Seed Dormancy and Germination of *Echinochloa oryzicola* VASING.: An Observation through Respiration and Several Enzyme Activities

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Introduction

Persistence of annual weeds is often related to a large number of the dormant seeds buried in soil. Dormancy enables weeds to survive and maintain the longevity as a seed bank in soil despite frequent soil disturbances and weedings. It is indicated that dormancy is the single most important characteristic of weeds which enables them to persist and flourish, and germination is critical in establishment of weed infestation (NAS, 1968). Ecological behaviors of many weeds such as the possession of seed dormancy, germination requirements, periodicity of germination and its relationship with environmental stimuli suit the purpose for their establishment and propagation at their habitats, and these are often theoretically defined as adaptation. However, our physiological and biochemical understanding of such ecological adaptation of weeds is not limited only to scientific interest, but is also important for a new approach in herbicide technology and control measures.

Echinochloa oryzicola VASING. is a gramineous and representative rice paddy weed that produces large seeds (spikelets), 3 to 5 mg per seed, with innate or primary dormancy. The seed dormancy in fields was suggested to be

terminated with the low temperatures from the late autumn to early spring, the fluctuating temperatures in spring and with the flooding that accompanied the beginning of rice cultivation (MIYAHARA, 1972). Seeds awakening from dormancy can germinate under oxygen-deficient conditions (MIYAHARA, 1972 ; KATAOKA and KIM, 1978 ; KENNEDY *et al.*, 1980). and an anaerobic condition actually stimulates germination of seeds with relatively shallow dormancy (YAMASUE and UEKI, 1979). The seed dormancy could be artificially broken with respiratory inhibitors such as KCN and NaN_3 (SHIMIZU and UEKI, 1972). These combined findings indicate the importance of respiration- and/or oxygen-related reactions in the physiological mechanism of the dormancy and germination of seeds which germinate in fields flooded with paddy water.

This paper reports the first part of a series of experiments made to elucidate the ecophysiological mechanisms of seed dormancy and germination of *Echinochloa oryzicola*. Specific objective of the experiments reported here was to characterize the dormant and non-dormant seeds in respiration and its related enzyme activity during the imbibition under an optimum germination condition.

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Fig. 1. Percent germination of the dormant and non-dormant seeds of *Echinochloa oryzicola* used for the respiration measurement (A) and for the enzyme extraction (B).

Materials and Methods

Plant Material. Dormant and non-dormant seeds were obtained from the same uniformly inbred line of *Echinochloa oryzicola* grown in the experimental field of Kyoto University. This line was originated from one individual plant collected at the Konosu Branch Station of the National Agricultural Research Center. The dormant seeds were those stored under a dry condition at -21°C after harvesting, and the non-dormant seeds were prepared, as described by MIYAHARA (1972), by incubating the dormant seeds in flooded soil at 4°C for several months. Figure 1 shows the germination pattern of the seeds used for the respiration measurement and enzyme extraction. Data were the mean of duplicated 150-seed samples imbibed at 30°C in the light.

Respiration Measurement. Pattern of the change of the dormant and non-dormant seeds in respiration was determined in air with a manometric method using O_2 -Uptester C (Taiyo Sci. Inc. Co., Ltd.). One hundred and fifty seeds were sterilized with 2% NaOCl for 10 min, rinsed thoroughly with distilled water and

imbibed with 5ml of distilled water in 6-cm petri dish under the germination condition at 30°C in the light. The O_2/CO_2 exchange was measured at 3, 6, 12, 24, 54 and 72 hr after the beginning of imbibition. Data were the mean of duplicated measurements.

Preparation of Enzyme Extracts. The following procedure was used for all the enzyme extracts. Two hundreds of both dormant and non-dormant seeds, which had been fully imbibed at 4°C , were sterilized and imbibed at 30°C under the above procedure, and sampled at 0, 3, 6, 12, 24 and 48 hr. The samples were homogenized in 3.1 ml of cold extraction medium with Polytron (Kinematica, Switzerland) at full speed for about 45 sec. with cooling by ice bath. The extraction medium contained 0.1M potassium phosphate buffer (pH 7.2), 10 μl of Triton X-100 and 200 mg of polyvinyl-pyrrolidone. The homogenate was centrifuged at 32,000 $\times g$ for 30 min. The resulting supernatant was adjusted to 35% (v/v) glycerine with 75% glycerine solution and stored at -85°C as the crude enzyme solution until enzyme assays.

Enzyme Assays. Enzymes assayed with the

crude enzyme solution were glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.43), alcohol dehydrogenase (EC 1.1.1.1), isocitrate dehydrogenase (EC 1.1.1.42), cytochrome c oxidase (EC 1.9.3.1), polyphenol oxidase (EC 1.10.3.1), catalase (EC 1.11.1.6) and peroxidase (EC 1.11.1.7). Activities of glucose-6-phosphate dehydrogenase and 6-P-phosphogluconate dehydrogenase were determined by the rate of reduction of NADP measured by A_{340} . Three assays were carried out, the first containing 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 0.3 mM Glucose-6-P, 0.3 mM 6-P-gluconate, 0.2 mM NADP and 50 μ l enzyme extract in 3 ml. The second contained all the above except glucose-6-P, and the third contained only the buffer and 50 μ l enzyme extract. The difference in the reduction rate of NADP between the first and second assays was used to calculate glucose-6-phosphate dehydrogenase activity, and the difference between the second and third assays was used for 6-phosphogluconate dehydrogenase. Activity of alcohol dehydrogenase was determined by the rate of reduction of NAD in the 3 ml assay medium containing 200 mM Tris-HCl (pH 8.5), 0.3 mM NAD, 17 mM ethanol and 100 μ l enzyme extract.

Isocitrate dehydrogenase activity was also determined by reduction of NADP. Two assays were carried out, one containing 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 2 mM DL-isocitrate, 0.2 mM NADP and 100 μ l enzyme extract in 3 ml. The second contained only the buffer and 100 μ l enzyme extract. The difference in the NADP reduction rate between the two assays was used to calculate isocitrate dehydrogenase activity. Activity of cytochrome c oxidase was determined by the rate of oxidation of reduced cytochrome c measured by A_{550} , using a dual wavelength spectrometer (Shimadzu UV 3000). The assay medium contained 10 mM potassium phosphate buffer (pH 6.9), 1 mM EDTA, 5 μ M reduced cytochrome

c and 50 μ l enzyme extract.

Polyphenol oxidase activity was measured by the rate of O_2 consumption in 3 ml of 0.1 M Tris-HCl (pH 8.0) containing 20 mM phloroglucinol. The measurement was started by the addition of 100 μ l enzyme extract using an oxygen electrode (Rank Brothers Engineering, England). Air-saturated distilled water was used to take a standard of the oxygen level at 237 μ M at 30°C. The oxygen electrode was also used for the determination of catalase activity. The activity was measured by the rate of O_2 liberation after 50 μ l enzyme extract addition into the assay medium containing 0.1 mM potassium phosphate buffer (pH 7.2) and 6 mM H_2O_2 in 4.05 ml. Activity of peroxidase was determined by the rate of oxidation of guaiacol measured spectrophotometrically by A_{470} . The assay medium contained 40 mM acetate buffer (pH 4.0), 1 mM guaiacol, 4.7 H_2O_2 and 50 μ l enzyme extract in 2.7 ml.

All the above enzyme assays were carried out at 30°C except that for cytochrome c oxidase at 25°C. Each of the seed samplings and enzyme extractions was duplicated except for isocitrate dehydrogenase activity with no duplication, and there was little difference between the first and second samplings in the pattern of change in enzyme activities. One representative data is shown in the following. Total protein content in the crude enzyme extracts was determined by Bio-Rad Protein Assay Kit using bovine γ -globulin as a standard.

The specific activity of an enzyme used here was defined as units of the enzyme activity per mg of the total protein content.

Ethanol Determination. One hundred of both dormant and non-dormant seeds were imbibed and sampled by a procedure identical to the above for the enzyme extracts. The samples were homogenized in 2 ml of cold acetone and centrifuged at 32,000 xg for 30 min. Two μ l of the resulting supernatants was injected into a gas chromatograph (Shimadzu GC-7A) equip-

ped with a flame ionization detector. The column used was a 0.32- by 210-cm coiled glass column pack with Porapak Q (50–80 mesh). Temperatures of the injection port and column oven were 250 and 200°C, respectively. Supplies of nitrogen, air and hydrogen were 50 ml/min, 0.6 kg/cm² and 0.7 kg/cm², respectively. Ethanol excreted into the imbibition water was also quantitatively determined by injecting 2 μ l of water directly to the gas chromatograph. No determination was done for ethanol vaporizing from the seeds and imbibition water since our preliminary trial had shown that the amount was negligible when compared with those in seeds and excreted. Data were the mean of duplicated sampling.

Results and Discussion

The seed materials used for the respiration measurement and enzyme extraction showed relatively uniform germination (Figure 1 A and B), and in particular, the latter seeds showed highly synchronized germination: 88% of the seeds protruded the shoot and coleorhiza during 12 to 24 hr in the imbibition, and 100% had root growth at 48 hr. As a nature of weeds, weed seeds often exhibit germination over a long period even under a laboratory condition. This asynchronized germination makes physiological data difficult to interpret because in such an experiment we have to handle a batch of seeds with individual variation in the speed of germination. From this point of view, the seed samples of *Echinochloa oryzicola* VASING. used here were appropriate to analyze for respiration and enzyme activities. No germination was observed in the dormant seeds for either respiratory measurement or enzyme extraction.

Patterns of the changes in the respiration are shown in Figure 2. The dormant seeds rapidly increased both O₂ uptake and CO₂ liberation during the first 12 hrs, and then after reached a plateau with little change. The

Fig. 2. Pattern of the change in O₂/CO₂ exchange of the dormant (A) and non-dormant seeds (B) of *Echinochloa oryzicola* under the imbibition at 30°C in the light.

non-dormant seeds also increased respiration up to 6 hr, but then decreased for once until 24 hr. During the period from 12 to 24 hr CO₂ liberation exceeded O₂ uptake, and the respiratory activity in CO₂/O₂ exchange was two- to four-fold smaller than that of the dormant seeds. After this period the O₂ uptake rose almost continuously throughout germination. The plateau in O₂ uptake from 12 to 24 hr appeared to end about the time when the seed coats were broken and free gas exchange started as pointed out for pea seeds (SPRAGG and YEMM, 1959; MAYER and POLJAKOFF-MAYBER, 1982). Protrusion of the shoot and coleorhiza started about 48 hr in some seeds (Figure 1A). The RQ values calculated are shown in Figure 3. The values were near 3.0 for non-dormant seeds at the respiratory plateau from 12 to 24 hr. RQ values are often related to the substrate oxidized in respiration. But, as evidence by the increased activity of alcohol dehydrogenase and ethanol formation during the period prior to the protrusion of the shoot and coleorhiza (Figures 4 and 5), these increased RQ values were due to CO₂ liberation from the alcohol fermentation; the caryopsis may be so compactly enclosed by the floral lemma and glumes that it is hardly allowed to respire the external

Fig. 3. Change of the dormant (○) and non-dormant seeds (●) of *Echinochloa oryzicola* in RQ during the imbibition at 30°C in the light.

O₂ to satisfy the increased energy requirement for germination before breakage of the outer barriers.

Pattern of the change in the respiratory enzyme activities of the dormant and non-dormant seeds is shown in Figure 4. The activity is expressed by a relative activity on percent basis using the largest activity of a sample as 100. In the non-dormant seeds before imbibition at 30°C the activity of glucose-6-phosphate dehydrogenase was almost two-fold higher than that in the dormant seeds, and increased continuously throughout germination, whereas in the dormant seeds the activity decreased to the initial level after an increase for once from 6 to 12 hr. The activity of 6-phosphogluconate dehydrogenase changed in a pattern similar to the preceding enzyme in the non-dormant seeds. Patterns of changes in these two enzyme were almost identical to those found in dormant and non-dormant seeds of *Avena fatua* (KOVACS and SIMPSON, 1976). Glucose-6-phosphate dehydrogenase is the regulatory enzyme for the oxidative pentose phosphate pathway that ROBERTS and SMITH (1977) pointed out for the metabolic control in loss of seed dormancy. Early involvement of this pathway has been commonly reported for seed germination of various species including *E. oryzicola* during anaerobic germination (KEN-

NEDY *et al.*, 1980).

There was no significant change either in alcohol dehydrogenase or cytochrome c oxidase during 48 hrs in the dormant seeds (Figure 4). However, in the non-dormant seeds these two showed contrasting patterns of activity changes. Activity of the former enzyme rapidly increased up to 12 hr and then decreased sharply to almost nothing at the end of imbibition. The period from 12 to 24 hr coincided with the time when the shoot and coleorhiza broke the puter barriers (Figure 1). In contrast, the activity of cytochrome c oxidase started to increase from 24 hr. These two enzyme activity patterns suggested that at about 24 hr there was a transient phase in the respiratory pathway from alcohol fermentation to the conventional electron transport chain. In the dormant seeds the rate of gaseous exchange was virtually unchanged after an initial increase due to the exposure to 30°C and the RQ values were maintained at nearly 1.0 (Figures 2 and 3). Thus, it appeared that the seeds respired mainly through the conventional electron transport chain.

During 48 hrs in imbibition no change were observed in the activities of polyphenol oxidase, catalase and peroxidase in the dormant seeds (Figure 6). In the non-dormant seeds, activities of these three oxidases increased continuously and reached a level about two-fold higher than those in the dormant seeds at 48 hrs imbibition. The role of polyphenol oxidase was indicated in seed dormancy of several species in relation to the seed coat impermeability to water and oxygen. Mature seeds of wild pea (*Pisum elatius*) have seed coats rich in *o*-dihydroxyphenols and impermeable to water, and germinate only after abrasion, but no such requirement exists for the cultivated pea (*P. sativum*) (MARBACH and MAYER, 1975). Catechol oxidase, a polyphenol oxidase, rises sharply during seed development and dehydration of the wild pea, and the contact between the oxidase and its substrate was

Fig. 4. Pattern of the change in respiratory enzyme activities of the dormant and non-dormant seeds of *Echinochloa oryzicola* during the imbibition at 30°C in the light. The relative activity was calculated on the % basis of the largest activity being 100.

Fig. 5. Pattern of the change in ethanol formation by the dormant and non-dormant seeds of *Echinochloa oryzicola* during the imbibition at 30°C in the light. Seeds and imbibition water were analyzed separately. No detectable ethanol was found in the imbibition water with the dormant seeds.

suggested to be enhanced by rupturing of the cell membrane. But such a control mechanism of germination was lost in the cultivated pea. In *E. oryzicola*, the non-dormant seeds had lower activity of polyphenol oxidase than the dormant seeds at 0 hr (Figure 6), and the polyphenol content of dormant seeds gradually decreased as the level of dormancy decreased (YAMASUE and UEKI, unpublished). Also, the dormancy could be broken with KCN, an inhibitor for the copper-containing enzyme (SHIMIZU and UEKI, 1972). Thus, it might be indicated that the reaction between polyphenol oxidase and its substrates had a role in a loss of the seed dormancy in regard to the seed coat impermeability to water and/or oxygen. But, this may not be the case since the seed coat impermeability to water did not appear to be a causal factor of the dormancy and the non-dormant seeds showed anaerobic germination (YAMASUE *et al.*, 1985; YAMASUE and UEKI,

1986).

Catalase is usually regarded as a detoxifying enzyme to break H_2O_2 down to H_2O and O_2 in aerobic organisms, and peroxidase also does so in the presence of electron donors such as phenols and amines (BEEVERS, 1961). In *Amaranthus albus* L. and *Lactuca sativa* L., catalase and peroxidase activity were assayed and found to increase during seed germination (HENDRICKS and TAYLORSON, 1974). From the fact that thiourea, sodium nitrite and hydroxylamine salts, which break seed dormancy of the species, inhibit the activity of catalase in extracts of the seeds. HENDRICKS and TAYLORSON (1974) suggested for the mechanism of germination promotion by these chemicals that metabolically derived hydrogen peroxide, spared from decomposition by catalase inhibition, oxidized reduced NADPH required as the oxidant in the pentose pathway. In the dormant seeds of *E. oryzicola*, the specific activity of catalase was thousands-fold higher than that of peroxidase (Figure 6). When the localization is disregarded, it is therefore suggested that H_2O_2 derived was broken down mainly by catalase rather than peroxidase in the seeds. If the shift of H_2O_2 breakdown to peroxidase is important for loss of dormancy as suggested by the above authors, the catalase activity smaller in the non-dormant seeds than in the dormant seeds (Figure 6) might be worth noting. In fact, KCN, which is remarkably effective in breaking dormancy of this species, inhibited catalase and increased peroxidase activity *in vivo* before germination (HASEGAWA *et al.*, 1986).

Objective of the experiments reported herein was to characterize the dormant and non-dormant seeds of *Echinochloa oryzicola* in respiration and its related enzyme activity. In the dormant seeds, there were virtually no changes observed in either the gas exchange or enzyme activity throughout the imbibition period, and the seeds were quite quiescent in every aspect determined. However, activities of the enzyme

Fig. 6. Pattern of the change in oxygen-related enzyme activities of the dormant and non-dormant seeds of *Echinochloa oryzicola* during the imbibition at 30°C in the light. The relative activity was calculated on the % basis of the largest activity being 100.

of the enzyme assayed, alcohol dehydrogenase in particular, were relatively high when compared with those of the non-dormant seeds at 0 hr. This may indicate that those enzymes were pre-existing, not a limiting factor in the activity *per se* and the enzyme operations were somehow blocked *in vivo* as seen by little ethanol production even at a high activity of alcohol dehydrogenase *in vitro* (Figures 4 and 5). In the non-dormant seeds, it was apparent that the seeds respired through alcohol fermentation prior to the protrusion of the coleoptile and/or coleorhiza which would expose the true seed (caryopsis or fruit) to the external oxygen, and after this protrusion the respiratory pathway was shifted to the conventional electron transport chain linked to the Krebs cycle. Thus, we could characterize the non-dormant

seeds as those having the ability to satisfy the energy requirement for the embryonic growth alcohol fermentation at the early stage of germination. In addition, the fluctuation of alcohol dehydrogenase activity observed (Figure 4) appears to be partially regulated by a proteinaceous inactivator present in the seeds (YAMASUE and UEKI, unpublished) as in seedlings of *Oryza sativa* L., *Zea mays* L. and *Psium sativa* L. (SUZUKI and KYUWA, 1972; Ho and SCANDALIOS, 1975; SHIMOMURA and BEEVERS, 1983). But, since the majority of seeds of *Echinochloa oryzicola* germinate in fields under flooded water deficient in oxygen, further evidence is required of the importance of anaerobic respiration for growth of the coleoptile and radicle under an oxygen deficient condition, and we also need to clarify the behavior of

catalase and glucose-6-P dehydrogenase during the transient period from the dormant to non-dormant state in both laboratory and field soils.

Summary

As the first part of a series of experiments to elucidate the ecophysiological mechanisms of seed dormancy and germination of *Echinochloa oryzicola* VASING., and observation was made through respiration and several enzyme activities during imbibition at 30°C in the light. Those determined were the pattern of changes of the dormant and non-dormant seeds in CO₂/O₂ exchange, ethanol production and activities of G6PDH, 6PGDH, ADH, ICDH, cytochrome c oxidase, polyphenol oxidase, catalase and peroxidase. In the dormant seeds, there were virtually no changes observed throughout the imbibition period, and the seeds appeared to respire to a small extent through the conventional electron transport chain since the RQ values were maintained at nearly 1.0. However, in the non-dormant seeds both RQ values and ADH activity increased sharply until protrusion of the coleoptile and/or coleorhiza, and after this period cytochrome c oxidase activity started to increase throughout germination and the RQ values approached 1.0. It was, therefore, suggested that the non-dormant seeds respire through alcohol fermentation during the early imbibitional period, and then through the conventional electron transport chain after the breakage of the outer barriers. The sharply increased activity of ADH observed at an early stage of imbibition could be a key metabolic character prior to the visible sign of germination: protrusion of the coleoptile and/or coleorhiza.

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タイヌビエ種子の休眠と発芽

——呼吸および呼吸関連酵素活性の変動——

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摘 要

タイヌビエ種子の休眠・発芽における生態的挙動に対する生理・生化学的機構 (physiological ecology or ecophysiology) を解明しようとする研究の第一歩として、休眠および休眠覚醒種子の生理的特徴を呼吸およびその関連酵素の活性から検討した。供試種子は農研センター鴻巣試験地由来のタイヌビエから採集したもので、休眠種子は採種後 -21°C に貯蔵してその休眠性を維持させ、またこの一部の種子を 4°C 土壌埋没処理によって休眠覚醒種子を得た (Figure 1)。測定項目は 30°C 、明条件の発芽床に置床したときの CO_2/O_2 交換、エタノール生成、呼吸関連酵素 (G 6 PDH, 6PGDH, ADH, ICDH, cytochrome c oxidase, polyphenol oxidase, catalase, peroxidase) 活性の変動であった。休眠種子は置床全期間を通じて CO_2/O_2 交換量、すべての酵素活性においてはほとんど変動がなく、呼吸代謝的にも全く静止状態にあると考えられた。また、RQ 値が 1.0 付近に維持され、cytochrome c oxidase 活性も低いレベルで存在することから (Figures 3, 4)、休眠種子は通常の電子伝達系で僅かながら好気呼吸していることが示唆された。しかし、測定された酵素の活性 (Figures 4, 6)、とくに ADH 活性は休眠覚醒種子の置床 0 hr に比べ比較的大きく、休眠種子内では酵素活性そのものが制限要因でなく、何らかの機作で酵素反応が静止しているものと考えられた。

しかし、休眠覚醒種子においては、置床直後から RQ 値、ADH 活性などが急激に増大し (Figures 3, 4)、多量のエタノールが生成されるが (Figure 5)、その後鞘葉、根鞘の突出によって外被が破られ外部酸素が利用可能となると ADH 活性は低下し、代って cytochrome c oxidase 活性が増大し始め、RQ 値も 1.0 に近づいた (Figures 3, 4)。したがって、休眠覚醒種子は花被、果皮などを含む外被によって胚に対する酸素の供給が制限されており、置床後から鞘葉などの突出までの幼芽の生長に必要なエネルギーはアルコール発酵系によって獲得されることが示唆され、この置床初期における ADH 活性の急激な増減は発芽の肉眼的観察以前におこる重要な生理的形質と考えられた。