Tubercle disease of sugar beet roots (*Beta vulgaris*) found in Poland is neither caused by *Xanthomonas beticola* nor by tumorigenic *Agrobacterium/Rhizobium*

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Abstract

Symptoms of tubercle disease known also as *Xanthomonas* gall were noticed in Poland in 2014–2017. Roots with disease symptoms showed the reduction of sucrose content compared to the healthy ones. In the literature, there are two gall diseases described: tubercle disease (also known as *Xanthomonas* gall) and crown gall, which is caused by *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*). None of the bacterial strains isolated from the malformed tissues were identified as the bacterial pathogen responsible for causing *Xanthomonas* gall or crown gall. The result was confirmed by searching for the presence of T-DNA, which was not found. Our conclusion is that the tubercle disease observed in Poland is not caused by *R. radiobacter*.

Keywords *Xanthomonas* gall · *Rhizobium radiobacter* · *Agrobacterium tumefaciens* · Tubercle disease · Sugar beet

Symptoms of *X. beticola* disease were first documented on sugar beet roots about one hundred years ago in the United States and have been observed only occasionally since then. Despite this, the disease is frequently described, especially in European literature (Benada et al. 1984; Kozyrovskaya et al. 1984; Nyvall 1989; Drycott 2006; Lazarev 2009; Harveson et al. 2009). However, in recent times, symptoms of the disease called “tuberculosis” were observed on sugar beet roots in 2014 in the central part of Poland (supplementary materials, photos 1–5). The symptoms included unusual tumor-like deformations located on the roots’ head. Some roots had galls that covered the

entire surface and were larger and more irregular than previously described galls (supplementary materials, photos 1–5). These symptoms were observed again in Poland from 2015 to 2017. Each year diseased roots were collected for chemical analysis which was done in the laboratory of Kutnowska Hodowla Buraka Cukrowego (KHBC, Kutnowska Sugar Beet Breeding) by VENEMA automatic system. The analysis of the roots’ quality showed that the reduction of sucrose content was 1.0–1.5% (in 2015) and 3.8–6.2% (in 2016) when compared to healthy roots. In 2017, sucrose content in malformed roots was about 15%. We did not find the causal agent responsible for the disease, and we suggest that the currently known pathogen of the tuberculosis called also “*Xanthomonas* gall”—*X. beticola*, does not exist (Moliszewska et al. 2016). Therefore, our search was targeted to discover other potential bacterial pathogens which could be responsible for the disease symptoms observed.

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Laboratory investigations for pathogens

We have isolated bacteria present in the deformed root tissues. Tissues were macerated in phosphate-buffered saline (PBS), and the homogenate was applied onto nutrient agar (NA, BioMaxima), nutrient agar with an addition

of 1% saccharose (NAS, BioMaxima), nutrient agar with an addition of 0.5% yeast extract (YNA, BioMaxima), *Pseudomonas* agar (PsA, Biocorp), malt extract agar (MEA, Biocorp), agar with yeast extract, dextrose and calcium carbonate (YDC, BioMaxima) and agar with 2% sucrose and peptone (SPA, BioMaxima). The incubation was conducted in 25 ± 2 °C for 48 h. The preliminary identification of isolated mono-bacterial cultures, with characteristic yellow colonies, was carried out on the basis of macroscopic and microscopic description and by API tests (ID32 GN and Api 20E) according to the producer recommendations (BioMerieux). For bacteria identification, we used also partial sequencing of the 16S rDNA obtained by PCR method with primers: forward—20F (5'-AAGTGAAGAGTTTGATCCCTG-3') and reverse—17R (5'-GACTTCACCCAGTCAT-3') and with the use of Direct PCR Kits—Terra PCR Direct Polymerase Mix (Clontech, Mountain View, CA, USA = Takara Bio USA). The primers 20F and 17R for PCR assay were designed by BLAST tool (NCBI) to obtain their partial 16S rRNA gene fragments of different *Xanthomonas* genus strains with a predicted PCR product length of 1500 bp. According to the results, we have identified *Pantoea agglomerans* and *Pseudomonas plecoglossicida*/*P. putida*. The other isolated endophytic bacteria were identified with the following primers: 27F (5'-AGAGTTTGATCTTGCTCAG-3') and 1492R (5'-ACGGTTACCTTGTTACGACTT-3') (Kim et al. 2012). Isolated bacteria were identified to genera and species like: *Pseudomonas putida*, *P. moraviensis*, *P. fulva*, *P. plecoglossicida*, *Microbacterium phyllosphaerae*, *M. foliorum*, *Bacillus subtilis*, *B. megaterium*, *B. amyloliquefaciens*, *Brevundimonas vesicularis*, *Brevibacterium casei*, *Arthrobacter* sp., *A. phenanthrenivorans*, *Phyllobacterium myrsinacearum*, *Rhodococcus globerulus*, *Rhodococcus erythropolis*, *Stenotrophomonas chelatiphaga*, *S. rizophila*, *Peanarthrobacter aureus*, *Acinetobacter johnsonii*, *Mesorhizobium* sp., *Mycobacterium* sp., *Bosea* sp., *Kocuria* sp., *Pseudoclavibacter* sp. None of the isolated bacteria were identified as *Xanthomonas* sp. and *Agrobacterium*/*Rhizobium radiobacter* was also not detected.

Pathogenicity experiments

To check, if bacteria isolated from malformed roots have pathogenic properties, we have prepared the inoculation experiments.

Experiment 1

For this purpose, 1-month-old roots of sugar beets, grown in phytotron in pots filled with sterile bentonite (pH $\approx$ 7), were inoculated by injuring with pure bacteria cultures of

representative strains (*Pseudomonas* sp. and *P. agglomerans*). No disease symptoms were observed after 2 months of culturing.

Experiment 2

Additionally, we cultured sugar beets in pots where dried galls from diseased sugar beet roots, collected in July 2016, were crushed and used for inoculation. Sugar beet roots were injured in the part above the soil surface using scalpel to simplify infection. The experiment lasted 6 months. No disease symptoms were observed in this experiment.

Experiment 3

The experiment was conducted on the experimental field (on the KHBC area) on which “tuberculosis” symptoms were observed in previous years and were also observed in the current year. In the field experiment, 10-week-old plants were treated by pure cultures of *P. agglomerans* obtained in 2015 from diseased roots. We used 5 ml of the culture (cfu = 1×10^6 /ml) for each plant in the row consisting of 30 plants. The experiment was consisted of five replicates, with no treated control. To avoid a false result, we counted all plants present with visible symptoms of the roots' malformations. During harvest, plants with those symptoms were counted once more and the number of diseased plants was compared. We observed no significant differences in the number of diseased plants and disease occurrence between infected and uninfected plants in this experiment.

Additional laboratory investigations for pathogens

Additionally, in 2015 sugar beets from two locations and in 2016—from three locations, in which the disease was observed in both years, were tested for crown gall causal agents. To check if the deformations were caused by tumorigenic bacteria belonging to *Agrobacterium*/*Rhizobium* genus, isolation of bacteria was carried out on selective media Mg + TE and 1A + 2E (Puławska et al. 2016). Obtained bacterial colonies were pre-identified as *Agrobacterium*/*Rhizobium* by 23S rDNA-based multiplex PCR (Puławska et al. 2006) and identified as possessing Ti plasmid (necessary for tumor formation on plants) by *tms2* gene-based PCR (Puławska and Sobiczewski 2005). Additionally, selected isolates were identified by sequence analysis of 16S rDNA. Isolated bacteria were identified to genera and species like: *Pseudomonas lurida*, *Phyllobacterium ifriqiense* and different species of *Rhizobium* characteristic for soil environment. We also isolated

bacteria belonging to *Agrobacterium skierniewicense* (Puławska et al. 2012a) and *A. nepotum* (Puławska et al. 2012b), but none of the isolates was tumor-inducing.

Conclusion

Our conclusion is that the tubercle disease (syn. *Xanthomonas gall*) is not caused by bacterial pathogens described in the literature, especially not by *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*). Additional investigations for the causal agent of the disease are requested. Simultaneously, we found that investigations for endophytic bacteria and their role in sugar beets are interesting and hopeful for future research.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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