

PRODUCTION AND ANTIBACTERIAL ACTIVITY ASSESSMENT OF SALMOCIN IN TRANSGENIC TOBACCO PLANTS

Shcherbak Nataliia¹, Dzuh Maryna^{1,2}, Myroslav Parii^{1,2}, Rudas Volodymyr¹, Ovcharenko Olga¹, Balko Oleksandr³, Balko Olga³, Kuchuk Mykola¹

¹Institute of Cell Biology and Genetic Engineering, NAS of Ukraine, Kyiv, Ukraine

²Ukrainian Research Institute of Plant Breeding, Kyiv, Ukraine

³Institute of Microbiology and Virology named after D. K. Zabolotny, NAS of Ukraine, Kyiv, Ukraine

Salmonella remains one of the leading causes of foodborne infections, causing substantial economic losses in livestock production and posing a serious threat to food safety. In Europe, salmonellosis consistently ranks among the most common zoonotic diseases: in 2023 alone, more than 78,000 laboratory-confirmed cases were reported, with increasing incidence observed in many countries (Nadi et al., 2020). Moreover, *Salmonella* is becoming an increasing challenge in the context of antibiotic resistance, highlighting the need for alternative approaches to pathogen control. According to the European Centre for Disease Prevention and Control, resistance of *Salmonella* to fluoroquinolones (one of the key classes of antibiotics used for the treatment of salmonellosis) is increasing in most countries. Salmocins are antibacterial proteins produced by bacteria (bacteriocins) that selectively kill pathogenic *Salmonella* serotypes. Like other bacteriocins, salmocin is considered a potential alternative to conventional antibiotics, at least for prophylactic purposes, and its use may help prevent the spread of bacterial infections. Salmocins have previously been produced in plants using transient expression systems (Schneider et al., 2018; Hahn-Löbmann et al., 2019). Their functionality was confirmed, and the recombinant bacteriocins were recognized by the U.S. Food and Drug Administration as GRAS (Generally Recognized As Safe) and approved for use as antibacterial agents (GRAS Notices (fda.gov)).

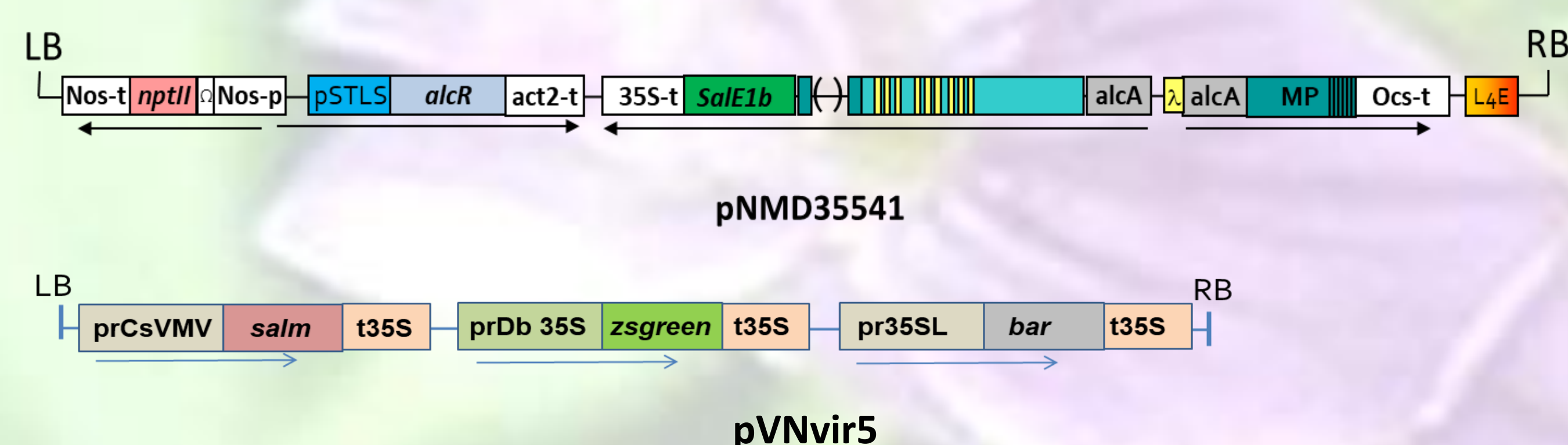


Fig. 1. Expression vectors used for the genetic transformation of tobacco carrying inducible (pNMD35541) and constitutive (pVNvir5) salmocin expression cassettes.

RB, LB – right and left T-DNA borders; *nptII* – neomycin phosphotransferase II gene; 35S t – cauliflower mosaic virus (CaMV) 35S terminator; nos pro, nos t – nopaline synthase promoter and terminator; ocs t – octopine synthase terminator; SalE1b – salmocin E1b gene; alcR – gene encoding the ethanol-responsive transcription factor AlcR; *alcA* – *Aspergillus nidulans* alcohol dehydrogenase promoter activated by the ethanol-bound AlcR transcription factor; prDb35S – dual CaMV 35S promoter; pr CsVMV – Cassava Vein Mosaic Virus promoter

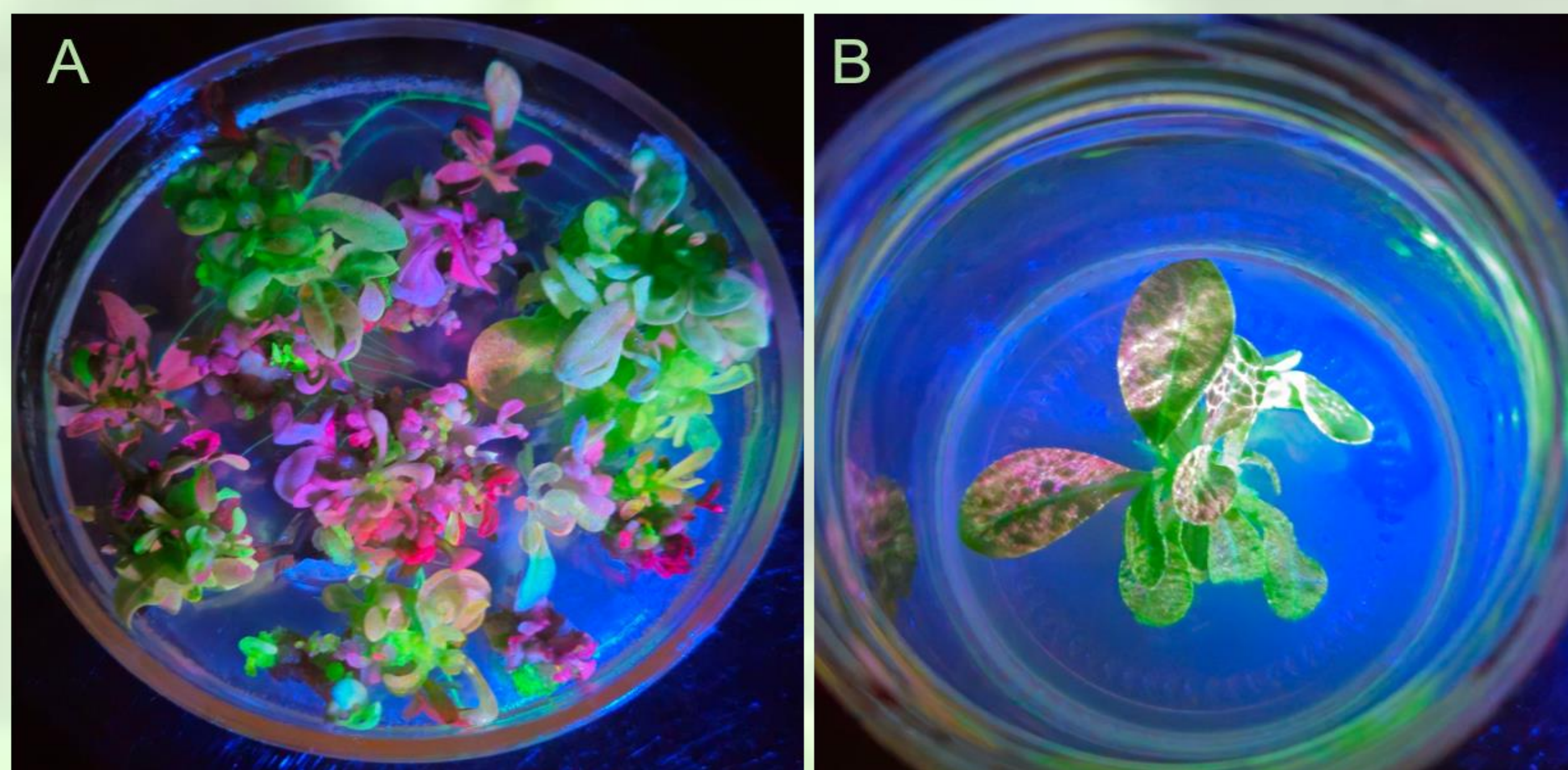


Fig. 2. Regeneration of transformed tobacco plants on selective medium supplemented with 5 mg/L phosphinothricin, 16 weeks after transformation (A) and rooted transgenic tobacco plant expressing both *zsgreen* and *salmocin* genes (B) under blue light illumination (490–495 nm)

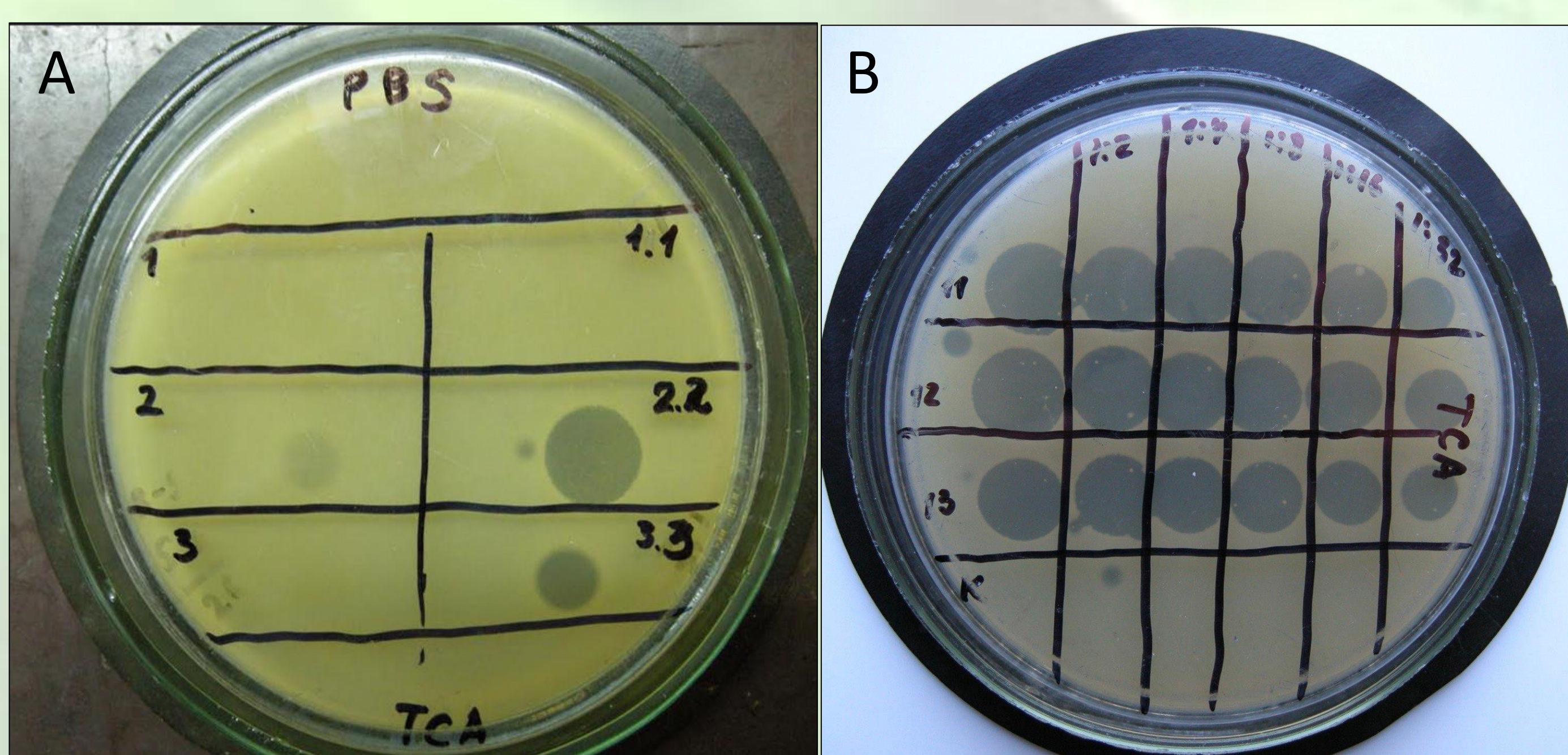


Fig. 4. Antimicrobial activity of transgenic plants extracts against *Salmonella enterica*: A – plants extracts obtained from non-induced and ethanol-induced leaves of two independent transgenic tobacco lines (extracts from induced plants are indicated by doubled numbers. Samples 1 and 1.1 represent extracts from non-transgenic control plants); B – extracts of transgenic tobacco with constitutive salmocin expression: from left to right, serial two-fold dilutions of the extracts: 1:2, 1:4, 1:8, 1:16 and 1:32.

Conclusions: Extracts from transgenic tobacco plants expressing the salmocin gene exhibited antibacterial activity against *Escherichia coli* XL1-Blue and *Salmonella enterica*. Tobacco lines carrying the constitutive expression construct demonstrated consistently high antibacterial activity and allowed rapid selection of high-expressing transformants using the ZsGreen reporter. Transgenic lines harboring the ethanol-inducible expression system also exhibited antibacterial activity, although with greater variability among individual lines. In the most active inducible lines, a measurable antibacterial effect was detected even in the absence of ethanol induction, suggesting a low level of basal transgene expression.

The use of plants as biofactories for the production of pharmaceutical proteins offers several significant advantages over conventional expression systems, including bacterial, yeast or mammalian cell cultures, making this approach particularly promising for the development of antimicrobial products. Plant-based systems eliminate the risk of contamination with bacterial toxins and human pathogens and can be readily scaled up without substantial material costs. Another important advantage is the ability to accumulate recombinant antibacterial proteins in plant biomass in a stable and functionally active form, while maintaining antibacterial activity after drying and long-term storage of plant material, as demonstrated in our previous work with colicin M (Schcherbak et al., 2023). The aim of our work was to generate transgenic tobacco plants accumulating recombinant salmocin using both inducible and constitutive expression systems.

Methods: Transgenic tobacco plants were obtained via *Agrobacterium*-mediated transformation with vectors containing salmocin E1b coding gene. Vectors for ethanol-inducible salmocin expression in plants were kindly provided by Nomad Bioscience GmbH (Halle, Germany). For constitutive salmocin expression, a vector with the salmocin E1b gene under the control of the CsVMV promoter using Golden Gate cloning technology was constructed. In addition to the target gene, the vector also contained the reporter gene ZsGreen (Fig. 1).

Agrobacterium-mediated transformation of tobacco was carried out using leaf disks of aseptic plants as explants. After 48 h of co-cultivation in diluted *Agrobacterium* suspension supplemented with 100 mM acetosyringone, explants were placed on regeneration medium MS supplemented with BAP (1 mg / l) and NAA (0.1 mg / l) and 5 mg/l of phosphinothricin or 100 mg/l of kanamycin for selection. As shoot appeared, each one was removed and placed on the MS medium for rooting. Well established plants were transferred into the soil and grown in the greenhouse.

We used PCR analysis with primers specific for target gene (Table 1) to confirm the presence of the salmocin gene in the genome of obtained plants. Genomic DNA was isolated from plant leaves using CTAB method. PCR was carried out in 20 µl reaction volume containing 50 ng DNA, 200 µM each of forward and reverse primers, 200 µM dNTPs and 1 U Taq DNA polymerase (Thermo Scientific). Amplification was performed on a Mastercycler® personal (Eppendorf, USA).

The products of the amplification were separated on a 1% (w/v) agarose gels. Antimicrobial activity of salmocin-containing transgenic plants extracts versus laboratory *E. coli* strains XL1-Blue and *Salmonella enterica* was determined by soft agar overlay assay. Each aliquot (25 ml) of LB softagar medium (~45°C) is supplemented with 250 µl bacterial culture of OD₆₀₀=1.0, mixed and immediately poured on pre-poured LB plate. 5 µl of the plant extract was applied to the surface of the solidified medium.

In plants carrying the ethanol-inducible expression system, the tobacco leaves were pre-incubated in chambers containing 4% ethanol and maintained for 3 days to allow accumulation of the recombinant product.

Table 1. Primers used for PCR-based molecular analysis in this study.

	Primer sequences	Annealing temperature	PCR product size
SalE1b-F	5'-ATGAGCGATAACACCAATTGCTTACTACG-3'	59°C	1584 bp
SalE1b-R	5'-TTAGATCCACAGTGCCCTGTCAGCTTC-3'		

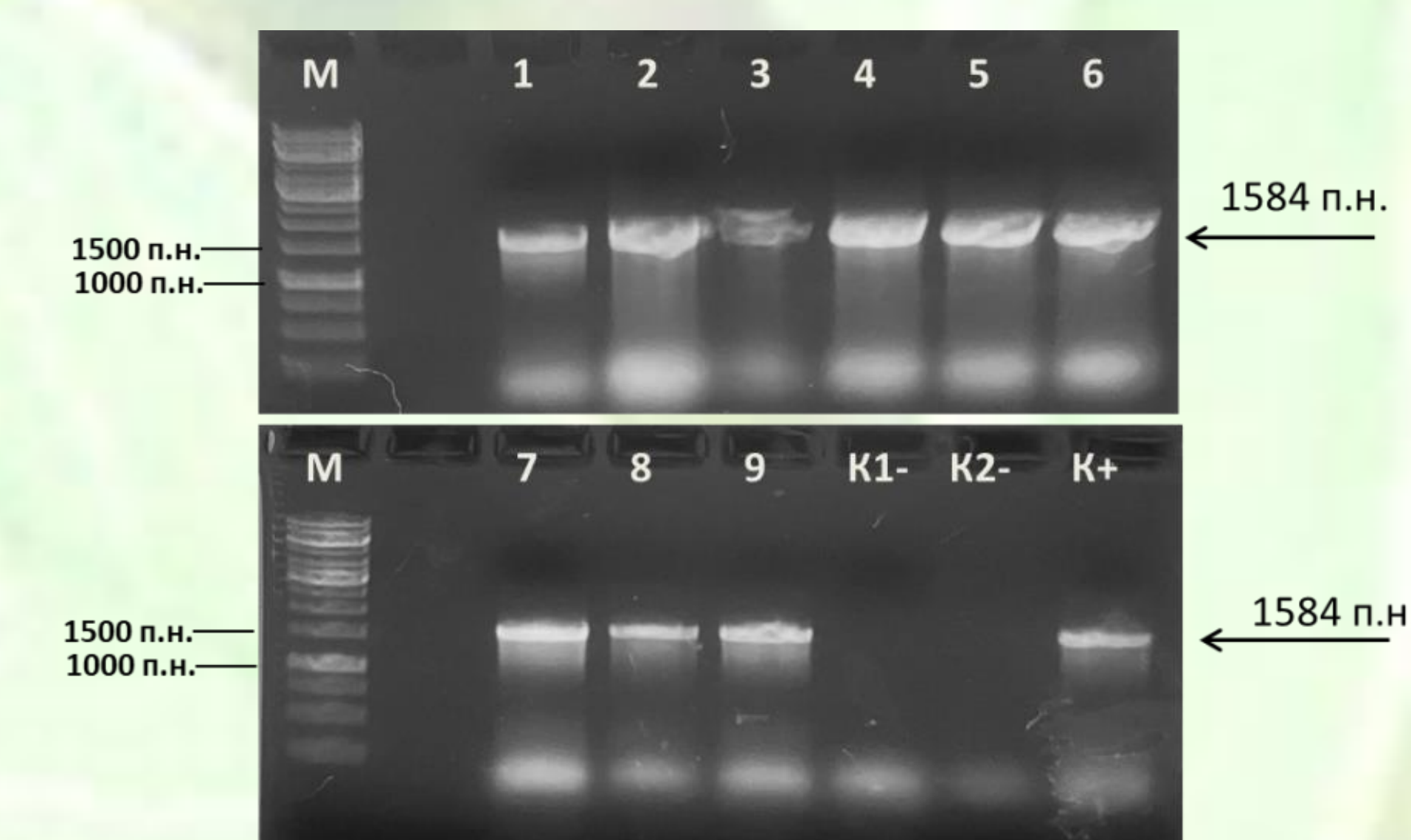


Fig. 3. PCR analysis of transgenic plants containing the salmocin E1b gene: M - DNA mass ladder *O'GeneRuler™ 1 kb DNA Ladder* (Thermo Scientific); K - negative control with DNA from non-transformed plant; K+ - positive control, plasmid pNMD35541 DNA; 1-9 - independent transgenic tobacco with primers to salmocin E1b

Results: In our work transgenic plants of tobacco were obtained via *Agrobacterium*-mediated transformation with vectors carrying the salmocin gene. Transgenic plants were selected for their ability to grow on a nutrient medium containing phosphinothricin or kanamycin. Regenerated plants formed roots after transfer to the MS medium. The transgenic nature of obtained plants was confirmed using the PCR method (Fig. 3).

The presence of the reporter protein ZsGreen in the vector with constitutive salmocin expression allowed us to selectively screen transgenic tobacco plants with high levels of recombinant protein expression by assessing fluorescence intensity under blue light illumination (Fig. 2). Transgenic tobacco lines selected according to ZsGreen fluorescence intensity demonstrated high antibacterial activity. As shown in Figure 4B, the inhibition zones were comparable among all tested lines and remained largely unchanged, exhibiting only a minor decrease at the highest extract dilution (1:32).

Transgenic tobacco lines harboring the inducible expression system showed greater variability in antibacterial activity against *Salmonella* compared to lines with constitutive recombinant salmocin expression. For induction the transgenic tobacco leaves were pre-incubated in chambers containing 4% ethanol and maintained for 3 days to allow accumulation of the recombinant product. In the most active transgenic lines, a measurable antibacterial effect was observed even in the absence of ethanol induction, suggesting a low level of basal (leaky) transgene expression (Fig. 4A). Nevertheless, transgenic tobacco plants carrying the ethanol-inducible system showed the ability to accumulate recombinant salmocin at a specific time point following ethanol induction, which is convenient for the subsequent isolation and purification of the recombinant protein. On the other hand, the presence of the reporter protein ZsGreen in the vector with constitutive salmocin expression allowed us to selectively screen transgenic tobacco plants with high levels of recombinant protein expression by assessing fluorescence intensity under blue light illumination. Such a vector design could facilitate the development of transgenic lines in recalcitrant crop species by providing rapid visual selection of successfully transformed and highly expressing events.

References.

- Nadi, Z., et al. (2020) Evaluation of antibiotic resistance and prevalence of common *Salmonella enterica* serovars isolated from foodborne outbreaks. *Microchem J* 155:104660. <https://doi.org/10.1016/j.microc.2020.104660>
- Schneider, T., et al. (2018). Plant-made *Salmonella* bacteriocins salmocins for control of *Salmonella* pathogens. *Scientific Reports*, 8(1), 4078. <https://doi.org/10.1038/s41598-018-22465-9>
- Hahn-Löbmann, S., et al. (2019). Colicins and salmocins – New classes of plant-made non-antibiotic food antibacterials. *Frontiers in Plant Science*, 10, 437. <https://doi.org/10.3389/fpls.2019.00437>
- Shcherbak N., et al. (2023) Accumulation Of Colicin M Protein and Its Biological Activity in Transgenic Lettuce and Mizuna Plants. *Frontiers in Plant Science*, 14:1271757. [doi: 10.3389/fpls.2023.1271757](https://doi.org/10.3389/fpls.2023.1271757)