Rhodococcus fascians, an Emerging Threat for Ornamental Crops

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ABSTRACT

The actinomycete *Rhodococcus fascians* is a biotrophic pathogen that is capable of deregulating plant development and provoking the formation of multiple shoots. Naturally occurring infections have been reported for 43 families of mostly herbaceous plants. Because of its broad host range and its capacity to disfigure plants, *R. fascians* causes important local losses to the ornamentals industry. As global movement of plants is expanding and no efficient eradication measures are available, *R. fascians* infection is becoming an emerging threat to herbaceous nurseries worldwide. To facilitate detection and identification of possible targets for control procedures, several fundamental aspects of the interaction of *R. fascians* with model plants have been studied. Elucidation of the colonization strategy and the early steps of the interaction might shed new light on the epidemiology of the disease, whereas elaborate knowledge on the virulence determinants of the bacterium might allow new diagnostic tools to be developed. On the other hand, evaluation of the plant response to *R. fascians* infection might yield fundamental insights into plant growth and meristem formation. Here, we present an overview of the current in-the-field and primary knowledge available on this plant-pathogen interaction.

1. INTRODUCTION

The genus *Rhodococcus* belongs to the Corynebacterinae, a group of mycolic acid-containing organisms within the Actinomycetales. The suborder currently includes the genera *Tsukamurella*, *Dietzia*, *Corynebacterium*, *Williamsia*, *Turicella*, *Mycobacterium*, *Gordonia*, *Skermania*, *Rhodococcus*, and *Nocardia* (Gürtler *et al*. 2004). Rhodococci are described as aerobic, Gram-positive, non-motile, asporogenous, GC-rich nocardioform bacteria that exhibit mycelial growth with fragmentation into rod-shaped or coccoid elements (Bell *et al*. 1998). They are common throughout nature and have been isolated from very diverse sources, such as soils, rocks, boreholes, groundwater, marine sediments, animal dung, insect guts, and diseased animals and plants (Bell *et al*. 1998).

This widespread occurrence is reflected in an extensive catabolic diversity and unique enzymatic capabilities with applications in environmental, pharmaceutical, (agro)chemical, food, and energy sectors (van der Geize and Dijkhuizen 2004). The redundancy of the pathways involved and their dissemination on large, often linear, plasmids, underlie these capacities. Many of the functions are essential for the potential use in bioremediation through biodegradation of xenobiotic compounds and for the synthesis of useful and/or bioactive secondary metabolites. Besides the value of the *Rhodococcus* species in industrial or ecological applications, certain strains exhibit also a pronounced pathogenic behavior towards humans and animals with important veterinary and clinical implications (Gürtler *et al*. 2004).

Like the other members of the genus, *R. fascians* (Tilford 1936) Goodfellow 1984 has an industrial and ecological potential. The bacterium is successfully used in debittering of citrus juices for flavor improvement (Cánovas *et al*. 1997) and has been reported to control water transformation in media of biofilters during treatment of ethyl-acetate-contaminated waste streams (Hwang *et al*. 2002). The metabolic versatility of *R. fascians* is illustrated further by the production of volatiles that are chemotropic to *Wohlfahrtia magnifica*, a dipter that causes myiatic lesions in sheep (Khoga *et al*. 2002).

However, unlike other rhodococci, *R. fascians* is a biotrophic phytopathogen capable of infecting a wide array of primarily herbaceous hosts. Incidences of infection have been recorded throughout the world, concentrated largely in temperate regions (CBI Market Survey 2006), with some notable exceptions, including Greenland (Miteva *et al*. 2004) and tropical India (**Fig. 1**). Upon interaction with its host, the bacterium

Abbreviations: ABA, abscisic acid; **DNA**, deoxyribonucleic acid; **ELISA**, enzyme-linked immunosorbent assay; **GA**, gibberellic acid; **GUS**, β-glucuronidase; **HPLC**, high-performance liquid chromatography; **IAA**, indole-3-acetic acid; **IFAS**, indirect fluorescent antibody staining; **iP**, isopentenyladenine; **IPT**, isopentenyltransferase; **MI**, mitotic index; **mRNA**, messenger ribonucleic acid; **PCR**, polymerase chain reaction; **qRT-PCR**, quantitative reverse-transcriptase-PCR; **RNA**, ribonucleic acid

Fig. 1 Geographical distribution of *R. fascians* incidences.

provokes – depending on the plant species – thickened, fleshy stems (fasciation), multiple etiolated stems (witches' broom), abundant buds crowded into a compressed area, or a profusion of partially or fully expanded leaves in a compact mass (leafy gall). Other symptoms, less frequently encountered in general, but specific to particular hosts, include abnormal or attenuated root growth, distorted bulbils of bulbous crops, formation of fleshy lumps of amorphous tissue, flower abortion, and/or stunting (Goethals *et al*. 2001).

These symptoms are due to a complex sequence of events between host and pathogen. In the following sections, we will give an overview of the diverse aspects of *R. fascians*-induced plant responses, specifically focusing on the epidemiology of the disease in ornamentals and on the plant side of the interaction.

2. HOST RANGE AND SYMPTOM DEVELOPMENT UPON INFECTION WITH *R. FASCIANS*

R. fascians is capable of infecting an exceptionally broad range of plant hosts (Anonymous 2005; **Table 1**). Naturally occurring infections have been documented for 164 species in 43 families, comprising both monocotyledonous and dicotyledonous plants. Most hosts are herbaceous, but a few woody species have been either inoculated and found susceptible or were naturally infected: *Justicia*, *Carica* spp., *Euphorbia pulcherrima*, *Populus*, and *Hebe* spp. (Miller *et al*. 1980; Cooksey and Keim 1983; Elia *et al*. 1984; Hu *et al*. 1992; Vereecke *et al*. 2000). It is anticipated that new hosts will continue to be discovered.

Upon interaction with its host, the bacteria typically cause neoplastic shooty outgrowths (**Fig. 2**) by producing growth-modulating hormones, such as auxins and cytokinins, hereby disturbing the plant's hormone balance. Mainly young plant tissues with high meristematic potential are sensitive to the morphogenic signals. Consequently, dormant axillary meristems become active and generate shoots whose further outgrowth is inhibited by these bacterial signals (Vereecke *et al*. 2000). Concomitantly, cortical cells from the stem and petioles re-enter the cell cycle and start dividing to form new shoot primordia (de O. Manes *et al*. 2001), while cells neighboring the vascular tissue in leaves can also give rise to ectopic shoots (Vereecke *et al*. 2000). The ultimate consequence of the continuous repetition of these processes is the formation of a dense gall of meristematic tissue differentiating into shoot primordia. For maintenance of the leafy gall structure, the presence of metabolically active bacteria is required, indicating that the constant delivery of bacterial morphogens sustains the symptoms (Vereecke *et al*. 2000, 2002).

Ornamentals are high-value crops in an industry that is international in scope. The European Union and the United States of America are the greatest consumers of herbaceous material, with conservative estimates for the value of plants purchased annually being $10.2 billion and $11.6 billion, respectively (Anonymous 2005; United States Department of Agriculture 2006). Within the past two decades, production of rooted and unrooted cuttings has shifted from Europe and North America to facilities in Central America, China, Kenya, Uganda, South Africa, India, and Tanzania. Given this expansion in global movement of plants, it seems timely to revisit *R. fascians* epidemiology in terms of production settings.

3. EPIDEMIOLOGY OF THE DISEASE AND DIAGNOSTICS IN ORNAMENTALS

Because *R. fascians* disfigures plants, it is an important pathogen to the ornamentals industry that specializes in plants highly valued for their esthetic appeal. The first symptoms were mentioned in 1927 as a malady of sweet peas (*Lathyrus odoratus*), used as cut flowers (Brown 1927). Soon thereafter, the causal bacterium was described and found to provoke fasciation on sweet pea, "cauliflower" symptoms on strawberry

Table 1 Alphabetic list of greenhouse-grown plants susceptible to *R. fascians*.

Scientific name	Family	Scientific name	Family
Acanthus mollis	Acanthaceae	*Kalanchoe blossfeldiana*	Crassulaceae
Althaea rosea = *Alcea rosea*	Malvaceae	*Lathyrus odoratus*	Fabaceae
Antirrhinum majus	Scrophulariaceae	*Lavatera* sp.	Malvaceae
Argyranthemum sp.	Asteraceae	*Leucanthemum maximum*	Asteraceae
Aster × *frikartii*	Asteraceae	*Leucanthemum* × *superbum*	Asteraceae
Begonia × *tuberhybrida*	Begoniaceae	*Lilium longiflorum*	Liliaceae
Campanula × 'Sarastro'	Campanulaceae	*Lilium regale*	Liliaceae
Catharanthus roseus	Apocynaceae	*Lilium speciosum*	Liliaceae
Cheiranthus allionii = *Erysimum* × *marshallii*	Brassicaceae	*Nemesia* sp.	Scrophulariaceae
Cheiranthus cheiri = *Erysimum cheiri*	Brassicaceae	*Nierembergia* sp.	Solanaceae
Chrysanthemum indicum	Asteraceae	*Oenothera berlandieri*	Onagraceae
Chrysanthemum × *morifolium*	Asteraceae	*Papaver somniferum*	Papaveraceae
Chrysanthemum sp.	Asteraceae	*Perlargonium domesticum*	Geraniaceae
Consolida ambigua = *Consolida ajacis*	Ranunculaceae	*Pelargonium* × *hortorum*	Geraniaceae
Coreopsis sp.	Asteraceae	*Pelargonium zonale*	Geraniaceae
Cosmos atrosanguineus	Asteraceae	*Pelargonium* sp.	Geraniaceae
Dahlia sp.	Asteraceae	*Petunia* × *hybrida*	Solanaceae
Dahlia variabilis = *D. pinnata*	Asteraceae	*Petunia* sp.	Solanaceae
Delphinium ajacis = *Consolida ajacis*	Ranunculaceae	*Phlox* sp.	Polemoniaceae
Dianthus barbatus	Caryphyllaceae	*Primula juliae*	Primulaceae
Dianthus caryophyllus	Caryphyllaceae	*Primula* sp.	Primulaceae
Dianthus sp.	Caryphyllaceae	*Schizanthus grandiflora*	Solanaceae
Digitalis lanata	Scrophulariaceae	*Schizanthus pinnatus*	Solanaceae
Erysimum asperum	Brassicaceae	*Schizanthus retusus*	Solanaceae
Euphorbia pulcherrima	Euphorbiaceae	*Sedum spurium* = *Phedimus spurius*	Crassulaceae
Fuchsia sp.	Onagraceae	*Tagetes erecta*	Asteraceae
Gaura sp.	Onagraceae	*Tagetes patula*	Asteraceae
Gladiolus sp.	Iridaceae	*Tanacetum coccineum*	Asteraceae
Gloriosa sp.	Liliaceae	*Tiarella* sp.	Saxifragaceae
Gypsophila paniculata	Caryophyllaceae	*Tropaeolum majus*	Tropaeolaceae
Helianthus annuus	Asteraceae	*Tulipa gesneriana*	Liliaceae
Heliopsis helianthoides	Asteraceae	*Verbascum densiflorum* = *V. thapsiforme*	Scrophulariaceae
Heuchera sanguinea	Saxifragaceae	*Verbascum nigrum*	Scrophulariaceae
Hosta sp.	Liliaceae	*Verbascum* sp.	Scrophulariaceae
Iberis gibraltarica	Brassicaceae	*Verbena* sp.	Verbenaceae
Iberis sempervirens	Brassicaceae	*Veronica spicata*	Scrophulariaceae
Impatiens walleriana	Balsaminaceae	*Viburnum opulus*	Caprifoliaceae
Ipomoea purpurea	Convolvulaceae	*Viola* sp.	Violaceae

(*Fragaria* x *ananassa*), and leafy galls on *Chrysanthemum*, carnation (*Dianthus* sp.), and *Schizanthus* (Lacey 1936a; Tilford 1936). Over the next few decades, disease inflicted by *R. fascians* was reported on dozens of new hosts in 16 states of the USA, Germany, Denmark, and Sweden (Lacey 1936b; Baker 1950). Since then, *R. fascians* has been isolated from a continually expanding number of hosts from nearly all continents in the world (CBI Market Survey 2006; **Fig. 1**).

From the above, it is clear that *R. fascians* is a persistent, if intermittent, problem in the ornamentals industry. Since its first report in the literature, the bacterium still generates losses of varying proportions in horticultural crops, with certain hosts being particularly susceptible. Under conditions of natural infection, *Leucanthemum* x *superbum* (Baker 1950) and *Begonia* (Faivre-Amiot 1967) were both reportedly infected with 100% incidence. In our experience, growers have had to dispose of entire crops of *Veronica* hybrids, especially 'Royal Candles', *Leucanthemum* cultivars 'Esther Reed' and 'Becky', and various cultivars of *Monarda* due to *R. fascians* infection. Losses in individual nurseries can be severe (Miller *et al*. 1980; Vantomme *et al*. 1980; Putnam and Miller 2007), necessitating vigorous roguing and implementation of extensive sanitation measures to eradicate the bacterium from affected facilities. Shooty symptoms may be induced by *Agrobacterium tumefaciens*, eriophyid mites, phytoplasmas, herbicides, introduction of dwarfing genes into specific cultivars, or plant growth regulators used to stimulate a highly branched, compact structure in the production of herbaceous material (Putnam and Miller 2006; **Fig. 3**). Therefore, plants infected with *R. fascians* are often erroneously classified into one of these categories, which might lead to an underestimation of the actual financial impact of *R. fascians* in the field.

The most important way of spreading the disease in production facilities seems to be infected propagation material, including plants, cuttings, and seeds (Lacey 1936a, 1939; Baker 1950; Digat 1977). *R. fascians* has been described also to move in irrigation water in field situations (Baker 1950) and to travel via water in greenhouses. The bacteria are known to reside in soil, and healthy plants can become diseased when grown in infested soil (Lacey 1936a; Tilford 1936). Experimentally, aphids were shown to transmit *R. fascians* from diseased to healthy plants (van Hoof *et al*. 1979), but currently there is no evidence of insect transmission in greenhouse or field settings.

Diagnosing symptomatic plants with abnormal organogenesis is best achieved by combining multiple methods that rely on different detection and identification methodologies. The most secure method to confirm the diagnosis is the isolation of the bacterium from infected plants, but a negative culture may not mean absence of the pathogen because *R. fascians* is often difficult to isolate (Lacey 1961). Typically, after removal of surface debris by brief washing in sterile water, orange-yellow colonies can be recovered from symptomatic tissue macerated in saline, streaked to solid media and incubated at 28°C for 3 to 5 days. Non-virulent isolates of *R. fascians* may be present, necessitating confirmation of pathogenicity via molecular probing or inoculation to an indicator host. Successful isolation depends on viability of the pathogen, reduction of contaminating organisms, use of differential or semi-selective media (Kado and Heskett 1970; Takayama *et al*. 1985), and skill. For

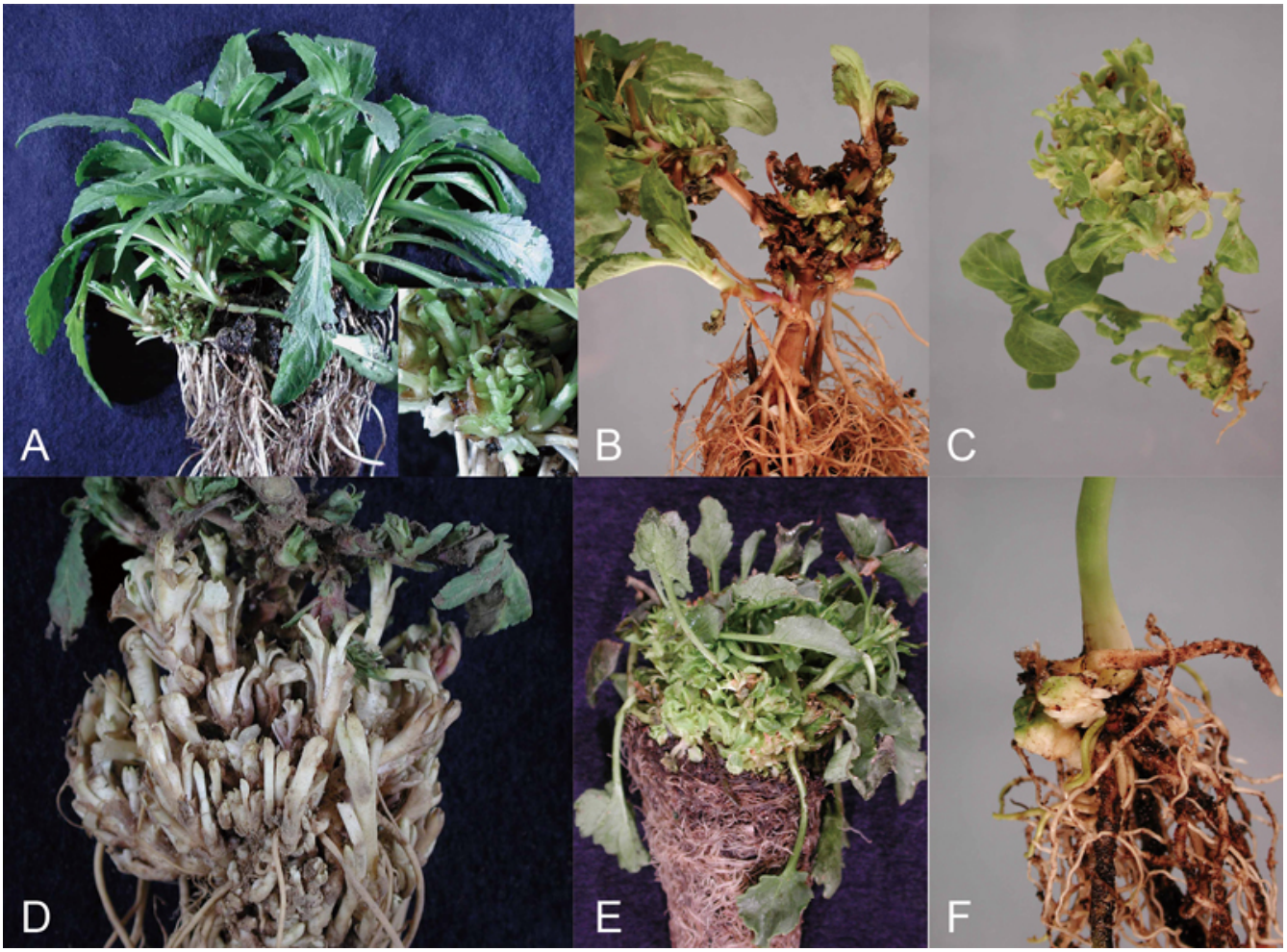

Fig. 2 Symptoms on greenhouse-grown plants infected with *R. fascians*. (**A**) *Leucanthemum* sp. (**B**) *Verbana* "Shauna Ann". (**C**) *Petunia* sp. (**D**) *Veronica* "Royal Candles". (**E**) *Campanula* sp. (**F**) *Hosta* sp.

details, the reader is referred to the original publications. Although the method is time consuming because of the incubation period, the advantages of culturing are the extreme sensitivity for pathogen detection in diseased tissues (Stead 1992) and the possibility for further manipulation and analysis of the bacterium.

Serological approaches are a good alternative for isolation. Indirect fluorescent antibody staining (IFAS) assays have been developed and used to test production material (Angiboust 1975; Digat 1977). Extracts of plants are spotted to microscope slides, treated with fluorochrome-conjugated antiserum, and visually evaluated with a microscope fitted with the proper lenses and filters. Compared to culturing, such assays allow more rapid testing of large numbers of plants, although they are also labor intensive. An enzyme-linked immunosorbent assay (ELISA) would enable testing of many thousands of plants daily, be less expensive than either culturing or IFAS, and could be more easily automated. Unfortunately, ELISA for *R. fascians* has not been developed commercially. Serological assays however can be problematic when bacterial populations are low in the typical samples spotted to microscope slides or microtitre plates, and require the presence of an organism that carries the epitope to which the antibody is raised. Consequently, naturally occurring variants may be missed or other organisms that exhibit the same antigenic sites may yield false positives (de Boer *et al*. 1988).

The polymerase chain reaction (PCR), a molecular detection method, has permitted development of sensitive and specific assays for *R. fascians*, which have been described in detail (Stange *et al*. 1996; Gális *et al*. 2005a). Sequences unique to pathogenic isolates of the bacteria may be targeted and selectively amplified, facilitating relatively rapid sample processing. Because detection does not rely on viability of the bacteria, PCR assays can work well with plant tissues that are unsuitable for isolation. This method also allows quantification of the pathogen, although many plants have inhibitors that interfere with the assay requiring optimization with each species. Sample cost on a per unit basis is however higher for PCR than with culturing or serological assays, and specialized expertise is essential for accurate interpretation of assay results.

Upon recovery of putative *R. fascians* colonies further identification is required. Determinative biochemical tests are described in Bradbury (1986), Goodfellow (1984) and Hu *et al*. (1992). Carbon source utilization profiles can be determined easily and rapidly in a microtitre plate format with commercial systems (e.g. Biolog Inc., Hayward CA, USA; bioMérieux, Durham, NC, USA). Analysis of cellular fatty acid methyl esters via gas chromatography has been widely used for specific and rapid identification of bacteria (MIDI, Inc., Newark, DE, USA). Both methods have limitations however (Harris-Baldwin and Gudmestad 1996; Oka *et al*. 2000; Massomo *et al*. 2003) and neither should be used to the exclusion of other confirmatory procedures. Isolation of genomic DNA and analysis of 16S rRNA gene sequences can help to clarify situations where conflicting or ambiguous results are obtained with other techniques. None of the identification methods discussed above will distinguish between non-pathogenic and virulent isolates. Although amplification via PCR of typical virulence determinants will give a good indication, host inoculation is the ultimate means of establishing identity and pathogenicity. Pea (*Pisum sativum*), sweet pea (*Lathyrus odoratus*), *Nicotiana* spp. and

Fig. 3 Gall-like symptoms induced by different agents. (**A**) *Dahlia* sp. infected with *R. fascians*. (**B**) *Dahlia* sp. infected with *A. tumefaciens*. (**C, D**) *Lavendula* sp. and *Geranium* sp. treated with growth regulators, respectively.

Arabidopsis thaliana have been adopted as bioindicators (Lacey 1936a; Tilford 1936; Kim and Woodward 1985; Vereecke *et al*. 2000). Because symptoms on these hosts often differ from those observed on the plant of isolation, it is advisable to include that host in the test. Symptom development does not require wounding (Lacey 1939), so successful inoculations can be made using a bacterial suspension in which the plants are soaked (Lacey 1936a; Tilford 1936). Inoculation via vacuum infiltration, spotting, and soil infestation are also effective (Tilford 1936; Lacey 1939; Faivre-Amiot 1967; Vereecke *et al*. 2000).

4. DISEASE MANAGEMENT

Chemical control strategies for bacterial diseases in plants have typically involved preventive topical applications of various compounds, often a copper-containing product or – once the bacterium is present in a crop – use of an antibiotic, such as streptomycin, oxytetracycline, gentamicin, or oxolinic acid. Agricultural use of antibiotics is however not allowed in some countries, and is being reexamined in others due to concerns regarding development of antibiotic-resistant populations (McManus *et al*. 2002). Copper compounds may help to prevent establishment of *R. fascians* on plants, but would not eliminate the bacterium once symptoms have developed. Although *R. fascians* has an epiphytic phase, it is not limited to the surface layer of plants (Cornelis *et al*. 2001), and any material applied post-infection would need some level of systemicity in order to reach and eradicate sub-epidermal bacteria.

The best means of control would be immune plant material, but so far none has been identified. Currently, sanitation is paramount in order to prevent introduction of the pathogen into a greenhouse. Novel plantings and new plant material for propagation should be segregated from both production and stock plants until they are found to be pathogen-free. A hierarchical level of sanitation and culture indexing should be

established to ensure plant health, with the greatest level of biosecurity obtained for the mother stock (source germplasm). Such a system has been successfully used to keep *Pelargonium* cuttings disease-free (Angiboust 1975; Digat 1977). Plants bulked up for sales need be assayed on a less intense schedule, but ensuring pathogen-free material requires monitoring during all phases of production, including storage and distribution. Source and origin (e.g. where the material comes from and from what line it is propagated) should be tracked for groups of plants to allow trace-backs should an infected plant be found.

Plant material should be grown so as to minimize the chance of infection and to limit spread once *R. fascians* is introduced. In the greenhouse, freely draining benches should be used to obviate puddling of water under pots or flats, because the bacteria may be carried in water. Flood irrigation should not be used, especially when growing particularly susceptible hosts, such as *Veronica* or *Leucanthemum*. Diseased plants should be removed and immediately destroyed. Neighboring plants, especially those with foliage in physical contact with affected plants, should also be culled. Pots, flats, and trays should be new or thoroughly washed and sanitized with sodium hypochlorite, quaternary ammonium, or another efficacious surface disinfectant.

Physical treatments to destroy the pathogen have been successful in some instances. Hot water treatment of *Lilium* bulbs rid the material of the pathogen when bulbs were held at 39°C for 2 hours, especially when 0.5% commercial formalin was added to the tank (Kruyer and Boontjes 1982). However, treating *Leucanthemum* root divisions with hot water was injurious to the plants (Baker 1950). A suggestion of treating *Begonia* for 10 minutes at 50°C (van Hoof *et al*. 1979) would surely be lethal for leaf cuttings, but may be efficacious for tubers. Thermotherapy has been used to clear *Tropaeolum majus* seed of *R. fascians* (Baker 1950). Seeds were allowed to imbibe for 1 hour in cool water, then immersed in 52°C water for 30 minutes, cooled, and dried overnight. This regimen was used successfully for four years on seed produced in the field. Care must be taken when employing hot water treatments to ensure even temperature distribution throughout the tank. Hot spots may damage plant material and cool corners may not achieve killing temperature for a sufficient duration. Immersion times and temperatures must be determined for each crop.

The efficacy of topical chemical seed treatments (such as mercuric chloride) suggests that the bacteria reside on the seed coat rather than under it. Therefore, hydrogen peroxide, acidified hydrogen peroxide, or household bleach (sodium hypochlorite) might be an alternative to surface disinfest seeds that would not tolerate extended periods at high temperature. Immersion times and concentrations would have to be determined for each crop as well.

5. THE VIRULENCE DETERMINANTS OF *R. FASCIANS*

For efficient colonization and successful symptom induction, *R. fascians* is strictly dependent on the presence of a linear virulence plasmid, designated pFiD188 (for fasciation-inducing) for strain D188 (Crespi *et al*. 1992; Cornelis *et al*. 2001). Some *R. fascians* strains have additional extrachromosomal elements, but these circular plasmids are not involved in pathogenicity (Murai *et al*. 1980). pFiD188 is a conjugative linear plasmid of 200 kb that structurally resembles catabolic plasmids of other *Rhodococcus* strains. It contains four conserved R regions that are involved in plasmid maintenance and three unique U regions that are implicated in the interaction with the host (Francis *et al*. 2007; **Fig. 4**). Below, we describe briefly the two best studied virulence loci, *fas* and *att*, both located in region U1 and identified via insertion mutagenesis (Crespi *et al*. 1992, 1994).

The *fas* operon is essential for virulence because *fas* mutants lose all ability to cause disease. It consists of six genes, one of which is homologous to isopentenyltransferase (*ipt*) genes of Gram-negative hyperplasia-inducing bacteria, and is involved in the biosynthesis of cytokinins (Crespi *et al*. 1992). The other *fas* genes encode a P450-monooxygenase and its accessory electron transport chain, a putative cytokinin oxidase, and a lysine decarboxylase (Crespi *et al*. 1994). Although the structure of the *fas* molecules remains to be resolved, the central role of cytokinins in the pathology can be anticipated for several reasons. The shooty appearance of infected plants clearly points to a deregulated cytokinin signaling, which is supported by the ability to partially mimic *R. fascians*-induced deformations by exogenous addition of cytokinins (Thimann and Sachs 1966). No fewer than 11 different cytokinins have been identified in the supernatant of several *R. fascians* isolates (Helgeson and Leonard 1966; Klämbt *et al*. 1966; Rathbone and Hall 1972; Armstrong *et al*. 1976; Eason *et al*. 1996) and there is a strict correlation between the occurrence of the *ipt* gene and virulence (Stange *et al*. 1996). The importance of the *fas* operon is further emphasized by the tight control of transcription and translation, mediated by different regulatory proteins that integrate multiple environmental signals, such as pH, carbon and nitrogen sources, and availability of oxygen (Temmerman *et al*. 2001). The most important *fas*-activating signal, however, is an autoregulatory compound generated by enzymes encoded by the second virulence locus, the *att* operon (Maes *et al*. 2001). This molecule is produced when the bacteria sense the plant and when conditions favorable for infection are met. The *att* genes are only expressed by the epiphytic population at the onset of the pathogenic interaction (Cornelis *et al*. 2001). At later time points, other unknown control mechanisms ensure continuous *fas* gene expression and, hence, *att* mutants exhibit an attenuated virulence phenotype. The sequence of the nine genes of the *att* operon reveals functions in arginine (*attA*, *attB*, and *attH*) or β-lactam (*attD*, *attE*, and *attF*) and fatty acid (*attC* and *attG*) biosynthesis (Maes *et al*. 2001). Based on these homologies, the *att* autoregulator has been hypothesized to be an antibiotic-like molecule.

Despite the crucial role of the *fas* operon, virulence is not restored upon introduction of a cosmid containing the *fas* locus into a plasmid-free *R. fascians* strain (Crespi *et al*. 1992), implying that additional bacterial signals are required for pathogenicity. In this context, *R. fascians* has been shown to secrete indole-3-acetic acid (IAA) in a plant-dependent manner (Vandeputte *et al*. 2005). The biosynthetic machinery is encoded by the chromosome, but no data are currently available on the relevance of this bacterial signal for virulence.

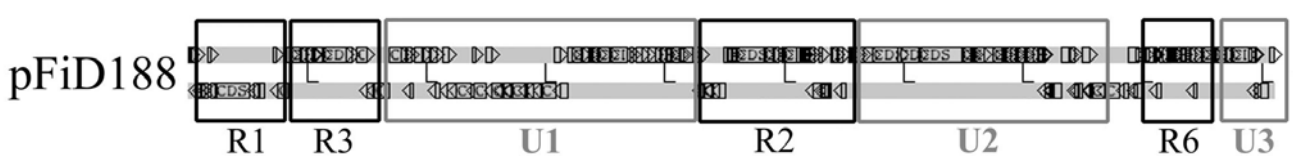

Fig. 4 Schematic representation of pFiD188, the linear virulence plasmid of *R. fascians* strain D188. Conserved R and unique U regions are indicated.

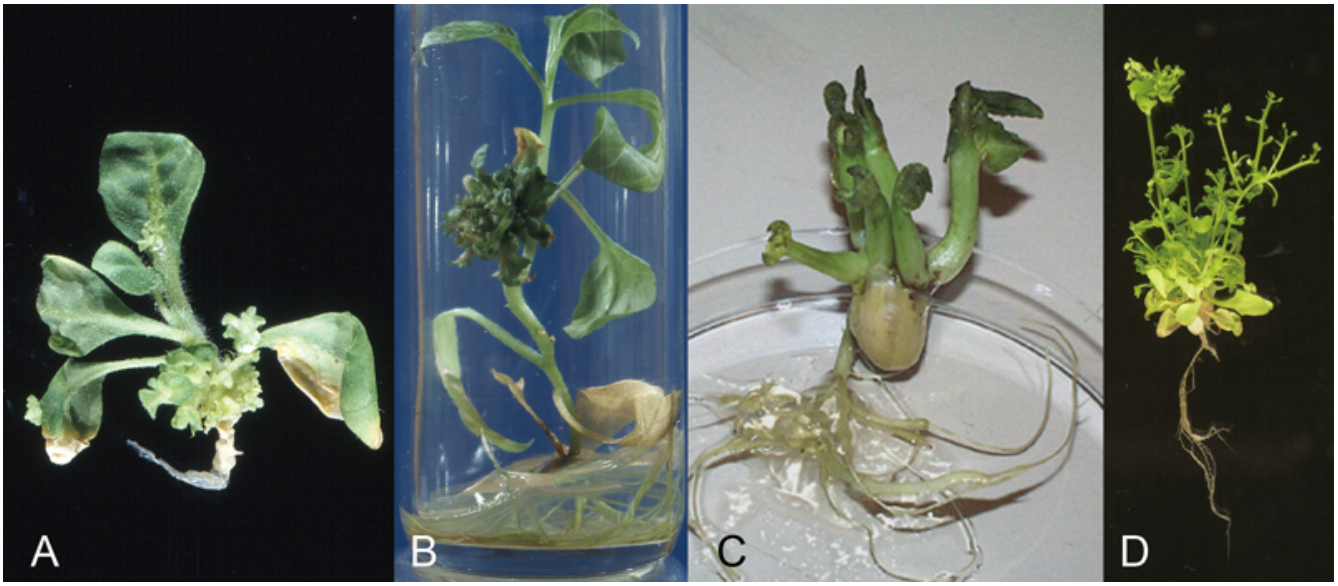

Fig. 5 Symptoms on model plants infected with *R. fascians*. (**A**) *Nicotiana tabacum*. (**B**) *Atropa belladonna*. (**C**) *Pisum sativum*. (**D**) *Arabidopsis thaliana*.

The induction of neoplastic outgrowths on plants is not unique for *R. fascians*. Gram-negative phytopathogens, such as *A. tumefaciens* (Nester and Kosuge 1981), *Pseudomonas savastanoi* (Comai *et al*. 1982), and *Pantoea agglomerans* (Manulis and Barash 2003), all induce galls on their hosts. However, the latter are unstructured tissues resulting from the proliferation of undifferentiated cells, whereas cell proliferation induced by *R. fascians* is combined with differentiation to a leafy organ. Interestingly, similar bacterial signals – such as cytokinin and auxin – seem to play a role in all hyperplasia-inducing interactions (Costacurta and Vanderleyden 1995) yet the outcome of infection is very different, suggesting that other pathways are modulated in the host. The molecular techniques used to unravel these pathways in the host upon *R. fascians* infection are summarized in what follows as well as what is currently known on the reaction of model plants (**Fig. 5**).

6. DISTURBANCE OF THE HORMONAL LANDSCAPE OF THE PLANT UPON INFECTION WITH *R. FASCIANS*

Several studies have focused on the *in planta* changes of cytokinin and auxin levels during this plant-pathogen interaction (Balázs and Sziráki 1974; Eason *et al*. 1996; Vereecke *et al*. 2000; de O. Manes *et al*. 2001; Gális *et al*. 2005a, 2005b; Depuydt *et al*. 2008). Unexpectedly, no significant amount of cytokinins could be detected in infected *Nicotiana tabacum* (tobacco) tissue by an immunoaffinity chromatography method with a broad-spectrum antibody (de O. Manes *et al*. 2001). Using high performance liquid chromatography (HPLC) followed by a radio-immunoassay-based quantification, Eason *et al*. (1996) found a modest increase in the isopentenyladenine (iP) content in pea plants infected with virulent *R. fascians* strains but, more importantly, a decrease in the level of iP and zeatine nucleotides was apparent upon infection. Similarly, upon infection of pea with the virulent *R. fascians* strain 602, the total content of free bases and ribosides, *O*-glucosides, and nucleotides was severely reduced over time (Gális *et al*. 2005a). These results demonstrate that, despite the cytokinin-linked phenotypes and the central role of bacterially produced cytokinins (Thimann and Sachs 1966; Crespi *et al*. 1992), infected plant tissues do not contain elevated levels of standard cytokinins. The reason for this finding might be a balance between the delivery of standard cytokinins by the bacteria and the subsequent turnover by the plant that would not result in a net accumulation of cytokinins. Alternatively, the bacteria might produce specialized cytokinins that have not been identified to date.

Additional phenotypic alterations that cannot be correlated with cytokinin action, such as cell swelling, secondary differentiation of vascular tissues, and lateral root initiation, point toward an involvement of auxins during the interaction. In infected *Atropa belladonna* plants, a 40-fold increased IAA level was measured by a semi-quantitative analysis via ELISA (Vereecke *et al*. 2000). Similar data were obtained by the more sensitive gas chromatography–mass spectrometry method on infected tobacco plants (de O. Manes *et al*. 2001). *R. fascians* synthesizes and secretes IAA by the indole-3-pyruvic acid pathway (Vandeputte *et al*. 2005), but the contribution of bacterial IAA in symptom development remains to be assessed.

Although the mere production of auxin and cytokinin would be sufficient to change the endogenous cytokinin:auxin ratio in infected plants leading to shoot organogenesis, there is growing evidence for an involvement of other phytohormones during the *R. fascians*-host interaction. A differential display screen on infected tobacco implied a role for gibberellic acid (GA) and abscisic acid (ABA) metabolism. In short, this PCR-based technique allows detection of up- and down-regulated genes by systematic amplification of the 3' terminus of the mRNAs with an anchored oligo-dT primer and an arbitrary upstream primer. The amplification products that are labeled with radioisotopes are visualized by denaturing polyacrylamide gel electrophoresis and autoradiography (Liang and Pardee 1998). When compared to control infections with the plasmid-free non-pathogenic strain D188-5, in tobacco inoculated with the *R. fascians* strain D188, four up-regulated genes have been identified, whose differential expression has been confirmed in infected *Arabidopsis* shoots by quantitative reverse-transcriptase-PCR (qRT-PCR). The gene products were highly homologous to a senescence-associated protein, a proline dehydrogenase, a GA2-oxidase, and a P450 mono-oxygenase. The latter belongs to a family of enzymes that are reported to inactivate ABA (Kushiro *et al*. 2004), while GA2-oxidases are involved in deactivation of GA (Lester *et al*. 1999). Moreover, in pea, the effects of *R. fascians* infection can be counteracted with exogenous addition of GA, supporting the role of a putative low GA regime during pathogenesis (Rousseaux 1975). Lowered GA and ABA levels *in planta* are assumed to activate axillary meristems and to break dormancy, two processes correlated with *R. fascians* symptomatology (Simón-Mateo *et al*. 2006).

7. *R. FASCIANS* AFFECTS THE CELL CYCLE AND THE TRANSCRIPTOME OF THE HOST

The first visible effects of *R. fascians* infection are the proliferation of shooty tissue and the re-activation of cell division in cortical cells. The impact of *R. fascians* on the plant cell cycle has been evaluated with different approaches. β-glucuronidase (GUS) converts the colourless 5-bromo-4-chloro-3-indolyl-β-D-glucuronide substrate to a non-soluble blue dichloro-dibromo-indigo dye and allows visualization of expression patterns when the gene is fused to a promoter of interest (Jefferson *et al*. 1987). Histochemical analysis of infected transgenic *Arabidopsis* plants that carry promoter-*GUS* fusions of the cyclin genes *CYCB1;1* and *CYCA2;1* and the cyclin-dependent kinase gene *CDKA;1* showed first an enhanced expression of *CYCA2;1*, later followed by a strong induction of *CYCB1;1* and *CDKA;1*. These three cell cycle genes are associated with cell division and the competence to divide (Hemerly *et al*. 1993; Ferreira *et al*. 1994; Shaul *et al*. 1996). The effect of infection on *CYCB1;1* expression was confirmed in tobacco where it was correlated with *de novo* division of outer cortical cells of the stems, leading to the formation of shoot primordia (de O. Manes *et al*. 2001). Reactivation of cell division is typically linked with *CYCD3* expression; indeed, an RNA gel blot analysis with *NtCYCD3;2* as probe revealed a 4-fold increase in *CYCD3;2* transcript levels of infected tobacco plants when compared to control plants. Although the mechanism by which *R. fascians* triggers cell division is not known, the observation that *CYCD3* and *CDKA;1* expression can be activated by cytokinin treatment (Hemerly *et al*. 1993; de O. Manes *et al*. 2001) suggests that the induction of these genes is probably triggered by the *fas* molecule(s).

The effect of *R. fascians* on cell cycle progression has been explored by inoculating S-phase synchronized cell cultures of tobacco cv. 'Bright Yellow 2' and by following the mitotic index (MI) over time. Depending on the treatment, a broadening of the MI peak (Temmerman *et al*. 2001) or an acceleration of the cell cycle (Vandeputte *et al*. 2007) was observed. These effects depended on *fas* and, interestingly, differed completely from the outcome of exogenous cytokinin addition. Vandeputte *et al*. (2007) further looked into this modulation of cell cycle progression via a cDNA-amplified fragment length polymorphism transcript profiling. This technique starts with digestion of the generated cDNA by two restriction enzymes (a tetracutter and a hexacutter). After ligation of adapters at the end of these fragments, a first PCR amplification is carried out, followed by a selective PCR amplification with primers that contain one or more selective nucleotides. The obtained fragments are then separated on high-resolution gels and a global view of gene expression is generated (Breyne *et al*. 2003). Of the 38 differentially expressed transcript-derived fragments, six were linked with a faster entry into mitosis (Breyne and Zabeau 2001; Breyne *et al*. 2003; Vandeputte *et al*. 2007). Through qRT-PCR, two of these transcripts were shown to be also up-regulated upon infection of tobacco plants. One of these genes encodes a putative apyrase, an enzyme known to detoxify high levels of cytokinins, supporting again the overproduction of this phytohormone (Vandeputte *et al*. 2007).

From the above it is clear that many pathways are modulated in the host upon interaction with *R. fascians*. Nevertheless, it is also evident that currently only fragmentary information is available on the response of the host to infection. Two reports address genome-wide changes in gene expression that occur in the plant, as visualized by the differential display technique (Nouar *et al*. 2003; Simón-Mateo *et al*. 2006). In *A. belladonna*, expression profiles were monitored at the later stages of infection. After confirmation by RT-PCR, six genes differentially expressed upon infection with strain D188 were identified. The three up-regulated transcripts showed homology to proteins involved in defense response: a pathogenesis-related protein, a putative chitinase-like protein, and a β-1,3-glucanase enzyme. The three down-regulated genes corresponded to a multicystatin protein, a miraculin protein, and a metallothionein-like protein. Although these genes can be involved in plant defense, it has been hypothesized that they participate in plant developmental processes because their down-regulated expression is correlated with the presence of viable bacteria in leafy gall tissue (Nouar *et al*. 2003). Despite the extensive differential display screens, this methodology only identified a few genes whose expression is changed upon infection. Recently, microarray hybridizations with samples taken at several stages during the infection of *Arabidopsis* with *R. fascians* have been analyzed (unpublished results). On the microarray chips, 90% of all *Arabidopsis* genes are represented with gene-specific tags. Competitive hybridization with mRNAs from plants infected with strain D188 and strain D188-5 will reveal differentially expressed genes and identify pathways involved in the disease.

8. METABOLIC CHANGES UPON *R. FASCIANS* INFECTION

Assessment of the amino acid composition in leafy gall tissue of infected *Datura innoxia* revealed that all, except phenylalanine and lysine, are strongly reduced when compared with normal plant tissue (El-Wakyl and Blakeny 1980). These differences might be accounted for by the compromised ability of leafy gall cells to produce amino acids or by the utilization of these compounds by the bacteria. Modulation of the metabolism of the host to generate a specific niche seems indeed to be the driving force of this plant-bacterium interaction (Vereecke *et al*. 2000).

The content in phenolic compounds in ethanolic and aqueous extracts from uninfected plants and leafy galls of tobacco were compared by means of HPLC. Caffeic acid, a cinnamoyl analog, and 7-methyl esculin have been detected only in tobacco leafy galls. Interestingly, the latter compound is specific for proliferating tissue of tobacco because it could be detected in crown galls induced by *A. tumefaciens* but not in leafy galls induced on *Helianthus annuus* or *Artemisia annua*. Coumarins, such as 7-methyl esculin, have been reported to function as plant growth regulators, so that a regulatory role in plant cell division could be envisioned (Vereecke *et al*. 1997). Finally, HPLC analysis of extracts from leafy galls formed on the medicinal plant *Pratia nummularia* assessed polyacetylenes (diosmin and linarin), flavonoids (lobetyol, lobetyolin, and lobetyolinin), and coumarins (o-coumaric acid, esculin, and esculetin) (Li *et al*. 2003; Lin *et al*. 2004). However, no remarkable changes in these secondary metabolites could be measured upon infection with *R. fascians*.

9. POTENTIAL BIOTECHNOLOGICAL APPLICATIONS OF *R. FASCIANS* IN PLANT PROPAGATION

Despite its pathogenic character, the strong shoot-inducing capacity of *R. fascians* offers opportunities for putative applications in plant propagation and transformation procedures. Because *R. fascians* induces cell differentiation and organogenesis, it is anticipated that plant propagation can be improved by applying *R. fascians* or, ideally, the morphogens secreted by the bacterium. Besides the advantage that no addition of exogeneous plant hormones is required, laboratory tests indicate that the risk of genetic or epigenetic variation is low and that no vitrification problems are to be expected. Therefore, the method would comprise fewer steps making it more time and cost effective. Because of

its very broad host range, *in vitro* plant propagation of a wide range of plants – including trees and medicinal and crop species – could potentially benefit from co-cultivation with *R. fascians* (Vereecke *et al*. 2000). A second potential application of *R. fascians* might be in the generation of transgenic plants. Many important crop species have a low regeneration frequency and are, therefore, recalcitrant to genetic transformation. Even though further research is still necessary, it is tempting to assume that *R. fascians* or derived signals could improve the regeneration capacity of certain plant species, allowing their genetic modulation.

10. CONCLUSION

R. fascians has caused problems in nurseries since the beginning of the last century. Modern propagation practices and the increased global movement of plant material steadily strengthen the impact and the occurrence of the losses. Knowledge is available on the spread of the pathogen in production settings and diagnostic tools, designed to prevent introduction of the bacterium into nurseries, are being refined. Nevertheless, once the disease is established, the grower can only depend on extensive sanitary measures to eradicate the bacteria. Researchers have identified key aspects of the bacterium and the interaction with host plants. Upon contact with a host, the bacteria autoregulate the production and secretion of morphogens that will alter the developmental pathways of the plant and modulate the metabolic content of the host. While changes in the hormone landscape are probably directly responsible for shoot amplification, alterations in secondary metabolites and amino acids might reflect defense reactions of the plant and/or niche establishment. Most of these changes are resulting from a major impact of the bacterium on the transcriptome of the host, an aspect that has been addressed only recently. At present, only a fragmentary description is available of the molecular features of the interaction. Notwithstanding the problematic image of *R. fascians* in the ornamental sector, the impact of this organism on shoot amplification might eventually be turned into a tool for plant propagation. A closer contact between growers, researchers from plant clinics, and fundamental scientists, all confronted with *R. fascians*, could define strategic insights and lead to a more synergistic approach to deal with the pros and contras of this organism in the field.

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