

MANAGEMENT OF PIERCE'S DISEASE OF GRAPE BY INTERFERING WITH CELL-CELL COMMUNICATION IN *XYLELLA FASTIDIOSA*

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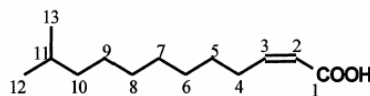
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ABSTRACT

Xylella fastidiosa (*Xf*) produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF) that changes its gene expression in cells as they reach high numbers in plants. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption as a means of controlling Pierce's disease. We have investigated both the role of DSF-production by *Xf* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, performed genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control. *Xf* mutant strains that overproduce DSF cause disease symptoms in grape, but only at the site of inoculation and the cells do not move within the plant as do wild-type strains. Thus elevating DSF levels in plants should reduce movement of *Xf* in the plant and also reduce the likelihood of transmission by sharpshooters. We identified bacterial strains that can interfere with *Xf* signaling both by producing large amounts of DSF or by degrading DSF. We have identified the genes needed to degrade DSF and when they were transferred to and over-expressed in other strains they conferred the ability of these strains to degrade DSF. When co-inoculated into grape with *Xf*, both DSF-producing strains and DSF-degrading strains greatly reduced the incidence and severity of disease in grape. Given that DSF overabundance appears to mediate an attenuation of virulence in *Xf* we have transformed grape with the *rpjF* gene of *Xf* to enable DSF production in plants; such grape plants produce at least some DSF and are much less susceptible to disease. Higher levels of expression of DSF have been obtained in plants by targeting the biosynthetic enzymes to the chloroplast. Studies are underway to determine if DSF produced by rootstocks can move to scions and confer disease control.

INTRODUCTION

Xylella fastidiosa (*Xf*) colonizes the internal tissues of grape and other hosts, forming a biofilm inside the plant. Given that there is a very strong correlation between the number of xylem vessels heavily colonized by *Xf* and disease symptoms in grape (9, 24, 28) we thus think of Pierce's disease as a result of uncontrolled proliferation of *Xf* in the plant, thereby causing disruption in water flow. Such an endophyte presumably acquires most of its nourishment from the dilute solutes present in the xylem sap. A key determinant of success for an endophyte such as *Xf* is the ability to move within the plant since its success would be dependent upon moving from one xylem vessel to another, presumably through pits, where it could intercept increasing amounts of xylem sap; the population size in the plant would be proportional to the number of xylem vessels into which it had moved. *Xf* possess traits such as endoglucanases and polygalacturonases that allow it to degrade pit membranes and hence move within the plant. Work by Roper et al. (29) has shown that polygalacturonases are required for movement in the plant, presumably by contributing to pit membrane degradation; other enzymes such as endoglucanases also probably contribute. While occasional paths that transit significant distances within the plant occasionally occur (34) enabling passive movement of *Xf*, it appears that *Xf* cells introduced into a given vessel are usually constrained by the anatomy of the vessel and must actively move by altering the plant (19,20).



We have found that the virulence of *Xf* is strongly regulated in a cell density-dependent fashion by a process similar to quorum sensing (the *rpj* locus) which involves a small signal molecule. Numerous species of bacteria communicate using small molecules, such as *N*-acyl homoserine lactones, small peptides, butyrolactone derivatives or fatty acids, as signals. The signals, which increase in concentration with population density, typically coordinate the expression of genes involved in exploitation of a host organism. The virulence of many pathogens is greatly reduced when the ability to produce signaling compounds is disrupted by mutation. *Xf* and *Xanthomonas campestris* pv. *campestris* (*Xcc*) are very closely related pathogens. *Xcc* makes a farnesoic acid derivative, cis-11-methyl-2-dodecenoic acid called diffusible signal factor (DSF). We now have shown that *Xf* makes a molecule that is recognized by *Xcc* but probably slightly different than the DSF of *Xcc*. In striking contrast to that of *Xcc*, *rpjF*- mutants of *Xf* blocked in production of DSF, exhibit dramatically increased virulence to plants, however, they are unable to be spread from plant to plant by their insect vectors since they do not form a biofilm within the insect. These observations of increased virulence of DSF-deficient mutants of *Xf* are consistent with the role of

this density-dependent signaling system as suppressing virulence of *Xf* at high cell densities. Our observations of colonization of grapevines by *gfp*-tagged *Xf* are consistent with such a model. We found that *Xf* normally colonizes grapevine xylem extensively (many vessels colonized but with only a few cells in each vessel), and only a minority of vessels are blocked by *Xf*. Importantly, *rpfF*- mutants of *Xf* plug many more vessels than the wild-type strain. We thus believe that *Xf* has evolved as an endophyte that colonizes the xylem; blockage of xylem would reduce its ability to multiply since xylem sap flow would cease and thus the DSF-mediated virulence system in *Xf* constrains virulence. That is, *Xf* would benefit from extensive movement throughout the plant where it would partially colonize xylem vessels but would have evolved not to grow too excessively within a vessel, thereby plugging it and hence blocking the flow of necessary nutrients in the xylem sap. Given that the DSF signal molecule greatly influences the behavior of *Xf* we are investigating various ways by which this pathogen can be “confused” by altering the local concentration of the signal molecule in plants to disrupt disease and/or transmission. We thus are further exploring how DSF-mediated signaling occurs in the bacterium as well as ways to alter DSF levels in the plant. Our work has shown that the targets of Rpf regulation are genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel as well as adhesins that modulate movement. Our earlier work revealed that several other bacterial species can both positively and negatively interact with the DSF mediated cell-cell signaling in *Xf*. In this period we have extensively investigated both the role of DSF-production by *Xf* on its behavior within plants, the patterns of gene regulation mediated by DSF, the extent to which other endophytes can modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, obtained genetic transformation of grape and other hosts of *Xf* to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control.

OBJECTIVES

1. Identify bacteria that interfere with DSF-mediated cell-cell signaling in *Xf*, and conduct pathogenicity tests on grapevines colonized by DSF-interfering bacteria to determine potential for Pierce’s disease control.
2. Isolation of mutant strains of DSF-degrading and DSF-producing bacteria that can no longer interfere in cell-cell signaling to verify that disease control is linked to cell-cell signal interference.
3. Molecular identification of genes conferring DSF-degrading activity.
4. Engineer the grapevine endophytes to express genes conferring DSF-degradation and DSF-synthesis activities and test whether the resulting transgenic endophytes are capable of disease control.
5. Creation of grapevines expressing genes conferring DSF-degradation and DSF-synthesis activity to test for PD resistance.
6. Evaluate topical application of DSF-degrading and DSF-producing bacteria with penetrating surfactants for PD Control.

RESULTS

Understanding the pathway of cell-cell signaling mediated by DSF

Analysis of the genome sequence of *Xf* revealed that several genes encoding proteins potentially involved in intracellular signaling are present. In many pathogenic bacteria, intracellular signaling couples extracellular cell-cell signaling to regulate different cellular processes. The Rpf signaling component RpfG encodes a response regulator with domain which modulates the level of cyclic di-GMP. In many pathogenic bacteria, it has been reported that di-Cyclic GMP influence many virulence traits such as biofilm formation, motility, adhesion etc. In *Xf* we have identified an ORF (PD0279), which encodes a GGDEF domain protein predicted to be involved in the synthesis of di-cyclic GMP. *Xf* mutants in the GGDEF domain proteins were produced and initial analysis revealed that they are also altered in *in vitro* attachment and biofilm formation, much like the other cell-cell signaling mutants-*rpfF* and *rpfC*. Comparative transcription analysis of the GGDEF domain protein mutant revealed that both RpfF and RpfC are involved in its regulation. Interestingly, transcription analysis revealed that the GGDEF protein (and indirectly, di-Cyclic GMP) is involved in regulation of cell-cell signaling in *Xf* (Table 1). Mutants in this regulator highly over-express *rpfF* and hence express very high levels of DSF (Table 1). Mutants in the GGDEF protein, like *rpfC* mutants which over-produce DSF are severely reduced in their ability to incite Pierce’s disease to grape (Figure 1). This is consistent with our other results that show that DSF over-production attenuates virulence of *Xf* (Figure 1). We thus will be testing the DSF overproducing GGDEF mutant strain for PD control.

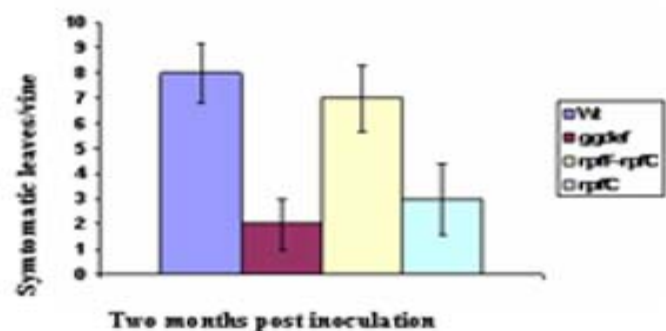


Figure 1. Severity of Pierce’s disease of grapevines inoculated with DSF overproducing strains of *Xf* (Wild type, *rpfC* and *ggdef* mutants).

Table 1. Transcriptional activity of genes in the *rpf* cluster of *Xf* as well as other genes contributing to virulence in *rpfF*, *rpfC* and GGDEF mutant backgrounds. * Relative quantification of RNA calibrated with the wild type *Xf* RNA as 1, and normalized to the 16S ribosomal.

Gene Name	* Fold change in expression		
	GGDEF mutant	<i>rpfF</i> mutant	<i>rpfC</i> mutant
<i>rpfG</i>	6.2	0.60	2.01
<i>rpfE</i>	3.65	0.33	1.6
<i>tolC</i>	1.78	6.53	3.80
<i>rpfB</i>	1.5	0.82	3.27
PD0279 (GGDEF domain protein)	n.d	6	3
<i>pglA</i>	3.0	5.83	6.80
<i>rpfC</i>	5.38	4.0	n.d
<i>rpfF</i>	10	n.d ¹	8.0

DSF negatively regulates twitching motility in *Xf*

Since *rpfF* mutants of *Xf* are hyper virulent and move more rapidly in the xylem vessel, we investigated whether DSF overproduction can influence twitching motility in *Xf*. Assay for the twitching motility (1) revealed that the *rpfF* mutant which does not produce any DSF, showed an increased colony fringe indicative of high twitching motility (Figure 2). However an *rpfC* mutant which is a DSF over-producing strain of *Xf* showed reduced colony fringe. Interestingly the GGDEF domain protein mutant, which also greatly over-produces DSF and is deficient in di-cyclic GMP synthesis and also altered in *rpfF* expression was severely reduced in twitching motility (Figure 2). This suggests that DSF regulates adhesion and motility at least partially via its indirect regulation of di-cyclic GMP levels. Combined with our other studies of those genes that are regulated by DSF accumulation in *Xf* as determined from microarray and quantitative RT-PCR studies, we have developed a model for DSF-mediated gene regulation in *Xf* (Figure 3).

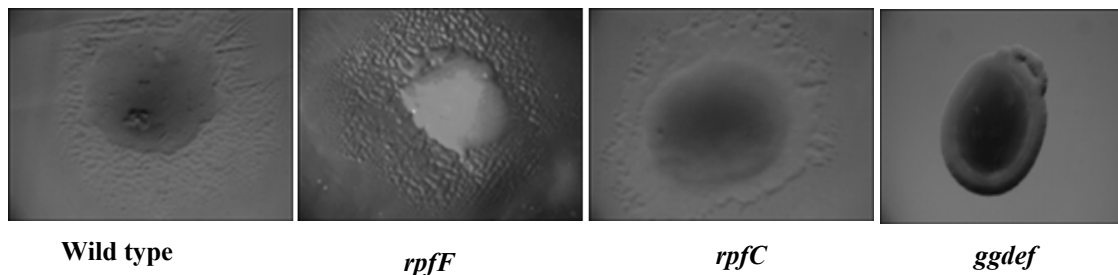


Figure 2. colonies of different *Xf* strains were spotted on cellophane placed on modified PWG media with low BSA. Twitching motility is indicated by the formation of a peripheral fringe.

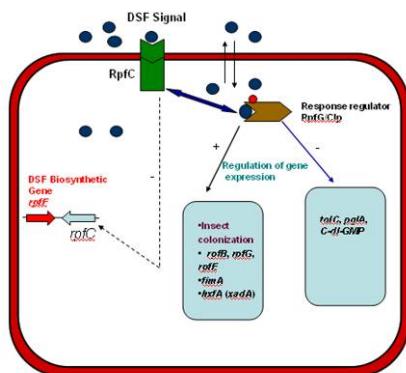


Figure 3. A proposed model for DSF-mediated cell-cell signaling regulation in *Xylella fastidiosa*. *rpfF* encodes the DSF synthase. RpfC encodes a hybrid two component sensor which can sense DSF. DSF levels negatively regulate the expression of *rpfF* by feed back which acts as a negative feed back. Expression of other virulence associated functions is regulated by a putative intracellular DSF sensor/response regulator (like-RpfG/Clp). DSF can diffuse inside the cell and is sensed by this putative intracellular DSF sensor/response regulator, which acts as positive regulator of functions associated with attachment-biofilm formation and other components of *rpf* regulon. The intracellular DSF sensor/response regulator, in the presence of RpfC, can negatively regulate the expression of other virulence associated genes like *pglA*, *tolC* and GGDEF domain encoding gene.

Production of DSF in transgenic plants for disease control.

We have expressed the *rpjF* gene in several different plant species to investigate whether DSF excess can lead to reduced disease caused by *Xf*. In addition to grape, we have transformed genes conferring DSF production into tobacco since this species is colonized by *Xf* and disease symptoms can be produced (Figure 4). Because transformation of tobacco is much quicker than grape, we have used studies of *Xf* infection of tobacco as a surrogate for studies in grape to speed our assessment of different ways to produce DSF in grape. The various mutants of *Xf* that are hyper and hypo virulence on grape yield similar reactions on tobacco (Figure 5).



Figure 4. Symptoms caused by *Xf* on SR1 Tobacco.

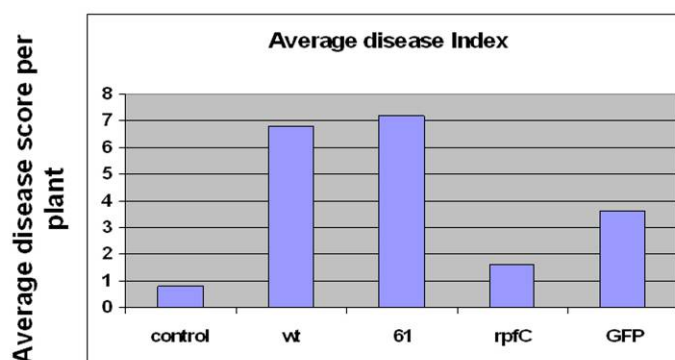


Figure 5 Disease caused by WT *Xf*, an *rpjF*- mutant (61), an *rpjC*- mutant, and a *gfp*-marked *Xf* strain.

Grape has been transformed at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis. Initially, we submitted a tested but un-optimized *rpjF* construct to the facility. The first transformed plants have been tested for DSF production. Initial assays reveal that DSF is rapidly degraded by damaged plant tissue during extraction procedures, making it hard to estimate the abundance of DSF within the plants. Therefore different assays are being developed to avoid this complication in assessing DSF abundance. Large numbers of clonal *rpjF*-expressing grapes have now been produced and inoculated with *Xf* to test for susceptibility to Pierce's disease. The *rpjF*-expressing grape are MUCH less susceptible to Pierce's disease. (Figure 6). The severity of disease was reduced over 10-fold compared to non-transformed plants. While *Xf* spread throughout non-transformed plants causing disease on petioles located great distances from the point of inoculation, disease was observed only very close to the point of inoculation in *rpjF*-expressing plants. We thus expect to find that *Xf* is limited in its movement in plants having high indigenous levels of DSF due to the expression of *rpjF*, in a manner similar to what we have observed in DSF-overproducing strains of *Xf*.

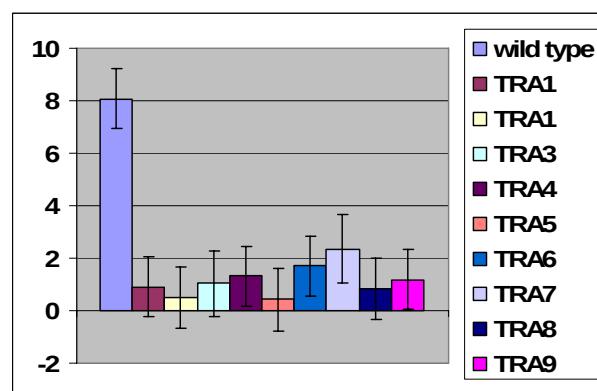


Figure 6. Disease severity (# symptomatic leaves/plant) on Freedom grape transformed with the *rpjF* gene encoding DSF production and inoculated with *Xf*.

We are thus very excited about the prospects of enhancing DSF levels in plants as a means of reducing disease. This might best be done in transgenic plants or perhaps in topical applications of DSF or analogs or also by expression in plants by other endophytic bacteria.

Enhancing the DSF levels in plants

Given that fatty acid synthesis in plants occurs primarily in the chloroplast and that DSF is presumably a fatty acid derivative, we have recently transformed tobacco and *Arabidopsis* with an *rpjF* gene that has been modified to direct the protein product to the chloroplast where fatty acid synthesis (and DSF synthesis) should be much enhanced compared to its production in the cytosol, the presumed location of RpfF in the current transformed plants. The *Arabidopsis* ribulose biphosphate carboxylase small subunit 78 amino acid leader peptide and mature N-terminal which is sufficient to target the protein to the chloroplast, has been fused with the RpfF protein of both *Xylella* and *Xcc*. Assay of DSF in transgenic SRI tobacco plants-where the RpfF is targeted to the chloroplast, indicates that the DSF levels as well as expression of *rpjF* are much higher as compared

to the plants in which the RpfF is expressed in the cytosol. Transcription analysis of the chloroplast targeted *rpjF* transformed plants indicates high level expression of the *rpjF* gene (Figure 7). We have generated seeds from the transgenic SRI tobacco plants and we are conducting pathogenicity assay with *Xf*.

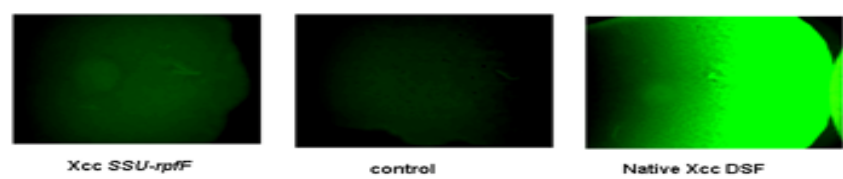


Figure 7. DSF extracted from transgenic tobacco SR1 plants expressing the chloroplast targeted RpfF, compare to nontransgenic SR1 and native DSF extracted from *Xcc*. The DSF is spotted at the right hand side on a filter disc and the *Xcc* DSF bioindicator is streaked on the left side of the spot. The green GFP fluorescence is indicative of DSF production.

We have also initiated transformation of grapes with the improved chloroplast targeted *rpjF* constructs- with the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis. Although RpfB is not required for DSF synthesis in *Xf*, it presumably aids in DSF synthesis by encoding long chain fatty acyl CoA ligase which might increase availability of the appropriate substrates for DSF synthesis by RpfF. We expect that co-expression of RpfB and RpfF in the chloroplast will further enhance the DSF levels in plants. We have produced transgenic Arabidopsis plants with such a construct and find evidence of high levels of DSF production. Pathogenicity assays with the *rpjF* mutant of *Xcc* indicated that the transgenic plants can complement the virulence of the nonpathogenic *rpjF* mutant of *Xcc* (Table 2). Importantly, transgenic plants expressing both *rpjB* and *rpjF* were more susceptible to the *rpjF* mutant of *Xcc*, indicating enhanced DSF levels.

Table 2. Disease severity from topical application of bacteria varying in DSF production to Arabidopsis. Bacteria were inoculated on different Arabidopsis genotypes transformed with *rpjF* or with both-*rpjB* and *rpjF*

<i>Arabidopsis</i> Genotype	<i>Xcc</i> strains	
	Wild type	<i>rpjF</i> ⁻
Col (wild type)	++++	-
<i>rpjF</i> transformed	++++	+
<i>rpjF</i> and <i>rpjB</i> transformed	++++	++

To test whether DSF is mobile within the plant we are performing grafting experiments in which DSF-producing tobacco transformed with the *rpjF* of *Xf* are used as rootstocks to which normal SR1 tobacco is grafted as a scion (Figure 8). In addition, a large number of greenhouse experiments are underway in which we have introduced purified DSF into plants, have sprayed purified DSF onto plants, and have applied DSF as a soil drench to plants. The results of these experiments will soon be available as further disease development occurs. Preliminary results indicate that direct introduction of purified DSF into plants reduced Pierce’s disease by over 50%.



Figure 8. Grafted tobacco plants into which *Xf* has been inoculated. A normal SR1 tobacco scion is grafted onto transgenic DSF-producing tobacco. The graft point is noted with blue tape, and *Xf* has been inoculated above the graft union. The plant is as yet asymptomatic.

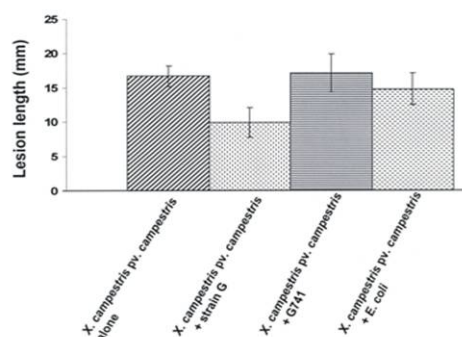
Producing DSF in bacterial endophytes. We have previously been successful in producing large quantities of DSF in endophytes like *Erwinia herbicola* and also in lab strains of *E. coli* (Table 3). We recently were able to transform a putative efficient endophyte of plants, *Rizobium etili* with both the *Xcc* and *Xf rpjF* (DSF biosynthetic gene) and have obtained production of DSF in this strain (Figure 9). This DSF-producing endophyte has been co-inoculated into grape to determine both its ability to move and multiply within grape as well as its ability to interfere with the disease process.

Table 3. Production of DSF by *E. coli* and *Erwinia herbicola* harboring cloned *rpfF* genes from *Xf* and *Xcc*.

Strains	Relative DSF production (Units)
<i>Xcc</i>	100
<i>Xf</i> Temecula	4-5
<i>E. coli</i> DH10B (ptrp <i>XccrpfF</i>)	3000
<i>E. coli</i> DH10B (ptrp <i>XfrpfF</i>)	100
<i>E. herbicola</i> (ptrp <i>XccrpfF</i>)	6000
<i>E. herbicola</i> (ptrp <i>XfrpfF</i>)	200

**Figure 9.** Endophytic strains of *R. etli* producing *Xcc* and *Xf* DSF. The GFP fluorescence produced by an *Xcc* DSF biosensor which is sprayed on the plates, is indicative of DSF production.

Further support for the role of *carAB*, required for production of carbamoyl phosphate (required for pyrimidine biosynthesis in bacteria) in the ability of bacteria to degrade DSF and to control disease was obtained. The *carAB* genes from *Pseudomonas* strain G (which is a highly efficient degrader of DSF and capable of biological control of Pierce's disease) were cloned and used to restore CarAB function in a *carAB* mutant of strain G and restored its ability to reduce disease caused by *Xf*. (Figure 10). These results suggest that it should be possible to enhance biocontrol by DSF-degraders by over-expressing CarAB.

**Figure 10.** Severity of Pierce's disease of grape co-inoculated with *Xf* strain STL and DSF degrading *Pseudomonas* strain G, *CarAB* mutant G741 of *Pseudomonas* strain G, or with mutant G741 complemented with pSC4 when measured 3 months after inoculation. The vertical bars represent the standard error of the mean of the number of symptomatic leaves per vine.

CONCLUSIONS

Several methods of altering DSF levels in plants, including direct introduction of DSF producing bacteria into plants, topical application of such bacteria to plants with surfactants, and direct application of DSF itself to plants appear promising as means to reduce Pierce's disease. Transgenic DSF-producing plants appear particularly promising and studies should soon indicate whether they could serve as a rootstock instead of a scion. While the principle of disease control by altering DSF levels has been demonstrated, more work is needed to determine how to achieve this in the most practical means.

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