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Inouye, Jun
Laboratory of Crop Science, Department of Agriculture, Kyushu University

Mukai, Jun-Ichiro
Laboratory of Sericulture Chemistry, Faculty Of Agriculture, Kyushu University

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Effect of 2-thiouracil on flower initiation in rice and wheat plants grown under aseptic conditions

Jun INOUYE* and Jun-Ichiro MUKAI†

In order to investigate biochemical changes involved in flowering, number of attempts on modifying flower initiation by application of nucleic acid base-analogues, i.e., 8-azaguanine and thiouracil, have been conducted.[1,2,3,7,8,9,10,11] In the case of rice plants, one of the most thermo-sensitive varieties, 2-thiouracil inhibited flower initiation under aseptic conditions.[3]

By using the aseptic culture methods, the present experiment was conducted to examine the effect of 2-thiouracil and its detecting in ribonucleic acid from treated rice plants. In a few experiments, the reversal of the effect of 2-thiouracil by uracil or its metabolic precursor orotic acid was also examined.

Materials and methods

The materials used in the experiments were two cultural varieties of *Oryza sativa* L., "Nōrin No 15" and "Nōrin No. 18", and a cultural variety of *Triticum aestivum* L., "Kōnosu No. 25." In rice plants, the former is one of the most thermo-sensitive varieties (insensitive to photoperiod) and the latter is one of the most photo-sensitive varieties (qualitative short day plant). On the other hand, "Kōnosu No. 25" is a quantitative long day variety in wheat plants.

Unless otherwise mentioned, the basic culture medium was a modified White's medium containing $MgSO_4$ 360 mg, $Ca(NO_3)_2$ 200 mg, Na_2SO_4 200 mg, KNO_3 80 mg, KCl 65 mg, NaH_2PO_4 16.5 mg, $MnSO_4$ 4.5 mg, $ZnSO_4$ 1.5 mg, H_3BO_3 1.5 mg, KI 0.75 mg, Fe-citrate 30 mg, sucrose 10 g, agar 6 g, and distilled water 1,000 ml.

The test tubes, 25 by 350 mm, each containing about 50 ml of the

* Laboratory of Crop Science, Faculty of Agriculture, Kyushu University.
† Laboratory of Sericultural Chemistry, Agricultural Chemistry Institute, Kyushu University.

culture medium were autoclaved at 1.0 kg/cm^2 overpressure for 20 min. To obtain uniform growth, well matured seeds of medium size were selected. Rice seeds were husked. Seeds were sterilized by immersing them in 75% alcohol for 30 sec (rice seeds) or 3 min (wheat seeds), then in 10% calcium hypochlorite for 20 min and finally in 3% hydrogen peroxide for 20 min. One seed was sown in each tube.

Then, 2-thiouracil was dissolved in a 1/15 M phosphate buffer and the solution was filtered through the Toyo asbestos sterilizing film (No. 85). Reversal reagents, uracil and orotic acid, were added to the culture medium before autoclaving. The effect of 2-thiouracil was tested in the following two ways. (i) The sterilized seeds were dried in a evacuated desiccator under reduced pressure for about 2 hours. The desiccated seeds were immersed in the 10^{-2} M 2-thiouracil solution for about 24 hours at 30°C. After being washed with 3% hydrogen peroxide for 30 sec, the treated seeds were sown on the basic medium with or without reversal reagents. (ii) The sterilized seeds were sown on the autoclaved culture medium (with or without reversal reagents) to which 2-thiouracil solution was added.

After sowing, the seeds were kept at 30°C for 24 hours in the dark, and thereafter at 25-20°C under natural light in a phytotron. In some experiments, the plants were cultured under the respective conditions of long day (continuous light) and short day (8 hours light). Under the day-time condition, sunlight was the only source of light. During night-time, a 20 watt day-light fluorescent tube was employed to hang 0.3 meter above the plants.

The plants were examined for flower initiation 50-120 days after the start of the experiments. The effect of the treatments was evaluated by the number of leaves formed before the initiation of flower primordia.

Experimental Results

1. Effect of 2-thiouracil on flower initiation in thermosensitive rice plants "Nōrin No. 15"

After 24 hours, incubation at 30°C in the dark, the plants were cultured under sunlight. The observation of flower primordia was conducted 50 days after sowing. The results are presented in Table 1.

As shown in Table 1, 2-thiouracil inhibited the flower initiation. When 2-thiouracil was added to the medium, the floral inhibition by this reagent was more significant than in the case of seed-treatment. The floral inhibition was accompanied by both acceleration of the plastochrone up to 6 leaf stage and malformation of the leaves. Simul-

taneous application of uracil or orotic acid in medium reversed the floral inhibition to some extent, but hardly alleviated the anomalies in vegetative growth (Fig. 1). No promotive effect on flowering of either uracil or orotic acid alone was observed.

Table 1. Effect of 2-thiouracil on flower initiation in thermo-sensitive rice plants "Nōrin No. 15."

Concentration of 2-thiouracil in medium (M)	Concentration of uracil in medium (M)	Concentration of orotic acid in medium (M)	Number of plants	Number of leaves formed before flower initiation
	0	0	10	6.0±0.00
	5×10^{-5}	0	9	5.9±0.30
0	10^{-4}	0	10	5.9±0.30
	0	5×10^{-5}	13	6.5±0.45
	0	10^{-4}	8	6.2±0.53
3×10^{-5}	0	0	9	5.9±0.30
	0	0	17	8.3±1.50
	5×10^{-5}	0	12	7.0±0.71
5×10^{-5}	10^{-4}	0	12	6.4±0.86
	0	5×10^{-5}	14	7.8±1.05
	0	10^{-4}	9	7.1±0.83
	0	0	11	11.8±1.28
	5×10^{-5}	0	12	10.5±1.79
10^{-4}	10^{-4}	0	16	8.7±1.45
	0	5×10^{-5}	12	10.7±2.25
	0	10^{-4}	11	8.9±1.44
2×10^{-4}*				
10^{-4}†	0	0	13	8.1±0.88
	10^{-4}	0	15	7.5±0.76

* No seed germinated in this lot.
† 2-thiouracil was given to the desiccated seeds.

2. Effect of 2-thiouracil on flower initiation in photo-sensitive rice plants "Nōrin No. 18"

After incubation for 24 hours at 30°C in the dark, the plants were cultured under short day (8 hours light) condition. The plants were examined for flower initiation 120 days after the start of the experiment. Table 2 shows the results.

The results presented in Table 2 are similar to those obtained in

the preceding experiment (Table 1) in which the thermo-sensitive rice plants were treated. In this photo-sensitive rice plants, however, some of plants which were cultured on 5×10^{-5}M 2-thiouracil medium

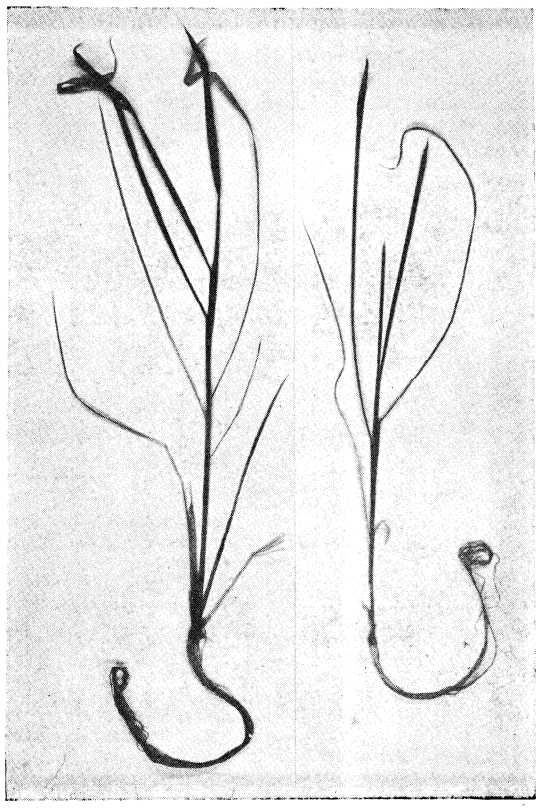

Fig 1. Growth of the thermo-sensitive rice plant as affected by 2-thiouracial added to the medium (50 days after sowing at 25 20°C). Left, 5×10^{-5}M 2-thiouracil and 10^{-4}M uracil; right, 10^{-4}M uracil.

and the plants cultured on 10^{-4}M 2-thiouracil medium were vegetative at dissected. As in the experiment of the thermo-sensitive rice plants, the inhibition of flower initiation by 2-thiouracil was accompanied by malformation of the leaves. Application of uracil reversed the flower inhibition to some extent, but hardly alleviated the anomalies in vegetative growth. Uracil alone had a slight accelerating effect on flowering.

Table 2. Effect of 2-thiouracil on flower initiation in photo-sensitive rice plants "Nōrin No. 18."

Concentration of 2-thiouracil in medium (M)	Concentration of uracil in medium (M)	Number ot plants	Number of leaves formed before flower initiation*
0	0	19	11.3±1.29
	10^{-4}	20	10.6±0.75
10^{-5}	0	10	13.1±0.94
5×10^{-5}	0	6	14.0±0.81
		11	(13.2±0.83)
10^{-4}†	0	8	(14.1±0.78)
10^{-2}‡	0	12	12.9±1.18
	10^{-4}	14	12.1±0.91

* The number of leaves of plants without flower primordia is enclosed in parentheses.

† No seed germinated in this lot.

‡ 2-thiouracil was given to the desiccated seeds.

3. Effect of 2-thiouracil on flower initiation in quantitative long day wheat plants "Kōnosu No. 25"

Agar media containing 1, 5 or 10% sucrose (with the other minerals of White's solution) were used for examination of inhibitory effect of 2-thiouracil on flower initiation in wheat plants. After incubation for 24 hours at 30°C, the plants were cultured under short or long day conditions. The plants were examined for flower initiation 30-60 days after the start of experiments. The results are presented in Table 3, a, b.

When added to the medium, 2-thiouracil inhibited the flower initiation under both short and long day conditions. The inhibitory effect of this reagent appears to be more significant under short day condition than under long day condition. It was particularly effective at 2×10^{-4}M concentration. The inhibitory effect of 2-thiouracil was more obvious on sucrose 5% medium. 2-Thiouracil at the concentrations lower than 3×10^{-5}M had no effect on flower initiation. 2×10^{-4}M 2-thiouracil inhibited the growth of leaves and the elongation of roots (Fig. 2). The leaves, however, were normal in shape. Seed-treatment of 2-thiouracil had no significant effect on flower initiation. Uracil had no effect on flower initiation.

The plants cultured on sucrose-enriched media developed flower primordia at lower node than those cultured on sucrose-deficient media under both long and short day conditions.

Table 3. Effect of 2-thiouracil on flower initiation in quantitative long day wheat plants "Kōnosu No. 25."

a. Long day.

Concentration of sucrose in medium (%)	Concentration of 2-thiouracil in medium (M)	Concentration of uracil in medium (M)	Number of plants	Number of leaves formed before flower initiation
1	0	0	12	8.1±1.08
	5×10^{-5}	0	8	8.6±1.06
	10^{-4}	0	12	8.5±1.13
	2×10^{-4}	0	6	10.2±1.22
	4×10^{-4}†			
	10^{-2}‡	0	6	8.7±0.52
		10^{-4}	18	8.7±0.84
5	0	0	9	7.3±0.50
		10^{-4}	12	7.1±0.29
	5×10^{-5}	0	14	7.3±0.67
	10^{-4}	0	14	8.1±0.75
	2×10^{-4}	0	18	9.1±0.87
	10^{-2}‡	0	21	7.4±0.62
		10^{-4}	16	7.3±0.81
10	0	0	5	5.4±0.55
		10^{-4}	4	5.5±0.58
	5×10^{-5}	0	10	5.7±0.67
	10^{-4}	0	6	5.3±0.58
	2×10^{-4}	0	7	7.0±1.15
	10^{-2}‡	0	10	5.5±0.53
		10^{-4}	9	5.3±0.50

4. Failure of detecting 2-thiouracil incorporation into ribonucleic acids from treated rice plants (Nōrin No. 15)

To study biochemically the mode of inhibitory or malforming actions of 2-thiouracil on the growth of rice and wheat plants, an attempt was made to correlate the observed effects to the alteration of the concerned RNA molecules caused by the analogue incorporation.

The plants grown on the agar media containing 0.6 mg of 2-thiouracil (with the other modified White's solution and sucrose 1%) were harvested at 6 leaf stage. The plants were washed with tap water and then cut into two parts, *i.e.*, stems and leaves, and roots respectively. Preparation of ribonucleic acids was made of each of these samples by a phenol method described by Kirby.[5] Approximate yields of RNAs were 0.31, 0.24, and 0.47 mg/g of stems and leaves, roots, and the

b. Short day.

Concentration of sucrose in medium (%)	Concentration of 2-thiouracil in medium (M)	Concentration of uracil in medium (M)	Number of plants	Number of leaves formed before flower initiation*
	0	0	3	9.0±0.00
	5×10^{-5}	0	4	8.8±1.50
	10^{-4}	0	7	9.0±1.00
1	2×10^{-4}	0	3	(10.0±0.00)
	4×10^{-4}†			
		0	10	7.8±0.75
	10^{-2}‡	10^{-4}	26	7.6±0.58
	0	0	10	7.8±0.75
	5×10^{-5}	0	15	8.3±0.75
	10^{-4}	0	18	8.7±0.84
5	2×10^{-4}	0	12	10.7±0.89
		0	11	8.1±0.61
	10^{-2}‡	10^{-4}	14	7.9±0.99
	0	0	5	6.8±0.45
		10^{-4}	5	6.8±0.45
	5×10^{-5}	0	8	7.8±0.53
10	10^{-4}	0	9	8.6±1.13
	2×10^{-4}	0	2	9.0±0.00
		0	14	7.0±0.60
	10^{-2}	10^{-4}	4	7.0±0.00

*The number of leaves of plants without flower primordia is enclosed in parentheses.

†No seed germinated in this lot.

‡2-thiouracil was given to the desiccated seeds.

whole body (control plants).

The final precipitate of purified RNAs thus obtained was dehydrated with ethanol, and then analysed for thiouracil as follows.

In one instance, the dried RNA sample was subjected to N-HCl hydrolysis, followed by paper chromatography using tert-BuOH-6N-HCl-H_2O (70 : 13.3 : 16.7).[4] The spot corresponding to uridylic and, if present, thiouridylic acids was eluted with water, concentrated in a vacuum desiccator, and in turn analysed by paper electrophoresis using 0.1 M borate buffer, pH 9.1. The result was negative for thiouridylic acid.

On another occasion, the sample was hydrolysed with 0.3 M KOH overnight at room temperature, neutralized with perchloric acid, and

then taken to paper chromatography using ammoniacal 70% iso-propanol.[6] The unresolved pyrimidine mononucleotide group was then

Fig. 2. Growth of wheat plant as affected by 2-thiouracil added to 10%-sucrose medium under long day condition (30 daysafter sowing at 25-20°C). Left control; right, 2×10^{-4}M 2-thiouracil.

analysed by electrophoresis in the same manner as above for the analogue. The result was again negative.

It is clear from these results that the extent to which 2-thiouracil was incorporated in RNA is, if any, below the level of detection. Replacement by 5% at least must take place in order that the analogue be detected in the ways employed here.

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