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The Effects of Preservation Temperatures and Periods on Hairy Roots Inducing Ability of *Agrobacterium rhizogenes*

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The preservation of bacteria by long-term and short-term storage techniques are employed routinely because bacteria strains often lose desired properties and characteristics after repeated subculture. Gram-negative, soil phytopathogenic bacterium *Agrobacterium rhizogenes* is an efficient and widely used mediator of gene transfer to wide varieties of plants by inciting hairy root disease. The ability of *A. rhizogenes* to induce hairy roots is determined by a virulent Ri plasmid[1]. Continuous subculture or long term storage may result in the loss of plasmid and reduce the pathogenicity[2]. It is necessary to find convenient methods for maintaining the pathogenicity, not only survival, of *A. rhizogenes*.

Several methods are available for preservation of *Agrobacterium* species including L-Dry[3], frozen[4], lyophilization[5] and sterile water[6]. However, each method has its limitations. For example, the equipment for lyophilization and L-Dry is expensive and cultures cannot be returned to storage for reuse. Storage in sterile water carries a risk of mutation which resulted in change of morphological characters[6]. Freezing at −80°C may be a suitable alternative, and if possible, freezing at −20°C or storing at 4°C will be most helpful for most laboratories because the only major equipment is a household freezer.

In this experiment, *A. rhizogenes* was preserved at −80°C, −20°C, 4°C or 25°C. After 3 or 6 months of preservation, the pathogenicity was checked by comparing the hairy root inducing ability on tobacco.

A. rhizogenes strains IFO 14554, 14555, 15188, 15189, 15190, 15191 and 15192 were obtained from the Institute of Fermentation, in Osaka, Japan[7] and MAFF 301726, 301727, 210265, 210266, 210267, 210268 and 210269, from the Ministry of Agriculture, Forestry and Fishery, in Tsukuba, Japan[8]. These strains were regrown in YEB medium[9] for 2 days at 25°C. One m*l* of this suspension was inoculated to 40 m*l* of YEB medium and grown until $OD_{600}=0.8$. For the group of −80°C and −20°C, 40 m*l* of bactrial suspension was pelleted by centrifuging at 3500 rpm. After being washed with 10 mM $MgSO_4$, the bacteria pellet was resuspended with 20 m*l* HMFM (Hoghness Modified Freezing Medium)[2], distributed into Cryotube at 1.7 m*l*/tube and immediately frozen at −80°C or −20°C. For the group of 4°C and 25°C, one loop of the suspension ($OD_{600}=0.8$) was inoculated onto slant solid YEB medium, cultured at 25°C in the dark for 4 days and then stored at 4°C or 25°C. After preservation for 3 or 6 months, bacteria were regrown by culturing 0.5 m*l* sample of −80°C and −20°C or one loop of the bacteria suspension of 4°C and 25°C, respectively, into YEB medium for 3 days. One m*l* of the regrown culture were incubated to 40 m*l* YEB medium. When OD value reached to about $OD_{600}=0.8$, the bacterial suspensions were used for inoculation onto tobacco leaf segments to confirm the pathogenicity.

Plant material of tobacco (*Nicotiana glutinosa* var. Wisconsin Havana) were prepared by surface

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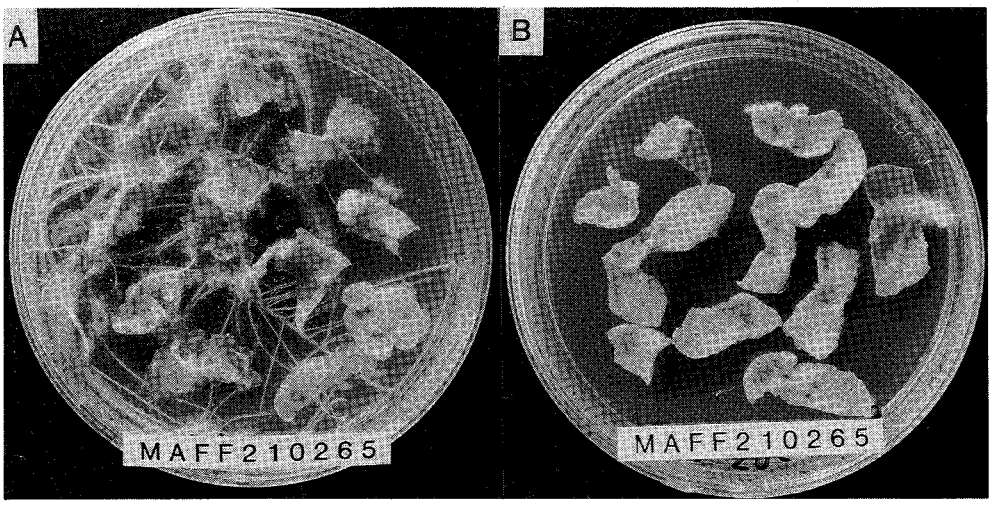

Fig. 1 Induction of hairy roots on tobacco leaf explants 20 days after inoculated with strain MAFF 210265 of *A. rhizogenes* preserved at (A) −80°C for 6 months and (B) 25°C for 3 months.

sterilizing seeds with 0.5% sodium hypochlorite for 20 min. and aseptically germinated on MS medium[10]. Leaves of 4 weeks old plantlets were cut into segments of about 1.5×0.7 cm^2 and dipped into bacteria suspension for inoculation. After shaking for 10 min., the inoculated segments were blotted dry and transferred to sterile water wetted filter paper. After 3 days of cocultivation, explants were transferred to hormone-free MS medium containing 500 mg/l cefotaxime and cultured under 16 hrs light at 25°C. Pathogenicity of these preserved *A. rhizogenes* were investigated by recording the percentage of leaf explants regenerating hairy roots 20 days after inoculation.

Adventitious roots were observed about 7 days after transferring to MS medium containing cefotaxime on some inoculated tobacco leaf explants. However, in many cases of preservation, pathogenicity was reduced or lost and *A. rhizogenes* failed to induce adventitious roots. After storage at −80°C for 6 months, strain MAFF 210265 still kept a high ability to induce hairy roots on tobacco (**Fig. 1-A**). In contrast, the same strain being preserved at 25°C for 3 months remarkably reduced the ability to induce hairy roots (**Fig. 1-B**). The adventitious roots were proved te be transformed hairy roots by opine assay, and from these tobacco hairy roots, MAFF 301726, 301727, 210265, 210266, 210267, 210268, 210269, IFO 14554 and 14555 were analyzed to be mikimopine type, while IFO 15188, 15189, 15190, 15191, and 15192, to be mannopine type (data not shown).

Preservation temperature and periods had a remarkable influence on the ability to induce tobacco hairy roots (**Table 1**). Bacteria preserved at −80°C for 6 months kept high pathogenicity when observing tobacco hairy roots induction. As to −20°C, preservation for 6 months reduced the pathogenicity showing large variation among the bacteria strains, although 3 months, of preservation did not significantly affect the pathogenicity. As to 4°C and 25°C, a wide range of pathogenicity was observed on various strains preserved for 3 months, and most of the strains lost the ability to induce hairy roots after 6 months of preservation.

In conclusion, −80°C was the most suitable preservation temperature for *A. rhizogenes* in this experiment. Pathogenicity was reduced after preserving at −20°C for 6 months. For short term preservation, 4°C was better than 25°C, although the maximum period might be 3 months. Survival of *A. rhizogenes* could not guarantee the existence of pathogenicity because the preserved *A. rhizogenes* reduced or lost the ability to induce hairy roots regardless of normal growth on nutrient

Table 1. Effects of preservation temperatures and periods on tobacco hairy roots inducing ability of *A. rhizogenes*.

strain	−80°C	−20°		4°C		25°C	
	6 months*1	3 months	6 months	3 months	6 months	3 months	6 months
MAFF 301726	100± 0*2	100± 0	53± 3	82± 8	0	70± 0	0
MAFF 301727	100± 0	100± 0	64± 0	100± 0	11± 1	100± 0	0
MAFF 210265	82± 2	100± 0	25±13	73± 3	0	20±10	11± 1
MAFF 210266	90±10	100± 0	22± 3	55± 5	20± 0	54± 5	0
MAFF 210267	100± 0	100± 0	100± 0	100± 0	0	100± 0	20± 0
MAFF 210268	100± 0	82± 2	54± 5	64± 6	0	55± 5	0
MAFF 210269	100± 0	90±10	47± 3	90±10	0	84± 6	0
IFO 14554	100± 0	80±10	40±10	100± 0	13± 0	84± 6	13± 0
IFO 14555	100± 0	100± 0	40±10	100± 0	30±10	100± 0	30±10
IFO 15188	100± 0	100± 0	20± 0	60±10	16± 4	47± 6	16± 4
IFO 15189	100± 0	70±10	0	64±17	16±15	17± 3	16±15
IFO 15190	91± 1	100± 0	16± 4	46± 4	0	64± 8	0
IFO 15191	100± 0	100± 0	25± 5	54± 4	0	85±14	0
IFO 15192	90±10	100± 0	0	36± 3	0	55± 5	0

*1 Preservation periods of 3 or 6 months.

*2 Percentage of hairy roots induced explants ± S. E. recorded 20 days after inoculation. Each value is the mean of three repeated experiments and each treatment consisted of 10 replicates.

medium.

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