operations were performed in three parallel measurements at 20°C , 65% relative humidity in dim green light (515–560 nm) and wavelength was controlled by two filters 515 nm long-wavelength and 560 short-wavelength (Coherent, Inc.).

Measurement of UBC

Two-dimensional imaging system. The experimental imaging set-up is shown in Fig. 1. It is a two-dimensional imaging system based on an ICCD (Intensified Charge Couple Device, 576-S/1, Princeton Instruments Inc.) detector, which consists of a micro-channel plate (MCP) image intensifier and a cooled CCD. The output of the intensifier is coupled to the CCD with optical fibres. The combination of the high gain of the MCP and the low reading-noise of the CCD brings about a sensitive detector able to respond to a single photon. The typical dark current at the temperature of -50°C is 4–8 electrons/s/pixel. The spectral response range of the ICCD is 400–1060 nm. To efficiently collect the UBC from the sample, we used a quality camera lens (focus 50 mm, F 1.4) coupling to the ICCD detector.

Single photon counting measurements. The single photon counting technique (14) was used in order to

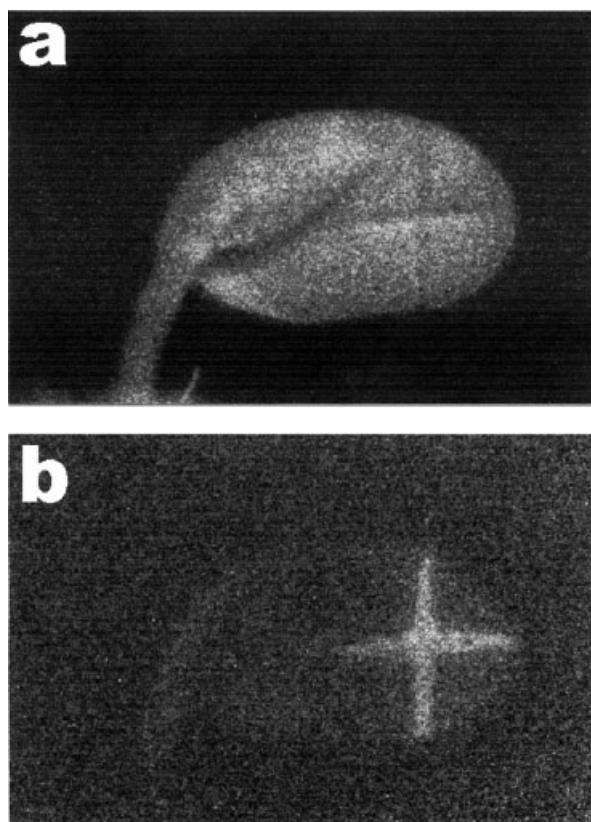


Figure 2. Topography (a) and UBC image (b) of a wounded germinating soybean cotyledon.

detect and analyse UBC. The system consists of a temperature-controlled light-tight sample chamber ($20 \pm 2^\circ\text{C}$, $65 \pm 3\%$ of the relative humidity), a single

photon counting photomultiplier tube (PMT; MP962, Perkin Elmer Optoelectronics, Wiesbaden, Germany) and a computer-controlled photon counter module. The spectral sensitivity of the PMT's photocathode is 185–850 nm and the typical quantum efficiency is 20%. The dark count rate is about 25 counts/s (cps).

The results of measurements presented in the text were the average UBC intensity of three replicate. Dark counts (scattering background and dark current) were subtracted from this signal.

RESULTS AND DISCUSSION

UBC from wounded germinating soybean

Fig. 2(a) shows the topography of a green soybean sprout photographed in dim green light. One of its cotyledons was removed and a cross was cut with a knife. The cut was about 0.5 mm deep, 4.0 mm long. Water was supplied to the soybean sprout through the wet absorbent cotton at the root. Fig. 2(b) is the UBC image of this soybean sprout. The acquisition time was 1 h. The background noise has been subtracted from the image. The UBC at the cut is far stronger than in the intact parts of the plant.

Fig. 3 is the time-dependent intensity of the UBC kinetic curves from wounded and intact soybean cotyledons obtained with the SPC device. The intensity of UBC from intact green soybean cotyledons exhibited low intensity [signal-to-noise (S:N) ratio 1.5]. However, from wounded green soybean cotyledons, the UBC

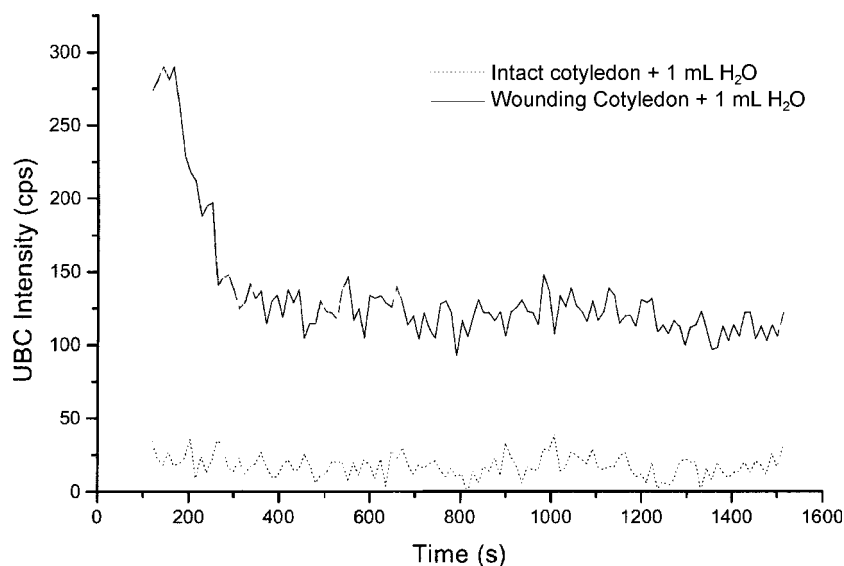


Figure 3. UBC kinetics curves from wounded soybean cotyledon and intact soybean. The wounded and intact soybean cotyledons were each placed in a quartz sample dish and immersed in 1 mL pure deionized water each. All curves are the means of three replicates.

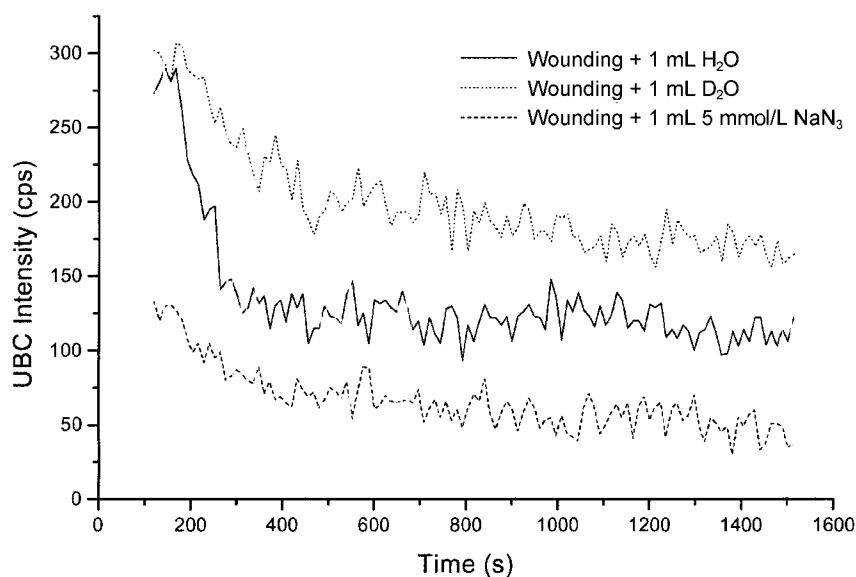


Figure 4. UBC kinetics curves from the effect of NaN₃ and D₂O on wounding induced in germination soybean cotyledon. The wounded soybean cotyledons were each placed in a quartz sample dish and immersed in 1 mL pure deionized water and 5 mmol/L NaN₃, D₂O. All curves are the means of three replicates.

intensity was initially very high (S:N ratio 11) and then decayed quickly. After about 5 min the intensity decreased slowly and then reached a stationary state (S:N ratio 5).

It is commonly accepted that the UBC of living systems results from the electronically excited triplet state peroxidation of unsaturated fatty acids, or from ¹O₂ and the excited triplet state carbonyls of the dismutation of peroxyradicals generated in biological metabolism. The time-dependent variations in emission intensity suggests that some physiological and biochemical changes must have taken place in the wounded tissues. Physiological and biochemical changes might result in the production of ¹O₂ and excited triplet state carbonyls in wounded soybean cotyledons. We believe that this phenomenon arises from a defence response of the soybean cotyledon to wounding. In order to confirm ¹O₂ generation in wounded soybean cotyledons, we designed the following experiments.

Effect of NaN₃ and D₂O on wounding-induced UBC

The UBC kinetic curves from NaN₃ and D₂O on wounding-induced UBC are shown in Fig. 4. NaN₃ can be used as a quencher of ¹O₂. Wounding-induced UBC could be suppressed by sodium azide (NaN₃) (inhibited by 58.0%). In order to confirm that the light emission of rice seeds arises from ¹O₂, we performed a complementary experiment using histidine (which can react with ¹O₂ with high efficiency) and observed quenching of the light emission.

In order to confirm the ¹O₂ formation further, the effect of D₂O on the wound-inducing UBC was observed. The UBC intensity was amplified markedly by 56.4%. The solvent D₂O is often used (15, 16) due to the long lifetime of singlet oxygen (−68 μs), which facilitates measurements as compared to the solvent H₂O (lifetime −3.5 μs). Thus, biological responses are often compared by replacing H₂O in the system with D₂O, as a test for ¹O₂ generation (15).

The main sites of ROS production in the plant cell during abiotic stress are the organelles with highly oxidizing metabolic activities or with sustained electron flows—chloroplasts, mitochondria and microbodies (17). The reactive nature of ROS makes them potentially harmful to all cellular components. Fortunately, plants have the capacity to cope with these ROS by eliminating them with an efficient ROS-scavenging system (18). Under moderate stress conditions, the radical are efficiently scavenged by this antioxidant defence system. Our results showed that the intensity of UBC from intact soybean cotyledons was very low (Fig. 3) because there is a balance of the generation and elimination of ROS in normal physiological conditions (the level of ¹O₂ generation is very low).

However, during periods of more severe stress, the scavenging system may become saturated by the increased rate of radical production. Excessive levels of ROS result in damage to the chloroplasts and mitochondria, ultimately leading to severe cellular damage (11). Recent work has established the rapid production of active oxygen species (termed the oxidative burst) as an important plant response to pathogen infection (19, 20).

The oxidative burst is an early localized defence response that involves the production of potentially cytotoxic quantities of H_2O_2 and O_2 (19). From our results, the intensity of UBC from wounded soybean cotyledons was higher than in the intact cotyledons, especially during the first phase (Fig. 3). We suggest that the oxidative burst also occurs during mechanical wounding of plant tissue. UBC was inhibited by catalase during the early phase in wounded soybean cotyledons (data not shown), and this suggests that H_2O_2 was generated in the early wounding phase.

ROS production in plant chloroplast and mitochondria can be a source of ROS under specific stress conditions (21). ROS reaction induced by chloroplast and mitochondrial damage due to wounding could be the primary step that initiates a stimulated burst of lipid peroxidation, leading to $^1\text{O}_2$ generation, and when singlet oxygen and ROS decay from excited states, photon emission will be generated. The mechanism leading to UBC probably involves energy transfer from excited species to molecules in chloroplasts and mitochondria, and further investigations are needed to classify the mechanism.

CONCLUSIONS

In conclusion, we have observed that the UBC images of soybean sprouts with mechanically wounded cotyledons were significantly enhanced as compared with those of the non-injured parts of the cotyledons. SPC measuring results showed that the UBC intensity of wounded cotyledons was initially very high and reached a stationary state after about 5 min. Wounding-induced emission could be suppressed by treatment with sodium azide (NaN_3). Deuterium oxide amplified the emission intensity. These results revealed that singlet oxygen was a main cause of the emission during the wounding phase. We suggest that the main process probably involved chloroplast and mitochondrial damage at the site of wounding and led to ROS generation, e.g. H_2O_2 , $^1\text{O}_2$.

Morphological detection of ultra-weak bio-chemiluminescence is non-invasive and provides real-time imaging. If combined with the time-dependent single photon counting analysis, it could become a method of assessing the resistance of plants to adverse conditions, and is expected to be used widely in agriculture.

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REFERENCES

1. Scott RQ, Usa M and Inaba H. Ultraweak emission imagery of mitosing soybeans. *Appl. Phys. B* 1989; **48**: 183–185.
2. Ichimura T, Hiramatsu M, Hirai N and Hayakawa T. Two-dimensional imaging of ultraweak emission from intact soybean roots. *Photochem. Photobiol.* 1989; **50**: 283–286.
3. Yoda B, Goto Y, Sato K, Saeki A and Inaba H. Ultraweak chemiluminescence of smokers' blood. *Arch. Environ. Health* 1985; **40**: 148–150.
4. Hideg E and Inaba H. Dark adapted leaves of paraquat-resistant tobacco plants emit less ultraweak light than susceptible ones. *Biochem. Biophys. Res. Commun.* 1991; **178**: 438–443.
5. Sohkhichi S, Usa M, Nagoshi T, Kobayashi M, Watanabe N, Watanabe H and Inaba H. Two-dimensional imaging and counting of ultraweak emission patterns from injured plant seedlings. *J. Photochem Photobiol. B* 1991; **9**: 211–217.
6. Allen RC. Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. *Biochem. Biophys. Res. Commun.* 1972; **47**: 679–684.
7. Makino T, Kato K, Lyozumi H, Honzawa H, Tachiiri Y and Hiramatsu M. Ultraweak luminescence generated by sweet potato and *Fusarium oxysporum* interactions associated with a defense response. *Photochem. Photobiol.* 1996; **64**: 953–956.
8. Salin LM and Briges MS. Chemiluminescence in wound root tissue. *Plant Physiol.* 1981; **67**: 43–46.
9. Avels BF, Leather RG and Forrence EL. Plant chemiluminescence. *Plant Physiol.* 1978; **62**: 696–698.
10. Wu HG, Michler CH, LaRussa L and Davis JM. The pine *Pschi4* promoter directs wound-induced transcription. *Plant Sci.* 1999; **142**: 199–207.
11. Breusegem FV, Vranova E, Dat JF and Inze D. The role of active oxygen species in plant signal transduction. *Plant Sci.* 2001; **161**: 405–414.
12. Pearce G, Strydom D and Johnson S. A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science* 1991; **253**: 895–897.
13. Ryter SW and Tyrrell RM. Singlet molecular oxygen ($^1\text{O}_2$): a possible effector of eukaryotic gene expression. *Free Rad. Biol. Med.* 1998; **24**(9): 1520–1534.
14. Chen WL, Xing D, Wang J and He YH. Rapid determination of rice seed vigour by spontaneous chemiluminescence and singlet oxygen generation during early imbibition. *Luminescence* 2002; **17** (in press).
15. Pennell RI and Lamb C. Programmed cell death in plants. *Plant Cell* 1997; **9**: 1157–1168.
16. Bolwell GP and Wojtaszek P. Mechanisms for the generation of reactive oxygen species in plant defense-broad perspective. *Physiol. Mol. Plant. Pathol.* 1997; **51**: 347–366.
17. Dat J, Vandenabeele S, Vranova E, Montagu MV, Inze D and Breusegem FV. Dual action of the active of the active oxygen species during plant stress response. *Cell. Mol. Life Sci.* 2000; **57**: 779–795.
18. Slooten L, Montagu MV and Inze D. Manipulation of oxidative stress tolerance in transgenic plants. In *Transgenic Plant Research*, Lindsey K (ed.). Harwood Academic: Amsterdam, 1998; 241–262.
19. Chandra S, Martin GB and Low PS. The Pto-kinase mediates a signaling pathway leading to the oxidative burst in tomato. *Proc. Natl Acad. Sci. USA* 1996; **93**: 13393–13397.
20. Scheel D. Oxidative burst and the role of reactive oxygen species in plant-pathogen interactions. In *Oxidative Stress in Plants*, Inze D, van Montagu M (eds). Harwood Academic: London, 2001; 137–153.
21. Camp WV, Inze D and Montagu MV. The regulation and function of tobacco superoxide dismutases. *Free Radic. Biol. Med.* 1997; **23**(3): 151–120.