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Effect of endophytic bacteria isolates on growth and oxidative stress injury of transgenic tobacco shoots *in vitro*

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Abstract

Transgenic plants have a great potential for production of high-value molecules, including peptides, for medical or industrial applications. Cultivation of plant tissues in a contained environment, such as plant tissue culture *in vitro*, could effectively address safety issues associated with transgenic plants as well as strict manufacturing standards. However, unfavourable *in vitro* conditions can often lead to elevated stress levels and suppressed growth of *in vitro* tissues urging the need to improve plant adaptation to *in vitro* conditions. As the beneficial effect of endophytic bacteria on the growth of *in vitro* cultures has been described previously, the aim of the present study was to assess the effect of growth-promoting endophytic bacteria isolates of the *Bacillus cereus* group on the growth of transgenic tobacco shoots *in vitro* and the accumulation of recombinant protein (RP) construct comprising a green fluorescent protein and a collagen mimicking peptide conjugate. The research revealed that transgenic tobacco shoots had reduced growth and increased oxidative injury levels compared with the non-transgenic parental line. The inoculated bacteria isolates colonised plant tissues and were sustained at densities of 0.6–9.0 × 10^5 CFU g^{-1} of the shoot fresh weight for at least several passages. Enhanced, 18% to 30% higher, biomass accumulation compared to the uninoculated transgenic tobacco shoots, and 14% to 25% lower levels of the oxidative lipid injury marker malondialdehyde (MDA) were detected for three-week-old shoots co-cultivated with isolates of *Bacillus mycoides* Nt.10.1, *B. toyonensis* Nt.18 and *B. wiedmannii* Nt.3.2. *B. wiedmannii* isolate Nt.14.2 reduced oxidative stress injury symptoms but had no effect on shoot growth. Co-cultivation with endophytic isolates had no effect on the expression of the recombinant protein.
These results suggest that co-cultivation with growth-promoting endophytic bacteria provides a viable solution to improve the growth of transgenic plant tissues used for recombinant protein production *in vitro*.

Keywords: adaptation, antibiotic treatment, *Bacillus* spp., recombinant protein, transgenic plants.

Introduction

Plant molecular farming is a branch of biotechnology with a focus on the production of high-value molecules in plants, including heterologous polypeptides (Schillberg, Finnern, 2021). As the demand for recombinant proteins for medical use or industrial enzyme applications continues to rise, plant-based protein expression systems are becoming an attractive alternative to the conventional expression hosts such as yeast, bacteria, or mammalian cell culture (De Martinis et al., 2016; Tschofen et al., 2016; Menary et al., 2020). The use of transgenic plants in industrial production processes has been partially constrained by the regulations imposed on the release of genetically modified crops and strict manufacturing standards for pharmaceutical production (Fischer et al., 2013; Hundleby et al., 2018). However, in many instances, tissue culture cultivation in a closed environment could provide an effective solution (Schillberg et al., 2013).

Shoot culture *in vitro* based on axillary bud proliferation is commonly used for propagation of clonal plants such as ornamentals, vegetables, and agronomic crops (Read, Preece, 2014). In plant cell culture, plant tissues or organs are grown *in vitro* on artificial media under aseptic and controlled conditions. The limited capacity of the *in vitro* environment to reproduce natural conditions of plant tissues often leads to an imbalance of physiological equilibrium and stress (Cassells, Curry, 2001). Therefore, much effort has been dedicated to optimising the synthetic cultivation media including the composition of mineral nutrients and plant growth regulators.

In plant growth modulation and adaptation to stress under natural conditions, an important role plays plant-associated microbiome; however, its importance for plant tissue adaptation *in vitro* has rarely been addressed. In this respect, special attention should be paid to plant genetic engineering techniques that use bacterium-to-plant DNA transfer machinery of *Agrobacterium* for genetic transformation of plant tissues *in vitro* and involve treatment with one or several antibiotics for an extended period (Ozawa, 2009). To have a detrimental effect on endophytic bacteria residing in plant tissues, antibiotic treatment has been demonstrated (Tamošiūnė et al., 2022).

Plants and their associated microorganisms constitute a holobiont that evolves as a unit, constantly adapting to the dynamic abiotic and biotic environment (Guerrero et al., 2013). Endophytes are a class of endosymbiotic microorganisms that live in internal plant

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tissues (Brader et al., 2017). Their role in enhancing plant growth due to the production of siderophores and phytohormones, such as gibberellins, auxin, and ethylene, and enzymes, such as 1-aminocyclopropane-1-carboxylic acid-deaminase, as well as priming of disease resistance has been extensively documented (Hardoim et al., 2015; Miliute et al., 2015; Le Cocq et al., 2017; Rho et al., 2018). Therefore, reduced microbial diversity is often linked to elevated stress levels or higher disease susceptibility of the plant (Sessitsch, Mitter, 2015; Berg et al., 2017). Indeed, several studies have shown the beneficial effect of endophytic bacteria on the growth and the adaptation of an *in vitro* culture. Botta et al. (2013) demonstrated the growth-promoting effect of endophytic *Azospirillum brasilense* and *Gluconacetobacter diazotrophicus* on a tomato (*Lycopersicon esculentum*) grown *in vitro*. Similarly, our previous studies have shown a growth-promoting effect in apple (*Malus* × *domestica*) and tobacco (*Nicotiana tabacum*) shoots induced by co-cultivation with endophytic *Bacillus* spp. or *Pseudomonas fluorescens* strains (Tamošiūnė et al., 2018; Andriūnaitė et al., 2021). Drought stress-reducing activity of endophytic *Bacillus* and *Pseudomonas* spp. strains has been described in grapevine (*Vitis vinifera*) (Salomon et al., 2014). The application of *Pseudomonas* and *Arthrobacter* spp. strains to stimulate growth and metabolite accumulation in coneflower (*Echinacea purpurea* and *E. angustifolia*) plants *in vitro* has been described by Maggini et al. (2019). *Rhodopseudomonas palustris* and *Microbacterium testaceum* co-cultivation with sweet cherry (*Prunus avium*) shoot culture has been shown to stimulate the rooting of genotypes that are difficult to propagate (Quambusch et al., 2016).

Cultivated tobacco (*Nicotiana tabacum* L.) has been commonly used for genetic transformation as a model species in functional genomics studies (Ganapathi et al., 2004; Sierro et al., 2014) and for practical application in molecular farming using *in vitro* techniques or contained greenhouse practices (Colgan et al., 2010; Tremblay et al., 2010; Moustafa et al., 2016). Recently, a study by Tamošiūnė et al. (2022) demonstrated that antibiotic treatment results in a substantial decrease of the endophytic bacteria population in tobacco culture *in vitro*, and this effect is associated with reduced growth and adaptive capacity of the tobacco tissue culture *in vitro*. Endophytic bacteria play an important role in the modulation of plant growth and adaptation. Our previous study (Andriūnaitė et al., 2021) demonstrated a growth-promoting effect of endophytic bacteria of the *Bacillus cereus* group on tobacco shoot culture *in vitro*. Considering these findings, it could be presumed that supplementing a diversity-deprived microbiome of a transgenic plant tissue with growth-promoting endophyte species could improve the growth of the tissue and its adaptation to the *in vitro* environment.

Therefore, the aim of the experiment was to assess the effect of four bacterial isolates of the *Bacillus cereus* group on the growth and stress levels as well as the accumulation of recombinant protein (RP) in a transgenic tobacco (*Nicotiana tabacum* L.) shoot culture *in vitro*.

Materials and methods

The research was carried out at the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry in 2019–2021.

DNA construct and plant transformation. The DNA construct used for plant transformation encoded a chimeric recombinant protein (RP) composed of a polyhistidine (6 × His) purification tag and a basic green fluorescent protein (avGFP) (Prasher et al., 1992) followed by SplB protease cleavage site (Trp-Glu-Leu-Gln) (Dubin et al., 2008) and short collagen mimicking peptide sequence Cys-Gly-$(Pro-Lys-Gly)_4$$(Pro-Hyp-Gly)_4$$(Asp-Hyp-Gly)_4$ (kindly provided by Ferentis Ltd., Lithuania).

For the construction of the plant transformation vector, GoldenBraid 2.0 (GB) DNA assembling system (Sarrion-Perdigones et al., 2013) and DNA parts provided in the Orzaez Lab GoldenBraid 2.0 kit (Addgene Headquarters, USA) were used. The RP construct sequence was produced by synthesis in the pMA-RQ plasmid (Thermo Fisher Scientific, USA) and was designed to include BsaI recognition and adapter sequences required for multipartite assembly. The transcription unit included Cauliflower mosaic virus (CaMV) 35S promoter and terminator (GB parts Nos. GB0030 and GB0036, respectively) and was assembled using pDGB3alpha1 vector according to the standard protocol provided by GB-cloning online tool (Vazquez-Vilar et al., 2015). Briefly, 1 × ligase buffer, 10 U of BsaI, 4 U T4 DNA ligase, and 75 ng of each DNA vector were mixed in 10 µL reaction volume and incubated 2 min at 37°C and 5 min at 16°C for 25 cycles. One microliter of the cloning reaction was used to transform electrocompetent *Escherichia coli* DH5alpha cells (McLab, USA), and positive clones were selected on lysogeny broth (LB) agar (Bertani, 1951) supplemented with 50 µg mL^{-1} kanamycin, 0.5 mM isopropyl β-d-1-thiogalactopyranoside, and 40 µg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. The DNA vector was purified using GeneJet Plasmid Miniprep kit (Thermo Fisher Scientific), and the construct was confirmed by sequencing. An electrocompetent *Agrobacterium tumefaciens* GV3101(pM90) strain (kindly provided by Dr Vaiva Kazanavičiūtė, Vilnius University) was transformed with the purified plasmid, and positive clones were selected on LB agar supplemented with 50 µg mL^{-1} kanamycin, 50 µg mL^{-1} gentamycin, and 30 µg mL^{-1} rifampicin.

Agrobacterium-mediated tobacco leaf explant transformation. Tobacco shoots were maintained on solid Murashige and Skoog (MS) medium supplemented with 0.75 mg L^{-1} benzylaminopurine (BAP), 30 g L^{-1} sucrose, and 0.8% agar in a climatic chamber (Sanyo Electric Co. Ltd., Japan) at 25 ± 3°C temperature under fluorescent lamp illumination at 150 µmol m^{-2} s^{-1} intensity and with a 16/8 h light/dark photoperiod. For callus induction, tobacco leaves were excised and pre-cultured for two days in 9 mm Petri dishes with the agarized MS medium with 30 g L^{-1} sucrose, 0.1 mg L^{-1} kinetin, and 1.0 mg L^{-1} 1-naphthyl acetic acid (NAA).

Agrobacterium-mediated tobacco leaf explant transformation was carried out according to the modified methodology described previously by Horsch et al. (1989) and Pathi et al. (2013). Transformed *Agrobacterium* was cultured in LB medium and resuspended at 0.8 optical density (OD) at 600 nm in regeneration medium (RM) composed of MS medium supplemented with 30 g L^{-1} sucrose, 2 mg L^{-1} BAP, and 0.2 mg L^{-1} NAA. The bacterial suspension was incubated at 23°C temperature for 3 h with gentle agitation on a shaker. Tobacco leaves were cut transversely and longitudinally 1–2 times with a scalpel soaked in the bacterial suspension resulting in 5–8 mm pieces. With a small amount of bacterial suspension in the syringe, the leaf fragments were damaged with a needle making at least 2–3 punctures each. The explants were transferred to a 50 ml tube with the prepared bacterial suspension and incubated for 30 min at 28°C temperature with gentle shaking. After incubation leaf explants were dried briefly on sterile filter paper, transferred on solid RM medium and incubated at 23°C temperature in the dark. After two days of co-cultivation, the explants were washed in liquid RM medium supplemented with 1 g L^{-1} timentin for 3 min, rinsed in the antibiotic-free RM medium and washed 4 times with RM medium supplemented with 300 mg L^{-1} timentin by gentle stirring for 1 h. The explants were dried briefly on sterile filter paper and transferred to solid RM medium supplemented with 400 mg L^{-1} timentin. The explants were kept under the same conditions as the tobacco shoots *in vitro* and transferred onto a fresh cultivation medium once per week. To select the transformed tissues, kanamycin was added to the medium at 100 mg L^{-1} starting from the 2nd sub-cultivation, and timentin concentration was reduced to 250 mg L^{-1} starting from the 3rd sub-cultivation. avGFP fluorescence was used to monitor the formation of transformed callus, which could be separated from the leaf explants after eight sub-cultivations or later. After 8–16 weeks, the transgenic tobacco shoots *in vitro* regenerated from the transformed callus were kept under

the same conditions as shoots of the non-transgenic parental tobacco line.

Endophytic bacteria isolates and co-cultivation with tobacco shoots in vitro. Isolates of *Bacillus mycoides* Nt.10.1, *B. toyonensis* Nt.20.2, *B. wiedmannii* Nt.3.2 and Nt.14.2 used in the experiment were previously obtained from greenhouse-grown tobacco leaves (Andriūnaitė et al., 2021). A transgenic tobacco shoot culture *in vitro* was maintained as described above. Four-week-old shoots were transferred to a fresh medium and used for bacterial inoculation the next day. Shoot inoculation experiments were carried out as described previously by Tamošiūnė et al. (2018). Briefly, the bacterial inoculum was grown in LB medium at 25°C temperature to an exponential growth phase. Bacteria were sedimented via centrifugation and resuspended in MS medium at a concentration of ~10^7 colony-forming units (CFU) mL^{-1}. Several nodes of the shoot petiole were inoculated with 3 µL of the bacterial suspension. For the control treatment, MS medium without bacteria was used. The inoculated shoots were maintained as described above and transferred to a fresh medium every four weeks. The shoots were cultivated for at least three passages, and fresh weight (FW) was assessed three weeks after transfer to a fresh medium. To evaluate the effect of the transformation procedure on plant growth, non-transgenic line was used as a referral group. The density of endophytic bacteria in shoot tissues was estimated using serial dilution and plating as described by Andriūnaitė et al. (2021).

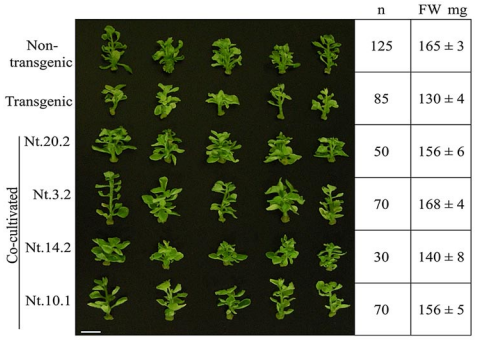
Assessment of oxidative stress injury. To estimate oxidative injury of cellular membranes in the tobacco shoot tissues, a quantitative analysis of the accumulation of malondialdehyde (MDA), a product of lipid peroxidation, was used. The analysis was carried out as described by Hodges et al. (1999) and Jagendorf and Takabe (2001). Tobacco shoots were combined into samples of approximately 0.2 g weight, ground to powder using liquid nitrogen, extracted with 50 mM Tris-HCl pH 7.4, containing 1.5% of polyvinylpolypyrrolidone for 30 min at 4°C, and centrifuged at 10,000 g for 15 min at 4°C. An equal volume of tissue extract was mixed with 0.5% thiobarbituric acid in 20% trichloroacetic acid, heated at 95°C for 30 min, cooled on ice, and centrifuged at 10,000 g for 5 min. The absorbance measured at 532 nm was corrected by subtracting the absorbance value at 600 nm. MDA concentration was estimated using $\varepsilon = 156\ mM^{-1}\ cm^{-1}$.

Recombinant protein (RP) accumulation analysis. Tobacco shoots were ground to powder using liquid nitrogen, and the total cell protein sample was prepared using phenol extraction and precipitation with ammonium acetate (Isaacson et al., 2006). Samples were solubilised in 100 mM Tris-HCl pH 7.4, 2% sodium dodecyl sulphate (SDS), and protein concentration was determined using Roti-Quant universal reagent (Carl Roth GmbH & Co KG, Germany). Protein samples were diluted in Laemmli buffer (BioRad Laboratories, Inc., USA) and separated by SDS PAGE on 5–12% polyacrylamide gel using Mini Protean system (BioRad). Proteins were transferred to a PVDF (polyvinylidene difluoride) membrane using a semi dry electroblotting system (Biometra Ltd., Germany). Proteins were visualized using SYPRO Ruby protein gel stain (Thermo Fisher Scientific). The membrane was blocked overnight at 4°C temperature in 50 mM Tris HCl pH 7.6, 150 mM NaCl (TBS) supplemented with 4% skim milk powder (TBSM). The membrane was incubated for 2 h with primary poly-His tag-specific mouse monoclonal antibodies (Thermo Fisher Scientific) or RbcL-specific rabbit polyclonal antibodies (Agrisera AB, Sweden) at 1:10,000 dilution in TBSM. The membrane was washed three times for 10 min each with TBS supplemented with 0.1% Tween 20 (TBST) and incubated for 2 h with a secondary HRP-conjugated antibody (BioRad) at 1:5,000 in TBSM. Following four 10 min washes with TBST, the signal was detected using the Clarity Western ECL substrate (BioRad). For image processing, SYPRO Ruby stained membranes and ECL films were scanned using scanner Typhoon FLA900 (GE Healthcare, USA), and software ImageQuant TL (GE Healthcare) was used.

Statistical analysis. The co-cultivation experiments were repeated 3–4 times. The mean values of FW and MDA concentration were estimated using 30 to 125 replicates from different experiments. For the analysis of bacterial density in shoot tissues, two replicates were used for each dilution, and the experiment was repeated at least twice. For RP accumulation analysis, protein extraction was performed from a pooled sample of 5–10 shoots; 3–5 biological replicates from different experiments were used. To normalise the RP accumulation densitometry data, the abundance of the house-keeping gene ribulose bisphosphate carboxylase large chain (RbcL) protein products was used. To combine data from different experiments, the data was normalised to the calibrator sample, which was included once or twice in each gel. To establish statistically significant ($p < 0.05$) differences between the means of all experimental groups including non-transgenic and transgenic lines and inoculated shoots, one-way analysis of variance (ANOVA) and Tukey post-hoc test (Prism, GraphPad software Ltd.) were used. Data are presented as the mean and standard error of the mean.

Results and discussion

After *Agrobacterium*-mediated transformation, cultivation for 8–16 weeks on the medium supplemented with timentin and kanamycin was required to select a homogeneous tobacco shoot culture transformed with the RP construct. After an extensive antibiotic treatment, the selected transgenic tobacco line showed a substantial increase in the oxidative stress injury levels and reduced biomass accumulation (Figures 1 and 2). The biomass of three-week-old tobacco shoots was reduced 18 ± 2%, and the accumulation of MDA increased 21 ± 3%. The observed growth-suppressing effect in shoots was two times higher than that previously observed in the non-transgenic tobacco shoots treated with timentin only (Tamošiūnė et al., 2022). It could be proposed that the larger negative effect is related either to the additional impact of transgenesis on the shoot physiology or is a consequence of the cumulative antimicrobial action of the two distinct types of antibiotics on the shoot endophytic microbiome.

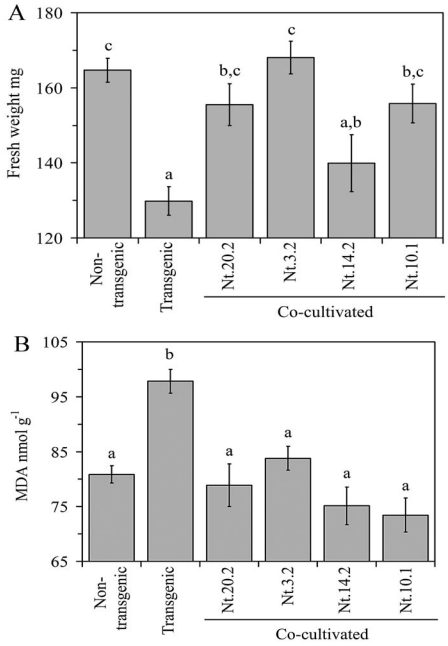


	n	FW mg
Non-transgenic	125	165 ± 3
Transgenic	85	130 ± 4
Nt.20.2	50	156 ± 6
Nt.3.2	70	168 ± 4
Nt.14.2	30	140 ± 8
Nt.10.1	70	156 ± 5

Note. Representative samples of the tobacco shoots are shown in the left panel and the number of replicates (n), and tobacco shoot fresh weight (FW) data (the mean ± standard error) are presented in the table on the right; a scale bar represents 10 mm.

Figure 1. Effect of co-cultivation with *Bacillus cereus* group endophytic bacteria on three-week-old transgenic tobacco shoots *in vitro* morphology and biomass accumulation

To assess the potential of endophytic bacteria to restore growth and the adaptive capacity properties of transformed *in vitro* tissues, further experiments were carried out. The microbiome of the transgenic tobacco shoots was supplemented with pure isolates of endophytic bacteria of the *Bacillus cereus* group, which have previously been shown to assert growth-regulating properties on the non-transgenic parental line of tobacco shoot culture *in vitro* (Andriūnaitė et al., 2021). Colonisation and survival in plant tissue is an essential property of endophytic bacteria. Therefore, the initial



Note. Data presented as the mean ± standard error; FW – fresh weight; ANOVA test $p < 0.001$ (A, B); pairwise values were $p < 0.05$ (A) and $p < 0.001$ (B) between all significant differences indicated by different letters.

Figure 2. Effect of co-cultivation with *Bacillus cereus* group endophytic bacteria on three-week-old transgenic tobacco shoots *in vitro* biomass accumulation (A) and malondialdehyde (MDA) content (B)

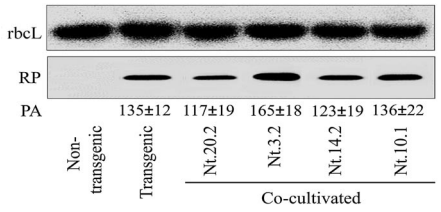
analysis aimed to evaluate the survival of endophytic bacteria in transgenic tobacco shoot *in vitro* tissues during a prolonged co-cultivation period. It was estimated that after more than 10 passages on fresh cultivation medium, the bacterial density in transgenic tobacco shoots *in vitro* tissues co-cultivated with *B. mycoides* isolate Nt.10.1, *B. toyonensis* isolate Nt.20.2, *B. wiedmannii* isolates Nt.3.2 and Nt.14.2 was $6 \pm 1 \times 10^4$, $5 \pm 2 \times 10^5$, $9 \pm 1.6 \times 10^5$, and $2 \pm 1 \times 10^5$ CFU g^{-1} FW, respectively.

Previous studies have demonstrated that endophytes can colonise a variety of plant tissues including the intercellular spaces of cell walls and xylem vessels of plant roots, stems, and leaves. In different tissues, the density of endophytic bacteria varies from hundreds to 10^{10} cells per gram of plant tissue (Chi et al., 2005). Typically, a higher density of endophytic bacteria is found in plant roots and other below-ground tissues compared to the above-ground tissues. Therefore, in the present study, the density of endophytic bacteria observed in tobacco shoots *in vitro* is within the limits commonly observed for the above-ground plant tissues (Oliveira et al., 2002; Tiwari et al., 2010).

Furthermore, the effect of four endophytic bacteria isolates of the *Bacillus cereus* group on the growth of the transgenic tobacco shoot culture was assessed. The four isolates used in the experiment have previously been shown to have a distinct effect on the growth of a non-transgenic tobacco shoot culture *in vitro* (Andriūnaitė et al., 2021). Co-cultivation with *B. mycoides* isolate Nt.10.1 and *B. toyonensis* isolate Nt.20.2 resulted in an enhanced growth of the tobacco shoots, while the closely related *B. wiedmannii* isolates Nt.3.2 and Nt.14.2 had contrasting properties. Whereas enhanced tobacco shoot growth was observed for isolate Nt.3.2, isolate Nt.14.2 had no significant effect. In the present study, the endophytic isolates showed similar growth modulating properties. Co-cultivation with isolates Nt.3.2, Nt.10.1 and Nt.20.2 resulted in an 18% to 30% increase in shoot biomass accumulation compared to the uninoculated control of transgenic shoots, whereas shoot weight was comparable to the non-transgenic parental line. Isolate Nt.14.2 had little effect on shoot weight as compared to the uninoculated control of transgenic line (Figures 1 and 2A). However, it is notable that the absence of a growth-stimulating effect of isolate Nt.14.2 was not due to the inability to colonise or survive in shoot tissues, as the density of the bacterium in the shoot tissues was similar to the growth-stimulating isolates. The results confirmed a strain-specific interaction between the plant host and the two closely related *B. wiedmannii* isolates Nt.3.2 and Nt.14.2 with contrasting shoot growth regulating properties.

MDA accumulation analysis revealed that co-cultivation of transgenic tobacco shoots with the endophytic bacteria isolates effectively reduced oxidative stress injury (14% to 25%) to the same level as non-transgenic parental line (Figure 2B). Interestingly, this effect was similar irrespectively of the growth-promoting properties of the isolates, and isolate Nt.14.2 presented no exception. This implies that the improved adaptation and the reduced level of oxidative stress injury are not sufficient to enhance shoot growth, at least in the case of isolate Nt.14.2. Therefore, the co-cultivation effect might be conferred by an isolate-specific combination of distinct biochemical mechanisms. It is also likely that the growth-promoting isolates have the ability to biosynthesize growth regulators and siderophores or are involved in the fixation of nitrogen that have previously been described as growth-promoting properties of endophytes (Hardoim et al., 2015; Miliute et al., 2015; Le Cocq et al., 2017; Rho et al., 2018), which could play an important role under *in vitro* conditions. However, the mechanisms behind the growth-enhancing and adaptation-modulating properties of the endophytic bacteria isolates from the *Bacillus cereus* group remain elusive and require more detailed analysis.

To ensure high yields and effective purification of RP, it is important to consider factors affecting the transgene expression level. In addition to the genetic and epigenetic factors, as well as protein stability, environmental factors could have an important effect on RP accumulation (Fujiuchi et al., 2016). In addition, symbiotic or pathogenic microorganisms produce a variety of compounds that could be sensed by plants and cause them to undergo a reprogramming of metabolic pathways resulting in altered morphogenesis or an activation of the defence and stress-related responses (Oldroyd, 2013), which could indirectly affect the production of the RP. Therefore, in the present study, the effect of co-cultivation with endophytic bacteria on the RP accumulation in the transgenic tobacco shoots was assessed. The immunoblot analysis revealed similar RP abundance levels in the tobacco shoots co-cultivated with distinct endophytic bacteria isolates and uninoculated transgenic shoots (Figure 3). These results suggest that the physiological changes leading to enhanced adaptation and growth of plant tissue colonised with the *Bacillus cereus* group isolates did not interfere with the biological functions involved in RP expression.



Note. Total protein visualisation using immunoblot produced using antibodies specific to 53 kDa ribulose bisphosphate carboxylase large chain (RbcL) and polyhistidine-tag of the 33 kDa recombinant protein (RP); protein abundance (PA) presented as a percentage value normalised to sample average (the mean ± standard error).

Figure 3. Results of immunoblot analysis of the RP expression in three-week-old transgenic tobacco shoots *in vitro*

In summary, current experiment revealed that co-cultivation with endophytic bacteria could alleviate the negative effect on the growth and stress levels

of transgenic tobacco shoots *in vitro* following the transformation procedure. This approach could be used to improve RP production *in vitro*. The application of growth-promoting bacteria isolates led to an improved adaptive capacity and higher biomass accumulation comparable to the levels of the non-transgenic parental line. This effect appeared to be defined by a combination of isolate-specific traits.

To unveil the scope of the biochemical mechanisms involved in endophyte interaction with the plant host, further studies would be required. The results of such studies would pave the way for knowledge-driven design of the inoculum of endophytic bacteria.

Conclusions

1. *Agrobacterium*-mediated transformation involving extensive antibiotic treatment resulted in elevated levels of oxidative stress injury and reduced biomass accumulation in transgenic tobacco shoots *in vitro*.

2. Colonisation of transgenic tobacco shoot tissues with *Bacillus mycoides* isolate Nt.10.1, *B. toyonensis* isolate Nt.18, and *B. wiedmannii* isolate Nt.3.2 reduce oxidative lipid injury levels and enhance the accumulation of shoot biomass to the level characteristic of the non-transgenic parental line.

3. *B. wiedmannii* isolate Nt.14.2-inoculated transgenic tobacco shoots sustain a significant bacterial density, which results in reduced oxidative stress injury symptoms, but the isolate co-cultivation has no effect on shoot growth.

4. The results confirmed the presence of strain-specific interaction between the plant host and the two closely related *B. wiedmannii* isolates Nt.3.2 and Nt.14.2 with contrasting shoot-growth-regulating properties.

5. Transgenic tobacco shoots co-cultivation with endophytic bacteria isolates of the *Bacillus cereus* group did not interfere with plant biological functions involved in protein expression and did not affect the accumulation of the recombinant protein (RP) in the transgenic tobacco shoot tissues.

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Endofitinių bakterijų izoliatų poveikis transgeninio tabako ūglių augimui ir oksidacinio streso pažaidai *in vitro*

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Santrauka

Transgeniniai augalai atveria perspektyvas didelės pridėtinės vertės junginių, tarp jų ir medicininės arba pramoninės paskirties peptidų, gamybai. Augalų audinių auginimas izoliuotoje aplinkoje, pavyzdžiui, jų kultivavimas *in vitro*, pateikia efektyvų sprendimą, atitinkantį transgeninių augalų saugumui keliamus reikalavimus ir griežtus gamybos standartus. Tačiau padidėjusį audinių streso lygį ir sulėtėjusį augimą dažnai lemia nepalankios *in vitro* aplinkos sąlygos, todėl yra augalų adaptyvumą *in vitro* sąlygomis gerinančių priemonių poreikis. Ankstesnių tyrimų metu buvo nustatyta teigiama endofitinių bakterijų įtaka audinių augimui *in vitro*.

Tyrimo tikslas – įvertinti augimą skatinančių *Bacillus cereus* grupės endofitinių bakterijų izoliatų poveikį transgeninių tabako ūglių augimui *in vitro* ir rekombinantinio baltymo, sudaryto iš žalio fluorescuojančio baltymo bei kolageną imituojančio peptido, kaupimui. Tyrimo duomenis palyginus su netransgeninės motininės kontrolės linijos rezultatais nustatyta, kad transgeninio tabako ūgliams būdingas mažesnis augimo greitis ir padidėjęs oksidacinių pažeidimų lygis. Užkrėsti bakterijų izoliatai kolonizavo augalo audinius, o jų tankis buvo palaikomas 0,6–9,0 × 10^5 KFV g^{-1} žalios ūglių masės bent keletą persodinimų. Trijų savaičių amžiaus ūgliams, augintiems su *B. mycoides* Nt.10.1, *B. toyonensis* Nt.18 ir *B. wiedmannii* Nt.3.2 izoliatais, nustatytas 18–30 % biomasės kaupimo padidėjimas ir 14–25 % oksidacinio lipidų pažeidimo žymeklio malondialdehido (MDA) koncentracijos sumažėjimas, palyginus su neužkrėstu transgeninio tabako variantu. *B. wiedmannii* Nt.14.2 izoliatas sumažino oksidacinio streso pažaidos simptomus, bet neturėjo įtakos ūglių augimui. Endofitinių bakterijų izoliatai neturėjo įtakos rekombinantinio baltymo raiškai.

Tyrimo rezultatai leidžia teigti, kad endofitinių bakterijų inokuliatų panaudojimas gali būti perspektyvi priemonė, siekiant pagerinti rekombinantinių baltymų gamybai naudojamų transgeninių augalų audinių augimą *in vitro*.

Reikšminiai žodžiai: adaptacija, apdorojimas antibiotiku, *Bacillus* spp., rekombinantinis baltymas, transgeniniai augalai.