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Isolation of streptomycetes causing common scab from 3-years old potato samples from South America

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Abstract. In this paper, we aimed at assessing the best conditions for the isolation of actinobacteria from old potato samples. A set of media and pretreatments were tested. The optimal were chosen for the isolation of actinobacteria from potatoes from Peru, Chile and Argentina. Isolates were tested on the presence of thaxtomin phytotoxin by amplification of the respective gene. Phylogenetic position of strains was compared with their geographical origin, pathogenic potential and existence of common scab (CS) symptoms on potato sample. We demonstrated that RNAlater can be successfully used for the long-term preservation of potato peel for subsequent isolation of actinobacteria on R2A medium. Many streptomycetes were thaxtomin-positive, though they are distantly-related to described pathogens causing CS. Genus *Nocardia* was first reported to be thaxtomin-positive. Potentially pathogenic strains were isolated not only from infected potato but also from those lacking CS symptoms. Some strains from scabby potatoes were thaxtomin-negative.

Key words: common scab, streptomycetes, thaxtomin, potato disease.

INTRODUCTION

Common scab (CS) is one of the most widespread potato diseases caused by several species of streptomycetes, a genus of the phylum Actinobacteria, able to produce phytotoxins thaxtomins (Loria et al., 2006). Thaxtomin is the only known pathogenicity determinant in *Streptomyces*, and the presence or absence of an operon encoding thaxtomin synthetase *txtAB* is 100% correlated with pathogenicity in more than 100 isolates for which pathogenicity assays have been carried out (Wanner, 2009).

The disease is characterized by shallow, raised or deep corky lesions not only on potato (*Solanum tuberosum L.*) tubers, but also on roots of other crops, such as beet (*Beta*

vulgaris L.), carrot (Daucus carota L.), and turnip (*Brassica rapa L.*). The most widespread causal agent *Streptomyces scabies* also inhibits the growth of seedlings of radish (*Raphanus sativus L.*), alfalfa (*Medicago sativa L.*), cauliflower (*Brassica oleracea L.*), colza (*Brassica napus L.*), and turnip (Goyer et al., 2000).

There are different measures to control potato diseases, such as crop rotation (Simson et al., 2017) or selection of the resistant potato varieties (Razukas et al., 2009). However, isolation of the phytopathogenic microorganisms and the study of their resistance and ecological optima is of great importance for disease management. The most common method for the isolation of *Streptomyces scabiei* is plating a freshly collected homogenate from infected tissue or soil suspension from the infected field on water agar or semi-selective media for streptomycetes (Meng et al., 2011; Dees et al., 2013). However, when we used the classical conditions (such as adding nalidixic acid and cycloheximide for the elimination of gram-negative bacteria and fungi) and media for isolation of pathogenic streptomycetes from three-years-old samples of infected potato from South America, we encountered a problem. In most cases at different dilutions Actinobacteria on Petri dishes were over-competed by fast-growing non-filamentous bacteria and could not be isolated.

There are several reported conditions favoring actinobacteria, which can be used for selective isolation. For instance, spores of actinomycetes are more resistant to desiccation as compared to gram-negative bacteria (Kumar & Jadeja, 2016). Streptomycetes can also survive low water potential attributed to dry conditions or to a high concentration of substances in solution (Zviagintsev et al., 2007). Calcium may simulate the aerial mycelium formation and spore germination of streptomycetes including *S. scabiei* (Lambert & Manzer, 1991; Natsume et al., 2001). Adjusting of selective media or soil sample pretreatment with calcium carbonate facilitates the isolation of Actinobacteria (Alferova & Terekhova, 1988; Fang et al., 2017).

In this paper, we aim at assessing the optimal conditions for the isolation of streptomycetes causing common scab of potato from 3-years old potato samples from Peru, Chile and Argentina. The objectives: 1) to choose the optimal medium and pretreatments for selective isolation of actinobacteria and elimination of non-targeted bacteria from old potato samples; 2) to assess isolates in terms of the ability to produce thaxtomin.

MATERIALS AND METHODS

Isolation of Actinobacteria

Potato samples with and without common scab lesions from localities in Chili, Peru, and Argentina (Fig. 1) were collected in 2016 and stored as air-dried potato peel or 5 mm sliced potato-peel in RNAlater Stabilization Solution (Invitrogen) in a -70°°C freezer until the processing for isolation. No surface sterilization was applied to the samples. The set of pretreatments and inoculation procedures for the isolation of Actinobacteria is listed in Table 1.

To choose medium and pretreatment the most suitable for isolation of actinobacteria, dried potato peel (0.1 g) from one selected sample site was ground with mortar and pestle, serially diluted in sterile distilled water and aliquots of 100 µl of the diluted suspension was plated on freshly prepared agar media (in triplicates). The following isolation media were used: Gauze's agar 1 (Gauze et al., 1983); R2A agar

(Reasoner & Geldreich, 1985); Oatmeal agar (Gauze et al., 1983), and Water agar (WA) containing 20 g of agar per liter of water. To test the effect of 0.1% calcium carbonate and 3% agar, R2A and WA medium were supplemented accordingly. Further, the media will be referred to as Rc, R3, Wc, W3 respectively. Each of these media was supplemented with cycloheximide (300 mg L^{-1}) and nalidixic acid (20 mg L^{-1}) to prevent the growth of fungi and gram-negative bacteria.

For assessment of potato preservation form best suited for isolation of actinobacteria, R2A and WA medium were chosen for inoculation. Dried potato peel was serially diluted as described previously or 1 cm diameter piece was used for imprinting into the agar and then streaked out by inoculation loop. Potato peel preserved in RNAlater solution was plated by the serial dilution method.

Following 1-week incubation at 28 °C (2 weeks for WA medium), actinomycete- like and other non-actinobacterial colonies developed on the isolation media were counted.

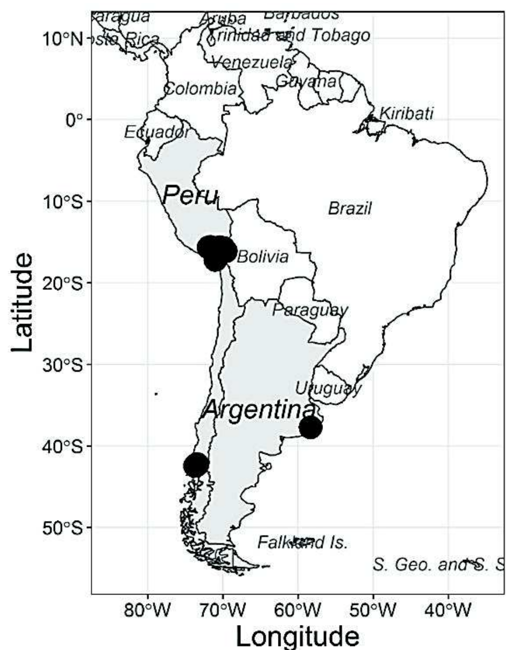

Figure 1. The geographic location of sampling sites in Chile, Peru, and Argentina.

Table 1. Selected conditions for the assessment of isolation of Actinobacteria from 3-years old potato samples

Tested condition	
Media	(a) Gauze-1 (b) Oatmeal Agar (c) R2A (d) Water Agar
Pretreatment and media adjustment	(a) Supplement medium with 0.1% CaCO3 (b) Higher agar content in medium (3%)
Choice of sample form for plating	(a) Dilution of suspension from dried potato peel (b) Imprint of dried potato peel (c) Dilution of sliced potato peel in RNA later solution

The best medium, sample form and pretreatment procedure was chosen for the inoculation of potato samples from 11 sites in Chile, 26 in Peru, and 3 in Argentina. The criteria for selection (in descendent by priority order): 1) ability to isolate actinobacterial strains from separated non-overcompeted colonies; 2) morphological diversity of actinobacteria colonies; 3) lower time of sample processing before the inoculation; 4) shorter incubation time.

Morphologically distinct colonies were selected and purified on the R2A medium. The purified cultures were preserved as glycerol suspensions (20%,v/v) at -70 °C.

Graphical and statistical analysis was performed in Excel and R software (R Core Team, 2019).

Phylogeny and pathogenicity

DNA from pure strains was extracted using a commercial kit according to the manufacturer (DNeasy PowerLyzer Microbial Kit, Qiagen). 16S rRNA gene was amplified with primers *16Seu27f* and *PH* (Bruce et al., 1992) and sequenced by the capillary Sanger sequencing (Macrogen Europe Inc., Amsterdam, the Netherlands). The resulted sequences were amplified with primers 16Seu27f and pH (Bruce et al., 1992) and aligned using SILVA Incremental Aligner v.1.6.0. (Quast et al., 2013) with GenBank sequences of 7 type strains of known-to-date pathogenic streptomycetes causing CS of potato. The best-fit model of nucleotide substitution was selected using jModelTest v.2.1. (Darriba et al., 2012). A phylogeny was inferred using maximum-likelihood analysis in FastTree 2.1.10. (Price et al., 2009). The phylograms were finalized using iTOL (Letunic & Bork, 2016).

Thaxtomin production ability was inferred as a positive PCR reaction targeting a fragment of the *txtAB* (*txtA* and *txtB*) gene using primers *stx1a* and *stx1b* (Flores-González et al., 2008). The thaxtomin-positive isolates later are referred to as *stx*-positive to emphasize primer used.

RESULTS AND DISCUSSION

Media and pretreatments

The results showing the bacterial colonies developed on different media is presented in Fig. 2. A non-parametric *Kruskal-Wallis test* showed that there was a statistically significant difference in ratios of Actinobacterial colonies to other Bacteria colonies (A:B) between the different media used for inoculation (*Chi-square* = 9.31, *p-value* = 0.025, *df* = 3), with a mean rank of A:B ratio score of 6.0 for Gauze, 4.5 for OA, 4.5 for WA and 11.0 for R2A medium. No actinomycete was observed on the OA medium and only 1-2 non-actinobacterial colonies. Although the OA medium is known to stimulate thaxtomin production and support the good growth of *S.scabiei* (Loria et al., 2006), it did not prove to be a good selective medium for actinobacterial isolation from potato.

The highest number of actinobacterial colonies with a relatively small number of non-mycelial bacteria were developed on R2A agar. In comparison with WA and mineral Gauze medium, R2A agar is ‘nutrient-rich’, but nutrients are diluted to mimic oligotrophic environment, as the medium was originally developed for the isolation of bacteria from the potable water (Reasoner & Geldreich, 1985). Surprisingly, Gauze 1 medium, traditional for the selective isolation of streptomycetes from the soil (Gauze et al., 1983), was not suitable for the development of actinobacteria, as plates were over-competed by non-actinomycetes. The soil streptomycetes are known to be oligotrophic and slow-growers which feed on recalcitrant carbon source (e.g. starch as in Gauze medium) (Zviagintsev & Zenova, 2001). It is possible that plant-associated actinobacteria, in contrast to soil inhabitants, require more nutrients because the inner and outer plant environment is rich in plant exudates. While Gauze is a defined medium

with starch as the only carbon source, the R2A medium contains small quantities of starch, yeast extract, peptone, casamino acids and pyruvate, which may be present in potato tuberosphere. There was a minimal number of colonies both of actinobacteria and other bacteria developed on WA in comparison with R2A, though actinobacterial colonies were not over competed by fast-growing bacteria and could be isolated.

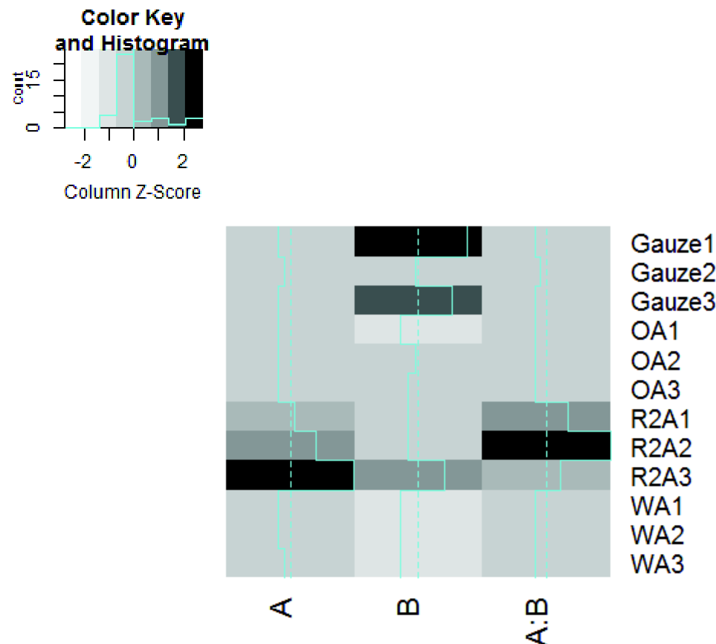

Figure 2. Heatmap of colony numbers of Actinobacteria (A), other bacteria (B), and their ratio (A:B) after inoculation of potato sample suspension from the same dilution on different media (Gauze, OA, R2A, and WA) in triplicates (numbers 1, 2, 3 near the respective medium).

A comparison of inoculation source, medium and medium modifications is presented in Fig. 3. We expected the best sample pretreatment to be homogenization of dried potato peel in a mortar, as streptomycetes spores are usually well preserved in air-dried soil samples (Doroshenko et al., 2005). Indeed, as we see in a heatmap (Fig. 3), the highest actinobacterial counts with low non-targeted bacteria colonies were registered from the inoculated suspension of the dried peel on R2A agar and R2A medium supplemented with 0.1% $CaCO_3$ (Rc).

However, R2A medium with higher agar content (referred to as R3) stimulated the growth of fast-growing bacteria from the same suspension used for the inoculation. The higher agar content equals to lower water potential in a medium (Buah et al., 1999), so we expected streptomycetes to have the advantage to develop in such an environment, as their spores are resistant to desiccation (Doroshenko et al., 2005). The competing fast-growing non-actinomycete bacteria that appeared on R3 plates are possibly more resistant to a higher water potential than streptomycetes. Moreover, actinobacteria produce exospores which are less resistant to many environmental factors in comparison with endospores of other bacteria (Abel-Santos, 2015).

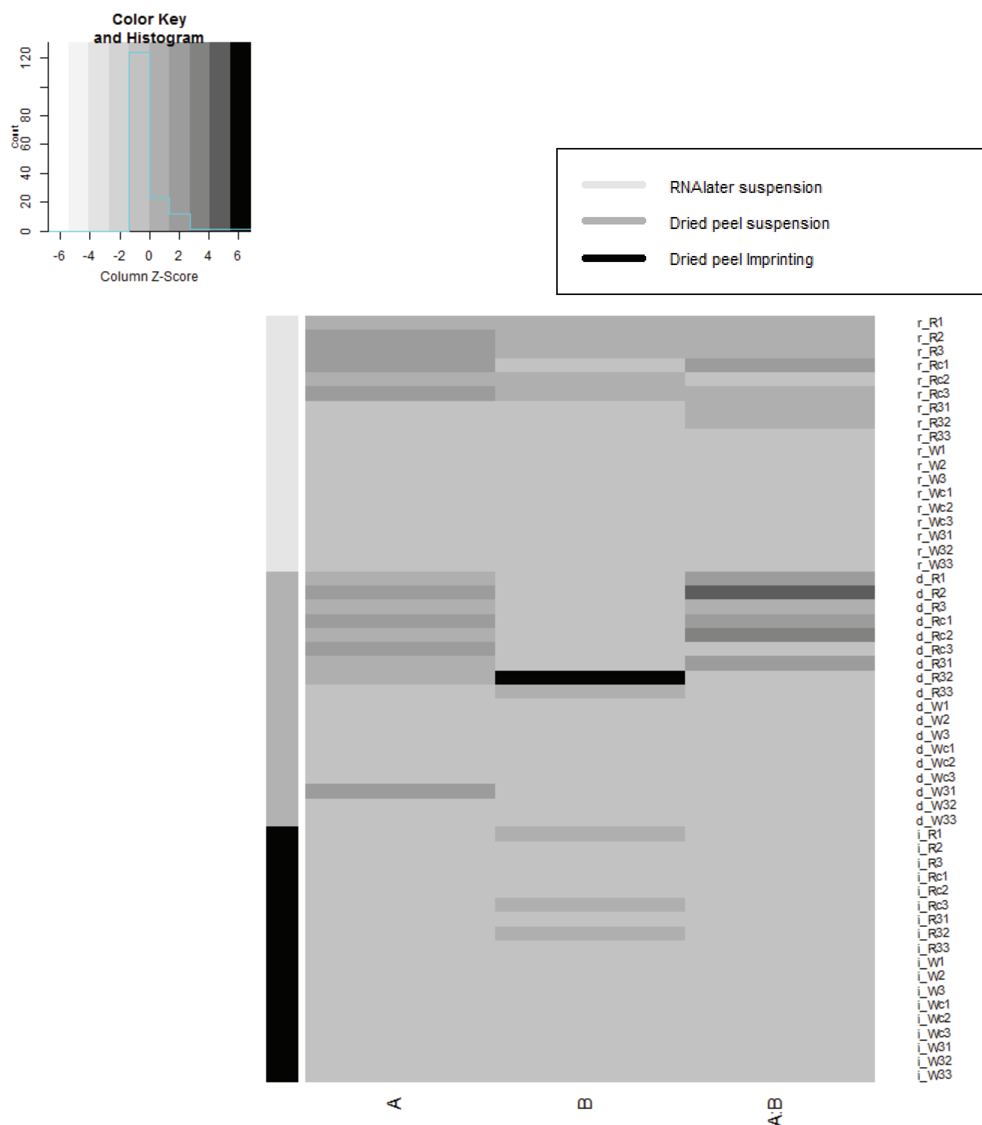

Figure 3. Comparison of different sample forms for plating (left bar) and media adjustments (rows labels). R stands for R2A agar; Rc – R2A with 0.1% CaCO3; R3 – R2A with 3% agar; W – WA. Numbers 1,2,3 near the respective medium indicates triplicates. Heatmap reflects colony numbers of Actinobacteria (A), other bacteria (B), and their ratio (A:B).

Imprinting of dried potato peel was the poorest method for streptomycetes isolation, as only non-actinomycetes grew on all tested media. It is possible that actinomycetes are not on the surface of the dried peel and need to be desorbed from inside by sample homogenization. Inoculation from RNAlater suspensions on R2A and Rc promoted the growth of actinobacteria more prominent, then other bacteria, though not so effectively as from dried peel suspension.

A *Kruskal-Wallis rank sum test* detected the significant difference in A:B ratios between three sample inoculation treatments (*Chi-squared* = 8.7794, *df = 2*, *p-value* = 0.0124). However, pairwise comparisons using *Wilcoxon rank sum test* as a *post-hoc test* with *False Discovery Rate p-value* adjustment (Benjamini & Hochberg, 1995) indicated the significant differences only between A:B ratios on plates inoculated from the Dry peel Imprints and the Dry peel Suspension ($p = 0.011$), and between plates inoculated from the Dry peel Imprints and the RNAlater Suspension ($p = 0.011$). Although we observed the differences between media adjustments on a heatmap, no significant differences were supported by the *Kruskal-Wallis rank sum test* between A:B ratios detected on control plates, and plates with 3% agar content or with CaCO3 supply (*Chi-squared* = 1.29, *df* = 2, *p-value* = 0.5246).

Water agar plating was inefficient for the isolation of streptomycetes from old potato samples in all cases, except one plate inoculated with the dried peel suspension, but not in replicates. A *Wilcoxon rank sum test* with continuity correction showed that there was a significant difference ($p < 0.001$) between scores given for the ratio of Actinobacteria over other Bacteria that appeared on an R2A medium compared to the Water Agar medium ($W = 306$, *p-value* = 3.036e-07). The median score for the R2A medium was 27.0 compared to 10.5 for WA. The effect size is approximately 0.854 which is very large according to Cohen's (1988) classification of effect sizes which is 0.1 (small effect), 0.3 (moderate effect) and 0.5 and above (large effect). It is possible that after such prolonged sample storage, to develop viable colonies bacteria need more nutrients, like in the R2A medium. Moreover, actinobacterial colonies developed on WA appeared to be morphologically identical, while R2A allowed actinobacterial differentiation (different forms and colors of colonies), which is useful in actinobacterial screening. WA requires 2 weeks for colonies to develop, while only 1 week of incubation is sufficient for the R2A medium. Thus, we believe that the WA medium is not the best choice for the isolation of pathogenic streptomycetes from potato samples, as it was commonly used so far.

Storage the peel in RNAlater solution were comparably efficient for the streptomycete isolation, while at the same time facilitating the manipulation with sample and greatly reducing the time for plating. This is the first report that RNAlater solution may be used for the long-term preservation of plant samples for subsequent isolation of actinobacteria.

As R2A medium and R2A medium supplemented with 0.1% CaCO3 were similarly efficient, we have chosen to use usual R2A without any supplement for subsequent isolation of actinomycetes from 3-years old potato samples from 40 different localities of South America. We used serial dilution from RNAlater solution for inoculation.

Actinobacterial isolates from South American potato samples

A total collection of 123 actinobacteria was isolated from 40 localities in Peru, Chile, and Argentina. The phylogenetic position and pathogenicity potential of strains are presented in Fig. 4. Most of the actinomycetes belong to the genus *Streptomyces*, while 19 to the genus *Nocardia*.

As could be expected, closely related strains come from the same regions. Surprisingly, many *stx*-positive strains were not closely related to known pathogens causing CS. Moreover, thaxtomin genes were never described in the genus *Nocardia* before. What is interesting, *stx*-positive nocardias were isolated exclusively from healthy

plants. On the one hand, it may be attributed to the horizontal gene transfer (HGT) of the pathogenicity island which bares genes for the thaxtomin production (Loria et al., 2006). On the other hand, little is known about the actinobacterial community on potato samples coming from these regions, probably the origin of the potato itself.

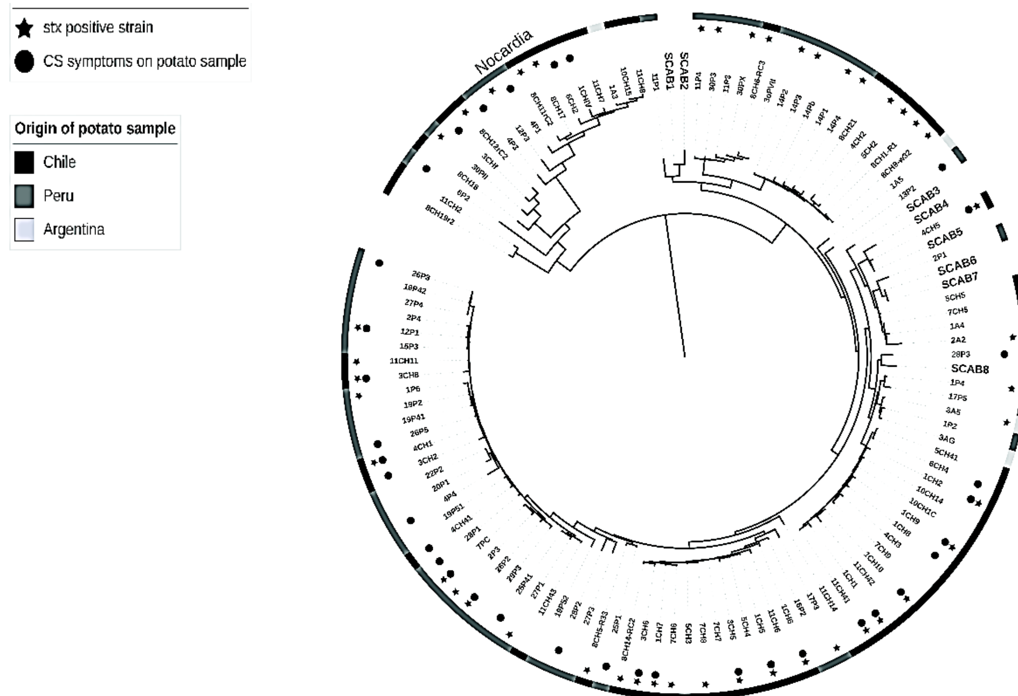

Figure 4. ML Phylogenetic tree based on the 16S rRNA gene of actinobacterial strains (belonging to the genus *Streptomyces* and *Nocardia*) isolated from 3-years old potato samples from different localities in South America. SCAB1-8 is designation of type strains of pathogenic streptomycetes causing common scab of potato (CS): 1 – *Streptomyces scabiei* ATCC 49173T (NR_025865.2); 2 – *S. niveiscabiei* KACC 20254T (NR_037095.1); 3 – *S. europaeiscabiei* KACC 20186T (NR_042790.1); 4 – *S. puniciscabiei* LMG 21391T (NR_025156.1); 5 – *S .reticuliscabiei* CFBP 4531T (NR_025293.1); 6 – *S. stelliscabiei* CFBP 4521T (NR_025294.1); 7 – *S. acidiscabies* ATCC 49003T (D63865.1); 8 – *S. turgidiscabies* ATCC 700248T (NR_040828.2).

Several thaxtomin-positive strains were isolated from potato with CS symptoms. However, many of them came from healthy potato, while many non-pathogenic strains were isolated from infected potato. The presence of scab causing streptomycetes in the tuberosphere of the healthy plant may be connected with suppressivity of soil, which has special bacterial communities protecting the plant (Kopecky et al., 2018). The absence of the pathogenic streptomycetes on infected potato may be due to poor sampling or due to another organism causing the infection.

It was reported previously that all of the known disease-causing *Streptomyces* species also have non-pathogenic members (Dees et al., 2013). In this study, we detected the presence of the pathogenicity determinant in the strains distantly-related to the so-far described pathogens and in actinobacteria coming from the “healthy” plant

environment. It is unclear if these observations may be attributed to the HGT and how it may affect disease epidemiology and plant protection. As we tested only the presence of the *txtAB* operon of the thaxtomin gene cluster, we do not know for sure that *stx*-positive strains are virulent. Future work should focus on the *in vitro* and *in vivo* virulence assays on a broad range of hosts to confirm the pathogenic potential of *Nocardia*, streptomycetes distantly-related to known pathogens and strains coming from the 'healthy' source. It is also challenging to compare pathogenicity gene pool of the 'healthy' and 'diseased' plant environments not only in terms of thaxtomin genes present but also other independent virulence factors (such as *nec1, tomA*, *fas* operon), which may be present on the same pathogenicity island and affect the disease.

CONCLUSIONS

Isolation of phytopathogenic actinobacteria from old plant samples may require different conditions than it is traditionally used for the selective isolation of actinomycetes from soils or freshly collected environmental samples. RNAlater solution may be effectively used for long-term storage of potato peel for subsequent isolation of actinobacteria. The optimal medium for the isolation may be R2A agar, which was first developed for bacterial isolation from potable water.

South America, the mother of the potato plant, is an interesting object to study bacterial communities in tuberosphere, their role in plant protection, and the origin of plant diseases. We analyzed actinobacteria coming from infected and healthy potato samples from different locations in Peru, Chile, and Argentina. Regardless of the presence of the CS symptoms, samples differed in a share of pathogenic actinobacteria: while from some samples all isolates were thaxtomin-positive, others contained only thaxtomin-negative isolates, and third both *stx*-positive and *stx*-negative actinobacteria. Genes for the thaxtomin production were detected in *Nocardia*, which have never been described to have genes for the thaxtomin production. It is obvious that complex actinobacterial community populates tuberosphere and affects the potato plant in different ways. On the other hand, there are many other potato diseases with common symptoms but different causes. Finally, horizontal gene transfer may play a role in pathogenesis.

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