Effect of Hyphal Homogenate of *Phytophthora infestans* on the Potato-Tuber Protoplasts

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富山宏平*・李 好植*・道家紀志*: 疫病菌磨砕汁液の
ジャガイモプロトプラストに対する影響

It has been reported that the time from penetration by an incompatible race of *Phytophthora infestans* to hypersensitive death of the penetrated potato-cell was very short^{1,2,3}). In the quickest case, the death of the infected cell occurred within about 30 min. These results suggested that some biologically active substances may be related to the hypersensitive death of the infected cell in the case of the incompatible host-parasite combination.

In a previous paper¹), effects of the hyphal homogenate and culture filtrate of *Phytophthora infestans* on the potato tuber slices and leaf tissues was reported. The toxic substances in homogenate and culture filtrates of *P. infestans* (race 0 and race 1) appeared to be the same as Saveleva and Rubin's endo- and exotoxins⁴). The toxicity of partially purified high-molecular substances of the endo- and exotoxins did not correlate with the host-parasite specificity. In the experiments reported here, we tried to test the effect of the fungal homogenate against the potato-tuber protoplasts, using different race-variety combinations.

Tubers of the potato cultivars Rishiri (R₁) and Irish Cobbler (r) were used. The former cultivar is incompatible with race 0 of *Phytophthora infestans* and compatible with race 1. The latter is compatible with both the races. When the tubers of the cultivar "Rishiri" are infected by race 0, the infected cells hypersensitively die very quickly.

At first, We examined various method to isolate protoplasts from potato leaves and tubers. Protoplasts could be isolated from leaves and also from tubers by using a little modified method of Takebe *et al.*⁵).

Maceration of the tubers was carried out by digesting pieces of the sliced tubers with macerozyme, a crude polygalacturonase (preparation from *Rhizopus* sp., Zen-Nippon-Biochemicals Co.). The maceration medium contained 1.0-0.5% macerozyme, 0.7 M D-mannitol and 0.3% potassium dextran sulfate, pH being adjusted to 5.8 with 2 N HCl. Four g (fr. wt.) of the pieces of sliced tubers were placed in 20 ml of the maceration medium in a 100 ml Erlenmeyer flask. The flask was shaken in a water bath at 26-27 C by a reciprocal shaker for 2 h. The sliced-tuber pieces were then placed in 0.7 M mannitol solution containing 2-6% (Rishiri) and 2% (Irish cobbler) cellulase (Zen-Nippon-Biochemicals Co.) and 0.1 mM CaCl₂ (pH adjusted to 5.4 with 2 N HCl). The flasks were then shaken overnight at 26-27 C, or otherwise, for 5 h at the same condition except that the concentration of the enzyme was higher than in the case of overnight shaking. The enzyme solutions were filtered

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through a milipore filter with $0.45\ \mu$ pore size before use. The protoplasts were rinsed five times with 0.7 M sterilized mannitol solution containing 0.1 mM CaCl_2 . The protoplast solution was finally added with ceporan to the concentration of 0.2-0.3%. All these treatments were done under the aseptic condition.

Race 0 and race 1 of *P. infestans* were grown for 12-20 days in a liquid or on an agar medium containing rye extract⁶⁾. The mycelia were separated from the medium and several times washed with water. Seven to ten g (fresh weight) of mycelia was homogenized three times in a French press cooled at -10°C (at beginning of the homogenization) with 0.01 M phosphate buffer at pH 7.1, and then treated with ultrasonic wave for 5 min. Alternatively, the mycelia grown on the rye extract agar (10-18 days) were ground in a mortar with liquid nitrogen and then lyophilized. One g of the powder was dissolved in 10 ml of 0.01 M potassium phosphate buffer, pH 7.0. The homogenate obtained by the former or the latter method was centrifuged at 3000 rpm for 5 min. The supernatant was centrifuged at $105,000\times g$ for 40 min. Lipid-like component floating on the supernatant was discarded. The supernatant was again centrifuged at $105,000\times g$ for 40 min and the lipid-like component was again discarded. The supernatant was dialyzed against water overnight at 0°C , and then concentrated to 2 ml by using a Sartorius membranefilter. Ceporan and D-mannitol were added to the concentrations 2 mg/ml and 0.7 M, respectively.

Forty μl each of protoplast solution and fungal component either the supernatant or the precipitate suspension were placed on the hollow made on a slide glass. The slides were incubated at $17-18^\circ\text{C}$ and the toxic effect was examined after a certain time.

The supernatant fraction of homogenate of race 0 gave the most injurious effect on the protoplasts of the cultivar "Rishiri" as compared with the precipitate fraction of both races and the supernatant fraction of race 1 (Fig. 1). Conversely, there was little difference between the effect of homogenates of race 0 and race 1 on the protoplast of the cultivar "Irish cobbler". There seemed to be an apparent correlation between the toxicities of the supernatants to the protoplasts and the host-parasite specificity. However, more careful examination is necessary to conclude that the

supernatant fraction is related to establishment of host parasite specificity. The protoplasts treated with the supernatant of the homogenate showed granular appearance of the cell membrane, deformation, and finally coagulation and browning of the cell content (Fig. 2).

To test the effect of heat treatment on the toxicity of the supernatant, the Wassermann test tubes containing the supernatant were heated in a water bath and then the resulted turbid solution was used for the assay. To test the effect of ethanol on the toxicity, the supernatant of fungal homogenate was treated twice with ethanol (final concentration 80%). The insoluble fraction was dissolved in water and tested for its toxicity. The supernatants lost their toxic activity after heat

Fig. 1. Percent survival of the protoplasts treated with ultracentrifuge-supernatants of the fungal homogenates (24 hr after treatments). Average of experiments repeated 6 times.

c: control (mannitol solution), R-0: homogenate of race 0, R-1: race 1. Arrows indicate standard deviations.

Fig. 2. Protoplasts obtained from potato tubers.

Left: Rishiri (treated and abnormal) Right: Rishiri (normal)

Table 1. Effect of heat treatment on the toxicity of the hyphal components to the protoplasts of cultivar "Rishiri" (percentages of abnormal protoplasts).

	Ultracentrifuge supernatant			Ethanol insoluble fraction of ultracentrifuge supernatant	
	unheated	heated at		unheated	heated at
		70 C	90 C		70 C
Mannitol solution	3.5	—	—	11.2	—
Race 0 homogenate	31.4	5.5	2.9	75.4	58.5
Race 1 homogenate	23.5	4.6	1.0	56.1	56.4

Remark : Average of experiments repeated three times.

treatment at 70 C or 90 C for 3 min (Table 1). When the supernatants of the fungal homogenates were treated with ethanol (final 80%), the precipitates were toxic to the protoplasts of both cultivars. The toxic principles in the ethanol-insoluble fraction seemed rather heat-stable (Table 1). These results suggested that the protoplasts would be useful to investigate the mechanism of specificity between host and parasite.

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