New Rice Seedling Test for Gibberellins—Microdrop Method

By YUTAKA MURAKAMI

Chief, 4th Laboratory, 1st Division of Physiology, Department of Physiology and Genetics, National Institute of Agricultural Sciences

The gibberellins were originally discovered by Japanese scientists as metabolic products of a fungus, *Gibberella fujikuroi*. They have a considerable effect to promote the growth of intact plants. In addition, the substances which are obtained from other sources and have similar effect have also been called gibberellins. The symbols A_1, A_2, etc. have been used to denote the characterized gibberellins.[2] Gibberellin-like substances are now known to be widely distributed in higher plants, and thus appear to be important in controlling many different phases of plant growth and development.

Many workers have provided various bioassays for detecting gibberellins in plant tissues. Seedlings of normal *Oryza sativa*,[3] dwarf mutants of *Zea mays*[7] and dwarf cultivars of *Pisum sativum*[1] have been used as the assay organism. The present writer[4),5)] devised a rapid and simple bioassay for gibberellins which was based upon the elongation response of the second leaf sheath of the dwarf cultivars of *Oryza sativa* to gibberellins. Evidence for the occurrence of bamboo gibberellin (GA_{19}) in rice shoots will be reported as an example of the use of this method.

Principle

In *Oryza sativa* there are many dwarf varieties or cultivars. Most of them are simple recessives. One dwarf variety 'Tan-ginbozu' contains no gibberellins in shoots.[8] The Tan-ginbozu dwarf responds well to gibberellins such as A_5, A_9 and A_{20} which have no OH group at the C–2 position of the gibbane ring, but the gibberellin-producing dwarf, Waito-C, much less to them (Table 1). However, as shown in Fig. 1., both dwarfs respond equally well to gibberellin A_3 from 0.1 mμg per plant. Such specificity between two dwarfs can be used as a 'multiple plant assay' to detect gibberellins with similar structures.

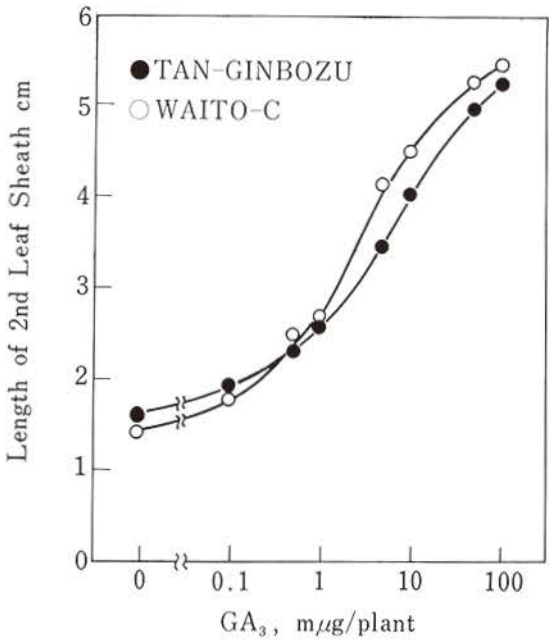
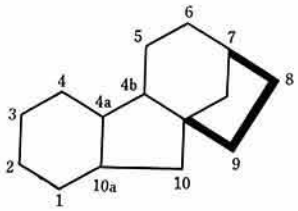


Fig. 1. Growth response curve for gibberellin A_3 by using two dwarfs, Tan-ginbozu and Waito-C.



Gibbane ring

Table 1. Relative activity of gibberellins on the elongation of 2nd leaf sheath of rice at the concentration of 10 mμg per plant

Gibberellins	Rice variety	
	Tan-ginbozu	Waito-C
A_1	100	80
A_2	10	10
A_3	100	100
A_4	30	20
A_5*	30	5
A_7	10	20
A_8	1	0.5
A_9*	50	1
A_{18}	8	3
A_{20}*	90	1

Gibberellins with * indicate the lack of OH group at the C—2 position of the gibbane ring.

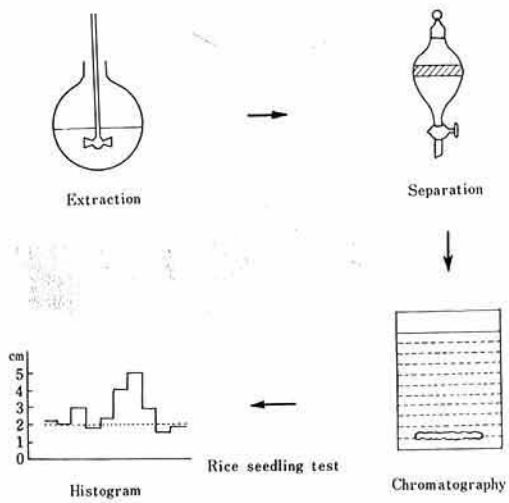


Fig. 2. Techniques for extraction, fractionation and bioassay of gibberellins.

Methods

The techniques to examine gibberellins in plants are illustrated in Fig. 2.

1) Gibberellin extraction and fractionation

The plant material was ground in a mixer with eight times its fresh weight of 70% acetone and the mixture was allowed to stand overnight at room temperature. The homogenate was filtered through cheesecloth and filter paper. The filtrate was evaporated on a rotary evaporator, and then the remaining aqueous phase was adjusted to pH 2.5 with phosphoric acid and partitioned against ethyl acetate three times. This ethyl acetate was three times partitioned against 1 M phosphate buffer at pH 7.0. The buffer was acidified to pH 2.5 with phosphoric acid and three times partitioned against ethyl acetate. The acidic ethyl acetate fraction was dried over anhydrous sodium sulfate and evaporated to dryness prior to further purification by thin-layer chromatography (TLC). These procedures are shown in Fig. 3.

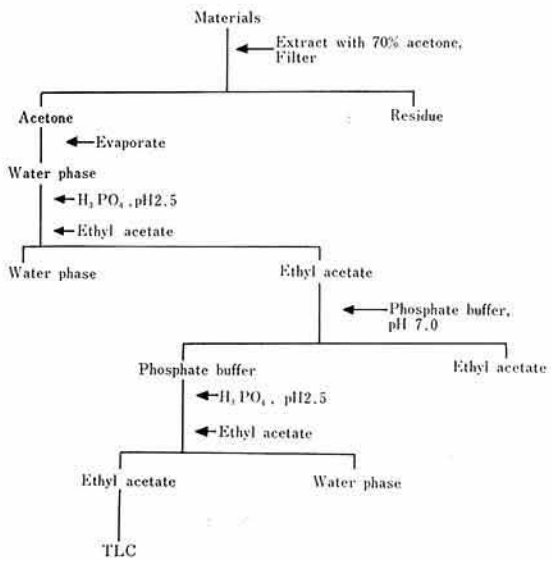


Fig. 3. Flow sheet of procedures for extraction and separation of gibberellins from plant shoots.

2) Thin-layer chromatography and recovery of material from thin-layer chromatograms

The acidic ethyl acetate fraction was reduced to dryness, redissolved in a small volume of acetone and applied as a band to the origin of a 20 × 20 cm silica gel (G) thin-layer plate which was about 0.5 mm thick. The plates were developed in the following solvent systems: Isopropyl ether/acetic acid (95 : 5, v/v); chloroform/ethyl acetate/acetic acid (60 : 40 : 5, v/v); isopropanol/acetic acid (95 : 5, v/v); isopropanol/water (4 : 1, v/v); isopropanol/ammonia (28%)/water (10 : 1 : 1, v/v); n-butanol/1.5 N ammonia (3 : 1, v/v). The solvent was permitted to run a distance of 10 cm on the plate. After drying, the plates were divided into 10 equal zones between origin and solvent front (the first zone was further subdivided into two zones). Each zone was scraped off into small beakers and eluted three times with 2 ml of 50% acetone. The eluates were taken up into a small glass tube (1.8 × 3.5 cm), reduced to dryness using a hair dryer, and redissolved in 0.1 ml of 50% acetone for bioassay.

3) Bioassay

The method of microdrop application was used for the treatment of rice seedlings with test substances (Fig. 4).

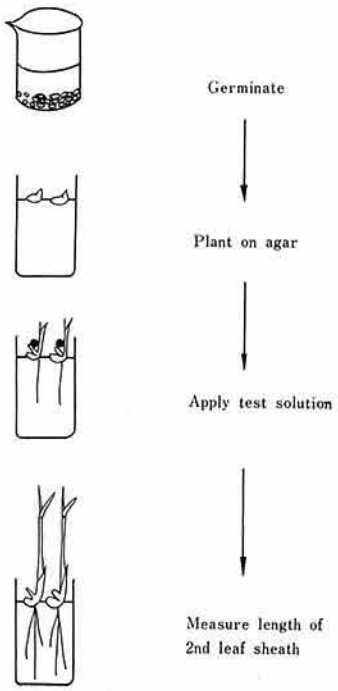


Fig. 4. Procedure of microdrop method.

Rice seeds were submerged in water at 32°C for 2 days; at this time the coleoptile had emerged. The germinated seeds were selected

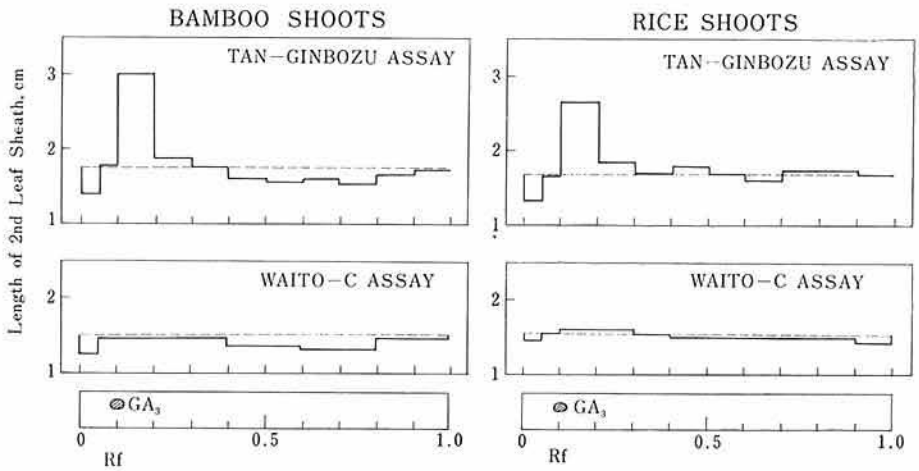


Fig. 5. Comparative rice assays of thin-layer chromatograms loaded with extracts of bamboo and rice shoots.

About 50 g fresh weight equivalent of each extract was chromatographed on silica gel (G) in the solvent system, isopropylether acetic acid (95 : 5, v/v).

for uniformity, and planted in group of five into cylindrical bottles of 2.8 cm in diameter and 6 cm in depth which were filled with 0.9 per cent water-agar. The bottles were placed in a tall Petri dish of about 18 cm in diameter and 12 cm in depth, and they were then incubated for 45 hours in a cabinet under continuous fluorescent light (about 5,000 lux) at 32°C. When the second leaf emerged from the first leaf, eluates were applied in a single 1 μl droplet to the surface of each coleoptile with a micropipette. The treated seedlings were then grown for 3 more days under the same condition as before. The length of the second leaf sheath was then measured with a ruler. The results of gibberellin activity were shown in the form of histograms indicating average of 5 test plants. Assay responses were not regarded as positive unless they were 10% or greater than over the controls.

Application for examination of gibberellin in rice shoots

Rice (var. Kotake-tamanishiki) was grown in the paddy field and harvested at the age of about 60 days. Young bamboo shoots (*Phyllostachys edulis*) were obtained at a local market. From these plant materials the acidic ethyl acetate fractions were prepared and bioassayed using Tan-ginbozu and Waito-C dwarfs as described above.

The results of gibberellin-like activity are summarized in the form of histograms showing the length of the second leaf sheath of rice (Fig. 5). In the upper histograms of bamboo and rice shoots, which were bioassayed using the Tan-ginbozu dwarf, one peak of gibberellin activity was detected at the same zone, Rfs 0.1~0.2. The active zone lies close to the known position of gibberellin A_3. The lower histograms indicate that the Waito-C dwarf was unresponsive to the extract of this zone. As the Waito-C dwarf responds to gibberellin A_3 almost in the same manner as the Tan-ginbozu dwarf (Fig. 1), the activity present in the zone at Rfs 0.1~0.2 may not be due to gibberellin A_3.

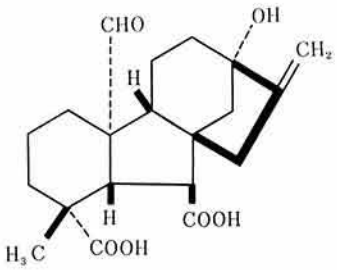


Fig. 6. Structure of bamboo gibberellin (GA_{19}).

Recently Murofushi *et al.*[6] isolated bamboo gibberellin from water extract of bamboo shoots and its structure was established as Fig. 6. The trivial name of bamboo gibberellin is gibberellin A_{19} (GA_{19}). GA_{19} has no OH group at the C–2 position of gibbane ring. As already described, the Waito-C dwarf is weakly responsive to those gibberellins having no OH group at the C–2 position of the gibbane ring. Thus the eluates from bamboo shoots did not affect the growth of Waito-C dwarf at all.

Table 2. Comparison of gibberellin activity in extracts from rice and bamboo shoots following thin-layer chromatography in five different solvent systems

Solvent system	Rf of GA_3	Plant material	Zone of growth promotion* Rf
1	0.2	Rice	0.1 —0.2
		Bamboo	0.1 —0.2
2	0.9	Rice	0.8 —1.0
		Bamboo	0.8 —1.0
3	0.75	Rice	0.7 —0.9
		Bamboo	0.7 —0.9
4	0.65	Rice	0.05—0.2
		Bamboo	0.05—0.2
5	0.4	Rice	0.05—0.1
		Bamboo	0.05—0.1

* Bioassay was carried out by using the Tan-ginbozu dwarf.

Solvent systems:

1) Chloroform/ethyl acetate/acetic acid (60 : 40 : 5, v/v).
2) Isopropanol/acetic acid (95 : 5, v/v).
3) Isopropanol/water (4 : 1, v/v).
4) Isopropanol/ammonia (28%)/water (10 : 1 : 1, v/v).
5) n-Butanol/1.5 N ammonia (3 : 1, v/v).

Because of the apparent similarity between gibberellins from extracts of bamboo shoots and from those of rice shoots, further examinations of extracts were made by using different chromatographic solvent systems and bioassay. The results are summarized in Table 2. It can be seen that the growth response to the Tan-ginbozu dwarf was obtained by the same zones of chromatograms in rice and bamboo. The active zones of the chromatograms did not correspond to the known position of gibberellin A_3, when the solvent systems containing ammonia were used. The high activity in the Tan-ginbozu assay, the low activity in the Waito-C assay, and the Rf evidence all suggested the presence of GA_{19} in rice shoots.

References

1) Brian, P. W. and Hemming. H. G.: The effect of gibberellic acid on shoot growth of pea seedlings. *Physiol. Plantarum* **8**, 669–681 (1955).
2) MacMillian, J. and Takahashi, N.: Proposed procedure for the allocation of trivial names to the gibberellins. *Nature* **217**, 170–171 (1968).
3) Murakami, Y.: The effect of the extract of immature bean seeds on the growth of coleoptile and leaf of rice plant. *Bot. Mag.* Tokyo **70**, 376–382 (1957).
4) Murakami, Y.: A new rice seedling test for gibberellins, 'Microdrop Method', and its use for testing extracts of rice and morning glory. *Bot. Mag.* Tokyo **81**, 33–43 (1968).
5) Murakami, Y.: A new rice seedling test for gibberellins, 'Microdrop Method'. *Shokubutsu No Kagaku Chosetsu* (*Chem. Regul. Plant*) **4**, 78–83 (1969).
6) Murofushi, N., Iriuchijima, S., Takahashi, N., Tamura, S., Kato, J., Wada, Y., Watanabe, E. and Aoyama, T.: Isolation and structure of a novel C_{20} gibberellin in bamboo shoots. *Agr. Biol. Chem.* **30**, 917–924 (1966).
7) Phinney, B. O.: Dwarfing genes in *Zea mays* and their relation to the gibberellins. In Plant Growth Regulation (Iowa State Univ. Press, Ames, Iowa) 487–501 (1961).
8) Suge, H. and Murakami, Y.: Occurrence of a rice mutant deficient in gibberellin-like substances. *Plant & Cell Physiol.* **9**, 411–414 (1968).