Mycoscience 42: 241–245, 2001

A substance inducing teliospore production in wheat leaf rust, *Puccinia recondita* f. sp. *tritici*

Tomoo Hosoe[1], Tatsuya Sano[1], Hideo Nishikawa[1], Koohei Nozawa[1]*, Ken-ichi Kawai[1], Yuichi Yamaoka[2], Keizo Katsuya[2], Tsuyoshi Sato[3], Hiroshi Hagiwara[3], and Hatsuo Saito[3]

[1] Faculty of Pharmaceutical Sciences, Hoshi University, Ebara 2–4–41, Shinagawa-ku, Tokyo 142–8501, Japan;
[2] Institute of Agriculture and Forestry, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan;
[3] National Agricultural Research Center, Tsukuba, Ibaraki 305-8666, Japan.

Received 27 November 2000
Accepted for publication 12 March 2001

A substance inducing teliospore production in *Puccinia recondita* f. sp. *tritici* was found in water and methanol extracts of wheat leaves with telia of the wheat leaf rust just before harvest time. Methanol (MeOH) and water extracts from uninfected wheat leaves also showed telia-inducing activity. However, the MeOH and water extracts from wheat leaves covered with telia showed much stronger activity than those from uninfected wheat leaves. We obtained a fraction (0.2 mg) showing activity at 2 ng/ml by purification of the water extract.

Key Words——*Puccinia recondita* f. sp. *tritici*; teliospore; teliospore production; wheat leaf rust.

Puccinia recondita Roberge ex Desm. f. sp. *tritici* Eriksson causing wheat leaf rust is a heteroecious macrocyclic species, living on wheat (*Triticum aestivum* L.) in a uredinial-terial state and on *Thalictrum* spp. in a spermagonial-aecial state. Urediniospores produced on wheat leaves infect leaves several times during the growing season and contribute to damage to the crop. At the end of the growing season, which is early June in the Kanto area (central Honshu island) of Japan, telia are produced on leaves. Telia production prepares the fungus for the season when wheat plants are not available. Teliospores lack the ability to infect wheat, so development of the disease ceases.

Teliospores are commonly resting or winter spores and are capable of surviving unfavorable periods (Cummins and Hiratsuka, 1983). They are generally produced on host plants when the plants approach maturity (Takahashi et al., 1965; Agrios, 1997). Teliospore production is induced by unfavorable conditions for rust growth, such as incubation of urediniospore-infected host plants at lower temperature (Waters, 1928; Yeh et al., 1981), insertion of a dark period during incubation (Waters, 1928; Völker and Boyle, 1994), infection with mycoparasites like *Aphanocladium album* (Biali et al., 1972), and application of culture filtrate extracts of *A. album* (Forrer, 1977) and Ajoene (Völker and Boyle, 1994). However, there is no report on substances inducing teliospore production in rust-infected leaves. We assumed that a chemical substance inducing teliospores was present in the infected wheat leaves at harvest. We report here a substance inducing teliospore production of *P. recondita* that is present in infected wheat leaves with many telia.

* Author to whom correspondence may be addressed.
Telephone: +81–3–5498–5789. Fax: +81–3–3787–0036. E-mail: nozawa@hoshi.ac.jp

Materials and Methods

Collection of wheat leaves and stems infected with rust Leaves and stems of wheat (*Triticum aestivum* L. cultivar Norin No. 61) infected with wheat leaf rust were collected from the experimental field of the National Agricultural Research Center, Ministry of Agriculture, Forestry and Fishery, Tsukuba, Ibaraki. To survey the period of production of the substance inducing teliospore production, samples were collected at three different growth stages of wheat in 1995. Wheat leaves and stems at the flowering stage with numerous uredinia were collected on May 16 (Sample A), leaves and stems at ripening stage (just before harvest) with abundant telia were collected on May 30 (Sample B), and leaves and stems at dead-ripe stage were collected on June 10 (Sample C). In 1996, wheat leaves with numerous telia of *P. recondita* just before harvest were collected on June 14 through 18 (Sample E) in the same field. Leaves of uninfected wheat of the same cultivar just before harvest (Sample D) were also collected from a wheat field at the University of Tsukuba, Tsukuba, Ibaraki on June 14 for comparison.

Extraction of a substance inducing teliospore production and sample preparation Wheat leaves or stems of Samples A, B, and C were air-dried and crushed into small pieces. Two grams each of leaves and stems were extracted stepwise with dichloromethane (CH_2Cl_2), acetone, and methanol (MeOH), each for 24 h. After

evaporation at reduced pressure, each extract was dissolved in 0.5 ml of MeOH and added to a water blank to give three dilutions of 1: 10, 1: 20, and 1: 40 (Table 1). Leaves (4 g) of infected (Sample E) and uninfected (Sample D) wheat were extracted twice with MeOH and water (each 200 ml) for 24 h, after removal of fat with CH_2Cl_2 and acetone in turn. Each extract was filtered and evaporated at reduced pressure, and the residue was dissolved in distilled water at concentrations of 0.3, 3.0, and 15 mg/ml.

Separation of the MeOH and water extracts The MeOH and water extracts from Sample E were each diluted with water (10 ml) and filtered through filter paper to remove insoluble substances. The filtrate was acidified to pH 2 with 4 M HCl and extracted twice with ethyl acetate (EtOAc) (50 ml). The organic layer was dried with sodium sulfate (Na_2SO_4) and evaporated until dry. Each residue was separated by chromatography on a column of HP21 resin (DIAION) (resin volume, 100 ml in water; inside diameter of column, 22 mm) by elution with water [50 ml / fraction (fr)] at a flow rate of 0.4 ml/min (Fig. 3). Active frs 6 to 8 were combined, concentrated and chromatographed on an ion exclusion column (Shodex Rspak C-2011, 20 × 500 mm) by elution with 1 mM $HClO_4$ at a flow rate of 3.2 ml/min.

Isolation of a chemical substance inducing teliospore production The dried wheat leaves (3.6 Kg) with numerous telia of sample E were crushed, extracted with CH_2Cl_2 and acetone to remove fat, and then extracted in turn with MeOH (20 L, twice) and water (45 L, twice). Half of the dried water extract (240 g) was dissolved in water (700 ml), then filtered through a filter paper to remove insoluble substances. The filtrate was acidified to pH 2 with 4 M HCl and extracted twice with EtOAc (2.5 L). The organic extract was dried with Na_2SO_4 and evaporated to dryness. The residue was suspended in water (150 ml), then centrifuged at 3850 g for 20 min. The supernatant was separated by chromatography on a column of HP21 (resin volume, 2 L in water; inside diameter of column, 75 mm) by elution with water (10 L, 1 L/fr) at a flow rate of 4.5 ml/min, and then each fr was evaporated under reduced pressure to dryness (Fig. 4). Active frs 7 and 8 (330 mg) were subjected to centrifugal liquid-liquid partition chromatography (CPC) at 160 g, using a Sensyu SSC-3160 pump and SANKI CPC LLB-M, with a solvent system of water-butanol (1:1) (mobile phase: butanol) at a flow rate of 5 ml/min. This resulted in an active fr of 19.5 mg, which was further separated by HPLC on a Senshu Pak AQUASIL SS-5251 (20 × 250 mm) into four frs at a flow rate of 8 ml/min: MeOH for 13 min (15.9 mg), 50% MeOH for 10 min (0.2 mg) and 20 min (1.1 mg), then 30% MeOH for 40 min (3.2 mg) (Fig. 4).

Bioassay Seeds of wheat (Cultivar Norin No. 16) were sown in soil in plastic pots (150 × 50 × 100 mm) and placed in a controlled growth cabinet at 22°C under 12 h light (15,000 lux): 12 h dark conditions for 6 d. Seedlings were inoculated with urediniospores of wheat leaf rust race 1A by the brushing method and kept in a moist chamber at 22°C in the dark for 1 d, then returned to the growth cabinet and incubated under the above conditions. Eight d after inoculation, cotyledons with uredinia were cut into segments (4 cm long), and each segment was put into a glass vial (5 ml) with a 2-ml sample solution in distilled water and incubated in the growth chamber under the above conditions for 10 d. The

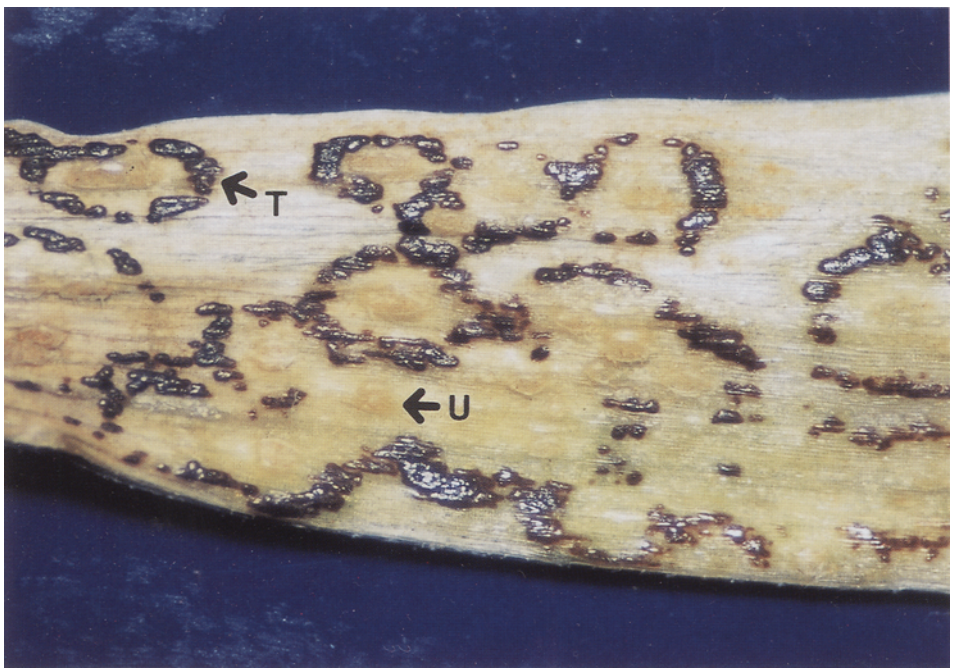


Fig. 1. Telia induced on a leaf segment. Note that telia completely encircle a green island (assessed as +++; strong). T, telia; U, uredinia.

Fig. 2. Teliospores produced in the induced telia.

cotyledon segments were observed under a dissecting microscope to compare production of telia. The activity of sample solution was assessed as strong (+++: telia completely encircling a green island (Fig. 1)), medium (++: telia partially encircling a green island), weak (+: a few telia present in or around a green island) or negative (−: no telia formation). Teliospores produced in telia on cotyledon segments were confirmed with a light microscope (Fig. 2).

Results

Survey of a substance inducing teliospore production The MeOH extracts of infected wheat leaves and stems from three samples collected at different growth stages of wheat were used for bioassay. Among them, the MeOH extract of infected wheat leaves with abundant telia collected just before harvest (Sample B) showed the strongest activity for inducing teliospore formation (Table 1). On the other hand, the extracts of wheat leaves without telia at the flowering stage (Sample A) and of dead wheat leaves with telia at dead-ripe stage (Sample C) had only weak activity. The extract of stems from the samples also showed weak activity.

Both the MeOH extract and water extract from wheat leaves with abundant telia of *P. recondita* collected in 1996 (Sample E) also showed very strong activity. Extracts from uninfected wheat leaves of the same cultivar just before harvest (Sample D) showed weak activity (Table 2). The water extract from Sample E showed the strongest activity of all the extracts used in the present study.

Separation of the MeOH and water extracts Both MeOH and water extracts from Sample E showed the following behavior. When the extract was dissolved in water and extracted with EtOAc at pH 2, the activity was found in the organic phase. When the EtOAc extract was suspended in water again, the active substance moved into the aqueous phase. The EtOAc extract was separated

Table 1. Teliospore-inducing activity of MeOH extracts from dried leaves (2 g) and stems (2 g) of infected wheat collected in the flowering stage (Sample A), just before harvest (Sample B), and dead wheat past harvest (Sample C).

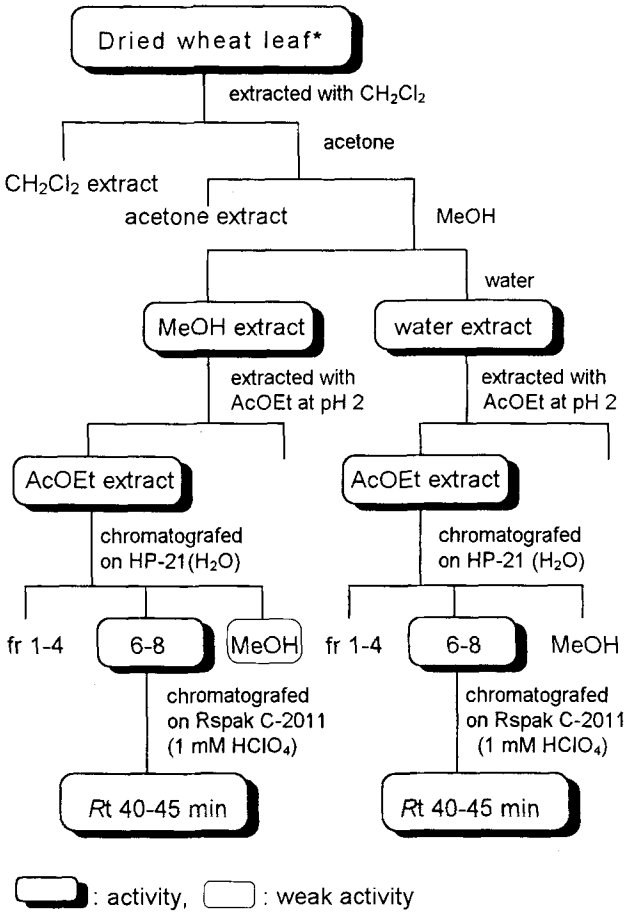
Sample	Organ	Dilution ratio of extract		
		X 10	X 20	X 40
A	Leaf	+, −	−, −	−, −
	Stem	−, −	−, −	−, −
B	Leaf	+++, +	+++, +++	+, −
	Stem	+, −	+, −	+, −
C	Leaf	+, +	+, −	−, −
	Stem	+, −	−, −	−, −

Note: All CH_2Cl_2 and acetone extracts showed no activity. Two samples were tested from each extract.

Table 2. Activity of extracts from uninfected(Sample D) and infected wheat leaves (Sample E).

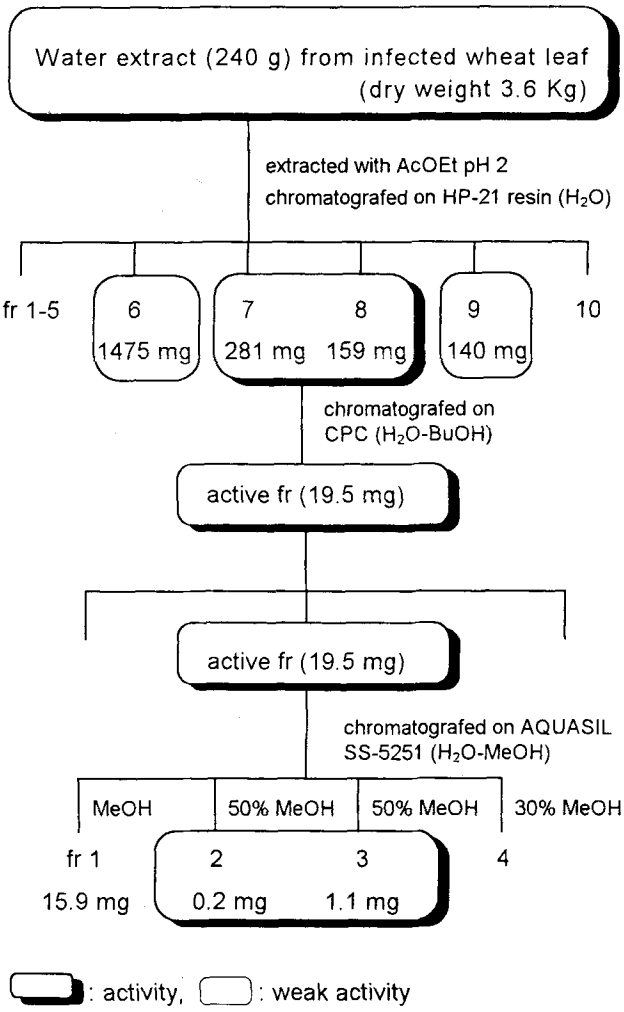
Sample	Solvent	Extract weight		
		0.3 mg	3 mg	15 mg
D	MeOH	−, −	+, −	ND
D	water	−, −	+, −	++, −
E	MeOH	+, −	+, +	ND
E	water	++, +	+++, +++	ND

ND: Null decision for the growth of the mold.



* 4g in Sample E

Fig. 3. Separation of active fractions in MeOH and water extracts of wheat leaves.



The fractionation without frame showed no actiivity.

Fig. 4. Isolation of chemical substance inducing teliospore production.

by column chromatography on HP21 resin and eluted with water to give an active fr in the eluent at 2.5 to 4 times the column volume. The activity appeared in frs at 40–45 min with separation by HPLC using an ion exclusion column (Fig. 3).

Isolation of a chemical substance inducing teliospore production The extraction of dried leaves (3.6 kg) from infected wheat just before harvest (Sample E) gave a water extract (240 g) showing strong telia-formation activity (+++) (Fig. 1) at 0.2 mg/ml. The water extract was then extracted with EtOAc at pH 2 and chromatographed on an HP21 resin column. Elution with water gave two frs, 281 mg and 159 mg, showing strong activity at 1 μg/ml and 25 μg/ml, respectively. The combined active frs were further subjected to CPC using a solvent system of H_2O-butanol (mobile phase: butanol) and gave a fr (19.5 mg) showing strong activity at 0.3 μg/ml in t_R between 70 and 120 min. This fr was purified by HPLC (AQUASIL SS-5251) and eluted with a solvent system of H_2O-MeOH. Fr 2 (0.2 mg) and 3 (1.1 mg) eluted with 50% MeOH showed strong activity at 2 and 10 ng/ml, respectively (Fig. 4).

Discussion

We have found that a substance inducing teliospore production is present in wheat leaves infected with wheat leaf rust, *P. recondita* f. sp. *tritici*. Extraction and bioassay of infected wheat at different stages showed that the active substance was accumulated in wheat leaves and stems with abundant teliospores just before harvest (Table 1). MeOH and water extracts from wheat leaves with abundant telia showed significantly stronger activity than those from the uninfected wheat leaves (Table 2). Thus, the production of the substance may be stimulated by rust infection.

In the present study, wheat cotyledons were used in the bioassay tests. Telial formation on young seedlings is generally rare, although some strains of wheat leaf rust might produce telia on the first leaves of seedlings 20 to 25 d after inoculation (Takahashi et al., 1965). In fact, telia were not produced on cotyledon segments with uredinia immersed in distilled water as a control.

Producton of telia on cotyledons within 10 d after applying sample solution was considered to be caused by active substances in the sample solution.

We consider that the active substances in the MeOH and water extracts were the same compound because they showed the same behavior in the process of separation (Fig. 3). Since the active fr moved into the organic phase under acidic conditions in the process of purification, it is clear that the active substance is an acidic compound.

Forrer (1977) reported that organic extracts of fungus-free culture filtrate of *A. album* induced precocious teliospore formation of *P. graminis* f. sp. *tritici* on wheat. The substance inducing precocious teliospore formation seemed to be metabolites from *A. album*, but the active compound was not isolated.

Biali et al. (1972) cited the following four potential advantages of induction of telia by using *A. album*. (i) The induction of telia in rust isolates which rarely produce this stage permits a more dependable identification of species than by urediniospore characters. (ii) The obtaining of teliospores in such isolates might permit studies on the genetics of virulence in these rusts, utilizing the sexual stage, if basidiospores proved infective. (iii) Earlier induction of telia in rusts which normally do produce them would save time of research workers. (iv) As urediniospores are 'repeating spores' in the macrocyclic rusts causing epiphytotics, early induction of teliospores in the field might herald the end of an outbreak for that season, unless new urediniospore showers from other areas complicate matters. The substance obtained in the present study has the same potential. It will further provide important information on the mechanisms of switching urediniospore production in teliospores of rust fungi on host plants.

It has recently become apparent that the use of synthetic agricultural chemical is potentially harmful to nature. Our active substance present in infected wheat has the potential to replace the agricultural chemicals required at present. We believe that the complete characterization of the teliospore-inducing substance could lead to the control of infection by the wheat leaf rust.

Acknowledgements—We thank Miss S. Kondo, Institute of Agriculture and Forestry, University of Tsukuba, for help in collection of infected wheat leaves. We also thank Mr. S. Hanamura and Mr. T. Hamaguchi, Tsumura & Co., for help in extracting infected wheat leaves with water.

Literature cited

Agrios, G. N. 1997. Plant pathology. 4th ed., Academic Press, San Diego, California, U.S.A.

Biali, M., Dinoor, A., Eshed, N. and Kenneth, R. 1972. *Aphanocladium aibum*, a fungus inducing teliospore production in rusts. Ann. Appl. Biol. **72**: 37–42.

Cummins, G. B. and Hiratsuka, Y. 1983. Illustrated genera of rust fungi. Revised ed. APS Press, St. Paul, Minnesota, U.S.A.

Forrer, H. R. 1977. Der Einfluss von Stoffwechselprodukten des Mycoparasiten *Aphanocladium album* auf die Teleutosporenbildung von Rostpilzen. Phytopath. Z. **88**: 306–311.

Takahashi, K., Yamada, M. and Takahashi, H. 1965. Teleutospore formation of leaf rust, *Puccinia recondita* f. sp. *tritici* on young seedling of wheat. 1. Isolate-variety relation and some characters of teleutospores. Ann. Phytopath. Soc. Japan **30**: 54–61.

Völker, K. and Boyle, C. 1994. Bean rust as a model system to evaluate efficiency of teliospore induction, especially in the potential mycoherbicide *Puccinia punctiformis*. Weed Res. **34**: 275–281.

Warter, C. W. 1928. The control of teliospore and urediniospore formation by experimental methods. Phytopathology **18**: 157–213.

Yeh, C. C., Tschanz, A. T., and Sinclair, J. B. 1981. Induced teliospore formation by *Phakopsora pachyrhizi* on soybeans and other hosts. Phytopathology **71**: 1111–1112.