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Germination Response in Wheat Grains to Dihydroactinidiolide, a Germination Inhibitor in Wheat Husks, and Related Compounds

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On the basis of our recent findings that the germination of intact wheat grains with glumes (husks) belonging to dormant varieties was restrained as compared with that of dehusked grains, we have explored the identities of germination inhibitors in the glumes, resulting in the characterization of dihydroactinidiolide (**1**) and some aromatic compounds. A related natural product, tetrahydroactinidiolide (**2**), showed similar activity. The present study has demonstrated that the sensitivity in inhibition response of germination of the grains to **1** and **2** declined during after-ripening, in parallel with changes in germinability; the sprouting of after-ripened seeds on a whole spike was preventable by exogenous application of **2** in laboratory conditions, and germination of after-ripened grains was delayed by more than two weeks by the action of **1** or **2**. The term "pseudodormancy" is proposed for the phenomenon of delay of germination caused by the inhibitor. After accumulation of additional evidence on inhibition response of actinidiolide-type natural products, structurally related to inhibitor **1**, a mechanism concerning germination inhibition by **1** or **2** is proposed on the basis of the concept of nonbonding interaction with the inhibitors at an active site of an acceptor.

KEYWORDS: ***Triticum aestivum*; actinidiolide-type; dihydroactinidiolide; tetrahydroactinidiolide; germination; growth; inhibition; after-ripened seeds; pseudo-dormancy**

INTRODUCTION

Preharvest sprouting is one of the main problems encountered in cereal grain production, particularly when seed maturation takes place under damp conditions. Although it is known that embryo maturation is regulated and precocious germination is prevented by abscisic acid (ABA) in many species, including wheat, during grain development (*1*−*3*), involvement of ABA in dormancy after maturation and desiccation of grains is uncertain (*3*−*5*), and the underlying physiological basis of dormancy is poorly understood. On the basis of our recent findings that the germination of mature wheat grains with glumes (intact seeds) belonging to dormant varieties are restrained as compared with those of the dehusked grains at the after-ripening, we have explored the germination inhibitors in the glumes, resulting in the characterization of dihydroactinidiolide **1** (**Figure 1**) and some aromatic compounds as the active constituents. All of the characterized compounds inhibited germination in mature wheat grains at 500 ppm in aqueous solution. The related compound, tetrahydroactinidiolide **2**, was as active as **1**, whereas no noticeable difference in activity was observed among both enantiomers and the DL-form of compounds **1** and **2**. In addition, clear synergistic relations were evident between **1** and aromatic compounds, suggesting that the characterized active compounds operate synergistically as sprouting inhibitors (*6*).

From the viewpoint of agricultural importance, it is of particular interest to examine the detailed responses to inhibition of germination and growth in wheat grains in the presence of the inhibitors, **1** and **2**. This study has investigated the germination and growth of after-ripened seeds (grains) in laboratory conditions regulated by the action of **1** or **2**.

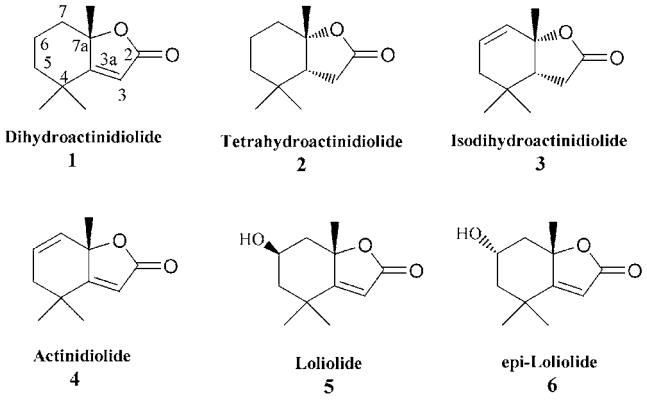

Figure 1. Germination inhibitor **1** in wheat glumes and its related natural products.

MATERIALS AND METHODS

General Experimental Procedures. The structure and purity of all the described compounds were supported by NMR, HRMS

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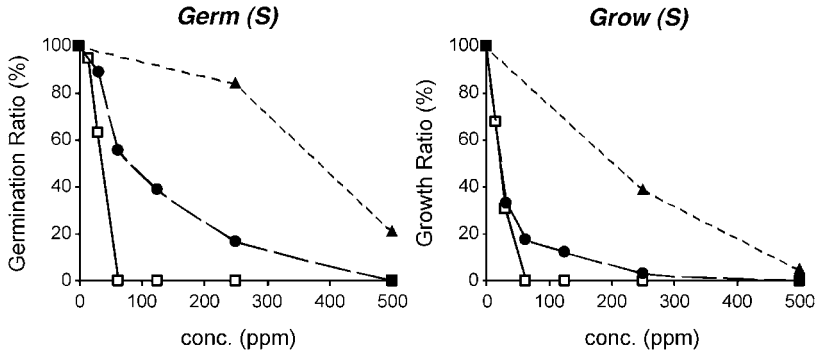

Figure 2. Difference in germinability of wheat grains *RL4137*, stored for different periods, in aqueous solutions of **2** under different concentrations (500 ppm corresponds to 2.75 mM). Germination [Germ (S)] and growth [Grow (S)] ratios of sprouts of the grains stored at 20 °C for ca. 3 (□) and 5 months (●) after harvesting and those stored for 2 years at 5 °C (▲).

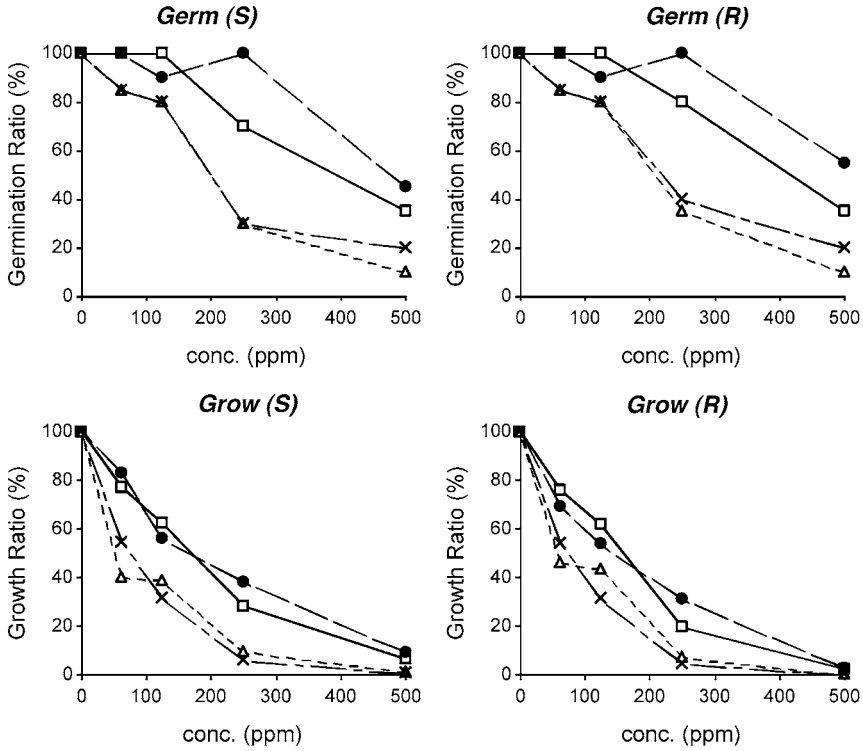

Figure 3. Time course dependency on sensitivity in fully after-ripened grains, *Hokushin*, toward germination of sprouts [Germ (S)] and roots [Germ (R)] and growth of sprouts [Grow (S)] and roots [Grow (R)] under different concentrations of **1** (500 ppm is 2.78 mM). After 5 months' storage at 20 °C, the grains were stored for 1 further month (□) or 2 months (●) at 20 °C, and also the grains from the same lots were kept for 1 month (△) or 2 months (×) at −30 °C.

and HPLC analyses. The NMR spectra (^{1}H and ^{13}C) were recorded on JEOL JMN-LA 500 (500 MHz) and JMN-LA 400 (400 MHz) spectrometers. GC-MS spectra were recorded on a JEOL JMS-700QQ instrument, and possible structures were derived by library search with Wiley 6. HRMS spectra were measured with a Hitachi M-80B spectrometer. For HPLC analyses, a Waters Associates 490E chromatograph was used. The column was a 300 × 7.8 mm i.d. with flow rate of 1.0 mL/min using H_2O/MeOH for μ-Bondapak C18 and hexane/EtOAc for silica gel as the mobile phase, respectively. Peaks were detected by measurement of the absorption at 230 nm using UV detector and refractive index (RI) detector equipped with a model D-2500 chromatointegrator (Hitachi, Tokyo). The germination assay was performed in a Sanyo MIR-151 incubator at 15 °C under continuous lighting with 15W fluorescent lamp. Water for the assay was prepared by distillation and deionization from Advantec GS-200 apparatus.

Chemicals. DL-1-Phenylethyl alcohol is commercially available. DL-Tetrahydroactinidiolide **2** was prepared by our previously described procedure (*7*), or more conveniently obtained as follows from homogeranic acid. After a mixture of homogeranic acid (2.3 g) and trifluoroacetic acid (TFA) (30 mL) was refluxed for 30 min, toluene (30 mL) was added, the volatile materials were removed by evaporation under reduced pressure, and the resultant residue was extracted with ether. The ether solution was evaporated after washing with water and brine, followed by drying over Na_2SO_4. SiO_2 column chromatography, eluted with hexane:EtOAc (7:1) to (4:1), provided DL-**2** (1.7 g, 74%). DL-Dihydroactinidiolide **1** was prepared from DL-**2** by Mori's procedure (*8*). Solubility of **1** and **2** in water is high enough to make more than 10^3 ppm aqueous solution by treatment with ultrasonic agitation. L-Loliolide **5** and D-epiloliolide **6** were supplied by Professor M. Katsumura of Kwansei University, Osaka (Japan) (*9*). The related compounds

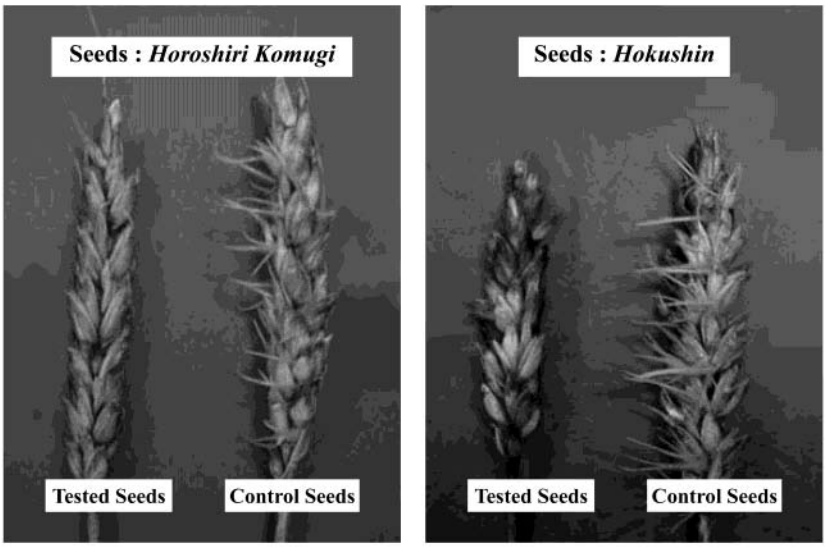

Figure 4. The germination inhibition of after-ripened seeds by the action of inhibitors. Each of a whole seed on a spike was immersed in an aqueous solution containing 200 ppm each of **2** and 1-phenylethyl alcohol (tested seeds) or in water without any sample (control seeds).

3, **4**, and **5** were prepared in DL-forms following the reported procedure (*10*), starting from 2,4,4-trimethyl-2-cyclohexen-1-one (*11*). The structure of synthesized compounds was supported by the following spectroscopic evidence.

DL-Isodihydroactinidiolide **3** (4,4,7a-trimethyl-3a,4,5,7a-tetrahydrobenzofuran-2(3*H*)-one): δ_H (300 MHz, $CDCl_3$) 0.95 (3H, s), 1.04 (3H, s), 1.54 (3H, s), 1.76−1.86 (1H, m), 2.01−2.07 (1H, m), 2.21 (1H, *t*, $J = 9.4$), 2.38−2.61 (2H, m), 5.67 (1H, d, $J = 10.2$), and 5.77−5.83 (1H, m).

DL-Actinidiolide **4**, mp 37−39 °C: δ_H (400 MHz, $CDCl_3$) 1.29 (3H, s), 1.33 (3H, s), 1.60 (3H, s), 2.14 (1H, br d, $J = 17.5$), 2.28 (1H, m), 5.72 (1H, s), 5.76 (1H, m) and 5.88 (1H, dd, $J = 9.8, 1.2$).

DL-Loliolide **5**, mp 140 °C: δ_H (300 MHz, $CDCl_3$) 1.26 (3H, s), 1.46 (3H, s), 1.53 (1H, dd, $J = 14.5, 3.7$), 1.73 (1H, dd, $J = 14.5, 4.1$), 1.77 (3H, s), 1.98 (1H, dt, $J = 14.0, 2.6$), 2.07 (1H, d, $J = 2.8$), 2.45 (1H, dt, $J = 14.0, 2.5$), 4.32 (1H, m), and 5.67 (1H, s). δ_C (125 MHz, $CDCl_3$) 26.5 (q), 27.0 (q), 30.6 (q), 36.0 (s), 45.6 (t), 47.2 (t), 66.6 (d), 87.1 (s), 112.7 (d), 172.2 (s), and 183.0 (s). HRMS calcd for $C_{11}H_{16}O_3$: 196.1099. Found: 196.1096.

Identification of Dihydroactinidiolide in Wheat Bran Including Embryos. After a mass of wheat bran including embryos (340 g) of *RL 4137* variety was kept in distilled water (1.5 L) at 60 °C for 30 h under argon atmosphere, the bran was removed by filtration through four layers of gauze. The bran was again treated with water under the same conditions. The combined aqueous layers were submitted to steam distillation at 60 °C under reduced pressure, and the aqueous distillate was extracted with CH_2Cl_2 (500 mL × 2). The CH_2Cl_2 solution was dried over Na_2SO_4 before evaporation at 40 °C under 600 hPa to obtain residue (25 mg). After the residue was passed through a short SiO_2 column eluted with hexane:EtOAc (10:1), the eluate was condensed and then analyzed by HPLC and GC-MS. Although a distinct peak at the retention time of 33 min corresponding to **1** was not detected in the normal phase silica gel HPLC eluted with hexane:EtOAc (20:1), the presence of **1** in the eluate was confirmed by GC-MS, which exhibited a peak showing the fragmentation patterns corresponding to **1**. The peaks corresponding to aromatic compounds characterized from the extracts of glumes were not detected in the GC-MS spectra.

Plant Materials. The wheat variety *RL4137* of a spring wheat and *Hokushin* and *Horoshiri komugi* varieties of winter wheats were harvested at 40−45 days after anthesis from field plots at Tokyo Agriculture University Farm, Abashiri, Hokkaido, and Hokkaido Prefectural Plant Genetic Resources Center, Takikawa, Hokkaido (Japan), on August 26 and July 19, 2001, respectively. Seed moisture content of the examined mature seeds was ca. 15−20% at harvesting. These varieties existed in the deep primary dormant state just after harvest. After the samples were kept for about 3 months at room temperature, partial after-ripening took place, and the germination ratios were ca. 60% and 80% on the basis of the examined total grains in the cases of *RL4137* and *Hokushin*, respectively. The germination ratio of *RL4137* increased to ca. 80−90% and those of *Hokushin* and *Horoshirikomugi* to ca. 100% after being kept at room temperature for a further 2 months (total 5 months after harvesting). These intact seeds were kept as a whole spike in a refrigerator at −30 °C, and hand-threshed grains were provided prior to use for the germination assay.

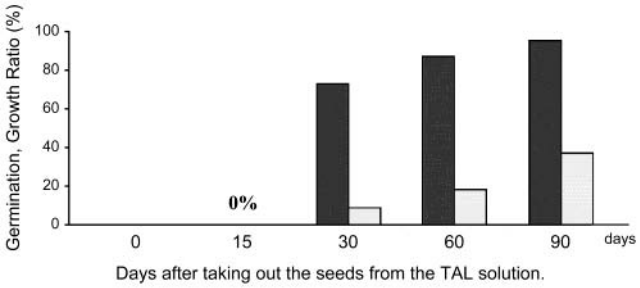

Figure 5. Response in germination (black bar) and growth (gray bar) of after-ripened grains, *Hokushin*, on treatment with **2**.

Germination and Growth Assay. In germination tests, 20 hand-threshed grains, collected by random sampling after elimination of small light grains, were placed with the embryo side up on a double thickness of 5.5 cm filter paper disks which had been soaked with 4 mL of the test solution in a Petri dish. The dishes were transferred to a transparent chamber, the bottom of which was covered with a sheet of wet filter paper, and then incubated at 15 °C under lighting for 4 days. Germination was defined as the appearance of a shoot (sprout) or distinct rootlets (root), and the number and average length (mm) of germinated grains in the solution with or without sample were recorded after 4 days. The test solution contained a sample in distilled and deionized water with a specific concentration. The 10^3 ppm

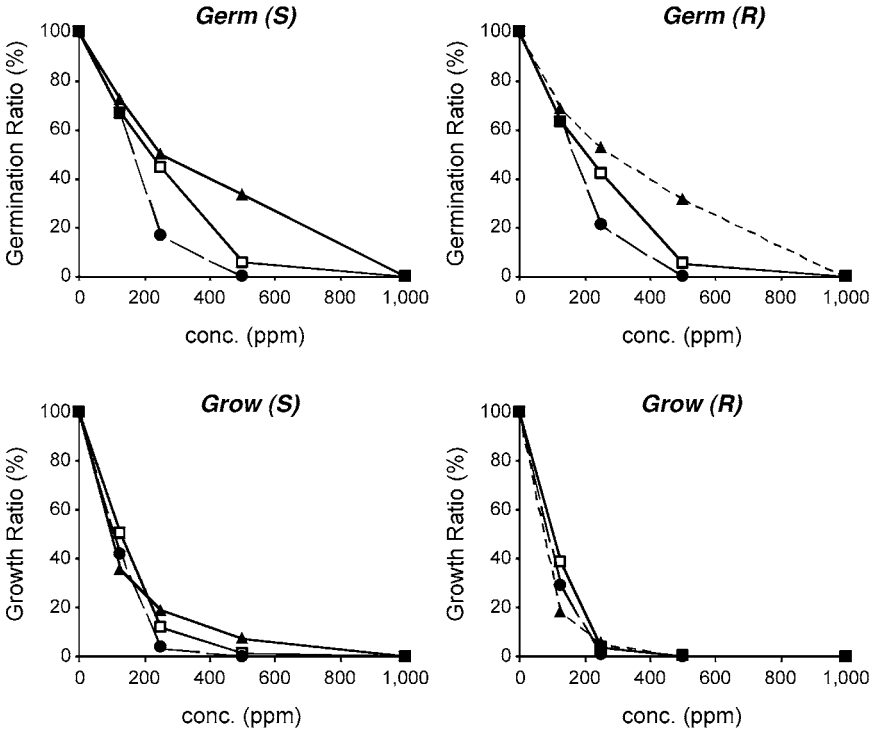

Figure 6. Inhibition activities of actinidiolide and its dihydro derivatives to germination and growth of wheat grains, *RL 4137* possessing ca. 85% germinability. The germination of sprouts [Germ (S)] and roots [Germ (R)] and growth of sprouts [Grow (S)] and roots [Grow (R)] were examined under variable concentrations (ppm) of actinidiolide **4** (□), dihydroactinidiolide **1** (●), and isodihydroactinidiolide **3** (▲) in aqueous solutions.

aqueous solution of **1** and **2** corresponds to 5.56 and 5.49 mM, respectively. The determination of the number and average length (mm) of the germinated grains in the solution without any sample was referred to as a controlled experiment for the estimation of germination and growth ratios (%) of the examined sample. All the assay experiments were carried out two or three times, the average deviations being less than ± 10% in each experiment. The grains *RL4137* with different levels of dormancy were provided by varying storage periods and were employed for the experiment in **Figure 2**. The fully after-ripened *Hokushin* variety, stored at room temperature for 5 months after harvesting, was used for the experiments in **Figures 3**, **4**, and **5**. In the germination inhibition experiment in **Figure 4**, a whole spike was immersed in an aqueous solution containing 200 ppm each of **2** (1.1 mM) and dl-1-phenylethyl alcohol (1.6 mM) and kept for 24 h at room temperature. Another spike of the same variety was similarly immersed in water containing no compound as a control treatment. After being taken out of the solution, the individual spike was wiped with a paper towel, and then kept in a humid chamber at 16 °C for 4 days. In the experiment in **Figure 5** using fully after-ripened grains of *Hokushin*, the grains were soaked in 10^3 ppm solution (5.49 mM) of **2** for 4 days at room temperature, during which the soaked grains remained ungerminated. After being taken out of the solution and then wiped with paper towels, the grains were kept under desiccation at room temperature in a net box for 90 days, during which period the germination of the grains was examined after 15, 30, 60, and 90 days. The grains of *RL4137* possessing ca. 85% germinability were employed for the experiments in **Figures 6** and **7**.

RESULTS AND DISCUSSION

Germination Inhibition Response in Grains Possessing Different Levels of Dormancy. It is well documented that grain dormancy differs markedly among genotypes at maturity, and a variable period of after-ripening is necessary for grains to become fully germinable (*12*). The variety *RL4137*, a highly dormant genotype, exists in the deep primary dormant state just after the harvesting. After storage for about 3 months at room temperature, ca. 60% of the grains germinate due to partial after-ripening. The germination ratio increases to ca. 85% after storage for further 2 months (total 5 months after harvesting). During the course of the assays in the present study, a difference in inhibition response to **1** and **2** was observed among the grains stored for different periods. The germination and growth of the grains in the different after-ripening processes were examined in aqueous solutions of **2** under variable concentrations, the results being shown in **Figure 2**. The ca. 60% germinant grains showed high response to **2**, and no germination was observed at 60 ppm (330 μM). On the other hand, considerable decrease was observed when solutions of **2** were applied to ca. 85% germinant grains. The dramatic diminution of the responsiveness was recorded in the experiment with the grains stored for 2 years at 5 °C. In addition to a similar observation in the same wheat with **1**, a dormant variety, *Hokushin*, exhibited the same response to the compounds **1** and **2** with respect to the correlation between after-ripening period and sensitivity. Furthermore, it was also apparent that the time-course dependency on sensitivity change in wheat grains was arrested when the grains were stored in a freezer at −30 °C. An example of *Hokushin* is shown in **Figure 3**, in which the inhibition response to fully after-ripened grains was almost the same within experimental deviations among the grains stored for 1 and 2 month(s) at −30 °C and was solely dependent on the concentration of the solution of **1**. In the case of grains stored at room temperature for 1 and 2 months, the sensitivity to the inhibitors decreased, depending on storage periods. It is of interest to note that freeze-storage procedure is reported to arrest after-ripening of dormant grains (*13*, *14*). This

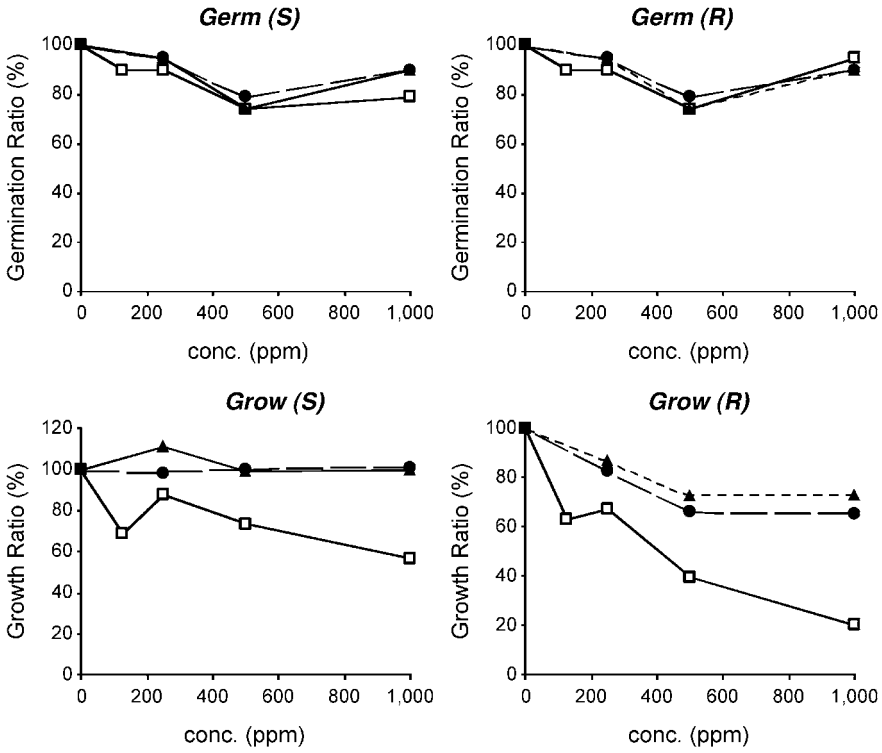

Figure 7. Inhibition responses to germination and growth of wheat grains, *RL 4137* possessing ca. 85% germinability, by DL-loliolide and its isomers. The germination of sprouts [Germ (S)] and roots [Germ (R)] and growth of sprouts [Grow (S)] and roots [Grow (R)] were examined under different concentrations (ppm) of DL-loliolide **5** (□), D-epiloliolide **6** (●), and L-loliolide **5** (▲) in aqueous solutions.

freeze-storage procedure allowed the provision of a continuous supply of uniform material without any reduction of sensitivity toward the inhibitors.

Germination Inhibition of Fully After-Ripened Grains by the Inhibitors. Preharvest sprouting is a phenomenon of germination caused by rain falling onto seeds on a spike in field and is associated with inadequate grain dormancy. From the agricultural viewpoint, it is of particular interest to examine preventability of germination of the seeds on a spike by exogenous application of the present inhibitors. As a representative example, an inhibition experiment was carried out with a mixture of **2** and 1-phenylethyl alcohol, which was revealed to have a synergistic relation (*6*). The difference in germination between tested and control seeds is shown in **Figure 4**, in which no seeds on the spike treated with the inhibitors germinated at all while vigorous germination was observed in the control treatment. The seeds of two dormant varieties, *Horoshiri-komugi* and *Hokushin*, which had lost their dormancy before the experiment, exhibited the same phenomenon.

It is of importance to clarify whether the complete inhibition of germination of the tested seeds in **Figure 4** was caused by fatal damage due to supposed phytotoxic nature of the inhibitors. To gain insight into the inhibitory action of the inhibitors **1** and **2**, a germination inhibition experiment was carried out in a solution of **2** as high as 10^3 ppm (5.49 mM) concentration, using fully after-ripened dormant grains of *Hokushin*, which showed ca. 100% germination in distilled water after 4 days. The dormancy-released grains were soaked in the aqueous solution of **2** for 4 days at room temperature, during which no grains germinated at all. The grains were then kept at room temperature in a net box under desiccation for 90 days, during which period the germination of the grains was examined after 15, 30, 60, and 90 days. No grains germinated after storage for 15 days, while more than 75% of the grains germinated after 30 days and ratios of germination and growth increased depending on the storage time, as shown in **Figure 5**. The experimental evidence in **Figures 4** and **5** supports clearly that the tested grains were fully alive under the examined conditions. In addition, the evidence indicates that the dormancy-released grains in **Figure 5** became dormant for at least more than 15 days, and more than 75% of the grains were released from their dormant state after storage for 30 days under desiccation at room temperature. It should be noted that the growth is still regulated even after 90 days storage. The high concentration of the employed 10^3 ppm solution of **2** does not mean the minimum concentration for the appearance of the observed inhibition response. To evaluate the role of the inhibitors in nature, further experiments are necessary under lower concentrations of the inhibitors **1** and **2** with or without the aromatic compounds present in the glumes (*6*).

It is often observed that fully after-ripened seeds become dormant when kept under the physiologically abnormal conditions, and this state of dormancy is called secondary dormancy. The dormancy induction observed in the present study seems different from secondary dormancy, and the term of pseudo-dormancy is proposed for such a dormant state of seeds as the germination inhibitor is involved in dormancy induction of fully after-ripened seeds.

Germination Inhibition by Compounds related to 1. It was of interest to examine the sprouting inhibition activities of the related actinidiolide-type natural products **3**−**6** to evaluate the structure−activity relationship, which may assist us to speculate on the mechanism concerning germination inhibition by **1** or **2**. The germination and growth of the grains, *RL4137* possessing ca. 85% germinability, were examined by using DL-actinidiolide **4** and two kinds of DL-dihydro derivatives, **1** and **3**. The results are shown in **Figure 6**. The similar responses on germination and growth of sprouts and roots were observed among the

compounds examined, in which the inherent **1** exhibited the strongest activity. Neither compound **3** nor **4** was detected from the extracts of wheat glumes in our previous study (*6*). It has been documented that L-loliolide (**5**), detected from leaves of *Equisetum arvense*, possesses inhibitory activity toward lettuce seed germination (*15*). In addition, L-loliolide (**5**) and D-epiloliolide (**6**) were isolated from the brown alga *Sargassum crassifolium* and shown to be potent germination inhibitors at low concentrations (*16*). These evidences suggest that loliolides **5** and **6** have potent sprouting inhibition activity toward wheat grains. To our surprise, however, DL- and L-loliolides **5** and D-epiloliolide **6** showed the least activity (**Figure 7**), disclosing that the activity declines markedly by introduction of a hydroxyl group on the cyclohexane ring of the original molecule **1**.

Proposed Mechanism of Germination Inhibition by 1 and 2. The manifestation of germination inhibition of wheat grains may be caused by taking **1** or **2** into an acceptor existing at the growing point of the embryo of the grain. There may be two possible ways for the acceptor to bind with **1** or **2**: one is some sort of chemical bond formation between lactone carbonyl of the molecules and a functional group of the acceptor such as NH_2 or OH group, forming amide or ester linkage; the other being caused by nonbonding interaction between **1** or **2** and the acceptor through hydrophobic and hydrophilic associations. The acceptor possesses two kinds of active sites: one possesses a hydrophilic site associated with the lactone group; the other holds a hydrophobic site able to interact with the trimethylcyclohexane ring of the molecules.

The present study has demonstrated a clear correlation between level of germination inhibition caused by **1** and **2** and their concentrations, suggesting the existence of an equilibrium between associated and dissociated forms among the acceptor of the grains and the compounds in an aqueous solution. The former causes the inhibition while the germination takes place from the latter, in which the grains exist free from **1** or **2**. The high concentration of **1** or **2** enriches the associated form in aqueous solution and hence prevents germination. The nonbonding interaction seems more plausible on the basis of the consideration of the existence of the equilibrium. If chemical bond formation had taken place, the binding might be irreversible. The experiment in **Figure 7** showed that the compounds **5** and **6**, in which a hydrophilic hydroxyl group is attached on the respective cyclohexane ring, showed almost no response to the inhibition, and this evidence may be explained by considering the preferential association of the hydroxyl group, instead of the lactone moiety, with the hydrophilic site of the acceptor, resulting in no formation of the association suitable to inhibit germination.

When **1** or **2** was applied to the dormant genotypes, *RL4137* and *Hokushin*, under different periods of after-ripeness, sensitivity changes were observed (**Figure 2**). This difference in sensitivity of mature grains may correspond to the association ability of the acceptor with **1** or **2**, in which *K* values, the equilibrium constants, vary depending on the storage periods of the grains, that is, the sensitivity change in germination response seems to be related to differences in association sensitivity to the inhibitors, **1** and **2**.

With respect to the induction of dormancy by plant hormones, abscisic acid (ABA) has often been suggested to play a central role in developing seeds (*1*, *17*, *18*); however, its involvement in dormancy of mature grains lacks firm experimental evidence, and endogenous ABA levels are not well correlated with germinability (*19*). Although existence of the inhibitor **1** was qualitatively confirmed in the extracts of mature wheat bran, consisting of embryo, seed coat and pericarp, by the aid of GC-MS analysis in our preliminary experiment, the amounts of **1** seems too small to induce the dormancy of mature grains. The synergistic assistance of aromatic compounds may be absent in the embryo since any aromatic compounds, identified from the glumes, was not detected in the bran. It may be suggestive that the inhibitor **1** plays a role of prevention of sprouting in the field by exogenous supply from wet glumes of mature seeds under falling rain. The glumes have been qualitatively revealed to contain relatively larger amounts of the inhibitors **1** and aromatic compounds although further study is necessary to analyze quantitatively the amounts of the inhibitors in wheat seeds. The activity of **1** may be enhanced in field by the synergistic assistance of the aromatic compounds. It should be noted that germination of mature rice grains was similarly prevented by exogenously applied inhibitors **1** and **2**. This evidence suggests the general essentiality of **1** and aromatic compounds to the germination inhibition in mature cereal seeds.

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