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Development of Bioassay for Screening of Resistant Roses against Root Rot Disease Caused by *Pythium helicoides* Drechsler

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Rose root rot disease caused by *Pythium helicoides* has caused serious economic losses in rose production in Japan. The use of resistant rootstock against root rot disease is a valuable strategy toward legal restrictions and consumer concerns against fungicide application. So, the establishment of a bioassay for the selection of resistant roses is very important. In this study, the utility of the original inoculation method by a self-made Ebb & Flow watering system was examined using a bioassay for the selection of resistant roses. *Rosa multiflora* 'Matsushima No. 3' and *R.* 'Nakashima 91' were used as resistant and susceptible plant materials, respectively. Rooted cuttings were inoculated by soaking them with a zoospore suspension for 1 h in the situation of them being planted in a plug tray without transplanting, and then zoospore-containing nutrient solution was given from the tank of the Ebb & Flow system 4 times every day, for 2 min each time. Although the conventional phytopathological bioassay could not distinguish between 'Matsushima No. 3' and 'Nakashima 91' in terms of disease severity, the resistibility of 'Matsushima No. 3' and susceptibility of 'Nakashima 91' to root rot disease were confirmed through the original bioassay developed in this study, and the reproducibility was high. Therefore, it could be concluded that this bioassay was suitable for the screening of resistant varieties. Histological observation indicated that the density of hyphae in cortical cells of 'Matsushima No. 3' was less than that of 'Nakashima 91'. Hyphae penetrated into the endodermal tissue in 'Nakashima 91', whereas they were inhibited from expanding to cortical cells of 'Matsushima No. 3'.

Key Words: bioassay, *Pythium helicoides*, resistance, root rot disease, rose.

Introduction

The Ebb & Flow watering system for potted plant production was introduced to Japan in 1985 for water economy, and it spread immediately. The recirculation of nutrient solution was further introduced to the Ebb & Flow watering system around 1990 from the viewpoint of environmentally-friendly agriculture. Root rot disease has been found in this system since August 1996, and *Pythium helicoides* was isolated from the rotted root of *Rosa* 'Nakashima 91' in potted miniature rose production (Kageyama et al., 2002). The same root rot disease has also been observed in rock-wool culture for cut roses and has spread all over the country. We proved that metalaxyl, oxadixyl, and echlomezole were effective chemicals against root rot disease, but the use of fungicides is strictly regulated by the Agricultural Chemicals Regulation Law in Japan, and growers refrain from the use of fungicides based on the desire for environmentally-friendly agriculture. The use of resistant rootstock against root rot disease is showing signs of encouragement in the field of integrated pest management.

In 1998, root rot disease spread in greenhouses involved in rock-wool culture for cut rose production of *R.* 'Dukat' in Gifu Prefecture. Plants which were grafted on *R. multiflora* 'Matsushima No. 3' showed no disease symptoms, but others with their own root and grafted on *R.* × *odorata* died. We have reported that 'Matsushima No. 3' was susceptible to crown gall disease. From the results mentioned above, it was suggested that 'Matsushima No. 3' might have resistance to *P. helicoides* and be suitable for use as a root rot-resistant rootstock.

Development of the proposed bioassay is important to select resistant rootstocks. A bioassay to confirm the infection of *P. helicoides* has been established by our research group (Kageyama et al., 2002). However, it is difficult to apply this phytopathological bioassay to the

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selection of resistant roses against root rot disease, because this bioassay is not suitable to evaluate exact resistance and to test many plants. So, a bioassay for the selection of resistant roses against root rot disease was newly developed in this study, and the reproducibility and precision of the bioassay were evaluated using 'Matsushima No. 3' and 'Nakashima 91', which were estimated as resistant and susceptible, respectively.

Materials and Methods

P. helicoides B-5, which was isolated from the rotted roots of *R.* 'Lavender Parade' and had a high pathogenicity (Kageyama et al., 2002), was placed on the center of corn meal agar (CMA) medium, and 1 cm pieces of autoclaved grass blades were placed on the CMA medium around the *Pythium*. After incubation at 25°C for 24 h, the colonized grass blades were transferred to 9 cm autoclaved Petri dishes containing 10 mL of autoclaved water (distilled water : pond water = 2 : 1, v/v). The grass blades were incubated for 24 h at 25°C again, and then zoospores were collected from mycelium by the method of slowly pouring the suspension into a beaker through gauze (Waterhouse, 1967). The concentration of zoospores was adjusted to 6.0×10^3 per mL for inoculation.

As plant materials, cuttings of 'Matsushima No. 3' and 'Nakashima 91' were put in plug trays with 200 plug cells filled with autoclaved soil (BM-2, Berger Horticultural Products Ltd., Quebec, Canada) : perlite = 1 : 1, v/v), and then cuttings were rooted at 25°C, with over 90% relative humidity, and 4000 lx for 18 h per day. After incubating for 3–4 weeks, rooted cuttings were transferred to a growth chamber which was kept at 30–35°C and 3000 lx for 24 h per day and acclimated for a week. Rooted cuttings in plug trays were cultured by the self-made Ebb & Flow system, which consisted of two cultivation trays (300 mm × 630 mm × 90 mm), a water pump controlled by an electric timer, and a nutrient solution tank of 45 L (Fig. 1). Nutrient solution for cut roses in rock-wool culture was supplied to the cultivation trays 4 times every day for 2 min from the nutrient solution tank.

After acclimation, rooted cuttings in plug trays were soaked in zoospore suspension (4 L per cultivation tray) for 1 h, and the zoospore suspension was then drained into the tank with the nutrient solution. The inoculated cuttings in the plug trays were grown for 7 days in the same conditions as per acclimation. Non-inoculated cuttings were used as a control. A treatment had 30 cuttings with three experimental repetitions.

To compare the developed bioassay mentioned above with a phytopathological bioassay, rooted cuttings were also inoculated by Kageyama's method (Kageyama et al., 2002). Rooted cuttings transplanted to 6 cm pots with inoculated soil were grown at 30°C in natural light in a growth chamber for 7 days. The treatment had 10 cuttings with five experimental repetitions.

Root rot was evaluated at 3, 5, and 7 days after inoculation. Root symptoms were visually estimated at 3, 5, and 7 days after inoculation using a disease index from 0 to 3: 0 = no root rot symptom was observed (healthy and white root), 0.5 = a part of the root was slightly brown, 1 = a part of the root was brown, 1.5 = 50% of the root was brown, 2 = 75% of the root was brown, 2.5 = the root was completely brown but the plant survived, and 3 = the root was completely brown and the plant died. Root rot severity was calculated as:

Root rot severity
= Σ(disease index × number of plants at each severity)/(maximum disease index × total number of plants) × 100

After the evaluation of root rot severity at 3, 5, and 7 days after inoculation, the re-isolation of *P. helicoides* from roots was tested. The roots were washed with tap water and excess water was removed with filter paper. They were then cut into 5 mm segments and ten root segments were plated on *Pythium* selective medium (AP2) containing CMA amended with 5 mg·L^{-1} pimaricin, 100 mg·L^{-1} agrimycin, and 50 mg·L^{-1} pentachloronitrobenzene (Kageyame and Ui, 1980). Root segments were incubated at 35°C in the dark for 24 h.

Re-isolation rate
= Σ(root pieces from plants with *Pythium* isolated/total root pieces)/number of plants × 100

Roots evaluated for root rot severity were classified as white, partly brown, and completely brown and were sliced in serial sections (80–100 μm) by a plant microtome (NK system, Osaka, Japan). Sections were stained with cotton blue and observed under an optical microscope.

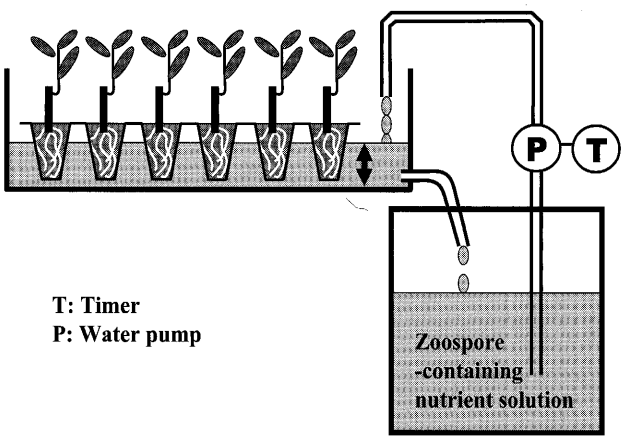

Fig. 1. A self-made Ebb & Flow watering system was used for the evaluation of root rot disease. Rooted cuttings in plug trays were soaked with the zoospore suspension for 1 h, and then zoospore-containing nutrient solution was circulated in the plant bed and tank 4 times every day for 2 min each time.

Results

Root browning was observed after inoculation (Fig. 2). Most non-inoculated roots were white and healthy (Fig. 2A), and inoculated roots at 3 days after inoculation were observed to be slightly brown. Primary root rot was observed at the root-tip or the middle of roots (Fig. 2B), and roots with a higher disease index increased according

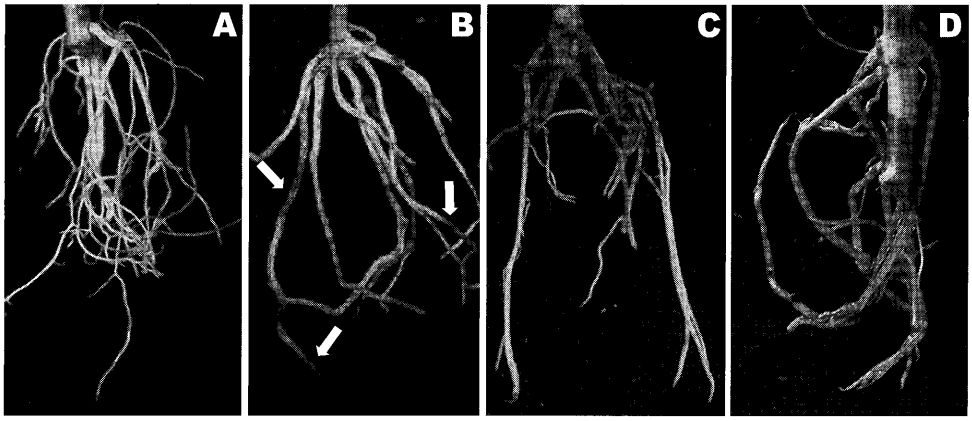

Fig. 2. Root browning caused by *Pythium helicoides*. A: A healthy, white root evaluated as 0 in the disease index, B: A partly-browned root evaluated as 1.0, and primary root rot was observed at the root-tip or the middle of the root (arrows), C: A half-browned root at 5 days after inoculation in 'Nakashima 91', D: All the root was browned at 7 days after inoculation in 'Nakashima 91'.

Table 1. Root rot severity and re-isolation of inoculated and non-inoculated *Rosa multiflora* 'Matsushima No. 3' and *R.* 'Nakashima 91'.

	Root rot severity in new bioassay						Root rot severity in Kageyama's bioassay[z]	
Days after inoculation	3		5		7		7	
Cultivar	Root rot severity	Re-isolation (%)	Root rot severity	Re-isolation (%)	Root rot severity	Re-isolation (%)	Root rot severity	Re-isolation (%)
Non-inoculation								
Matsushima No. 3	0.0±0.0[y]	0.0	0.9±0.8	0.0	0.9±0.8	0.0	8.7±19.4	0.0
Nakashima 91	0.0±0.0	0.0	0.0±0.0	0.0	0.0±0.0	0.0	7.2±11.3	0.0
Inoculation								
Matsushima No. 3	2.0±0.8	98.6	3.7±1.5	100	4.8±2.7	100	72.0±19.7	94.8
Nakashima 91	10.3±4.6	97.8	19.0±5.7	100	25.8±2.8	100	61.3±13.9	95.6
F-test[x]	NS		*		**		NS	

[z] Data from five experimental repetitions with 10 cuttings. Inoculation was performed according to Kageyama's pathogenicity test (Kageyama et al., 2002).
[y] Means±SE (n=10).
[x] NS, *, and ** represent non-significance, and significance at $P=0.05$ and $P=0.01$, respectively.

to days after inoculation (Fig. 2B–D).

Root rot severity of inoculated and non-inoculated 'Matsushima No. 3' and 'Nakashima 91' at 3, 5, and 7 days after inoculation are shown in Table 1. Root rot severity in non-inoculated 'Nakashima 91' was 0 regardless of days after inoculation, and all roots were healthy and white. Those in non-inoculated 'Matsushima No. 3' at 3, 5, and 7 days after inoculation were 0.0, 0.9, and 0.9 respectively, and roots of 'Matsushima No. 3' were observed to be partly brown. However, this browning of roots was not due to infection with *P. helicoides*, because no *P. helicoides* was re-isolated from brown roots.

Root rot severity in inoculated 'Matsushima No. 3' increased slightly from 2.0 to 4.8 according to days after inoculation, and *P. helicoides* was re-isolated from most roots. However, there was no significant difference among root rot severities at 3, 5, and 7 days after inoculation and also between root rot severity in inoculated and non-inoculated roots at 7 days after inoculation.

In inoculated 'Nakashima 91', root rot severity was already 10.3 at 3 days after inoculation and increased to 25.8 at 7 days after inoculation. As the re-isolation rate was approximately 100, root browning was due to infection with *P. helicoides*. Comparing the root rot severity of inoculated 'Matsushima No. 3' with 'Nakashima 91', there was a significant difference at 5 days after inoculation and highly significant difference at 7 days after inoculation.

Root rot severity in Kageyama's bioassay is shown in Table 1. Regardless of non-inoculation, root rot severities were over 7.0 in two roses and standard errors were high. In inoculated 'Matsushima No. 3' and 'Nakashima 91', root rot severities were 72.0 and 61.3 at 7 days after inoculation, respectively, and many roots were completely brown. The level of root rot severity, however, was not definite in every experiment because of high standard errors.

Percentages of each disease index of inoculated and

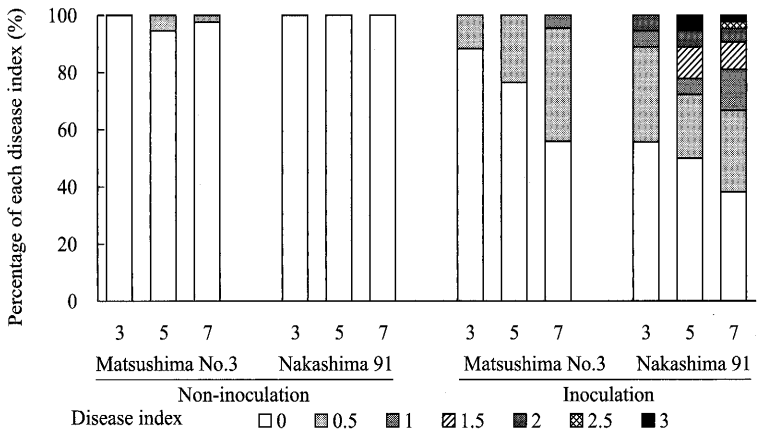

Fig. 3. Percentage of each disease index in non-inoculated and inoculated *Rosa multiflora* 'Matsushima No. 3' and *R.* 'Nakashima 91' with *Pythium helicoides* at 3, 5, and 7 days after inoculation.

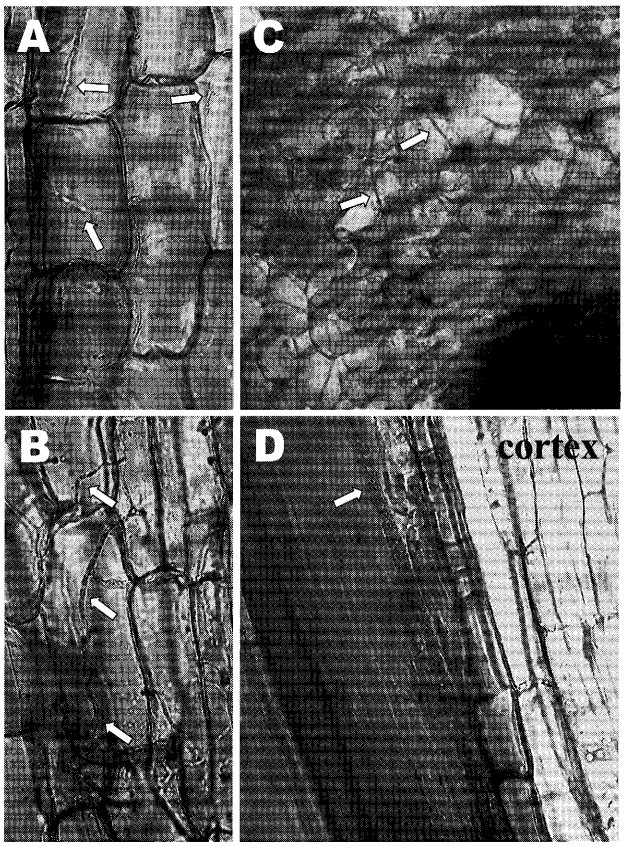

Fig. 4. Hyphal expansion into the cortex and endodermis at 7 days after inoculation. A: Hyphal invasion into cortical cells in the slightly-browned root of 'Matsushima No. 3', B: Hyphal expansion into cortical cells in the slightly-browned root of 'Nakashima 91', C: Hyphal expansion into cortical tissue in the completely-browned root of 'Nakashima 91', D: Hyphal penetration into an endodermal cell in the completely-browned root of 'Nakashima 91'. Arrows: hypha.

non-inoculated 'Matsushima No. 3' and 'Nakashima 91' are shown in Fig. 3, respectively. In inoculated 'Nakashima 91', the percentage with a disease index of 0.5 was about 30% regardless of days after inoculation, and that of over 1.0 increased according to days after inoculation. The total percentage with a disease index over 0.5 increased from 44.5% at 3 days after inoculation to 62.0% at 7 days after inoculation. The percentage with a disease index of 0.5 in inoculated 'Matsushima No. 3'increased from 11.8% to 39.5% according to days after inoculation, and that with a disease index of 1.0 was 4.7% at 7 days after inoculation. The other disease index, which indicated severe root rot symptoms, was not detected, and root rot in 'Matsushima No. 3' showed only an slight symptoms.

From the observation of non-inoculated root tissue by an optical microscope, no hyphae were found in white roots of both 'Matsushima No. 3' and 'Nakashima 91'. On the inoculated roots, however, hyphae were observed in cortical tissue. In the partly brown roots of 'Nakashima 91' and 'Matsushima No. 3', hyphae expanded into the cortical tissue of roots and were observed in tissue cells (Fig. 4A, B). Although the density of hyphae in the cortical tissue of 'Nakashima 91' was found to be higher than that of 'Matsushima No. 3', the difference of hyphal density could not be evaluated quantitatively. In the white part of roots adjacent to the browned part, hyphae were also observed in the cells of cortical tissue in the same way as the partly-browned root, and the hyphal density in the cortical tissue of 'Nakashima 91' was more than that in 'Matsushima No. 3'. Hyphae in the completely-browned roots of 'Nakashima 91' spread around the cortical tissue (Fig. 4C) and also penetrated to the endodermis (Fig. 4D). Although hyphae in the cortical tissue of 'Nakashima 91' expanded more and more according to the severity of the symptoms, expansion in the cortical tissue of 'Matsushima No. 3' was inhibited.

Discussion

In order to evaluate phytopathological pathogenicity, the development of bioassays is important. In root rot disease caused by *P. helicoides*, we have developed a bioassay for the purpose of distinguishing infected roses and evaluated the pathogenicity of isolates of *P. helicoides* (Kageyama et al., 2002, 2003). This bioassay, which does not need special equipment and

obtains a high disease index, had valuable characteristics for phytopathological evaluation.

For the control of root rot disease, the use of resistant rootstock was effective from the viewpoint of environmentally-friendly agriculture, and development of the bioassay for the selection of resistant roses was important. We firstly attempted to select resistant varieties by the phytopathological method; however, both 'Matsushima No. 3' and 'Nakashima 91' indicated a high disease index, as shown in Table 1, and no difference in root rot severity was observed between them, even though they were estimated as resistant and susceptible varieties, respectively. A possible reason could be injury to the root by transplanting rooted cuttings to pots with inoculated soil. This may have led to an excessive disease severity because hyphae could penetrate easily into the root from the wound. In addition, although the size of the pot was sufficient in the case of testing a few varieties, a growth chamber with a big capacity is necessary to evaluate many varieties. Therefore, this conventional phytopathological bioassay has some problems in its application for the selection of resistant roses.

The developed bioassay in this study could evaluate the normal response to the disease of the variety, because rooted cuttings were not transplanted to pots and roots were not damaged. As shown in Table 1, the root rot severity of susceptible 'Nakashima 91' was significantly higher than that of resistant 'Matsushima No. 3'. In addition to the absence of injury, the soaking with zoospore suspension for 1 h and the repeated watering with the zoospore-containing nutrient solution also increased the chances for zoospores to adhere to roots, and the reproducibility was high, as shown by the low standard errors. Regarding the water content in plug trays, the Ebb & Flow system was introduced as a watering system to maintain the soil moisture after inoculation, and it promoted the attachment, encystment, and germination of zoospores on roots. These improvements in the inoculation method could bring about a high repeatability.

P. helicoides belongs to the group of high temperature *Pythium* species, and a high infection rate has been shown in high-temperatures over 30°C (Kageyama et al., 2002). But, a temperature over 30°C markedly stressed plant materials, and so the rose roots turned brown in spite of there being non-inoculated plant materials in the preliminary experiment. Therefore, we reduced the stress on plants by acclimatization for a week. Although the disease index in non-inoculated 'Nakashima 91' was 0 and all roots were white, 2.4–5.6% of roots had a disease index of 0.5 in non-inoculated 'Matsushima No. 3' (Fig. 3). This result suggested that 'Matsushima No. 3' had a lower heat tolerance than 'Nakashima 91'.

The disease severity of inoculated 'Nakashima 91' clearly increased according to days after inoculation, and 'Nakashima 91' was decided to be a susceptible rose to root rot disease. In 'Matsushima No. 3', the disease severity increased slightly according to days after inoculation (Table 1 and Fig. 3). Although it has been suggested that 'Matsushima No. 3' might show resistance to *P. helicoides* from field observations, 'Matsushima No. 3' was not truly resistant but field resistant or tolerant based on our results. Generally, a truly resistant variety would prevent hyphae from penetrating into the cortical tissue, as reported by Xi et al. (2000). Hyphae, however, were also observed in the cortical tissue of the slightly-browned roots in 'Matsushima No. 3' (Fig. 4), and 'Matsushima No. 3' could not prevent the hyphae of *P. helicoides* from penetrating into the cortical tissue. Therefore, 'Matsushima No. 3' would be defined as field resistant or tolerant, as reported by Widmer et al. (1998).

In comparison with 'Matsushima No. 3' and 'Nakashima 91' from the viewpoint of hyphal expansion into root tissue, hyphae extended into the cortex in 'Matsushima No. 3', whereas hyphae penetrated into the endodermis in 'Nakashima 91'. The roots in which hyphae had penetrated into the endodermis turned completely brown and had a disease index over 2.0. We supposed that there was no penetration into the endodermis in 'Matsushima No. 3' because it did not have a disease index over 1.5. The low disease index in 'Matsushima No. 3' might indicate that the cortical cells prevented hyphae from elongating into the cortex.

In the developed bioassay, rooted cuttings were inoculated in the situation of them being planted in plug trays without transplanting to pots, and the self-made Ebb & Flow system consisted of two cultivation trays. Therefore, it was possible for 20 rose varieties with 20 cuttings to be bioassayed at once using two plug trays with 200 plug cells. The development of an effective bioassay for root rot disease was one of the necessary steps for breeding disease-resistant varieties. From the results of this study, it could be concluded that this bioassay was suitable for the screening of resistant varieties, because all procedures for the bioassay were put into practice in a controlled laboratorial space, and the results showed good reproducibility. Recently, many diseases caused by zoosporic pathogens such as *Pythium* and *Phytophthora* species have been reported in ornamental plants (Hüerli et al., 2004; Tojo et al., 2006; Zhang et al., 2003). This developed inoculation method for the selection of resistant roses against *P. helicoides* can be successfully applied to other crops.

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