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In Vitro Testing of Rose Rootstocks Resistance to Crown Gall Disease

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Summary

Fifteen rose rootstocks which were cultured and propagated in vitro were inoculated with the strain GOU1 of *Agrobacterium tumefaciens* as a test for resistance to the crown gall disease.

Rosa multiflora and *R. multiflora* 'K2' were highly susceptible, 95% and 90% of the stems forming large tumors, 6.61 and 3.62 (mm × mm), respectively. Three varieties of *R. canina*, 'Pfänder', 'Superbe', and 'Brögs Stachellose', and a native one became infected but formed small tumors (0.5 mm × mm).

In *R. rugosa* and *R. canina* 'Superbe' 40% of the infected shoots formed tumors during the first week after inoculation. Thus, these species have a low resistance to tumor formation, whereas *R. virginiana* formed no tumor during the first week after inoculation; 73% of the shoots produced tumors 3 weeks after inoculation. Therefore, *R. virginiana* is subject to infection but resistant to tumor formation.

Key Words: crown gall disease, in vitro inoculation, resistance, rootstock, rose.

Introduction

Crown gall, caused by *Agrobacterium tumefaciens* Conn., is devastating disease of roses. It has been difficult to control this disease by soil disinfection and agricultural chemicals. Pre-inoculation with *Agrobacterium radiobacter* strain 84 imparts resistance to this disease; the number of successful cases are impressive, but some applications were unsuccessful (Moore, 1979).

Utilizing resistant varieties is the most desirable method to control crown gall disease. Boelema (1969) selected 'ISU 60-5' as a resistant rootstock from seven rootstocks. Ohta (1993), however, reported that 'ISU 60-5' was not resistant. This disagreement in resistance of 'ISU 60-5' is attributed to the use of difference of strains of *A. tumefaciens* and varying environments after inoculation (Ohta, 1993). Additionally the growing conditions of the rose plant, i.e. season, soil, nutrient conditions, and method of inoculation, affect resistance. Brown (1923) found that disease incidence differed among seasons. To avoid these environmental effects on resistance, we developed an universal in vitro inoculation method for testing resistance to crown gall disease in rose (Zhou et al., 1996). Using this method, we selected 'PEKcougel' as a resistant variety out of 24 cut-rose and miniature rose varieties (Zhou et al., 1999). In this study, we report the variability in resistance among 15 rose rootstocks to crown gall disease.

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Materials and Methods

Plant materials

The 15 rose rootstocks used are listed in Table 1. The shoot tips of these rootstocks were cultured in vitro on MS medium (Murashige and Skoog, 1962), containing 3% sucrose, 0.2% gelrite, 1×10^{-7} M gibberellic acid A_3 (GA_3), and 1×10^{-5} M or 3.2×10^{-6} M benzylaminopurine (BAP) (Table 1). All rootstocks made good growth and formed no callus on these media.

Pathogenic bacteria

The strain GOU1, of *A. tumefaciens*, was isolated from a rose tumor in Gifu, Japan (Zhou et al, 1999) and used as a source of inocula. The isolation method for pathogenic bacteria was as follows: fresh and actively growing galls were surface-sterilized for 10 min. in a 1% (v/v) sodium hypochlorite solution containing 0.01% (w/v) tween-20. The sterilized galls were diced (5 × 5 mm) and then homogenized in 0.1% (w/v) peptone solution. Single colony isolates were obtained from this suspension by culturing on medium 2E (Brisbane and Kerr, 1983). Isolates were then subcultured on YEB medium containing Bacto Beef Extract (Difco Laboratorise) 5 g · liter^{-1}, Bacto Yeast Extract (Difco Laboratorise) 1 g · liter^{-1}, $MgSO_4 \cdot 7H_2O$ 0.493 g · liter^{-1}, peptone 5 g · liter^{-1}, sucrose 5 g · liter^{-1}, and

Table 1. The rose rootstocks studied and the benzylaminopurine (BAP) concentrations in the growth medium[z].

Rose rootstock	BAP concentration (M)
Rosa multiflora	1.0×10^{-5}
R. multiflora 'K2'	1.0×10^{-5}
R. coriifolia froebelii	1.0×10^{-5}
R. rugosa	1.0×10^{-5}
R. eglanteria	1.0×10^{-5}
R. virginiana	1.0×10^{-5}
R. canina	1.0×10^{-5}
R. canina 'Pfänder'	1.0×10^{-5}
R. canina 'Inermis'	1.0×10^{-5}
R. canina 'Brögs Stachellose'	3.2×10^{-6}
R. canina 'Heinsohns Rekord'	3.2×10^{-6}
R. canina 'Superbe'	1.0×10^{-5}
R. 'Kuiper'	1.0×10^{-5}
R. 'Uniform'	1.0×10^{-5}
R. 'Veendam'	3.2×10^{-6}

[z] Basal medium details are given in the text.

agar 15 g · liter^{-1} . The pH was adjusted to 7.2. The gall induction by bacterial isolates was tested on tomato and rose by the needle prick inoculation method. The PCR analyses methods described by Haas et al. (1995), Sachadyn and Kur (1997), and Sawada et al. (1995) were used to confirm that the bacterial isolates were *A. tumefaciens*. The bacteriological characteristics of the isolates were identified by the following tests: colony morphology on D-1 agar medium, production of 3-ketolactose, levan formation, growth at 35 ℃ , and tolerance to NaCl.

Inoculation method

Excised shoots were cultured in vitro for 6 weeks, cut into 10-15 mm lengths each having 2-4 leaves, and inoculated with *A. tumefaciens* strain GOU1 that had been cultured for 24 hr at 25 ℃ on YEB medium. Twenty shoots per rootstock species except *R. virginiana* which had 15 shoots, were inoculated as above. The inoculated shoots were cultured for 6 weeks on media suitable for each rootstock (Table 1) at 25 ℃ under a 16-hr photo period. The number of that shoots formed tumors and tumor sizes were recorded weekly after inoculation. The disease incidence is given as a percentage of the shoots with at least one tumor among the inoculated shoots. The size of a tumor was calculated as the product of its maximum length and its maximum width (mm × mm).

Results and Discussion

After inoculation, white or pale green tumors formed on diseased shoots (Fig. 1). The disease incidence 6 weeks after inoculation ranged from 65% in *R. canina* 'Brögs Stachellose' to 95% in *R. multiflora* (Table 2). The mean number of tumors per shoot varied from 1.0 in *R. virginiana* to 3.0 in *R. coriifolia froebelii* (Table 2). Shoots of *R. multiflora* formed the largest tumors.

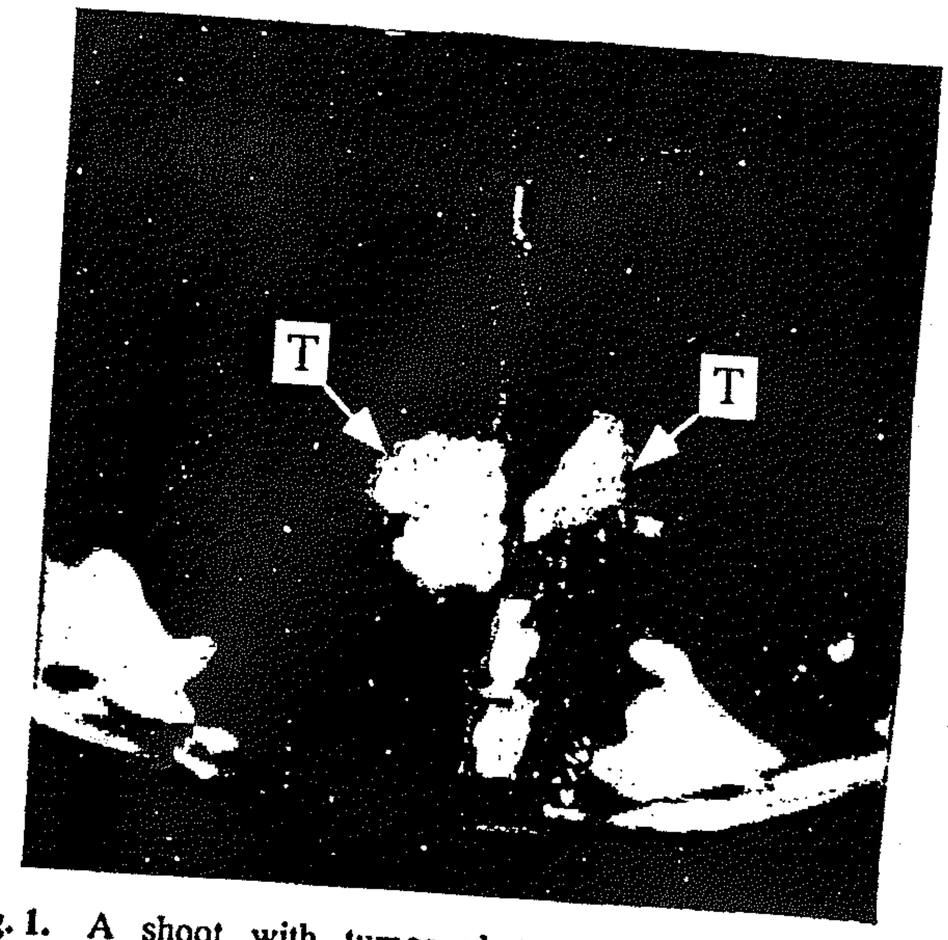

Fig. 1. A shoot with tumor photographed 6 weeks after inoculation.
T: tumor.

The data (Table 2) reveal differences to susceptibility in *A. tumefaciens* strain GOU1 among the six native rose species. *R. multiflora* was the most susceptible, 95% succumbing and forming large tumors (6.61 mm × mm). *R. coriifolia froebelii* exhibited a high disease incidence (90%), but formed small tumors (0.45 mm × mm). Disease incidence in *R. rugosa*, *R. eglanteria*, *R. canina* and *R. virginiana* ranged from 73 to 85 %; tumor size was under 0.83 (mm × mm) (Table 2).

Boelema (1969) found that *R. multiflora* was highly susceptible to crown gall disease, which was confirmed by our in vitro experiment. Therefore, we decided that our in vitro inoculation method yields reliable and consistent results.

Comparison among varieties of *R. multiflora* and *R. canina* in this experiment revealed that the disease incidence was high for *R. multiflora* 'K2' and for the native variety, and the tumor size of both was also extremely large, although there was significant difference between the native variety and 'K2'.

'K2' is a variety used for rootstock that was selected from *R. multiflora* at Kanagawa Horticultural Experimental Station, Japan. Ohta (1993) reported that 'K2' was resistant to *A. tumefaciens* because it formed no tumor. However, Okayama et al. (1988) observed that 'K2' became infected and formed tumors after inoculation with *A. tumefaciens*, whereas we found 'K2' with tumors in commercial rootstock nurseries. Those differences may have been caused by different inoculation methods and growing conditions of plant, or by different strains of *A. tumefaciens*. A suspension of concentrated pathogenic bacterial inoculum induced

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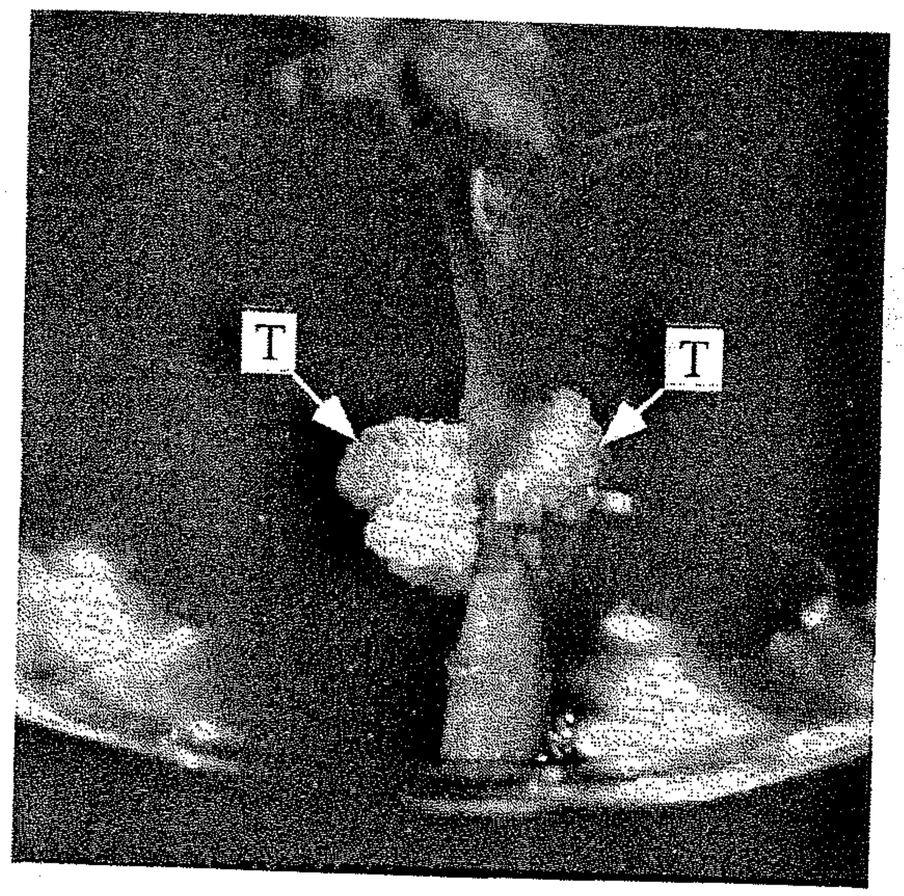

Fig. 1. A shoot with tumor photographed 6 weeks after inoculation.
T: tumor.

'Brögs Stachellose' to 95% in *R. multiflora* (Table 2). The mean number of tumors per shoot varied from 1.0 in *R. virginiana* to 3.0 in *R. coriifolia froebelii* (Table 2). Shoots of *R. multiflora* formed the largest tumors.

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Table 2. Response of rose rootstocks to *Agrobacterium tumefaciens* strain GOUI 6 weeks after inoculation.

Rose rootstock	Disease incidence[z] (%)	Mean number of tumors[y]	Tumor size[x] (mm × mm)
Rosa multiflora	95	2.5	6.61 ± 12.83a[w]
R. multiflora 'K2'	90	2.2	3.62 ± 4.32b
R. canina 'Pfänder'	90	2.5	0.29 ± 0.31c
R. canina 'Inermis'	90	2.1	0.74 ± 0.85c
R. coriifolia froebelii	90	3.0	0.45 ± 0.27c
R. canina 'Heinsohns Rekord'	85	1.7	0.73 ± 0.57c
R. canina 'Superbe'	85	2.3	0.41 ± 0.26c
R. rugosa	85	2.3	0.79 ± 0.61c
R. eglanteria	80	1.8	0.46 ± 0.49c
R. 'Kuiper'	80	1.5	0.69 ± 0.49c
R. 'Uniform'	80	2.1	0.32 ± 0.33c
R. 'Veendam'	75	1.5	0.45 ± 0.34c
R. canina	75	1.7	0.40 ± 0.34c
R. virginiana	73	1.0	0.83 ± 0.55bc
R. canina 'Brögs Stachellose'	65	1.2	0.46 ± 0.45c

[z] The percentage of shoots with tumors among all inoculated shoots. The number of inoculated shoots were 15 in *R. virginiana*, and 20 in the other rootstocks.
[y] Mean number of tumors per shoot (shoots without tumors excluded).
[x] Tumor size were calculated as the product of the maximum length and the maximum width.
[w] Means ± S. E. s with different letters are significantly different at the 5% level.

higher rates of disease incidence than that of a dilute suspension (Ohta, 1993). In this experiment, the use of bacterial cultures without dilution for inoculum resulted in a high disease incidence and large tumors in 'K2'.

Comparing varieties in *R. canina*, the disease incidence varied from 90% in *R. canina* 'Pfänder' to 65% in *R. canina* 'Brögs Stachellose'. The size of tumors was equally small among varieties of this species (Table 2), but that in *R. canina* was statistically smaller than that of *R. multiflora*.

Tumor development is related to plant growth and development. Stomp et al. (1990) reported that the difference of gall size among *Pinus* species was due to stump sprouting, which was regulated by an endogenous auxin, and/or cytokinin. In our trial, shoots of *R. multiflora* produced many lateral shoots, whereas those of *R. canina* had less. The formation of lateral shoots is also regulated by an endogenous auxin and/or cytokinin. This characteristic may be related to the susceptibility to tumor development.

We reported in a previous paper (Zhou et al., 1999) that the resistance in rose involved two processes. One is the resistance to tumor formation, which is expressed as the disease incidence, and the other is the resistance to tumor development, which is expressed by the size of tumors. No varieties in *R. canina* were resistant to tumor formation, as over 65% became infected. However, a native one and three of its varieties, 'Brögs Stachellose', 'Superbe', and 'Pfänder', were resistant to tumor development, growing to only 0.5 mm × mm. Two other varieties, 'Inermis' and 'Heinsohns Rekord', formed smaller tumors than did *R. multiflora* (Table 3).

The infection process of *A. tumefaciens* involves the binding of bacteria to the host plant cell, the transporting of T-DNA on the Ti-plasmid into the host plant cell, the integration of T-DNA into the chromosome, and autosynthesis of auxin and cytokinin (Parke et al, 1987; Braun, 1962). Since *R. canina* and these varieties had no resistance to tumor formation, it seems that *A. tumefaciens* bound to those cells, transported T-DNA, and integrated T-DNA into those chromosomes. However, varieties of *R. canina* which resist tumor development indicate that the autosynthesizing of phytohormones and the division of transformed tumor cells were inhibited. We are currently investigating this process of resistance in *R. canina*.

The period of tumor formation after inoculation differed among rootstocks (Fig. 2). In *R. virginiana*, no tumors formed in the first week, and 45% of the shoots formed tumors during the third week after inoculation; subsequently new tumor formation decreased (Fig. 2a). Over 40% of diseased shoots in *R. canina* 'Superbe' and *R. rugosa* formed tumors during the first week after inoculation, but the rate of infection decreased afterward (Fig. 2b). In the other rootstocks, few infections occurred after the first week, but 30-60% of the shoots formed tumors by the end of the second week (Fig. 2c). Brown (1923) reported that the period from inoculation

Table 3. Classification of resistance to crown gall disease among rose rootstocks.

Resistance to tumor formation	Rootstock	Resistance to tumor development	Rootstock
Low	*Rosa multiflora*	Low	*Rosa multiflora*
	R. multiflora 'K2'		*R. multiflora* 'K2'
	R. canina 'Pfänder'	Moderate to High	*R. virginiana*
	R. canina 'Inermis'		*R. rugosa*
	R. coriifolia froebelii		*R. canina* 'Inermis'
	R. canina 'Heinsohns Rekord'		*R. canina* 'Heinsohns Rekord'
	R. canina 'Superbe'		R. 'Kuiper'
	R. rugosa		*R. canina* 'Brögs Stachellose'
	R. eglanteria		*R. eglanteria*
	R. 'Kuiper'		*R.* 'Veendam'
	R. 'Uniform'		*R. coriifolia froebelii*
	R. 'Veendam'		*R. canina* 'Superbe'
	R. canina		*R. canina*
	R. virginiana		*R.* 'Uniform'
	R. canina 'Brögs Stachellose'		*R. canina* 'Pfänder'

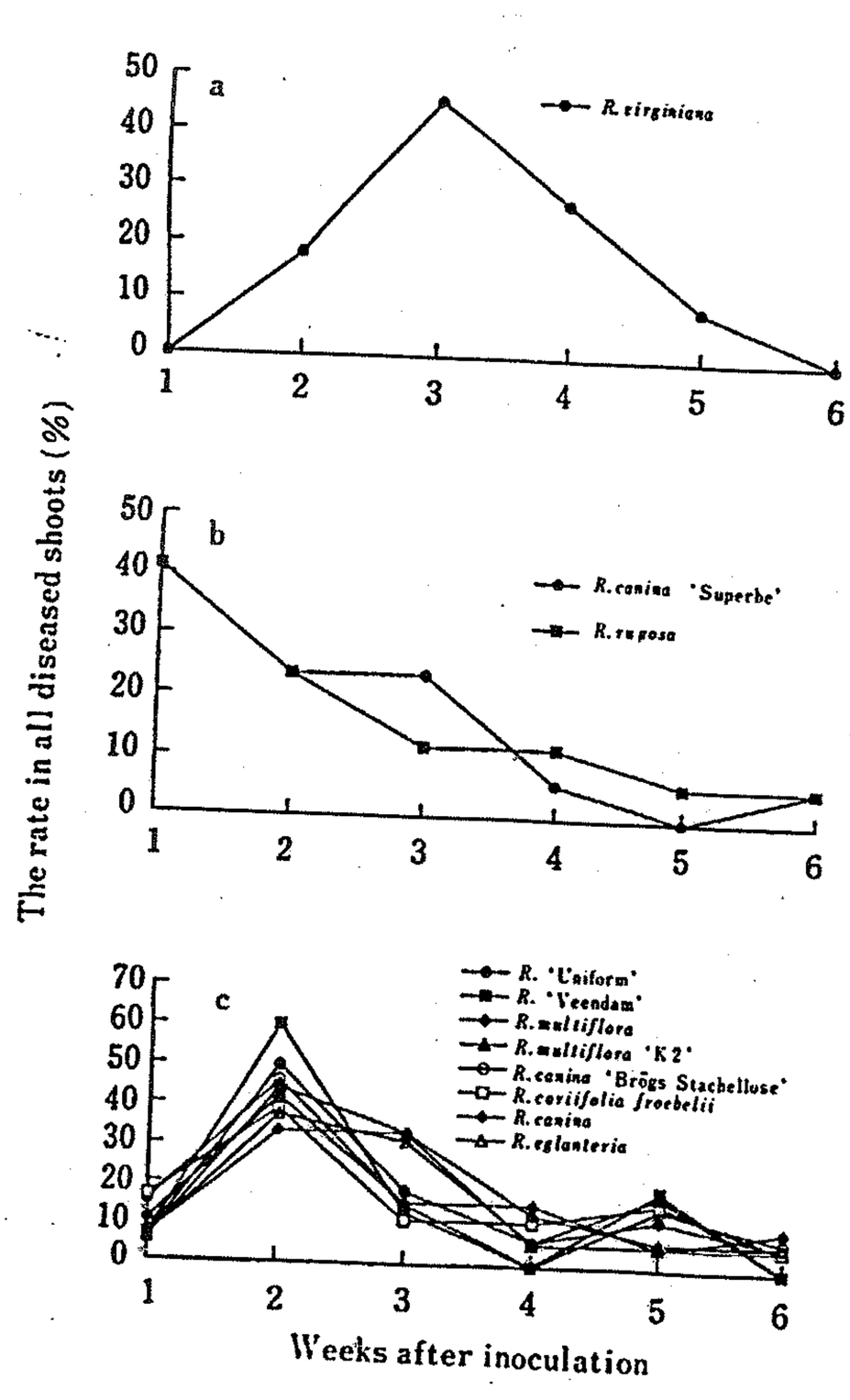

Fig. 2. The rate of new tumor formed per week after inoculation.

to tumor formation differed among cut rose varieties. This difference may be related to resistance to tumor formation. *R. virginiana*, therefore, may have some resistance to tumor formation, whereas *R. canina* 'Superbe' and *R. rugosa* may not.

Zhou et al. (1999) found that the disease incidence in cut roses varied between 0% to 100%, and the tumor size varied between 0.19 to 4.75 (mm × mm). In these species except *R. multiflora*, *R. coriifolia froebelii*, *R. rugosa*, *R. eglanteria*, *R. canina* and *R. virginiana*, 73 to 90 % of shoots became infected with tumor size under 0.83 (mm × mm). The narrow variation within these five species is contrary to that among commercial cut rose cultivars. Since these five species have not been commercialized (Wylie, 1954), the wide variations in susceptibility among cut roses and tumor size are derived from other *Rosa* species.

Utilizing these rootstocks may be effective against the crown gall disease. *R. multiflora*, however, is used widely as a rootstock in Japan, because it grows vigorously in Eastern Asia. Those of *R. canina* and other European rootstock varieties are rarely used because they have not adapted to the high temperatures and humidity in Japan. In a previous paper, we reported that 'PEKcougel', a hybrid-tea rose, had a strong resistance to crown gall disease. We are now crossing 'PEKcougel' and *R. multiflora* with the hope of obtaining a crown gall resistant variety of *R. multiflora*.

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In vitroでのバラ台木の根頭がん腫病抵抗性検定

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摘　要

In vitroで継代・維持しているバラ台木15系統を供試材料とし，根頭がん腫病菌(GOU1)を用い，in vitro検定法で根頭がん腫病に対する抵抗性を検討した．

バラ台木15系統のうち，*R. multiflora*および*R. multiflora* 'K2'ではそれぞれ95%，90%と高い発病率を示し，形成されたがん腫も6.61，3.62 (mm × mm)と大きく，病徴形成抵抗性 (Resistance to tumor formation)と病徴発達抵抗性 (Resistance to tumor development)ともに弱かった．*R. canina*およびそれに由来する3系統'Pfänder'，'Superbe'，'Brögs Stachellose'では発病率が高く，病徴形成抵抗性が低かったが，形成されたがん腫は0.5 (mm × mm)以下と小さく，病徴発達抵抗性が強かった．

*R. rugosa*と*R. canina* 'Superbe'はがん腫形成時期が早く，接種1週間後には40%ががん腫を形成し，病徴形成抵抗性が低かった．それに対して，*R. virginiana*は接種1週間後にがん腫が形成されず，ほとんどすべてのがん腫が3週間後から形成され，病徴形成過程において何らかの抵抗性を示すものと推定された．