PIRONETIN†, A NOVEL PLANT GROWTH REGULATOR PRODUCED BY *Streptomyces* sp. NK10958

I. TAXONOMY, PRODUCTION, ISOLATION AND PRELIMINARY CHARACTERIZATION

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A novel plant growth regulator, pironetin, was isolated from the culture broth of *Streptomyces* sp. NK10958. It was extracted from the culture broth with ethyl acetate and purified by column chromatographies. Pironetin showed 23% inhibition on the growth of rice plants without any loss of crop yield at 10 g/a on 9 days before heading.

Plant growth regulators for paddy rice are used for preventing from the lodging and loss of harvests. The lodging resistance of rice plants is owing to shortening of plant heights. In our screening of microbial secondary metabolites for plant growth regulators, we found a novel plant growth regulator from the culture broth of *Streptomyces* sp. NK10958 and named it pironetin (a pyrane ring in the structure, Fig. 1). In this report, we describe the taxonomy, production, isolation, physico-chemical and biological properties of pironetin. The structural elucidation by spectral analyses are reported in the preceding paper[1].

Materials and Methods

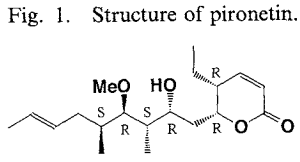
Taxonomy

The producing organism, NK10958, was isolated from a soil sample collected at Ageo, Saitama, Japan. The media and procedures used for the cultural and physiological characterization of strain NK10958 were according to the procedure of SHIRLING and GOTTLIEB[2]. Each culture was incubated at 27°C for 2 weeks before observation. The color names used in these studies were based on the Color Standard of Nihon Shikisai Co. Ltd.. Chemical composition of the cells was determined using the methods of BECKER *et al.*[3] and YAMAGUCHI[4]. Detailed observations of mycelial and spore morphologies were performed with the use of a light microscope and a scanning electron microscope (JEOL 25S III).

Production

A loopfull spores of the strain NK10958 was inoculated into 100 ml of a production medium consisted of glucose 2%, soy bean meal 2% and NaCl 0.3% (pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask, and cultured at 27°C for 2 days

Fig. 1. Structure of pironetin.



† Pironetin was originally called as NK10958.

on a rotary shaker (220 rpm). One milliliter of the above first seed culture was transferred to 100 ml of the same medium in a 500-ml Erlenmeyer flask and cultured under the same conditions. One hundred milliliters of the resulting second culture were transferred to 30 liter-jar fermentor containing 20 liters of the same medium. The fermentation was carried out at 27°C for 24 hours using the agitation rate of 200 rpm and the aeration rate of 20 liters/minute. Then 2 liters of the resulting third seed culture were transferred to a 200 liter-tank fermentor containing 100 liters of the production medium consisting of glucose 4%, soy bean meal 2% and NaCl 0.3% (pH 7.0 before sterilization). The fermentation was carried out at 27°C for 65 hours using the agitation rate of 200 rpm and the aeration rate of 80 liters/minute.

Isolation and Purification

The fermentation broth (200 liters) was separated into mycelial (30 kg) and filtrate (190 liters) by a filtration. The filtrate was applied on a column of Diaion HP-20 (Mitsubishi Chemical Ltd., 10 liters), and pironetin was eluted with acetone (30 liters) after washing the column with water. The acetone eluate was concentrated *in vacuo* to an aqueous solution. This aqueous solution was extracted with ethyl acetate. The extract was evaporated to dryness *in vacuo* yielding 54.0 g of crude oil. This crude oil was chromatographed on a silica gel (Merck, type 60, 1 kg) and eluted with the mixture of *n*-hexane and acetone (10 : 1). The active fractions were collected and evaporated to dryness. The residue was chromatographed on Sephadex LH-20 with acetone. The active fraction was collected and concentrated *in vacuo* and crystallized from *n*-hexane to give a colorless crystals of pironetin (5.0 g). The mycelial cake was extracted with MeOH (60 liters). The methanol extract was concentrated *in vacuo* to aqueous solution and the resulting solution was extracted three times with ethyl acetate. The combined extract was evaporated to dryness *in vacuo* to yield 69.0 g of crude oil. The crude oil was purified by the same procedure as above-mentioned to give a colorless crystals of pironetin (3.0 g).

Plant Growth Regulation Assay

Pironetin was formulated into 10% emulsifiable concentration (EC.; xylene 39%, iso-PrOH 39%, surfactant 12%). Each Wagner pot of 1/5,000 a was filled with paddy diluvium soil and manured (fertilizer application) and puddled with suitable water. Three rice seedlings of 2.5 leaf stage were transplanted in a depth of about 2 cm, and the pot was flooded in a depth of 3.5 cm. The pots were placed in a greenhouse. Each pironetin (10%EC) and the reference commercial compounds was diluted by water respectively and applied on the pots by submerged treatment on 21, 15, 9 or 5 days before heading at each dose shown in Table 4. The plant heights, weights and numbers of ear were measured at 65 days after heading. The treatments were replicated four times. The reference compounds, pacrobutazol and inabenfide were commercially purchased.

Results and Discussion

Taxonomy of Strain NK10958

Morphological observations were made with light and scanning electron microscopes (Fig. 2) on the culture grown at 27°C for 2 weeks on inorganic salts-starch agar. This strain showed *Rectiflexibiles* hyphae from branched aerial hyphae and no whirl. No sporangia, zoospores, vegetative mycelium spore and synnemata was observed. A matured spore chain comprised 20 or more spores ($0.6 \sim 0.8 \times 0.8 \sim 1.3$ μm) with a smooth surface. The cultural characteristics of strain NK10958 are summarized in Table 1. The aerial mass color was brownish white to pale pink. A slight brown soluble pigment was

Fig. 2. Scanning electron micrograph of a spore chain of strain NK10958 on inorganic salts-starch agar at 27°C for 2 weeks culture.

Bar represents 1 μm.

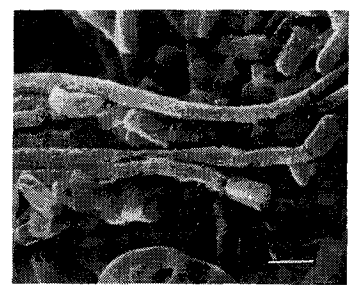


Table 1. Cultural characteristics of strain NK10958.

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Sucrose-nitrate agar	Moderate	Brownish white	Pale yellowish brown	Faint, brown
Glucose-asparagine agar	Good	Pinkish white~pale pink	Pale yellowish brown~yellowish brown	Faint, brown
Yeast extract-malt extract agar (ISP medium 2)	Good	Pinkish white~pale pink	Yellowish brown~pale brown	None
Oatmeal agar (ISP medium 3)	Moderate	Pinkish white~pale pink	Colorless~pale yellow	None
Inorganic salts-starch agar (ISP medium 4)	Moderate	Pale pink	Pale yellowish brown	Faint, brown
Glycerol-asparagine agar (ISP medium 5)	Good	Pale pink	Pale yellowish brown~yellowish brown	Faint, brown
Peptone-yeast extract iron agar (ISP medium 6)	Moderate	None	Light yellow	None
Tyrosine agar (ISP medium 7)	Good	Pinkish white~pale pink	Yellowish brown~pale brown	Faint, brown
Nutrient agar	Moderate	Pinkish white	Pale yellow brown	None

Observation after incubation at 27°C for 2 weeks. Color names from Guide to Color Standard, Nihon Shikisai Co., Ltd.

Table 2. Physiological characteristics of strain NK10958.

Temperature range for growth (°C)	10~37	Utilization of[a]	
Optimum temperature (°C)	27~37	D-Glucose	+
Reduction of nitrate	−	L-Arabinose	+
Hydrolysis of starch	+	D-Xylose	+
Coagulation of milk	+	D-Fructose	+
Peptonization of milk	+	Sucrose	+
Production of melanoid pigment		L-Rhamnose	+
Tyrosine agar	−	Inositol	+
Peptone-yeast extract-iron-agar	−	D-Mannitol	+
Tryptone-yeast extract broth	−	Raffinose	+
Liquefaction of gelatin	−	D-Galactose	+

+; Positive, −; negative.

[a] Basal medium: PRIDHAM-GOTTLIEB's carbon utilization medium (ISP No. 9).

observed without melanoid pigment. The physiological characteristics of strain NK10958 are summarized in Table 2. Hydrolyzed whole-cell of strain NK10958 contained LL-diaminopimelic acid. Accordingly, the cell wall of this strain was determined to be Type I. Based on the taxonomic properties described above, the strain NK10958 is considered to belong to the genus *Streptomyces*, and to be a strain of the red series of the PRIDHAM and TRESNER grouping[5]. The strain NK10958 was compared with *Streptomyces* species described in the literatures[5~12]. The strain NK10958 has been deposited in the National Institute of Bioscience and Human Technology (formerly The Fermentation Research Institute), Agency of Industrial Science and Technology, Japan under the accession No. FERM P-12317.

Production and Isolation

The fermentation broth was separated by filtration to mycerial cake (30 kg) and filtrate (190 liters). Five grams of pironetin were isolated from the filtrate, and 3.0 g of pironetin were isolated from the mycelial cake according to the isolation procedure summarized in Fig. 3.

Fig. 3. Isolation of pironetin.

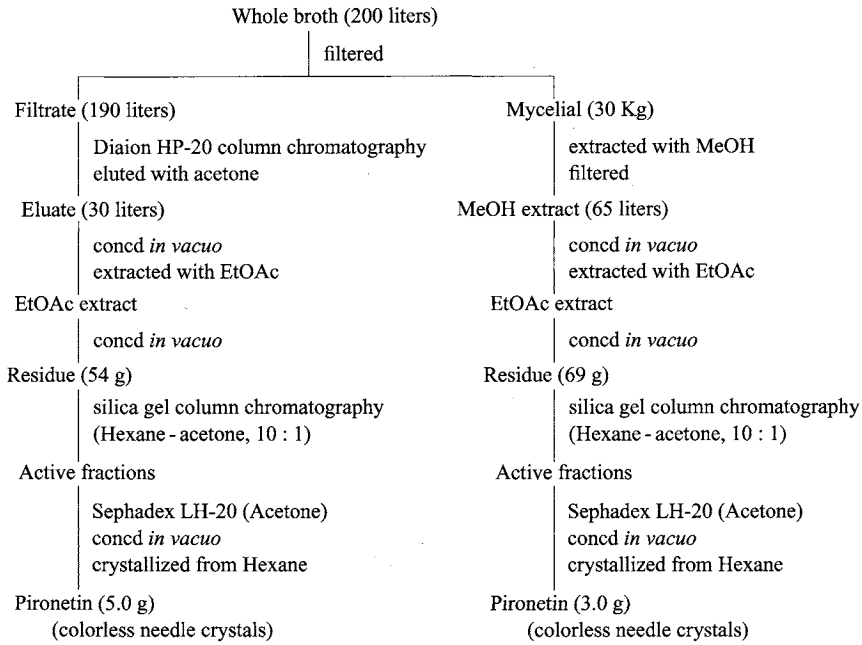


Table 3. Physico-chemical properties of pironetin.

Appearance	Colorless needle
MP	78~79°C
$[\alpha]_D^{20}$	−136.6° (*c* 1.0, $CHCl_3$)
Molecular formula	$C_{19}H_{32}O_4$
FAB-MS (*m/z*)	325 $(M+H)^+$, 347 $(M+Na)^+$
HRFAB-MS (*m/z*) Found:	325.2386 $(M+H)^+$
Calcd:	325.2379 for $C_{19}H_{33}O_4$
UV λ_{max}^{MeOH}	End absorption
IR ν_{max} (KBr) cm^{-1}	3511, 2966, 1728, 964
Rf* value	0.4
Solubility	Soluble: MeOH, EtOH, DMSO, Me_2CO, EtOAc
	Insoluble: H_2O

Rf*: Silica gel TLC (Kiesigel 60F 0.25 mm, Merck) was used with developing solvent hexane - acetone (5 : 2).

Physico-chemical Properties

The physico-chemical properties of pironetin are summarized in Table 3. Pironetin is soluble in methanol, ethanol, acetone, ethyl acetate and dimethyl sulfoxide, but insoluble in water. The spot on silica gel TLC plate was visible by vanillin-sulfuric acid reagent or sulfuric acid. The structure of pironetin was elucidated to be (5*R*,6*R*)-5-ethyl-5,6-dihydro-6-[(*E*)-(2*R*,3*S*,4*R*,5*S*)-2-hydroxy-4-methoxy-3,5-dimethyl-7-nonenyl]-2H-pyran-2-one (Fig. 1), on the basis of physico-chemical properties (Table 3), ^1H and ^{13}C NMR spectrometric and X-ray crystallographic analyses. The details of these studies will be reported in the preceding paper[1].

Biological Activities

Pironetin showed potent plant growth regulative activities against rice. The activities of pironetin are

Table 4. Plant growth regurative activities of pironetin.

Applycation timing (days before heading)	Compound	Conc (g/a)	Plant height (cm)	$\pm\sigma$	Relative height (%)	Total ears (number)	Total ear weight (g)	Weight (g/ear)	Relative weight (%)
21	Pironetin	10.00	63.63	5.55	86.07**	45	62.7	1.39	92.67
		2.50	77.00	3.22	104.15	44	70.9	1.61	107.33
		0.63	76.08	2.28	102.91	43	70.1	1.63	108.67
		0.16	75.24	3.16	101.77	44	67.0	1.52	101.33
	Pacrobutazol	1.80	60.23	2.94	81.47**	47	69.1	1.47	98.00
	Inabenfide	15.00	71.49	4.55	96.70*	43	69.9	1.63	108.67
12	Pironetin	10.00	63.63	4.36	86.07**	48	65.4	1.36	90.67
		5.00	75.51	4.04	102.14	40	64.7	1.62	108.00
		2.50	76.49	3.46	103.46	42	70.2	1.67	111.33
		0.63	75.47	2.48	102.08	40	68.3	1.71	114.00
		0.16	72.85	4.45	98.54	42	61.9	1.47	98.00
	Pacrobutazol	1.80	57.92	3.20	78.34**	44	60.1	1.37	91.33
	Inabenfide	15.00	69.34	3.39	93.79*	41	65.6	1.60	106.67
9	Pironetin	10.00	57.03	3.12	77.14**	43	66.7	1.55	103.33
		5.00	71.19	2.75	96.29*	47	71.3	1.52	101.33
		2.50	73.02	2.62	98.77	45	68.9	1.53	102.00
		0.63	74.38	2.54	100.61	46	70.8	1.54	102.67
		0.16	71.63	3.74	96.89	43	67.5	1.57	104.67
	Pacrobutazol	1.80	59.28	2.64	80.18**	46	64.5	1.40	93.33
	Inabenfide	15.00	67.40	2.60	91.17*	45	64.9	1.44	96.00
5	Pironetin	10.00	60.78	4.59	82.21**	45	67.5	1.50	100.00
		5.00	70.75	4.17	95.70*	39	63.1	1.62	108.00
		2.50	75.33	1.97	101.89	40	67.2	1.68	112.00
		0.63	75.32	2.57	101.88	45	65.9	1.46	97.33
		0.16	75.14	3.85	101.64	44	71.3	1.62	108.00
	Pacrobutazol	1.80	61.23	2.71	82.82**	41	59.8	1.46	97.33
	Inabenfide	15.00	69.72	3.30	94.31*	43	68.2	1.59	106.00
Control			73.93	2.70	100.00	42	62.8	1.50	100.00

* and ** Significant at $P=0.05$ and 0.01, respectively.

shown in Table 4. Pironetin showed 14~23% shortening of plant height at a dose of 10 g/a in our Wagner pot test. When pironetin was applied 5 or 9 days before the heading, there was no damage to yield of rice. Slight inhibitions to yield of rice were observed when pironetin was applied 12 or 21 days before the heading, but they were not significant. Pironetin was superior in the plant regulation effect and the yield of rice to the commercial plant growth regulators (pacrobutazol and inabenfide). Other biological activities will be reported separately.

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