X-band Electron Paramagnetic Resonance Investigation of Stable Organic Radicals Present under Cold Stratification in ‘Fuji’ Apple Seeds

Kouichi Nakagawa1,*, Kazuhiro Matsumoto2, Nattakan Chaiserm3 and Aroonsri Priprem3

1 Division of Regional Innovation, Graduate School of Health Sciences, Hirosaki University, 66-1 Hon-cho, Hirosaki 036-8564, JAPAN
2 Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga, Shizuoka 422-8529, JAPAN
3 Division of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, THAILAND

Abstract: We investigated stable organic radicals formed in response to cold stratification in ‘Fuji’ apple seeds using X-band (9 GHz) electron paramagnetic resonance (EPR) technique. This technique primarily detected two paramagnetic species in each seed. These two different radical species were assigned as a stable organic radical and Mn2+ species based on the g values and hyperfine components. Signal from the stable radicals was noted at a g value of about 2.00 and was strong and relatively stable. Significant radical intensity changes were observed in apple seeds on refrigeration along with water supplementation. The strongest radical intensity and a very weak Mn2+ signal were also observed for the seeds kept in moisture-containing sand in a refrigerator. Noninvasive EPR of the radicals present in each seed revealed that the stable radicals were located primarily in the seed coat. These results indicate that the significant radical intensity changes in apple seeds under refrigeration for at least 90 days followed by water supplementation for one week, can be related to cold stratification of the seeds.

Key words: EPR, apple seed, radical, antioxidants, dormancy, ROS

1 INTRODUCTION

Dormancy and germination of plants have been considered to be related to plant hormones for a long time. In the last decade, it was suggested that reactive oxygen species (ROS) are also related to various aspects of plant physiology, such as dormancy and germination1,2. It is, thus, possible that ROS, along with plant hormones, constitutes an alternative form of regulation of plant physiology. ROS are continuously produced during seed development, from embryogenesis to germination, as well as during seed storage. ROS can react with antioxidants, such as anthocyanins and polyphenols, and cellulose to produce stable organic radicals in plant seeds. Detection of free radicals using noninvasive electron paramagnetic resonance (EPR) technique can provide detailed information regarding the involvement of ROS-related free radicals in plant physiology.

Electron paramagnetic resonance is a sensitive and non-destructive technique to detect unpaired electrons (free radicals) in the samples at ambient temperature3,4. Several studies have used this method to investigate free radicals in naturally occurring samples3,5,6. The EPR methods can provide insights into the radical organization. Detailed studies of EPR line intensities, line widths, and line separations with various radicals might provide information on the radical generation processes and radical moieties. In addition, information on the distribution of radicals provides useful index of the antioxidant activities as a function of time.

In a previous study, the EPR and two-dimensional (2D) EPR imaging of radicals present in each seed indicated that stable radicals were primarily located in seed coat, with few radicals observed in the seed cotyledon5. In most cases, the pigmented seed coat shows a strong EPR signal3,5. The stable radicals could be the products of antioxidant reaction processes6,7. However, little is known about the effects of the presence of endogenous radicals in the seeds of apples. The EPR method could be useful for obtaining such information.

In the present study, stable organic radicals in chemically untreated apple seeds and seed parts were examined using X-band EPR. ‘Fuji’ apple seeds stored under various...
storage conditions were examined in relation to the physiological conditions of plants such as dormancy. The EPR spectroscopy detected two types of paramagnetic species in the seeds. We also discuss the localization and concentration of stable radicals within the seeds in relation to dormancy and antioxidant activity.

2 EXPERIMENTAL

2.1 Apple seeds samples

Experiments were carried out on the seeds of apples ('Fuji') harvested from a farm located in the far north (Hirosaki, Aomori Prefecture) of the main island of Japan in the beginning of November 2016. The apple seeds were stored under different conditions, as listed in Table 1.

The apples and apple seeds were stored (1) at room temperature (RM, 20°C) in a plastic bottle with a lid, (2) in a plastic bottle with a lid in a refrigerator (3°C), and (3) in a refrigerator (3°C) in a Petri dish with moisture-containing sand for 3 months. We supplied water to maintain the moisture in the sand for 3 months. Four to five seeds were analyzed. The seed coat and cotyledon were separated and dried for 24 h at RM (20°C) before the EPR measurements. Both the samples were used without subjecting them to any additional treatment. In addition, the spin concentration of the EPR signal was calculated based on the previously described methods.

A spin probe reagent, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL), was purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and used as received. TEMPOL was used to calculate the number of stable radicals in the samples.

2.2 EPR measurements

A JEOL RE-3X X-band (9 GHz) EPR spectrometer (JEOL Ltd., Tokyo, Japan) was used for continuous wave (CW) measurements. The system was operated at 9.43 GHz using a 100 kHz modulation frequency. All the CW EPR spectra were obtained in a single scan. The typical CW EPR settings were as follows: microwave power, 5 mW; time constant, 0.1 s; sweep time, 4 min; magnetic field modulation, 0.32 mT; magnetic field sweep width, 5–100 mT. For each measurement, the apple seeds were sequentially inserted into an EPR tube (outer diameter 5.0 mm, inner diameter 4.0 mm, Wilmad LabGlass, Buena, NJ, USA) and/or were attached to an EPR rod. The EPR signal intensity was divided by the sample weight.

3 RESULTS AND DISCUSSION

3.1 CW EPR of stable radicals in apple seeds

Figure 1 shows the EPR spectra of (A) whole apple seed kept in moisture-containing sand and incubated in the refrigerator, (B) seed kept at RM, and (C) seed kept in the refrigerator. The magnetic field center used was 310.0 mT with a 100 mT sweep width. The EPR spectrum was composed of two distinguishable signals. These signals were stable, and the results were consistent for at least a month.

The first signal was strong and reproducible. The relatively broad single peak observed at a g value of about 2.00 was indicative of stable organic radicals, indicating the possibility of the radicals being generated under scavenging conditions and that there were antioxidant-related organic compounds in the seed.

Table 1  ‘Fuji’ apple seeds kept under various conditions for three months.

| Storage conditions | Case (a) | Case (b) |
|--------------------|----------|----------|
| (1) Room temperature | Apples (seeds) in a bag | Seeds in a plastic bottle |
| (2) Refrigerator | Apples (seeds) in a bag | Seeds in a plastic bottle |
| (3) Refrigerator in moisture-containing sand | – | Seeds in a Petri dish with a lid |
The second signal was characteristic of the Mn$^{2+}$ paramagnetic center ($M_s = 5/2$-related sextet$^9$), 100% natural abundance of $^{55}$Mn isotope. The hyperfine coupling of the sextet was also consistent with the previously reported values for Mn$^{2+}$ $^9$. The apparent changes in the hyperfine couplings from low to high fields, with coupling being larger in high fields, were due to the overlap of multiple Mn$^{2+}$ centers. A similar EPR spectrum was previously reported in black pepper seeds $^3$. It is important to note that very weak Mn$^{2+}$ signal was observed for seeds kept in moisture-containing sand and incubated in refrigerator (Fig. 1 (A)). Therefore, one signal corresponded to stable organic radicals, whereas the other one was assigned to the Mn$^{2+}$ species. Thus, the observed signatures from these two different radical species were assigned as indicators of stable organic radicals and Mn$^{2+}$ species.

The EPR spectra obtained at $g$ values around 2.00 of the apple seed coat for the various storage conditions are shown in Fig. 2. Apple seeds were stored (A) in moisture-containing sand and incubated in refrigerator, (B) at RM, and (C) in refrigerator. The EPR radical intensity for the seed coat upon storage in moisture-containing sand and incubated in refrigerator was stronger than that for the seeds stored at RM and in refrigerator. Taking account of the weight of the seed coat, the intensities for the seed coat in case of storage at RM, and that in case of storage in refrigerator were similar (Fig. 1 (B) and (C), Fig. 2 (B) and (C)). Therefore, we used the normalized intensity for further EPR analyses. In addition, the spin (radical) concentration was estimated by comparison using a TEMPOL (known concentration) solution in a capillary tube (outer diameter 1.0 mm, inner diameter 0.9 mm). The number of spins per gram was approximately $3 \times 10^{18}$ (Fig. 2 (B)).

The normalized radical intensity as a function of time (weeks after apple harvest) is shown in Fig. 3. Apple seeds, which were taken out from the apples stored at RM, were used for the measurement. The procedures for EPR measurements are described in the Experimental section. The EPR measurements were conducted on each day. Since EPR signal intensity is proportional to the amount (weight) of the seed, we divided the signal intensity by the weight of the seed. The signal intensity of the radical increased with time after the harvest. At the beginning after the harvest, the signal intensity of the radical was rather less. The intensity increased with increase in the number of weeks after the harvest. The results might be related to ROS$^1$ and/or oxidation procedures in apples.

The EPR spectra obtained from the samples in which water was supplied for both the seeds (the refrigerated and incubated at RM) after the incubation in the refrigerator and RM for about 90 days are shown in Fig. 4. We also placed the seeds that had already been subjected to storage in refrigerator or at RM in a Petri dish containing moisture for about 90 days. The EPR signals increased in both the cases. In particular, EPR signal of the sample stored in refrigerator approximately doubled in intensity after one week compared with that stored at RM (Fig. 4). This result suggests that the radicals might be related to ROS$^1$. 

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**Fig. 2** EPR spectra obtained around the $g = 2.00$ region of an apple seed coat under various storage conditions. Apple seeds were stored (A) in moisture-containing sand and incubated in the refrigerator, (B) at ambient temperature, and (C) in the refrigerator. Each spectrum was obtained with a single scan. The arrow indicates the stable radical center.

**Fig. 3** Plot of normalized radical intensity as a function of weeks after apple harvest. The number of weeks refers to when the seeds were taken out from the apple after harvest for EPR measurements.
pre-germination processes of the seeds. Notably, the seeds containing moisture and stored in refrigerator germinated.

**Figure 5** shows the normalized radical intensities under various storage conditions: at RM, in refrigerator, and in moisture-containing sand and incubated in refrigerator. The EPR (radical) intensities of sample at RM and in refrigerator were similar, whereas very high intensity was observed for the seeds stored in moisture-containing sand and incubated in refrigerator. Notably, the weight of the seeds decreased by 20–30% during the storage in moisture-containing sand incubated in refrigerator and the EPR intensity of the organic radicals increased by 2–3 times in combination with much less Mn$^{2+}$ intensity (e.g., Fig. 1(A)). Thus, it is possible that the EPR results reflect the plant physiological condition, such as pre-germination stage.

The EPR spectra of the seed coat and the inner part

![Figure 4](image1.png)

**Fig. 4** Apple seeds were incubated in RM, refrigerator (water addition after 90 days incubation), and refrigerator in a Petri dish moisture-containing sand for about 90 days. The strong EPR signals correspond to incubation in refrigerator in sand.

![Figure 5](image2.png)

**Fig. 5** Bar plot of normalized radical intensities under various storage conditions: at room temperature, in the refrigerator, and in moisture-containing sand and incubated in the refrigerator.

![Figure 6](image3.png)

**Fig. 6** (A) The apple seeds were incubated in refrigerator. EPR spectra of the seed coat and the inner part (cotyledon) of the seed were presented. (B) The apple seeds were incubated in refrigerator and added water for one week. EPR spectra of the apple seed coat and cotyledon of the seed. Each spectrum was obtained in a single scan and was slightly shifted due to slightly different frequency. Both Y-axes are the same scale. An EPR rod was used for the seed coat measurements because the coat does not fit in an EPR tube.
‘Fuji’ apple seed radicals by EPR

(cotyledon) of the apple seeds are shown in Fig. 6. The EPR spectra for apple seeds incubated in refrigerator and for apple seeds incubated in refrigerator with water for one week, respectively, are shown in Fig. 6(A) and (B). The seed coat and the inner part of the seed were analyzed separately. The EPR results show that the stable radicals are located mostly in seed coat. The results obtained are consistent with a previous observation. It is interesting to note that EPR intensities for both seed coat and cotyledon (Fig. 6(B)) increase after water supplementation to seed.

There are two factors that influence the dormancy of apple and pear seeds: low temperature and moisture. However, the mechanisms of the breaking of dormancy are not completely understood. Recently, it was reported that alleviation of ROS and reactive nitrogen species (RNS) play important roles in the dormancy under prolonged cold stratification for 70–90 days. In fact, in the present experiment, the apple seeds kept in moisture-containing sand and incubated in refrigerator showed a strong EPR signal of radical. The procedures to measure ROS, for example, superoxide and hydrogen peroxide, are complex because there is a possibility that the measurement process itself can actually change the level of ROS. EPR techniques are non-destructive and do not alter the plant samples for the experimental protocols. EPR measurements can detect significant differences between dry (incubation at RM) and moist seeds during the cold accumulation process.

The stable organic radicals in seeds can be produced during the scavenging of ROS and/or RNS within the seeds. It is important to note that the radicals in the seeds can be indirect indicator of ROS. ROS and RNS are involved in the regulation of various processes in plants. For example, ROS react with phenolic compounds and produce stable radicals in the seed. In addition, the weak MnII signal of the seed coat might indicate dormancy-related antioxidant activity during the storage. Thus, the stable organic radicals are an indicator of antioxidant-related activity in the seed.

In summary, X-band EPR detected two different paramagnetic species in apple seeds. The EPR spectra showed that the stable radicals were mostly located in the seed coat, but few were also present in the cotyledon. Thus, the present study showed that EPR could be a useful method to reveal, in detail, the change of stable radical in relation to ROS status during dormancy and the subsequent germination process.

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