Identification of the causal agents of crazy root disease on hydroponically cultivated cucumber plants in Poland

Michał Warabieda · Artur Mikiciński · Marcin Oleszczak · Joanna Puławska

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Abstract In April 2019, hydroponically cultivated cucumber plants with characteristic symptoms of crazy root disease were found in two different commercial production cucumber greenhouses in Poland. Due to excessive and inappropriate root growth, this disease led to a reduction in yield and deterioration of the general conditions of infected plants. Bacteria isolated from the roots were subjected to a morphological evaluation, as well as molecular, biochemical and pathogenicity tests. To identify the bacteria causing the disease, *Agrobacterium*-like colonies were subjected to PCR with primers complementary to the pathogenicity-related genes located on the crazy root-inducing plasmid (Ri-plasmid): the *virD2A* + *virD2E* primers complementary to the *virD2* gene and the *rolBF* + *rolBR* primers complementary to the *rolB* gene. The pathogenicity of the isolated strains was studied in sunflowers and cucumbers. Twelve strains positive for the Ri plasmid, as determined by PCR, and pathogenic to sunflowers were identified based on sequence analysis of the 16S rRNA and *recA* genes. One strain was classified as belonging to the genus *Pararhizobium*, three to *Rhizobium*, and eight to *Agrobacterium* biovar 1, with the highest similarity to genomospecies G3. The results of

the analyses suggest that these strains may belong to a new, thus far, undescribed species. To confirm this hypothesis, further phylogenetic studies are required.

Keywords Ri-plasmid · Hairy root · Root mat · Detection · Phylogeny

Introduction

The occurrence of crazy root disease, also known as a root mat or hairy root, is responsible for significant economic losses, especially in hydroponic vegetable production. This disease infects dicotyledons and was first described as a soilborne disease in apples (Riker et al., 1930) at the beginning of the twentieth century. In recent years, crazy roots have been noted mainly in tomatoes and cucumbers but have also been found in melons and aubergines in the United Kingdom, France (Weller et al., 2000), New Zealand, Greece, Japan (Sawada & Azegami, 2014), Russia (Ignatov et al., 2016) and Belgium (Bosmans et al., 2015, 2017).

Based on reports of crazy roots in different plant species, it is known that the bacteria causing this disease carry root-inducing plasmids (Ri-plasmids). Bacteria that cause this disease and belong to the genus *Agrobacterium* have been classified mainly as *Agrobacterium* bv 1 genomospecies: G1, G3, G7, G8, and G9 (Sawada & Azegami, 2014; Bosmans et al., 2015, 2017; Ignatov et al., 2016). Among other species known to possess causal agents of crazy root disease,

M. Warabieda (✉) · A. Mikiciński · J. Puławska
Department of Plant Protection, The National Institute of Horticultural Research, ul. Konstytucji 3 Maja 1/3,
96-100 Skierniewice, Poland
e-mail: michal.warabieda@inhort.pl

M. Oleszczak
Intermag Sp. z o.o., Olkusz, Poland

Rhizobium rhizogenes (formerly *Agrobacterium* biovar 2) and *Allorhizobium vitis* (formerly *Agrobacterium vitis* or *Agrobacterium* biovar 3) have also been reported, and both species are also known to be causal agents of crown gall disease (Weller et al., 2000).

Agrobacteria belong to the family *Rhizobiaceae* and naturally occur in soils. They can cause crazy root disease or crown gall disease in a variety of plant species or be non-pathogenic (Puławska, 2010; Bosmans et al., 2015). Crazy root-inducing (rhizogenic) strains are mainly acknowledged to cause losses in cucumber and tomato hydroponic production. Tumorigenic bacteria cause financial decreases in plant nurseries, such as fruit trees, roses (*Rosa* spp.), walnuts (*Juglans* spp.), almonds (*Prunus dulcis*), and grapevines (*Vitis vinifera*) (Campillo et al., 2012).

Interestingly, other α -*Proteobacteria*, such as *Ochrobactrum* and *Sinorhizobium*, which also possess a Ri-plasmid, can cause symptoms of crazy root disease. Although a previous study showed that the pathogenicity tests of these bacteria were positive for the inoculation of cucumber cotyledons (Weller et al., 2004), their ability to cause disease symptoms was not observed in the whole-plant host test. This results was probably because not only genes located on the plasmid but also genes present on the bacterial chromosome are responsible for attachment to plant tissue and are involved in the transformation process and the further development of symptoms of crazy roots (Weller et al., 2005).

The characteristic trait of crazy root disease is uncontrolled, intensive and extended root proliferation. Anomalous roots are covered with numerous hairs, which are responsible for hindering the absorption of both nutrients and water (Nilsson & Olsson, 1997). In addition, the symptoms of the disease can also include early flowering, wrinkled leaves, changes in apical dominance or a shortened internode length (Tepfer, 1984). The decreased vitality of such plants is also associated with greater susceptibility to secondary infections, which makes a cultivation more expensive.

The appearance of the symptoms of crazy root disease is closely associated with the transfer of a specific section, called T-DNA (transfer DNA), of the Ri-plasmid into the plant cell, its integration into host DNA and its subsequent expression. This process occurs in a similar manner to the phenomenon of the transfer and expression of a particular segment of the tumour-inducing plasmid (Ti plasmid) in tumorigenic *Agrobacterium* (Hooykaas & Beijersbergen, 1994);

however, in the case of the Ri plasmid, a different set of genes is located on T-DNA. pRi-derived T-DNA bears root oncogenic loci genes (*rol* genes), and the expression of these genes in plant cells is responsible for the induction of abnormal root development (Chilton et al., 1982). In general, the *rol* genes increase the sensitivity of plants to endogenous auxins – plant hormones that play a key role in the coordination of growth and development processes in plant life cycles (Shen et al., 1988). The precise function of the *RollA* protein has not been fully determined. However, the expression of the *rolA* gene is correlated with the reduction of certain hormone levels, such as ethylene, abscisic acid, auxin, gibberellin or cytokinin (Dehio et al., 1993). Expression of the *rolB* gene appears to be the main factor responsible for the formation of fast-growing, branched roots, which are characteristic of crazy root disease (Altamura, 2004). Moreover, high expression of *rolB* has been found in meristems within a vascular system (in the pericycle, xylem and phloem), indirectly influencing proliferation and differentiation of those cells (Altamura et al., 1991, 1994). The *rolC* gene (as well as *rolB*) is considered a factor responsible for the appearance of changes in secondary metabolism (Shkryl et al., 2008). The expression of *rolD* is thought to be connected with an earlier entry into the generative growth phase, resulting in an increased number of flowering meristems and generative auxiliary buds (Mauro et al., 1996; Falasca et al., 2010). The structure of the Ri plasmid is not consistent in all bacterial strains. The parts of the Ri plasmid that are present in all isolates are the opine catabolism region and the origin of replication (*ori*), which are regions that are essential for the pathogenicity virulence region (*vir*) (Otten, 2018). These plasmids can be transferred to non-pathogenic *agrobacteria* or *rhizobia*, which explains how pathogenic populations can easily develop (Kerr & Panagopoulos, 1977; Weller et al., 2004).

In April 2019, crazy root symptoms were observed in hydroponically cultivated cucumbers (*Cucumis* L.); abnormal roots grew across rockwool surfaces and within rockwool propagation cubes in greenhouses growing two cucumber producers in the neighbourhood of Kalisz and in two other greenhouses near Kraków, Poland (Supplementary Fig. S1). The roots of the obtained cucumbers were highly branched and covered with root hairs. The aim of our study was to determine the aetiology of the observed symptoms in cucumbers and to characterize their causal agent.

Materials and methods

Sample collection

Samples of cucumber plants with crazy root symptoms were collected from two greenhouses located nearby Kalisz and next two located nearby Kraków. From each greenhouse, 1–2 diseased plants were collected. The cucumbers were in BBCH 71–72 stage (first fruits). Roots were washed in tap water and surface sterilized by wiping them with 70% ethanol. Bacteria were isolated from the inner tissue of hairy roots (2–3 samples per each cucumber plant) on King's B (King et al., 1954), MG + Te (Mougel et al., 2001), 1A + 2E (Puławska & Sobiczewski, 2005) media.

Bacterial identification

Bacterial colonies with a morphology resembling agrobacteria were preidentified using multiplex PCR with primers complementary to 23S rDNA (Puławska et al., 2006) to classify them at the biovar/species level. Additionally, DNA from the bacterial colonies was subjected to PCR with the primers virD2A + virD2E (Haas et al., 1995), which are complementary to the *virD2* gene located on Ti and Ri plasmids for the detection of pathogenic isolates, and PCR amplification of the *rolB* gene with the primers rolBF (Bosmans et al., 2016) + rolBR (Weller & Stead, 2002) was performed to identify strains able to induce hairy root disease.

Pathogenicity testing

The isolates preidentified as *Agrobacterium/Rhizobium* spp. were tested on sunflower seedlings according to Puławska and Sobiczewski (2005) to determine their pathogenicity. Briefly, 7-day-old sunflower seedlings were wounded with a sterile scalpel, and the wound was inoculated with the tested bacteria (10^8 cfu). The inoculated seedlings were planted in soil in pots and grown in the greenhouse, temperature: 15,40 °C – 37,90 °C; HR: 18,1% - 90%. Plants were fertilized with multi-component fertilizer (Florovit, Inco, Poland) every week with dose recommended by the manufacturer and the presence of hairy roots was assessed after 30 days. Each bacterial strain was tested on four plants.

Cucumber plants Pacto F1 were propagated from seeds in propagation cubes (Skierniewice, Poland). Three weeks after sowing, the plants were inoculated

with strains containing Ri plasmids (positive in *rolB* and *virD* PCRs) (Table 1) and pathogenic to sunflowers. Inoculation was performed by making an approximately 2 cm incision in the stem of each plant with a sterile scalpel. Bacteria from a single bacterial colony (10^8 cfu) of the selected strain were directly applied to each wound, and then, the wound was carefully wrapped with parafilm. Control cucumber plants were wounded but not inoculated with bacteria. All the plants were grown in the same glasshouse room temperature: 14.00 °C – 31.14 °C; HR 31% –91%. The plants were fed with commercial fertilizer and were reviewed regularly as in test on sunflowers. Two months after inoculation, bacteria were isolated from plants that showed crazy root symptoms (Supplementary Fig. S1c) and were identified by PCR with the *rolB* primers to fulfil Koch's postulates.

Biochemical tests

Agrobacterium strains were subjected to analysis of the biochemical properties characteristic of the *Agrobacterium* genus: 3-ketolactose production, acid or alkali production from citrate and erythritol, growth and pigmentation in ferric ammonium citrate broth, oxidase reaction and activity in litmus milk (Moore et al., 2001).

Housekeeping gene sequencing and phylogenetic analyses

The 16S rRNA gene fragment of 12 selected strains (Table 1) was amplified with the primers fD1 and rP2 (Weisburg et al., 1991). The same primers were used for sequencing by the Genomed S.A. (Warsaw, Poland) commercial service. Identification of the nearest phylogenetic neighbours of the sequenced 16S rRNA was carried out using the EzTaxon database (<http://www.eztaxon.org/>) (Yoon et al., 2017). Because *recA* gene sequences are treated as a marker for the *Agrobacterium* and *Rhizobium* genera (De Lajudie et al., 2019) and because a complete database of *recA* sequences for type strains of all species in these genera has been established, we amplified and sequenced the fragments of this gene from all the tested isolates with the primers ArecAF and ArecR according to the originally described conditions (Puławska et al., 2016b). Two *recA* maximum likelihood phylogenetic trees were constructed with MEGA X (Kumar et al., 2018) (Fig. 1,

Table 1 Overview of isolated and tested bacterial strains

Strain	PCR	Test on sunflower		Test on cucumber	16 s rRNA based identification	GenBank accession number	
		Multiplex ^a	<i>virD2</i> ^b			16S rRNA	<i>recA</i>
O1.1.1	–	–	+	+	<i>Rhizobium metallidurans</i> ; ChimEc512	MW256431	MW251507
O1.1.5	1	+	+	+	<i>Agrobacterium radiobacter</i> ; ATCC 19358	MW256422	MW222382
O1.2.1	–	–	+	+	<i>Rhizobium metallidurans</i> ; ChimEc512	MW256430	MW251508
O1.2.3	–	–	+	+	<i>Rhizobium metallidurans</i> ; ChimEc512	MW256432	MW251509
O1.3.2	1	+	+	+	<i>Agrobacterium radiobacter</i> ; ATCC 19358	MW256423	MW222383
O1.3.3	–	–	+	+	<i>Pararhizobium herbae</i> ; CCBau 83011	MW256433	MW251510
O1.3.4	1	+	+	+	<i>Agrobacterium radiobacter</i> ; ATCC 19358	MW256424	MW222384
O2.1.2	1	+	+	+	<i>Agrobacterium radiobacter</i> ; ATCC 19358	MW256425	MW222385
O2.3.1	1	+	+	+	<i>Agrobacterium radiobacter</i> ; ATCC 19358	MW256426	MW222386
O2.3.2	1	+	+	+	<i>Agrobacterium radiobacter</i> ; ATCC 19358	MW256427	MW222387
O3.4	1	+	+	+	<i>Agrobacterium radiobacter</i> ; ATCC 19358	MW256428	MW222388
O4.1	1	+	+	+	<i>Agrobacterium radiobacter</i> ; ATCC 19358	MW256429	MW222389

^a PCR assay for detection of biovar 1 strains conducted according to Pulawska et al. (2006), 1 – means the result of multiplex PCR shows that bacteria belong to biovar 1 of *Agrobacterium*; “–” no amplification results

^b PCR assay for detection of virulence region of Ri plasmid fragment conducted according to Haas et al. (1995).

^c PCR assay for detection of root oncogenic loci region of Ri plasmid fragment conducted according to Weller and Stead (2002); Bosmans et al. (2016)

Supplementary Fig. S2). Dendrograms were constructed using the general time reversible evolutionary model with a gamma distribution and by assuming that a certain fraction of sites were evolutionarily invariable (G + I) as this was found to be the best substitution model (Kumar et al., 2018). One dendrogram with all the members of the genus *Agrobacterium* for cucumber strains belonging to *Agrobacterium* and a second dendrogram with type strains of all the validly published species of *Rhizobium* and *Pararhizobium* for strains classified as belonging to these genera based on the 16S rRNA analysis were constructed. The significance of the internal branches of the phylogenetic trees was estimated with 500 bootstrap replicates.

Results and discussion

Out of the 51 strains isolated from the tested cucumbers, 12 that were preidentified as *Agrobacterium/Rhizobium* were pathogenic when tested on sunflowers, and after re-isolation, the identity of the inoculated bacteria was confirmed by colony morphology and PCR with primers complementary to the *rolB* gene. All 12 strains causing abnormal root growth in the area of infection in sunflower seedlings were positive according to PCR with the *virD* and *rolB* gene primers, suggesting that these strains possessed Ri plasmids.

We used sunflower seedlings to test the pathogenicity of the bacteria because this plant species is very susceptible to a wide range of *Agrobacterium* and

Fig. 1 Maximum likelihood phylogenetic tree obtained from the sequence of chromosomal housekeeping gene *recA* for type strains of all *Agrobacterium* species and the strains from cucumber

characterized in this study. As an outgroup, the sequence of *recA* gene *Rhizobium rhizogenes* LMG 150^T was used to root the tree

Rhizobium strains, both tumorigenic and rhizogenic. The observed results suggested that the strains differed in terms of their virulence level because some of them produced more intensive symptoms than others.

Out of the 12 pathogenic on sunflower strains, only three were pathogenic toward cucumbers (Table 1). The infected cucumber plants developed numerous, highly branched roots, mainly from the infected wound site. The newly formed roots overgrew the old root ball and reached its surface. The factor that may influence the efficiency of transformation, and thus the appearance of disease symptoms, is the concentration of the bacterial suspension used in the experiment (Paz et al., 2006). However, in the case of our experiment, it was unlikely that the concentration of the bacterial suspension had an effect because we used a large number of bacterial cells to inoculate one plant. Nevertheless, there are examples for which the optimal concentration of the bacterial suspension depends on the plant species or cultivar and the bacterial strain (Hiei et al., 1997; Giovannini, 2006).

The ability of infection of a particular plant species or even cultivar by the individual bacterial strain may depend on the interactions between host plant cells and the strain and on the interaction between the plasmid and chromosomal machinery within the strain (Gelvin, 2003; Tiwari et al., 2007). For example, the Ti plasmid pTiBo542, in its natural host strain *Agrobacterium* sp. Bo542, shows limited tumorigenic potential when tested on different leguminous plant species. However, when placed in the strain C58 chromosomal background, pTiBo542 directs strong virulence toward soybeans and other legumes (Gelvin, 2003). The mechanism of this phenomenon is not well recognized, however some potential reasons like stress-induced changes in lipopolysaccharide (LPS) components were found as resulting in impaired virulence of rhizogenic and tumorigenic strains (Arafat et al., 2009).

Identification of the nearest phylogenetic neighbours based on the sequence of 16S rRNA showed that 8 strains belonged to *Agrobacterium*, 3 strains belonged to *Rhizobium* and 1 strain belonged to *Pararhizobium* (Table 1).

The phylogenetic analysis of *recA* allowed us to conduct a more precise classification of bacteria. The *Agrobacterium* strains showed the highest similarity to genomospecies G3 (Fig. 1); however, they created a separate phylogenetic lineage with a high bootstrap value, which suggests that they could belong to a new

genomospecies. To confirm this hypothesis, further studies with genomic sequencing should be performed. Bacteria belonging to G3, to our knowledge, have not been reported to cause crown gall but have been reported to be the causal agents of crazy root disease (Bosmans et al., 2015, 2017).

Due to allelic diversity, several genomospecies within biovar 1 (also called the *Agrobacterium tumefaciens* species complex) were distinguished based on their *recA* gene sequences, including genomospecies G3 (Costechareyre et al., 2010). Technological progress in the field of genomic sequencing has increased the importance of in silico techniques (Auch et al., 2010; Colston et al., 2014). On this basis, several genomic species with genomovar numbering were distinguished from the *Agrobacterium tumefaciens* species complex, and some of them were validly described as species, e.g., G2 as *Agrobacterium pusense* (Panday et al., 2011; Mousavi et al., 2015) or G14 as *Agrobacterium nepotum* (Puławska et al., 2012; Mousavi et al., 2015). To simplify the diversity problem of genetically separated species, the concept of assigning them to an ecological species has been developed (Lassalle et al., 2011). However, the strains assigned to *Agrobacterium* G3 have been isolated from different and independent environments, which can be problematic for scientists to acknowledge them as a single ecological species (Popoff et al., 1970; Bhullar et al., 2012; Bai et al., 2015; Jones et al., 2016).

The strains belonging to the *Agrobacterium* genus possessed characteristic biochemical features: they possessed the ability to produce 3-ketolactose from α -lactose and were oxidase positive. In litmus milk, they performed alkaline reactions; they grew in ferric ammonium citrate broth with pigmentation; they did not utilize erythritol, but they differed in terms of their citrate utilization. Three strains (O1.1.5, O2.3.1, O3.4) produced alkali, and 5 strains (O1.3.2, O1.3.4, O2.1.2, O2.3.2, O4.1) produced acid from citrate.

Three other pathogenic strains, O1.1.1, O1.2.1 and O1.2.3, belonging to the genus *Rhizobium* based on the *recA* analysis, created the phylogenetic lineage closest to *Rhizobium metallidurans*, while strain O1.3.3 was located near *Pararhizobium* species (Supplementary Fig. S2); however, for the strains in these two phylogenetic lineages, precise classification was not possible without whole genome sequencing. Neither *R. metallidurans* nor *Pararhizobium* strains have been previously reported as causing crazy root or crown gall

disease, although *P. polonicum* was initially isolated from the galls of fruit trees (Puławska et al., 2016a). Bacteria classified as *R. metallidurans* were first identified in a zinc mining site. These bacteria are described as being tolerant to high concentrations of heavy metals, such as zinc and cadmium (Grison et al., 2015).

Conclusions

Our study reports, for the first time in Poland, the results of the identification and characterization of bacteria causing crazy root disease in cucumbers grown hydroponically. We observed that the bacterial strains isolated from diseased cucumbers were characterized by different degrees of pathogenicity and virulence in both sunflowers and cucumbers.

The results showed that new bacterial species involved in the disease might belong to new, hitherto undescribed species. Further studies including additional phylogenetic markers and whole genome sequencing should be performed to determine the actual taxonomic position of these species.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10658-021-02340-6>.

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Code availability Not applicable.

Author contribution MW isolated bacteria, identified them using molecular biology tools, performed phylogenetic and biochemical features analyses and pathogenicity tests on cucumbers; AM performed pathogenicity tests on sunflowers; MO monitored the greenhouses, found and delivered diseased plants; JP is the author of the conception of the study and its design. The manuscript was written by MW and JP. All authors contributed to manuscript revision, read and approved the submitted version.

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Data availability The gene sequences generated and analysed during the current study are available under accession numbers: MW251507–10; MW222382–9; MW256422–33.

Declarations

Authors declare that the manuscript is entirely original and have not been, and will not be, published elsewhere if accepted for publishing in EJPP. The content and authorship of the submitted manuscript has been approved by all authors, and all prevailing local, national and international regulations and conventions, and normal scientific ethical practices have been respected. Authors declare no competing interests

Conflicts of interest/competing interests The Authors declare no conflict of interest.

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