Production of Antifungal Substances in Mulberry

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Silkworm rearing with mulberry shoots is widely popularized in Japan as a labor-saving technique. However, it is known that this technique often induces lowering of disease-tolerance of shoots or causes severe outbreak of shoot-killing diseases as the cut surface permits the entrance of diseases. In view of such a background, the authors have carried out a study on disease-resistance mechanism of mulberry. As a result it was found out that an antifungal substance, i.e. prohibition exists endogenously in the epidermal tissue of malberry shoots, and cortex and xylem tissues also produce antifungal substances, phytoalexins (PA), in response to the infection of pathogenic fungi, and that these antifungal substances are involved in the disease-resistance. Chemical structure of these substances was determined by the cooperation of Dr. M. Takasugi et al. of the Faculty of Science, Hokkaido University.

Prohibitin in shoot epidermis

Infection of *Stigmina mori* and *Fusarium solani* f. sp. *mori*, both of them cause shoot-death, occurs easily when inoculated through wounds although the infection is quite difficult without wounding. Therefore, mulberry shoot was divided into epidermis, cortex, xylem and pith, and each of them was subjected to acetone extraction. Antifungal activity was recognized only in the extract of epidermis. The extract showed the antifungal activity to many kinds of fungi including those pathogenic to mulberry shoots. The extract was separated into 3–7 active substances differing in Rf value by silicagel thin layer chromatography (TLC)—bioassay method[2], using ethylether as developing reagent. The amount and the number of these active substances varied with different cultivars, and were more with basal part than top part of a shoot, more with old shoots than newly sprouted shoots, and much more was detected in the epidermis of stems and roots. These substances are prenylflavon compounds (Fig. 1–1); kuwanon C, morucin, and new compounds, albanin A~H, and albafuran A, and B[2]. All these substances exhibited an antifungal activity to many kinds of fungi. Kuwanon C inhibited completely the growth of *Rosellinia necatrix* which attacks mulberry roots at a low concentration such as 14 ppm.

The similar experiment was conducted with shoot epidermis of other arboreal plants. Antifungal substances different each other were detected from each of 14 species out of 22 species examined (Fig. 2).

Phytoalexins produced in shoot cortex

When fungi invade into mulberry shoots through lenticel or wound on the shoot surface, cortex tissue beneath the epidermis begin to change brown in color. The browned portion gradually expands, but soon ceased to enlarge so that the death of whole plant can be avoided. This phenomenon was studied in relation to production of antifungal substance, and the following results were obtained[1,5,8]. At the border between healthy portion and diseased portion, dark brown reaction zone was formed, and PA accumulated in that zone.

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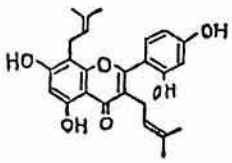
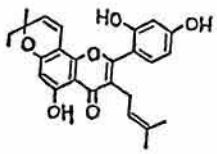
1) Epidermis

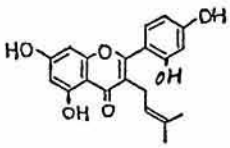
kuwanon C



morusin

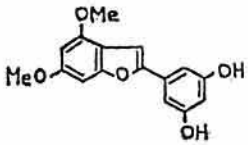


albanin A

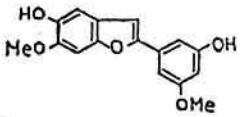


2) Cortex

moracin A

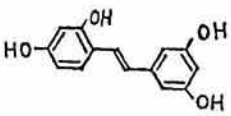


moracin B



3) Xylem

oxyresveratrol



4′-prenyloxyresveratrol

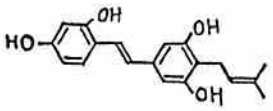


Fig. 1. The structure of main antifungal substances produced in mulberry shoot.

Irrespective of the difference of fungus species inoculated, PA was produced. Within the temperature range of 5–30°C, the higher the temperature, the faster was the production of PA. At 5°C, PA was produced slowly although no browning occurred in shoot cortex. At high temperature, the browning was limited to only surface layer of cortex and PA was produced prior to the browning and accumulated only in the browned tissue. Production of PA requires living tissue, and is induced not only by living fungi but also by heat-stable and dialysable substance contained in the filtrate of fungus culture solution. Virulency of different species of the genus *Fusarium* which causes twig blight of mulberry was found to be proportional to their tolerance to PA. Namely, the degree of tolerance to PA was in the order of *F. solani* f. sp. *mori* > *F. lateritium* f. sp. *mori* > *F. roseum*. This result shows that *F. roseum* which is susceptible to PA can hardly be pathogenic to mulberry shoots; and thus offers an evidence for the fact that this fungus gives only few damages in spite of its dense distribution in mulberry fields.

PA produced in shoot cortex was identified as 26 new compounds which have 2-phenylbenzofuran skeleton, a rare example in natural products, and named moracin A~Z[9,11] (Fig. 1–2). These substances showed a wide spectrum of inhibitive activity to many kinds of fungi and bacteria including pathogens to plants. Growth of *Diaporthe nomurai*, which causes die-back of mulberry, was completely inhibited by moracin A at 14 ppm.

PA of shoot cortex was found in 18 species of arboreal plants including 7 species of

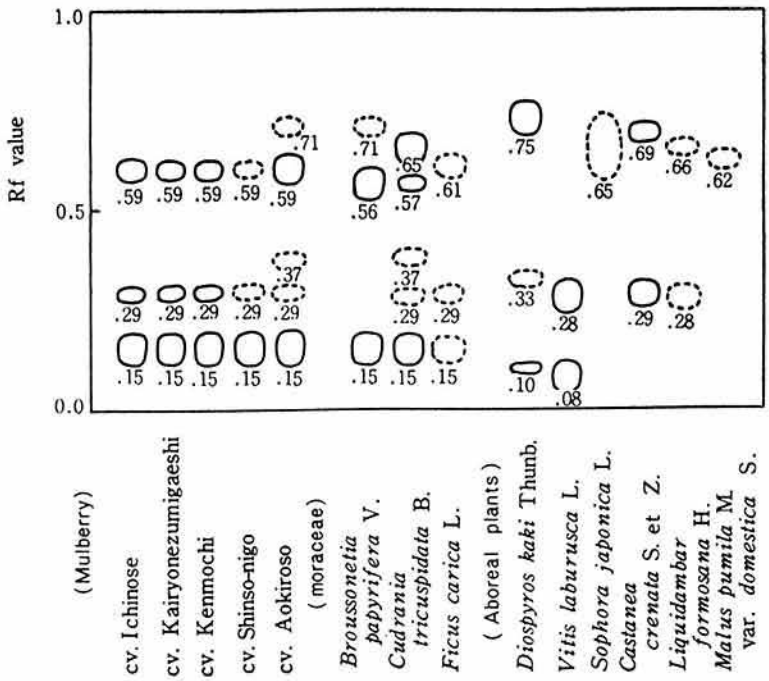


Fig. 2. Detection of antifungal substances in shoot epidermis of tree plants by the thin-layer chromatogram (TLC)-bioassay method.

Acetone extracts from epidermis of tree plants were spotted on TLC plate and developed in ethylether. Antifungal activity (inhibition zone) was checked by the bioassay method using the spray of conidiospores of *Bipolaris leersiae*.

Moraceae. Thus it was made clear that PA production is not specific to mulberry (Fig. 3).

Phytoalexins produced in xylem

When mulberry shoots are cut for harvesting, the xylem is directly exposed to the entrance of fungi, and die-back of shoots begins. However, when a lateral shoot below has grown to bear many leaves, the lesion stops expanding at the portion of the xylem adjacent to the base of the lateral shoot. It was found that an extremely large amount of PA has accumulated in that portion[3] (Fig. 4). As to the PA produced in xylems, the following findings were made from several experiments[4]. Xylem of new shoots grown in June produces PA by inoculation of fungi, but not by cutting wound only. On the contrary, xylem of shoots grown in the preceding year or over-wintering shoots produces PA by cutting alone. Production of PA requires living tissue block larger than a definite size. Within the temperature range from 5° to 30°C, the higher the temperature, the faster the PA production and browning, with the former occurring ahead of the latter. The PA was fractionated as a group of three antifungal spots by the TLC-bioassay method, and they were identified as oxyresveratrol, 4′-prenyloxyresveratrol (Fig. 1–3) and moracin M, respectively[10].

PA was not detected from healthy xylem

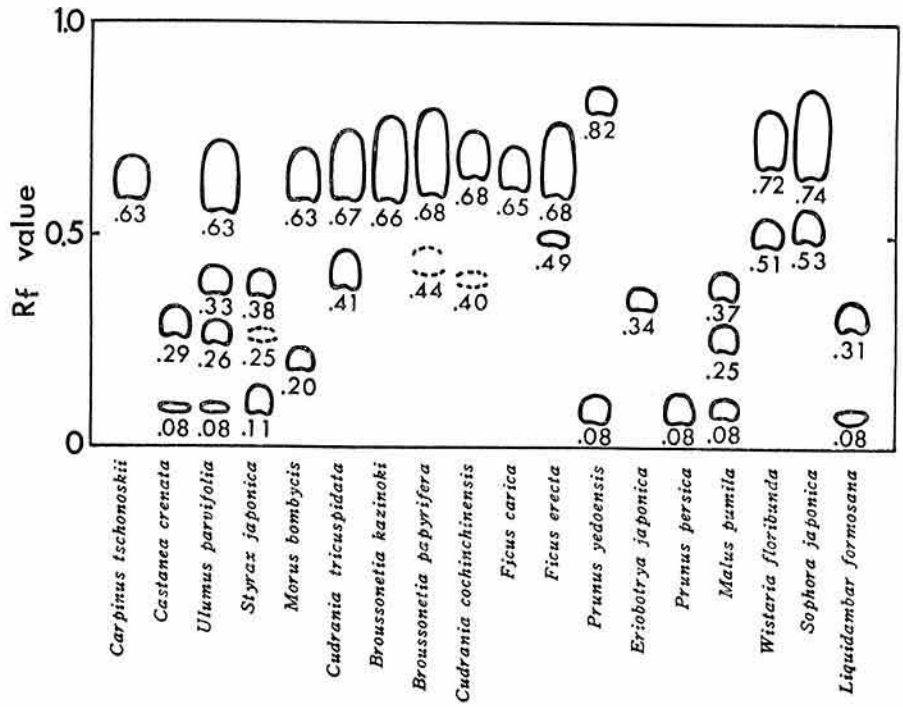


Fig. 3. Detection of phytoalexins produced in shoot cortex of 18 tree plant species inoculated with *F. solani* f. sp. *mori*.
The detection was made by the TLC-bioassay method using *Bipolaris leersiae*.

of 1–2 year-old shoots. However, shoots older than 3–4 years showed reddish tissue at the center of xylem, from which only oxyresveratrol was detected with its content amounting to 1.7% on dry weight basis.

These antifungal substances showed complete inhibition to many kinds of fungi pathogenic to plants at the concentration of 56–560 ppm.

Infected part of xylem was examined with 7 arboreal species of Moraceae, and extracts of the xylem expressing light-yellowish brown or brown reaction zone showed antifungal activity in all of the 7 species examined. Particularly, *Morus lhou* and *Cudrania tricuspidata* exhibited strong activity[4] (Plate 1).

Phytoalexins produced in leaves and roots

In mulberry leaf sections, finely chopped, and kept in a humid chamber at 20°C after inoculation of *F. solani* f. sp. *mori*, PA began to be produced from 12 hr after the inoculation, reaching the peak in 2–4 days, and then decreased. The PA produced was less in upper leaves, but more in middle or lower leaves, and it was recognized as 1–2 antifungal spots in the TLC-bioassay method. The major PA in leaf blade was named chalcomoracin ($C_{39}H_{36}O_9$)[12].

Cortex and xylem of roots produced antifungal substances simply by cutting, and these substances were isolated as 1–5 antifungal

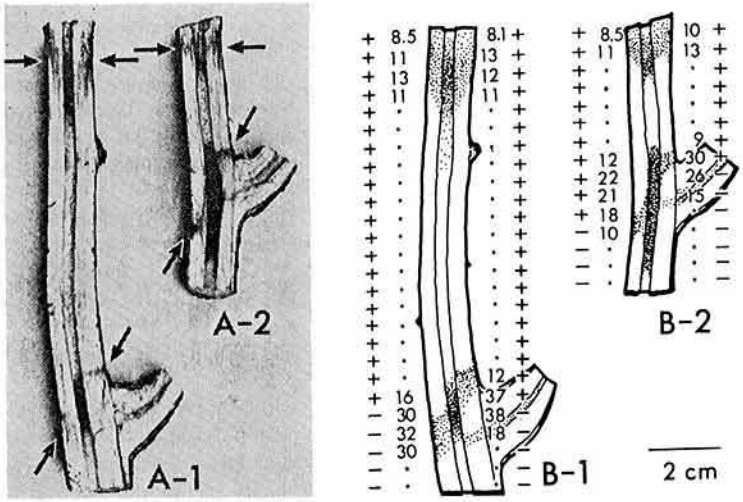


Fig. 4. Invasion of hyphae, formation of the brownish reaction zone and the accumulation of antifungal substances in the xylem of mulberry shoots

The cut surface of the middle part of shoot was inoculated with *Fusarium solani* f. sp. *mori* in a field on April 7th, and examination was carried out on July 15 after the buds had grown to lateral shoots.

A: The tree was cut vertically for observation. Note the two reaction zones shown with arrows.

B: Invasion of hyphae was shown with + (re-isolation) or— (no-isolation). Antifungal activity of an extract (0.1 g/ 0.5 ml of 50% acetone) from each part of the xylem was shown by diameter (mm) of the inhibition zone by the cup method using *Biporaris leersiae*. The diameter of the cup was 8 mm. • : non-inhibition.

Cultivar for A-1 and B-1: Ichinose, that for A-2 and B-2: Kenmochi.

spots by the TLC-bioassay method. Their Rf value was similar for cortex and xylem.

It was also found that 6 tree species of Moraceae produced antifungal substance, and its Rf value was different among species, and slightly different between cortex and xylem of the same species.

Conclusion

Surface of mulberry plants, especially that of their roots are exposed to various pathogenic micro-organisms. It is thought that mulberry plants may have some protective mechanisms. Standing on this viewpoint, each tissue of mulberry plants was examined to find out antifungal substance. As a result, several kinds of flavonoide were isolated from the epidermis. Thus, it is inferred that the epidermis can protect the plants from the invasion of pathogenic fungi, not only acting as a physical barrier, but also containing these antifungal substances endogenously localized in the thin layer of epidermal tissue.

No antifungal component was detected in the cortex and xylem of healthy mulberry shoots. However, when they were infected by fungi, the cortex produced moracin A–Z; antifungal "phytoalexins" (PA) which is not detected in healthy tissues, while the xylem produced two kinds of stilben compound. It seems that mulberry shoots protect themselves from the further invasion of fungi beyond the site of invasion, by producing PA

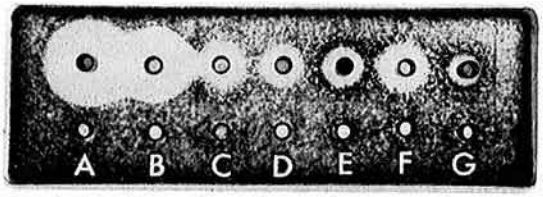


Plate 1. Detection of antifungal activity of extracts taken from the browned xylem tissues of shoots of plants belonging to Moraceae.

The antifungal activity of each acetone extract (10 volumes) taken from browned xylem (upper cups) near the cut surface, and from the intact xylem (lower cups) was tested by the cup method using *Bipolaris leersiae*.

A: *Morus lhou*, B: *Cudrania tricuspididata*, C: *Broussonetia kazinoki*, D: *B. papyrifera*, E: *Ficus erecta*, F: *C. cochinchinensis*, G: *F. carica*.

in response to a stimulus of PA-inducing factor excreted from fungi, and/or by accumulating a large quantity of PA in the browned tissue prior to the invasion of fungi.

Infected leaf blade of mulberry was found to produce chalcomoracin. Thus, the PA produced by the same plant was markedly different with different tissues. This phenomenon has not been reported so far. From the plant pathological standpoint, this finding is very important in relation to tissue-specificity of infection. Furthermore, it was made clear that the protective mechanism found in mulberry is commonly observed in many other arboreal plants[3,6].

Development of new pesticide by the use of these antifungal substances or their derivatives, and search for PA-inducing substances and their use as agents for promoting disease resistance of plants are expected in future.

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