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# Endophytes for plant protection: the state of the art

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# SESSION 3

# Host-Endophyte Interactions

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# 3-1 Association of plant damage with increased populations of deleterious endophytes following use of Benlate systemic fungicide

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## ABSTRACT

According to classical concepts of plant microbial ecology, microbes living inside plants are either pathogens or nonpathogenic beneficial or neutral symbionts. Hence, a common concept is that endophytic bacteria do not cause damage to the host plant, partly because they colonize at population densities lower than those of pathogenic bacteria. Evidence is growing, however, that a clear demarcation between pathogens and neutral or beneficial endophytes is not always present. A model is presented wherein a pertubation in rhizosphere and endorhizal microbial ecology, resulting from use of systemic fungicides, leads to increased populations of deleterious endophytic bacteria. In vegetatively propagated perennial plants, the deleterious endophytes consitute latent infections which can result in distorted growth or restricted plant development and can be passed to daughter plants produced via cuttings or rhizomes of the damaged plants. Examples will be presented in three plant systems: banana, citrus, and leatherleaf fern. In each example, applications of the systemic fungicide Benlate resulted in increased populations of endophytic fluorescent pseudomonads and total culturable bacteria. With citrus and leatherleaf fern, Benlate treatment also resulted in higher percentages of virulent endophytic bacteria as shown in the hypersensitive reaction in tobacco and the production of pectinolytic enzymes in the potato slice assay. Collectively the results and the emerging microbial ecology model indicate that the development of systemically active agrichemicals should include assessment of effects on endophytic bacteria.

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## INTRODUCTION

The term “endophytes” refers to microorganisms living inside plants, and the term has evolved often to include the connotation of microorganisms that are not harmful. For example, in the background material for this symposium (Anonymos 2013), it was stated that endophytes are “microbes that colonize living, internal tissues of plants without causing any immediate, apparent negative effects”. This definition was then immediately followed by the following qualifying comments. “Growing without symptoms here means ‘growing without visible damages’. While such a symptomless nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggests they can also be aggressive saprophytes or opportunistic pathogens” (Anonymous 2013). Opportunistic pathogens are microorganisms that typically exist in the plant environment without causing damage, but when there is a change in the system, such as reduced competition from indigenous microbes or stress on the plant host, the population of the opportunists increase and plant damage is seen.

In this article, the effects of Benlate systemic fungicide on endophytes and the corresponding effect on plant growth are presented and discussed. A review of previous work on leatherleaf fern (*Rumohra* adiantiformis) is presented first. Then selected results of new studies on banana and citrus are presented. A model is presented whereby Benlate is the trigger for a series of microbial changes that result in increased populations of deleterious endophytes and damage to plants.

## REVIEW OF PUBLICATIONS RELATED TO FERN DISTORTION SYNDROME (FDS) AND THE ROLE OF BENLATE AND DELETERIOUS ENDOPHYTIC BACTERIA IN INCITING FDS

Leatherleaf fern (*Rumohra adiantiformis*) is a tropical ornamental plant of high economic value due to production of highly symmetrical, pyramidal-shaped, dark-green fronds used in flower arrangements. Costa Rica is a major producer of leatherleaf fern and exports the product to markets in North America and Europe. Growers began reporting an increase in irregularly shaped fronds in the mid-1990s (Kloepper *et al.* 2010), and production decreased substantially as a result. In 2010, the problem was described for the first time in the literature (Kloepper *et al.* 2010) and was termed fern distortion syndrome (FDS). The main diagnostic symptom of FDS is distorted growth of fronds, which can range from a slight bending of the frond tip to severe twisting and loss of the symmetrical shape of the frond. Other symptoms associated with FDS that make fronds unmarketable include thickening of mature fronds, uneven patterns of sporulation, and the presence of yellow, red, or bronze streaking on the pinnae of fronds. Below-ground symptoms of FDS may include reduced root growth, small-diameter rhizomes, and internal discoloration of the rhizomes.

Prior to the description of FDS in 2010, distortions of leatherleaf fern had been noted in Florida, and their appearance was reported to coincide with the use of Benlate DF systemic fungicide (Mills *et al.* 1996). Kremer *et al*. (1996) compared fluorescent pseudomonads from the rhizosphere of Benlate-treated or nontreated ferns for their capacity to be "allelopathic" (deleterious) in a lettuce seed bioassay. Their results showed that 80% of the pseudomonad strains from Benlate-treated plants were deleterious compared to 6% of strains from control plants. These two studies suggested that rhizosphere communities of deleterious pseudomonads triggered by Benlate DF might be the cause of distorted ferns. A modification of this suggestion was investigated in the 2010 study (Kloepper *et al.* 2010) by examining the association between FDS and endophytic fluorescent pseudomonads and total culturable bacteria. Paired samplings of rhizomes from symptomatic and healthy-appearing ferns in six commercial ferneries in Costa Rica revealed that endophytic populations of fluorescent pseudomonads were significantly greater at all six locations and populations of total culturable bacteria were greater at five of the six locations in symptomatic than in healthy-appearing plants.

In 2012, results of two-year greenhouse studies on the effects of Benlate on leatherleaf fern were published (Kloepper *et al.* 2012). The trials were conducted to test the hypotheses that 1) Benlate treatment of leatherleaf fern leads to long-term increases in populations of deleterious fluorescent pseudomonads endophytically colonizing rhizomes; and 2) endophytic colonization of rhizomes following treatment with Benlate is associated with FDS symptoms. To avoid the possibility of latent infections inside rhizomes of field-collected ferns, commercial tissue culture plants were used in this study. Treatment with Benlate WP and Benlate DF resulted in increased populations of pseudomonads inside rhizomes compared to controls after 24 months. At this time, ferns treated with Benlate had reduced frond weight per plant. Benlate treatments also resulted in significant increases in the severity of FDS using a rating scale that assessed the degree of frond deformations. Benlate also led to increases in the number of newest fronds with a twisted rachis and decreased diameter of rhizomes, which are two additional symptoms of FDS. Dwarfing of plants (severe stunting) was exhibited by half of the plants treated with Benlate 50 DF but by none of the control plants.

In the same study (Kloepper *et al.* 2012), the development of FDS symptoms by Benlate treatments corresponded with significant increases in the endophytic populations both of fluorescent pseudomonads and total aerobic culturable bacteria in both studies. Also, assessing the incidence of rhizomes containing detectable populations of endophytic pseudomonads showed that 75-90% of rhizomes on plants treated with Benlate contained fluorescent pseudomonads compared to 20% of control rhizomes. There was also a marked increase in pectinolytic enzyme activity of endophytic pseudomonads inside rhizomes of Benlate-treated plants compared to control plants. DNA sequencing of fluorescent pseudomonads isolated from inside rhizomes or petioles indicated that Benlate treatments resulted in pronounced shifts in phylogenetic clusters of endophytes. Overall, the two-year study showed that treatment of leatherleaf fern with Benlate 50 WP and Benlate 50 DF

caused a sequence of long-term deleterious effects that were associated with increased populations of fluorescent pseudomonads that were functionally and phylogenetically distinct from bacteria inside control plants.

Confirmation that the main symptoms of FDS are caused by endophytic fluorescent pseudomonads was presented in 2013 (Kloepper *et al*. 2013). A collection of 47 strains of fluorescent pseudomonads was used in this study: 17 strains isolated from inside rhizomes of healthy-appearing ferns grown in Florida in a fernery with no history of Benlate use, 14 strains from the surface of roots and rhizomes of symptomatic plants in Costa Rica, and 16 strains from inside rhizomes of symptomatic plants in Costa Rica. Phylogenetic analysis of the strains revealed that they fit into five clusters and that there were clear differences between strains from healthy ferns and strains from symptomatic ferns. The same strains were characterized for three traits that have been reported to be related to virulence: elicitation of the hypersensitive reaction (HR) in tobacco leaves (Mathesium *et al.* 2003), production of pectinolytic enzymes (Berg *et al.* 2005), and production of indole acetic acid (IAA) (Preston 2004). Differences in the frequency of HR and potato slice, but not IAA, were noted between strains from symptomatic and healthy ferns. None of the pseudomonads isolated from inside rhizomes of healthy plants without a history of Benlate use elicited HR. In contrast, HR was elicited by 77% of the strains isolated from inside rhizomes or from the rhizosphere of diseases symptomatic ferns. Pectinolytic enzyme production was exhibited by 46% of the strains from symptomatic plants but from none of the pseudomonads from healthy plants.

In the same study (Kloepper *et al.* 2013), micropropagated ferns from tissue culture were grown for one year to produce rhizomes free of possible latent infections of endophytic bacteria from fields. The rhizomes were inoculated by dipping into 9 different treatments: water (control) and two concentrations of four mixtures of bacteria. Bacterial mixtures included the group of endophytic bacteria from rhizomes of healthy plants and three groups from the plants exhibiting FDS: two groups of bacteria from inside rhizomes and one group from the rhizosphere. At 12 months after inoculation, all the main symptoms of FDS were recreated with the strains of fluorescent pseudomonads isolated from diseased but not from healthy plants. Surprisingly, many of the secondary symptoms of FDS were also recreated by the pseudomonads from diseased plants, including thickening of older fronds, reduced overall growth sometimes resulting in dwarfing of plants, the presence of red or yellow streaks on the pinnae of fronds, an irregular pattern of sporulation, reduced size of new rhizomes, and internal discoloration of rhizomes. Ferns inoculated with pseudomonads from healthy plants had none of these symptoms. In fact, inoculation with bacteria from healthy plants significantly increased one parameter of plant growth, frond width, compared to the water control. This finding shows that some endophytic bacteria in rhizomes of healthy plants are beneficial to plant development, which is consistent with studies on plant growth-promoting rhizobacteria.

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## EXPERIMENTAL WORK ON BANANA

### Methods

An experiment was conducted to determine the effects of Benlate on plant growth and populations of endophytic bacteria in banana. Banana (*Musa acuminata*) 'Dwarf Cavendish' plantlets from tissue culture were grown for 3 months in a 1:1 mixture of field soil and sand in a greenhouse prior to applying treatments. The experiment was a randomized complete block with 6 treatments, each with 10 replicate plants. Treatments were foliar sprays or drenches of water (controls) and two formulations of the commercial fungicide Benlate from DuPont—Benlate 50 DF and Benlate 50 WP. Benlate applications were made at 1.9 g/L, which is equivalent to the label rate of 2 lbs per acre in 125 gallons of water. Foliar sprays were applied by spraying leaves to the point of run-off. Drench applications were made by applying 50 ml per pot. Treatments were applied three times at monthly intervals. The experiment was conducted twice: once for destructive sampling 6 months after the first application of treatments and once for destructive sampling 15 months after applications.

Destructive sampling was done by removing each plant from the pot and washing roots. In experiment one, treatment effects on plant growth were determined by measuring plant height, shoot fresh weight (stems + leaves), and root dry weight. In experiment two, effects on plant growth were determined by measuring the stem diameter, stem height, stem weight, weight of the main rhizome, and weight of the new (daughter) rhizomes. In both experiments, populations of endophytes inside stems were determined by surface disinfesting stem tissue by soaking in 80% ethanol for 2 min and then in 20% bleach for 2 min followed by rinses in sterile distilled water. After trituration in mortars, dilution plating was performed on 50% King's medium B for fluorescent pseuduomonads and on 10% tryptic soy agar for total culturable bacteria. After incubation for 48-72 hours at room temperature, colonies were counted to determine mean log cfu/g.

### Results

Both formulations of Benlate, applied as drenches and foliar sprays, resulted in significant reductions in all of the measured parameters of plant growth at 6 and 15 months after the first application (Tables 1A and 1B). At 15 months, significant growth reductions were noted in the diameter, height, and weight of the stem as well as the weight of the original rhizome and new (daughter) rhizomes (Table 1B and Figure 1). These decreases in plant growth after Benlate applications were associated with increases in the endophytic populations of fluorescent pseudomonads and total aerobic bacteria (Tables 1A and 1B).

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Table 1A. Effect of Benlate on endophytic bacteria and growth of Banana 6 months after first application of Benlate

| Treatment | Plant height (cm) | Shoot fresh weight (g) | Root dry weight (g) | Populations of endophytic bacteria inside stems (log CFU/g) Fluorescent pseuduomonads | Total aerobic bacteria |
|---|---|---|---|---|---|
| 1. Benlate 50 WP foliar spray | 31.8* | 341.4* | 13.05* | 1.56 | 4.43* |
| 2. Benlate 50 DF foliar spray | 32.7* | 364.6* | 11.13* | 2.78* | 4.54* |
| 3. Water control foliar spray | 42.0 | 636.9 | 24.82 | 0.53 | 3.47 |
| 4. Benlate 50 DF drench | 32.1* | 386.4* | 11.54* | 2.37* | 4.62* |
| 5. Benlate 50 WP drench | 32.2* | 380.2* | 12.90* | 2.48* | 4.70* |
| 6. Water control drench | 42.0 | 650.1 | 22.92 | 0.59 | 3.36 |
| $LSD_{0.01}$ | 2.2 | 48.1 | 4.35 | 1.53 | 0.51 |

Values shown are means of 10 plants per treatment.
* Indicates significantly lower value than the corresponding water control at $P = 0.01$

Table 1B. Effect of Benlate on endophytic bacteria and growth of Banana 15 months after first application of Benlate

| Treatment | Stem diameter (cm) | Stem height (cm) | Stem weight (g) | Main rhizome weight (g) | Weight of new rhizomes (g) | Populations of endophytic bacteria inside stems (log CFU/g) Fluorescent pseudo-monads | Total aerobic bacteria |
|---|---|---|---|---|---|---|---|
| 1. Benlate 50 WP foliar spray | 6.13* | 50.5* | 956* | 386* | 207* | 2.58* | 4.95* |
| 2. Benlate 50 DF foliar spray | 5.80* | 48.5* | 850* | 324* | 178* | 2.47* | 4.89* |
| 3. Water control foliar spray | 8.19 | 58.9 | 1375 | 0680 | 253 | 0.79 | 3.56 |
| 4. Benlate 50 DF drench | 6.06* | 49.5* | 813* | 373* | 171* | 2.45* | 4.86* |
| 5. Benlate 50 WP drench | 5.81* | 47.4* | 775* | 306* | 178* | 2.56* | 4.97* |
| 6. Water control drench | 8.25 | 59.8 | 14563 | 669 | 283 | 0.81 | 3.63 |
| $LSD_{0.01}$ | 0.64 | 2.72 | 169 | 88 | 75 | 0.93 | 0.60 |

Values shown are means of 10 plants per treatment.
* Indicates significantly lower value than the corresponding water control at $P = 0.01$

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Figure 1. Effect of Benlate on growth of banana plants 6 months after first spray with Benlate DF (left) and water (right); lower photo is close-up of stem, showing smaller diameter on Benlate-treated banana.

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## EXPERIEMNTAL WORK ON CITRUS

### Methods

An experiment was conducted to determine the effects of Benlate on plant growth and populations of endophytic bacteria in citrus. Citrus plants used were orange variety Valencia budded onto Swingle rootstock. Plants were obtained from a commercial citrus nursery in Florida and were transplanted into citrus pots (25 cm square X 35 cm deep) containing a 1:1 mixture of field soil and sand. The experiment was a randomized complete block with 6 treatments, each with 8 replicate plants. Treatments were foliar sprays or drenches of water (controls) and two formulations of the commercial fungicide Benlate from DuPont—Benlate 50 DF and Benlate 50 WP. Benlate WP and DF were applied at the same rate and the same monthly frequency as in the banana test. The experiment was conducted twice, once for destructive sampling 7 months after the first application of treatments and once for destructive sampling 16 months after applications.

In experiment one, treatment effects on plant growth were assessed at 5.5 months after the first application of treatments by measuring the cumulative length of new shoot growth and stem caliper. At 7 months after treatment, plants were removed from pots and roots were washed. Roots were scanned with a WinRhizo root analyzer, and three aspects of root architecture were measured for each plant: total root length, surface area, and total number of root tips. At the same time, the endophytic population densities of fluorescent pseudomonads and total aerobic bacteria inside stems were determined as described for the banana experiment.

In experiment two, some plants began blooming about one year after the first application of treatments. The number of blooms and small fruits per plant were counted 15.5 months after the first application of treatments. The experiment was destructively sampled at 16 months after the first application of treatments. Roots were washed, and fresh weights of shoots and roots were measured. In addition, treatment effects on root architecture and on populations of endophytic bacteria in stems were determined as in experiment one.

### Results

In experiment 1 (Table 2A), both formulations of Benlate applied as foliar sprays and as drenches resulted in significant reductions in growth of shoots, as determined with length of new shoots, stem caliper, and reductions in growth of roots as measured by reduced total root length, root surface area, and total number of root tips per plant. Examples of reduced growth of stems and roots following applications of Benlate are shown in Figures 2 and 3. Mean populations of endophytic bacteria, both fluorescent pseudomonads and total aerobic bacteria, were significantly greater in all treatments with Benlate than in the water-treated control plants.

In experiment 2 (Table 2B), at 15.5 months after the first applications of Benlate, control plants were blooming and setting fruit. Blooming was significantly reduced by all treatments

with Benlate, and no fruits were present on the Benlate-treated plants. At 16 months (Table 2C), weights of shoots and roots were significantly lower for all Benlate treatments than for controls. Analysis of root system architecture revealed the same results as in experiment 1, that Benlate treatments had reduced total root length, root surface area, and total number of root tips, compared to controls. Also at 16 months after treatment, population densities of endophytic bacteria inside stems of Benlate-treated plants were significantly greater from those of control plants.

Table 2A. Citrus test 1, effect of Benlate on endophytic bacteria and growth of Valencia orange at 5.5 and 7 months after first application of Benlate

| Treatment | Measurements of plant growth at 5.5 months | | Measurements of root architecture at 7 months | | | Populations of endophytic bacteria inside stems (log CFU/g) at 7 months | |
|---|---|---|---|---|---|---|---|
| | Length of new shoot growth (cm) | Stem caliper (mm) | Total root length (cm) | Root surface area ($cm^2$) | Total no. of root tips | Fluorescent pseuduomonads | Total aerobic bacteria |
| 1. Benlate 50 WP foliar spray | 57.7* | 5.4* | 411* | 415* | 547* | 1.56 | 4.43* |
| 2. Benlate 50 DF foliar spray | 61.4* | 5.6* | 383* | 394* | 499* | 2.78* | 4.54* |
| 3. Water control foliar spray | 98.7 | 6.6 | 651 | 649 | 771 | 0.53 | 3.47 |
| 4. Benlate 50 DF drench | 60.2* | 5.1* | 387* | 404* | 494* | 2.37* | 4.62* |
| 5. Benlate 50 WP drench | 51.6* | 5.0* | 353* | 381* | 485* | 2.48* | 4.70* |
| 6. Water control drench | 98.0 | 6.5 | 634 | 660 | 787 | 0.59 | 3.36 |
| $LSD_{0.01}$ | 15.1 | 0.6 | 48.5 | 57.0 | 61.1 | 1.53 | 0.51 |

Values shown are means of 8 plants per treatment.
* Indicates significantly lower value than the corresponding water control at $P = 0.01$

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Table 2B. Citrus test 2, effect of Benlate on number of blooms and fruit on Valencia orange 15.5 months after first application of Benlate

| Treatment | Number of blooms and buds per plant | Number of fruit per plant |
|---|---|---|
| 1. Benlate 50 WP foliar spray | 2.00* | 0* |
| 2. Benlate 50 DF foliar spray | 1.25* | 0* |
| 3. Water control foliar spray | 19.88 | 9.25 |
| 4. Benlate 50 DF drench | 0.38* | 0* |
| 5. Benlate 50 WP drench | 0.38* | 0* |
| 6. Water control drench | 26.38 | 9.0 |
| $LSD_{0.01}$ | 16.1 | 6.47 |

Values shown are means of 8 plants per treatment.
* Indicates significantly lower value than the corresponding water control at $P = 0.01$

Table 2C. Citrus test 2, effect of Benlate on endophytic bacteria and growth of Valencia orange 16 months after first application of Benlate

| | | | Measurements of root architecture | | | Populations of endophytic bacteria inside stems (log CFU/g) | |
|---|---|---|---|---|---|---|---|
| Treatment | Shoot fresh weight (g) | Root fresh weight (g) | Total root length (cm) | Root surface area ($cm^2$) | Total no. of root tips | Fluorescent pseudo-monads | Total aerobic bacteria |
| 1. Benlate 50 WP foliar spray | 94.5* | 81.2* | 480* | 885* | 503* | 4.43* | 4.93* |
| 2. Benlate 50 DF foliar spray | 96.2* | 74.6* | 515* | 871* | 549* | 4.62* | 5.08* |
| 3. Water control foliar spray | 175.4 | 133.3 | 985 | 1531 | 1008 | 0.70 | 2.94 |
| 4. Benlate 50 DF drench | 88.3* | 90.2* | 432* | 962* | 515* | 4.77* | 4.92* |
| 5. Benlate 50 WP drench | 95.4* | 83.0* | 482* | 975* | 506* | 4.78* | 5.05* |
| 6. Water control drench | 183.0 | 150.3 | 993 | 1600 | 1040 | 0.50 | 3.84 |
| $LSD_{0.01}$ | 20.7 | 17.3 | 137 | 262 | 94 | 0.93 | 0.60 |

Values shown are means of 8 plants per treatment.
* Indicates significantly lower value than the corresponding water control at $P = 0.01$

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Figure 2. Valencia orange 5.5 months after first drench with Benlate DF (left) and water (right)

Figure 3. Effect of Benlate on root growth of Valencia citrus 7 months after first application of Benlate. Right = drench with Benlate DF; left = water control.

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Figure 4 Effect of Benlate on formation of citrus fruit 15.5 months after first application of Benlate. Upper photo is a water control plant; lower photo was treated with Benlate

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## DISCUSSION

The genus *Pseudomonas* contains a large diversity of species and strains within species (Preston 2004). Within the fluorescent pseudomonads there are many biological control strains, some pathogenic strains, and some strains reported to be deleterious to plants. Preston (2004) pointed out that the distinction between saprophytes and pathogens is not always clear-cut because both live on and inside plant tissues where there are frequent opportunities for genetic recombination. Such recombination can occur after horizontal gene transfer, thereby conferring new phenotypic traits (or suites of traits) to bacteria sharing an ecological habitat (Berg *et al.* 2005). Therefore, in the rhizosphere or inside plants, bacterial genes for production of virulence factors may move among different phylogenetic groups. The expression of virulence factors can relate to population density of the bacterial strains. Examples of virulence factors that are regulated by plant cell density via quorum sensing are elicitation of the hypersensitive response (Mathesius *et al.* 2003), production of IAA (Preston 2004), and production of cell wall degrading enzymes including pectinase (Berg *et al.* 2005; Laasik *et al.* 2006). Quorum sensing also has been reported to regulate horizontal gene transfer and bacterial colonization of hosts (Berg *et al.* 2005), and some virulence factors can also help endophytes colonize plants. For example, pectinolytic enzymes have been reported to facilitate entry of rhizosphere bacteria inside plants by hydrolyzing pectic substances located between plant cell walls (Okon and Vanderleyden 1997).

As discussed above, in three different perennial plant systems, leatherleaf fern, banana, and citrus, applications of Benlate systemic fungicide led to increased populations of endophytic bacteria, including fluorescent pseudomonads, and to decreased growth or distortions in growth of the host plant. We propose the following as an emerging model to account for these observations. In healthy plants, the native microflora in the rhizosphere and inside roots or rhizomes includes groups of fluorescent pseudomonads, and likely other bacterial genera that contain genes for production of virulence and colonization factors. Under normal growth conditions, populations of these bacteria are kept below the level necessary for widespread production of the virulence factors because of competition with the native bacterial and fungal microflora of the rhizosphere and endorhiza. Changes in the balance of the native microflora of the rhizosphere and inside roots or rhizomes result from perturbations in the growing conditions, such as the application of the systemic fungicide Benlate. These microbial changes trigger population increases of bacteria containing the virulence and colonization traits. As their populations increase, quorum sensing activates expression of the colonization and virulence factors as well as horizontal gene transfer of virulence genes to other closely related phylogenetic groups of bacteria. Over time in perennial plant systems, there is a shift in the bacterial community inside roots, rhizomes, or stems with a concomitant development of symptoms such as stunting of root and shoot growth or deformed growth of plants. This model is consistent with reports that the rhizosphere is a reservoir of opportunistic pathogens that cause human infections, reviewed by Berg *et al.* (1005).