Rhizogenic agrobacteria in hydroponic crops: epidemics, diagnostics and control

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Complete List of Authors:	Bosmans, Lien Moerkens, Rob Wittemans, Lieve De Mot, Rene Rediers, Hans Lievens, Bart
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ABSTRACT

Rhizogenic *Agrobacterium* biovar 1, harbouring a Ri-plasmid (root-inducing plasmid), is the causative agent of hairy root disease (HRD) in the hydroponic cultivation of tomato and cucumber. The disease is characterized by extensive root proliferation leading to strong vegetative growth and, in severe cases, substantial losses in marketable yield. *Agrobacterium* biovar 1 is a heterogeneous group of agrobacteria and includes (at least) ten genomospecies, among which (at least) four genomospecies (G1, G3, G8 and G9) have been associated with HRD in hydroponically grown vegetables. In this review, we have synthesized the current knowledge on rhizogenic *Agrobacterium* biovar 1, including infection process, current taxonomic status, genetic and phenotypic diversity, detection methods and strategies for disease control. With regard to the latter, symptom reduction and prevention of infection through cultivation methods and chemical disinfection (e.g. by the use of chlorine-based disinfectants and hydrogen peroxide) have been discussed. Additionally, we elaborate on biocontrol strategies. Recent research has lead to the identification of a phylogenetically related clade of *Paenibacillus* strains that have antagonistic activity against rhizogenic *Agrobacterium* biovar 1 strains, holding great potential for HRD control. Finally, possible directions for future research are proposed.

Key words: *Agrobacterium* biovar 1, control, crazy roots, extensive root development, hairy root disease.

48 SYMPTOMS AND GEOGRAPHIC OCCURRENCE

49 Hydroponics involves growing plants in a mineral nutrient solution instead of soil and is
50 an established method of producing vegetables such as tomato, cucumber, pepper and
51 lettuce, as well as ornamental crops (e.g. herbs, roses and foliage plants). A major
52 advantage of hydroponics, as compared with field grown crops, is that the crop is
53 isolated from the soil, which often engenders problems of diseases, pests, salinity, poor
54 structure and/or drainage (Litterick *et al.*, 2004). Nevertheless, since the early 1990s
55 hydroponically grown cucumber and melon plants, and later also eggplants and tomato
56 crops, have been affected by a root disorder known as ‘hairy root disease’ (HRD; also
57 known as ‘hairy roots’, ‘crazy roots’ or ‘root mat’). HRD is characterized by extensive
58 root proliferation within the rockwool cube and across the rockwool slab surface (Fig.
59 1), leading to strong vegetative growth with reduced fruit production. Additionally,
60 affected roots are often prone to secondary infections, caused by e.g. *Pythium* and
61 *Pseudomonas* species (Weller *et al.*, 2006). Occasionally, plants showing HRD grow
62 normally and yield is unaffected. Nevertheless, in most cases yield is severely reduced,
63 e.g. up to more than 10% in tomato production (Bosmans L., unpublished results). Due
64 to the persistent nature of HRD, an infested greenhouse is very likely to be re-infested
65 in subsequent seasons, with increasing economic losses year by year. So far, the disease
66 has been reported in several European countries, including Austria, Belgium, Denmark,
67 France, Greece, the Netherlands, Poland, Switzerland, and United Kingdom (Ludeking
68 *et al.*, 2013). A survey performed in Flanders (Belgium) in 2014 indicated that 33% of
69 the tomato, cucumber and eggplant producers were confronted with HRD (Moerkens *et*
70 *al.*, 2014). In the Netherlands, nearly half of the hydroponic growers has to deal with
71 HRD (data of 2012; Ludeking *et al.*, 2013). Since 2013, also cucumber and tomato
72 crops in the Russian Federation have been affected by HRD where it has become one of

the major problems in the greenhouse vegetable industry (Ignatov *et al.*, 2016). In addition, hairy roots has been reported in Japan, New Zealand and the USA (Sawada & Azegami, 2014).

INFECTION PROCESS

Although HRD is generally associated with pathogenic *Rhizobium rhizogenes* strains (formerly *Agrobacterium rhizogenes*; Chandra, 2012; Escobar & Dandekar, 2003; Lindström & Young, 2011), the disease on hydroponic crops is generally caused by *Agrobacterium* biovar 1 strains (*Agrobacterium radiobacter*) harbouring a root-inducing Ri plasmid (also referred to as ‘rhizogenic agrobacteria’) (Weller *et al.*, 2000; 2004; 2006, Weller & Stead, 2002). Strains that do not harbor an Ri plasmid are considered avirulent (Gelvin 2003; 2009). Symptoms arise following transfer and expression of a portion of the Ri plasmid, i.e. the so-called ‘T-DNA’ (‘transferred DNA’), to the plant genome in a manner similar to that seen by tumour-inducing Ti plasmids in *Agrobacterium tumefaciens* (Hooykaas & Beijersbergen 1994; Van Larebeke *et al.*, 1974). The T-DNA on the Ri plasmid is defined by the so-called left and right border, comprised of 25bp direct repeats.

Ri plasmids can be classified according to the opines (i.e. a class of carbohydrate derivatives that serve as a nutrient source for the agrobacteria) that are produced by transformed cells (Wetzel *et al.*, 2014). Mannopine- and cucumopine-type Ri plasmids contain a single T-DNA, whereas two T-DNA regions (TL-DNA (left T-DNA) and TR-DNA (right T-DNA), separated from each other by non-transferred DNA) have been identified in agropine-type Ri plasmids. The left T-DNA (TL-DNA) is about 20 kb in length and encodes up to 18 potential genes, depending on the bacterial strain (Slightom

97 *et al.*, 1986; Nemoto *et al.*, 2009). However, only 4 of the 18 loci have an essential role
98 in the formation of hairy roots (White *et al.*, 1985). These loci were named root locus
99 genes A-D (*rolA-D*), which render plant cells more sensitive to endogenous auxins,
100 leading to extensive proliferation of the roots (Nemoto *et al.*, 2009; Shen *et al.*, 1988).
101 The right T-DNA (TR-DNA) contains genes involved in auxin and agropine
102 biosynthesis (Chandra, 2011). The single T-DNA of mannopine- and cucumopine-type
103 Ri plasmids shows homology to the TL-DNA and also contains the *rol*-locus
104 responsible for induction of hairy root formation, but not the genes involved in auxin or
105 opin biosynthesis (Brevet & Tempe, 1988; Chandra, 2012).

106 Transfer of *Agrobacterium* T-DNA to the plant cell has been the subject of
107 several reviews (e.g. Gelvin, 2009; Hwang *et al.*, 2015; Lacroix & Citovsky, 2016;
108 Sheng & Citovsky, 1996; Tzfira & Citovsky, 2006). The gene transfer is mediated by
109 the co-operative action of a number of virulence (Vir) proteins encoded by genes also
110 present on the plasmid, collectively referred to as the virulence (*vir*) genes (Tzfira &
111 Citovsky, 2006; White & Nester, 1980). The multi-step process of T-DNA transfer and
112 insertion has been the focus of intense study, but some steps are still poorly understood.
113 Briefly, following attraction to wounded host cells the bacteria loosely bind to the host
114 cell surface, where they start synthesizing cellulose filaments that stabilize the binding,
115 resulting in a tight association between *Agrobacterium* and the host cell. This process is
116 facilitated by two chromosomal *Agrobacterium* genes, *chvA* and *chvB*, that are involved
117 in the synthesis of extracellular β -1,2-glucans, allowing *Agrobacterium* to attach to
118 plant hosts (Giri & Narasu, 2000). Subsequently, phenolic compounds secreted by the
119 wounded plant cells (e.g. acetosyringone, lignin, flavonoid precursors) as well as sugar
120 molecules and a low pH activate the VirA protein, the sensor of a 2-component
121 regulatory system (Lacroix & Citovsky, 2013). The activated VirA protein then acts as a

122 kinase and phosphorylates the cytosolic response regulator VirG (Lee *et al.*, 1995 Wise
123 & Binns, 2015). Phosphorylated VirG acts as a transcriptional activator by binding to a
124 12-bp vir box located upstream of the transcription start sites of the *vir* genes
125 (Czarnecka-Verner *et al.*, 2016). The VirD2 endonuclease in association with the VirD1
126 topoisomerase make a single-stranded cut at the T-DNA border sequence (Wang *et al.*,
127 1987). Next, the 5' end of the T-DNA is covalently bound to VirD2 protein and is
128 removed by strand displacement (Chandra, 2012). In order to protect the excised single
129 stranded T-DNA from cytoplasmic nucleases of the host cell and to adapt it for nuclear
130 import, the T-DNA is coated with the single-stranded DNA binding protein VirE2
131 (Citovsky *et al.*, 1989). Next, the T-DNA complex is directed to a type IV secretion
132 system (T4SSs), which forms a transmembrane channel through which the T-DNA
133 complex is transferred into the cytoplasm of the host plant cell (Chandran Darbari &
134 Waksman, 2015; Dumas *et al.*, 2001; Jakubowski *et al.*, 2004; Sakalis *et al.*, 2014;
135 Zupan *et al.*, 2007).

136 Subsequently, the T-DNA complex is delivered into the host nucleus through
137 interaction with host proteins. Importin α , which is part of the host nuclear import
138 machinery, binds to the Nuclear Localization Signals (NLS) in VirD2 and facilitates
139 import of VirD2 into the host nucleus. VirE2 proteins are bound indirectly to importin
140 α , through the VIP1 host protein (VirE2 Interacting Protein 1), which acts as an adaptor
141 protein between VirE2 and importin α (Citovsky *et al.*, 2004; Lacroix & Citovsky,
142 2013; Tzfira *et al.*, 2001; 2002). Once the T-DNA complex has arrived in the nucleus,
143 the accompanying proteins are removed, presumably by the host ubiquitin-proteasome
144 system, the complementary strand of the T-DNA is synthesized and the double stranded
145 fragments are finally integrated into the plant host genome (Lacroix & Citovsky, 2016).
146 Subsequent expression of T-DNA genes results in synthesis and excretion of opines,

147 which are utilized by *Agrobacterium* as a nutrient source (Dessaux *et al.*, 1992).
148 Additionally, expression of the *rolA-D* genes render the plant cells more sensitive to
149 plant auxines leading to the typical HRD symptoms. Plant assays have shown that HRD
150 symptoms generally develop five to eight weeks after infection. Nevertheless,
151 sometimes three to four months are needed to develop symptoms.

152

153 TAXONOMY

154 Agrobacteria are Gram-negative, rod-shaped bacteria belonging to the class
155 Alphaproteobacteria and the family Rhizobiaceae, whose members represent a wide
156 diversity of (plant) pathogens and non-pathogens including beneficial bacteria. The
157 taxonomy of agrobacteria was historically based on disease symptoms: bacteria
158 inducing crown galls were named *Bacterium tumefaciens* (Smith & Townsend, 1907);
159 root-inducing bacteria were named *Phytomonas rhizogenes* (Riker, 1930); and non-
160 pathogenic agrobacteria were named *Bacillus radiobacter*. Based upon similarity in
161 morphology and physiology among these bacteria, Conn (1942) proposed a new genus,
162 *Agrobacterium*, comprising the species *Agrobacterium tumefaciens* (Smith &
163 Townsend, 1907), *Agrobacterium rhizogenes* (Riker *et al.*, 1930) and *Agrobacterium*
164 *radiobacter*. *A. tumefaciens* (type species) was the name given to *Agrobacterium* strains
165 capable of inducing tumorigenic reactions in a wide range of host plant species. *A.*
166 *rhizogenes* comprised strains capable of causing hairy roots in host plants, and *A.*
167 *radiobacter* harboured non-pathogenic *Agrobacterium* strains (Conn, 1942).
168 Afterwards, a number of additional species have been allocated to the genus, including
169 *Agrobacterium larrymoorei* (infecting *Ficus* spp.), *Agrobacterium rubi* (infecting *Rubus*
170 spp.) and *Agrobacterium vitis* (infecting *Vitis* spp.) (Bouzar *et al.*, 1995). As
171 pathogenicity traits of *Agrobacterium* depend on the type of plasmid hosted by the

bacteria, it became clear that phytopathological properties were not valid for species delineation (for a review, see van Berkum & Eardly, 1998). Using a polyphasic approach, various authors have recognized three large clusters in the genus *Agrobacterium* (Table 1), corresponding to biovars 1, 2, and 3, as defined by Keane *et al.* (1970). Biovars are determined by chromosomal genes and not plasmids, and therefore better reflect phylogenetic relationships. As a result, tumorigenic, rhizogenic and non-pathogenic strains can be found within the same biovar. More specifically, biovar 1 contains strains of *A. tumefaciens*, *A. rhizogenes* and *A. radiobacter*, including the type strains of *A. tumefaciens* and *A. radiobacter*. Biovar 2 also contains strains of all three species, including the type strain of *A. rhizogenes*. Biovar 3 contains *A. tumefaciens* and *A. vitis* strains (Ophel & Kerr, 1990; Panagopoulos *et al.*, 1978).

In modern bacterial taxonomy, criteria to delineate taxa include genomic information in addition to, for example, morphological, physiological and biochemical features. Homogenous genomospecies is the ultimate criterion to validly delineate a *bona fide* species (Stackebrandt *et al.*, 2002). Meeting this criterion, *A. larrymoorei*, *A. rubi* and *A. vitis* (biovar 3) have been validated as *bona fide* species (Bouzar & Jones, 2001; Ophel & Kerr, 1990). *Agrobacterium* biovar 1, however, is heterogeneous, including (at least) ten genomospecies currently called genomovar G1 to G9 (Mougel *et al.*, 2002) and G13 (Portier *et al.*, 2006), suggesting that biovar 1 comprises multiple species that have yet to receive a Latin binomial name. Remarkably, the type strains of the formerly described species “*A. radiobacter*” and “*A. tumefaciens*” were found to belong to the same genomospecies (G4). As a result, these species names are synonymous and should be renamed as “*A. radiobacter*” for antecedence reasons (Young *et al.*, 2006). Costechareyre *et al.* (2010) proposed that the group of closely related taxa corresponding to biovar 1 should be collectively called the “*A. tumefaciens*

species complex” in order to avoid confusion with genomic species G4 (which must be validly named *A. radiobacter*) (Costechareyre *et al.*, 2010). For the International Committee on Systematics of Prokaryotes (ICSP) subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium* this seems to be a good interim solution until genomospecies can be formally named (Lindström & Young, 2011).

Based on 16S ribosomal RNA (rRNA) gene phylogenies Young *et al.* (2006) proposed the genus *Agrobacterium* to be incorporated in the genus *Rhizobium*, but had not obtained a general agreement (Su *et al.*, 2006). As a result, the ICSP subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium* (Lindström & Young, 2011) decided to only transfer *A. rhizogenes*, representing the most distantly related species, into the genus *Rhizobium* as *Rhizobium rhizogenes*. Consequently, *R. rhizogenes* is now a legitimate name for biovar 2 (Costechareyre *et al.*, 2010). Interestingly, all strains currently known to induce hairy roots in hydroponic crops have been identified as *Agrobacterium* biovar 1 strains (Fig. 2) (Bosmans *et al.*, 2015), whereas strains collected from infected plants in the soil belong to biovar 2. Nevertheless, Weller *et al.* (2004) have shown that Ri plasmids may be readily transferred to non-pathogenic agrobacteria or rhizobia, suggesting that novel pathogenic populations with novel characteristics may arise.

GENETIC AND PHENOTYPIC DIVERSITY

Whereas the genetic diversity of tumour-inducing agrobacteria has been studied extensively before (Llop *et al.*, 2003; Pulawska & Kaluzna, 2012), it is only since recently that the genetic and phenotypic diversity of rhizogenic *Agrobacterium* biovar 1 strains causing HRD on hydroponic crops has been assessed (Bosmans *et al.*, 2015; Xiao *et al.*, 2015). These studies showed that rhizogenic *Agrobacterium* biovar 1 strains

222 have substantial genetic diversity, especially for chromosomal loci such as the *trpE*
223 gene (encoding the anthranilate synthase component I). For loci from the Ri plasmid,
224 such as *rolB* and *virD2*, genetic diversity was lower, indicating that the chromosomal
225 housekeeping genes and the Ri plasmid-borne loci have a different evolutionary history
226 (Bosmans *et al.*, 2015). This is in agreement with the fact that Ri plasmids can be easily
227 transferred to other agrobacteria or rhizobia carrying no Ti or Ri plasmids (Weller *et al.*,
228 2004). Furthermore, it has been suggested that recombination events could have
229 contributed to the evolutionary dynamics of rhizogenic *Agrobacterium* biovar 1
230 populations (Bosmans *et al.*, 2015). A genomospecies analysis based on phylogeny of
231 *recA* and *rpoB* sequences, encoding the DNA repair protein RecA and the β subunit of
232 RNA polymerase, respectively, revealed that members of at least four genomospecies
233 may occur on hydroponically grown cucurbit and tomato crops, including
234 genomospecies G1, G3, G8 and G9 (Bosmans *et al.*, 2015). While these studies have
235 undoubtedly contributed to a better understanding of the genetic structure within
236 rhizogenic agrobacteria, further research using more strains from diverse geographic
237 origins is needed to have a more comprehensive view on the population structure.
238 Rhizogenic *Agrobacterium* biovar 1 strains have also been shown to differ substantially
239 at the phenotypic level (Fig. 3). For example, although the majority of strains grow well
240 between 22 and 37°C, some strains are also able to grow at 4°C and/or at 44°C.
241 Furthermore, the majority of strains grow well between pH 5 and pH 9 but some strains
242 are able to grow at more extreme values, i.e. as low as pH 3 and as high as pH 11.
243 Altogether, this shows that rhizogenic agrobacteria can thrive well under a broad range
244 of environmental conditions. Interestingly, most strains are able to form biofilms in
245 laboratory assays. Furthermore, strains have been identified that possess catalase

246 activity, enabling them to resist hydrogen peroxide exposure which is a commonly used
247 disinfectant in hydroponic horticulture (Bosmans *et al.*, 2015).

248

249 DETECTION AND QUANTIFICATION

250 Early detection, accurate identification and reliable quantification of pathogenic strains
251 are cornerstones for effective disease management. Detection and identification of
252 rhizogenic agrobacteria have been classically performed using plating on semi-selective
253 media (Brisbane & Kerr, 1983; Schroth *et al.*, 1965) followed by fatty acid profiling or
254 PCR detection of the Ri plasmid (Haas *et al.*, 1995). Selective media to isolate
255 agrobacteria (Brisbane & Kerr, 1983) are generally based on the particular ability of
256 agrobacteria to resist toxic compounds such as selenite (Na_2SeO_3) or tellurite (K_2TeO_3)
257 salts. On tellurite-amended media, colonies exhibit a typical circular glistening
258 morphology with a characteristic black color and a metallic shine (Brisbane & Kerr,
259 1983; Schroth *et al.*, 1965). A major disadvantage of isolation-based detection and
260 identification, however, is time-consuming and the risk of getting false positives due to
261 lack of medium selectivity (Brisbane & Kerr, 1983). Therefore, alternative methods
262 based on molecular approaches are increasingly developed and implemented for direct
263 detection and quantification of the bacteria. Weller & Stead (2002) described the
264 development of a TaqMan-based quantitative real-time PCR (qPCR) assay to detect
265 *Agrobacterium* biovar 1 strains from diverse environmental samples after enrichment in
266 a selective medium. More recently, Bosmans *et al.* (2016a) developed a SYBR Green-
267 based qPCR assay based on the *rol* locus of the Ri plasmid for detection and
268 quantification of rhizogenic agrobacteria in hydroponic systems. The assay allowed the
269 authors to detect less than one bacterial cell per mL (Bosmans *et al.*, 2016a), illustrating
270 its power for routine detection and quantification of rhizogenic *Agrobacterium* biovar 1

271 strains. Importantly, the assay can be used for pathogen assessment before symptoms
272 develop or even before infection takes place, enabling to take preventative control
273 measures before plants become infected (Bosmans *et al.*, 2016a).

274

275 **MANAGEMENT PRACTICES**

276 Once the T-DNA has been integrated into the plant genome, HRD cannot be controlled
277 by curative means. Instead, preventative actions should be taken before infection can
278 occur. Major strategies to prevent HRD include cultivation methods and chemical
279 disinfection by chlorine-based disinfectants or hydrogen peroxide (H₂O₂). Besides,
280 biocontrol strategies using antagonistic strains are under investigation.

281

282 **Cultivation methods**

283 Excessive root proliferation favors vegetative growth over generative (reproductive)
284 growth, decreasing fruit setting, fruit size and yield. To have sufficient and balanced
285 fruit setting and yield, a good balance between vegetative and generative growth is
286 necessary. During vegetative growth, a plant uses the products from photosynthesis to
287 grow more leaves and stems, while a generative plant is using these products to produce
288 more fruit. However, fruit size and yield decrease if plants become either too generative
289 or vegetative (Van Ieperen, 1996). Therefore, in order to reduce yield losses caused by
290 rhizogenic agrobacteria, actions can be taken that benefit reproductive growth to correct
291 for the excessive vegetative growth caused by the agrobacteria. This can be achieved by
292 various cultivation methods, including choice of rootstock, crop variety (cultivar),
293 growth substrate, irrigation strategy, etc. Most greenhouse tomatoes and cucurbits are
294 grafted onto a rootstock, protecting the plants against diseases and abiotic stress.
295 However, different rootstocks may have a different effect on plant growth favoring

296 either vegetative or generative growth, and thus yield and fruit quality (Abdelmageed
297 & Gruda, 2009; Spornberger & Pieber, 2007). Likewise, the crop variety is known to
298 have an impact on plant growth (Berg, 2009). Hydroponic crops are grown in a
299 substrate medium like rockwool, coco fibre, perlite or peat, each having its own
300 characteristics and requiring a specific irrigation strategy. Both the substrate medium
301 and irrigation strategy are known to have a great effect on whether a plant grows
302 primarily vegetative or generative (Aghdak *et al.*, 2016), as well as on disease
303 development. For example, hydroponic eggplants grown on perlite have been shown to
304 develop less symptoms of HRD compared to rockwool (Wittemans, L., unpublished
305 results). Furthermore, irrigating at a lower frequency and using higher quantities of
306 feeding solution per irrigation cycle was found to decrease HRD symptoms more on a
307 rockwool substrate than on coco fibre. Increasing the irrigation frequency and using
308 lower quantities of nutrient solution on the other hand resulted in similar disease
309 severity at the end of the growing season (Floury, 2010). A common method applied in
310 French, Belgian and Dutch greenhouses that are infested with rhizogenic agrobacteria is
311 removing the upper part of the plastic cover of the growth substrate. Preliminary trials
312 in practical conditions indicated that this method may reduce fruit yield loss caused
313 by HRD (Moerkens R., unpublished data). The underlying mechanisms of this
314 observation, however, remain unclear so far, but it is reasonable to assume that light or
315 oxygen may be important factors. A common method for stimulating reproductive
316 growth is leaf pruning, by which more energy becomes available for flower production,
317 fruit set, growth and development (Kim *et al.*, 2014). While not yet been tested to
318 control HRD, pruning of young leaves from the top of the plant may yield an adequate
319 solution. Nevertheless, it has to be noted that this is a time- and labor-consuming
320 process. Alternative cultivation methods that may be able to reduce HRD may be found

321 in the production of tree fruit. Here, excessive tree vigor can cause a lot of shoot growth
322 which leads to competition for nutrients and assimilates between shoots and fruits.
323 Treatments like root pruning and trunk incisions are used to finetune growth control,
324 flower bud development, fruit set and fruit quality (Maas, 2008). Additionally, the pH
325 of the nutrient solution has been found to be an important factor in balancing vegetative
326 and reproductive growth in roses (Roosta & Rezaei, 2014). However, the pH of the
327 nutrient solution can only vary within a limited range to prevent reduced availability of
328 nutrients for the plants.

329

330 **Chemical disinfection**

331 In most hydroponic systems the nutrient solution is recirculated and reused. The initial
332 water used can be sourced from diverse water supplies such as ponds, lakes, rivers and
333 reservoirs (Stewart-Wade, 2011). However, as these water sources can harbor plant
334 pathogens, they may present a possible source of pathogens entering the crop (Hong &
335 Moorman, 2005). Moreover, when contaminated nutrient solution is recirculated, it
336 becomes a source of infection for the other plants in the irrigation system.

337 Currently, a variety of techniques are used to disinfect the nutrient solution
338 and/or irrigation pipes, including biocide application and exposure of the nutrient
339 solution to ultraviolet (UV) light (Pozos *et al.*, 2004). Cationic surfactants such as
340 benzalkonium chloride (BC), cetyltrimethylammonium bromide (CTAB), and Phytan
341 20, a quaternary ammonium compound (a mixture of alkyl dimethyl benzyl ammonium
342 chloride and alkyl dimethyl ethyl benzyl ammonium chloride), have been shown to
343 eliminate 100% of tumorigenic agrobacteria in water suspensions treated at 7, 5, and 2
344 ppm, respectively. Sodium hypochlorite eliminated 100% of the *A. tumefaciens*
345 population at 0.5 ppm (Yakabe *et al.*, 2012). As chlorine-based disinfectants may be

346 degraded to potentially toxic, mutagenic and/or carcinogenic products posing potential
347 residue problems, in particular in the case of chlorate, the majority of growers have
348 shifted to apply H₂O₂ to control HRD, which is less hazardous for the environment.
349 However, as several rhizogenic *Agrobacterium* biovar 1 strains are catalase-positive and
350 able to tolerate H₂O₂ (Bosmans *et al.*, 2015), H₂O₂ concentrations as high as 100 ppm
351 are needed to significantly reduce rhizogenic agrobacteria populations containing
352 catalase-positive strains. For catalase-negative strains, a treatment with 50 ppm H₂O₂
353 resulted in a significant reduction of the population in both biofilm and water (Bosmans
354 *et al.*, 2016c). However, greenhouse experiments have shown that H₂O₂ concentrations
355 in the water circuit decrease with distance from the application point and are
356 consistently lower at the end of the irrigation circuit, most likely because it reacts with
357 organic material in the (biofilms of the) piping system (Bosmans *et al.*, 2016c).
358 Interestingly, the lower hydrogen peroxide concentrations measured at the end of the
359 irrigation circuits correlate with a higher disease incidence at the end of the irrigation
360 circuit, illustrating the need for monitoring actual H₂O₂ concentrations in the water
361 circuit and/or H₂O₂ applications at additional sites throughout the irrigation system.

362

363 **Biological control**

364 There is an increasing demand from both government and consumers to reduce the use
365 of chemical pesticides and produce pesticide residu-free vegetables (Whipps &
366 Lumsden, 2001). Indeed, as from January 2014 all EU professional growers are
367 obligated to apply Integrated Pest Management (IPM) (Directive 2009/128/EC), in
368 which biological, mechanical and chemical control methods are combined rationally
369 with a minimum use of chemical means (EUCIPM, 2014), reinforcing the need for
370 environmentally-friendly and sustainable alternatives. In this regard, much attention is

371 given to biocontrol organisms (BCO) because of the ability of such antagonistic
372 organisms to suppress plant diseases with a narrow activity spectrum and less
373 environmental impact than chemical pesticides or disinfectants, and the possibility to be
374 integrated with other control methods (Raaijmakers *et al.*, 2002). Especially rhizosphere
375 bacteria are generally considered ideal BCO of soilborne plant pathogens because of
376 their effective colonization of the rhizosphere providing a front-line defence against
377 pathogen attack, their versatility to protect plants under different conditions, and
378 production of antimicrobial compounds (Sharma *et al.*, 2009). Research has shown that
379 *A. tumefaciens* can be successfully controlled with an avirulent *A. rhizogenes* strain
380 without Ri plasmid. This strain (K84) produces agrocin 84 which has a selective
381 toxicity against *A. tumefaciens* (Penyalver *et al.*, 2000). Studies indicate that K84 also
382 produces agrocin 434 which would be active against *A. rhizogenes* (Penyalver *et al.*,
383 2000). Nevertheless, agar overlay assays have shown that K84 is not able to inhibit the
384 growth of rhizogenic *Agrobacterium* biovar 1 strains *in vitro* (Bosmans, L., unpublished
385 results). Furthermore, it cannot be ruled out that this avirulent K84 strain may receive
386 the Ri plasmid from pathogenic strains via horizontal gene transfer (Weller & Stead,
387 2002), and thereby become rhizogenic. For this reason, the use of strain K84 is currently
388 not authorized in Europe.

389 Recently, a large collection of diverse bacterial strains has been screened for
390 antagonistic activity against rhizogenic *Agrobacterium* biovar 1 strains using the agar
391 overlay assay (Bosmans *et al.*, 2016d). Interestingly, only *Paenibacillus* strains showed
392 antagonistic activity and phylogenetic analysis showed that antagonistic activity was
393 restricted to a particular *Paenibacillus* clade, representing the species *Paenibacillus*
394 *illinoisensis*, *Paenibacillus pabuli*, *Paenibacillus taichungensis*, *Paenibacillus tundrae*,
395 *Paenibacillus tylopili*, *Paenibacillus xylanexedens* and *Paenibacillus xylanilyticus* (Fig.

4). When a mixture of two strains identified as *P. xylanexedens* was evaluated for its biocontrol potential under greenhouse conditions, 17 weeks after artificial infection of tomato plants HRD incidence was found to drop with 30% compared to the control treatment where no antagonistic strains were applied, suggesting high biocontrol potential of the inoculum (Bosmans *et al.*, 2016d). Although these results are highly promising, it can be assumed that biocontrol efficacy can even be enhanced by regular application of the BCO. However, it remains to be investigated whether these antagonistic strains also affect biofilm formation or are able to control agrobacteria that are residing in biofilms. Preliminary characterization of the antagonistic compounds of both strains revealed that they are water-soluble and have low molecular weight. Furthermore, Bosmans *et al.* (2016d) suggested an important role of Ca^{2+} to produce and/or secrete these antimicrobial compounds, or to make rhizogenic agrobacteria vulnerable for these compounds (Bosmans *et al.*, 2016b). Further research is needed to fully characterize these compounds, including their identity, production and mode of action.

411

412 CONCLUSION AND PERSPECTIVES

Since its first appearing in hydroponic cucumbers in the UK at the beginning of the 1990s, HRD caused by rhizogenic *Agrobacterium* biovar 1 has rapidly spread in hydroponic cultivation of cucurbits, tomatoes and eggplants worldwide. Symptoms caused by rhizogenic agrobacteria can differ in terms of severity, ranging from no symptoms to heavily proliferated roots. So far, the host and bacterial factors as well as the abiotic factors that play a role in symptom development and severity of the disease remain unknown. Functional studies, e.g. through the use of transcriptomics and/or

420 mutants, may help increasing our understanding of the host responses to *Agrobacterium*
421 infection. Future work could also be aimed at correlating the different genotypes and/or
422 phenotypes of *Agrobacterium* biovar 1 with differences in pathogenicity as well as
423 management strategies. Advantageously, these studies can benefit from recent whole
424 genome sequencing initiatives allowing in depth characterization of chromosomal and
425 plasmid DNA sequences (Slater *et al.*, 2009). A preliminary analysis of the sequenced
426 genome of a rhizogenic *Agrobacterium* biovar 1 strain (NCPBP 2659 isolated from
427 *Cucumis sativus*) resulted in a genome assembly of 5,277,347 bp with a GC content of
428 59.8% (Franco *et al.*, 2016). Genome sequencing of representative biovar 1 isolates may
429 allow correlating genetic diversity with phenotypic differences in, for example,
430 virulence characteristics or biofilm formation capacity. Efforts for HRD control have
431 focused on several strategies to prevent HRD, including cultivation methods, chemical
432 disinfection and biological control. While these methods might work separately, a
433 combination of different methods would probably result in a better protection. Further
434 research could also be aimed at identifying resistance sources and the development and
435 implementation of resistant cultivars or rootstocks, e.g. in combination with suitable
436 BCOs, protecting the crops from infection with a minimum use of chemicals.

437

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441

442 **Conflict of Interest**

443 No conflict of interest declared.

444

For Peer Review

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714 **FIGURES LEGENDS**

715

716 **Figure 1.** Hairy root disease on 4-month old hydroponically grown tomato plants. Left:
717 Healthy roots; right: extensive root proliferation caused by rhizogenic *Agrobacterium*
718 biovar 1.

719

720 **Figure 2.** Maximum likelihood consensus tree from concatenated sequences of four
721 housekeeping genes (16S ribosomal RNA gene, *recA*, *rpoB* and *trpE*) of all
722 *Agrobacterium* biovar 1 and biovar 2 strains for which the sequences are present in
723 Genbank (accessed October 2016). Node support values (bootstrap percentages, based
724 on 1000 simulations) $\geq 90\%$ are shown next to the branches. Bayesian inference
725 posterior probabilities $\geq 90\%$ and Neighbour-joining bootstrap node support values $\geq$
726 90% are denoted by asterisks and daggers, respectively. The small phylogram is
727 included to illustrate branch length heterogeneity (scale bar = 0.05 nucleotide
728 substitutions per site). *Rhizobium tropici* LMG 9503^T was used as an outgroup to root
729 the tree. All strains inducing hairy roots in hydroponic crops (indicated with a red dot)
730 fall within the *Agrobacterium* biovar 1 clade (having LMG 187 as a representative
731 strain), whereas strains causing hairy roots in soil-grown plants (indicated with a blue
732 dot) group together within *Agrobacterium* biovar 2 (containing LMG 150^T as a
733 reference strain). Strains from unknown host/origin are not marked with a dot.

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738 **Figure 3.** Beanplots illustrating the phenotypic diversity (growth temperature, pH,
739 biofilm formation and resistance to hydrogen peroxide (300 ppm)) within a collection of
740 rhizogenic *Agrobacterium* biovar 1 isolates. Plots were made using all agrobacteria
741 studied by Bosmans *et al.* (2015). Each bean represents a one-dimensional scatter plot,
742 showing its distribution as a density shape. The solid line represents the average value
743 for each group; the dotted line represents the mean among the different groups.

744

745 **Figure 4.** Phylogenetic positioning of *Paenibacillus* strains showing antagonistic
746 activity against rhizogenic *Agrobacterium* biovar 1 strains. A maximum likelihood tree
747 was constructed based on 16S ribosomal RNA gene sequences (1390 bp) for all type
748 strains of all *Paenibacillus* species currently described (163 species) and a number of
749 additional *Paenibacillus* strains tested (Bosmans *et al.*, 2016d). Only a particular clade
750 of phylogenetically related paenibacilli was found to have antagonistic activity against
751 rhizogenic agrobacteria (indicated with an orange dot), while strains that were less
752 related to this clade did not (indicated with a blue dot). Strains without coloured dots
753 were not tested for antagonistic activity against *Agrobacterium* biovar 1. Major
754 bootstrap values ($\geq 85\%$; 1000 replicates) are shown at the nodes of the tree.

FIGURES

Figure 1. Hairy root disease on 4-month old hydroponically grown tomato plants. Left: Healthy roots; right: extensive root proliferation caused by rhizogenic *Agrobacterium* biovar 1.

Figure 2. Maximum likelihood consensus tree from concatenated sequences of four housekeeping genes (16S ribosomal RNA gene, *recA*, *rpoB* and *trpE*) of all *Agrobacterium* biovar 1 and biovar 2 strains for which the sequences are present in Genbank (accessed October 2016). Node support values (bootstrap percentages, based on 1000 simulations) $\geq 90\%$ are shown next to the branches. Bayesian inference posterior probabilities $\geq 90\%$ and Neighbour-joining bootstrap node support values $\geq 90\%$ are denoted by asterisks and daggers, respectively. The small phylogram is included to illustrate branch length heterogeneity (scale bar = 0.05 nucleotide substitutions per site). *Rhizobium tropici* LMG 9503^T was used as an outgroup to root the tree. All strains inducing hairy roots in hydroponic crops (indicated with a red dot) fall within the *Agrobacterium* biovar 1 clade (having LMG 187 as a representative strain), whereas strains causing hairy roots in soil-grown plants (indicated with a blue dot) group together within *Agrobacterium* biovar 2 (containing LMG 150^T as a reference strain). Strains from unknown host/origin are not marked with a dot.

Figure 3. Beanplots illustrating the phenotypic diversity (growth temperature, pH, biofilm formation and resistance to hydrogen peroxide (300 ppm)) within a collection of rhizogenic *Agrobacterium* biovar 1 isolates. Plots were made using all agrobacteria studied by Bosmans *et al.* (2015). Each bean represents a one-dimensional scatter plot, showing its distribution as a density shape. The solid line represents the average value for each group; the dotted line represents the mean among the different groups.

Figure 4. Phylogenetic positioning of *Paenibacillus* strains showing antagonistic activity against rhizogenic *Agrobacterium* biovar 1 strains. A maximum likelihood tree was constructed based on 16S ribosomal RNA gene sequences (1390 bp) for all type strains of all *Paenibacillus* species currently described (163 species) and a number of additional *Paenibacillus* strains tested (Bosmans *et al.*, 2016d). Only a particular clade of phylogenetically related paenibacilli was found to have antagonistic activity against rhizogenic agrobacteria (indicated with an orange dot), while strains that were less related to this clade did not (indicated with a blue dot). Strains without coloured dots were not tested for antagonistic activity against *Agrobacterium* biovar 1. Major bootstrap values ($\geq 85\%$; 1000 replicates) are shown at the nodes of the tree.

TABLES

Table 1. Classification of *Agrobacterium* strains.

Old classification ^a	Biovar classification ^b	New classification ^c
<i>Agrobacterium tumefaciens</i>		
<i>Agrobacterium radiobacter</i>	Biovar 1: tumorigenic (<i>A. tumefaciens</i>), rhizogenic (<i>A. rhizogenes</i>) and avirulent (<i>A. radiobacter</i>) strains, includes type strains of <i>A. tumefaciens</i> and <i>A. radiobacter</i>	<i>Agrobacterium tumefaciens</i> species complex (LMG 187 ^T)
<i>Agrobacterium rhizogenes</i>	Biovar 2: tumorigenic (<i>A. tumefaciens</i>), rhizogenic <i>A. rhizogenes</i>) and avirulent (<i>A. radiobacter</i>) strains, includes type strain of <i>A. rhizogenes</i>	<i>Rhizobium rhizogenes</i> (LMG 150 ^T)
<i>Agrobacterium rubi</i>	-	<i>Agrobacterium rubi</i> (LMG 156 ^T)
<i>Agrobacterium vitis</i>	Biovar 3: tumorigenic on <i>Vitis</i> (<i>A. tumefaciens</i> and <i>A. vitis</i>)	<i>Agrobacterium vitis</i> (LMG 8750 ^T)
<i>Agrobacterium larrymoorei</i>	-	<i>Agrobacterium larrymoorei</i> (LMG 21410 ^T)

^a As published in the International Journal of Systematic Bacteriology (International Journal of Systematic and Evolutionary Microbiology).

^b Based on a polyphasic classification approach.

^c Based on Lindström and Young (2011). The type strain (LMG collection, Laboratory of Microbiology, Ghent University, Ghent, Belgium) is given between brackets.