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Selection of 'Koshu' Grape Callus Resistant to Culture Filtrate of the Pathogenic Fungus, *Glomerella cingulata*

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Somaclonal variants obtained by *in vitro* selection may provide a quick and useful means of obtaining agriculturally useful variants. Plant tissue and cell culture techniques have made it possible to select toxin substances-tolerant[1,2], pathogen-resistant[3,4], and herbicide-resistant plants[5,6]. In cases in which transgenic systems can not be established, the selection of mutant cells offers a very powerful, straightward method for obtaining resistants.

Ripe rot disease (Japanese: *banpu* or *osogusare*) caused by *Glomerella cingulata* is the main grape disease in Japan. Phenylacetic acid and indoleacetic acid, known as plant growth regulators and fungal metabolites, have been reported to be the phytotoxic substances in *G. cingulata*[7]; but, what other disease mechanisms operate in ripe rot disease are unknown. The selection of ripe rot-resistant cells should prove useful for the production of disease-resistant plants. We here report the successful selection of the callus that resistant to the culture filtrate of *G. cingulata* from suspension-cultured somatic cells of 'Koshu' grapes using a plating-culture method.

Suspension-cultured grape cells were obtained from callus of *Vitis vinifera* L. cv. Koshu that was induced at 28°C in the dark from sliced green berries on Murashige-Skoog (MS) medium (pH 5.5) supplemented with 3% sucrose, 3 mg/*l* kinetin, 10 mg/*l* naphthaleneacetic acid (NAA), and 0.8% agar. For the cell suspension culture, about 2 g of fresh callus was inoculated in 40 m*l* of liquid MS medium (pH 5.5) containing 10 mg/*l* NAA and 0.01 mg/*l* benzylaminopurine (BAP) contained in a 100 m*l* Erlenmeyer flask. The cultures were agitated at 25°C in the dark on a gyratory shaker (100 rpm) and subcultured every 20 days. Before plating, cells that have been subcultured to 2-week periods were separated by filtering them through 250 μm and 94 μm mesh Miracloth, but not all the cells obtained were single ones even after filtration.

In preliminary experiment to establish the best conditions for plating-culture, filtered cells were suspended in a 2-fold concentration of predetermined optimum medium; Nitsch & Nitsch (1969)[8] (pH 5.2) containing 10 mg/*l* NAA, 0.01 mg/*l* zeatin, and 3% sucrose. One milliliter of the filtered cells and 1 m*l* of the jelling agent (agar (Wako), purified agar (Difco), agarose (Difco), sodium alginate (Wako) or Gellan gum (Wako)) that contained 2 mg of activated charcoal were mixed in a petri dish (30×15 mm) which then was sealed with parafilm and incubated at 25°C in the dark. The number of cells in the medium was adjusted to an initial concentration of about 1×10^4 cells/m*l*. Of the ten different conditions tested, the combination of 0.3% Gellan gum and activated charcoal in the medium was the most satisfactory for cell growth in the plating-culture method, as in grape protoplast culture[9] (data not shown). About 3.3% of the initial cell concentration (1×10^4 cells/m*l*) developed into small colonies more than 2 mm in diameter after 2 weeks of incubation. Abundant colonies developed on the optimum medium at the initial concentration of 1×10^5 cells/m*l*, but few developed at 1×10^3 cells/m*l*.

The fungal isolate from 'Koshu' grapes with ripe rot (**Fig. 1-A**) was identified as *G. cingulata*.

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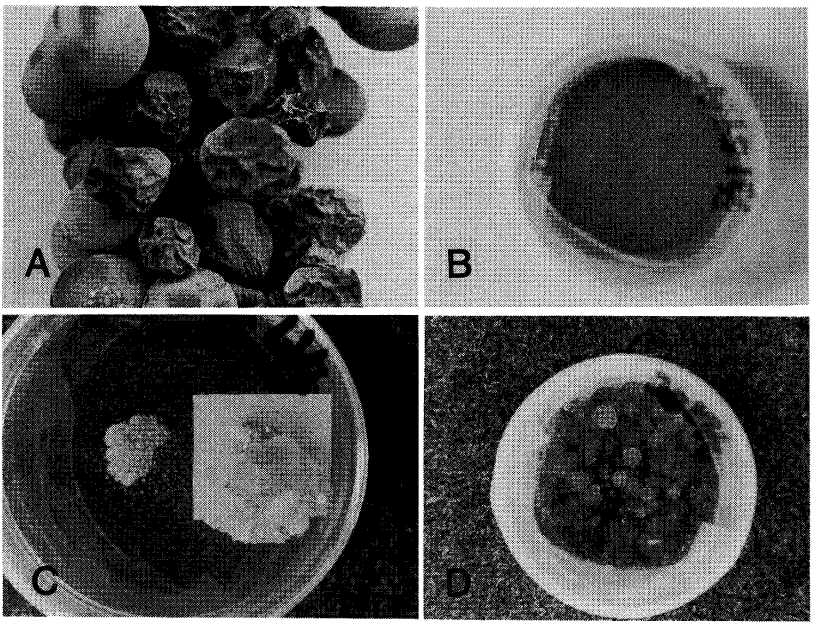

Fig. 1 (A) Ripe rot (Japanese: *banpu* or *osogusare*) induced by *G. cingulata* in 'Koshu' grapes.
(B) Complete inhibition of cell growth at the first 2-week selection. A 5% concentration of the *G. cingulata* filtrate prepared from a 40-day culture was added to the mixed medium. Colony formation on the plating culture medium was checked after 2 weeks.
(C) Nurse culture of callus generated from selected cells that resistant to the culture filtrate of *G. cingulata*.
(D) Colony formation from 'Koshu' grape cells on a mixed *G. cingulata* filtrate medium. A 10% concentration of *G. cingulata* filtrate prepared from a 40-day culture was added to the mixed medium. After 3-weeks of incubation, cells were derived from colonies formed by the first selected cells.

The fungus was incubated at 27°C in the dark in diluted 'Koshu' grape juice (°Brix 7, pH 3.5) as a static culture for 0, 10, 20, 30 and 40 days. Culture filtrates prepared by crude filtration and centrifugation were sterilized by filtering them through a 0.22 μm membrane. These filtrates were used as the selective agent by adding them at concentrations of 0-10% to the plating culture medium under the optimun condition; the combination of Gellan gum, activated charcoal and an initial cell concentration of 1×10^4 cells/m*l*. The effects of the period of incubation and the concentration of the *G. cingulata* filtrate on the inhibition of cell colony formation respectively are shown in **Figs. 2** and **3**. Colony formation was not inhibited completely by the filtrates of the 5% of 10, 20 and 30 -day- or 0-3% of 40-day-incubated *G. cingulata*. But, complete inhibition of cell growth took place on plates with 5% or 10% of the *G. cingulata* filtrate prepared from the 40-day culture during the first two weeks of incubation (**Figs. 1-B**, and **2**). After 3 weeks, growth of one to three colonies was seen on five plates of the same plating culture with the 5% 40-day-incubated *G. cingulata* filtrate. The classification of the cells that resistant to the culture filtrate of *G. cingulata* was based on the growth of the colonies at less than 3 weeks of plating culture. Colonies that grew on the selection medium were transferred immediately to a nurse culture on filter paper placed over a callus culture grown on MS agar medium containing 15 mg/*l* NAA and 3 mg/*l* kinetin (**Fig. 1-C**). Resistant cells from the second selection grew even at a 5% or 10% concentration of *G. cingulata* filtrate (**Figs. 1-D** and **3**).

By this method, we could successfully select 'Koshu' grape cells resistant to culture filtrate of

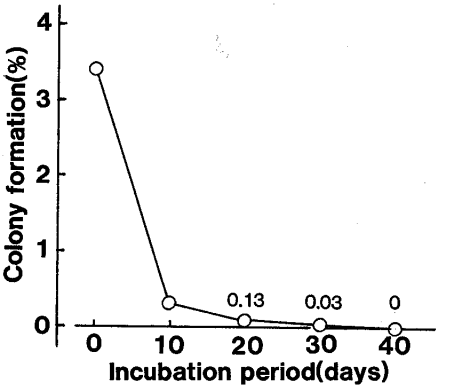

Fig. 2 Inhibitory effects of the incubation period of the *G. cingulata* filtrate on colony formation by 'Koshu' grape cells.
A 5% concentration (0.1 m*l* of filtrate added to 2 m*l* of culture medium) of the culture liquid from 0- to 40-day incubations of *G. cingulata* was used. After 2 weeks, colony formation on the plating culture medium was checked in 10 duplicates.

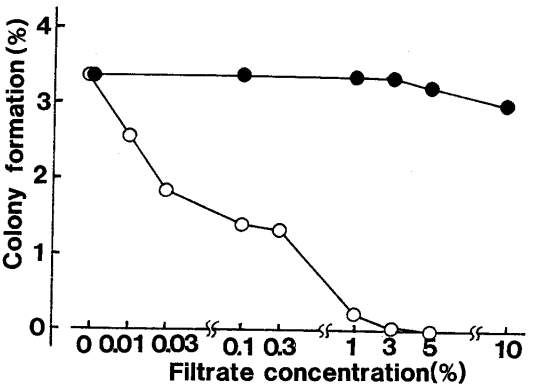

Fig. 3 Inhibitory effect of the *G. cingulata* filtrate concentration on colony formation by 'Koshu' grape cells.
A 0 to 10% concentration of the culture liquid filtrate from 40-day-incubated *G. cingulata* was added to the culture medium. After 2 weeks, colony formation on the plating culture medium was checked in 10 duplicates.
○——○ First selection: snspension-cultured cells obtained from callus tissue.
●——● Second selection: suspension cells obtained from cells in colonies formed from the first selection cells.

pathogenic fungus, *G. cingulata*. Further research on plants regenerated from selected callus cultures is necessary to obtain ripe rot-resistant grapevines.

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