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An Effective Culture of Ri Plasmid-induced Grape Hairy Roots†

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The breeding of transgenic grapes with the Ti or Ri plasmid is of interest in terms of promoting disease tolerance. Grape plantlets with the β-glucuronidase (GUS) gene have already been reported in earlier research as producing useful transgenic plants [1-4]. However, regenerations from transgenic tissues have been achieved in only a limited variety of grapes and in a defined part of the grape [1-4]. Furthermore, these regenerations are not consistently reproducible. Many problems must be solved before transgenic procedures using grapes can be employed in practice. It has been reported that Ri plasmid induction of the hairy root is better for breeding than that of the Ti plasmid, which requires regeneration via the callus [5]. Regeneration of a plantlet from the grape callus is generally considered to be more difficult than regeneration from the hairy root [6]. However, using conventional plating methods, the culturing of grape hairy roots was inefficient, laborious and tedious to perform, and appeared to be unsuccessful due to slow growth, lack of branching (a cause of diminished yields), and browning, resulting in cessation of growth. We investigated various methods of culturing hairy roots which were applicable to regeneration tests.

Two plantlets from the shoot tips of *Vitis vulpina* and *V. vinifera* L. Kosyu, obtained from Yamanashi-Fruit Tree Experimental Staion or the experimental vineyard of Yamanashi University, respectively, were used as the plant materials. They were maintained on 1/2 MS medium [7] solidified with 0.2% gellan gum (Kelco, USA) containing 0.05 mg/l naphtaleneacetic acid (NAA) and 1.5% sucrose at 25°C under 8,000 lux for 14 h and dark for 10 h. *Agrobacterium tumefaciens* R1000 [8] (pRiA 4 b)/pBI121 was used throughout the study. The plasmid pBI121 had the neomycin phosphotransferase II (NPT II) gene and the GUS gene [9]. The strain was inoculated into 5 ml of LB medium, containing 100 mg kanamycin/l (pH 7.2), with one loopful of seed culture from a stock culture that had been stored at −80°C, and precultured and agitated overnight at 28°C. Next, 100μl of the preculture was inoculated into a liquid medium (30 ml/100-ml conical flask) of the same composition as the preculture medium and cultured until late logarithmic phase. Segments (2 cm long) of stems excised from subculured plantlets grown for two months *in vitro* were infected through immersion in bacterial culture for 60 sec. The segments were then transferred to a filter paper for initial drainage and were subsequently co-cultured with *Agrobacterium* for 48 h in darkness at 22°C on petri dishes (ϕ9 cm) containing 20 ml of MS medium (sucrose; 3%, w/v) solidified with gellan gum (0.2%, w/v), with a filter paper placed on the surface. After co-cultivation, each explant was transferred to the medium described above, which contained Claforan (500 mg/l, 15 lots per plate, Hoechst Japan). The cultures were incubated in darkness (22°C) for 3-5 weeks. The roots that had been induced, and had reached 2 cm in length were excised and transferred to a fresh medium of the same composition. The new growth after this process was again transferred to a new plate containing the same medium. The procedures were repeated 3-4 times to remove bacteria and select for transformants. The transformed roots obtained were evaluated by the presence of GUS as determined using a histological assay according to the procedure described by Jefferson [10]. Positively stained roots were used as transgenic materials.

However, the transformed roots of the two varieties tested were not only slow-growing (0.05-0.10 cm/day) but no branching occurred on the plating medium as shown in **Fig. 1**. Therefore, considerable time was needed to obtain a specified amount of the root in a defined culture space. We then examined various culture conditions for the promotion of growth rate and branching. The following culture conditions were found to give superior results: The roots were acclimatized to a liquid medium of MS (40 ml/100-ml conical flask) containing 0.01% activated charcoal, 3% sucrose, and plant hormones (0.1 mg/l indole-3-butyric acid (IBA) plus 0.1 mg/l kinetin for *V. vulpina*; 0.1 mg/l NAA plus 0.1 mg/l benzylaminopurine (BAP) for Kosyu) at 80 rpm rotary agitation with an inoculum of 0.1 g (2 cm long × 5 lots) for 3 weeks in darkness at 27°C. During this period, almost no growth could be seen. However, when the acclimatized roots were

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transferred to a fresh medium of the same composition, vigorous new growth was observed using the same culture conditions as those described above with an increase to 100 rpm agitation. Though the cause of this growth improvement has not been sufficiently investigated yet, the transformed roots initially added (0.1 g) grew to 1.2-1.4 g in 10 days (**Fig. 2**), after which elongation of about 9 mm/day occurred with branching at 5 to 7 mm intervals for each variety (**Fig. 3**). Repeating the subcultures of Kosyu roots acclimatized to the liquid culture enabled us to increase yields more than ten-fold every 10 days, using 0.1 g of root as the initial amount in the liquid medium. However, on the plating medium, using the same initial amount, the growth in 10 days was no more than double. Growth obtained from the 10-day culture of the first subculture after acclimatization to the liquid culture was 6 times greater than when only the plating method was used. Similar results were obtained with *V. vulpina.*

If activated charcoal was not added to the medium, the samples turned yellowish in 3-4 days and eventually became brown with growth being completely interrupted. Activated charcoal was also effective against browning of grape protoplasts in culture [6].

The effect of combinations of plant hormones on growth was examined. NAA, 2, 4-dichlorophenoxyacetic acid (2, 4-D), and IBA were used as auxins, each at concentrations of 0, 0.05, 0.1, and 0.5 mg/*l*. BAP, Kinetin, and Zeatin were used as cytokinins, at the same concentrations as the auxins. The best combinations obtained were those reported above for each variety. Compared with the results for hormone-free growth, the growth of *V. vulpina* had increased by 1. 30-fold and that of Kosyu 1.25-fold in 10-day cultures with an average of five trials. The effect of agitation speed (0, 50, 100, and 150 rpm) and the method of agitation (rotary and reciprocal) on growth rates were also examined. The optimal results with both varieties were obtained at 100 rpm rotary agitation. Reciprocal agitation gave a growth rate of 63% of that obtained with rotary agitation (mean of three trials with Kosyu). Physical damage due to collision against the culture vessel appeared to occur with reciprocal agitation, since partial browning occurred throughout the transformed roots.

However, the roots obtained were still thin (ϕ0.5-0.

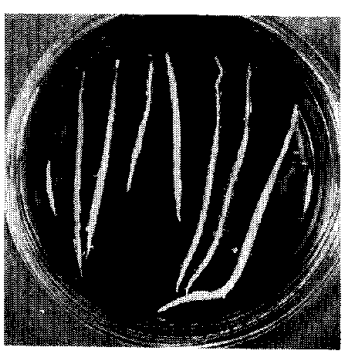

Fig. 1 No branching of transformed grape roots cultured on the plating medium.
Transformed roots from Kosyu were cultured for 10 days on MS plating medium at 25°C in the dark in petri dishes (ϕ9 cm) containing 20 m*l* of MS medium (sucrose; 3%, activated charcoal; 0.01%, w/v) solidified with gellan gum (0.2%, w/v).

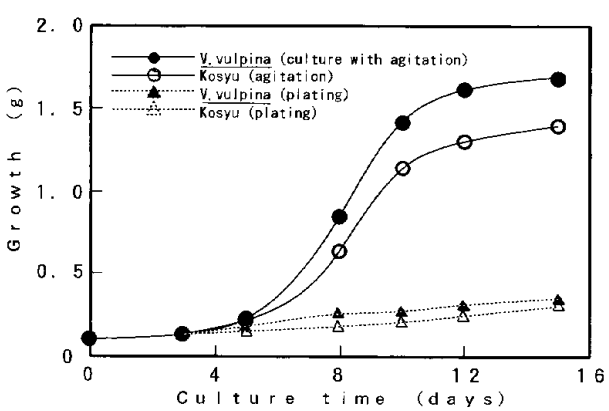

Fig. 2 Comparison of liquid culture and plating culture for the growth of transformed grape roots.
The roots acclimatized to a MS liquid medium were cultured as described in the text. With plating cultures, the transformed roots induced from the two varieties were cultured at 27°C in the dark, on medium that had the same composition as the liquid culture but had been solidified with gellan gum (0.2%, w/v).

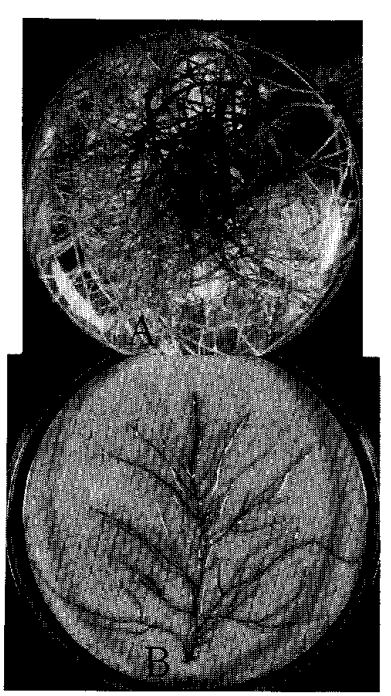

Fig. 3 Branching of transformed grape roots in the liquid culture.
Culture conditions were the same as those described in **Fig. 2.** A: Liquid culture of transformed grape (Kosyu) root. B: Branching of transformed grape (Kosyu) root.

7 mm) and they were not thickened by further culturing. In this case, necrosis also occurred with all samples transferred to the regeneration media reported by Nishimaki *et al.* [2]. (MS plate solidified with 0.2% gellan gum containing 0.2 mg/*l* 2-naphthoxyacetic acid (NOA) and 0.5 mg/*l* *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (4PU)). In an attempted to thicken them, the roots obtained through liquid culture were recultured through transfer to the MS plate media containing NAA (0.025 mg/*l*) and BAP (0.025 mg/*l*). The tested samples were grown to ϕ1.5-2.5 mm during a three-week period with two transfers per week. These thick roots showed virtually no necrosis upon transfer to the regeneration plates. However, the hormone balance (0.2 mg/*l* NOA and 0.5 mg/*l* 4PU) of the regeneration medium caused callus formation in the root samples tested. Through further culturing on an MS plate containing 2% sucrose, 0.3 mg/*l* 4PU, and 0.2% gellan gum under 5,000 lux at 24°C for 30 days, green tissues similar to adventitious buds had differentiated in 12 out of 17 samples tested. However, further differentiation was not observed.

As mentioned above, both the growth rates and the mass in both varieties tested were greatly improved by this combined method of using liquid and plated cultures as compared with the results using only the plating method. As an example, in a 65-day culture, transformed roots fitted for application of differentiation tests were obtained in at least 100 to 150-fold amounts by transferring them five times to a new medium with this method (*i.e.*, subculturing twice with a liquid culture, one transfer from a liquid to a plating culture, followed by two transfers using a plating culture). This is compared to results obtained using the plating method of subculturing every week for 8 times, using 0.1 g of root as the initial amount. Mass production of a root fitted for regeneration tests would have enabled us to investigate many conditions for regeneration at the same time.

The GUS activity in the root tissues was different in each lot, however, *rol* and GUS coding genes were identified in all of the chromosomal DNA samples (tested with 9 lots) prepared using the CTAB method [11], PCR and the Southern method [12] according to Kiyokawa *et al.* [13]. The transformed roots were identified as hairy roots by the existing *rol* genes. It was also verified that the genes transformed were not lost by these procedures.

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