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Root Colonization by Beneficial *Pseudomonas* spp. and Bioassay of Suppression of Fusarium Wilt of Radish.

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유용 *Pseudomonas* 종의 근면점유와 무우 Fusarium시들음병의 억제에 관한 생물학적 정량

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ABSTRACT: Fusarium wilt of radish (*Raphanus sativus* L.) is caused by the *Fusarium oxysporum* f. sp. *raphani* (FOR) which mainly attacks *Raphanus* spp. The pathogen is a soil-borne and forms chlamydospores in infected plant residues in soil. Infected pathogen colonizes the vascular tissue, leading to necrosis of the vascular tissue. Growth promoting beneficial organisms such as *Pseudomonas fluorescens* WCS374 (strain WCS374), *P. putida* RE10 (strain RE10) and *Pseudomonas* sp. EN415 (strain EN415) were used for microorganisms-mediated induction of systemic resistance in radish against Fusarium wilt. In this bioassy, the pathogens and bacteria were treated into soil separately or concurrently, and mixed the bacteria with the different level of combination. Significant suppression of the disease by bacterial treatments was generally observed in pot bioassy. The disease incidence of the control recorded 46.5% in the internal observation and 21.1% in the external observation, respectively. The disease incidence of *P. putida* RE10 recorded 12.2% in the internal observation and 7.8% in the external observation, respectively. However, the disease incidence of *P. fluorescens* WCS374 which was proved to be highly suppressive to *Fusarium* wilt indicated 45.6% in the internal observation and 27.8% in the external observation, respectively. The disease incidence of *P. putida* RE10 mixed with *P. fluorescens* WCS374 or *Pseudomonas* sp. EN415 was in the range of 10.0-22.1%. On the other hand, the disease incidence of *P. putida* RE10 mixed with *Pseudomonas* sp. EN415 was in the range of 7.8-20.2%. The colonization by FOR was observed in the range of 2.4-5.1$\times 10^3$/g on the root surface and 0.7-1.3$\times 10^3$/g in the soil, but the numbers were not statistically different. As compared with 3.8$\times 10^3$/g root of the control, the colonization of infested ROR indicated 2.9$\times 10^3$/g root in separate treatments of *P. putida* RE10, and less than 3.8$\times 10^3$/g root of the control. Also, the colonization of FOR recorded 5.1$\times 10^3$/g root in mixed treatments of 3 bacterial strains such as *P. putida* RE10, *P. fluorescens* WCS374 and *Pseudomonas* sp. EN415. The colonization of FOR in soil was less than that of FOR in root part. Based on soil or root part, the colonization of ROR didn't indicate a significant difference. The colonization of introduced 3 fluorescent pseudomonads was observed in the range of 2.3-4.0$\times 10^7$/g in the root surface and 0.9-1.8$\times 10^7$/g in soil, but the bacterial densities were significantly different. When growth promoting organisms were introduced into the soil, the population of *Pseudomonas* sp. in the root part treated with *P. putida* RE10 was similar in number to the control and recorded the low numerical value as compared with any other treatments. The populatoin density of *Pseudomonas* sp. in the treatment of *P. putida* RE10 indicated significant differences in the root part, but didn't show significant differences in soil. The population densities of infested FOR and introduced bacteria on the root were high in contrast to those of soil. *P. putida* RE10 and *Pseudomonas* sp. EN415 used in this experiment appeared to induce the resistance of the host against Fusarium wilt.

KEYWORDS: Bioassay, Beneficial organisms, Root colonization, Radish, Fusarium wilt, Population density

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Fusarium wilt diseases are responsible for yield losses on numerous crops. Since the use of agrochemicals are adversely affecting the quality of the products and of the environment, the development of alternative ways to control disease is a high priority (Lyon *et al.*, 1995).

Biological control of soil-borne plant pathogens with bacteria has been studied as an alternative or complementary approach to physical and chemical disease control measures for over 70 years (Wellers, 1988). Some soils are known for their natural supppressiveness to *Fusarium* wilts. The soil microflora is responsible for the natural suppressiveness of these soils (Alabouvette, 1986; Louvet *et al*, 1976; Scher and Baker, 1980; Smith and Snyder, 1972). When the biological control organisms were introduced to the disease conducive soil, the *Fusarium* wilt was significantly suppressed (Alabouvette, 1986; Kloepper *et al.*, 1980; Paulitz *et al.*, 1987; Leeman, 1995). However, the inoculation of disease suppressing microorganisms to the disease conducive soil never reach the level of suppression observed in the natural suppressive soils, and the effects are often inconsistent (Weller, 1988). A multitude of factors could account for the inconsistent performance of biocontrol agents, due to the complex interactions among the *Pseudomonas* strain, the pathogen, the plant and the environment (Schippers, 1992; Weller, 1988).

Studies on the population dynamics of introduced bacterial strains demonstrated that their population densities on roots of single strains tended to decrease in time (Bakker *et al*, 1987; Weller, 1984). Thus, the inefficient root colonization by introduced strains of *Pseudomonas* spp. was considered as a major cause of the inconsistent results obtained under field conditons (Schippers, 1992; Weller, 1988).

The importance of root colonization by fluorescent pseudomonads for biocontrol of soilborne plant pathogens was emphasized by Bull *et al.* (1991), who demonstrated an inverse linear relationship between the population size of *P. fluorescens* 2-79RN 10 on seminal root of wheat and suppression of take-all by *Gaeumannomyces graminis* var. tritici. Suppression of *Fusarium* wilt of radish by pseudomonads through fluorescent siderophore mediated competition for ferric iron was reported by Scher and Baker (1982). Growth promoting effects on radish by fluorescent pseudomonads were first published by Kloepper and Schroth (1978) and later by Geels *et al* (1985).

In the Netherlands, *Fusarium* wilt of radish was a problem in continuous cropping of radish in green houses. In this study, the mechanisms responsible for the beneficial effect of separate or mixed treatment of three different bacterial strains on disease suppression to *Fusarium* wilt of radish were evaluated on different levels of disease incidence and the relation between root colonization and suppression of *Fusarium* wilt by fluorescent pseudomonads in rhizosphere was investigated as well.

Materials and Methods

Radish cultivar

The radish (*Raphanus sativus* L.) cultivar Saxa Nova (S and G Seeds V.B., Enkhuizen, the Netherlands) that was moderately resistant to fusarium wilt, was used in this experiment.

Bacterial strains and growth media

A wild type strain of *Pseudomonas fluorescens* WCS374 (WCS374) was a plant growth promoting and disease suppressing rhizobacterium isolated from the potato rhizosphere (Geels and Schippers, 1983). One strain of *P. putida* RE010 (RE10) was isolatd

from endophyte of radish and *Pseudomonas* sp. EN415 (EN415) was isolated from endorhizosphere of tomatoes (van Peer and Schippers, 1989). For the preparation of bacterial suspensions, strains were cultured on KB-agar (King *et al.*, 1954) for 48 hr at 27°C, harvested in 0.1 M $MgSO_4 \cdot 7H_2O$ and washed twice by centrifugation (5,000 g, 10 min.).

Preparation of inoculum of *Fusarium oxysporum* f .sp. *raphani*

Fusarium oxysporum f. sp. *rhaphani* (FOR), the causal agent of *Fusarium* wilt of radish, was grown in an aerated 2% malt extract medium. After 9 days of growth at room temperature, the cultures were filtered through glass wool to remove mycelial mats of the culture. Microconidia left in the filterate were pelleted by centrifugation (5,000 g,10 min.) and washed twice with 0.01 M $MgSO_4 \cdot 7H_2O$. The conidial density of FOR was determined by direct observation using a haemocytometer.

Pot bioassay

For a bioassay to test suppression of *Fusarium* wilt of radish by 3 bacterial strains, the potting soil was mixed with non-sterile river sand in a 2:1 ratio (v/v). This mixture was sieved (5 mm mesh). Conidial suspensions of FOR were introduced into this soil at the density of 10^5 conidia per gram of soil. The infested soil was incubated for 5 days in the dark under controlled conditions with a 16 hr period at 24°C followed by an 8 hr period at 20°C. For bacterization, serial dilutions of suspensions (2×10^9 cfu/m*l* and 2×10^{10}) of WCS 374, RE10 and EN415 were introduced into autoclaved soil (20 m*l* per 600 g) and stored for 24 hr at 5°C. The mixing ratio of FOR infested soil, bacterized soil, and non-sterile river sand were mixed at the rate of 1:1:5 ratio (w/w/w). The spore density of FOR in this soil mixture was approximately 10^4 cfu/g soil, as determined by dilution plate technique on Komada agar plates (Komada, 1975). The bacterial strains were introduced in sandy soil at different levels of densities and combinations of 10^5 and 10^7 cfu/g soil. In making a pot soil, the pots were composed of different bacteria and mixtures. Treatments were as follows: the control, RE10, WCS374, EN415, RE10+WCS 374, RE10+EN415, WCS374+EN 415, and RE10+WCS374+EN415. PVC pots (110 mm high, 140 mm diameter) were filled with the soil mixture. Radish seeds (*Raphanus sativus*) were sown at the depth of 1 cm. Each treatment consisted of nine replicates of 10 plants. Plants were grown under relative humidity of 70% and light conditions of 16 hr at 24°C and then under relative humidity of 70% and dark conditions of 8 hr at 20°C. Plants were watered twice a week. Once a week, plants were watered with 200 ml of nutrient solutions (Raaijmakers, 1994).

Disease severity

Suppression of radish wilt caused by *Fusarium oxysporum* f. sp. *raphani* (FOR) was observed in pot bioassay with three bacterial strains of *Pseudomonas putida* RE010 (RE10), *P. fluorescens* WCS374 (WCS374) and *Pseudomonas* sp. EN415 (EN415). Typical disease symptoms, chlorosis, necrosis or death were observed and internal symptoms was scored. After root harvesting, the cross sections of taproots were made for scoring internal wilting symptom. The distinct symptom inclued browinsh discolorations of the vascular system. In the bioassays, the number of diseased radish plants were determined approximately 27 days after plant emergency. Beside scoring external wilting symptoms, the cross-sections of the tap root were made at 2 cm below the tuber base and examined for brownish discoloration of the vascular system. For each treatment, the percentage of diseased plants was determined from nine replicates of ten plants.

Enumeration of the population of introduced bacterial strains and FOR

For the preparation of soil suspensions, two grams of the bacterized soil from each pot was suspende d in 10 ml sterile 0.1 M $MgSO_4 \cdot 7H_2O$ and shaken vigorously for 60 sec in glass test tubes containing 2.5 g of glass beads (0.17 mm diameter). Plants were harvested from the PVC pots after loosening the soil surrounding the plants. Loosely adhering soil was removed from the roots by gentle shaking. Rhizoplane (root) suspensions were prepared from randomly sampled root segments (0.5 g) from each pots at the depth of 0-8 cm from the stem base. These segments were suspended in 5 ml sterile 0.1 M $MgSO_4 \cdot 7H_2O$ and shaken vigorously for 30 sec in glass test tubes containing 2.5 g of glass beads (0.17 mm diameter). Aliquots (0.1 ml) of serial dilutions of soil and rhizosphere suspensions were mixed homogeneously through selective agar such as Komada and KB media for estimating the number of colony forming units (CFU). Samples were also plated on Komada agar (Komada, 1975) modiefied as described by Gams and van Laar (1982) for estimating the total number of CFU of *Fusarium oxysporum*, and on KB media (King *et al*, 1954) for introduced fluorescent *Pseudomonas* spp. Root colonization was expressed as the value of the numer of CFU per gram of fresh root weight and soil sample in a pot soil. Root colonization was carried out in two replications of the experiment.

Results

A percentage of external disease symptom was showed in Table 1. As for an investigation of external symptoms, the rate of disease incidence was observed in the treatment of WCS374 strain showing 27.8% of diseased plant as compared with 21.1% of the control, but the rate of disease incidence was significantly lower than any other bacterial treatment. Especially, strain RE10 or mixed strains of RE10 + WCS374 + EN415 lowered the disease rate at the level of 7.8% (Table 1). On the other hand, the disease incidence of radish based on internal symptom was the highest (45.6%) in separate treatments of strain WCS374, and this was similar to that of the control (46.5%). Also, strain RE10 showed the low percentages of disease incidence, indicating 12.2% in separate treatments of strain RE10 and 23.3% in concurrent treatments of mixing strain RE10 with strain WCS374 and EN415, respectively. A significant reduction of the diseased plant indicated 12.2% in separate treatments of

Table 1. Comparison of external and internal disease incidence caused by *F. oxysporum*. f. sp. *raphani* in radish plant with different bacterial treatments in pot bioassay for 4 weeks of culture

Treatment	Disease rate (%)[a]			
	External	symptom	Internal	symptom
Control	21.1±12.7	ab	46.5±18.8	a
RE10	7.8±8.3	be	12.2±6.70	cd
WCS 374	27.8±21.1	a	45.6±25.1	ab
EN 415	12.2±8.3	b	26.7±12.3	c
RE 10+WCS 374	10.0±11.2	bd	18.6±13.1	cd
RE 10+EN 415	11.1±10.5	bc	22.1±12.0	ce
WCS 374+EN 415	13.3±12.3	ac	23.3±14.1	cd
RE 10+WCS 374+En 415	7.8±8.3	be	20.2±10.0	cd

[a] Value within tests denoted by the same letter are not significantly different according to DMRT ($P<0.05$)

strain RE10 and 18.6% in mixed treatments of RE10 + WCS374. Correlation between external and internal disease symptom was analyzed in Fig. 1. There was a significant linear relationship (R^2=0.843) between external and internal symptom, suggesting that internal symptom was highly correlated with external symptom or disease incidence.

The root colonization by the introduced bacterial strains of RE10, WCS374 and EN 415 against pathogen FOR was observed in pot bioassay. The population densities of FOR in root of radish and pot soil were shown in Table 2. In comparison with 3.8×10^3/g root of the control, FOR populations in root were the highest in mixed treatments of three bacterial strains such as RE10 + WCS374 + EN415, indicating 5.1×10^3/g root, and also were relatively high in separate treatments of strain WCS374 + EN415 indicated 2.4×10^3/g root whereas the separate treatments of strqain RE10 indicated 2.9×10^3/g root. FOR populations in soil were high in mixed treatments of three bacterial strains such as Re10, WCS 374 and EN415 and recorded 1.3×10^3/g soil. However, the reduction of FOR populations were most remarkable in the results of separate treatments of strain EN415 indicating 0.7×10^3/g soil or in those of mixed treatments of strain RE10 and WCS374 indicating 0.8×10^3/g soil. FOR populations counted were not significantly different in root or soil (Table 2). The correlation of the numbers of FOR between root and soil was analysed for their relationship (Fig. 2). There was no a significant relationship (R^2=0.028) between the root and soil.

The number of cell density of introduced fluorescent *Pseudomonas* spp. was observed in radish plant and soil (Table 3). The number of pseudomonads colonized on root tissues was significantly different in the treatments. Low number of bacteria was observed in separate treatments with strain RE10, indicating 2.3×10^7/g root whereas high number was observed in mixed treatments with strains RE10 + EN415,indicating 4.0×10^7/g root) (Table 3). On the other hand, the density in soil was in the range of $0.9\text{-}1.8\times0^7$/g soil, but there was no a significant relationship in each treatment. Compairing the number of density in root and soil, many numbers were populated on root surface (Table 3). Correlation of the introduced bacteria between root and soil was analyzed in Fig. 3. There was no relation-

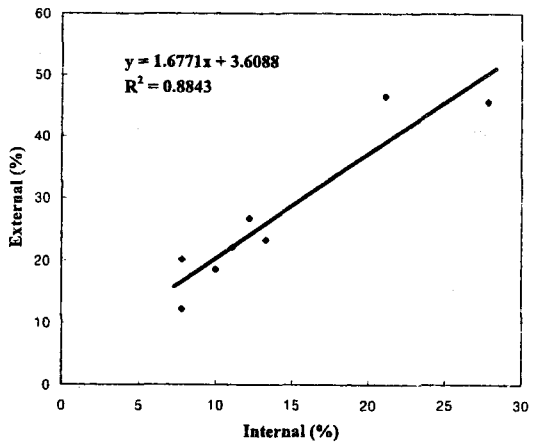

Fig. 1. Correlation of disease incidence between internal and external symptoms.

Table 2. Population of introduced *F. oxysporum.* f. sp. *raphani* in radish root with soil with different treatments of 3 bacterial strains

Treatment	cfu $\times10^3$/g[a]	
	Root	Soil
Control	3.8±1.4[a]	1.1±0.5[a]
RE10	2.9±2.2[a]	1.3±0.4[a]
WCS 374	4.5±2.7[a]	1.0±0.5[a]
EN 415	3.2±1.9[a]	0.7±0.4[a]
RE 10+WCS 374	3.9±1.8[a]	0.8±0.5[a]
RE 10+EN 415	3.2±1.1[a]	1.2±0.5[a]
WCS 374+EN 415	2.4±1.3[a]	1.0±0.2[a]
RE 10+WCS 374+En 415	5.1±3.4[a]	1.3±0.8[a]

[a] Value within tests denoted by the same letter are not significantly different according to DMRT ($P<0.05$)

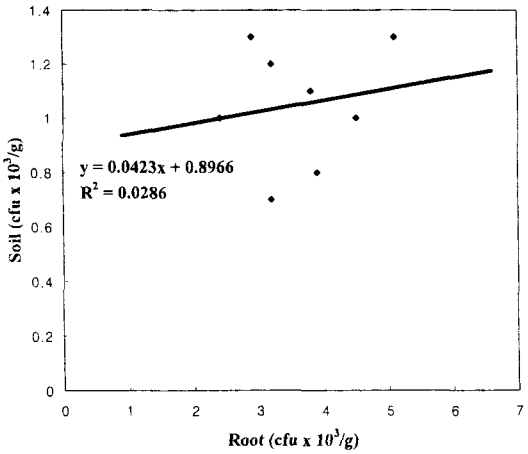

Fig. 2. Correlation of propagle densities of *F. oxysporum* f. sp. *ruphani* between root and soil.

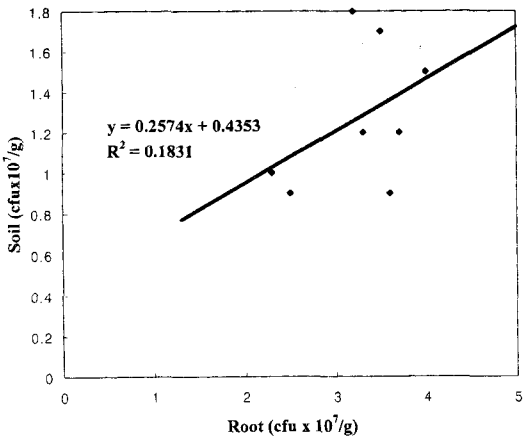

Fig. 3. Correlation of fluorescent *Pseudomonas* spp. between root and soil.

Table 3. Population of introduced *Pseudomonas* f. sp. in radish root and soil

Treatment	cfu × 10^7/g[a]	
	Root	Soil
Control	2.5±0.6[b]	0.9±0.4[a]
RE10	2.3±0.4[b]	1.0±0.6[a]
WCS 374	3.2±0.9[a]	1.8±1.5[a]
EN 415	3.5±1.1[a]	1.7±2.0[a]
RE 10+WCS 374	3.7±0.8[a]	1.2±0.8[a]
RE 10+EN 415	4.0±0.6[a]	1.5±2.1[a]
WCS 374+EN 415	3.6±0.9[a]	0.9±0.4[a]
RE 10+WCS 374+En 415	3.3±0.8[a]	1.2±0.8[a]

[a] Value within tests denoted by the same letter are not significantly different according to DMRT ($P<0.05$)

ship (R^2=0.18) between root and soil condition.

Discussion

Various mechanisms have been implicated in suppression of soil-borne plant pathogens by particular strains of fluorescent *Pseudomonas* spp., including antibiotics production (Fravel, 1988; Keel *et al.*, 1992; Thomashow and Weller, 1988), induced resistance (van Peer *et al.*, 1991), and siderophore mediated competition for iron (Bakker *et al.*, 1993; Loper and Buyer, 1991: Schipper *et al.*, 1987). There are generally common assumptions that efficient root coloniziation by beneficial organisms is critical to suppression of soil-borne diseases. A comparable relationship has been demonstrated between rhizosphere population densites (Bull *et al.*, 1991; Xu and Gross, 1986) and rhizoshere conditions (Parke, 1991; Kim and Lee, 1994; Loper *et al.*, 1984).

Disease incidence observed in based on internal and external symptoms was generally low in the treatment of bacterial strains by separate treatments of RE10 or mixed treatments with strains EN415 and WCS374 ranging from 7.8% to 1.2% comparing to 21.1% of the control, but strain WCS374 was exceptionally high, indicating the rate of 27.8% in case of internal symptom (Table 1, Fig. 1). Among the tested strains in this study, strain RE10 and one isolate of strain EN415 induced partial resistance against fusarium wilt caused by FOR, whereas strain WCS374 did not reduce disease incidence. This result suggests that the induction of resistance by *Pseudomonas* spp. depends on specific strain traits. Leeman (1995) proved that bacterial strain WCS 374 induced resistance against

FOR in a bioassy using a rockwool system in his experiment. In this experiment, strain WCS374 did not induce the resistance in radish growth, rather severe disease symptoms were developed in external and internal part. At the inoculum densities, a reduction of inoculm densities reduced disease incidence and increased the relative disease reduction by WCS358r or B243 about twofold (Baayen and de Maat, 1987). Leeman (1995) observed a population change of fluorescent *Pseudomonas* strain WCS374 from 1.6×10^8 at the begining of the experiments to 8.5×10^7/g fresh weight of root, and the changes also was observed in treated pathogens as FOR. In the present study, similar trends were also observed (Table 2, 3; Fig. 2, 3). Reduction of disease incidence of radish against FOR was obtained by separate treatment of strain RE10, and mixed treatmetns of three bacterial strains in a pot bioassay.

In this experiment, most of the tested bacteria were colonized and reduced the disease development when treated separately and mixed or combined with pathogen FOR, although their proliferation in rhizosphere were not the same extent. This suggests that bacterial competition is partially responsible for the inhibition of pathogen's growth (Table 2, 3; Fig. 2. 3), (Hartel *et al.*, 1991; 1993; Han *et al.*, 1996). Han *et al.* (1996) proved that *P. cepacia* antagonistic to the pathogen, *F. solani* was a good competitor as compared with *B. cereus*. The possible mechanisms could be interpreted as a difference of growth pattern of microorganisms in rhizosphere. *P. cepacia* has more fast generation time and greater nutritional versatility than *B. cereus* or pathogen, *F. solani*. Therefore, it may reduce the amount of nutrient available for some microorgnisms that have slow characteristics in their generation time (Palleroni, 1984; Hartel *et al.*, 1993; Williamson and Hartel, 1991; Li and Alexander, 1986). It is likely assumed that growth and colonization of some organisms in root or soil is a consequence of obtaining proper nutrients and multiplying their species. Growth rate may be important in competion for limiting nutrients (Raaijmakers, 1994; Curl and Truelove, 1986).

Proliferation or germination of microbial population in soil environment has been attributed to reserved nutrients or nutritionally deprived soil environment which is maitained by microbial competition (Ko and Lockwood, 1967; Yoder and Lockwood, 1973; Lee *et al*, 1985). Lockwood (1975) mentioned that the importance of exogeneous nutrients on the germination or increase of population had been also dependent on nutritional conditions in environment.

In this experiment, population densities of introduced fluorescent *Pseudomonas* sp. and FOR in root were not much influenced by themselves. It suggests that nutrient level in soil could possibly limit a population density. Further study have to propel how the introduced beneficial organisms will be strongly colonized on the root zone and long survived in soil condition in competing with established populations of indigenous microorgansisms which may prevent or alter interactions of introduced organisms in field condition.

The results show that root colonization with beneficial bacteria induces a set of plant defense mechanisms that culminate in the elaboration of barrier and the creation of a specific environment that adversely affects pathogen growth and development.

적 요

무우품종(*Raphanus sativus* L.) Saxa Nova에 시들음병을 일으키는 *Fusarium oxysporum* f. sp. *raphani* (FOR)에 대해 저항성을 증가시켜 방제효과를 얻기위하여 식물성장을 증진시키는 것으로 알려진 *Pseudomonas florescens* WCS374 (WCS

374), *P. putida* RE10 (RE10) 및 *Pseudomonas* sp. EN415 (EN415)을 병원균처리 토양에 단독 또는 혼합처리하여 4주간 폿트배양한 후 무우에 나타나는 외부 및 내부병징을 조사하여 처리세균에 의한 병억제 효과를 측정하였다.

내부 및 외부병징으로 대조구는 각각 46.5% 및 21.1%를 나타내었고, RE10처리는 내외병징이 각각 12.2%와 7.8%로 발병이 억제됨을 알수있었다. 그러나 발병억제력이 높다고 알려진 WCS374는 내외 병징이 각각 45.6%와 27.8%로 나타났다. 한편 RE10균주를 WCS374 또는 EN415 균주와 혼합하여 처리할 경우 내외 병징이 10.0-22.1% 정도이었고 EN415와 혼합처리하면 7.8-20.2%의 병징을 나타낸다. FOR의 뿌리점유율은 뿌리에서 2.4-5.1 $\times 10^3$/g였고 토양내 분포수는 0.7-1.3$\times 10^3$/g이었다. 대조구는 뿌리에서 3.8$\times 10^3$/g 였으며 RE10의 처리는 2.9$\times 10^3$/g로 분포수가 적었고, 3종 세균의 혼합처리는 5.1$\times 10^3$/g으로 많이 관찰되었으나, 처리간에 통계적 차이는 없었다. 토양에서 관찰된 FOR은 뿌리부분 보다 그 분포 수가 적었다. 처리된 3가지 세균은 뿌리에서 2.3-4.0$\times 10^7$/g 범위이고, 토양에서는 0.9-1.8$\times 10^7$/g 으로 뿌리에서 관찰된 수 보다 적게 분포하였다. 뿌리부분에서 대조구나 RE10의 처리토양은 형광성 *Pseudomonas* spp.의 분포수가 적고 처리 간에는 통계적 차이를 나타냈으나, 토양조사에서 이세균은 큰 차이를 나타내지 않았다. 뿌리에 처리된 세균과 FOR의 분포수는 토양에 처리된것과 대조하였을 때 많았으며,이 실험에 사용된 RE10 과 EN415은 *Fusarium*시들음병에 대한 기주의 저항성을 유도하는 것으로 생각된다.

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