Amounts of Alcohol and Carbon Dioxide Produced in the Anaerobic Respiration of Seeds of *Nelumbo nucifera* and Others

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Abstract

No alcohol was detected in new and old seeds of *Nelumbo*, while old seeds contained small amounts of alcohol in three other species. Seeds of *Nelumbo*, rice and wheat were incubated at 35° in syringes filled with boiled distilled water, and the amounts of alcohol and CO₂ produced were measured. *Nelumbo* seed showed more active metabolism than the other two. The total amount of CO₂ produced was more than the theoretical amount to be expected from the usual alcoholic fermentation. These facts may be mainly due to the previous observation that some redox substances participate in the anaerobic respiration.

On the anaerobic respiration of the seed of *Nelumbo nucifera*, the writer^{1,2)} has studied previously. Under anaerobic conditions seed may produce ethanol and CO₂ through alcoholic fermentation of endogenous hexose. Numerous papers³⁻⁹⁾ have been presented concerning the respiration in germinating seeds of higher plants. However, the relation between the amounts of ethanol and CO₂ produced has not yet been satisfactorily elucidated. The present study deals with the amounts of ethanol and CO₂ produced during the anaerobic respiration of *Nelumbo*, rice and wheat.

Material and Methods

The sample mainly used in this work was one year-old *Nelumbo* fruit collected at Kamakura. The fruit-coat was peeled off and the seed was soaked in an Usuplun solution (0.5%). After 1 hr. seed coat was removed and the naked seed was treated with a new Usuplun solution for 10 min., followed by washing thoroughly with boiled distilled water. Each of the sterilized *Nelumbo* seeds was put in a syringe (30 ml capacity) which had been filled with 20 ml boiled distilled water. Grease was applied beforehand between the piston and cylinder of the syringe, whose opening end was fitted with a rubber cap. A small amount of gas remaining between the cotyledons was removed by pulling the piston, then the gas was thoroughly excluded from the syringe. And the seeds were incubated at 35° in the dark.

In all the procedure care was taken to keep off microbial contamination. At the end of each experiment, microbial contamination of the medium was examined by pouring 1 ml of it on agar plates containing 5% glucose; the colonies formed were counted after 24 hr. incubation at 27°. In almost all incubation media with seeds of *Nelumbo*, rice or wheat, no excess counts were found beyond the level of the

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control with fresh boiled water. In cases, where a slight turbidity was noticed in incubation medium, the data were discarded.

Experiments on the seeds of rice (*Oryza sativa*) and wheat (*Triticum aestivum*) were conducted in the same way as in the above. The former were collected from a field at Kamakura, and the latter at Yokohama. Each syringe contained 780 mg seeds (roughly corresponding to the dry weight of one *Nelumbo* seed).

Volume of the gas formed in a syringe was read. When it was too small to be read, it was estimated by comparing with air bubbles of known volumes contained in other syringes. It was confirmed in the previous study²⁾ that the gas evolved from *Nelumbo* seeds during incubation under similar anaerobic conditions consists of CO₂ at least 92%. The liquid medium in the syringe was poured out, the seed being washed and incubated further boiled distilled water. To the medium taken out baryta water was added. Precipitate of BaCO₃ was collected by centrifugation and weighed after drying as usual. The total amount of CO₂ thus assayed was represented as the volume under standard conditions.

The supernatant fluid was distilled and alcohol was determined by colorimetric method¹⁰⁻¹⁴⁾ by using ceric ammonium nitrate. Since the alcohols other than ethanol also react with this reagent, gas chromatography¹⁴⁾ was applied to the distillate to confirm that no other alcohols than ethanol was contained. The incubation medium was extracted by a mixture of ether-pentane (2:1, v/v) for 40 hrs., and the extract was washed with 5% HCl and 10% Na₂CO₃ solutions successively, followed by drying with anhydrous sodium sulfate. After evaporation of the solvents, the residue was subjected to gas chromatography using TEA (Triethanolamine) for packing and N₂ as carrier gas.

The theoretical amount of CO₂ to be produced on alcoholic fermentation was calculated according to the equation: $C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$.

Results

Alcohol content of freshly harvested and older seeds of some angiosperms is shown in Table 1.

Table 1. Alcohol content of new and older seeds (mg/g fresh wt.).

	Newly harvested seeds	Older seeds (age)
<i>Nelumbo nucifera</i> (Indian lotus)	0	0(1 year)
"	0	0(4 years)
<i>Oryza sativa</i> (rice)	0	3.7 (7 months)
<i>Triticum aestivum</i> (wheat)	0	0.06 (10 months)
<i>Glycine Max</i> (soybean)	0	0.3 (1 year)

Seeds of *Nelumbo*, rice and wheat were bottled up in syringes together with boiled distilled water and incubated for 3 days at 35°. Total amount of CO₂ produced in 3 days was measured and the amount of CO₂ corresponding to the alcohol formed was also determined. The results are shown in Table 2.

During the incubation for 3 days, most of *Nelumbo* seeds germinated and expanded their cotyledons and ca. 40% of rice grains swelled to germinate, while wheat seeds remained almost unchanged.

Table 2. CO₂ produced from seeds of *Nelumbo*, rice and wheat anaerobically incubated in water at 35° for 3 days.

	CO ₂ -gas evolved (ml)	CO ₂ dissolved in water (ml)	Total CO ₂ (ml)	CO ₂ calculated from alcohol produced (ml)
<i>Nelumbo nucifera</i>	6.6	5.2	11.8	8.1
Rice	0	5.8	5.8	3.8
Wheat	0	2.8	2.8	0

Fig. 1. Daily production of CO₂ from *Nelumbo* seed incubated at 35° in a syringe filled with boiled distilled water.
E: CO₂-gas evolved; T: Total CO₂; A: Theoretical amount of CO₂ calculated from the amount of ethanol formed.

In another experiment, *Nelumbo* seeds were incubated in the same way as mentioned above, and CO₂ and alcohol were measured every day, incubation water being renewed every time. The results are shown in Fig. 1, in which E is the amount of CO₂ as gas bubbles and T denotes the total CO₂, gaseous plus liquid. A is the theoretical amount of CO₂ calculated from the amount of ethanol formed, assuming the substrate of alcoholic fermentation as hexose.

The daily production of CO₂ as represented in Fig. 1 was summed up for 3 days and was expressed in mg for the purpose of comparing with the decrease in the dry weight of seed during the same period. The results are shown in Table 3.

The results of gas chromatographic analysis are represented in Table 4. Evidently, no alcohol other than ethanol was detected in the incubation medium of *Nelumbo* seed.

Table 3. Dry weight of *Nelumbo* seed before and after experiments, and production of CO₂ and ethanol during 3 days' incubation.

Dry weight of seed before exp. (mg)	Dry weight after exp. (mg)	CO ₂ evolved as gas (mg)	CO ₂ dissolved in liquid phase (mg)	Ethanol produced (mg)
640	530	1.9	33.4	28.4

Table 4. Gas chromatographic analysis of alcohols (p. p. m.)
in incubation medium of *Nelumbo* seed.

	After 1 day	After 3 days
Methanol	—	—
Ethanol	212.5	342.0
iso-Propanol	—	—
n-Propanol	—	—
iso-Butanol	—	trace*
n-Butanol	—	—
iso-Amyl alcohol	—	trace*
n-Amyl alcohol	—	—

* The estimate being less than 0.001 ppm.

Discussion

Alcohol was not found in fresh seeds of four species of angiosperms examined, but small amounts of alcohol were detected in older ones. In *Nelumbo* seed, however, no measurable amount of alcohol was found in 1 or even in 4 year-old ones. When incubated anaerobically in water, *Nelumbo* seed produced more alcohol than rice and wheat seeds. CO₂ production also was much more with *Nelumbo* seed than others. Generally the amount of CO₂ produced was larger than that to be theoretically expected from the amount of alcohol produced by alcoholic fermentation (Table 2 and Fig. 1). This disagreement seems to be due to the production of CO₂ from some endogenous substances or secondary consumption of ethanol by the seed.

In the previous paper,¹⁾ the writer reported that *Nelumbo* seeds contained dehydroascorbic acid (about 1.6 mg/seed), which showed a marked decrease in amount upon germination resulting in a simultaneous increase in ascorbic acid. Besides, it was found that oxidized glutathione¹⁶⁾ (about 1 mg/seed) was also contained in *Nelumbo* seed and that this decreased upon germination giving rise to an increase in its reduced form. By these reactions, substrates may be oxidized under an anaerobic condition. But the amounts of these redox substances are too small to account for the excess of CO₂ observed, some other sources seem to be responsible for this reaction.

Taylor⁷⁾ has pointed out that in the fermentation of rice and wheat seeds the quotient C₂H₅OH/CO₂ is not always to 1, probably due to other type of fermentation.

As seen in Table 3 the amount of CO₂ and ethanol produced was less than the dry weight decrease. This discrepancy may be elucidated by the fact the soluble substances diffused out of the seed into surrounding water as Erigin⁶⁾ demonstrated in the germination of rice and wheat.

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