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Germination and Growth Inhibitors from Wheat (*Triticum aestivum* L.) Husks

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On the basis of our findings that the germination of intact wheat seeds (with husks) belonging to dormancy varieties was restrained as compared with that of the dehusked seeds (grains), the germination inhibitors in the husks were explored. The water-soluble extracts from the husks were separated by the aid of inhibition assay experiments, resulting in the characterization of 2-phenylethyl alcohol **1**, 4-vinylphenol **2** and its 2-methoxy derivative **3**, and dihydroactinidiolide **4**, all of which showed clear inhibition of germination at 500 ppm in aqueous solution. The related compounds 1-phenylethyl alcohol **5** and tetrahydroactinidiolide **6** were as active as **1** and **4**, while no noticeable difference in activity was detected among both enantiomers and the DL-form of compounds **4**−**6**. Clear synergistic relations were observed between **4** and **1** and also **4** and **3**. Since the present inhibitors have been isolated from various kinds of seed plants, they may be responsible for the general germination inhibition in the seed plants.

KEYWORDS: ***Triticum aestivum*; inhibitors; germination; growth; dihydroactinidiolide; 2-phenylethyl alcohol; 2-methoxy-4-vinylphenol; tetrahydroactinidiolide**

INTRODUCTION

Preharvest sprouting is one of the main problems encountered in cereal seed 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 seed development (*1*−*3*), involvement of ABA in dormancy after maturation and desiccation of seeds is uncertain (*4*, *5*), and the underlying physiological basis of dormancy is poorly understood. It was suggested that, in addition to ABA, other endogenous constituents in wheat may mediate dormancy and germination (*6*−*8*); however, definitive compounds other than ABA have not yet been identified from wheat seeds, to our knowledge. Recently, a large difference in germination between intact and dehusked rice seeds was observed (*9*); that is, the germination of the seeds covered with husk (intact seeds) is strongly inhibited as compared with that of dehusked seeds (grains) of the *indica* type. In the case of the *japonica* type, the opposite situation was observed, in which the germination of dehusked seeds is more profoundly inhibited. On the basis of these observations, we have been much interested in the elucidation of the role of husks toward germination inhibition in rice seeds (*10*, *11*). Although it is well documented that the germination of wheat seeds is largely dependent on the wheat varieties, the effect of wheat husk on the germination resistance has not been fully studied. We, therefore, focused our attention on the role of wheat husks, expecting that one possible factor responsible for the difference in germination resistance is the presence of inherent chemical inhibitors in the husk. This investigation demonstrates that the husks of wheat seeds possess several kinds of inhibitors, which become synergistically operative as germination inhibitors.

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MATERIALS AND METHODS

General Experimental Procedures. The structure and purity of all the described compounds were supported by NMR, high-resolution mass spectrometry (HRMS), and high-performance liquid chromatography (HPLC) analyses. The ^{1}H and ^{13}C NMR spectra were recorded on JEOL JMN-LA 500 (500 MHz) and JMN-LA 400 (400 MHz) spectrometers. Gas chromatography−mass spectrometry (GC−MS) spectra were recorded on a JEOL JMS-700QQ instrument, and possible structures in the extraction study were derived by a library search with Wiley 6. HRMS spectra were measured with a Hitachi M-80B spectrometer. The silica gel and Cosmosil gel used for MPLC (Eyela RI-20UV, Nikkyou Technos Co. Japan) were Merck 60F_{254} (60−230 mesh) and Nacalai Tesque Cosmosil 140C18-OPN, respectively. For HPLC analyses, a Waters Associates 490E chromatograph was used. The column was a 300 × 7.8 mm i.d. μ-Bondapak C18 and silica gel in tandem. Peaks were detected using UV and refractive index (RI) detectors equipped with a model D-2500 chromatointegrator (Hitachi, Tokyo). Optical rotations were recorded on Jasco DIP-370 equipment.

The germination assay was performed in a Sanyo MIR-151 incubator at 15 °C under continuous lighting with a 15 W fluorescent lamp. Water for the assay was prepared by distillation and deionization from an Advantec GS-200 apparatus. The usual workup in the preparation of

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chemicals involved dilution of the reaction mixture with water, extraction with an organic solvent such as ether or CH_2Cl_2, and evaporation after washing the organic layers with water and brine, followed by drying over Na_2SO_4. Lipase PS "Amano", a lipolytic enzyme manufactured from culture of *Pseudomonus* sp., and pig liver esterase (PLE) were supplied by Amano Enzyme Inc. (Nagoya, Japan). All chemicals used for the preparation of active compounds were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan) and purified if necessary by the standard procedure.

Chemicals. 2-Phenylethyl alcohol **1**, D-, L-, and DL-1-phenylethyl alcohol **5**, and ABA are commercially available and were purchased from Tokyo Kasei Organic Chemicals Co., Ltd. (Tokyo, Japan). 4-Vinylphenol **2** and its 2-methoxy derivative **3**, stabilized with 2,6-di-*tert*-butyl-4-methylphenol, are commercially available. In the present study, these compounds were synthesized according to the patented procedures (*12*). 2-Methoxy-4-vinylphenol **3** was synthesized by the following procedure: Vanillin acetate (522 mg, 99%) was obtained by acetylation of vanillin (413 mg, 2.71 mmol) with acetic anhydride (0.51 mL, 543 mmol) in pyridine (5 mL) for 3.5 h at room temperature. The zinc powder was first activated by warming a mixture of Zn powder (703 mg, 11.0 mmol) and acetyl chloride (0.02 mL, 0.27 mmol) in dimethylformamide (DMF) (3 mL) at 50 °C for 15 min under an argon atmosphere. A mixture of 4-acetoxy-3-methoxybenzaldehyde (522 mg, 3 mmol) and dibromomethane (0.28 mL, 4.03 mmol) was gradually dropped into the activated Zn powder in DMF. After being stirred at 50 °C for 30 min, acetic anhydride (0.25 mL, 2.7 mmol) was added to the mixture, and the reaction mixture was further stirred for 2.5 h at 50 °C. After being cooled to 0 °C, aqueous saturated NH_4Cl solution (3 mL) was added to the cooled mixture. The Zn powder was removed by filtration through a glass filter. The Zn powder and aqueous solution were extracted with ether (30 mL × 3). After the usual workup of the ether solution, the crude residue was passed through a silica gel (30 g) column eluted with hexane/EtOAc (20:1) to obtain 4-acetoxy-3-methoxyvinylbenzene (337 mg, 65% from vanillin) as a colorless oil. A mixture of 4-acetoxy-3-methoxy-vinylbenzene (105 mg, 0.55 mmol) and 2 N KOH−MeOH (2 mL) was then stirred at room temperature for 1 h; the mixture was acidified with 2 N aqueous HCl solution under ice cooling and then extracted with CH_2Cl_2 (10 mL × 3). After the usual workup of the CH_2Cl_2 solution, pure 2-methoxy-4-vinylphenol **3** (72 mg, 88%) was obtained by SiO_2 column chromatography eluted with hexane/EtOAc (10:1). 4-Vinylphenol **2** was synthesized by the following procedure: 4-Hydroxybenzaldehyde (5 g, 41 mmol) was converted to 4-acetoxybenzaldehyde (6.4 g, 95%) with acetic anhydride (11.6 mL, 120 mmol) in pyridine (150 mL). 4-Acetoxyvinylbenzene (400 mg, 44%) was provided from 4-acetoxybenzaldehyde (1 g, 6 mmol) by the action of dibromomethane (0.64 mL, 9.2 mmol) and acetic anhydride (0.58 mL, 6.1 mmol) in the presence of Zn powder (1.5 g, 23 mmol), activated with acetyl chloride (0.04 mL, 0.56 mmol) in DMF (5.3 mL). Since the compounds **2** and **3** are fairly unstable and apt to polymerize, the benzene solution of the corresponding acetoxyvinylbenzene derivatives was stored in a freezer at −20 °C. The final step of the alkaline hydrolysis was carried out prior to the assay experiments.

DL-Tetrahydroactinidiolide **6** was prepared by the previously described procedure (*13*). dl-Dihydroactinidiolide **4** was prepared from DL-**6** by Mori's procedure (*14*). Both enantiomers of **6** and **4** were prepared based on the procedure developed by Mori's group (*15*), starting from the dl-form of 2,4,4-trimethyl-2-cyclohexen-1-ol (*16*). Optical resolution of the DL-form was carried out by application of lipase PS "Amano" in isopropyl ether at the first step and then PLE in 0.1 M phosphate buffer (pH 7.0) to the partly resolved acetate. The resolved D- and L-alcohols had $[\alpha]^{22}_D$ +82.5° (c = 1.0, MeOH) and −86.6° (c = 1.0, MeOH), respectively, showing 90 and 94% enantiomeric excess (ee), estimated based on the integral ratios of the methyl signals in the NMR spectra of the corresponding Mosher esters. The sequential reactions from the resolved alcohols to the respective enantiomers followed the published procedure. Optical rotations ($[\alpha]^{23}_D$) of **6** submitted for the present assay experiments were +57.6° (c = 0.93, $CHCl_3$; lit. (*14*) +63.5° (c = 0.95, $CHCl_3$)) and −56.6° (c = 1.05, $CHCl_3$; lit. (*14*) −66.1° (c = 0.97, $CHCl_3$)), respectively, and those for D- and L-**4** ($[\alpha]^{23}_D$) for the assays were +101° (c = 0.50, $CHCl_3$; lit. (*14*) +121° (c = 1.00, $CHCl_3$)) and −98.4° (c = 0.71, $CHCl_3$; lit. (*14*) −121° (c = 1.05, $CHCl_3$)), respectively.

Table 1. Relative Germination Ratios of Intact Seeds on the Basis of Dehusked Seeds

variety (dormancy at harvest)	germination (%)	
	sprouts	roots
Lancer (dormancy)	17	24
RL4137 (dormancy)	64	53
Menyou (nondormancy)	100	89

Plant Materials. All of the field-grown wheat varieties used in the present study were harvested when the moisture content of the examined matured seeds was ca. 16%. For the extraction study, the air-dried wheat spikes of dormancy variety *Kwankei W421*, harvested in June 1998 in the field of the National Ministry of Agriculture and Forestry at Tsukuba (Japan), were mechanically separated into grains and husks. The husks were shredded to small pieces by a commercially available juicer prior to the present extraction experiments. In the exploratory experiment in **Table 1**, concerning the effect of husks toward germination inhibition, three kinds of wheat varieties, *Lancer*, *RL4137*, and *Menyou*, were kept at room temperature for 5 months after harvesting (Tsukuba, Japan) in order to release the dormant seeds from their dormancy. The wheat dormancy varieties, *RL4137* and *Nourin 61*, harvested at 43 days after antheses of August 2001 in the fields of the Tokyo University of Agriculture at Abashiri, Hokkaido (Japan), and the dormant variety *Hokushin*, obtained from the Preservation and Evaluation Section, Hokkaido Prefectural Plant Genetic Resources Center (Japan), were used for the comparisons of germination inhibition activities. All varieties existed in a deep primary dormant state just after the harvesting. After the samples were kept for about 4 months at 20 °C, after-ripening germination took place, and the germination ratios were ca. 60% and 70% on the basis of the examined total grains in the cases of *RL4137* and *Nourin 61*, respectively. The germination ratio of *RL4137* increased to ca. 85% after being kept at room temperature for a further 2 months (total of 6 months after harvesting). *Nourin 61* and *Hokushin* showed more than 95% germinability after 6 months keeping. These intact seeds were kept as a whole spike in a freezer at −30 °C, and hand-threshed grains were provided prior to use for the germination assay.

Extraction and Isolation. Distilled water (1 L) was added to the wheat husks of *Kwankei W421* (200 g), preshredded to small pieces, and the mixture was kept at 60 °C for 48 h under an argon atmosphere. The replacement with argon gas was necessary to prevent formation of mold. The husks were removed by filtration through a four-layered gauze and treated again with water (1 L). The combined aqueous solutions were submitted to steam distillation at 60 °C under reduced pressure using an aspirator to separate the steam distillate and residue. The distillate was extracted with CH_2Cl_2 (500 mL × 3), from which 30 mg of steam distillate residue was obtained after drying with Na_2SO_4 and careful evaporation of the combined CH_2Cl_2 layers. The residue of the steam distillation was extracted with ether (200 mL × 2), providing 900 mg of ether extract residue upon evaporation of the volatile materials after drying with Na_2SO_4. The steam distillate residue (30 mg) was fractionated by Cosmosil gel flash chromatography with 300 mL each of H_2O first and then H_2O/acetone in the ratios of 5:1, 5:2, 5:3, and 1:1, respectively. The acetone from each eluate was evaporated at 75 °C under normal pressure, and the resulting aqueous layer of each fraction was extracted with CH_2Cl_2 (50 mL × 2) to obtain 6 mg (100%), 5 mg (11%), 9 mg (11%), 4 mg (0%), and 4 mg (0%), respectively. The percent shown in parentheses of each yield indicates the germination ratio of sprout at 10^3 ppm in aqueous solution.

For the isolation study, the active fractions were collected by repeated water extraction of the husks followed by steam distillation. The fraction of 5:2 eluate (8 mg) was separated by HPLC with a μ-Bondapak C-18 column (300 × 7.8 mm i.d.; flow rate of 1.0 mL/min; RI detector) using H_2O/MeOH (10:3) as the mobile phase to isolate 2-phenylethyl alcohol **1** (2 mg). The 5:3 fraction (9 mg) was similarly separated by HPLC with the same conditions using H_2O/MeOH (3:2) to isolate 4-vinylphenol **2** (2 mg) and 2-methoxy-4-vinylphenol **3** (2 mg),

respectively. The GC−MS and ^{1}H NMR of the remainder (3 mg) obtained by the HPLC separation suggested the existence of dihydroactinidiolide **4**. The ether extracts (900 mg) of the residue of steam distillation were a complex mixture, which was submitted to repeated flash chromatography with a Cosmosil gel column eluted first with H_2O/MeOH (5:2) to give an active fraction (50 mg) from 200 g of husks. The germination ratio (%) of root and sprout at 10^3 ppm was 20 and 11%, respectively. HPLC analysis of the active fraction showed no clear peak corresponding to ABA. Separation of the active fraction resulted in the isolation of only coniferyl aldehyde (4-hydroxy-3-methoxy-cinnamyl aldehyde, 1.8 mg) and its methoxy derivative (4-hydroxy-3,5-dimethoxy-cinnamyl aldehyde, 0.8 mg), the germination ratios (%) of sprout being more than 80% at 10^3 ppm.

Steam Distillation and HPLC Analysis of ABA. A solution of ABA (20 mg) in H_2O (300 mL) was submitted to steam distillation at 60 °C under reduced pressure, and the residue was extracted with CH_2Cl_2 to obtain ABA (18 mg, 90%) after the usual workup. HPLC analysis of ABA was carried out with a μ-Bondapak C18 column, the same column used for the separation of active compounds, eluted with H_2O/MeOH (2:1) at the flow rate of 1.0 mL/min. The peak corresponding to ABA was only observed with a retention time of 37 min on UV detection at 250 nm.

Germination and Growth Assay. In germination tests, 20 hand-threshed seeds (grains), collected by random sampling, 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 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. The test solution contained the sample in distilled and deionized water with the concentration in ppm (parts per million). 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. In the solution without any sample, 85% of the total of 20 grains of *RL4137* germinated after 4 days on the average, and these seeds were employed for the experiments in **Figures 2** and **3**. 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. In the exploratory experiment shown in Table 1, the determination of the number of the germinated sprouts and roots of the hand-threshed seeds was referred to as the controlled experiment to calculate the germination ratios of the intact seeds. All the assay experiments were carried out two or three times, the average deviations being less than ±10% in each experiment.

RESULTS AND DISCUSSION

At the outset of the present investigation, the effect of wheat husk on the germination and growth of the seeds was examined to obtain the result shown in **Table 1**. The germination of the intact seeds (with husks) was restrained by the existence of the husks in the cases of *Lancer* and *RL4137*, both of which are dormancy varieties. The germination restraint due to the existence of husks was almost negligible in the case of the nondormancy variety *Menyou*. The inhibition of the former two varieties may be attributable to the existence of water-soluble inhibitors in the husks, which percolated into the water during the germination experiment. The reason for negligible restraint of germination of the variety *Menyou* is obscure at present. After certification of the existence of germination inhibitors in the husks of both varieties of *Lancer* and *RL4137* in preliminary experiments, husks of *Kwankei W421*, a type of dormancy variety easily available in Japan, were extracted with warm water for the large-scale isolation of the inhibitory substances. The combined aqueous solutions were submitted to steam distillation under reduced pressure, and the distillate was extracted with CH_2Cl_2 to provide 30 mg of extracts from 200 g of the husk.

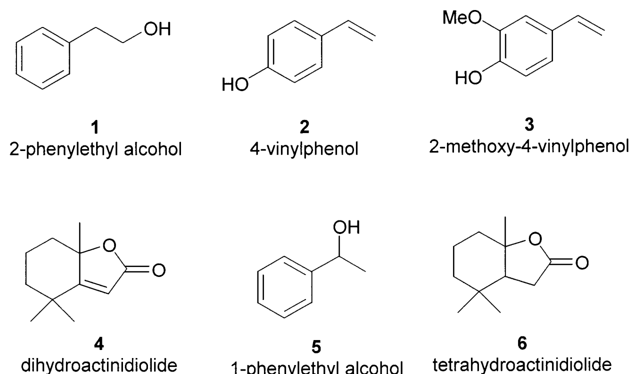

Figure 1. Natural and related germination inhibitors in wheat husks.

Table 2. Inhibition Activities of Natural and Related Compounds at 500 ppm[a]

compounds	germination (%)		growth (%)	
	sprouts	roots	sprouts	roots
1	53	53	16	9
2	83	94	49	31
3	12	12	5	3
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0

[a] The wheat grains *RL4137* with an 85% germination ratio were employed.

In pursuit of the active inhibitors from the aqueous layer, the steam distillation procedure was quite effective for the separation of minute amounts of active compounds from many kinds of water-soluble inactive components.

The germination of both roots and sprouts of the tested grains was completely inhibited by the extracts at 10^3 ppm in aqueous solution. The extracts were fractionated by MPLC on Cosmosil gel using water with increasing amounts of acetone as the mobile phases. The fraction of H_2O-only eluate showed no activity, while the other fractions had strong inhibition activities. The active fractions were further purified with HPLC to isolate compounds **1**, **2**, and **3**. The GC−MS of an active part of the residues obtained during HPLC separation suggested the existence of compound **4**, which was supported by the ^{1}H NMR spectrum of the mixture. The structures of compounds **1**−**4** were confirmed by comparisons of retention time on HPLC under several conditions, ^{1}H NMR, and GC−MS with respective authentic samples. To find the structural requirement for the activity, the activities of related compounds **5** and **6** were also examined. The inhibition activities of compounds **1**−**6** in **Figure 1** at 500 ppm are summarized in **Table 2**, demonstrating that all the natural products, particularly dihydroactinidiolide **4**, possessed a high potency of inhibition activity. It was revealed that the related tetrahydroactinidiolide **6** was as active as the original natural product **4** whereas the activity of 1-phenylethyl alcohol **5** was much higher than that of natural 2-phenylethyl alcohol **1**.

Since the absolute configuration of **4**, which has molecular asymmetry, could not be determined due to the minute amounts of available materials in the present study, both enantiomers were produced by synthesis and submitted to the germination assay for evaluation of each enantiomer. We expected that only one enantiomer would be active toward germination inhibition. For additional evaluation of the effect of asymmetry, both enantiomers of the relevant compounds **5** and **6** were also examined. The D-, L-, and DL-forms of 1-phenylethyl alcohol **5** were commercially available. Both enantiomers of **4** and **6** were

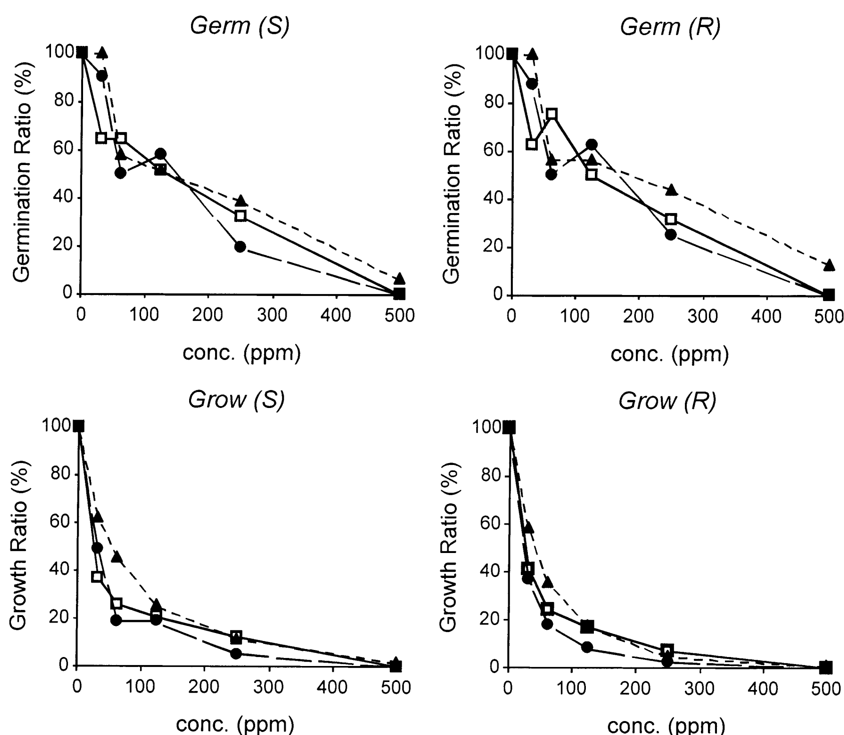

Figure 2. Germination ratios of sprouts [Germ (S)] and roots [Germ (R)] and growth ratios of sprouts [Grow (S)] and roots [Grow (R)] by D-**4** (●), L-**4** (▲), and DL-**4** (□) under different concentrations in ppm. The seeds of *RL4137* possessing an 85% germination ratio were employed.

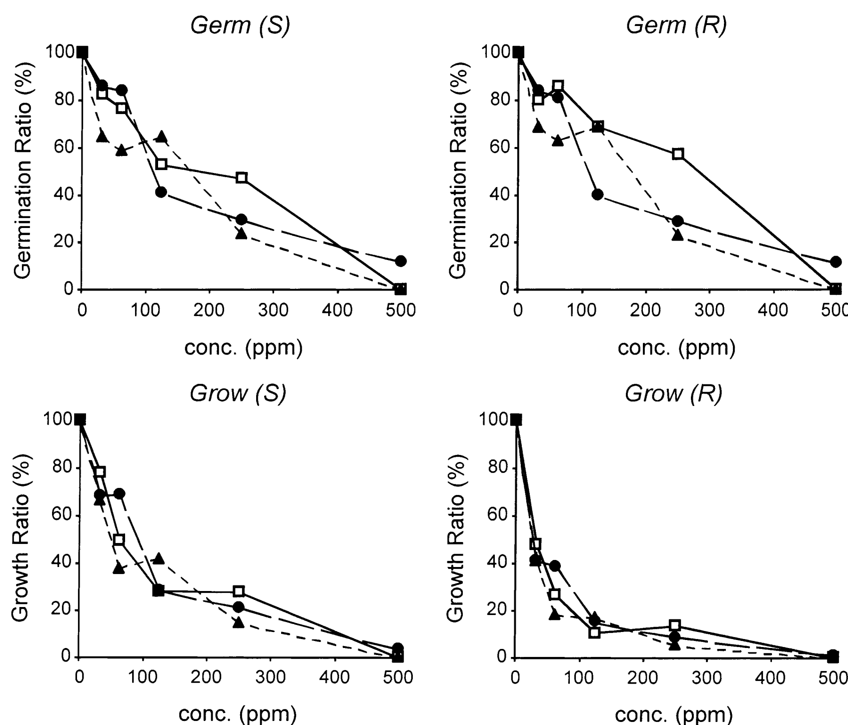

Figure 3. Germination ratios of sprouts [Germ (S)] and roots [Germ (R)] and growth ratios of sprouts [Grow (S)] and roots [Grow (R)] by D-**5** (●), L-**5** (▲), and DL-**5** (□) under different concentrations in ppm. The seeds of *RL4137* possessing an 85% germination ratio were employed.

prepared according to Mori's procedure starting from 2,4,4-trimethyl-2-cyclohexen-1-ol.

The germination and growth responses of sprout and root were examined using the D-, L-, and DL-forms of each compound **4**, **5**, and **6**, revealing no noticeable difference in inhibition activities among these three forms. The germination modes under variable concentrations of **4** and **5** are shown in **Figures 2** and **3** as representative examples. Thus, the absolute configuration of natural **4** could not be evaluated by the degree of response to the inhibition activities. Both enantiomers of the plant hormone ABA, structurally related to the present compounds **4** and **6**, possess almost the same activities toward biological assays of several kinds of plants (*17*).

During the separation procedure of crude steam distillate extracts, which were separated into five fractions by flash chromatography, the crude steam distillate showed a stronger activity than that of each fraction obtained by flash chromatography. This evidence suggested the possibility of the exist-

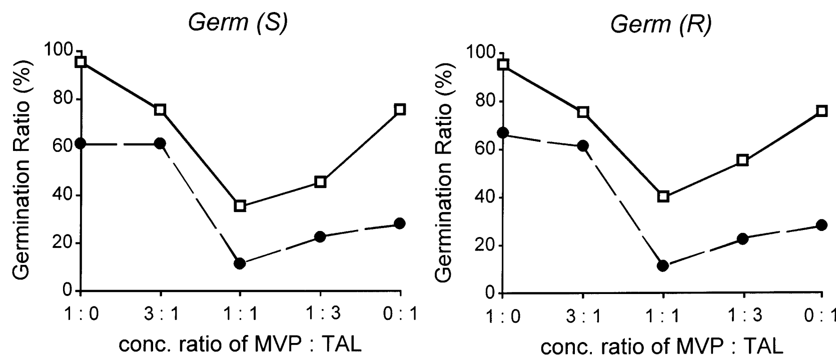

Figure 4. Germination ratios of sprouts [Germ (S)] and roots [Germ (R)] of *Hokushin* (□) and *Nourin 61* (●) in aqueous solutions of a mixture of **3** and **6** under different concentration ratios. The total concentration in each solution was set to 300 ppm by changing the amounts of **3** and **6**.

ence of synergistic relations between the active compounds in **Figure 1**. In fact, clear synergistic relations were observed between **3** and **6** as indicated in **Figure 4**. In the experiments, the total concentration in each solution was set to 300 ppm by changing the ratios of **3** and **6**, using 1:0, 3:1, 1:1, 1:3, and 0:1, respectively. The strongest activity was observed at a 1:1 ratio of **3** and **6** in dormancy varieties *Hokushin* and *Nourin 61*. Similar relations were seen between **4** and aromatic compounds **1**, **2**, **3**, and **5**. It cannot be ruled out, therefore, that the active compounds from the husks become synergistically operative as germination inhibitors in actual seeds in the field.

Thus, the present research has identified the germination inhibitors in husks of wheat seeds. A representative germination inhibitor, ABA, could not be detected by HPLC analysis from extracts of the residue obtained by steam distillation. All of the active compounds in **Figure 1** are quite common natural products, and co-occurrence of these compounds has also been reported in several plants (*18*). The phytoregulatory activities of individual compounds have been published, although no description of wheat seed germination inhibition has been found in the literature. The characterization of 2-phenylethyl alcohol **1**, a germination inhibitor of cress and lettuce seeds (*19*), was reported from *Aspergillus niger*. Compound **1** was shown to stimulate seedling growth at low concentrations, whereas at higher concentrations growth was inhibited (*20*). An aqueous extract of field-grown wheat (*Triticum aestivum* L.) reduced the germination and root length of pitted morning glory (*Ipomoea lacunose* L.) and common ragweed (*Ambrosia artemisiifolia* L.), the reduction being attributed to **3** (*21*). Compound **4** was first isolated from leaves of *Actinidia polygama* (*22*); other leaves such as tobacco (*23*) and teas (*24*, *25*) are rich sources of **4**. It is generally accepted that carotenoids and β-ionone are the biological precursor of **4** (*26*, *27*). Two reports describe the germination inhibition activity of **4** (*28*, *29*). Compound **4** shows stronger activity toward barley, lettuce, and rice as compared with abscisic acid (*30*). The related compound **6** was found in decomposing barley straw during reservoir algal control, and it is active for the reduction of general phytoplankton activity (*31*). Compound **6** was discovered in essential oils of *Ammomum* and *Alpinia* plants (*32*) and tobacco (*33*, *34*), although no report concerning its germination inhibition activity was published.

Since the present inhibitors have been isolated from various kinds of seed plants, they may be responsible for the general germination inhibition in these plants. This deduction is partly supported by our recent findings that **4** and **6** affect profoundly the germination and growth inhibition of rice seeds (unpublished results).

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